NutraSteward

Providing regulatory support for food and feed ingredients

April 7, 2022

Ms. Wasima Wahid
Division of Animal Feeds
Center for Veterinary Medicine
U.S. Food and Drug Administration
1225 Wilkins Avenue
Rockville
Maryland 20852

Dear Ms. Wahid,

Re: GRAS Notice for Oligosaccharides-Peptides Complex for Use in Food for Cats and Dogs [IFA-013-146]

In October 2021, Gnubiotics Sciences SA submitted a GRAS notice for the use of oligosaccharides-peptides complex as a source of amino acids, peptides and glycopeptides in food for cats and dogs of all life stages at a level not to exceed 1.5% by weight of the complete feed. After an initial review of the GRAS notice, the Food and Drug Administration (FDA) Center for Veterinary Medicine (CVM) declined to file the GRAS notice. In January 2022, Gnubiotics Sciences SA met with the CVM to discuss the decline to file letter.

In follow-up to the letter and meeting, Gnubiotics Sciences SA has revised the GRAS notice. The primary modifications are summarized below:

CVM Comment	**Summary of GRAS Notice Revisions**
CVM asked for the relationship between GNU100 and oligosaccharides-peptides complex to be clarified.	The section "Nomenclature" has been revised and moved from Part 7 to precede Part 1. The Nomenclature section states that GNU100 is the experimental name for oligosaccharides-peptides complex.
CVM asked if heparin is present in the starting materials.	The pharmaceutical industry has optimized the production process to heparin to extract as much as technically feasible from hydrolyzed porcine intestinal mucosa. Confirmation of the absence of any detectable levels of heparin in oligosaccharides-peptides complex by LC-MS/MS is now included in Section 2.4.7 (p. 33) of the GRAS notice.
CVM suggested that the notifier not use the same term glycans to refer to both free glycans and glycans that are attached to peptides.	The terms in the original GRAS notice of "glycans constituent of glycopeptides" or "glycans constituent (present as glycopeptides)" have been replaced by "glycans side chains of the glycopeptides" and "glycans side chains (prime constituent of glycopeptides)". Where glycans are not in the form of glycopeptides, i.e., mammalian milk oligosaccharides, the term "oligosaccharides" is used.

CVM Comment	Summary of GRAS Notice Revisions
CVM highlighted an inconsistency in the mineral content vs. variation in ash content.	The total ash content of oligosaccharides-peptides complex is around 13% (130,000 mg/kg product). By comparison, the mean total mineral content does not exceed 105,394 mg/kg depending on the lot tested. We have summed the mineral contents of the lots to show that they fall below the total ash content. Sulfur and sodium were measured as part of the ash content, but it is also noted that the bisulfite content is around 2,170 mg/kg. Recognizing that sulfur and other elements will be present as oxides and salts, the total ash content is, as expected, slightly higher than the reported total mineral content. See Section 2.4.6.
CVM highlighted a variation in aerobic plate count reported in the specifications, batch analysis and stability studies.	The levels of microorganisms for 2 additional lots of oligosaccharides-peptides complex are presented in the GRAS notice confirming conformance with the product specifications (Table 2.4). The absence of any increase in the levels of microorganisms is noted in the stability studies (see Section 2.6).
CVM noted that the results of the mutagenicity and target animal safety studies could not be assessed until the chemical identity of the notified substance is clear. CVM also reminded the notifier that it should clearly indicate whether the substance used in the mutagenicity and target animal studies is the same as the notified substance.	Throughout the GRAS notice, the test article name "oligosaccharides-peptides complex" is in bold (including in the toxicity summaries in the GRAS notice) to help identify when this is the substance under discussion. Where study reports included as appendices to the GRAS notice refer to GNU100, an explanation has been added to confirm that GNU100 is the experimental name for oligosaccharides-peptides complex and test item is the notified substance. Published data are also included in the GRAS notice in pigs on protein hydrolysate of porcine intestinal mucosa, the starting material to oligosaccharides-peptides complex. Similarly, the term protein hydrolysate of porcine intestinal mucosa is in bold throughout the GRAS notice to help identify when it is the substance under discussion. The section describing the swine studies has been revised to more clearly explain the test article is equivalent to the raw material, protein hydrolysate of porcine intestinal mucosa.

All confidential within the GRAS notice is highlighted in grey. A redacted version of the GRAS notice is also provided. Certain appendices are considered confidential and are watermarked as such within the appendix as well as being listed as confidential in Part 7 (List of Appendices).

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Gnubiotics Sciences SA is keen to work with the FDA to ensure the successful filing of the GRAS notice for oligosaccharides-peptides complex. Please do not hesitate to contact me if further information or clarification is required.

Yours sincerely,

(b)(6)

Dr. Elizabeth Lewis
Scientific & Regulatory Advisor
NutraSteward

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GRAS Notice for Oligosaccharides-Peptides Complex for Use in Food for Cats and Dogs

Gnubiotics Sciences SA
Biopole StartLab, Bat Alanine
Route de la Corniche 5
1066 Epalinges
Switzerland

April, 2022

GRAS Notice for Oligosaccharides-Peptides Complex for Use in Food for Cats and Dogs

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NOMENCLATURE

Gnubiotics intends to market **oligosaccharides-peptides complex** for use in cat and dog food under the trade name "GNUbiome FI". **Oligosaccharides-peptides complex** is the product obtained from **protein hydrolysate of porcine intestinal mucosa** after physical processing to selectively isolated the **glycopeptides and peptides** component.

Protein hydrolysate of porcine intestinal mucosa is the product obtained from physical processing of hydrolyzed porcine intestinal mucosa, a by-product of the heparin production process. The source porcine intestinal mucosa is collected from pigs fit for human consumption. Physical processing is conducted to reduce the levels (b) (4) from hydrolyzed porcine intestinal mucosa. **Protein hydrolysate of porcine intestinal mucosa** is a recognized feed material in the EU and Canada, and is often marketed under the common name dried porcine solubles (DPS).

Oligosaccharides-peptides complex contains free amino acids, peptides and glycopeptides. Glycopeptides comprise glycans side chains which are linked by *O*-glycoside bonds to peptides. These glucans side chains (prime constituent of glycopeptides) exist as a diverse range of oligosaccharides which are generally described in terms of their "core" and "extended core" structures.

The following terminology is used within the GRAS Notice to describe the different compounds:

Oligosaccharides-peptides complex	GRAS substance
	Experimental name – GNU100
	Trade name – GNUbiome FI
Protein hydrolysate of porcine intestinal mucosa	Raw material used by Gnubiotics to manufacture oligosaccharides-peptides complex
	Experimental name – GnuGlycoPro
	Other common names when marketed as a feed material – porcine solubles, dried; condensed porcine solubles (CPS) and dried porcine solubles (DPS)
Hydrolyzed porcine intestinal mucosa	Source of protein hydrolysate of porcine intestinal mucosa obtained as a by-product of the heparin manufacturing process
Porcine intestinal mucosa	Material extracted from pigs fit for human consumption at the slaughterhouse

GRAS Notice for Oligosaccharides-Peptides Complex for Use in Food for Cats and Dogs

PART 1. §570.225. SIGNED STATEMENTS AND CERTIFICATION

In accordance with 21 CFR §570 Subpart E consisting of §570.203 to 280, Gnubiotics Sciences SA (hereafter referred to as "Gnubiotics"), herby informs the United States (U.S.) Food and Drug Administration (FDA) that they are submitting a Generally Recognized As Safe (GRAS) notice for **oligosaccharides-peptides complex**.

1.1 Name and Address of Organization

Gnubiotics Sciences SA
Biopole StartLab, Bat Alanine
Route de la Corniche 5
1066 Epalinges
Switzerland

1.2 Name of the Notified Substance

The notified substance is **oligosaccharides-peptides complex**.

1.3 Intended Conditions of Use

Oligosaccharides-peptides complex is intended for use as a source of amino acids, peptides and glycopeptides in the diet of cats and dogs. It will be included in food for cats and dogs of all life stages at a level not to exceed 1.5% by weight.

1.4 Statutory Basis for the Conclusion of GRAS Status

Pursuant to 21 CFR §570.30(a) and (b), **oligosaccharides-peptides complex** as manufactured by Gnubiotics, has been concluded to have GRAS status for use as a source of amino acids, peptides and glycopeptides in food for cats and dogs of all life stages under the conditions described in Part 1.3, on the basis of scientific procedures.

1.5 Premarket Exception Status

Gnubiotics herby informs the U.S. FDA of the view that **oligosaccharides-peptides complex** is not subject to the premarket approval requirements of the Federal Food, Drug and Cosmetic Act (FFDCA) based on Gnubiotics conclusion that the notified substance is GRAS under the conditions of intended use as described in Part 1.3 above.

1.6 Availability of Information

The data and information that serve as the basis for this GRAS notification will be made available to the U.S. FDA for review and copying upon request during customary business hours at the offices of:

Gnubiotics Sciences SA
Biopole StartLab, Bat Alanine
Route de la Corniche 5
1066 Epalinges
Switzerland

In addition, upon request, Gnubiotics will supply the U.S. FDA with a complete copy of the data and information either in an electronic format that is accessible for the Agency's evaluation or on paper.

1.7 Freedom of Information Act, 5 U.S.C. 552

In Gnubiotics view, all data and information presented in Parts 2 through 7 of this notice, with the exception of the text highlighted in grey in Sections 2.2 to 2.7 and 6.3, and Appendices 001 to 014, and 016 to 033, do not contain any trade secret, commercial or financial information that is privileged or confidential, and therefore, all data and information presented therein are not exempt from the Freedom of Information Act, 5 U.S.C. Section 552.

1.8 Certification

Yemi Adesokan hereby certifies that to the best of his knowledge, all data and information presented in this notice constitutes a complete, representative and balanced submission, which includes all unfavorable as well as favorable information known to Gnubiotics and pertinent to the evaluation of the safety and GRAS status of oligosaccharides-peptides complex.

Signed,

(b)(6)

April 1, 2022

ADEYEMI ADESOKAN _____ _____

Dr. Yemi Adesokan, CSO Date

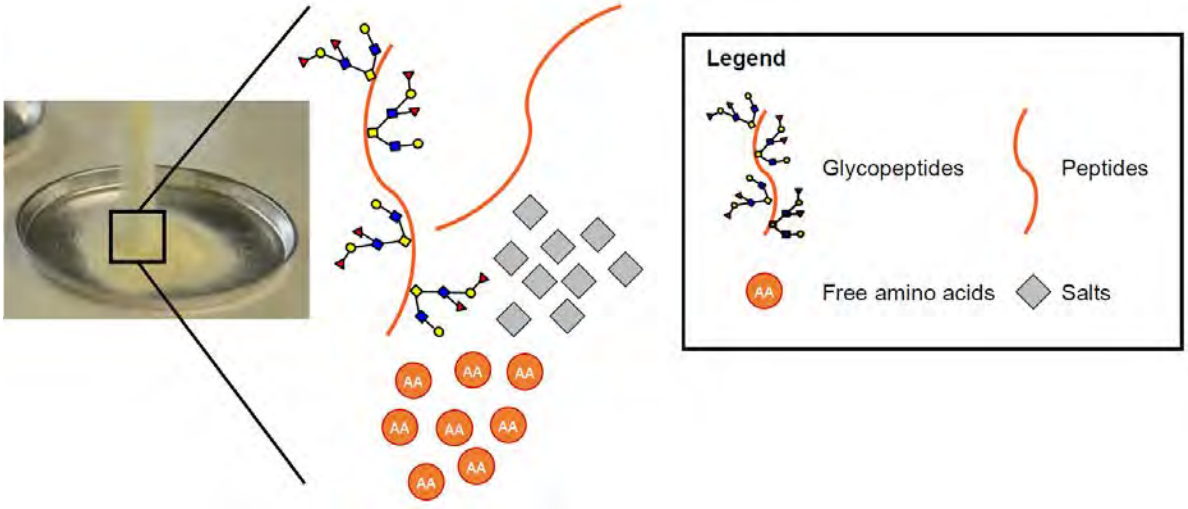
PART 2. §570.230. IDENTITY, METHOD OF MANUFACTURE, SPECIFICATIONS AND PHYSICAL OR TECHNICAL EFFECT

2.1 Identity

2.1.1 Identity of the GRAS Substance

Oligosaccharides-peptides complex is the product obtained by physical processing of **protein hydrolysate of porcine intestinal mucosa** to selectively isolate the peptides and glycopeptides components. It is comprised of (b) (4) glycopeptides and peptides, (b) (4) free amino acids and not more than (b) (4). The ingredient is a white to yellow hygroscopic powder with a moisture content of not more than 9%. A representative picture, including a schematic of the major components of the ingredient, is provided in Figure 2.1.

Figure 2.1: Representative Picture of Oligosaccharides-Peptides Complex



2.1.2 Source of Oligosaccharides-Peptides Complex

The source of **oligosaccharides-peptides complex** is **protein hydrolysate of porcine intestinal mucosa** which is currently marketed as a feed material (protein source) for pigs, poultry and fish in the European Union (EU) (see Section 2.1.3). The porcine mucosa from which **protein hydrolysate of porcine intestinal mucosa** is derived is obtained from pigs entering the food chain in the EU.

2.1.3 Regulatory Status of Protein Hydrolysate of Porcine Intestinal Mucosa (Source of Oligosaccharides-Peptides Complex)

United States

There is currently no listing in the Association of American Control Officials (AAFCO) Official Publication (OP) or Title 21 of the Code of Federal Regulations (21 CFR) specifically describing products derived from hydrolyzed porcine intestinal mucosa. Notably, there is a definition for animal digest, the material resulting from chemical and/or enzymatic hydrolysis of clean and undecomposed animal tissue, which is

commonly used as both a source of highly digestible protein and a flavor in pet food kibble (Ingredient Definition 9.68; AAFCO, 2021a; Purina, 2021).

European Union

Protein hydrolysate of porcine intestinal mucosa, which is the raw material used by Gnubiotics for the production of **oligosaccharides-peptides complex** is a recognized feed material for use as a nutrient source in animal food in the EU. (b) (4) It has been listed since 2018 in the industry-maintained Feed Materials Register and is defined as: "*hydrolyzed protein from porcine intestinal mucosa. It is obtained by enzymatic digestion from porcine mucosa. Source of animal protein*" (007909-EN; EU Feed Materials Register, 2022). Gnubiotics has also listed **oligosaccharides-peptides complex** in the Feed Materials Register (008429-EN; EU Feed Materials Register, 2022).

Additionally, hydrolyzed animal protein, defined as the: "*polypeptides, peptides and amino acids, and mixtures thereof, obtained by hydrolysis of animal by-products, which can be concentrated by drying*" is a recognized feed material in the EU under Commission Regulation (EU) No 68/2013 on the Catalogue of Feed Materials (EC, 2013; Entry 9.6.1).

Canada

In Canada, porcine solubles, dried (or dried porcine solubles, DPS) is listed in Schedule IV, Part II of the Feed Regulations (Entry 5.6; CFIA, 2022) for use as a protein feed and described as: "*the product obtained after the extraction of heparin for human use from enzymatically digested porcine mucosa and small intestines which have been heat-treated, condensed and dried with or without carrier. It shall be labeled with guarantees for minimum % crude protein, minimum % crude fat, maximum % moisture, maximum % crude fiber and maximum % ash. The carrier shall also be listed on the label*".

Porcine solubles, dried (or DPS) is the material described herein as **protein hydrolysate of porcine intestinal mucosa** for use as the source of **oligosaccharides-peptides complex**. As mentioned above, the feed material is generally utilized in the dry form for ease of handling and can be co-dried with a carrier. Thus, in Canada there is an established history of use of the source of **oligosaccharides-peptides complex** in animal feed.

2.1.4 Composition of Porcine Intestinal Mucosa

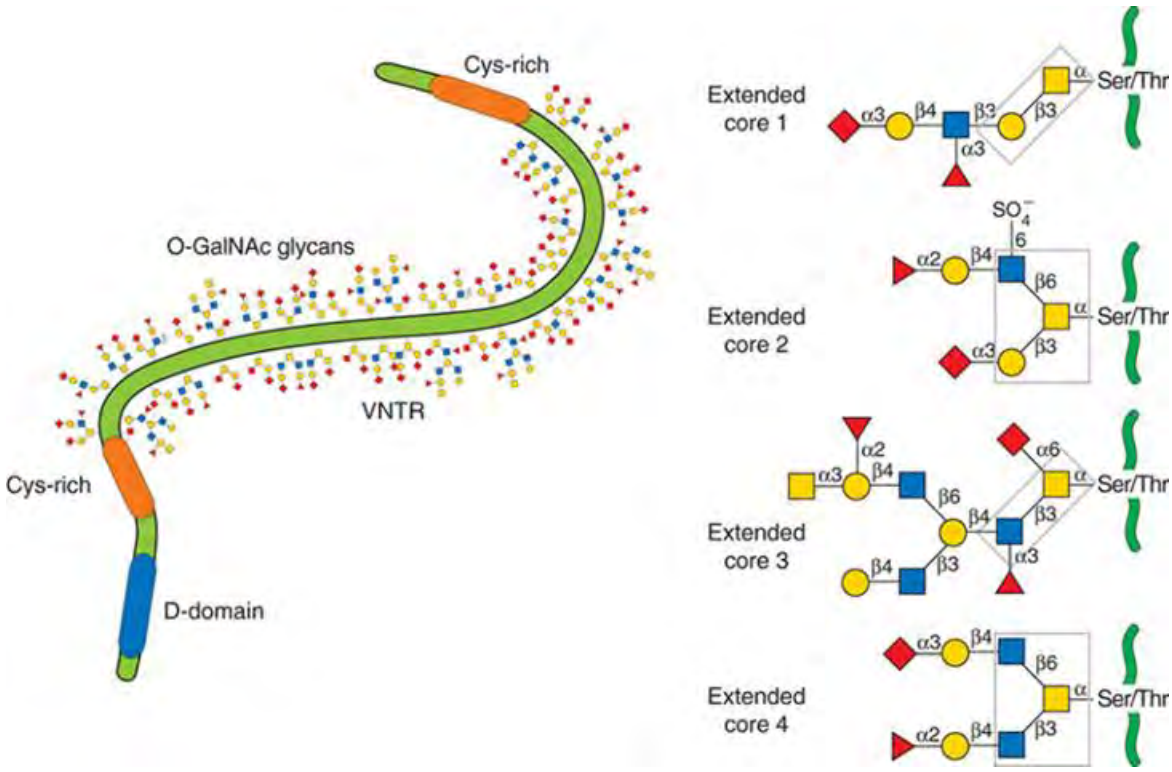
The amino acids, peptides and glycopeptides comprising **oligosaccharides-peptides complex** are derived from porcine intestinal mucosa.

The main components of porcine intestinal mucosa are mucins, which are large extracellular glycoproteins ranging in molecular weight from 0.5 to 20 MDa (Bansil and Turner, 2006). These glycoproteins comprise around 80% glycans (a diverse range of oligosaccharide cores) and 20% polypeptides (protein core), linked through *O*-glycosidic bonds. The glycans constituents of porcine glycopeptides are primarily composed of *N*-acetylgalactosamine (GalNAc), *N*-acetylglucosamine (GlcNAc), fucose (Fuc), galactose (Gal) and *N*-acetylneuraminic acid (sialic acid) along with minor

amounts of mannose (Man), glucose (Glc) and sulfate. In general, the glycans contain between 5 and 15 monomers displaying moderate branching and are linked by *O*-glycosidic bonds to the hydroxyl chains of serine and threonine arranged in what is described as "bottle brush" configuration around the protein core (b) (4); Brockhausen and Stanley, 2017). Any of these sugars can be the unit directly linked to the hydroxyl residues of the amino acids but the most common modifying sugar unit in mammalian mucins, including porcine mucins, is GalNAc resulting in *O*-GalNAcylation (also known as mucin-type glycosylation). The glycans can exist as a diverse range of structures known as "cores" (Mantle and Allen, 1981; Karlsson *et al*., 1997; Darula and Medzihradszky, 2018). A schematic depicting a model of mammalian-derived mucin glycoproteins and the 4 most common glycan core structures in porcine-derived mucin (cores 1 to 4; the 2 or 3 residues within the grey boxes represent the core glycan structure) is presented in Figure 2.2. The diagram also highlights the diversity and branching of the glycan cores beyond these core structures (extended core structures), although the example is for human respiratory and colonic mucins, rather than porcine mucins specifically.

Compositional analysis of a glycoprotein isolated from porcine gastric mucosa after pepsin hydrolysis revealed a protein content of 15% and carbohydrate content of 55% (b) (4) It was recognized that the recovery was not 100% even after accounting for a 10% moisture content, which was potentially due to limitations with the assay used to quantify the levels of individual monosaccharides. The individual constituents of the glycans component were determined to be GalNAc (55%), GlcNAc (33%), Gal (9%), Fuc (2%) and Sia (1.7%). It is reasonable to assume that the glycoproteins of porcine intestinal mucosa display similar compositions.

Figure 2.2: Representation of Mammalian-Derived Glycoprotein (Mucin) and Glycan Core Structures (Reproduced from Brockhausen and Stanley, 2017)



VNTR = variable number tandem repeat region; a region rich in serine, threonine and proline which is highly *O*-glycosylated; D-domain = a distinct structural unit identified in mucins; *O*-GalNAc glycans = glycans linked to the protein core by *O*-GalNAcylation, which is the most common type of mammalian *O*-glycosylation; the 2 or 3 residues within the gray boxes represent the characteristic glycan core structure assigned the nomenclature 1 to 4; α2/3 and β3/4/6 represent the linkages between the sugar monomer units [α-(1,2)-, β-(1,3)-linkages etc.]; the branching and structures beyond these cores will vary between mammalian sources, and the above extended cores are provided only as an example and are taken from human respiratory and colonic mucins.

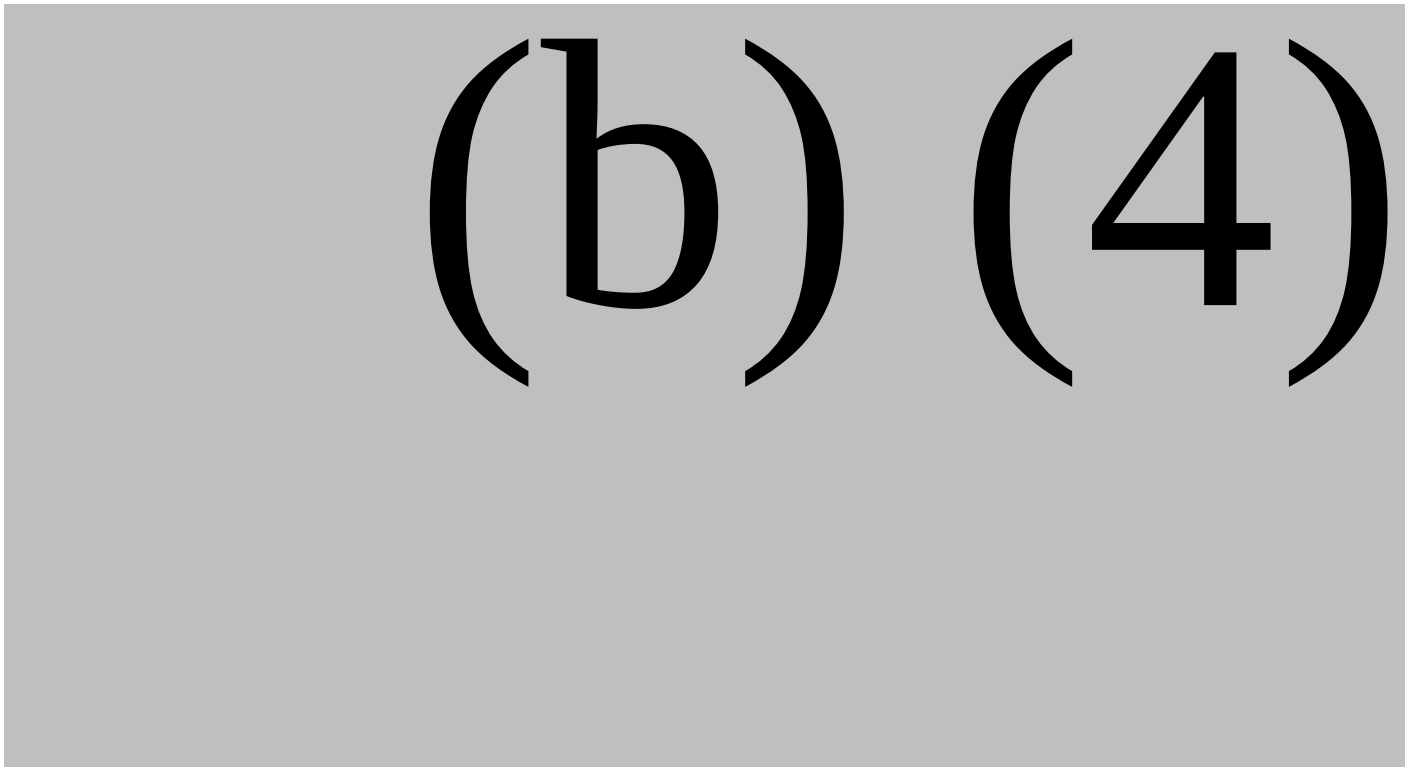
The monomers are represented by the established symbol nomenclature for glycans (SNFGs):

= GalNAc; = Gal; = GlcNAc; = Sia; =Fuc.

GalNAc = *N*-acetylgalactosamine; Gal = galactose; GlcNAc = *N*-acetylglucosamine; Sia = sialic acid; Fuc = fucose.

2.1.5 Hydrolysis Products of Glycoproteins from Porcine Intestinal Mucosa

Protein hydrolysate of porcine intestinal mucosa which is the source of **oligosaccharides-peptides complex**, (b) (4)



2.1.6 Composition of Protein Hydrolysate of Porcine Intestinal Mucosa

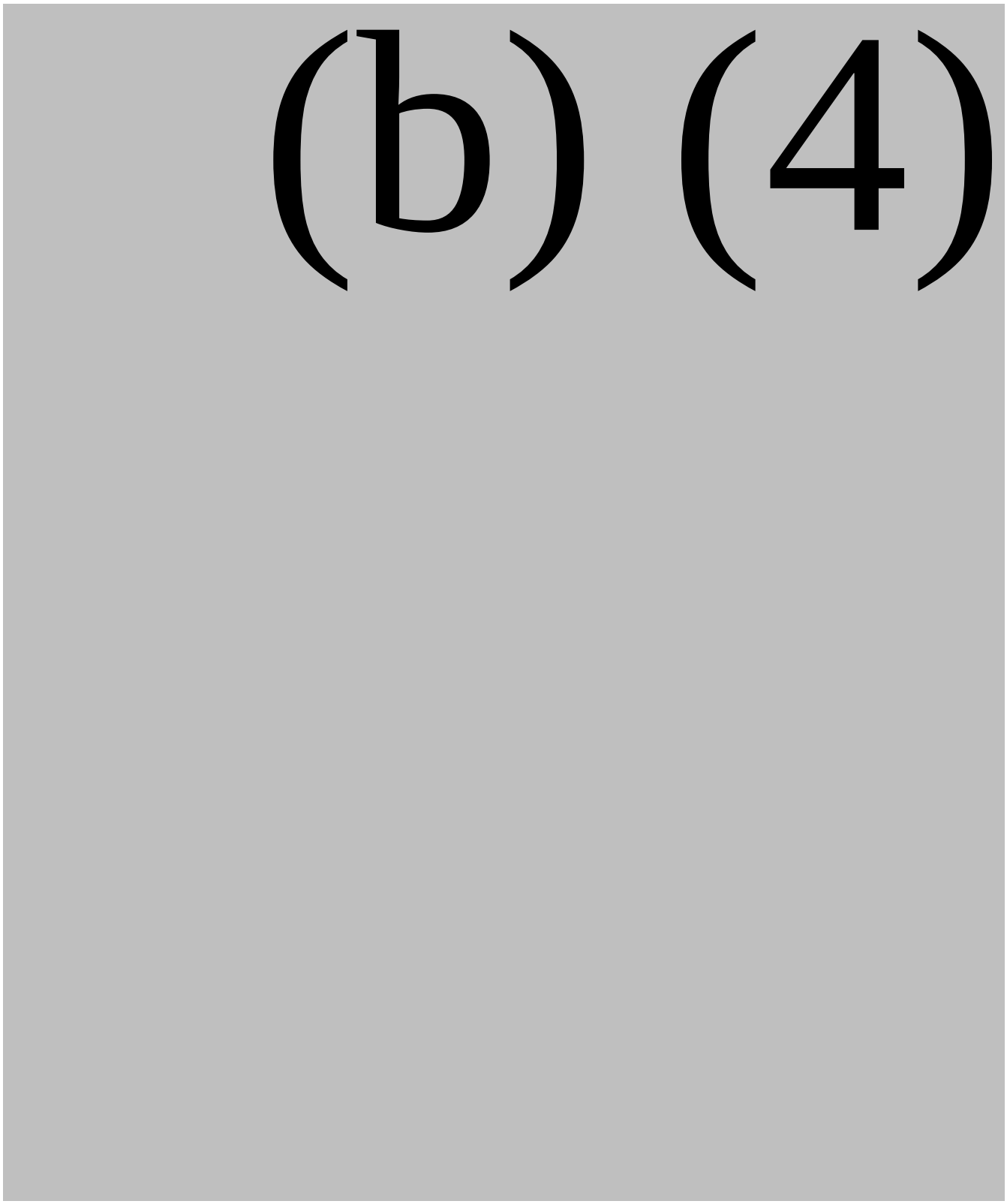
The hydrolysis products of porcine intestinal mucosa are subject to physical processing, in particular, (b) (4), to yield **protein hydrolysate of porcine intestinal mucosa**. This ingredient is marketed directly for use as a protein source in the feed of pigs, poultry and fish in the EU, and is used as the source of **oligosaccharides-peptides complex**.

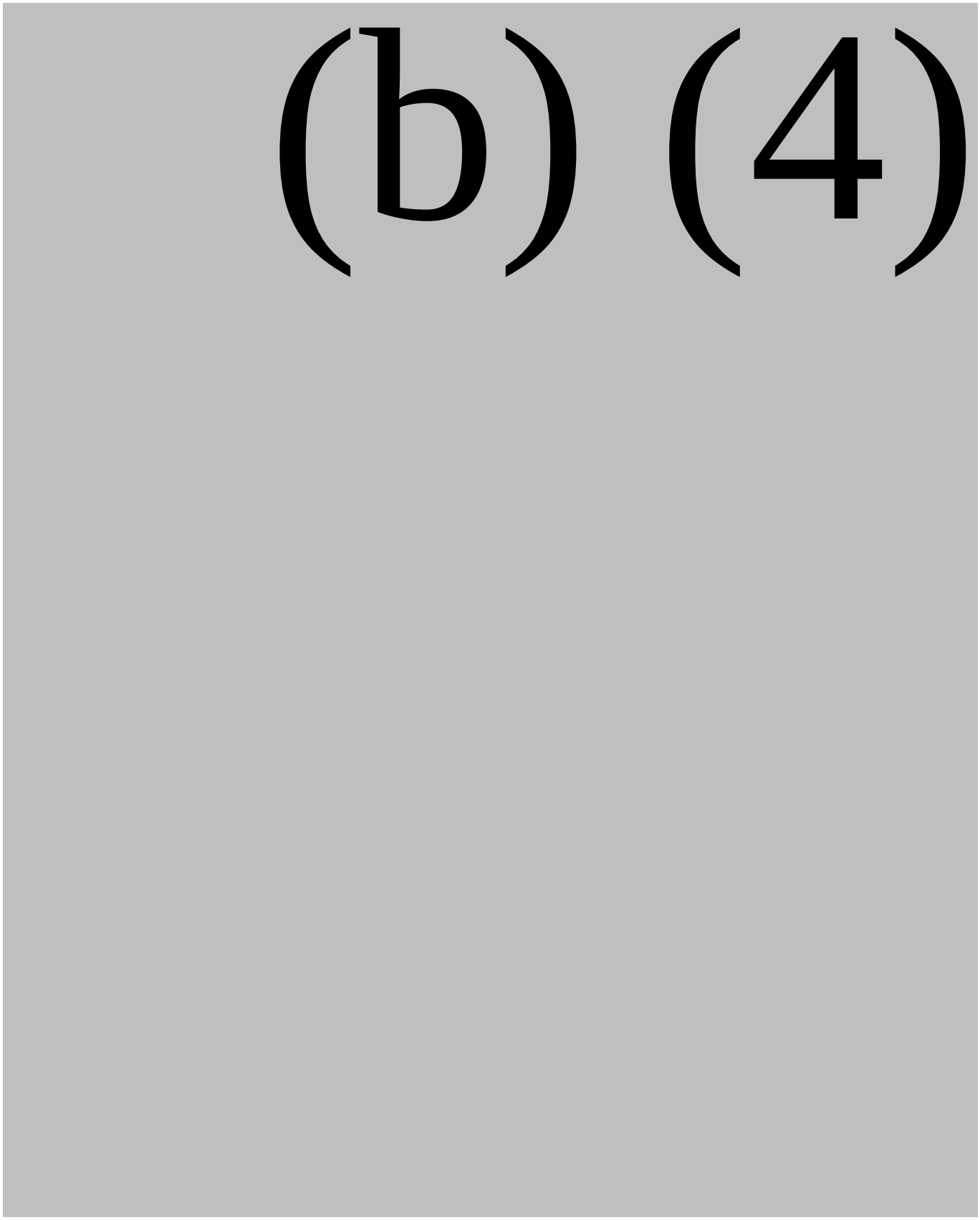
Protein hydrolysate of porcine intestinal mucosa intended for use as a feed ingredient is marketed on the basis of its free amino acid and peptides content. As such, the glycopeptides content can vary between manufacturers and lots, and is not routinely measured. (b) (4)

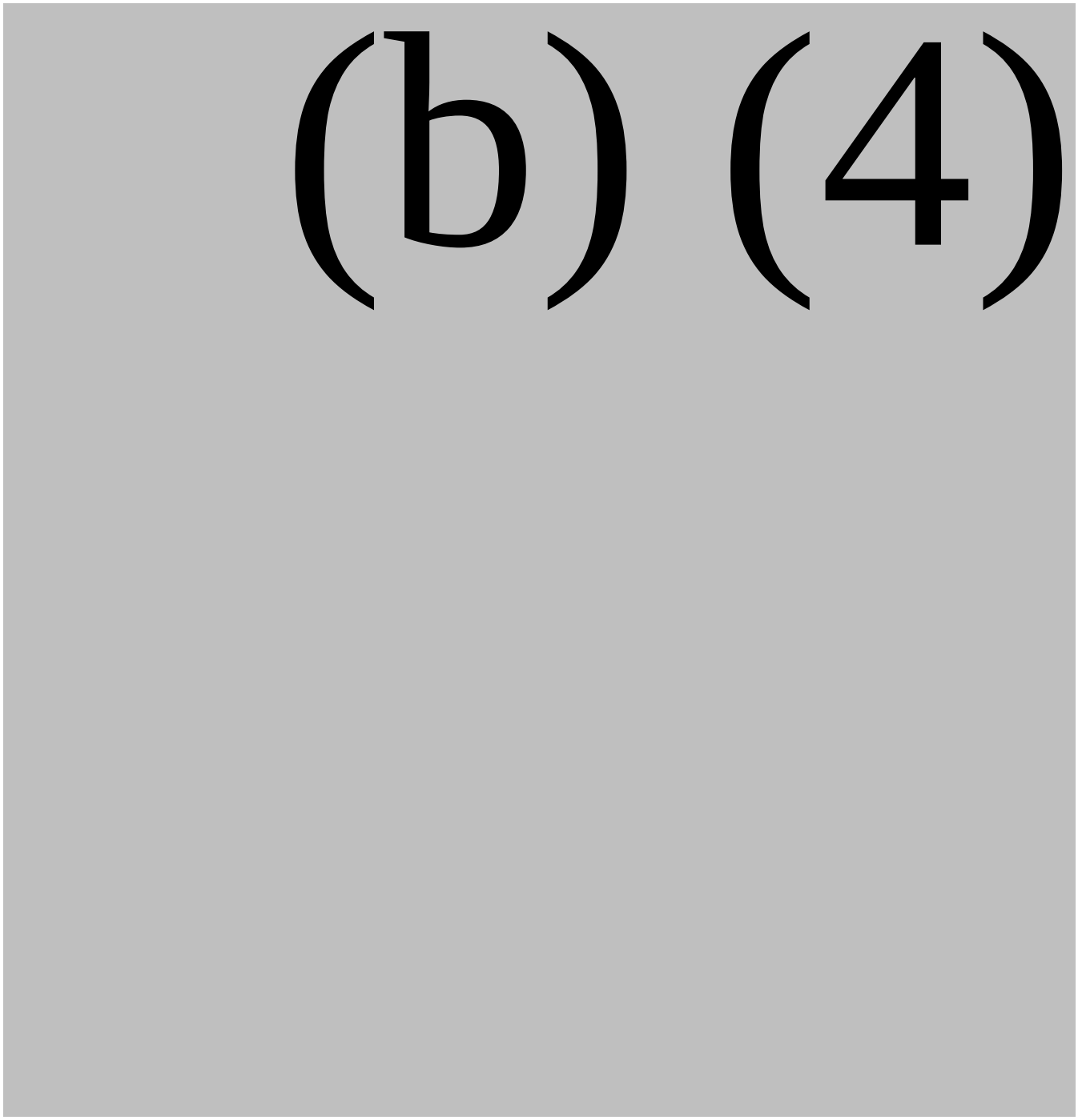
2.1.7 Composition of Oligosaccharides-Peptides Complex

Gnubiotics (b) (4) processes to the **protein hydrolysate of porcine intestinal mucosa** in order to remove some of the low molecular weight substances (b) (4) and increase the content of glycopeptides and peptides. No chemical transformation occurs and the composition of the glycopeptides and peptides components will reflect the glycoproteins of porcine intestinal mucosa.

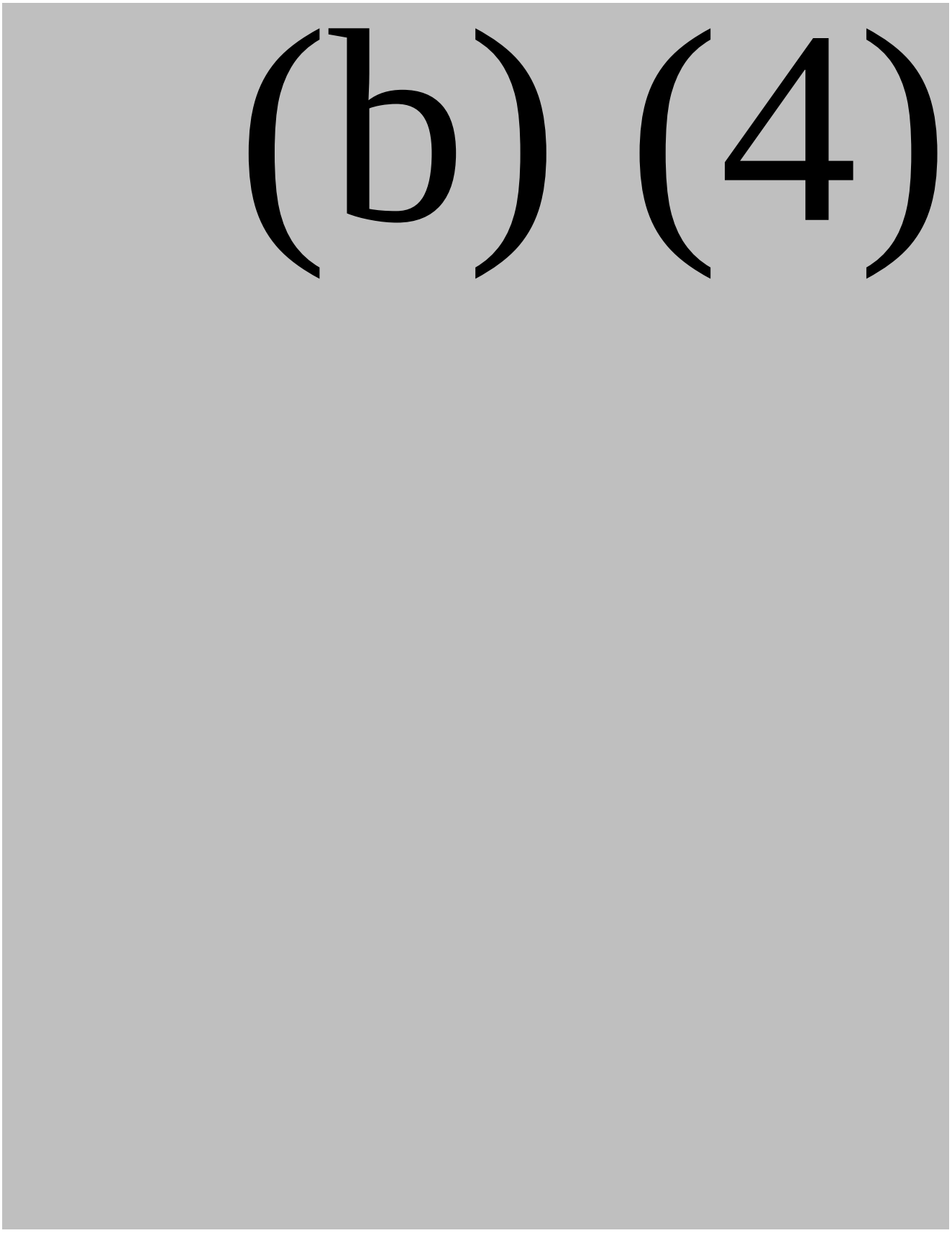
2.2 Method of Manufacture




(b) (4)


(b) (4)

(b) (4)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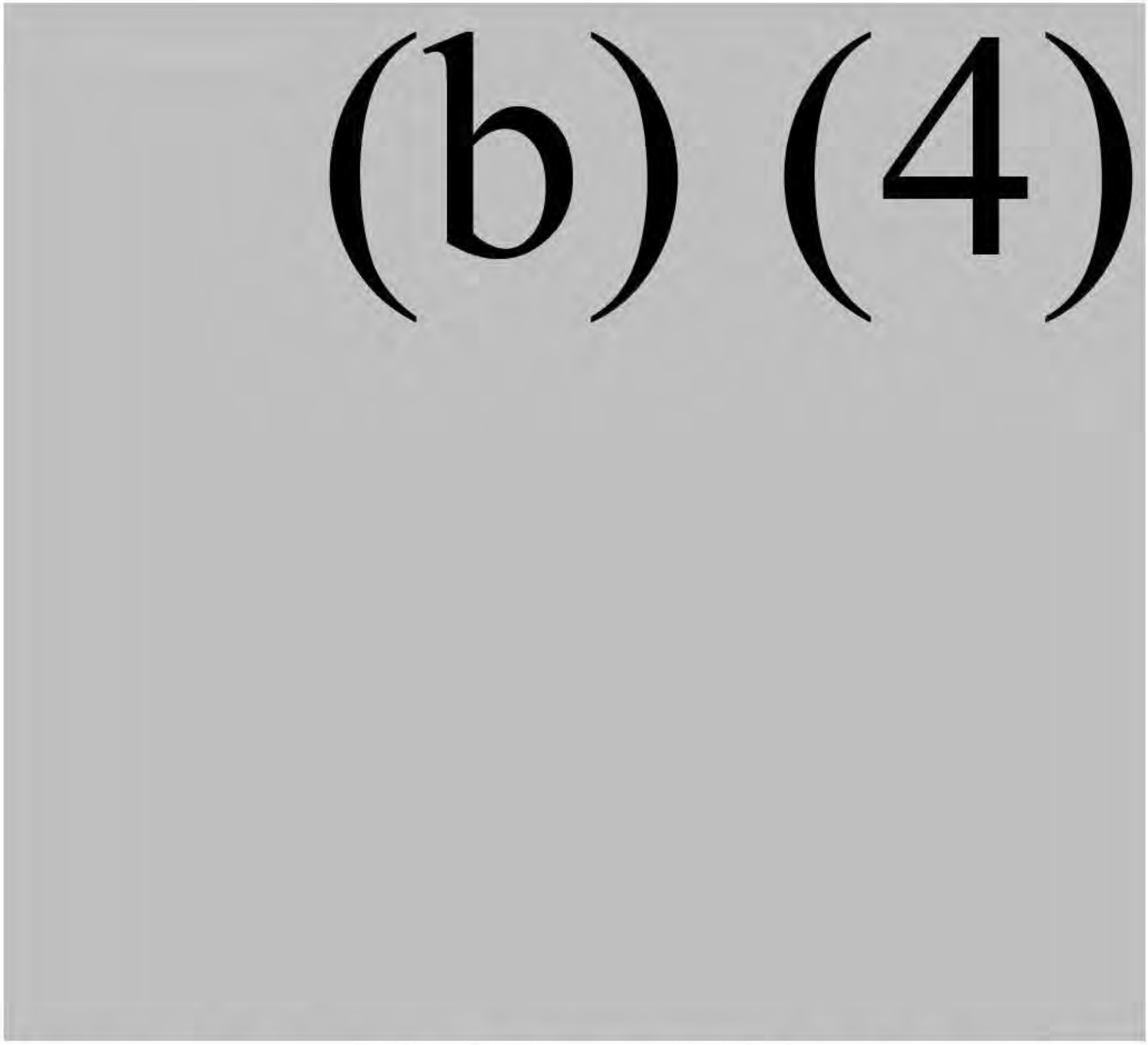


Oligosaccharides-peptides complex will be manufactured in accordance with cGMP and a HACCP plan will be in place. It will also comply with the requirements of the Food Safety Modernization Act (FSMA) for imported animal food. (b) (4)

All aspects of the manufacturing process comply with Regulations (EC) No 1069/2009 and (EC) No

142/2011 on the manufacture, handling and transportation of animal by-products in the EU (EC, 2009a and 2011). The controls in place are consistent with Regulation (EC) No 183/2005 laying down requirements for feed hygiene in the EU (EC, 2005).

2.3 Product Specifications and Analytical Data for Oligosaccharides-Peptides Complex

2.3.1 Product Specifications

Appropriate feed-grade specifications have been established for **oligosaccharides-peptides complex** and are presented in Table 2.3. Copies of the methods of analysis are provided in Appendices 015A to 015J.

(b) (4)

The ash content is specified not to exceed (b) (6).

Maximum limits are proposed for arsenic, lead, cadmium and mercury of 1, 1, 0.5 and 1 mg/kg, respectively in **oligosaccharides-peptides complex**. These limits fall below, or are equal to, the maximum tolerance level in complete feed suggested by AAFCO in the official guidelines for contaminants in individual mineral feed ingredients (AAFCO, 2021b) for arsenic, lead, cadmium and mercury of 50, 30, 0.5 and 2 mg/kg, respectively.

Acceptable feed grade microbiological criteria are established for **oligosaccharides-peptides complex** which verify that appropriate feed hygiene practices were followed (total aerobic plate count, and yeasts and molds) and the absence of contamination with pathogenic microorganisms (*Salmonella* and *Escherichia coli*).

All methods of analysis are internationally recognized or follow validated internal procedures.

Table 2.3: Proposed Product Specifications for Oligosaccharides-Peptides Complex		
Parameter	**Specification**	**Method of Analysis**
Physical Properties		
Appearance	White to yellow powder	Visual inspection
pH (2% aqueous solution)	5.0 to 7.0	Ph. Eur. 2.2.3 (Appendix 015A)
Composition		
Moisture	≤9%	Ph. Eur. 2.5.32 (Appendix 015B)
Ash	(b) (4)	Ph. Eur. 2.4.14 (Appendix 015C)
Free amino acids		Ph. Eur. 2.2.56 (Appendix 015D)
Glycopeptides and peptides		Calculation: 100 – (moisture + ash + free amino acids) (Appendix 017E)
Heavy Metals		
Arsenic	≤1 mg/kg	prEN 15763:2009 (ICP-MS) (Appendix 015F)
Lead	≤1 mg/kg	prEN 15763:2009 (ICP-MS) (Appendix 015F)
Cadmium	≤0.5 mg/kg	prEN 15763:2009 (ICP-MS) (Appendix 015F)
Mercury	≤1 mg/kg	prEN 15763:2009 (ICP-MS) (Appendix 015F)
Microbiology		
Total aerobic plate count	≤10,000 CFU/g	ISO 4833-1:2003 (Appendix 015G); or Ph. Eur. 2.6.12 (Appendix 015H)
Yeast and mold	<100 CFU/g	ISO 21527-2:2008 (Appendix 015I); or Ph. Eur. 2.6.12 (Appendix 15H)
Salmonella	Negative in 25 g	Ph. Eur. 2.6.13 (Appendix 015J)
Escherichia coli	<10 CFU/g	Ph. Eur. 2.6.13 (Appendix 015J)

Abbreviations: CFU = colony forming units; ICP-MS = inductively coupled plasma – mass spectrometry; ISO= International Standardization Organization; Ph. Eur. = European Pharmacopoeia; prEN = provisional European Standard.

2.3.2 Compliance with Product Specifications

Analytical data for 6 independently produced batches of **oligosaccharides-peptides complex** representative of the commercial product are presented in Table 2.4. (b) (6) Three (3) of the batches were manufactured as part of a pilot study conducted by the contract production facility in order to optimize the process (Batches 4799190701, 4799190702 and 4799190703). The raw materials, processing aids and conditions were identical to those that will be applied on a commercial scale. The only exception is that the batches of **oligosaccharides-peptides complex** were retained prior to packaging for further testing (e.g., further compositional analysis and stability study testing). The additional 3 batches (Batches 4799210301, 4799210302 and 4799210303) of oligosaccharides-complex oligosaccharides were manufactured under commercial conditions

The results of the analyses demonstrate that **oligosaccharides-peptides complex** conforms with the physical, compositional, heavy metals and microbiological specifications, and exhibits acceptable batch to batch variation. Across the 6 batches tested, the glycopeptides and peptides content varied from 40 to 45% (mean 42%). The free amino acid content varied from 38 to 42% (mean 40%) and the ash

content from 12 to 15% (mean 13%). The moisture content did not exceed 6% in any of the batches tested.

Table 2.4: Results of Analysis of 6 Representative Batches of Oligosaccharides-Peptides Complex								
Parameter	**Unit**	**Specification**	**Analytical Data**					**Mean[1]**
			Batch 4799190701	**Batch 4799190702**	**Batch 4799190703**	**Batch 4799210301**	**Batch 4799210302**	**Batch 4799210303**	
Physical Properties									
Appearance									-
pH (2% solution)									5.4
Composition									
Moisture									5
Ash									13
Free amino acids									40
Glycopeptides and peptides									42
Heavy Metals									
Arsenic									-
Lead									-
Cadmium									-
Mercury									-
Microbiology									
Total aerobic plate count									-
Yeast and mold									-
Salmonella									-
Escherichia coli									-

(b) (4)

Abbreviations: "-" = not tested; CFU = colony forming units.

[1] Mean of the 6 independently produced batches of oligosaccharides-peptides complex.

2.4 Further Compositional Analysis of Oligosaccharides-Peptides Complex

2.4.1 Proximate Composition

The moisture, crude protein, ash and fat contents were determined for 3 representative batches of **oligosaccharides-peptides complex** and the results are summarized in Table 2.5. (b) (6) The carbohydrates content can be calculated by difference to complete the mass balance of **oligosaccharides-peptides complex.**

The mean crude protein content across the 3 batches tested was (b) (4) representing the primary compositional component of **oligosaccharides-peptides complex.** (b) (4) The carbohydrates content was estimated to be around (b) (4) and represents the glycans which are linked through *O*-glycosidic bonds to the peptides in the glycopeptides component. No significant levels of fat or fiber were detected in the 3 batches of **oligosaccharides-peptides complex.** The remainder of the ingredient is primarily composed of ash (b) (4) and moisture (b) (4)

Table 2.5: Proximate Composition of 3 Representative Batches of Oligosaccharides-Peptides Complex

Parameter	Analytical Data (%)			Mean
	Batch 4799190701	Batch 4799190702	Batch 4799190703	
Crude protein (Nx6.25)	(b) (4)			71.2
Carbohydrates (by difference)				10.2
Fat				0.2
Ash				14
Moisture				4
Total				***100***

2.4.2 Molecular Weight Distribution

(b) (4) . The molecular weight distribution was analyzed for 3 representative batches of **oligosaccharides-peptides complex** and the results are provided in Table 2.6. (b) (4) The results of the analysis of the 3 batches indicate that 99.7% of **oligosaccharides-peptides complex** has a molecular weight below (b) (4). The remaining 0.3% fraction of **oligosaccharides-peptides complex** displayed a molecular weight between (b) (4). The molecular weight of porcine intestinal mucins is reported to fall in the range 0.5 to 20 MDa (Bansil and Turner, 2006) and these findings are consistent with hydrolysis to glycopeptides, peptides and free amino acids.

Table 2.6: Molecular Weight Distribution of 3 Representative Batches of Oligosaccharides-Peptides Complex					
Molecular Weight	**Analytical Data (%)**			**Mean**	**SD**
	Batch 4799190701	**Batch 4799190702**	**Batch 4799190703**		
>10,000 Da	(b) (4)				0
5,000 to 10,0000 Da					0.058
2,000 to 5,000 Da					1.27
1,000 to 2,000 Da					1.08
<1,000 Da					1.55
Total					-

Abbreviations; Da = Daltons; ND = not detected; SD = standard deviation.

2.4.3 Composition of the Free Amino Acids Component

Oligosaccharides-peptides complex contains around (b) (4) **free amino acids** (see Section 2.3.2). The free amino acid profiles of 3 representative batches of **oligosaccharides-peptides complex** are summarized in Table 2.7. The variation in the content of any individual amino acid between the 3 batches was less than 1% indicating that there is no significant batch to batch variation in the free amino acid profile. (b) (6)

(b) (4)

Table 2.7: Composition of the Free Amino Acid Component of 3 Representative Batches of Oligosaccharides-Peptides Complex					
Amino Acid	**Analytical Data (% w/w)**			**Mean**	**SD**
	Batch 4799190701	**Batch 4799190702**	**Batch 4799190703**		
Proline	(b) (4)			2.4	0.16
L-Aspartic acid				3.6	0.11
L-Threonine				2.6	0.04
L-Serine				2.7	0.07
L-Glutamic acid				3.3	0.50
L-Glycine				2.5	0.07
L-Alanine				3.7	0.10
L-Valine				3.1	0.06
L-Methionine				0.9	0.09
L-Isoleucine				1.8	0.22
L-Leucine				2.8	0.47
L-Tyrosine				0.3	0.04
L-Phenylalanine				2.3	0.05
L-Histidine				1.3	0.14
L-Ornithine				1.1	0.21
L-Lysine				4.1	0.12
L-Arginine				1.0	0.13
L-Tryptophan				NR	NR
Total				***39.32***	-

Abbreviations: NR = not reported; SD = standard deviation.

2.4.4 Composition of the Peptides Component

The composition of the peptides and free amino acids components (combined), were analyzed for 3 representative batches of **oligosaccharides-peptides complex** and the results are summarized in Table 2.8. (b) (6).

(b) (4)

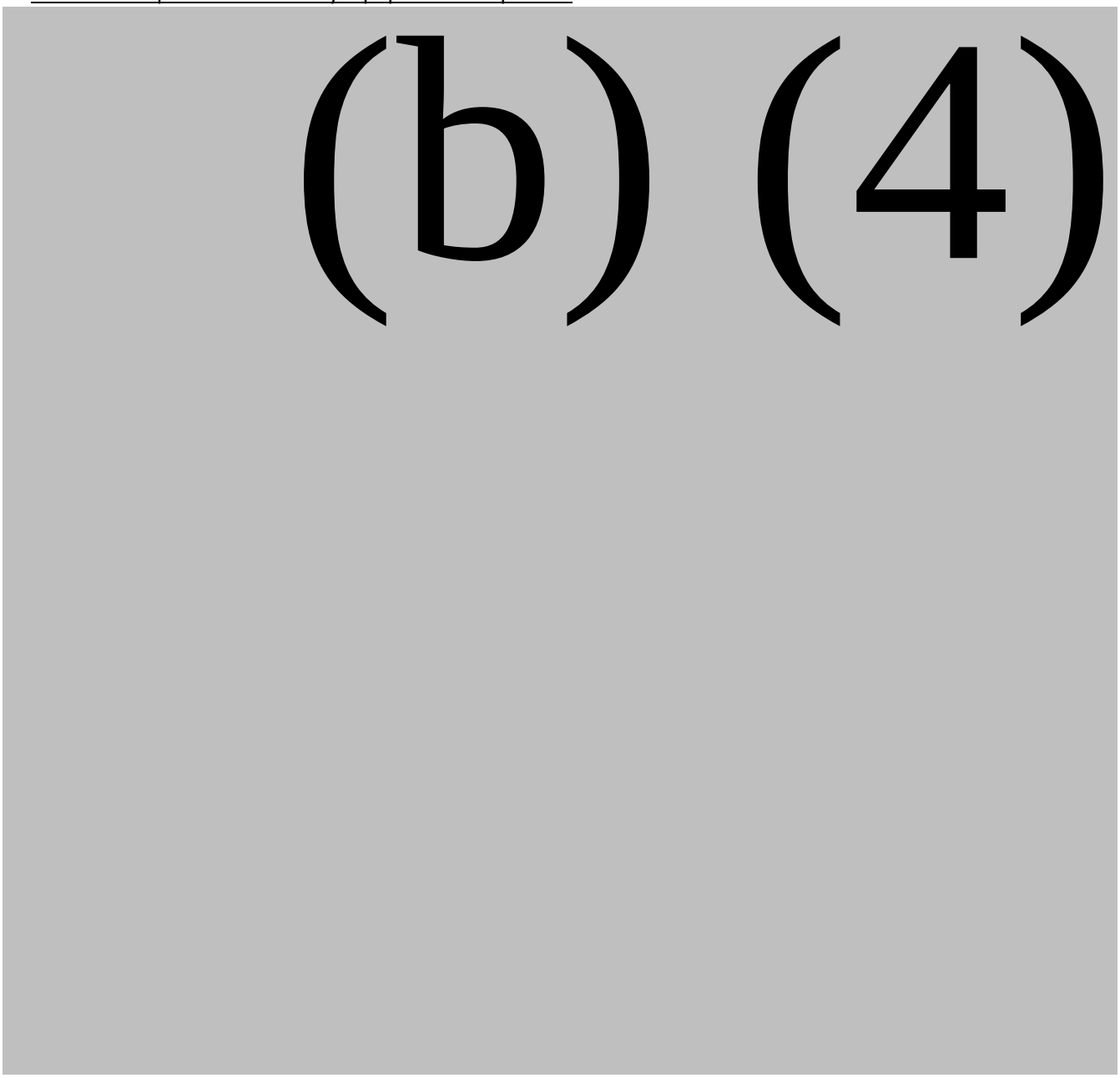
(b) (4)

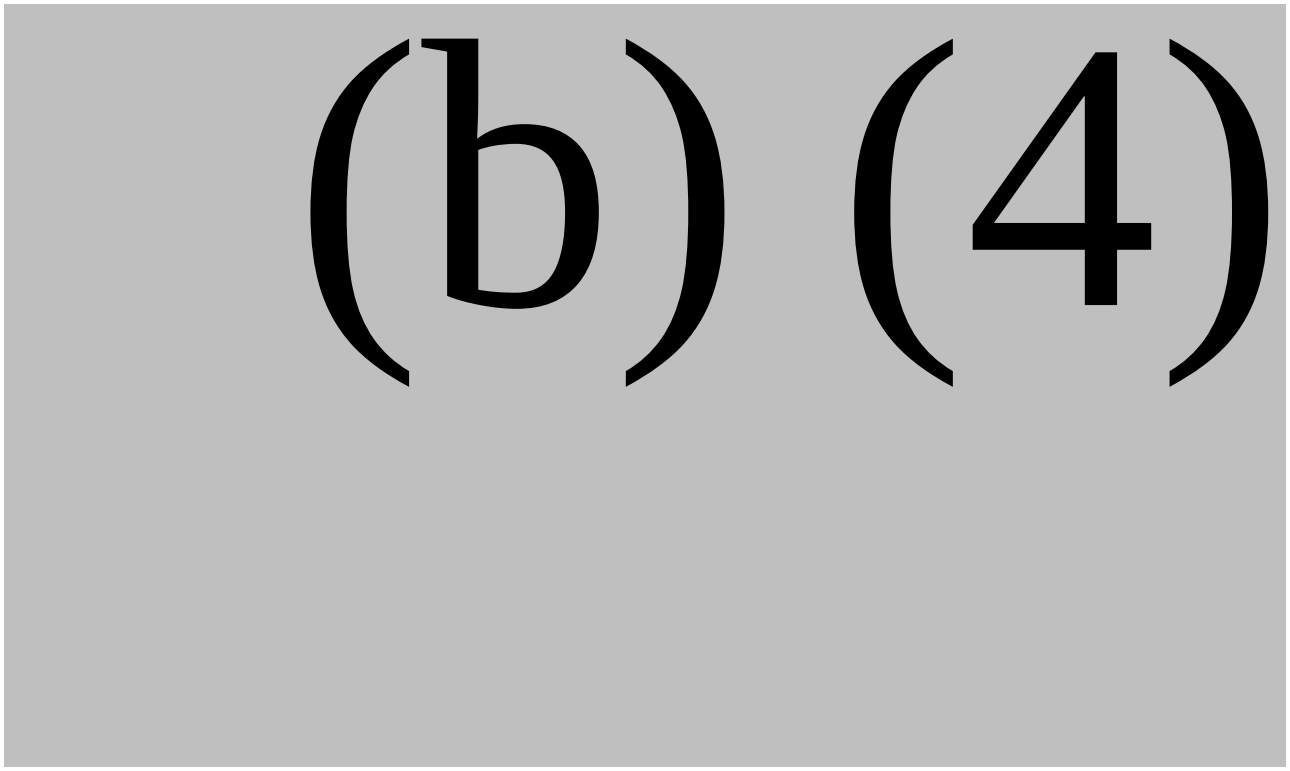
Table 2.8: Composition of the Free Amino Acid and Peptides Components (Combined) of Oligosaccharides-Peptides Complex

Amino Acid	Analytical Data (% w/w)				
	Batch 4799190701	Batch 4799190702	Batch 4799190703	Mean	SD
Proline	(b) (4)			4.4	0.15
L-Aspartic acid				5.9	0.01
L-Threonine				3.5	0.06
L-Serine				3.2	0.06
L-Glutamic acid				9.7	0.08
L-Glycine				5.2	0.10
L-Alanine				4.5	0.02
L-Valine				3.8	0.14
L-Methionine				1.1	0.13
L-Isoleucine				2.3	0.28
L-Leucine				3.6	0.59
L-Tyrosine				0.7	0.04
L-Phenylalanine				2.8	0.08
L-Histidine				1.5	0.15
L-Cystine				0.6	0.10
L-Ornithine				0.6	0.10
L-Lysine				5.6	0.16
L-Arginine				2.3	0.04
L-Tryptophan				0.7	0.10
Total				***61.3***	-

Abbreviations: SD = standard deviation.

2.4.5 Composition of the Glycopeptides Component

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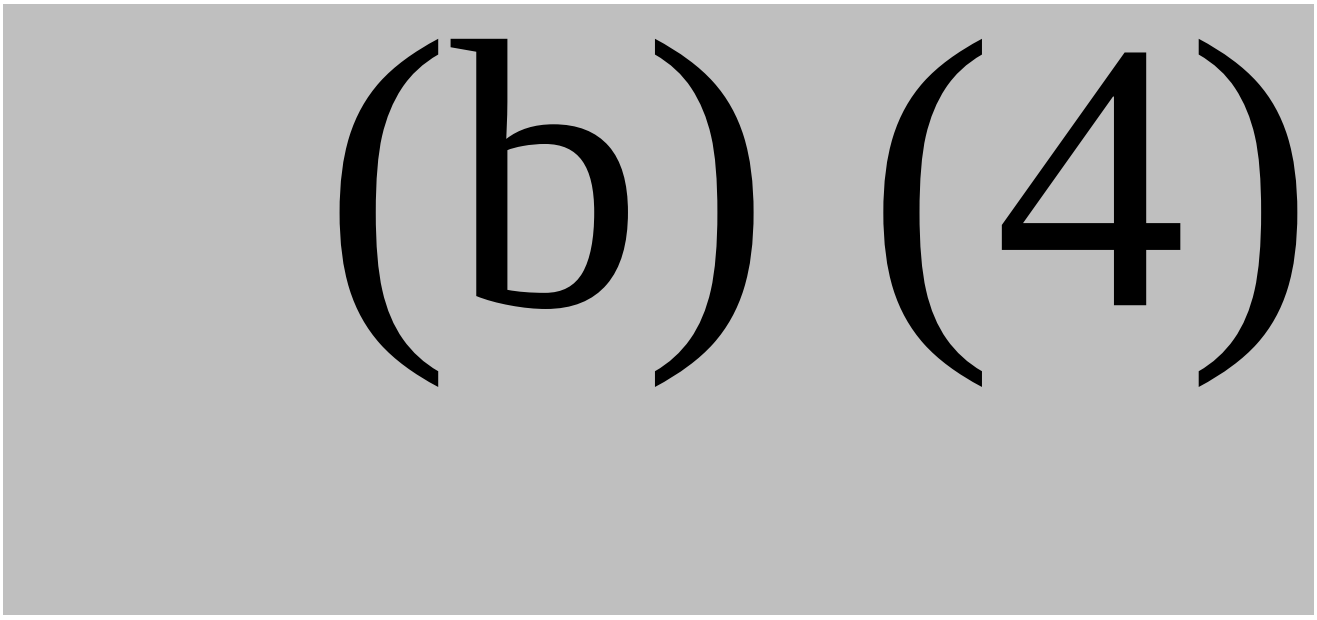
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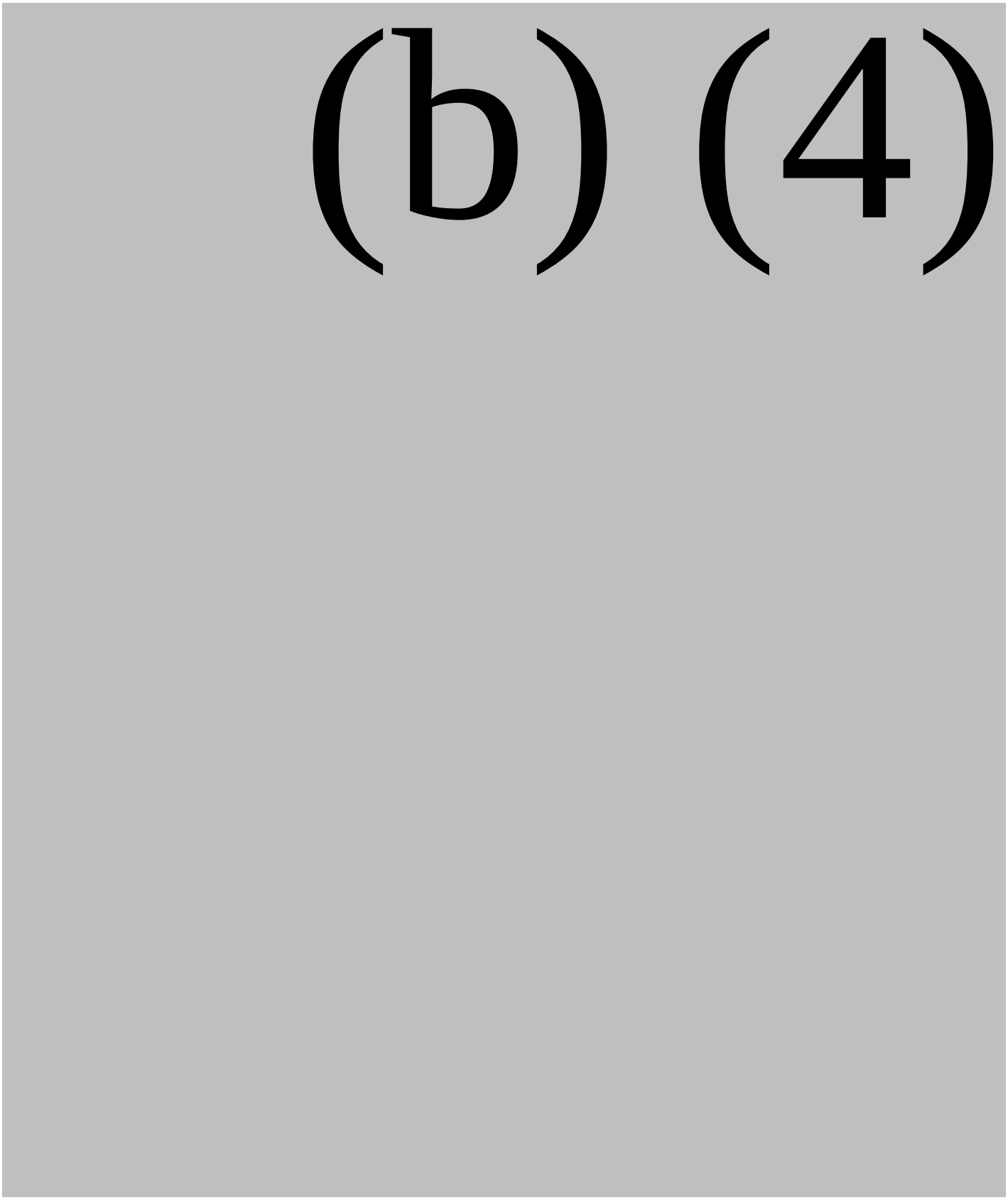
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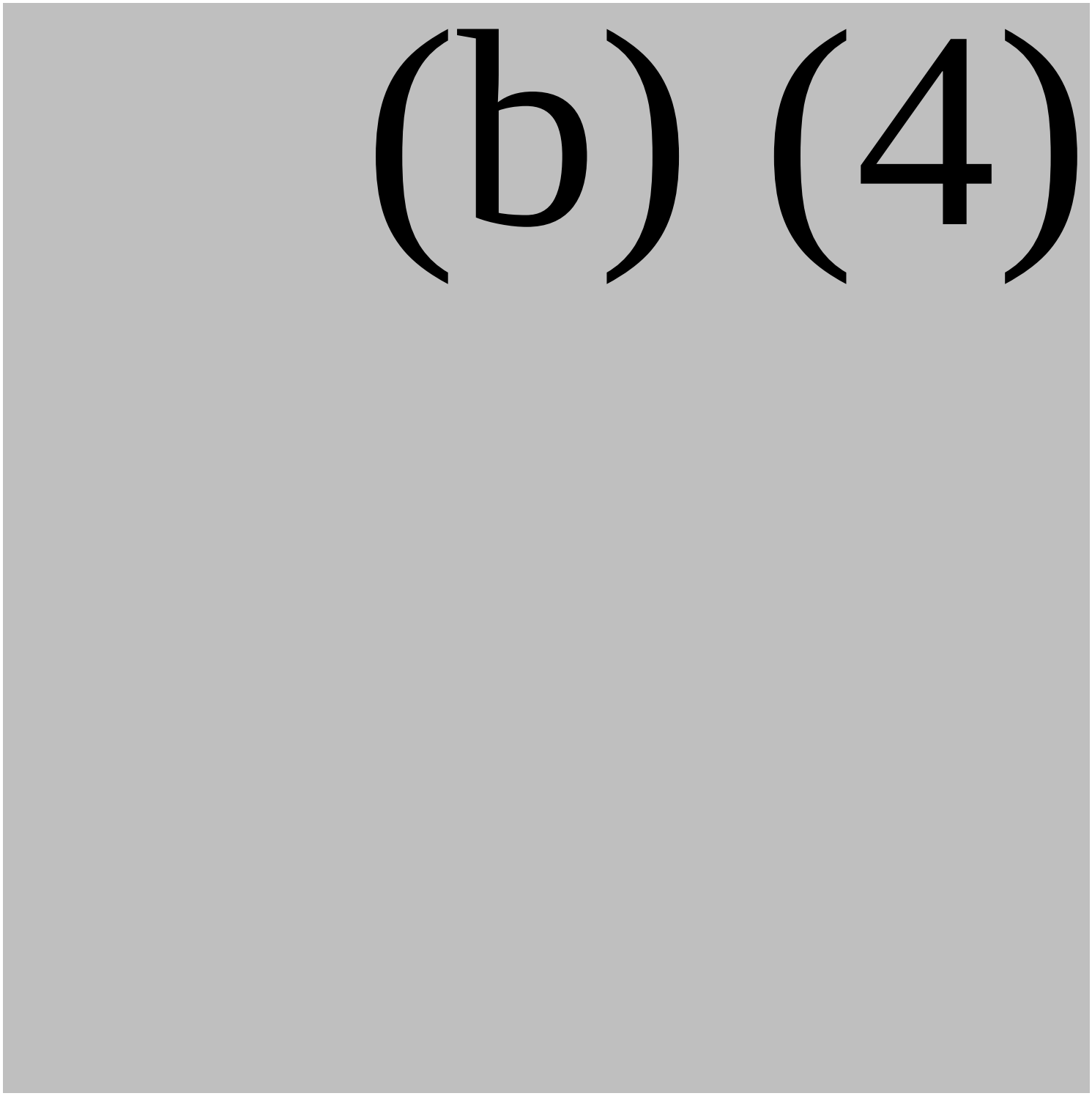
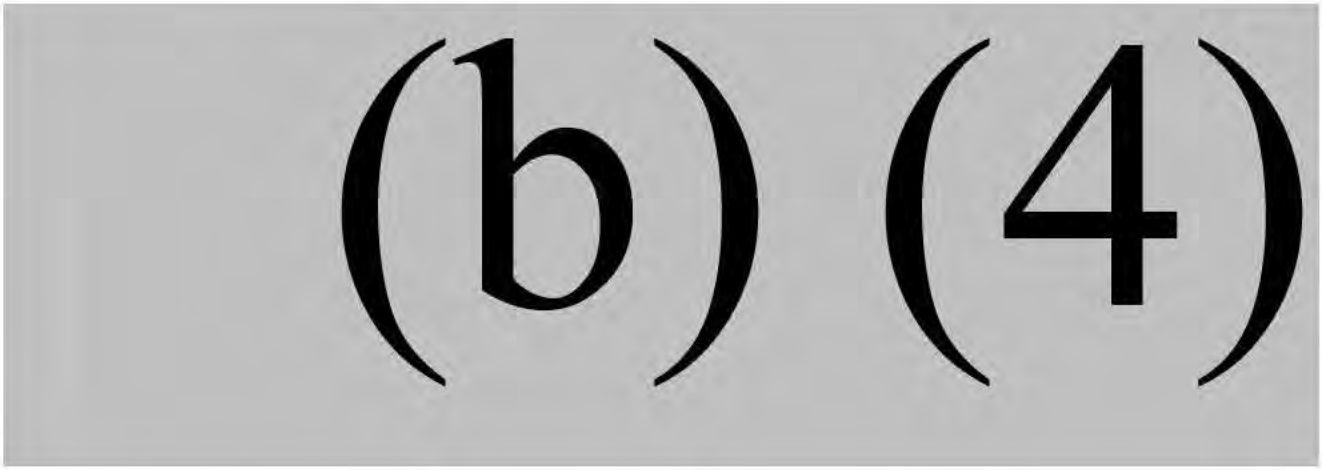


(b) (4)



2.4.6 Mineral Profile

The product specifications for **oligosaccharides-peptides complex** set a maximum limit for ash of (b) (4) The mean ash content of 6 representative batches of **oligosaccharides-peptides complex** was (b) (4) see Section 2.3.2).

The mineral content of 3 representative batches of **oligosaccharides-peptides complex** was analyzed and the results are presented in Table 2.11. (b) (6) There are some differences in the levels of individual minerals identified, but in general batch to batch variation was relatively low (estimated to be <15% for the majority of minerals).

Oligosaccharides-peptides complex is obtained from protein hydrolysate of porcine intestinal mucosa. (b) (4) . Across the 3 batches tested, the mean sodium and sulfur contents were (b) (4), respectively, which are likely to reflect the use of sodium bisulfite as a preservative during the collection of porcine intestinal mucosa from pigs at the slaughterhouse.

Additionally, the mean phosphorus content was (b) (4) which is expected to arise from the porcine tissue source. On isolation at the slaughterhouse, the porcine mucins will be primarily composed of glycoproteins but will also contain residual amounts of lipids, phospholipids, protein and salts (Bansil and Turner, 2006) which may contribute to the phosphorus content. The level of phosphorus in porcine processed animal proteins for use as ingredients in feed is reported to be in the region of 42 g/kg as-fed (INRAE-CIRAD-AFZ Feed Tables, 2021) which is (b) (4) higher than the residual levels in **oligosaccharides-peptides complex** (b) (4).

Table 2.11: Mineral Profile of 3 Representative Batches of Oligosaccharides-Peptides Complex				
Parameter	**Analytical Data (mg/kg)**			**Mean**
	Batch 4799190701	**Batch 4799190702**	**Batch 4799190703**	
Sodium (Na)	(b) (4)			31,967
Sulfur (S)				28,433
Calcium (Ca)				333
Iron (Fe)				117
Potassium (K)				6,900
Magnesium (Mg)				743
Phosphorus (P)				10,167
Selenium (Se)				0.8
Zinc (Zn)				94
Manganese (Mn)				3.6
Copper (Cu)				6.0
Total				***78,764***

As mentioned in Section 2.2.1, the mucosa scraped from the intestines of the pigs is preserved by the addition of sodium bisulfite (b) (4)). During the production of protein hydrolysate of porcine intestinal mucosa, (b) (4) is performed which is specifically designed to remove sodium sulfite along with any other salts. However, given the highly soluble nature of sodium salts, low levels of residual sulfite which are technically unavoidable may carry over into the **oligosaccharides-peptides complex**. The sulfites content of one representative batch of **oligosaccharides-peptides complex** was measured and reported to be (b) (4) expressed as sulfur dioxide, (b) (4) (b) (4). (b) (6)

Sodium bisulfite is GRAS for use as a chemical preservative in animal food in accordance with good manufacturing or feeding practices but may not be added to meat or food recognized as a source of vitamin B1 (thiamine) (21 CFR §582.3739; U.S. FDA, 2020a). **Oligosaccharides-peptides complex** incorporated into cat and dog food at a maximum proposed level of 1.5% by weight, will potentially provide 32.6 mg bisulfite/kg complete feed. The potential impact on the thiamine levels of the complete food for cats and dogs is evaluated in Section 6.3.5.

2.4.7 Potential for the Presence of Heparin Residues

(b) (4)

No traces of (b) (4) were identified above detection limits in any of the compositional studies on the glycans side chains conducted by Gnubiotics.

[4] (b) (4)

2.5 Further Quality and Contaminant Analysis

2.5.1 Freshness Profile

The total volatile base nitrogen (TVB-N) value and biogenic amines content of 3 representative batches of **oligosaccharides-peptides complex** were analyzed and the results are summarized in Table 2.12. The (b) (6).

Biogenic amines are formed during processing and transport of protein-rich ingredients as a result of microbial decarboxylation of the corresponding amino acids (Gou *et al.*, 2010; Naila *et al.*, 2010; EFSA, 2011a). There are currently no regulatory limits on the amount of biogenic amines in animal feed but a level of 300 mg/kg in finished pet food is generally considered acceptable in the U.S. (MidWest, 2021). **Oligosaccharides-peptides complex** is intended for use as a nutritional ingredient in cat and dog food at levels of up to 1.5% in the finished pet food (see Section 3.1). The sum of the histamine, tyramine, putrescine and cadaverine levels of **oligosaccharides-peptides complex** varied from 1,800 to 2,400 mg/kg across the 3 batches tested. Under the conditions of intended use of **oligosaccharides-peptides complex**, the contribution of these biogenic amines to the total biogenic load of pet food will be around 27 to 36 mg/kg which is around 8 to 10% of the 300 mg/kg level considered acceptable. Although these biogenic amines are not the only ones that may be present in **oligosaccharides-peptides complex**, they are recognized along with phenylethylamine to be of particular safety concern (EFSA, 2011a). Thus, **oligosaccharides-peptides complex** has the potential to be a source of biogenic amines but on the basis that the intended use level is relatively low (maximum 1.5%), the overall contribution to the finished pet food is not expected to pose a safety concern.

Table 2.12: TVB-N Value and Biogenic Amines Content of 3 Representative Batches of Oligosaccharides-Peptides Complex

Biogenic Amines	Unit	Analytical Data		
		Batch 4799190701	Batch 4799190702	Batch 4799190703
TVB-N value	mg/100 g	(b) (4)		
Histamine	mg/kg			
Tyramine	mg/kg			
Putrescine	mg/kg			
Cadaverine	mg/kg			
Sum of histamine, tyramine, putrescine and cadaverine	***mg/kg***			

Abbreviations: TVB-N = total volatile base nitrogen.

2.5.2 Dioxins and Dioxin-Like Polychlorinated Biphenyls (PCBs)

The results of analysis for dioxins and dioxin-liked PCBs for one representative batch of **oligosaccharides-peptides complex** are summarized in Table 2.13. (b) (6) . In the EU, maximum limits are set under Directive 2002/32/EC for the sum of dioxins of 0.75 ng WHO-PCDD/F-TEQ/kg and sum of dioxins and dioxin-like PCBs of 4.0 ng WHO-PCDD/F-PCB-TEQ kg (12% moisture content) in feed materials of animal origin (EC, 2002 – as amended). The dioxins and furans, and dioxins and dioxin-like PCBs level reported in the one batch of **oligosaccharides-**

peptides complex fell well below these maximum limits set by EU legislation and are not expected to pose a safety concern.

Table 2.13: Dioxins and Dioxin-like PCBs of Oligosaccharides-Peptides Complex		
Parameter	**Unit**	**Analytical Data**
		Batch 4799190701
Dioxins and furans TEQ-WHO (upper-bound, only PCDD/F	ng WHO-PCDD/F-TEQ/kg	(b) (4)
Sum of dioxins and dioxin-like PCBs TEQ-WHO (upper-bound)	ng WHO-PCDD/F-PCB-TEQ kg	

Abbreviations: PCB = polychlorinated biphenyls; PCDD = polychlorinated-p-dioxins; PCDF = polychlorinated dibenzofurans; TEQ = toxic equivalence; WHO = World Health Organization.

2.6 Shelf-Life and Stability Data

2.6.1 Shelf-Life Study

The shelf-life of **oligosaccharides-peptides complex** is 20 months stored under cool conditions (<20°C), in the original unopened packaging and in the absence of excessive humidity. The shelf-life is supported by the results of an accelerated study conducted over 6 months at 40°C, 75% relative humidity (RH), and an ongoing real-time study at 25°C, 60% RH using 3 independently produced batches of **oligosaccharides-peptides complex**. (b) (6).

The results of the stability study after 6 months of storage under accelerated conditions are summarized in Table 2.14. The 3 batches of **oligosaccharides-peptides complex** continue to conform with the product specifications at the 6-month time point. As mentioned in Section 2.3.2, the 3 batches of oligosaccharides-peptides used in the study were retained prior to packaging for additional testing (i.e., additional compositional and stability testing) and were not stored or handled in the same manner as a commercial product (i.e., with greater exposure to air). Thus, the aerobic plate counts exceeded the specifications at manufacture. However, consistent with the low moisture content of **oligosaccharides-peptides complex**, no microbial growth was observed over the 6-month storage period under accelerated conditions. Thus, the available data indicate that microbial growth is not expected to pose a concern during storage of **oligosaccharides-peptides complex**.

Table 2.14: Results of a Stability Study Under Accelerated Conditions for 3 Batches of Oligosaccharides-Peptides Complex (40°C, 75% RH)					
Parameter	**Unit**	**Specification**	**Analytical Data for Batch 4799190701**		
			T = 0 Mo	T = 3 Mo	T = 6 Mo
Moisture	%				
Free amino acids	%				
pH (2% solution)					
Total aerobic plate count	CFU/g				
Salmonella	CFU/25 g				
Escherichia coli	CFU/g				
Parameter	**Unit**				
Moisture	%				
Free amino acids	%				
pH (2% solution)					
Total aerobic plate count	CFU/g				
Salmonella	CFU/25 g				
Escherichia coli	CFU/g				
Parameter	**Unit**				
Moisture	%				
Free amino acids	%				
pH (2% solution)					
Total aerobic plate count	CFU/g				
Salmonella	CFU/25 g				
Escherichia coli	CFU/g				

(b) (4)

Abbreviations: CFU = colony forming units; Mo = month; RH = relative humidity.

The results of the stability study after 20 months of storage under real-time conditions are summarized in Table 2.15. The 3 batches of **oligosaccharides-peptides complex** comply with the product specifications at the 20-month time point. As mentioned above, the aerobic plate count was above specification limits at the start of the study but was not observed to increase during the 20-month storage period. These findings are consistent with the relatively low and stable moisture content of the batches of **oligosaccharides-peptides complex** (b) (4) and no safety concerns regarding microbial growth are anticipated under the recommended storage conditions over the 20-month shelf-life.

Table 2.15: Results of the Real-Time Stability Study for 3 Batches of Oligosaccharides-Peptides Complex (25°C, 60% RH)						
Parameter	**Unit**	**Specification**	**Analytical Data Batch 4799190701**			
			T = 0 Mo	**T = 6 Mo**	**T = 12 Mo**	**T = 20 Mo**
Moisture	%	(b) (4)				
Free amino acids	%					
pH (2% solution)	-					
Total aerobic plate count	CFU/g					
Salmonella	CFU/25 g					
Escherichia coli	CFU/g					
Parameter	**Unit**					
Moisture	%					
Free amino acids	%					
pH (2% solution)	-					
Total aerobic plate count	CFU/g					
Salmonella	CFU/25 g					
Escherichia coli	CFU/g					
Parameter	**Unit**					
Moisture	%					
Free amino acids	%					
pH (2% solution)	-					
Total plate count	CFU/g					
Salmonella	CFU/25 g					
Escherichia coli	CFU/g					

Abbreviations: CFU = colony forming units; Mo = month; RH = relative humidity.

2.6.2 Stability in Cat and Dog Food

Oligosaccharides-peptides complex is primarily composed of free amino acids (b) (4) glycopeptides and peptides (b) (4) and ash (b) (4) Analyzing these components once added to complete feed is challenging on the basis that they are all present naturally in cat and dog food (i.e., amino acids, peptides, monosaccharides) or cannot be readily extracted (glycopeptides). Stability studies were not therefore conducted. However, taking into account the composition of the ingredient, no significant degradation or impact on the organoleptic properties of the finished food is anticipated under the conditions of intended use of **oligosaccharides-peptides complex**.

2.7 Role of Oligosaccharides-Peptides Complex in the Diet

The requirements for the identity, method of manufacture, specifications and physical or technical effect part of a GRAS notice for a feed substance are laid down in 21 CFR §570.230 Subpart E (U.S. FDA, 2020b). In accordance with 21 CFR §570.230(d) relevant data and information bearing on the physical or other technical effect the notified substance is intended to produce, including the quantity of notified

substance required to produce such an effect must be included only when necessary to demonstrate safety.

Oligosaccharides-peptides complex is intended for use as a nutritional ingredient in food for cats and dogs at a level not to exceed 1.5% by weight. The ingredient provides a supplementary source of highly digestible free amino acids and peptides ((b) (4) as well as fermentable fiber in the form of glycopeptides ((b) (4) but will not replace other nutrients in the diet. The contribution of each of the components of oligosaccharide-peptides complex to the nutritional value of the diet of cats and dogs is described in Sections 2.7.2 to 2.7.5 below, and the overall impact on safety is considered in Section 2.8.

2.7.1 Oligosaccharides-Peptides Complex as a Source of Peptides and Amino Acids

AAFCO has established nutrient profiles for cat and dog growth and reproduction, and maintenance diets, which include recommended minimum amino acid contents (AAFCO, 2021c). The AAFCO profiles for cats and dogs are compared in Tables 2.16 and 2.17, respectively with the typical amino acid content of the free amino acids and peptide components of **oligosaccharides-peptides complex** under the intended conditions of use of not more than 1.5% in the diet by weight (on an as-fed basis). As mentioned above, a portion of the peptides in **oligosaccharides-peptides complex** is in the form of glycopeptides which are less likely to be susceptible to hydrolysis by proteolytic enzymes or to be available to the animal. Therefore, for the purposes of the comparison, only the free amino acids and peptides components of **oligosaccharides-peptides complex** are considered an available source of amino acids for cats and dogs.

Overall, **oligosaccharides-peptides complex** will contribute to the amino acid requirements of cats of all life stages under the intended conditions alongside other normal protein sources in the diet (see Table 2.13). However, the ingredient will generally provide no more than 10% of the requirement for any individual amino acid when included in the diet at the maximum inclusion level of 1.5%. Thus, the amino acid requirements of the animal will largely be met by the primary protein sources in the diet (e.g., meat and fish meal).

Table 2.16: AAFCO Nutrient Profile for Cat Growth and Reproduction, and Maintenance Diets (DM Basis) vs. Peptides and Free Amino Acids Profile of Oligosaccharides-Peptides Complex			
Amino Acid	**AAFCO Nutrient Profile (Minimum Content; %)[1]**		**Contribution by Oligosaccharides-Peptides Complex (1.5% in the Diet)[2]**
	Growth and Reproduction	**Adult Maintenance**	
Arginine	1.24	1.04	(b) (4)
Histidine	0.33	0.31	
Isoleucine	0.56	0.52	
Leucine	1.28	1.24	
Lysine	1.20	0.83	
Methionine	0.62	0.20	
Methionine-cystine	1.10	0.40	
Phenylalanine	0.52	0.42	
Phenylalanine-tyrosine	1.92	1.53	
Threonine	0.73	0.73	
Tryptophan	0.25	0.16	
Valine	0.64	0.62	
Crude Protein	***30.0***	***26.0***	

Abbreviations: AAFCO = Association of American Feed Control Officials; DM = dry matter.
[1]Taken from the AAFCO Official Publication (2021c) - AAFCO Cat and Dog Nutrient Profiles (Chapter 4);
[2]Calculated from the amino acid contents of the free amino acids and peptides components of 3 batches of oligosaccharides-peptides complex on a DM basis (Section 2.4.3) and assuming an inclusion level of 1.5% in the complete feed of cats and dogs

[(b) (4)].

Similarly, **oligosaccharides-peptides complex** will contribute to the amino acid requirements of dogs of all life stages under the intended conditions alongside other normal protein sources in the diet (see Table 2.14). However, the ingredient will generally provide no more than 15% of the requirement for any individual amino acid when included in the diet at the maximum inclusion level of 1.5%. Thus, the amino acid requirements of the animal will largely be met by the primary protein sources in the diet (e.g., meat and fish meal).

Table 2.17: AAFCO Nutrient Profile for Dog Growth and Reproduction and Maintenance Diets (DM Basis) vs. Peptides and Free Amino Acid Profile of Oligosaccharides-Peptides Complex			
Amino Acid	**AAFCO Nutrient Profile (Minimum Content; %)[1]**		**Oligosaccharides-Peptides Complex (1.5% in the Diet)[2]**
	Growth and Reproduction	**Adult Maintenance**	
Arginine	1.0	0.51	(b) (4)
Histidine	0.44	0.19	
Isoleucine	0.71	0.38	
Leucine	1.29	0.68	
Lysine	0.90	0.63	
Methionine	0.35	0.33	
Methionine-cystine	0.70	0.65	
Phenylalanine	0.83	0.45	
Phenylalanine-tyrosine	1.30	0.74	
Threonine	1.04	0.48	
Tryptophan	0.20	0.16	
Valine	0.68	0.49	
Crude Protein	***22.5***	***18.0***	

Abbreviations: AAFCO = Association of American Feed Control Officials; DM = dry matter.
[1]Taken from the AAFCO Official Publication (2021c) - AAFCO Cat and Dog Nutrient Profiles (Chapter 4);
[2]Calculated from the mean total amino acid content of the free amino acids and peptides components of 3 batches of oligosaccharides-peptides complex on a dry-basis (Section 2.4.3) and assuming an inclusion level of 1.5% in the complete feed of cats and dogs

[(b) (4)

2.7.2 Oligosaccharides-Peptides Complex as a Source of Minerals

Oligosaccharides-peptides complex contains up to (b) (4) ash and while not intended for use as a source of minerals, may contribute to the overall profile of the complete cat and dog food. The AAFCO profiles for cats and dogs are compared in Tables 2.18 and 2.19, respectively with mineral profile of **oligosaccharides-peptides complex** under the intended conditions of use of not more than 1.5% in the diet by weight (on an as-fed basis).

With the exception of sodium, oligosaccharides-peptides complex will not make a significant contribution to the mineral requirements of cats of all life stages, providing less than 5% of the daily requirements of any individual element (see Table 2.18). (b) (4)

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At the maximum intended use level of 1.5% in the diet, **oligosaccharides-peptides complex** are estimated to contribute around 25% of the requirements of sodium of cats. The European Pet Food Manufacturers Association (FEDIAF) has published nutritional guidelines for cats which indicate that a sodium level of 1.5% DM in the diet is not expected to pose a safety concern (FEDIAF, 2020). By comparison, **oligosaccharides-peptides complex** at the intended use level of 1.5% will provide 0.05% sodium which represents only 3% of the maximum safe level established by FEDIAF. It is reasonable to conclude that the level of sodium in **oligosaccharides-peptides complex** will not pose a safety concern to cats and can be taken into account by formulators when the ingredient forms part of the complete food.

Table 2.18: AAFCO Nutrient Profile for Cat Growth and Reproduction, and Maintenance Diets (DM Basis) vs. Mineral Profile of Oligosaccharides-Peptides Complex

Amino Acid	Unit	AAFCO Nutrient Profile (Minimum Content; %)[1]		Oligosaccharides-Peptides Complex (1.5% in the Diet)[2]
		Growth and Reproduction	Adult Maintenance	
Calcium	%	1	0.6	(b) (4)
Phosphorus	%	0.8	0.5	
Potassium	%	0.6	0.6	
Sodium	%	0.2	0.2	
Magnesium	%	0.08	0.04	
Iron	mg/kg	80	80	
Copper	mg/kg	15	5	
Manganese	mg/kg	8.4	7.6	
Zinc	mg/kg	75	75	
Selenium	mg/kg	0.3	0.3	

Abbreviations: AAFCO = Association of American Feed Control Officials; DM = dry matter.
[1]Taken from the AAFCO Official Publication (2021c) - AAFCO Cat and Dog Nutrient Profiles (Chapter 4);
[2]Calculated from the mean total amino acid content of 3 batches of oligosaccharides-peptides complex on a dry-basis (Section 2.4.3) and assuming an inclusion level of 1.5% in the complete feed of cats and dogs

(b) (4)

With the exception of sodium, **oligosaccharides-peptides complex** will not make a significant contribution to the mineral requirements of dogs of all life stages, providing less than 5% of the daily requirements of any individual element (see Table 2.19). At the maximum intended use level of 1.5% in the diet, **oligosaccharides-peptides complex** are estimated to contribute around 17% of the sodium requirements of growing and reproducing dogs, but 67% of the requirements of adult dogs. Similar to cats, FEDIAF indicates in its nutritional requirements for dogs that a sodium level of 1.5% DM in the diet is not expected to pose a safety concern (FEDIAF, 2020). As mentioned above, **oligosaccharides-peptides complex** will provide 0.05% sodium when included in the diet at a level of 1.5% which represents only 3% of the maximum safe level established by FEDIAF. Thus, the level of sodium in **oligosaccharides-peptides complex** will not pose a safety concern to dogs and can be taken into account by formulators when the ingredient forms part of the complete food.

Table 2.19: AAFCO Nutrient Profile for Dog Growth and Reproduction, and Maintenance Diets (DM Basis) vs. Mineral Profile of Oligosaccharides-Peptides Complex				
Amino Acid	**Unit**	**AAFCO Nutrient Profile (Minimum Content; %)[1]**		**Oligosaccharides-Peptides Complex[2]**
		Growth and Reproduction	**Adult Maintenance**	
Calcium	%	1.2	0.5	(b) (4)
Phosphorus	%	1.0	0.4	
Potassium	%	0.6	0.6	
Sodium	%	0.3	0.08	
Magnesium	%	0.06	0.06	
Iron	mg/kg	88	40	
Copper	mg/kg	12.4	7.3	
Manganese	mg/kg	7.2	5.0	
Zinc	mg/kg	100	80	
Selenium	mg/kg	0.35	0.35	

Abbreviations: AAFCO = Association of American Feed Control Officials; DM = dry matter.
[1]Taken from the AAFCO Official Publication (2021c) - AAFCO Cat and Dog Nutrient Profiles (Chapter 4);
[2]Calculated from the mean total amino acid content of 3 batches of oligosaccharides-peptides complex on a dry-basis (Section 2.4.3) and assuming an inclusion level of 1.5% in the complete feed of cats and dogs – no adjustment was made for dry matter content of the complete food on the basis that the moisture content is normally low

(b) (4)

2.7.3 Oligosaccharides-Peptides Complex as a Source of Glycopeptides

Oligosaccharides peptides complex is estimated to contain in the region of 20% glycopeptides (see Section 2.4.5. The glycopeptides component consists of glycans linked by *O*-glycosidic bonds to peptides. Each of these prime constituents (glycans side chains and peptides chains, respectively) represents around 50% by weight of the glycopeptides component, equivalent to 10% of **oligosaccharides-peptides complex.**

As further described in Section 3.1, the glycopeptides component which will act as a source of fermentable fiber for cats and dogs. A number of ingredients such as fructooligosaccharides, chicory and mannooligosaccharides have an established history of use as sources of fermentable fiber in cat and dog foods and have been the subject of studies in the published literature (e.g., Swanson *et al.*, 2002a and b; Zentek *et al.*, 2002, Barry *et al.*, 2010; Kanakupt *et al.*, 2011; Pinna *et al.*, 2018). AAFCO ingredient definitions have been established for fructooligosaccharides and inulin as summarized in Table 2.20 in recognition of their established role as fermentable fiber sources in the diet of animals in the U.S.

Table 2.20: Examples of Other Polysaccharides as Nutritional Ingredients in Animal Feed in the U.S.	
Ingredient	**AAFCO Ingredient Definition**
Fructooligosaccharides (FOS)	Carbohydrate product composed of short chain fructose units bound by β-2,1 linkages attached to a terminal glucose unit. The final product must contain a minimum of 80% fructooligosaccharide on a dry weight basis
Inulin	Polysaccharide product obtained from plant sources such as chicory (*Cichorium intybus*, L.), agave (Agave *azul tequilana*), and Jerusalem artichoke *(Hellianthus tuberosus*) by hot water extraction. It is intended as a source of soluble, fermentable fiber. It must contain not less than 90% inulin. It may contain products of partially hydrolyzed inulin.

Oligosaccharides-peptides complex will provide an alternative source of fermentable fibers to these recognized nutritional ingredients in the U.S. In the numerous studies identified in the published literature in which conventional oligosaccharide ingredients were evaluated as sources of fermentable fiber content of cat and dog food, dietary levels of up to 1% were generally reported to exert a positive nutritional effect on digestive health with no adverse effects on digestibility of fecal characteristics (e.g., Sparkes *et al.*, 1998; Hussein *et al.*, 1999; Swanson *et al.*, 2002a and b; Propst *et al.*, 2003; Middelbos *et al.*, 2007; Biagi *et al.*, 2010; Faber *et al.*, 2011). By comparison, at the maximum intended use of **oligosaccharides-peptides complex** as an ingredient in animal feed at levels of up to 1.5% in the diet, cats and dogs will consume around 0.3% glycopeptides. These levels of glycopeptides inclusion are towards the lower end of the amounts of other oligosaccharides such as fructooligosaccharides generally established to have utility in the diet of cats and dogs. However, these conventional counterparts are generally comprised of relatively simple structures which lack the diversity displayed by the glycopeptides derived from porcine intestinal mucosa (see Sections 2.1.4 and 2.4.5). This structural diversity, as further discussed below, is expected to contribute to the functionality of glycopeptides and result in nutritional utility at lower levels than are historically used for simpler oligosaccharide ingredients.

2.7.4 Comparison of the Glycans Side Chains of the Glycopeptides Component of Oligosaccharides-Peptides Complex and Mammalian Milk Oligosaccharides (MMOs)

As previously described, the glycopeptides component of **oligosaccharides-peptides complex** comprises glycans linked by *O*-glycosidic bonds to peptides. These glycans side chains, typical of mammalians glycans side chains in glycoproteins in general, comprise between 5 and 15 monomers of which the primary constituents are GalNAc, GlcNAc, Fuc, Gal, sialic acid along with minor amounts of Man, Glc and sulfate (b) (4); Brockhausen and Stanley, 2017). The glycan core structures of the glycopeptides components of **oligosaccharides-peptides complex** share many similarities with MMOs (Kunz *et al.*, 2000; Rostami *et al.*, 2014; Wrigglesworth *et al.*, 2020). These MMOs are important components of mother's milk serving as substrate for intestinal microbiota and supporting the development of the gastrointestinal (GI) system in neonates. In this respect, the glycopeptides component of **oligosaccharides-peptides complex** may be considered biologically similar to MMOs with the potential to play a nutritional role in supporting normal digestive function in cats and dogs of all life stages.

The identity and quantity of milk oligosaccharides (MOs) in mother's milk from different dog breeds was investigated by Rostami *et al*. (2014). Lactose, lactose-sulfate, 2 different trisaccharides composed of 3 hexose units, 3' sialyllactose (3'SL), 6'sialyllactose (6'SL), 2'fucosyllactose (2'FL), a tetrasaccharide composed of 2 hexoses, HexNAc and a deoxyhexose were identified in the milk samples. The oligosaccharide present in the highest amount was 3'SL, with levels starting at around 7.5 g/L and reducing to about 1.5 g/L over the first 10 days and 0.6 g/L for the remainder of the lactation period (40 days). In contrast, 6'SL levels reached between 0.3 and 0.6 g/L within the first few days of lactation and either reached a maximum at day 5 of lactation or remained constant thereafter. The levels of the different MOs varied between breeds, with for example, the tetrasaccharide only detected in the milk of Alaskan husky breeds and not in the Labrador retriever or Schnauzer breeds. Considering that MOs are highest early in the lactation period and reduce with time, and that the intake of milk will depend on the breed and size of the puppy, estimating exposure to these glycan components can be challenging. However, on a concentration basis only, assuming that the primary MOs represent at least 8 g/L of mother's milk in the first few days, the concentration in milk can be estimated to be in the region of 0.8% which is higher than the estimated intake of 0.3% glycopeptides from the maximum intended use level of **oligosaccharides-peptides complex** (1.5% by weight) in dog food for all life stages.

In another study by Wrigglesworth *et al*. (2020), the oligosaccharides in canine and feline milk were characterized. Three species, 3'SL, 6'SL and 2'FSL represented over 90% of the MOs content of canine milk samples, but a more diverse range of compounds were detected in feline milk, with up to 16 structures present at a relative abundance of >1% of the total. Difucosyllactose, 3'SL and lacto-*N*-neohexaose each represented greater than 10% of the MOs content of feline milk. The absolute values of the MOs in canine and feline milk were not reported by the authors and these data primarily provide evidence for the structural diversity of MOs for which the glycopeptides component of **oligosaccharides-peptides complex** can be considered a biological mimic from the perspective of its nutritional value.

The oligosaccharides in mother's milk of other feline species, the African lion and clouded leopard was also characterized by Senda *et al*. (2010). Similar to studies using other mammalian sources, the primary MOs identified included 2'FL, as well as a number of tetrasaccharides comprised of Gal, Glc, Fuc, GalNAc and sialic acid monomers.

Taken together, these data provide supporting evidence for the functionality of the glycopeptides component of **oligosaccharides-peptides complex** as a source of fermentable fiber in the diet of cats and dogs of all life stages at levels of 0.3% in the diet (equating to 1.5% of the ingredient).

2.7.5 *In Vitro* Fermentation Behavior of Oligosaccharides-Peptides Complex

A study was conducted to determine the fermentation potential of Gnubiotics' **oligosaccharides-peptides complex** in an *in vitro* system using healthy canine and feline fecal inocula (b) (4). (b) (6). The **oligosaccharides-peptides complex** (b) (4)

(b) (4) hus, the glycopeptides component will pass relatively intact to the lower digestive tract of cats and dogs where it is subject to fermentation by intestinal microflora (see Section 2.1.2). (b) (4) .

(b) (4)

(b) (4)

2.8 Critical Evaluation of the Utility of Oligosaccharides-Peptides Complex

Overall, **oligosaccharides-peptides complex** will provide a source of free amino acids (b) (4) peptides and glycopeptides (total (b) (4) in the diet of cats and dogs of all life stages. In practice, under the conditions of intended use, the ingredient provides a supplementary source of highly digestible **free amino acids** ((b) (4) and **peptides** (*ca*. 21%), as well as fermentable fiber in the form of **glycopeptides** (*ca*. 20%) but will not replace other nutrients in the diet. Analysis of the composition of **oligosaccharides-peptides complex** demonstrate that the ingredient will contribute to the amino acid content of cat and dog food but will not make a significant impact relative to the primary protein sources in the diet (e.g., meat and fish meal). A comparison of the mineral content of **oligosaccharides-peptides complex** with the nutrient requirements of cats and dogs under the conditions of intended use also indicate that the ingredient will make a significant contribution to sodium intakes by cats and dogs but will not pose a safety concern. The structural diversity displayed by the glycans side chains linked by *O*-glycosidic bonds to peptides in the glycopeptides component of oligosaccharide-peptides complex, shares many similarities to that of MMOs and by extrapolation, is expected to play a nutritional role in supporting digestive function in cats and dogs. The glycopeptides component was also found to be fermented in *in vitro* experiments using feline and canine microbiota.

Oligosaccharides-peptides complex provides a supplementary source of nutrients alongside, rather than as a substitute for, other protein and fermentable fiber sources in the diet. On this basis, there are no anticipated nutritional disadvantages associated with the intended use of **oligosaccharides-peptides complex** as a component in food for cats and dogs of all life stages at levels not to exceed 1.5% by weight. Moreover, beyond the compositional value as a supplementary source of amino acid, peptides and glycopeptides, the technical effect of **oligosaccharides-peptides complex** does not have any bearing on safety and no further evaluation of utility is warranted.

PART 3. §570.235 – TARGET ANIMAL AND HUMAN EXPOSURES

3.1 Intended Use and Use Levels in Cat and Dog Food

Oligosaccharides-peptides complex is intended for use as a source of highly digestible **free amino acids** and **peptides**, as well as a source of fermentable fiber in the form of **glycopeptides** in the diet of cats and dogs of all life stages at a level not to exceed 1.5% by weight. It can be incorporated as an ingredient in complete cat and dog food, or may be provided in the form of a supplementary food such as a top-dressing or treat.

The ingredient is comprised of glycopeptides and peptides ((b) (4) and free amino acids ((b) (4) obtained by physical processing of protein hydrolysate of porcine intestinal mucosa, which is a recognized feed material in the EU. The glycopeptides, peptides and amino acids components are derived from the proteolytic cleavage of the mucins (glycoproteins) of porcine intestinal mucosa.

Analytical data on 6 representative batches of **oligosaccharides-peptides complex** indicate that in practice, the glycopeptides and peptides, free amino acids and ash contents are around 42%, 40% and 13%, respectively (see Section 2.3.2). Further compositional analysis indicates that the glycopeptides and peptides components represent around 20% and 21%, respectively of **oligosaccharides-peptides complex**.

Glycopeptides are composed of glycans side chains displaying a diverse range of structures, which are linked to peptides by *O*-glycosidic bonds. As a consequence of their highly glycosylated nature, the peptide chains (prime constituents of the glycopeptides component) are less susceptible to hydrolysis by proteolytic enzymes. Thus glycopeptides are expected to remain largely intact during their passage through the GI tract (see Section 6.2).

3.2 Estimated Exposure by Cats and Dogs to Oligosaccharides-Peptides and Its Components

As mentioned above, **oligosaccharides-peptides complex** is intended for use as a source of free amino acids, peptides and glycopeptides in the diet of cats and dogs of all life stages at a level not to exceed 1.5% by weight. Exposure by animals will be primarily as a component of the complete feed but the ingredient may also be incorporated into treats. The estimated exposure by cats and dogs to the components of **oligosaccharides-peptides complex** under the conditions of intended use are summarized in Table 3.1.

Table 3.1: Exposure by Cats and Dogs to the Components of Oligosaccharides-Peptides Complex		
Component	**Content in Oligosaccharides-Peptides Complex (%)**	**Content in the Diet of Adult Cats and Dogs at the Maximum Use Level of 1.5% by Weight (%)**
Free amino acids	(b) (4)	0.53 to 0.68[3] (*ca.* 0.6)[4]
Glycopeptides and peptides		0.56 to 0.71[3] (*ca.* 0.6)[4]
Peptides[5]	*ca. 21%*	*ca. 0.3*
Glycopeptides[5]	*ca. 20%*	*ca. 0.3*
Ash	Max. (b) (4)	0.26[3] (*ca.* 0.2)[4]
		(b) (4)

[3]Calculation: specification (range or maximum) x 0.015 (%);
[4]Calculation: mean value x 0.015 (%);
[5]Individual peptides and glycopeptides contents determined by compositional analysis (see Sections 2.4.4 and 2.4.5).

The body weight (bw) and feed intake (FI) of cats and dogs can vary significantly between breeds, however, a cat weighing 4.6 kg and consuming 0.06 kg DM complete food daily containing 1.5% **oligosaccharides-peptides complex**, will be exposed to 0.9 g/day of the ingredient, equivalent to 0.2 g/kg bw/day. Similarly, a medium-sized dog weighing 7.7 kg and consuming 0.160 kg/DM complete food daily, will be exposed to 2.3 g/day of **oligosaccharides-peptides complex** or 0.3 g/kg bw/day.

3.3 Estimated Exposure by Humans to Oligosaccharides-Peptides Complex

Oligosaccharides-peptides complex is intended only for use in cat and dog food, and there will be no exposure by humans.

PART 4. §570.240. SELF-LIMITING LEVELS OF USE

The use of **oligosaccharides-peptides complex** is self-limiting based on its nutritional role as a supplementary fermentable fiber source in cat and dog food. Increasing the levels beyond those required to achieve the desired effect will result in detrimental physiological effects due to the high fiber content of the diet and presence of indigestible bulk matter in the lower intestine.

PART 5. §570.245. EXPERIENCE BASED ON COMMON USE IN FOOD BEFORE 1958

Not applicable.

PART 6. §570.250. NARRATIVE

6.1 Introduction

Oligosaccharides-peptides complex is intended for use as a source of highly digestible free amino acids and peptides, as well as a source of fermentable fiber in the form of glycopeptides in the diet of cats and dogs of all life stages at a level not to exceed 1.5% by weight. The primary application of **oligosaccharides-peptides complex** will be as an ingredient in complete cat and dog food, but the ingredient may also be incorporated into supplementary foods such as top-dressing sachets and treats.

The ingredient is comprised of glycopeptides and peptides (b) (4) and free amino acids (b) (4), obtained by physical processing of protein hydrolysate of porcine intestinal mucosa, which is a recognized feed material in the EU. The glycopeptides, peptides and amino acids components are derived from the proteolytic cleavage of the mucins (glycoproteins) of porcine intestinal mucosa.

Analytical data on 6 representative batches of **oligosaccharides-peptides complex** indicate that in practice, the **glycopeptides and peptides**, **free amino acids** and **ash** contents are around 42%, 40% and 13%, respectively (see Section 2.3.2). Further compositional analysis indicates that the glycopeptides and peptides components represent around 20% and 21%, respectively of **oligosaccharides-peptides complex** (see Sections 2.4.4 and 2.4.5). Greater than 99% of the species comprising **oligosaccharides-peptides complex** have a molecular weight below (b) (4).

Glycopeptides are composed of glycans side chains displaying a diverse range of structures, which are linked to peptides by *O*-glycosidic bonds. As a consequence of their highly glycosylated nature, the peptide chains (prime constituents of the glycopeptides component) are less susceptible to hydrolysis by proteolytic enzymes.

The safety of **oligosaccharides-peptides complex** is primarily based on (a) the known metabolic fate of free amino acids, glycopeptides and peptides by cats and dogs (Section 6.2); (b) published feeding studies in adult cats and dogs using Gnubiotics' **oligosaccharides-peptides complex** (Section 6.3) and (c) a basic battery of *in vitro* tests to evaluate the genotoxic potential (Section 6.4). Taken together these data can be considered pivotal to the safety determination.

Protein hydrolysate of porcine intestinal mucosa, the raw material used by Gnubiotics for the manufacture of **oligosaccharides-peptides complex**, also has an established history of use as a protein source in animal feed in the EU and Canada (see Section 2.1.3). A number of published studies in which animals were fed diets containing protein hydrolysate of porcine intestinal mucosa were identified in the published literature which provide supporting evidence for the safety of the further purified ingredient, **oligosaccharides-peptides complex** for cats and dogs (Section 6.5). Furthermore, MMOs are structurally similar to the glycans side chains of the glycopeptides component of **oligosaccharides-peptides complex**. Thus, the background consumption of these oligosaccharides by young cats and dogs provides supporting evidence of the safety the glycopeptides component of oligosaccharides-peptides for all life stages of animal (Section 6.6).

Corroborative evidence for safety is also provided by a battery of toxicity studies on individual oligosaccharide ingredients developed as structural mimics of MMOs and which are also structurally

similar to the glycans side chains of the glycopeptides component of **oligosaccharides-peptides complex** (Section 6.7).

6.2 Absorption, Distribution, Metabolism, Excretion (ADME) of the Components of Oligosaccharides-Peptides Complex

The ADME of each of the components of **oligosaccharides-peptides complex**, i.e., free amino acids, peptides and glycopeptides is considered in turn below.

6.2.1 ADME of the Free Amino Acids and Peptides Components

(b) (4) The full amino acid profile of the peptides and free amino acids components of the ingredient are described above in Sections 2.4.3 and 2.4.4.

An overview of general amino acid and peptide digestion and absorption in cats and dogs is provided below as described by Webb (1990), Yen (2004), Gitler (1964), Matthews (1975), and Gilbert *et al*. (2008). Following ingestion, the larger peptides are cleaved into smaller peptides by the enzyme pepsin, which is activated by the increase in stomach acidity that occurs during feeding. The peptides formed by gastric digestion are further hydrolyzed on the mucosal surface of the small intestine, by pancreatic enzymes, such as trypsin, chymotrypsin, and carboxypeptidases. This luminal digestion produces free amino acids, as well as small peptides and oligopeptides of more than 3 amino acids. At the intestinal mucosal membrane, further hydrolysis of oligopeptides occurs via an array of brush border peptidases, which break down oligopeptides into free amino acids and di- and tri-peptides.

The resultant mixture of free amino acids and small peptides is then transported into the mucosal cells by a number of specific carrier systems for individual amino acids and di- and tri-peptides (Gilbert *et al*., 2008). Individual amino acids are absorbed via sodium-dependent and independent amino acid transporters, whereas short peptides are absorbed though a proton coupled peptide transporter (PEPT1; Cho *et al*., 2013). Once absorbed, peptides may be hydrolyzed by epithelial intracellular peptidases or, if resistant, released intact across the basolateral membrane into the circulation. Absorbed amino acids pass into the liver, where a portion of the amino acids are taken up and used either for catabolic reactions to yield energy or for protein synthesis. The remainder pass through into the systemic circulation and are utilized by the peripheral tissues, especially muscle, which is the predominant site for metabolism of branched chain amino acids (valine, leucine and isoleucine). Active amino acid transport is prominent in the small intestine, but there is no evidence of active amino acid absorption in the colon except during the early neonatal period (Washabau, 2013).

In strict carnivores such as cats, a high rate of amino acid catabolism allows for a readily available source of energy, and as such, amino acids are continuously processed to yield glucose *via* hepatic gluconeogenic pathway (Rochus *et al*., 2013). Catabolism of amino acids occurs mainly in the liver and kidneys, but also in muscle tissue, in a two-stage process. In the first stage the α-amino group of an amino acid is enzymatically removed, yielding the corresponding α-keto acid analogue of the amino acid and ammonia. The resulting ammonia is converted to urea and transported to the kidney v*ia* the blood for excretion. In the second stage, the α-keto acid is degraded to an intermediate of the tricarboxylic acid pathway which results in the formation of energy in the form of adenosine triphosphate (ATP) and carbon dioxide (Moon, 1988). Muscle is the primary site for the metabolism of valine, leucine and isoleucine. Proteolysis in the muscle causes an increase in amino groups, the accumulation of which would be toxic due to an increase in ammonia; however, muscle tissue lacks the enzymes to convert ammonia to urea and instead, ammonia and glucose are metabolized to form alanine. Alanine is then released into the general circulation and taken up by the liver, where upon transamination, it can be converted to urea and the carbon skeleton of alanine can be used to generate glucose. Glucose can be released into the blood stream and taken up by the muscle tissue to form alanine, in the glucose-alanine cycle (Felig, 1973).

Cats, being true carnivores, have a higher dietary protein requirement compared to dogs and other omnivores. The reason for higher protein requirement of the cat has been attributed to the inability to regulate amino acids metabolizing enzymes in the liver. When most animals, including dogs, ingest a high protein diet, the activities of amino acid catabolizing enzymes in the liver increase to metabolize the increase ammonia generated upon catabolism of amino acids. In a study by Rogers *et al*. (1977), the activity of several catabolic enzymes of amino acid metabolism in adult cats fed either a high- or low-protein diet or fasted for five days was assessed. The results showed few changes in the hepatic enzyme activities between the three groups of cats, with hepatic enzyme activities remaining at high levels to reflect the high protein diet, even in fasted animals. The inability of cats to regulate the aminotransferases and urea cycle enzymes provides a safeguard against ammonia toxicity after ingestion of a high protein meal. Cats have specific dietary requirements for taurine and arginine, which is a consequence of low activities of two enzymes in the synthetic pathways of these two amino acids that have a negative multiplicative effect on the rate of synthesis (Morris, 2001; Verbrugghe *et al*., 2012).

6.2.2 ADME of the Glycopeptides Component

Glycopeptides comprise glycans linked by *O*-glycosidic bonds to peptides. These glycans side chains of the glycopeptides component (prime constituents) exist as a diverse range of structures in which GalNAc is the most common modifying sugar linking the glycans to the peptide chain. (b) (4) identified in Section 2.4.4. As a consequence of their highly glycosylated nature, the peptide chains (prime constituents) of glycopeptides are less susceptible to hydrolysis by proteolytic enzymes and the glycopeptides (b) (4); (b) (4); (b) (4)). These findings support that the glycopeptides component of **oligosaccharides-peptides complex** will be resistant to digestion and free to move to the

large intestine where they can act as a substrate for microbial fermentation (Pinna and Biagi, 2014; Oba *et al*., 2020a).

In mammals, endogenous mucins are generally hypothesized to serve as a major substrates for colonic microbial populations and to play a critical role in gut health (e.g., Yamada *et al*., 2019; Hino *et al*., 2020). As mentioned in Section 2.7.4, structurally similar *O*-glycans are also a component of mammalian milk supporting the development of the GI tract in infants and young animals (Pruss *et al*., 2020; Wrigglesworth *et al*., 2020).

Hino *et al*. (2020) conducted a series of 4 experiments in rats in order to evaluate the ability of mucin derived *O*-glycans to function as endogenous fiber and support large bowel SCFA production.

In an initial study, the amount of mucins entering the cecum was assessed using ileorectostomized rats in which the terminal ileum was anastomosed to the rectum in order to directly collect ileum effluent as feces. Wistar rats (n=12; mean bw 162 ± 0.713 g) were allowed free access to a control diet and water for 14 days. Feces were collected over the last 5 days of the experiment and analyzed for mucin and *O*-linked glycans content. The mean daily excretion of feces and mucin were 1.3 ± 0.037 g and 0.15 ± 0.0058 g, respectively. The *O*-glycan content was 286 ± 0.440 µmol/g mucin, indicating that 43.2 µmol of *O*-glycans entered the cecum daily.

In a second experiment, Wistar rats (n=6/group; mean bw 183 ± 1.41 g) were allowed free access to a control diet containing 0, 6 or 12 g/kg partially purified porcine stomach mucin (PM; classified as Type II and containing 1% sialic acid) for 14 days. PM supplementation was achieved by partially replacing an equal amount of corn starch in the diet. Fresh feces were collected for the last 3 days of the experiment and the mucinase activity measured. At the end of the experiment, the rats were anesthetized and decapitated, and the cecum collected. The cecum weight was determined and the contents were analyzed for SCFAs, mucin content, number of total bacteria and bacterial 16S ribosomal RNA (rRNA) genes. FI and cecum contents did not differ between animals fed diets containing PM or the control. PM consumption was estimated to be 0, 100 or 200 mg/day in the different treatment groups, equivalent to a PM-derived *O*-glycans intake of 0, 68 or 136 µmol/day. Cecal concentrations of acetate (+37%) and butyrate (+73%) were higher in rats fed the diets containing 12 g PM/kg relative to those fed the control or diet containing 6 g PM/kg. Likewise, propionate production was higher in rats fed 12 g PM/day compared to those fed 6 g/day. The mucin and *O*-glycans contents of the cecal contents did not differ among dietary treatments groups. Crypt length in the cecal tissue also did not differ between the groups, but the number of goblet cells was higher in rats fed the diet containing 12 g PM/kg than in those fed the control diet. The number of total bacteria was higher in rats fed diets containing 12 g PM/kg relative to the control or diets containing 6 g PM/kg. Feeding PM-containing diets resulted in an increase in the number of mucin-degrading bacteria identified in the cecum.

In another experiment, Wistar rats (n=6/group; mean bw 143 ± 1.19 g) were allowed free access to a control diet containing 0, or 10 g of GlcNAc, Fuc or sialic acid/kg for 14 days. Supplementation of *O*-glycan monosaccharide was performed by replacing an equal amount of cornstarch in the control diet. At the end of the experiment the rats were anaesthetized and decapitated in order to evaluate the weight and contents of the cecum. Cecum contents were analyzed for SCFAs, mucin content, number of total bacteria and bacterial 16S ribosomal RNA (rRNA) genes. FI was higher in rats fed the Fuc-

containing diet than those fed the other diets but bw was not observed to differ among treatment groups. Among the groups of rats fed diets containing *O*-linked glycan monosaccharides, only those fed GlcNAc-containing diets resulted in the generation of higher butyrate concentrations (+68%) and increased numbers of butyrate-producing bacteria compared to the group fed the control diet ($P<0.05$). Acetate (+30%) and Propionate (+30%) were also increased in animals receiving the GlcNAc diet compared to the control diet ($P<0.05$). There was an increase in the total number of bacteria in the cecum of rats fed the GlcNAc or sialic acid-containing diets relative to those fed the control diet.

In a final experiment, Wistar rats (n=6/group; mean bw 73 ± 0.74 g) were allowed free access to a control diet containing 0, 10 g of GlcNAc, or 15 g of purified PM/kg for 29 days. Supplementation of GlcNAc or PM was performed by replacing an equal amount of cornstarch in the diet. At the end of the experiment, the rats were anaesthetized and decapitated in order to measure cecum weight and contents. The cecum was analyzed for pH, organic acid contents, and immunoglobulin A (IgA) secreting plasma cells (IgA+) and regulatory T cells. Total RNA was isolated and analyzed for inflammatory cytokine gene expression. The cecal expression of inflammatory cytokines was reduced in rats fed diets containing GlcNAc and PM relative to the control group, with a significant decrease ($P<0.05$) in the expression of Tumor Necrosis Factor-a (−30%, −40%) and Interferon Gamma (−30%, −70%) observed. There were no differences in the expression of tight junction–related genes among the dietary groups. The populations of both IgA+ plasma cells and regulatory T cells were higher in rats fed the GlcNAc-containing diet than in those fed the control diet, whereas in rats fed the PM-containing diet, only the IgA+ plasma cell population was increased relative to those fed the control diet.

Collectively, the authors concluded that the observations from the series of experiments support the hypothesis that mucin-derived *O*-glycans function as endogenous fiber that facilitates symbiosis between the host and microbiota, via enhanced large-bowel SCFA-production. The effect can be largely attributed to the *O*-glycans monosaccharides component.

Although information on the absorption of porcine mucin glycans is limited to the information summarized above, there is a body of *in vitro* data pertaining to the absorption and metabolism of components of human milk oligosaccharides (HMOs), the findings of which can be extended to the digestion of *O*-glycans derived from porcine mucin glycans. Engfer *et al*. (2000), assessed the extent to which HMOs fractions are hydrolyzed in the GI tract using an *in vitro* digestion study with enzyme preparations of human and porcine pancreas and intestinal brush border membranes (BBMs). Preparations of human origin as well as from piglets and full-grown pigs were used to mimic the conditions of both the mature and immature GI tract. Purified individual oligosaccharides found in HMOs were used in the studies, specifically 2'FL, 3'FL as well as a mixture of lacto-*N*-tetraose and lacto-*N*-neotetraose, a mixture of lacto-*N*-fucopentaose I, II, III, and V, and lacto-*N*-difucohexaose I and II, all of which may be considered fractions of HMOs, were used as the test articles in the experiments along with maltodextrin as a control oligosaccharide. The HMOs fractions were digested for up to 20 hours using human pancreatic juice and also BBMs prepared from human or porcine intestinal tissue samples, and the concentrations of starting oligosaccharides and digestion products (Glu, Fuc and sialic acid) measured at regular intervals. Whereas the control oligosaccharide, maltodextrin was rapidly and completely hydrolyzed under the study conditions, the HMOs fractions were resistant to pancreatic juice and BBM hydrolases. The HMOs fractions were recovered intact after 20 hours and no hydrolysis

products were detected. Enzymatic determination of monosaccharides in the *in vitro* digests were performed in conjunction with mass spectrometry techniques to confirm that no terminal Fuc or core Glu units were released from the HMOs fractions. As a positive control for determining hydrolysis of the apparently non-digestible HMOs fractions, porcine pancreatic tissue homogenate containing the zymogens and intracellular, including lysosomal, enzymes were used along with the BBM preparations as an intestinal enzyme source. HMOs fractions were found to be rapidly digested with this pancreatic preparation with no intact oligosaccharides identified after 4 hours.

In another *in vitro* study by Gnoth *et al*. (2000), HMOs were isolated from human milk samples and separated into neutral or acidic compounds. The individual HMOs fractions were incubated with human salivary amylase, porcine pancreatic amylase, and brush border membrane vesicles (BBMV) isolated from porcine small intestine for up to 2 hours. The concentrations of starting HMOs fractions and their degradation products were analyzed after *in vitro* digestion. After a 2-hour incubation with BBMV, slight modifications of the HMO observed. Analysis revealed two new components within the neutral oligosaccharide fractions which were characterized as lacto-N-triose and galactose. The BBMVs were prepared without lysosomal fractions, the absence of which was confirmed with a lysosomal marker enzyme phosphatase. Therefore, the small changes observed after the digestion of HMO with BBMV, were not due to lysosomal enzymes and were due to minimal (<5%) digestion of the HMOs fractions.

Taken together, the results of the two *in vitro* studies using HMOs fractions support the conclusions that HMOs are not hydrolyzed to any significant extent by enzymes in the upper GI tract and are not expected to be absorbed into systemic circulation. The substantive majority of these HMOs will reach the large intestine and act as substrates for bacterial metabolism. By extrapolation, these findings support the findings of the studies in rats in which the structurally related *O*-glycans fraction of porcine stomach mucosa were also found to be resistant to digestion in the GI tract.

6.2.3 Overall Conclusions on the ADME of Oligosaccharides-Peptides Complex

The available ADME data support the conclusion that **oligosaccharides-peptides complex** will provide a highly digestible source of free amino acids (*ca*. 40%) and peptides (*ca*. 21%), as well as fermentable fiber in the form of glycopeptides (*ca*. 20%). The glycans side chains linked via *O*-glycosidic bonds to peptides in the glycopeptides component are structurally similar to the glycans identified in MMOs. The *O*-glycans side chains found in porcine-derived intestinal mucins as well as HMOs have been shown in *in vivo* and *in vitro* experiments, respectively to be resistant to digestion.

6.3 Studies in Adult Cats and Dogs using Oligosaccharides-Peptides Complex

The palatability, safety, and GI tolerance of Gnubiotics' **oligosaccharides-peptides complex** was evaluated in healthy adult cats and dogs. Preliminary palatability studies were conducted to confirm the acceptability of **oligosaccharides-peptides complex** to cats and dogs prior to conducting 26-week dietary feeding studies. Each of the studies is described in turn below. These studies have been published ((b) (4)) and are considered pivotal to the safety determination of **oligosaccharides-peptides complex** for the target animal. (b) (4)

Traditionally, the safety of additives which will be present in food at low levels, is evaluated by conducting a study in the target animal using graded dietary amounts of the test article, typically 0 (control), 1x (use-level), 3x and 5x (multi-fold use levels), in order to establish a margin of safety relative to the intended use level. However, the amount of a macronutrient which can be incorporated into the diet is limited before nutritional balances occur resulting in secondary consequences such as physiological effects (Borzelleca *et al.*, 1996; Munro *et al.*, 1996; EFSA, 2011b). Thus, when evaluating the tolerability of cats and dogs to **oligosaccharides-peptides complex**, the animals were fed diets containing graded levels of **oligosaccharides-peptides complex** up to the maximum intended use level of 1.5% by weight in the diet only.

6.3.1 Palatability Study in Adult Cats [Oba *et al.*, 2020b; (b) (4)

In a 2-day palatability trial, 20 adult domestic short-hair cats were housed individually and presented with test diets coded as diet A or diet B. The diets were prepared in an identical manner in which a mixed fat source (5% by weight) was sprayed onto the base food and then was, or was not, coated with 1% of Gnubiotics' **oligosaccharides-peptides complex**. Two (2) bowls, each containing 110 g of diet were offered once daily for the 2 days. Bowl placement was reversed daily to prevent left to right bias. Food consumption and first choice preference were recorded for each cat. A 17.58:1 total consumption ratio was observed for the 1% **oligosaccharides-peptides complex** diet *vs*. the control diet, demonstrating a strong preference ($P<0.05$) for this diet. According to a Wilcoxon signed rank test, the consumption difference between the 0 and 1% **oligosaccharides-peptides complex**-containing diets was significant ($P<0.0001$). Using data from both days, the 1% **oligosaccharides-peptides complex**-containing diet was approached first on 28/40 occasions and consumed first on 38/40 occasions.

6.3.2 26-Week Feeding Study in Adult Cats [Oba *et al.*, 2020b; (b) (4)

A study was conducted in adult cats to evaluate the safety and GI tolerance of Gnubiotics' **oligosaccharides-peptides complex** over a 26-week feeding period (Oba *et al.*, 2020b). Following a 2-week baseline period, 32 adult domestic short-haired cats (mean age of 1.9 ± 0.05 years; 12 males and 20 females; median initial weight of 4.6 ± 0.13 kg), were assigned into 1 of 4 dietary treatments based on bw and sex (8 cats/group; 3 male and 5 female). Cats were housed individually in cages during feeding times (8 to 10 am daily) and the fecal collection phases, in a temperature-, humidity-, and light-controlled room. At other times, cats were group-housed and able to socialize and exercise outside of their cages. Fresh water was available *ad libitum*. The animals were fed dry extruded treatment diets containing 0 (control), 0.5, 1.0 or 1.5% **oligosaccharides-peptides complex** for a period of 26 weeks. The diets contained approximately 35% protein, 15% fat, 7% ash, and 5% fiber, and were formulated to meet all AAFCO (2019) nutrient recommendations for adult cats. The ingredient and nutritional composition of the treatment diets are presented in Table 6.1.

Table 6.1: Composition of the Treatment Diets				
	Composition (%, as-is)			
	Diet 1 (Control; 0%)	Diet 2 (0.5%)	Diet 3 (1.0%)	Diet 4 (1.5%)
Ingredient				
Poultry by-product meal (low ash)	43.3	43.3	43.3	43.3
Brewer's rice (US #2)	39.8	39.3	38.8	38.3
Poultry fat	8.65	8.65	8.65	8.65
Corn, whole (US #2)	4.81	4.81	4.81	4.81
Palatant	1.92	1.92	1.92	1.92
Oligosaccharides-peptides complex	0.00	0.50	1.00	1.50
Salt (sodium chloride)	0.48	0.48	0.48	0.48
Potassium chloride, 50% K	0.43	0.43	0.43	0.43
Taurine	0.19	0.19	0.19	0.19
Mineral premixture[1]	0.18	0.18	0.18	0.18
Vitamin premixture[2]	0.18	0.18	0.18	0.18
Choline chloride	0.13	0.13	0.13	0.13
Nutritional Composition				
Dry matter (%)	93.7	92.6	93.1	93.4
Organic matter (% DM)	93.3	93.2	93.3	93.2
Ash (% DM)	6.69	6.77	6.73	6.74
Crude protein (% DM)	37.2	37.0	35.9	36.2
Acid-hydrolyzed fat (% DM)	17.6	17.3	15.7	16.8
Gross energy (kcal/g)	5.25	5.22	5.13	5.19

Abbreviations: DM = dry matter;

[1]Provided per kg diet: Mn (as MnSO4), 66.00 mg; Fe (as $FeSO_4$), 120 mg; Cu (as $CuSO_4$), 18.00 mg; Co (as $CoSO_4$), 1.20 mg; Zn (as $ZnSO_4$), 240 mg; I (as KI), 1.80 mg; Se (as Na_2SeO_3), 0.24 mg;

[2]Provided per kg diet: vitamin A, 5.28 mg; vitamin D3, 0.04 mg; vitamin E, 120.00 mg; vitamin K, 0.88 mg; thiamin, 4.40 mg; riboflavin, 5.72 mg; pantothenic acid, 22.00 mg; niacin, 39.60 mg; pyridoxine, 3.52 mg; biotin, 0.13 mg; folic acid, 0.44 mg; vitamin B12, 0.11 mg.

All cats were fed to maintain bw throughout the entire study and adjustments were made weekly if needed. A complete physical examination by a licensed veterinarian was conducted just prior to the start of the study and at study completion. Daily general health observations were noted throughout the study. Other variables associated with GI tolerance, such as emesis, poor physical appearance, and abnormal behavior were monitored and recorded. Food offered and refusals were measured daily to calculate intake. Skin and coat scores were evaluated prior to study initiation, then every 4 weeks and upon study completion. In weeks 2, 4, and 26, fresh fecal samples were collected for measurement of stool quality and immune and inflammatory markers, and in weeks 2 and 4 for microbiota and metabolites. Additionally, on weeks 0, 2, 4, 8, 12, 24 and 26, fresh fecal samples were collected for measurement of fecal pH and fecal scores. In week 4, a 5-day total fecal collection was conducted for the measurement of apparent total tract macronutrient digestibility. Blood samples were collected in weeks 0, 2, 4 and 26 for serum chemistry, taurine, hematology, and inflammatory marker analysis.

The treatment diets contained similar nutrient concentrations as expected and were highly digestible. The apparent total tract macronutrient digestibility of the treatment diets is summarized in Table 6.2.

The inclusion of graded levels of **oligosaccharides-peptides complex** did not affect the apparent total tract digestibilities of the treatment diets (P>0.05).

Table 6.2: Apparent Total Tract Digestibility of the Treatment Diets							
Parameter	**Unit**	**Digestibility**				**SEM**	***P*-Value**
		Diet 1 (Control; 0%)	**Diet 2 (0.5%)**	**Diet 3 (1.0%)**	**Diet 4 (1.5%)**		
DM	%	85.3	85.9	85.5	87.8	1.21	0.47
Organic matter	%	89.1	89.9	89.1	91.1	0.92	0.39
Acid-hydrolyzed fat	%	94.4	94.5	93.9	95.2	0.58	0.48
Gross energy	%	89.6	90.3	89.3	91.5	0.89	0.35
Crude protein	%	84.4	85.2	84.1	87.1	1.36	0.40

Abbreviations: SEM = standard error of the mean.
Digestibility data was measured from total feces collected over 5-consecutive days during week 4.

All cats remained healthy, had a good appetite and did not show any signs of GI intolerance throughout the study. The performance of the cats over the 26-week period is summarized in Table 6.3. There were no differences in FI, bw or body condition score (BCS) among the treatment groups (P>0.05). The hair of the cats remained in a state described as a medium level of reflectivity and softness for the duration of the study.

Table 6.3: Performance of Cats Provided Diets Supplemented with Oligosaccharides-Peptides Complex							
Parameter	**Unit**	**Treatment Diets**				**SEM**	***P*-Value**
		Diet 1 (Control; 0%)	**Diet 2 (0.5%)**	**Diet 3 (1.0%)**	**Diet 4 (1.5%)**		
Body weight	kg	5.0	4.4	4.4	4.6	0.25	0.38
Body condition score	-	7.1	6.1	6.5	6.6	6.6	0.39
Feed intake	g/day as-is	61.6	56.4	58.7	55.6	2.76	0.44
Feed intake ratio	(g/day)/bw	12.6	13.0	13.4	12.2	0.46	0.34
Calorific intake ratio (kg) ratio	(kcal/day)/bw	61.6	62.5	63.3	59.0	2.36	0.61

Abbreviations: bw = body weight; SEM = standard error of the mean.

Fecal scores, pH, and dry matter percentage and concentrations of IgA and calprotectin did not significantly differ among treatments. For most fecal metabolites (phenol, acetate, propionate, isobutyrate, isovalerate, valerate, and ammonia), there were no significant differences between treatments. However, cats fed diets containing 1.5% **oligosaccharides-peptides complex** displayed higher (P=0.02) fecal butyrate concentrations compared to those fed diets containing 0.5 or 1.0% **oligosaccharides-peptides complex.** These results are similar to data derived from a recent *in vitro* study using feline fecal inocula, whereby **oligosaccharides-peptides complex** increased the production of propionate and butyrate compared to the control diets (Oba *et al.*, 2020a). Also, cats fed diets

containing 1.5% **oligosaccharides-peptides complex** exhibited lower (*P*=0.02) fecal indole concentrations than those fed 0.5% **oligosaccharides-peptides complex**.

The composition of fecal microbiota was also assessed at weeks 2 and 4. Fecal samples were processed using high-throughput 16S rRNA gene PCR amplicons to determine the relative abundance of species. Further statistical analysis, including principal component analysis (PCA) and alpha-diversity measures were conducted to assess the differences among microbial profiles of the four groups. Alpha-diversity measures indicated that **oligosaccharides-peptides complex** did not affect species richness, Shannon diversity index or evenness.

The number of bacterial phyla and bacterial genera were determined as a mean % of total sequences in feces of cats fed the treatment diets. Similarities were observed among treatment groups at the phylum level, however, cats fed the control diet had a higher (*P*=0.003) relative abundance of Actinobacteria than cats fed 0.5% **oligosaccharides-peptides complex**. The relative abundance at the genus level was similar between treatments, except for *Peptococcus* and *Campylobacter*. The relative abundance of *Peptococcus* was higher (*P*<0.01) in cats fed diets containing 1.0% **oligosaccharides-peptides complex** than those fed the control at week 2 but this observation was not present at week 4. At week 4, *Campylobacter* was significantly lower (*P*<0.05) in fecal samples of cats fed 1.5% **oligosaccharides-peptides complex** than in other treatments. Overall, the composition of the microbial community was minimally changed on inclusion of **oligosaccharides-peptides complex** at levels of up to 1.5% in the diet and the microbial diversity was equivalent to animals fed the control diets.

There was no effect of treatment on serum chemistry, taurine, hematology inflammatory cytokines [TNF and IL-6], immunoglobulin E (IgE) and C-reactive protein (CRP) concentrations at weeks 0, 2, 4, 8, 12, 24 and 26. A few of the hematological measurements fell outside reference ranges for the cats, however, these differences were minimal in magnitude and were not treatment-level dependent. Thus, the investigators considered these differences not to be biologically significant or related to treatment.

Intestinal immunity (fecal IgA), inflammation (serum CRP, IL-6, and TNFα, fecal calprotectin), and allergy (IgE) markers were not affected by dietary treatment. The study authors also recognize that there are currently no immunological assays validated for cats. The values obtained from the assays were consistent with those from other studies in cats conducted at the same facility and to be indicative of the absence of any adverse effects of oligosaccharide-peptides complex on immune function. Taken together, the findings of the study indicate that **oligosaccharides-peptides complex** is well-tolerated by adult cats when fed at levels of up to 1.5% in the diet for a period of 26 weeks.

6.3.3 Palatability Study in Adult Dogs [(b) (4) *et al.*, 2021; (b) (4)

In a 2-day palatability trial, 20 beagle dogs were presented with two test diets. The diets were prepared in an identical manner in which a mixed fat source (5% by weight) was sprayed onto the base food and then was, or was not, coated with 1% of Gnubiotics' **oligosaccharides-peptides complex**. Dogs were offered two bowls per day, each containing 400 g of test diets. Food bowls were presented for 30 minutes each day, and to prevent left-right bias, bowl position was reversed on the second day of the test. Total daily food consumption and first choice preference were reported for each dog. A 3.6:1 total consumption ratio was observed for the 1% **oligosaccharides-peptides complex** diet compared with the

control (0%) diet, demonstrating a strong preference ($P<0.05$) for this diet. The data collected from both days indicate that the 1% **oligosaccharides-peptides complex** diet was consumed first on 31/40 occasions, compared to 9/40 for control diet.

6.3.4 26-Week Feeding Study in Adult Dogs [(b) (4)] *et al.*, 2021; (b) (4)

A study was conducted in adult dogs to evaluate the safety and GI tolerance of Gnubiotics' **oligosaccharides-peptides complex** over a 26-week feeding period (Lee *et al.*, 2021). Following a 2-week baseline period, 32 beagle dogs (1 to 8 years old; 5 males and 27 females; median initial weight of 7.6 ± 2.31 kg) were randomly assigned into 1 of 4 dietary treatments (8 dogs/group). Each dog was housed individually with *ad libitum* access to fresh water. In accordance with standard operating procedures and USDA welfare requirements, the dogs received regular exercise and socialization. The dogs were fed extruded diets containing 0 (control), 0.5, 1.0 or 1.5% of Gnubiotics' **oligosaccharides-peptides complex** for the 26-week study period. The diets were formulated to meet AAFCO (2019) nutrient recommendations for adult dogs at maintenance. The ingredient and nutritional composition of the treatment diets are summarized in Table 6.4.

Table 6.4: Composition of the Treatment Diets

	Composition (%, as-is)			
	Diet 1 (Control; 0%)	**Diet 2 (0.5%)**	**Diet 3 (1.0%)**	**Diet 4 (1.5%)**
Ingredient				
Poultry by-product meal (low ash)	43.27	43.27	43.27	43.27
Brewer's rice (US #2)	39.77	39.27	38.77	38.27
Poultry fat	8.65	8.65	8.65	8.65
Corn, whole (US #2)	4.81	4.81	4.81	4.81
Palatant	1.92	1.92	1.92	1.92
Oligosaccharides-peptides complex	0.00	0.50	1.00	1.50
Salt (sodium chloride)	0.48	0.48	0.48	0.48
Potassium chloride, 50% K	0.43	0.43	0.43	0.43
Taurine	0.19	0.19	0.19	0.19
Mineral premixture[1]	0.18	0.18	0.18	0.18
Vitamin premixture[2]	0.18	0.18	0.18	0.18
Choline chloride	0.13	0.13	0.13	0.13
Nutritional Composition				
Dry matter (%)	93.7	92.6	93.1	93.4
Organic matter (% DM)	93.3	93.2	93.3	93.2
Crude protein (% DM)	37.2	37.0	35.9	36.2
Acid-hydrolyzed fat (% DM)	17.6	17.3	15.7	16.8
Total dietary fiber (% DM)	9.3	8.5	8.1	8.6
Gross energy (kcal/g)	5.25	5.22	5.1	5.19

Abbreviations: DM = dry matter;

[1]Provided per kg diet: Mn (as MnSO4), 66.00 mg; Fe (as $FeSO_4$), 120 mg; Cu (as $CuSO_4$), 18.00 mg; Co (as $CoSO_4$), 1.20 mg; Zn (as $ZnSO_4$), 240 mg; I (as KI), 1.80 mg; Se (as Na_2SeO_3), 0.24 mg;

[2]Provided per kg diet: vitamin A, 5.28 mg; vitamin D3, 0.04 mg; vitamin E, 120.00 mg; vitamin K, 0.88 mg; thiamin, 4.40 mg; riboflavin, 5.72 mg; pantothenic acid, 22.00 mg; niacin, 39.60 mg; pyridoxine, 3.52 mg; biotin, 0.13 mg; folic acid, 0.44 mg; vitamin B12, 0.11 mg.

Dogs were fed once per day to maintain bw throughout the study. Daily food consumption was recorded to monitor intake, with adjustments made weekly if needed. A complete physical examination was conducted on all dogs by a licensed veterinarian at study initiation and at study completion. Coat and skin scores were evaluated prior to study initiation, every 4 weeks and at study completion.

Fresh fecal samples were collected in weeks 2 and 4 for fecal microbiota composition and fermentation metabolites (SCFAs, BCFAs, phenols, indoles and ammonia). In weeks 2, 4, and 26, fresh fecal samples were also collected for the measurement of fecal scores, pH, IgA and calprotectin. In week 4, in addition to the fresh fecal samples collected, total fecal samples were collected from each dog over a 5-day consecutive period to determine apparent total tract macronutrient digestibility.

Blood samples were collected at weeks 0, 2, 4, and 26 to measure serum chemistry, hematology, and markers of inflammation in the serum, which included CRP and inflammatory cytokines, interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α). Additionally, IgE was determined in the serum as a marker for allergic response.

Of the 32 dogs initially enrolled in the study, 1 of the dogs fed the diet containing 0.5% **oligosaccharides-peptides complex** was removed from the study on day 140 due to chronic food regurgitation. The dog maintained its bw throughout the study and the results of routine blood analysis was observed to be normal. The dog was diagnosed with megaesophagus and this condition was concluded not to be treatment-related. No other dogs presented GI discomfort or intolerance, and the animals remained healthy throughout the study.

The treatment diets contained similar nutrient concentrations as expected and were highly digestible. The apparent total tract macronutrient digestibility of the treatment diets is summarized in Table 6.5. The inclusion of graded levels of **oligosaccharides-peptides complex** did not affect the apparent total tract digestibilities of the treatment diets ($P>0.05$).

Table 6.5: Apparent Total Tract Digestibility of the Treatment Diets

Parameter	Unit	Digestibility				SEM	*P*-Value
		Diet 1 (Control; 0%)	Diet 2 (0.5%)	Diet 3 (1.0%)	Diet 4 (1.5%)		
DM	%	78.0	79.6	80.9	78.1	1.48	0.49
Organic matter	%	84.2	85.5	86.2	84.5	1.07	0.57
Acid-hydrolyzed fat	%	77.8	78.9	79.7	77.4	1.53	0.71
Gross energy	%	94.5	94.8	94.4	94.8	0.36	0.74
Crude protein	%	84.6	85.7	86.1	84.7	1.05	0.70

Abbreviations: SEM = standard error of the mean.
Digestibility data was measured from total feces collected over 5-consecutive days during week 4.

The performance of the dogs over the 26 week feeding period is summarized in Table 6.6. Mean daily food intake was 155 g/day and was not affected by the dietary levels of **oligosaccharides-peptides complex** ($P>0.05$). Likewise, the mean bw of the dogs was 7.7 kg and there were no differences between treatments ($P>0.05$).

Table 6.6: Performance of Dogs Provided Diets Supplemented with Oligosaccharides-Peptides Complex							
Parameter	**Unit**	**Treatment Diets**				**SEM**	***P*-Value**
		Diet 1 (Control; 0%)	**Diet 2 (0.5%)**	**Diet 3 (1.0%)**	**Diet 4 (1.5%)**		
Daily intake (g/day)	g/day	158.5	152.3	149.6	161.5	12.18	0.89
Body weight (kg)	kg	7.9	7.6	7.7	7.7	0.86	0.99

Abbreviations: SEM = standard error of the mean.

Fecal characteristics, including pH, fecal scores, and fecal DM percentage were not affected by treatment. Similarly, fecal fermentative metabolites were not altered by treatment. The dietary level of **oligosaccharides-peptides complex** did not influence fecal calprotectin or IgA except at week 4, where fecal IgA concentration was greater ($P<0.05$) in dogs fed the control diet than that fed the diet containing 1% **oligosaccharides-peptides complex**.

With regards to fecal microbiota, species richness and Shannon diversity index were not affected by treatment. At week 4, evenness tended to be greater ($P<0.10$) in dogs fed diets containing 1% **oligosaccharides-peptides complex** compared to those fed the control diet. PCA demonstrated that the microbial communities were not significantly affected by treatment at week 2. However, at week 4, dogs fed the diet containing 1% **oligosaccharides-peptides complex** tended ($P<0.10$) to differ.

The number of bacterial phyla and bacterial genera were determined as a mean % of total sequences in feces of dogs fed **oligosaccharides-peptides complex** supplemented diets. Similarities were observed among treatment groups at both the phylum and genus level. Of the 6 phyla detected, only 2 were altered by treatment. At week 4, relative abundance of Actinobacteria was higher ($P<0.05$) in dogs fed the control diet than those fed the other treatment diets. The relative abundance of Actinobacteria was also higher ($P<0.05$) in dogs fed 1.5% oligosaccharides-peptides in the diet compared to those fed 0.5% **oligosaccharides-peptides complex**. Dogs fed diets containing 0.5% **oligosaccharides-peptides complex** also displayed lower relative abundance of Firmicutes ($P<0.05$) than those fed diets containing 1.5% **oligosaccharides-peptides complex** at week 4. The relative abundance of Proteobacteria tended to be greater ($P<0.10$) in dogs fed 1.0% oligosaccharides-peptides in the diet at week 4.

At the genus level, microbiota was modulated over time and several microbial taxa were observed to alter at week 2 or week 4. Relative abundance of *Catenibacterium* was higher ($P<0.05$) in dogs fed diets containing 1.5% **oligosaccharides-peptides complex** compared to the other treatments at week 2, while dogs fed 1% or 1.5% oligosaccharides-peptides in the diet displayed higher ($P<0.05$) *Catenibacterium* than those fed 0% or 0.5% **oligosaccharides-peptides complex** at week 4. The abundance of *Megamonas* was decreased ($P<0.05$) in dogs fed the control diet relative to those fed the diets containing 1.5% oligosaccharides-peptides in week 2. There were net decreases of *Collinsella*, and *Turicibacter*, but these decreases were only observed among treatments and did not significantly differ from the dogs in the control group. Only a few specific taxa altered in abundance and the total magnitudes of these changes was small and were often different between treatments and were not related to oligosaccharides-peptides use level. Overall, these changes in the composition of the

microbial community were considered modest by the authors and to indicate that **oligosaccharides-peptides complex** is well-tolerated by dogs.

There were no effects of treatment on serum chemistry of hematology parameters. All serum metabolites fell within reference ranges for the dogs except for blood urea:nitrogen:creatinine ratio, which was slightly higher (P<0.05) than the range in dogs fed diets containing 1.0% **oligosaccharides-peptides complex** at week 2, and the control group and dogs fed 1.5% **oligosaccharides-peptides complex** in the diet at week 4. In the absence of a treatment level response, the effect was not considered biologically relevant. Cholesterol also tended to be lower (P<0.10) in dogs fed diets containing 1.5% **oligosaccharides-peptides complex** relative to dogs fed the control diet at week 26. Blood cell count values fell within reference ranges and only the percent monocytes (P<0.05) increased in the dogs fed diets containing 1.0% **oligosaccharides-peptides complex** at week 26. None of the other measured hematology parameters differed among treatment groups (P>0.05) and all values remained within reference ranges. Likewise, serum concentrations of IgE and CRP were comparable among treatment groups (P>0.05). Serum IL-6 and TNF-α concentrations were measured but the values were below the minimum detectable range (0.9 to 4.2 pg/mL for TNF-α and 0.1 ng/mL for IL-6). The investigators recognize there are no immunological assays validated for dogs but noted that the results were consistent with those of other studies conducted at the same facility. The findings were therefore, considered to be indicative of the absence of any adverse effects of **oligosaccharides-peptides complex** on immune function.

Taken together, the findings of the study indicate that **oligosaccharides-peptides complex** was well-tolerated by dogs at levels of up to 1.5% in the diet for 26 weeks.

6.3.5 Potential Interaction of Oligosaccharide-Peptides Complex with Dietary Vitamin B1 (Thiamine)

As mentioned in Section 2.4.6, **oligosaccharides-peptides complex** contains technically unavoidable levels of sodium bisulfite which carry-over from its use for the preservation of the mucosa scraped from the intestines of the pigs (*ca*. 1.5 to 2.5% by weight). The bisulfite level of a representative batch of **oligosaccharides-peptides complex** was determined analytically to be 2,170 mg/kg. **Oligosaccharides-peptides complex** incorporated into cat and dog food at a maximum proposed level of 1.5% by weight, will potentially provide 32.6 mg bisulfite (or 39.8 mg sodium bisulfite)/kg complete feed.

Thiamine in food, including canned and dry pet foods, is susceptible to degradation by sodium bisulfite used as a preservative in protein-based ingredients (Dwivedi and Arnold, 1973; Steel, 1997; Singh *et al*., 2005; Morin *et al*., 2021). Essentially, bisulfite acts as a nucleophile causing thiamine to be cleaved into its constituents, pyrimidine and thiazole, and therefore, irreversibly destroyed in the cat and dog food. The relationship between the extent of thiamine destruction and sodium bisulfite content of food is linear, with levels of 400 mg and 1,000 mg/kg expressed as sulfur dioxide (equivalent to 640 mg and 1,600 mg bisulfite/kg feed) reported to deplete the thiamine content by 55% and 95%, respectively in meat (Dwivedi and Arnold, 1973; Singh, 2005). By comparison, the bisulfite content of pet food from the intended use of **oligosaccharides-peptides complex** is approximately 33 mg/kg feed, which by extrapolation can be estimated to destroy around 3% of the thiamine content of food and therefore, not pose a significant safety concern to cats and dogs.

AAFCO nutrient profiles for maintenance diets for cats and dogs establish minimum thiamine concentrations of 5.6 mg and 2.25 mg/kg DM after processing, respectively, recognizing that up to 90% can be destroyed during processing (AAFCO, 2021c). The cat and dog diets used in the 26-week feeding studies were determined analytically to contain 33.7 mg thiamine hydrochloride/kg food (7% moisture), equivalent to 28.5 mg thiamine/kg DM which were significantly higher than the minimum concentrations recommended by AAFCO. The values reported were for the control diets which were used to prepare all treatment diets used in the studies, and were analyzed in October 2019. (b) (4) . Samples of the control and treatment diet containing the highest oligosaccharides-peptides content (1.5% by weight) from the study in dogs were re-analyzed for thiamine content in June 2021, after a period of around 18 months under uncontrolled conditions. (b) (4) The thiamine concentrations in the control diet and treatment diet containing 1.5% **oligosaccharides-peptides complex** were reported to be 19.7 mg and 21.4 mg/kg, respectively. Thus, only natural degradation of thiamine has occurred over the 18-month storage period with no observed differences between the control and treatment diets. These findings corroborate the estimates above derived from the published literature, that indicate no significant degradation of thiamine will occur in the presence of 33 mg bisulfite/kg complete feed (Dwivedi and Arnold, 1973; Singh, 2005).

Lastly, the degradation of thiamine by bisulfite present as a minor manufacturing impurity in **oligosaccharides-peptides complex** is expected to occur rapidly given the highly reactive nature of the ion on formulation of the treatment diets. The diets were manufactured at the start of the study and were stored in 40 lb. brown-craft bags containing a plastic liner. None of the diets were re-made during the study and therefore, any degradation of thiamine will have impacted the cats and dogs over the 26-weeks of feeding.

Clinical signs of thiamine deficiency are reported in the published literature to be displayed by cats within 30 to 40-days of feeding the nutritionally inadequate diet, with clinical manifestations including loss of balance and muscle weakness (Studdert and Labuc, 1991; Steel, 1997; Chang *et al.*, 2017). In dogs, the first symptoms of thiamine deficiency from nutritionally inadequate diets are usually gastrointestinal, including loss of appetite and associated weight loss. As the symptoms progress, neurological effects may be observed (Houston and Hullard, 1988; Studdert and Labuc, 1991; Singh, 2005). Although the effects may not be as pronounced in diets which are marginally rather than highly deficient in thiamine, if levels of the vitamin had fallen below the requirements of the cats and dogs, it is anticipated that some discernible changes in general behavior and health would have been observed over the 26-week feeding period.

Taken together, it is reasonable to conclude that the levels of sodium bisulfite present in **oligosaccharides-peptides complex** and which are a technically unavoidable consequence of the use of the additive to preserve the mucosal tissue following slaughter of the pigs, do not pose a safety concern for cats and dogs under the intended conditions of use.

6.4 Genotoxicity Studies

The mutagenic potential of Gnubiotics' **oligosaccharides-peptides complex** was evaluated in a basic battery of *in vitro* assays. These bacterial reverse mutation assays and a micronucleus assay are published (b) (4)) (b) (4)

Bacterial reverse mutation assays were conducted in accordance with OECD Guideline Test No. 471 using the treat and plate method. The first assay was conducted using *Salmonella typhimurium* tester strains TA98, TA100, TA1535, and TA1537, and *Escherichia coli* tester strain WP2 uvrA (pKM101) (b) (4)). The study consisted of two phases conducted in the presence and the absence of Aroclor 1254-induced rat liver S9-mix. The S9-mix was included at a concentration of 5% (v/v) in the initial mutation assay and at 10% (v/v) in the confirmatory assay. In an initial test, concentrations of **oligosaccharides-peptides complex** tested were 0, 52 ,164, 512, 1,600, and 5,000 µg/plate, while in the second confirmatory phase, the concentrations were 0, 492, 878, 1,568, 2,800, and 5,000 µg/plate. To verify a mutagenic response observed in the second confirmatory phase of the test in the tester strain WP2uvrA(pKM101), an additional experiment was performed using the same concentrations as in the second phase in the presence of 10% (v/v) S9-mix. No statistically significant increases in the number of revertants were observed in any of the tester strains treated with **oligosaccharides-peptides complex** at any concentration in the initial test. In the confirmatory assay, no increase in the number of revertants was observed in tester strains TA1535, TA1537, TA98, TA100 at any concentration with or without S9 mix.

In tester strain WP2uvrA(pKM101) without S9 mix, **oligosaccharides-peptides complex** did not elicit an increase in revertants that reached the threshold for a positive mutagenic response. However, in the presence of S9, **oligosaccharides-peptides complex** elicited a concentration-dependent increase in revertants, reaching a 2.3-fold increase at a concentration of 5,000 µg/plate. In the additional confirmatory experiment in tester strain WP2uvrA(pKM101) in the presence of S9, no increases in revertants were observed at any concentration. Based on the equivocal results (one positive and one negative result) the test was considered inconclusive for tester strain WP2uvrA(pKM101).

It has been established that biological materials capable of releasing amino acids can cause increases in the number of revertant colonies that are not related to a mutagenic mode of action, giving false positive results in bacterial reverse mutation assays (Thompson *et al.*, 2005). As **oligosaccharides-peptides complex** fit this criterion, a "treat and wash" variation of the bacterial reverse mutation test was also conducted with *Escherichia coli* tester strain WP2uvrA, with and without rat S9 mix (b) (4) This method was developed specifically to test peptide and amino acid containing materials. The following concentrations of **oligosaccharides-peptides complex** were tested: 0, 160, 310, 620, 1,200, 2,500, and 5,000 µg/plate. In the additional "treat and wash" assay, **oligosaccharides-peptides complex** did not elicit any increases in revertants in WP2uvrA at any concentration. As such, it can be concluded that **oligosaccharides-peptides complex** is not mutagenic in the bacterial reverse mutation and the positive result with S9 mix in the first test is due to the amino acids present in the test article and not an actual mutagenic response.

The *in vitro* mammalian cell micronucleus test was conducted in L5178Y $TK^{+/-}$ cells in accordance with OECD Guideline test No 487 ((b) (4) Based on a preliminary study, in which concentrations above 2,500 µg/mL were found to adversely impact osmolality but not affect cell viability, the concentrations of **oligosaccharides-peptides complex** that were selected were 0, 625, 1,250, and 2,500 µg/mL. Cells were treated with **oligosaccharides-peptides complex**, for 3 hours ("short-term exposure") in either the presence or absence of Aroclor 1254-induced rat liver S9-mix or for 24 hours ("long-term exposure") in the absence of S9-mix. Each treatment was coupled to an assessment of cytotoxicity at the same dose levels. Cytotoxicity was evaluated by determining the population doubling (PD) of cells. Micronuclei frequencies were analyzed in 1,000 mononucleated cells per culture (total of 2,000 mononucleated cells per dose). No cytotoxicity was induced at any of the tested dose levels, as evidenced by the absence of any statistically significant or dose-related decreases in the PD. **Oligosaccharides-peptides complex** did not induce any chromosome damage, or damage to the cell division apparatus in L5178Y $TK^{+/-}$ cells either in the presence or absence of a rat liver S9 mixture compared to the negative control. Likewise, **oligosaccharides-peptides complex** did not elicit any statistically significant increases in the frequency of micronucleated cells at any concentration. An incubation time of 24 hours was not found to influence genotoxicity, and the recovery period had no impact, as no significant chromosomal damage was observed.

Oligosaccharides-peptides complex was not mutagenic in the bacterial reverse mutation test with or without metabolic activation and did not elicit any chromosome damage or damage to the cell division apparatus in mammalian somatic cells *in vitro* with or without metabolic activation. These toxicity data provide critical evidence of the safety of **oligosaccharides-peptides complex** for cats and dogs over their life span.

6.5 Studies in Swine using Protein Hydrolysate of Porcine Intestinal Mucosa

6.5.1 Overview and Identification of the Test Articles

As mentioned in Sections 2.1.2 and 2.1.3, **protein hydrolysate of porcine intestinal mucosa**, the raw material in the manufacture of **oligosaccharides-peptides complex**, is currently marketed as a feed material (protein source) for pigs, poultry and fish in the EU. It is generally dried for use in feed for ease of handling and can be co-dried with a carrier. The ingredient is also recognized for use as a protein source in Canada and is listed under Schedule IV, Part II of the Feed Regulations (Entry 5.6, CFIA, 2022) under the common names "porcine solubles, dried" or "DPS".

Protein hydrolysate of porcine intestinal mucosa is of particular interest to the swine industry as a highly digestible source of protein for weaned piglets and lactating sows. In this respect, it is a specialty feed ingredient for use as an alternative to spray-dried plasma. A number of studies were identified in the published literature in which swine were fed diets containing **protein hydrolysate of porcine intestinal mucosa**. The ingredient was mainly fed in dried form and depending on the study, described as spray-dried porcine intestinal hydrolysate, porcine digestible proteins, DPS, condensed porcine solubles (CPS), dried porcine solubles or dried hydrolysate of pig intestines. Where protein hydrolysates of porcine intestinal mucosa was co-dried with a carrier (processed or unprocessed vegetable protein or co-products from wet milling of distillers grains, the test items were referred to as peptones. The carrier

will generally aid mixing into the feed but will not impact the nutritional value or composition of **protein hydrolysate of porcine intestinal mucosa**.

6.5.2 Compositional Comparison of Protein Hydrolysate of Porcine Intestinal Mucosa used as a Feed Material and the Gnubiotics' Raw Material

Protein hydrolysate of porcine intestinal mucosa is hydrolyzed porcine intestinal mucosa generated as a by-product of the heparin manufacturing industry which has been subject to physical processing to reduce the levels of salts and other particulates. It is marketed as a source of highly digestible protein in the form or free amino acids and peptides rather than on account of the glycopeptides content. Depending on the (b) (4) processes employed by different manufacturers of **protein hydrolysate of porcine intestinal mucosa**, the glycopeptides content may vary and will not be routinely monitored by these manufacturers.

Gnubiotics sets its own specifications for **protein hydrolysate of porcine intestinal mucosa** with the supplier to ensure a consistent product with the maximum amount of glycopeptides present for use in (b) (4) to **oligosaccharides-peptides complex**. Thus, Gnubiotics' raw material may be considered compositionally equivalent to **protein hydrolysate of porcine intestinal mucosa** marketed as a feed material in the EU and Canada except that it is in the liquid rather than dried form and has a standardized glycopeptides content.

6.5.3 Relevance of Studies using Protein Hydrolysate of Porcine Intestinal Mucosa to the Assessment of Oligosaccharides-Peptides Complex

(b) (6) . No chemical modifications or exogenous substances are introduced which alter the nutritional or functional properties. Thus, published studies in animals using **protein hydrolysate of porcine intestinal mucosa** are considered pertinent to the safety evaluation of **oligosaccharides-peptides complex** for cats and dogs.

The only studies identified using **protein hydrolysate of porcine intestinal mucosa** were in swine rather than the target species (cats and dogs). On the basis that physiologically, pigs and dogs share many similarities and can be expected to metabolize an ingredient rich in amino acids and peptides, and potentially containing low levels of glycopeptides in the same way, the studies were considered pertinent to the safety assessment. Of the studies identified, three studies evaluated digestibility, and one study assessed intestinal function (Sections 6.5.1 and 6.5.2). Another study considered gestation, lactation and growth (Section 6.5.3) and one study the performance of lactating sows (Section 6.5.4). The remainder of the studies were designed primarily to evaluate growth performance in weaned piglets rather than safety and therefore, measured only limited endpoints such as bw, FI and feed conversion ratio (FCR), as well as mortality (Section 6.5.5).

As mentioned above, in the studies **protein hydrolysate of porcine intestinal mucosa** was spray-dried or on a carrier, and marketed commercially under various product names including spray-dried porcine intestine hydrolysate, DPS, CPS and when on a carrier, peptone. The most common name used by the

study authors was DPS and for consistency with the publications, the test time is referred to as DPS rather than **protein hydrolysate of porcine intestinal mucosa** in the sections below.

6.5.4 Digestibility Studies

Kim et al., 2001

An experiment was conducted to determine the optimal inclusion ratio of spray-dried plasma protein (SDPP) and DPS for maximizing growth and improving immunity in piglets weaned at 21 days of age (Kim *et al*., 2001). The experiment included 150 cross bred piglets (Barrow, Landrace x Large white x Duroc; 6.01 ± 0.12 kg) in a completely randomized block design. Animals were fed diets based on corn/whey/SBM containing 0 (control), 6% SDPP, 6% SDPP + 6% DPS (S6D6), 6% SDPP + 3% DPS (S6D3), 3% SDPP + 6% DPS (S3D6) or 3% SDPP + 3% DPS (S3D3) for 3 weeks. Each treatment had 6 pens, with 5 piglets per pen. Two phase mash feeds were provided, during phase I (days 1 to 7) and phase II (days 8 to 21). Chromium oxide was included in the feed as a digestibility marker. Animals were given *ad libitum* access to feed and water and were housed in an environmentally controlled room. Body weight and feed intake were recorded weekly to calculate average daily gain (ADG) and average daily feed intake (ADFI) and gain-to-feed ratio (G:F). Four (4) piglets per treatment were used for a metabolic trial to determine nutrient digestibility (dry matter, crude protein, ether extract, crude Ash, Ca and P), as well as apparent amino acid digestibility. Blood samples were taken on day 0, 8 and 21 to determine porcine leukocyte population (CD4 positive T lymphocyte; CD8 positive T lymphocyte; granulocyte and monocytes). When considering the overall experimental period (days 0 to 21) piglets fed the S3D6 diet had statistically increased ADG and ADFI ($P<0.05$) vs. control but no significant differences were observed in G:F between treatments and control. The digestibility of DM and crude protein (CP) were higher in piglets fed the S6D6 diet than control. In terms of nutrient digestibility, no differences were observed among treatment groups ($P>0.05$). The apparent digestibility of amino acids over phase I and phase II were not significantly different to control except leucine was increased ($P<0.05$) in diets S6D6 and S6D3 in phase I. No correlation between the ratio of SDPP and DPS and the ratio of CD4 positive T lymphocyte and CD8 positive T lymphocytes were observed. The inclusion of DPS at 3% in combination with SDPP at 6% displayed better performance parameters and maintained low immunity status. Including DPS at a higher rate of 6% did not improve performance parameters and immunity level in weaned piglets.

Kim et al., 2000

A study to determine the ileal digestibility (ID) of amino acids and feeding values of spray dried plasma protein SPP and DPS in early weaned piglets was conducted by Kim *et al*. (2000). In the digestibility trial, 12 piglets (Landrace X Yorkshire X Duroc; 5.83 ± 0.51 kg and 18 days old) were housed in metabolic cages. Animals received 3 diets based on semi purified cornstarch and lactose that contained either 23.8% SDPP, 37% DPS or a N-free diet. The SDPP and DPS diets were formulated to contain 18.5% CP with vitamins and minerals exceeding the NRC (2012) recommendation. Four (4) piglets were assigned to each diet to collect ileal digesta for endogenous amino acid excretions. After one day of fasting, piglets were fitted with T-cannula in the terminal ileum and were fed a restricted amount of feed (5% of bw/day) 3 times per day. Diets were mixed with water and fed as a wetted mash, in order to improve feed intake. From the sixth day, digesta samples were collected for 3 days. The apparent and true ID

values of essential amino acids (except leucine, methionine and valine) were lower (P<0.01) in DPS than in SDPP. The average apparent ID of essential amino acids in SDPP and DPS were 75.63% and 71.30%, respectively, and the average true ID of essential amino acids was 84.83% and 80.51% respectively.

In the 7-day feeding trial, 90 piglets (Landrace X Yorkshire X Duroc; 6.70 ± 0.35 kg and 18 days old) were allocated to 3 treatments, with 10 piglets per pen and 3 pens per treatment. Animals were housed in an environmentally controlled room with *ad libitum* access to feed and water. Diets were based on extruded corn and SBM (CSF-extrudate; 47% corn 47% SBM and 6% animal fat) and were formulated to contain 3,500 ME/kg, 1.45% lysine, 20% dried whey, 15% milk replacer and 10% lactose. Diets were supplemented with either 6% SDPP, 6% DPS or a combination of 3% SDPP and 3% DPS. Performance parameters (ADG, ADFI, G:F) and apparent fecal digestibility were determined after 7 days. The ADG and G:F ratio were improved (P<0.01) in animals fed diets containing 6% SDPP than those fed 6% DPS in the diet. There was no difference in FI among treatment groups. Animals that received a combination of DPS and SDPP in the diet displayed comparable performance parameters to those fed SDPP only. The results of this study demonstrate that **protein hydrolysate of porcine intestinal mucosa** provides a highly digestible source of protein which can be utilized by early weaned piglets.

Sulabo et al., 2013

In another experiment using DPS and other protein-rich ingredients, 3 experiments were conducted by Sulabo *et al*. (2013) to determine apparent ileal digestibility (AID) and standardized ileal digestibility (SID) of CP and amino acids (AA), the concentration of digestible energy (DE) and metabolizable energy (ME), and standardized total tract digestibility (STTD) of phosphorus in dried fermentation biomass (DFB) and DPS products co-dried with other protein sources when fed to weanling piglets, and to compare these values to those obtained for fish meal. The two DPS products used were PEP50 (protein hydrolysate of porcine intestinal mucosa and dehulled soybean meal) and PEP2+ (produced by mixing protein hydrolysate of porcine intestinal mucosa with DFB and enzymatically processed, low-antigen SBM).

In the first experiment, 12 weanling barrows (G-Performer boars × Fertilium 25; initial bw 11.5 ± 1.1 kg; 36 ± 1 d of age) were equipped with a T-cannula in the distal ileum and allotted to a replicated 6 × 6 Latin square design with 6 diets and 6, 7-day periods in individual pens. Piglets were allowed 7 days to recover from the surgery before the experiment was initiated. One diet contained SBM (42%) as the sole source of CP and AA, 4 diets were based on SBM in combination with another protein source and included, SBM (21%) & DFB (13%), SBM (21%) & PEP50 (19%), SBM (21%) & PEP2+ (17%), or SBM (21%) & fish meal (16%). A nitrogen free diet (based on SBM) was used to measure basal endogenous ileal losses of CP and AA. Animals were housed in individual pens in an environmentally controlled room and had *ad libitum* access to feed and water. Piglet bw measurements were recorded at the beginning of each period and the amount of feed supplied each day was recorded. Ileal digesta samples were collected for 8 h on day 6 and 7 and the AID of CP and AA in SBM, DFB, DPS products (PEP50 and PEP2+), and fish meal by weanling piglets determined. The AID and SID of CP were less (P<0.05) in animals that received diets of SBM in combination with PEP2+ and PEP 50 than in SBM only. For indispensable AA, AID and SID of histamine, isoleucine, leucine, methionine, phenylalanine, threonine, and valine, and SID

of arginine were least (*P*<0.05) in SBM & PEP2+, compared with the other ingredients. AID of arginine and isoleucine, and SID of threonine were less (*P*<0.05) in SBM & PEP50 than in SBM only diets.

In the second experiment, 40 barrows (G-Performer boars × Fertilium 25; initial bw 12.8 ± 1.4 kg; 39 ± 2 d of age) were allotted to 5 dietary treatment groups with 8 piglets/treatment. A basal diet containing 96.4% corn and 4 diets containing corn (80.4%) & DFB (16%), corn (73.4%) & PEP50 (23%), corn (76.4%) & PEP2+ (20%), or corn (81.3%) & fish meal (18%) were formulated. Piglets were provided *ad libitum* access to feed and water. Animals were housed in metabolism cages with slatted floors that allowed for the total but separate collection of urine and fecal materials from each piglet. The experiment lasted 14 days. The initial 7 days were considered an adaptation period to the diet. Fecal and urine samples were collected in the following 7 days and analyzed for gross energy (GE). Following analysis of GE values for apparent total tract digestibility (ATTD) of energy and DE and ME were calculated. There were no differences in ATTD of GE among treatments. The DE and ME in PEP50 (4,758 and 4,512 kcal/kg DM for DE and ME, respectively) and DE in PEP2+ (4,935 kcal/kg DM), and ME (4,617 kcal/kg DM) were significantly greater (*P*<0.05*)* than in control diets (corn only diets; 4014 and 3846kcal/kg DM kcal/kg DM for DE and ME, respectively).

In experiment 3, 40 barrows (G-Performer boars × Fertilium 25; bw = 12.4 ± 1.3 kg; 37 ± 2 d of age) were randomly allotted to 5 dietary treatment groups with 8 piglets/treatment. A phosphorus-free diet and 4 diets in which the sole source of phosphorus was from DFB, PEP50, PEP2+, or fish meal were formulated. Vitamins and all minerals, except P, were included in the diets to meet or exceed requirements (NRC, 2012). Feed was provided in a daily amount equivalent to 3 times the maintenance energy requirement and divided into 2 daily meals. Markers included in the morning meals on days 8 and 13. Water was available at all times. Animals were housed in metabolism cages with slatted floors that allowed for the collection of fecal materials from each piglet. The basal endogenous loss of P was determined from piglets fed the P-free diet and ATTD and STTD of P in each diet were calculated. The STTD of phosphorus in PEP2+ (97.6%) and PEP50 (76.2%) were greater (*P*<0.01) than in fish meal (68.5%).

The AA content, concentration of DE and ME and digestibility of phosphorus was comparable between **protein hydrolysate of porcine intestinal mucosa** and existing fish meal and DFB counterparts. The results of these studies provide evidence for the utility of **protein hydrolysate of porcine intestinal mucosa** as a protein source for swine.

6.5.5 Study to Investigate Intestinal Function

González-Solé et al., 2020

A study comprising of 2 experiments was conducted by González-Solé *et al*. (2020) in which the effects of DPS, spray-dried plasma (SDP) or a combination of both on growth performance and intestinal function of weaned piglets was evaluated. A total of 180 male and female weaned commercial crossed piglets [(Landrace x Large White) x Pietrain; initial bw of 7.5 ± 1.15 kg] weaned at 28 days, were blocked according to initial weight into two blocks (heavy piglets: 8.6 ± 0.03 kg; light piglets: 6.4 ± 0.02 kg). Each block contained 9 pens of 10 animals and each treatment was randomly assigned with 6 pens used per treatment. Animals received a diet based on corn/wheat with a high content of soybean ingredients

containing 0 (control), 2% SDP or 2% DPS. SDP and DPS replaced part of the extruded soybean content of the diet. Diets were presented in mash form. Feed and water were available *ad libitum* throughout the study. Animals were weighed at days 0 and 14 and food disappearance was used to calculate ADG, ADFI and G:F. Animal fed DPS and SDP had significantly increased (P<0.05) ADG, ADFI and improved G:F vs. control. In the second experiment, a total of 198 male and female weaned commercial crossed piglets [(Landrace x Large White) x Pietrain; initial bw of 5.7 ± 0.60 kg] weaned at 21 days, were blocked according to initial weight into two blocks (heavy piglets: 6.3 ± 0.02 kg; light piglets: 5.1 ± 0.01 kg). Each block contained 9 pens of 11 animals and each treatment was randomly assigned with 6 pens used per treatment. Animals received a diet based on corn/wheat with a high content of soybean ingredients containing 0 (control), 3% SDP or a combination 1% SDP and 2% DPS. SDP and DPS replaced part of the extruded soybean content of the diet. Feed and water were available *ad libitum* throughout the study. Animals were weighed at days 0, 7, 14 and 35 post weaning, food utilization was recorded and used to measure parameters related to performance (ADG, ADFI and G:F). One (1) piglet per pen was euthanized on day 14 and a portion of jejunum and ileum tissues collected where intestinal function was assessed by quantifying the gene expression using an open array real-time PCR (polymerase chain reaction) platform. Although a numeric difference in bw was observed at day 35, no significant differences in ADFI, ADG or G:F were observed between animals receiving diets containing SDP or SDP-DPS. Ten genes in jejunum and five in ileum samples were differentially expressed among treatments (P<0.05). The highest expression was found in the animals fed diets containing SDP + DPS. These genes are involved in the barrier function of intestinal cells, the immune response, mucosal nutrient transport, digestion and metabolism of oxidation products. The combination of SDP + DPS upregulated genes related to the intestinal function without affecting growth performance. The underlying mechanisms that produced these effects are still unknown; however these effects were not considered adverse and support the ability of these protein ingredients to support digestive function in swine.

6.5.6 Gestation, Lactation and Growth Study

Figueroa et al., 2016

In a study by Figueroa *et al*. (2016) researchers evaluated whether DPS, when included in a diet free of dairy products and lactose may maintain the performance of weaned piglets, compared with animals fed diets containing significant amounts of whey and lactose (experiment 1) and whether pre- and post-natal exposure to DPS via the maternal diet may improve feed intake and performance of weaned piglets fed a diet containing DPS (experiment 2). In experiment 1, 2 different cereal-based diets were designed (meeting the NRC (2012) standards) and offered to piglets after weaning. Diets including lactose (lactose+) were given pre-starter diets (days 0 to 14) containing 142 g/kg sweet milk whey and starter diets (days 15-33) with 50 g/kg of sweet milk whey. The lactose-free group was offered an isoenergetic diet with 20 g/kg of DPS and 300 g/kg wheat in both the pre-starter and starter periods. Both diets were offered in mash form. A total of 240 piglets (Pietrain x [Large White x Landrace], average initial bw of 7.9 ± 1.2 kg and weaned at 28 days) were distributed into 4 blocks according to their bw (light: 6.1 ± 0.58 kg; middle-light: 7.6 ± 0.36 kg; middle-heavy: 8.5 ± 0.24 kg; and heavy: 9.4 ± 0.25 kg). Within each block, piglets were randomly distributed into 6 pens of 10 animals and balanced for bw. Pens were randomly assigned to 2 treatment groups according to a balanced distribution. Half of the pens (12 pens, 3 pens of each weight block) were fed *ad libitum* the lactose+ diet, and the rest of

the pens (n = 12) were fed the lactose-free diet during the whole period (33 days). Performance parameters (ADFI, ADG, and G:F) were measured on days 0, 7, 14, 21, 28, and 33 after weaning. Despite the preference in diet there was no difference in performance parameters between the two groups. In a sub-experiment choice and 1-feeder tests were performed in another group of animals (n=72) to evaluate the preference and acceptance for both diets. The lactose+ diet was preferred (*P*=0.039) to the lactose free diet as assessed by a 30 minute choice test. More of the lactose+ diet (*P*=0.001) was consumed than the lactose free diet in a 1-feeder test. In experiment 2, 240 animals were obtained from 26 sows (n = 13/treatment) that, during late gestation (14 days) and lactation (28 days), were fed an isoenergetic diet without DPS inclusion (n=120) or containing 20 g/kg of DPS (n=120). After weaning, all piglets received a feed containing 20 g/kg of DPS in the pre-starter and starter diets. Feed intake and body weight were measured weekly (days 0, 7, 14, 21, 28, and 33 after weaning) to calculate the ADFI, ADG, and G:F. Piglets coming from sows provided diets supplemented with 20 g/kg of DPS during late gestation and lactation had a tendency to display higher ADFI (*P*=0.077) and higher ADG (*P*=0.062) during the starter phase (15 to 33 days after weaning) than piglets coming from control sows.

6.5.7 Study in Lactating Sows

Johnston et al., 2003

A study conducted to determine the voluntary feed intake and performance of lactating Yorkshire x Landrace sows fed diets containing DPS was performed by Johnston *et al*. (2003). The experiment was conducted at 2 research centers wherein 119 lactating sows (n=66 and 53) were assigned to 3 diets during lactation. Sows were housed in confinement farrowing rooms on about day 110 of gestation. Animals were fed corn and SBM based diets formulated to contain 0.9% total lysine and included either 0 (control), 1.5% or 3% DPS. Animals were assigned to dietary treatments based on parity and expected farrowing date at each center. Sows received 2.04 kg daily of the control diet from entry to the farrowing room until farrowing. On the day of farrowing, feed offered to sows was switched to the appropriate dietary treatment. Sows were provided *ad libitum* access to the designated experimental diet throughout lactation. The amount of feed was adjusted daily to ensure that sows had continuous access to feed without accumulation at feeding stations. Unconsumed feed was weighed on days 9 and 18 postpartum and at weaning. Performance was measured by feed intake and sow weight. Additionally, sow back fat depth was determined on farrowing and weaning. Number and weight of individual piglets were recorded at birth while, total litter weight and number of piglets per litter were recorded on days 9 and 18 of lactation and at weaning. Dietary intake of DPS tended to increase (*P*<0.10) the amount of food consumed from day 0 through to weaning. Including DPS in the diet increased ADFI over the entire lactation period (6.03, 6.53, and 6.30 kg) for sows fed 0, 1.5, and 3.0% DPS, respectively. No significant effect of DPS on bw or backfat depth of the sow were noted. Litter size and weight on day 18 of lactation were not affected by concentration of DPS in the diet. DPS in the diet had no effect on milk production of sows and daily litter weight gain was 2.15, 2.10, and 2.00 kg for sows assigned to 0, 1.5, and 3.0% DPS-containing diets, respectively. There were no significant interactions between dietary treatments and research center or dietary treatments and farrowing group, suggesting that the effects of diet were consistent at both research centers and across farrowing groups. Overall, the DPS-containing diets were well-tolerated by lactating sows and tended to increase the ADFI of lactating sows.

6.5.8 Dietary Feeding Studies in Weaned Piglets

Nine (9) studies were identified in which the impact of DPS as a component of the diet on the performance of weaned piglets was evaluated. These studies are summarized in Table 6.7. Overall, the studies indicate that **protein hydrolysate of porcine intestinal mucosa** (spray-dried or on a carrier) may be incorporated into the diet of weaned piglets at levels ranging from 1 to 6% for a duration of 28 or 35 days without any adverse effects on performance.

Table 6.7: Summary of Feeding Studies Conducted in Weaned Piglets using Protein Hydrolysate of Porcine Intestinal Mucosa (Referred to as DPS)		
Reference and Objective	**Study Design**	**Key Findings Related to Safety and Utility**
Jones *et al.*, 2008 **Objective:** Effects of pepsoygen and DPS 50 on nursery pig diets	***Experiment 1***	
	Animals: 350 weaned piglets (13.4 lb IW), breed not stated (5 piglets/pen; 10 pens/treatment) **Test Article:** Fish meal, PepSoyGen (fermented SBM product), DPS **Treatment:** Negative control, 3% fish meal, 6% fish meal, 3.75% PepSoyGen, 7.5% PepSoyGen, 1.88% PepSoyGen + 1.88% DPS, 3.75% PepSoyGen + 3.75% DPS **Duration:** 35 days (14 days treatment diet + 21 days common diet)	From d 0 to 14, piglets fed increasing PepSoyGen and PepSoyGen in combination with DPS had improved (quadratic, P=0.01, linear, P=0.002, respectively) F/G. Average daily gain and F/G were improved (P=0.05 and P=0.03, respectively) for piglets fed diets containing PepSoyGen and DPS combinations compared with piglets fed diets containing fish meal. Feeding the combination of PepSoyGen and DPS improved ADG and ADFI (P=0.01 and P=0.02, respectively) compared with feeding only PepSoyGen.
	Experiment 2	
	Animals: 252 weaned piglets (15.0 lb IW), breed not stated (6 piglets/pen; 7 pens/treatment) **Test Article:** Fish meal, PepSoyGen, DPS **Treatment:** Negative control, 5% fish meal, 3.5% DPS, 6.0% PepSoyGen, 1.75% PepSoyGen + 1.75% DPS, 3.0% PepSoyGen + 2.5% fish meal **Duration:** 28 days (14 days treatment diet + 14 days common diet)	During the treatment period (d 0 to 14), piglets fed DPS alone or in combination with PepSoyGen had improved ADG and F/G (P<0.05) compared with piglets fed all other diets. Overall (d 0 to 28), piglets fed DPS from d 0 to 14 had improved ADG and F/G (P<0.05) compared with piglets fed the control diet. Additionally, piglets fed DPS had improved F/G (P<0.05) compared with piglets fed PepSoyGen and fish meal in combination.

Table 6.7: Summary of Feeding Studies Conducted in Weaned Piglets using Protein Hydrolysate of Porcine Intestinal Mucosa (Referred to as DPS)		
Reference and Objective	**Study Design**	**Key Findings Related to Safety and Utility**
Bregendahl and Zimmerman, 2001 **Objective:** Comparison of hydrolyzed intestinal by-products	**Animals:** 96 weaned piglets (6.4 ± 0.1 kg IW), breed not stated (4 piglets/pen; 8 pens/treatment) **Test Article:** Dried whey, DPS, CPS **Treatment:** Control (dried whey), 5% DPS, 5% CPS **Duration:** 35 days (21 days treatment diet + 14 days common diet)	Partially replacing dried whey with either DPS or CPS had no effect (P>0.05) on ADG, ADFI, or G:F during any of the 5 weeks. No effects (P>0.05) on cumulative ADG, cumulative ADFI, or cumulative G:F were observed. In week 3 only, G:F tended to improve (P=0.10) in pigs fed CPS compared with piglets fed dried whey. On a DM basis, ADFI in week 2 tended to be higher (P=0.10) in piglets fed DPS than in piglets fed CPS, although neither differed (P>0.10) from the ADFI observed with the dried-whey diet. The tendency (P=0.10) towards improved feed utilization in week 3 of piglets fed CPS compared with dried whey (control diet) was evident on both an as-fed and DM basis. Results of the trial indicated that porcine solubles could replace dried whey on a lysine basis in diets for weaned piglets.

Table 6.7: Summary of Feeding Studies Conducted in Weaned Piglets using Protein Hydrolysate of Porcine Intestinal Mucosa (Referred to as DPS)		
Reference and Objective	**Study Design**	**Key Findings Related to Safety and Utility**
Bregendahl *et al.*, 1999 **Objective:** Effect of spray-dried plasma and dried porcine solubles on the growth performance of weanling pigs raised in different health-status environments	**Animals:** 64 weaned piglets (6.4 ± 0.1 kg IW), breed not stated (1 piglet/pen; 8 pens/treatment) **Test Article:** SPD, DPS **Treatment:** Control, 5% SDP, 5% DPS, 5% SDP + 5% DPS **Environments:** Dirty: previously-used on-site nursery room that was not cleaned Clean: off-site nursery room that was cleaned and disinfected before the start of the trial. **Duration:** 35 days (14 days treatment diet + 21 days common diet)	Averaged over environments, ADG (P<0.008) and ADFI (P<0.006) were increased by SDP but not by DPS during treatment phase. ADG (P<0.03) and ADFI (P<0.04) were increased by SDP but not by DPS for the overall 5-week period. Piglets fed DPS utilized feed more efficiently (interaction, P<0.01) in the dirty environment than did DPS-fed piglets in the clean environment. Piglets fed DPS plus lactose performed as well as piglets fed dried whey (the control diet), however, no delayed effects of DPS on growth performance were observed, as had been reported previously. The data indicate that a combination of DPS and lactose can replace dried whey with no adverse effects on growth performance.
Cho *et al.*, 2010 **Objective:** Feeding value of dried porcine solubles for weaning pigs	***Experiment 1*** **Animals:** 150 weaned piglets (7.42 ± 0.68 kg IW), Hampshire x (Landrace x Yorkshire) (5 piglets/pen; 6 pens/treatment) **Test Article:** SPDD, DPS **Treatment:** Control,3% SDPP, 6% SDPP, 3% DPS, 6% DPS **Duration:** 28 days	The nutritional value of DPS relative to SDPP was 99 to 114% when appropriate adjustments for Lys, Met, and Thr were made.

Table 6.7: Summary of Feeding Studies Conducted in Weaned Piglets using Protein Hydrolysate of Porcine Intestinal Mucosa (Referred to as DPS)		
Reference and Objective	**Study Design**	**Key Findings Related to Safety and Utility**
Cho *et al.*, 2010 (continued) **Objective:** Feeding value of dried porcine solubles for weaning pigs	***Experiment 2***	
	Animals: 165 weaned piglets (6.30 ± 0.90 kg IW), Hampshire x (Landrace x Yorkshire) (5 or 6 piglets/pen; 6 pens/treatment) **Test Article:** Spray Dried Blood Cells (SDBC), DPS **Treatment:** Control, 3% SDBC, 1.5% DPS + 1.5% SDBC, 3% DPS, 5% DPS **Duration:** 28 days	There were no differences in ADG between piglets fed SDBC and various levels of DPS 30; there was some indication of a greater G:F with DPS 30.
	Experiment 3 (Preference Experiment)	
	Animals: 80 weaned piglets (6.30 ± 0.90 kg IW), Hampshire x (Landrace x Yorkshire) (4 piglets/pen; 20 pens) **Test Article:** DPS **Treatment:** Control, 2.5% DPS, 5% DPS **Duration:** 21 days	Piglets chose to eat more of the 2.5% DPS 30 diet ($P<0.05$) compared with either the 0 or 5% DPS 30 diets.
	Experiment 4 (Preference experiment)	
	Animals: 56 weaned piglets (6.30 ± 0.90 kg IW), Hampshire x (Landrace x Yorkshire) (4 piglet/pen; 14 pens) **Test Article:** SDPP, DPS 30, DPS 50RD **Treatment:** 5% SDPP, 2.5% SDPP + 2.5% DPS 30, 3% DPS 30, 1.5% DPS 50RD	Piglets chose to eat more of the 2.5% SDPP and 2.5% DPS 30 diets ($P<0.05$) compared with the 5% SDPP diet.

Table 6.7: Summary of Feeding Studies Conducted in Weaned Piglets using Protein Hydrolysate of Porcine Intestinal Mucosa (Referred to as DPS)		
Reference and Objective	**Study Design**	**Key Findings Related to Safety and Utility**
	Duration: 28 days	
Jones *et al.*, 2010 **Objective:** Effects of fermented SBM and speciality animal protein sources on nursery pig performance	***Experiment 1***	
	Animals: 252 weaned piglets (5.76kg IW), TR4 x 1050 (6 piglets/pen; 7 pens/treatment) **Test Article:** Fish meal, DPS, fermented SBM **Treatment:** Control, 5% fish meal, 3.5% DPS, 6% fermented SBM, 1.75% fermented SBM + 1.75% DPS, 3.0% fermented SBM + 2.5% fish meal **Duration:** 28 days (14 days treatment diet + 14 days common diet)	From d 0 to 14, piglets fed DPS alone or with fermented SBM had improved (P<0.05) ADG and G:F compared with piglets fed all other diets. Overall (d 0 to 28), piglets fed DPS had improved (P=0.01) ADG (421 vs. 383 g) and G:F (0.77 vs. 0.73) compared with piglets fed the control diet and had improved (P=0.03) G:F (0.77 vs. 0.74) compared with piglets fed the combination of fermented SBM and fish meal.
	Experiment 2	
	Animals: 350 weaned piglets (6.1kg IW), C22 x 1050 (5 piglets/pen; 10 pens/treatment) **Test Article:** Fish meal, DPS, fermented SBM **Treatment:** Control, 3% fish meal, 6% fish meal, 3.75% fermented SBM, 7.5% fermented SBM, 1.88% fermented SBM + 1.88% DPS, 3.75% fermented SBM + 3.75% DPS **Duration:** 35 days (14 days treatment diet + 21 days common diet)	Piglets fed the combination of fermented SBM and DPS had improved (P<0.05) ADG and G:F compared with piglets fed diets containing fish meal and had improved (P<0.05) ADG and ADFI compared with piglets fed diets containing fermented SBM. Feeding weaned piglets diets containing DPS, either alone or in combination with fermented SBM, can improve growth performance compared with those fed high concentrations of SBM or fish meal.

<table>
<tr><th colspan="3">Table 6.7: Summary of Feeding Studies Conducted in Weaned Piglets using Protein Hydrolysate of Porcine Intestinal Mucosa (Referred to as DPS)</th></tr>
<tr><th>Reference and Objective</th><th>Study Design</th><th>Key Findings Related to Safety and Utility</th></tr>
<tr>
<td>Myers et al., 2010

Objective:
An evaluation of peptone products and fish meal on nursery pig performance</td>
<td>Animals:
360 weaned piglets (11.8lb IW), C327 x 1050 (5 piglets/pen; 12 pens/treatment)
Test Article:
Spray Dried Animal Plasma (SDAP), Select menhaden fish meal (SMFM), PEP2+, PEP50, PEP-NS
PEP2+, PEP50 and PEP-NS (DPS products co-dried on different carriers and referred to as peptones)
Treatment:
<table>
<tr><th></th><th>Phase 1 (d0-8)</th><th>Phase 2 (d8-21)</th></tr>
<tr><td>1</td><td>2.5% SDAP</td><td>No specialty protein source</td></tr>
<tr><td>2</td><td>5% SDAP</td><td>3% SMFM</td></tr>
<tr><td>3</td><td>5% SDAP + 3%SMFM</td><td>6% SMFM</td></tr>
<tr><td>4</td><td>5% SDAP + 3% PEP2+</td><td>6% PEP2+</td></tr>
<tr><td>5</td><td>5% SDAP + 3% PEP50</td><td>6% PEP50</td></tr>
<tr><td>6</td><td>5% SDAP + 3% PEP-NS</td><td>6% PEP-NS</td></tr>
</table>
Duration:
35 days (8 days phase 1 + 13 days phase 2 + 14 days common diet)</td>
<td>During Phase 1, there were no differences in F/G among piglets fed any of the dietary treatments.

During Phase 2 (d8 to 21), piglets fed 6% PEP2+ had greater ($P<0.05$) ADG compared to those fed the negative control diet, 3% or 6% fish meal, with piglets fed PEP50 and PEP NS intermediate.

Piglets fed 6% PEP2+ had the greatest improvement ($P<0.02$) in F/G compared to piglets fed all other experimental diets.

Overall, piglets fed diets containing PEP2+ had increased ($P<0.03$) ADG and ADFI compared to piglets fed the negative control diet.

Piglets fed 3% PEP2+ during Phase 1 and 6% PEP2+ during Phase 2 had greater ($P<0.05$) ADFI compared to those fed 3% SMFM during Phase 1 and 6% SMFM during Phase 2.</td>
</tr>
</table>

Table 6.7: Summary of Feeding Studies Conducted in Weaned Piglets using Protein Hydrolysate of Porcine Intestinal Mucosa (Referred to as DPS)		
Reference and Objective	**Study Design**	**Key Findings Related to Safety and Utility**
Myers *et al.*, 2011 **Objective:** Evaluation of heparin production by-products in nursery piglet diets	**Animals:** 1,152 weaned piglets (12.3 ± 1.30lb IW), Newsham GPK35 x PIC380 (32 piglets/pen; 6 pens/treatment) **Test Article:** SMFM, poultry meal (PM), PEP2+, PEP50, PEP-NS **Treatment:** Negative control, 6% SMFM, 6% PM, 6% PEP2+, 6% PEP50, 6% PEP-NS **Duration:** 39 days (21 days treatment diet + 18 days common diet)	From d 0 to 21, piglets fed diets containing 6% SMFM, PM, PEP2+, or PEP-NS had improved (P<0.05) ADG and ADFI compared with those fed the negative control diet. Piglets fed diets containing 6% SMFM, PM, PEP2+, or PEP-NS had improved (P<0.05) F/G compared with piglets fed 6% PEP 50. From d 21 to 39, piglets previously fed diets containing 6% PEP2+ or PEP-NS had improved (P<0.05) ADG and ADFI compared with those previously fed the negative control diet. Overall (d 0 to 39), piglets fed diets containing 6% SMFM, PM, PEP2+, or PEP-NS had improved (P<0.05) ADG and ADFI compared with piglets fed the negative control diet.
Myers *et al.*, 2014 **Objective:** The effects of porcine intestinal mucosa protein sources on nursery pig growth performance	***Experiment 1*** **Animals:** 300 weaned piglets (5.4kg IW), PIC 327 x 1050 (5 piglets/pen; 12 pens/treatment) **Test Article:** PEP2, SDAP, SMFM **Treatment:** Negative control, 4% PEP2, 8% PEP2, 12% PEP2, positive control – 4% SDAP in phase 1 & 4% SMFM in phase 2 **Duration:** 25 days	From d 0 to 11, piglets fed SDAP had greater (P<0.05) ADG and G:F than piglets fed PEP2. From d 11 to 25, increasing PEP2 increased (quadratic; P<0.01) ADG and G:F, with the greatest response observed at 4%.

Table 6.7: Summary of Feeding Studies Conducted in Weaned Piglets using Protein Hydrolysate of Porcine Intestinal Mucosa (Referred to as DPS)		
Reference and Objective	**Study Design**	**Key Findings Related to Safety and Utility**
Myers *et al.*, 2014 (continued) **Objective:** The effects of porcine intestinal mucosa protein sources on nursery pig growth performance	***Experiment 2***	
	Animals: 960 weaned piglets (5.6kg IW), Newsham GPK35 x PIC 380 (32 piglets/pen; 6 pens/treatment) **Test Article:** SDAP, SMFM, PEP2+, PEP50, PEP-NS **Treatment:** Control, 6% SMFM, 6% PEP2+, 6% PEP50, 6% PEP-NS **Duration:** 21 days	From d 0 to 21, piglets fed diets containing SMFM, PEP2+, or PEP-NS had greater (P<0.05) ADG than piglets fed the control or 6% PEP50.
	Experiment 3	
	Animals: 180 weaned piglets (6.4kg IW), PIC 327 x 1050 (5 piglets/pen; 6 pens/treatment) **Test Article:** PEP-NS, SMFM **Treatment:** Negative control, 3% PEP-NS, 6% PEP-NS, 9% PEP-NS, 12% PEP-NS, 6% SMFM **Duration:** 14 days	Piglets fed increasing PEP-NS had improved (quadratic; P<0.01) ADG and G:F, with the greatest improvement observed in piglets fed 6% PEP-NS, similar to those fed 6% SMFM.

Table 6.7: Summary of Feeding Studies Conducted in Weaned Piglets using Protein Hydrolysate of Porcine Intestinal Mucosa (Referred to as DPS)		
Reference and Objective	**Study Design**	**Key Findings Related to Safety and Utility**
Zimmerman and Sparks, 1997 **Objective:** Evaluation of a by-product from hydrolyzed porcine small intestines as an ingredient in pig starters	***Experiment 1***	
	Animals: 80 weaned piglets (14.4lb IW), breed not stated (4 piglets/pen; 10 pens/treatment) DPS **Treatment:** Control, 5% DPS **Duration:** 28 days	The data indicate that DPS can be included at 5% of the diet. At this concentration, growth performance of pigs was not different from that of pigs fed the control diet.
	Experiment 2	
	Animals: 144 weaned piglets (14.8lb IW), breed not stated (6 piglets/pen; 6 pens/treatment) **Test Article:** Commercial DPS product (Protein Plus), experimental DPS product **Treatment:** Control, 9% lactose + 5% Protein Plus, 9% lactose + 6% experimental DPS, 18% lactose + 12% experimental DPS **Duration:** 28 days (14 days treatment diet + 14 days common diet)	During week one, the high level of inclusion of experimental hydrolysate decreased feed intake and average daily gain. In the second week, however, the low level of experimental hydrolysate stimulated feed intake, average daily gain, and feed efficiency. In weeks three and four, when all piglets were fed a common diet, piglets that previously had been fed Protein Plus or experimental DPS outperformed the piglets fed the control diet.

Table 6.7: Summary of Feeding Studies Conducted in Weaned Piglets using Protein Hydrolysate of Porcine Intestinal Mucosa (Referred to as DPS)		
Reference and Objective	**Study Design**	**Key Findings Related to Safety and Utility**
Zimmerman and Sparks, 1997 (continued) **Objective:** Evaluation of a by-product from hydrolyzed porcine small intestines as an ingredient pig starters	***Experiment 3***	
	Animals: ~100 weaned piglets, (13.9lb IW) breed not stated (4 or 5 piglets/pen; 4 or 5 pens/treatment) **Test Article:** Three different experimental DPS products, SDAP **Treatment:** Control, 6% of each DPS product, 5% SDAP **Duration:** 28 days (14 days treatment diet + 14 days common diet)	During the first week, piglets fed the diet containing spray-dried plasma consumed more feed and gained weight faster and more efficiently than piglets fed other diets. In the third and fourth weeks, when all piglets were fed a common diet, there was a trend for piglets that had previously consumed diets containing experimental hydrolysates to consume more feed and gain weight more rapidly than piglets that previously had been fed the control and plasma diets.
	Experiment 4	
	Animals: 84 weaned piglets (13.8lb IW), breed not stated (4 piglets/pen; 7 pens/treatment) **Test Article:** DPS **Treatment:** Control, 6% DPS for 2 weeks, 6% DPS for 4 weeks. **Duration:** 28 days	DPS did not improve performance of the weaned piglets in the first two weeks after weaning; but in weeks three and four, piglets that had been fed intestinal DPS and those that continued to be fed DPS grew more rapidly and consumed more feed than those fed the control diets.

Abbreviations: ADFI = average daily feed intake; ADG = average daily gain; CPS = condensed porcine soluble; DPS = dried porcine solubles; G:F = gain-to-feed ratio; IW = initial body weight; SBM = soybean meal; SDAP = spray dried animal plasma; SDBC = spray dried blood cells; SDP = spray dried plasma; SDPP = spray dried plasma protein; SMFM = select menhaden fish meal.

6.5.9 Overall Conclusions from the Studies in Swine

Collectively, a number of studies were identified in swine in which **protein hydrolysate of porcine intestinal mucosa** (spray-dried or on a carrier, and commercially often referred to as DPS) was demonstrated to be a highly digestible form of protein, which was not associated with any adverse effects on performance of growing, lactating and reproducing sows. The levels of inclusion of **protein hydrolysate of porcine intestinal mucosa** varied but were generally between 1 to 6% in feeding studies conducted in pigs. Assuming that **protein hydrolysate of porcine intestinal mucosa** is incorporated into the diet of swine at 3% DM, on a bw basis the intakes can be estimated for each category of animal as follows:

- For a weaned piglet with a FI of 0.88 kg DM/day and bw of 20 kg – 2.6 g/kg bw/day
- For a grower/finisher pig with a FI of 2.20 kg DM and bw of 60 kg – 2.2 g/kg bw/day
- For a lactating sow with a FI of 5.28 kg DM and bw of 175 kg – 1.8 g/kg bw/day

By comparison, the estimated intake of **oligosaccharides-peptides complex** by cats and dogs can be estimated by taking the mean bw and FI of the animals in the 26-week feeding studies above and an inclusion level of 1.5% in the diet (see Section 6.4):

- For a cat with a FI of 0.06 kg DM/day and bw of 4.6 kg – 0.2 g/kg bw/day
- For a medium (e.g., beagle) dog with a FI of 0.16 kg DM/day and bw of 7.7 kg – 0.3 g/kg bw/day

Thus, the exposure by growing, lactating and reproducing pigs to **protein hydrolysate of porcine intestinal mucosa** on a bw basis is approximately 10-fold higher than the anticipated intakes by cats and dogs to **oligosaccharides-peptides complex** under the maximum intended use level of 1.5% in the diet.

A similar calculation can be performed for kittens and puppies based on the dietary recommendations of commercial dry kitten and puppy food by brands such as PURINA[5]:

- For a kitten with a FI of 0.07 kg DM/day and bw of 1.3 kg – 0.8 g/kg bw/day
- For a puppy (small breed) with a FI of 0.054 kg DM/day and a bw of 1.4 kg – 0.6 g/kg bw/day

Thus, the exposure by growing pigs to **protein hydrolysate of porcine intestinal mucosa** on a bw basis is approximately 3 to 4-fold higher than the anticipated intakes by kittens and puppies to **oligosaccharides-peptides complex** under the maximum intended use level of 1.5% in the diet.

It is challenging to estimate the likely exposure by swine to the glycopeptides component of **oligosaccharides-peptides complex** on the basis these levels are not routinely measured and will be significantly lower in **protein hydrolysate of porcine intestinal mucosa**. However, it is reasonable to assume that there may be some exposure from products derived from manufacturing plants where the glycopeptides component is carried over at similar levels to that of the supplier of **protein hydrolysate of porcine intestinal mucosa** to Gnubiotics. Thus, these data provide supporting evidence for the safety of the source (**protein hydrolysate of porcine intestinal mucosa**) and in particular the free amino

[5] https://www.walmart.com/ip/Purina-Kitten-Chow-Nurture-Dry-Kitten-Food-Muscle-Brain-Development-Chicken-Recipe-14-lb-Bag/21623785 and https://www.walmart.com/ip/Purina-ONE-Natural-High-Protein-Dry-Puppy-Food-Plus-Healthy-Puppy-Formula-8-lb-Bag/10448981

acids and peptides components of **oligosaccharides-peptides complex** for all life stages of cats and dogs.

(b) (6)

No chemical transformation occurs and the composition of the glycopeptides and peptides components will reflect the glycoproteins of porcine intestinal mucosa. Thus, the studies in swine using the less pure source material, **protein hydrolysate of porcine intestinal mucosa**, can be extrapolated to support the safety of **oligosaccharides-peptides complex**.

6.6 Studies using MMOs with Structural Similarity to the Glycan Component of Oligosaccharides-Peptides Complex

As mentioned in Section 6.2.2, the glycopeptides component, which represents around 20% of **oligosaccharides-peptides complex**, will be resistant to hydrolysis by proteolytic enzymes in the GI tract but will be subject to microbial fermentation in the large intestine. The glycans side chains (prime constituent) of the glycopeptides component display similar structural diversity as MMOs and numerous studies have been conducted supporting the safety of these ingredients. Similar to other oligosaccharides, these MMOs are not metabolized or absorbed until being fermented in the large intestine.

6.6.1 MOs in Feline and Canine Milk

As mentioned in Section 2.7, feline and canine milk contain structurally diverse oligosaccharides, which are known to play an important nutritional role as fermentable polysaccharides in the development of digestive function by young animals. The glycans side chains (prime constituent) of the glycopeptides component in **oligosaccharides-peptides complex** exhibit similar structural diversity and in this respect may be considered biological mimics of these feline and canine MOs. On the basis of their structural similarities, the exposure by young cats and dogs to MOs in feline and canine milk provides supporting evidence for the tolerability to the target animals of the glycans side chains which are linked through *O*-glycosidic bonds to peptides in the glycopeptides component of **oligosaccharides-peptides complex**.

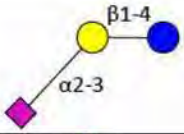
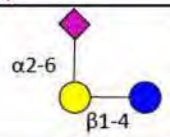
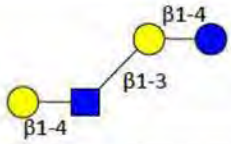
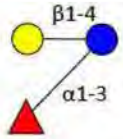
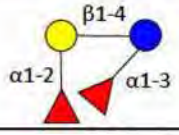
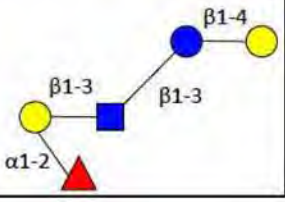
Estimating the exposure by puppies to MOs is challenging on the basis that (1) MO concentrations are highest in the early lactation period and reduce with time, and (2) the intake of milk will depend on the breed and size of the puppy. Rostami *et al*. (2014) conducted an investigation into the levels of individual MOs in canine milk at various time points over the lactation period. The results of the experiments are described in Section 2.7 and indicate that the concentration of the major individual MOs in canine milk is at least 8 g/L (0.8%) in the first few days of lactation. By comparison, under the conditions of intended use, **oligosaccharides-peptides complex** will provide around 0.3% glycopeptides to dogs of all life stages, which is approximately 2.5 times lower than the levels of MOs in canine milk of newborn puppies. Given the similarities in the structure of glycans from MOs and as side chains in glycopeptides as well as their function as fermentable fibers, it is reasonable to assume that the glycopeptides component of **oligosaccharides-peptides complex** will be well-tolerated by cats and dogs under the conditions of intended use.

6.6.2 Toxicological Studies on Individual Mimics of Milk Oligosaccharides

A number of individual oligosaccharides have been developed for use as ingredients in human infant formula intended to act as mimics for HMOs. On the basis that these individual oligosaccharides are structurally-related to the glycans side chains (prime constituent) of glycopeptides derived from porcine intestinal mucosa, toxicological information on these ingredients may be considered relevant to the assessment of the safety of **oligosaccharides-peptides complex** for use in cat and dog food.

Structural Comparisons of Glycans

A comparison the components comprising the glycans of glycopeptides in **oligosaccharides-peptides complex** and the individual HMOs mimics produced for use as ingredients in human infant formula is provided in Table 6.8. As previously noted (see Sections 2.7 and 2.8), there are many structural similarities between these individual HMOs mimics and the glycans (as glycopeptides) in **oligosaccharides-peptides complex**.

Table 6.8: Comparison of Monosaccharides from Oligosaccharides-Peptides Complex and HMOs Mimics Used as Test Articles in Toxicity Studies				
Monosaccharides from Oligosaccharides-Peptides Complex Glucose (Glc) ● Galactose (Gal) ○ N-Acetylgalactosamine (GalNAc) □ N-Acetylglucosamine (GlcNAc) ■ Sialic acid (Neu5Ac) ◆ Fucose (Fuc) ▲				
Human Milk Oligosaccharide	**Monosaccharide components**	**Structure**		**Reference**
	Sialic acid	Neu5Ac		Choi *et al.*, 2014
3'-Sialyllactose	Galactose, Glucose, Sialic acid	Neu5Acα2-3Galβ1-4Glc		Kim *et al.*, 2018; Parschat *et al.*, 2020
6'-Sialyllactose	Galactose, Glucose, Sialic acid	Neu5Acα2-6Galβ1-4Glc		Gurung *et al.*, 2018; Parschat *et al.*, 2020
Lacto-*N*-Neotetraose	Galactose, *N*-acetylglucosamine, Glucose	Galβ1-3GluNAcβ1-3Galβ1-4Glc		Coulet *et al.*, 2013; Prieto, 2005
2'-Fucosyllactose	Fucose, Glucose, Galactose	Fucα1-3Galβ1-4Glc		Coulet *et al.*, 2014; Phipps *et al.*, 2018; Phipps *et al.*, 2020;
Difucosyllactose	Fucose, Glucose, Galactose	Fucα1-2Galβ1-4[Fuc-(α1-3)]Glc		Phipps *et al.*, 2018
Lacto-*N*-Fucopentaose I	Galactose, *N*-acetylglucosamine, Glucose, Fucose	Galβ1-3[Fuc α1-2]GlcNAcβ1-3Galβ1-4Glc		Phipps *et al.*, 2020

Toxicity Studies

A battery of toxicity studies was identified in the published literature using a number of the different individual HMOs mimics of interest to the human infant formula industry. These studies were typically conducted in order to support regulatory acceptance of these ingredients for use in infant formula in the EU and U.S. and have been the subject of a number of novel foods assessments conducted by the EFSA (e.g., EFSA, 2015a and b) and GRAS notifications reviewed by the U.S. FDA (e.g., GRN 546, 571 and 81;

U.S. FDA, 2015a, b and 2019). Notably, although the primary use is in infant formula, there is also interest the use of these oligosaccharides in food supplements for young children (EFSA, 2015b). The HMOs mimics evaluated in the toxicity studies were 3'-sialyllactose (Kim *et al*., 2018), 6'-sialyllactose (Gurung *et al*., 2018), 2' -fucosyllactose (Coulet *et al*., 2014), Lacto-N-neotetraose (Coulett *et al*., 2013) or HMO mixtures (Parschat *et al*., 2020) and lacto-N-fucopentaose I and 2' -fucosyllactose (Phipps *et al*., 2020). The available short-term and subchronic toxicity studies are summarized in Table 6.9 and genotoxicity data in Table 6.10. Considering that the majority of these studies were conducted to assess the safety for inclusion in human infant milk formula the HMOs mimics were administered by gavage, starting at postnatal day 7 (PND 7).

Short-Term and Subchronic Toxicology Data

Overall, no adverse effects were observed in the short-term and subchronic toxicity studies. The No-Observed-Adverse-Effect-Levels (NOAELs) derived from 90-day oral toxicity studies in rats conducted in accordance with Good Laboratory Practice (GLP) and OECD 408 guidelines were the highest doses tested. These doses were generally 5,000 mg of the HMOs mimic/kg bw/day. By comparison, it is estimated that **oligosaccharides-peptides complex** contains in the region of 20% glycopeptides of which the glycans represents around 10% (see Section 2.4.3). The estimated intakes of glycopeptides by cats and dogs can be estimated by taking the mean bw and FI of the animals in the 26-week feeding studies above (see Section 6.4) and an inclusion level of 0.03% in the diet (based on 1.5% **oligosaccharides-peptides complex** inclusion level):

- For a cat with a FI of 0.06 kg DM/day and bw of 4.6 kg – 40 mg/kg bw/day
- For a medium (e.g., beagle) dog with a FI of 0.16 kg DM/day and bw of 7.7 kg – 60 mg/kg bw/day

Thus, the exposure by adult cat and dogs to the glycopeptides component is 125 and 83-fold lower, respectively, than the NOAELs derived from the toxicity data in rats.

Similarly, the estimated intakes of glycopeptides by kittens and puppies can estimated based on recommended feeding practices of food manufacturers such as Purina:

- For a 12-week old kitten with a FI of 0.07 kg DM/day and bw of 1.3 kg – 160 mg/kg bw/day
- For a 12-week old puppy (small breed) with a FI of 0.054 kg and bw of 1.4 kg – 100 mg/kg bw/day

Thus the exposure by kittens and puppies (small breed) to the glycopeptides component of oligosaccharides peptides component is 31 and 50-fold lower, respectively, than the NOAELs derived from the toxicity data in rats. Thus, although the exposure by kittens and puppies is higher than for adult animals, a significant margin of safety is still achieved relative to the derived NOAELs.

Notably, there was also one study conducted in Beagle dogs in which the highest use level of 2,000 mg/kg bw/day was well-tolerated when delivered by gavage (Kim *et al*., 2018).

Table 6.9: Short-Term and Subchronic Toxicity Studies using HMOs Mimics						
Species (Strain)	**Route of Administration**	**Dose**	**Test Article**	**Study Design (Quality system)**	**Reported Effects**	**Reference**
Mixture of 2'-Fucosyllactose and Difucosyllactose						
Rat [Crl:CD® (SD)] Juveniles (PND 7) (10/sex/group)	Oral gavage	0 (vehicle control), 1,000, 3,000, or 5,000 mg/kg bw/day	2'FL/DFL (92.2% purity; 2-FL 82.5% + DFL 9.7%)	90-day oral toxicity study followed by a 4 week recovery period (GLP & OECD Guideline Test No. 408)	No toxicological relevant findings were observed at any dose A NOAEL of 2'FL/DFL was determined to be 5,000 mg/kg bw/day, the highest dose used in the study	Phipps *et al.*, 2018
Mixture of Lacto-N-Fucopentaose I and 2'-fucosyllactose						
Rat [Sprague-Dawley] Juveniles (PND 7) (10/sex/mid and low dose groups; 15/sex/ vehicle control and high dose groups)	Oral gavage	0 (vehicle control), 1,000, 3,000, or 5,000 mg/kg bw/day	LNFP + 2'FL (91% purity; LNFP-1 59.4% + 2'FL 31.5%)	90-day oral toxicity study followed by a 4 week recovery period (GLP & OECD Guideline Test No. 408)	No toxicological relevant findings were observed at any dose A NOAEL of LNFP + 2'FLwas determined to be 5,000 mg/kg bw/day, the highest dose used in the study	Phipps *et al.*, 2020
Mixture of Mixed Human Oligosaccharides						
Rat [CD] (10/sex/group)	Diet	0 or 10 HMO mix	HMO mix (94.9% DW)[1] 2'-FL (47.1% DW), 3-FL (16.0% DW), LNT (23.7%DW), 3'-	90-day oral toxicity study followed by a 4 week recovery period	No toxicological relevant findings were observed at any dose The NOAEL for HMO MIX I in this study is 10% in the diet (equivalent to 5.67 g HMO MIX I/kg bw/day for males and 6.97 g HMO MIX I/kg bw/day for females)	Parschat *et al.*, 2020

Table 6.9: Short-Term and Subchronic Toxicity Studies using HMOs Mimics						
Species (Strain)	**Route of Administration**	**Dose**	**Test Article**	**Study Design (Quality system)**	**Reported Effects**	**Reference**
			SL (4.1% DW), 6'-SL (4.0% DW), other carbohydrates (5.1% DW)	(GLP & OECD Guideline Test No. 408)		
Studies on N-Acetyl-D-Neuraminic Acid						
Rat [Sprague-Dawley] (26/sex/group)	Diet	0, 0.5%, 1.0%, or 2.0%	N-Acetyl-D-neuraminic acid dihydrate (purity 98.6%)	13-week dietary toxicity phase preceded by an in utero phase (OECD Guideline Test No. 408)	No toxicological relevant findings were observed at any dose *N*-Acetyl-D-neuraminic acid was without maternal toxicity or compound-related adverse effects on female reproduction and on the general growth and development of offspring at a maternal dietary level of up to 2%, equivalent to a dose of 1895 mg/kg bw/day. During the subchronic phase, no compound-related adverse effects were observed in first generation rats at dietary levels of up to 2% (highest level tested), corresponding to doses of 974 and 1246 mg/kg bw/day in males and females, respectively.	Choi *et al.*, 2014
3'-Sialyllactose						
Rat [Sprague-Dawley] (5/sex/group)	Oral gavage	Single dose of 0, 5, 10, 15, or 20 g/kg bw	3'-sialyllactose sodium salt (purity 98.8%)	14 day acute oral toxicity study (GLP & FDA Redbook 2000, Chapter IV.C.2.))	No toxicological relevant findings were observed at any dose The lethal dose (LD50) of 3'- sialyllactose sodium salt was well above 20 g/kg bw/day, the highest dose tested	Kim *et al.*, 2018

Table 6.9: Short-Term and Subchronic Toxicity Studies using HMOs Mimics						
Species (Strain)	**Route of Administration**	**Dose**	**Test Article**	**Study Design (Quality system)**	**Reported Effects**	**Reference**
Rat [Sprague-Dawley] (10/sex/group)	Oral gavage	0, 500, 1,000, or 2,000 (mg/kg bw)	3'-sialyllactose sodium salt (purity 98.8%)	28-day oral toxicity study (GLP &OECD Guideline Test No. 407)	No toxicological relevant findings were observed at any dose A NOAEL of 3'-sialyllactose sodium salt was determined to be 2,000 mg/kg bw/day the highest dose used in the study	Kim *et al.*, 2018
Rat [Sprague-Dawley] (10/sex/group)	Oral gavage	0 (control; purified water), 500, 1,000, or 2,000 (mg/kg bw)	3'-sialyllactose sodium salt (purity 98.8%)	90-day oral toxicity study (GLP & OECD Guideline Test No. 408)	No toxicological relevant findings were observed at any dose The NOAEL of 3'-SL sodium salt was determined to be higher than 2000 mg/kg bw/day in the oral subchronic toxicity study in rats	Kim *et al.*, 2018
Dog [Beagle] (4 male and 4 female)	Oral gavage	3 dose changes (500 → 1,000 → 2,000 mg/kg) at 4 day intervals. Control animals received water at the same doses	3'-sialyllactose sodium salt (purity 98.8%)	Dose escalation single oral dose toxicity study	No treatment-related effects on clinical signs, bw, or food consumption in any animals in the dosing groups and ophthalmic examinations revealed no abnormalities Transient diarrhea was observed 4 hours after treatment in 2 males and 1 female in the 2000 mg/kg group The maximum tolerance dose of 3'-sialyllactose sodium salt was 2,000 mg/kg bw/day in male and female Beagle dogs, the highest dose tested in the study	Kim *et al.*, 2018

Table 6.9: Short-Term and Subchronic Toxicity Studies using HMOs Mimics						
Species (Strain)	**Route of Administration**	**Dose**	**Test Article**	**Study Design (Quality system)**	**Reported Effects**	**Reference**
6'-Sialyllactose						
Rat [Sprague-Dawley] (5/sex/group)	Oral gavage	0, 5, 10, 15, or 20 g/kg bw	6'-sialyllactose sodium salt (purity >98%)	Acute toxicity study (GLP & FDA Redbook 2000, Chapter IV.C.2.)	No toxicological relevant findings were observed at any dose A NOAEL of 6'-sialyllactose sodium salt was determined to be 20 g/kg bw/day the highest dose used in the study	Gurung *et al.*, 2018
Rat [Sprague-Dawley] (11/sex/group)	Oral gavage	0 (control; purified water), 1.0, 2.5, or 5.0 g/kg bw	6'- sialyllactose sodium salt (purity >98%)	90-day oral toxicity study (GLP & FDA Redbook 2000, Chapter IV.C.4.a)	No toxicological relevant findings were observed at any dose A NOAEL of 6'-sialyllactose sodium salt was determined to be 5.0 g/kg bw/day the highest dose used in the study	Gurung *et al.*, 2018
Lacto-N-Neotetraose						
Rat [Wistar IGS:Crl:WI Han rats] juveniles (PND 7) (5/sex/group)	Oral gavage	0, 1,000, 2,500 or 5,000 mg/kg bw/day	Lacto-N-Neotetraose (98.9%)	14-day dose range finding study (non GLP)	No toxicological relevant findings were observed at any dose The highest dose of 5,000 mg/kg bw/day Lacto-N-Neotetraose was considered suitable for the 28 and 90 day studies	Coulet *et al.*, 2013
Rat [Wistar IGS:Crl:WI Han rats], juveniles (PND 7) (10/sex/group	Oral gavage	0, 1,000, 2,500 or 5,000 mg/kg bw/day	Lacto-N-Neotetraose (98.9%)	28-day oral toxicity study (GLP & OECD Guideline Test No. 407)	No toxicological relevant findings were observed at any dose A NOAEL of Lacto-N-Neotetraose was determined to be 5,000 mg/kg bw/day for both male and female rats, the highest dose used in the study	Coulet *et al.*, 2013

Table 6.9: Short-Term and Subchronic Toxicity Studies using HMOs Mimics						
Species (Strain)	**Route of Administration**	**Dose**	**Test Article**	**Study Design (Quality system)**	**Reported Effects**	**Reference**
Rat [Wistar IGS:Crl:WI Han rats], juveniles (15 days old) (10/sex/group	Oral gavage	0, 1000, 2,500 or 5,000 mg/kg bw/day	Lacto-N-Neotetraose (98.9%)	90-day oral toxicity study (GLP & OECD Guideline Test No. 408)	No toxicological relevant findings were observed at any dose A NOAEL of Lacto-N-Neotetraose was determined to be 5,000 mg/kg bw/day for both male and female rats, the highest dose used in the study	Coulet *et al.*, 2013
Rat [Cri:CD®BR] Juvenilles	Oral gavage	0 (control), 10, 200 or 400 mg/kg/ day	Lacto-N-Neotetraose	28-day oral toxicity study	No toxicological relevant findings were observed at any dose A NOAEL of Lacto-N-Neotetraose was determined to be 400 mg/kg bw/day for both male and female rats, the highest dose used in the study	Prieto, 2005
2'-Fucosyllactose						
Rat [Wistar IGS:Crl:WI Han rats] juveniles (PND 7) (5/sex/group)	Oral gavage	0, 2,000, 5,000 or 7,500 mg/kg bw/day	2'-fucosyllactose (purity >99%)	14-day dose range finding study (non GLP)	Liquid (colored yellowish) feces, animals administered 2'-fucosyllactose, at doses of 5,000 and 7,500 mg/kg bw/day from days 1 to 3 and up to days 9 to 11 Deaths of 2 out of 5 females occurred at the highest 2' FL dose level. The cause of death could not be determined Females from the highest dose 2'FL had slightly lower bw than in control (-7.7%) The highest dose level for the 90 day toxicity study was set at 6,000 mg/kg bw/day	Coulet *et al.*, 2014

Table 6.9: Short-Term and Subchronic Toxicity Studies using HMOs Mimics						
Species (Strain)	**Route of Administration**	**Dose**	**Test Article**	**Study Design (Quality system)**	**Reported Effects**	**Reference**
Rat [Wistar IGS:Crl:WI Han rats] juveniles (PND 7) (10/sex/group)	Oral gavage	0 (vehicle control), 2,000, 5,000 or 6,000 mg/kg bw/day	2'-fucosyllactose (purity >99%)	90-day oral toxicity study (GLP & OECD Guideline Test No. 408)	No toxicological relevant findings were observed at any dose A NOAEL of 2'-fucosyllactose was determined to be 5,000 mg/kg bw/day for both male and female rats the highest dose used in the study	Coulet *et al.*, 2014

Abbreviations: 2'FL = 2'-fucosyllactose; 2'FL/DFL = 2'-fucosyllactose/difucosyllactose mixture; 3'FL = 3'-fucosyllactose; 3'SL = 3'-sialyllactose; 6'SL = 6'-sialyllactose; bw = body weight; DFL = difucosyllactose; DW = dry weight; GLP = good laboratory practice; HMO = human milk oligosaccharides; LNFP = lacto-N-fucopentaose; LNnT = lacto-N-neotetraose; LNT = lacto-N-tetraose; NOAEL = no observed adverse effect level; OECD = Organization for Economic Co-operation and Delevelopment; PND 7 = postnatal day 7.

[1] The aforementioned oligosaccharides were produced individually by fermentation, following the removal of the production strain and further purification and concentration processes.

Genotoxicity Studies

Negative findings were reported in bacterial reverse mutation and mouse lymphoma assays using the individual HMOs mimics. Likewise, the HMOs mimics were observed not be mutagenic or clastogenic as measured *in vitro* using the chromosome aberration assay or *in vivo* as assessed by the micronucleus test from mouse bone marrow cells. These findings further support the lifetime exposure by cats and dogs to the glycopeptides component of **oligosaccharides-peptides complex** under the conditions of intended use.

6.6.3 Overall Conclusions of the Toxicological Information on Individual HMOs Mimics

Taken together, the body of available toxicological information provides corroborative evidence that the glycans side chains (prime constituent) of the glycopeptides component of **oligosaccharides-peptides complex** will not be associated with any safety concerns under the conditions of intended use in food for cats and dogs of all life stages at levels not to exceed 1.5% by weight, equating to approximately 0.3% glycopeptides.

Table 6.10: Genotoxicity Studies using HMOs Mimics					
Assay (Quality system)	**Test System**	**Test Article**	**Concentration**	**Results**	**Reference**
In vitro					
Mixture of 2'-Fucosyllactose and Difucosyllactose					
Bacterial reverse mutation assay (FDA Redbook 2000: chapter IV.C.1.a, EPA effects guidelines OPPTS 870.5100 & OECD Guideline Test No. 471)	*Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537, and *Escherichia coli* strain WP2 uvrA (pKM101) in the presence or absence of S9 activation	2'FL/DFL (92.2% purity; 2-FL 82.5% + DFL 9.7%)	5, 15, 50, 150, 500, 1,500 or 5,000 µg/plate	The study author concluded that the test article was not mutagenic	Phipps *et al.*, 2018
Mammalian cell micronucleus (OECD Guideline Test No. 487)	Human peripheral blood lymphocytes in the presence or absence of S9 activation	2'FL/DFL (92.2% purity; 2-FL 82.5% + DFL 9.7%)	500, 1,000 or 2,000 µg/mL,	The study author concluded that the test article showed no evidence of mutagenic/clastogenic activity in this test	Phipps *et al.*, 2018
Mixture of Lacto-N-Fucopentaose I and 2'-fucosyllactose					
Bacterial reverse mutation assay (OECD Guideline Test No. 471)	*Salmonella typhimurium* strains TA98, TA100, TA1535, and TA1537, or *Escherichia coli* strain WP2 uvrA (pKM101)) in the presence or absence of S9 activation	LNFP + 2'FL (91% purity; LNFP-1 59.4% + 2'FL 31.5%)	500, 1,000, or 2,000 µg/mL	The study author concluded that the test article was not mutagenic	Phipps *et al.*, 2020
Mammalian cell micronucleus (OECD Guideline Test No. 487)	Human peripheral blood lymphocytes in the presence or absence of S9 activation	LNFP + 2'FL (91% purity; LNFP-1 59.4% + 2'FL 31.5%)	0.5, 5, 50, 500, 1,000, or 2,000 µg/mL	The study author concluded that the test article showed no evidence of mutagenic/clastogenic activity in this test	Phipps *et al.*, 2020

Table 6.10: Genotoxicity Studies using HMOs Mimics					
Assay (Quality system)	**Test System**	**Test Article**	**Concentration**	**Results**	**Reference**
Mixture of Mixed Human Oligosaccharides					
Bacterial reverse mutation assay (OECD Guideline Test No. 471)	Salmonella typhimurium strains TA98, TA100, TA102, TA1535, and TA1537 in the presence or absence of S9 activation	HMO mix (94.9 % DW)[1] 2'-FL (47.1% DW), 3-FL (16.0%DW), LNT (23.7%DW), 3'-SL (4.1%DW), 6'-SL (4.0%DW), other carbohydrates (5.1%DW)	5.0, 10.0, 31.6, 100, 316 or 600 mg HMO MIX l/plate	The study author concluded that the test article was not mutagenic	Parschat *et al.*, 2020
Mammalian cell micronucleus (OECD Guideline Test No. 487)	Human peripheral blood lymphocytes in the presence or absence of S9 activation	HMO mix (94.9 % DW) 2'-FL (47.1% DW), 3-FL (16.0%DW), LNT (23.7%DW), 3'-SL (4.1%DW), 6'-SL (4.0%DW), other carbohydrates (5.1%DW)	7.5, 15, 30 or 60 mg/mL	The study author concluded that the test article showed no evidence of mutagenic/clastogenic activity in this test	Parschat *et al.*, 2020

Table 6.10: Genotoxicity Studies using HMOs Mimics					
Assay (Quality system)	**Test System**	**Test Article**	**Concentration**	**Results**	**Reference**
Studies on N-Acetyl-D-Neuraminic Acid					
Bacterial reverse mutation assay (ICH guidelines for genotoxicity testing & OECD Guideline Test No. 471)	*Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537 and *Escherichia coli* strain WP2 uvrA in the presence or absence of S9 activation	N-Acetyl-D-neuraminic acid dihydrate (purity 98.6%)	50, 150, 500, 1,500, or 5,000 µg/plate of N-Acetyl-D-neuraminic acid dihydrate (purity 98.6%)	The study author concluded that the test article was not mutagenic	Choi *et al.*, 2014
Mammalian cell micronucleus (OECD Guideline Test No. 487)	Human peripheral blood lymphocytes in the presence or absence of S9 activation	N-Acetyl-D-neuraminic acid dihydrate (purity 98.6%)	1,690, 2,420, and 3,450 µg/mL	The study author concluded that the test article showed no evidence of mutagenic/clastogenic activity in this test	Choi *et al.*, 2014
3'-Sialyllactose					
Bacterial reverse mutation assay (OECD Guideline Test No. 471)	*Salmonella typhimurium* strains TA98, TA100, TA1535, and TA1537) and *Escherichia coli* strain WP2uvrA (pKM101) in the presence or absence of S9 activation	3'-sialyllactose sodium salt (purity 98.8%)	313, 625, 1,250, 2,500 or 5,000 µg/plate	The study author concluded that the test article was not mutagenic	Kim *et al.*, 2018
6'Sialyllactose					
Bacterial reverse mutation assay (FDA Redbook 2000: chapter IV.C.1.a)	*Salmonella typhimurium* strains TA97, TA98, TA100, TA102, and TA1535 in the presence or absence of S9 activation	6'-sialyllactose sodium salt (purity >98%)	100, 300, 625, 1,250, 2,500, and 5,000 µg/plate	The study author concluded that the test article was not mutagenic	Gurung *et al.*, 2018
Chromosomal aberrations assay	Chinese hamster lung (CHL) cells in the presence or absence of S9 activation	6'-sialyllactose sodium salt (purity >98%)	225, 450 or 900 µg/mL	The study author concluded that the test article showed no evidence of mutagenic/clastogenic activity in this test	Gurung *et al.*, 2018

Table 6.10: Genotoxicity Studies using HMOs Mimics					
Assay (Quality system)	**Test System**	**Test Article**	**Concentration**	**Results**	**Reference**
(FDA Redbook 2000: chapter IV.C.1.b)					
2'-Fucosyllactose					
Bacterial reverse mutation assay (OECD Guideline Test No. 471)	*Salmonella typhimurium* histidine auxotrophic strains TA98, TA100, TA1535, TA1537 and TA102 in the presence or absence of S9 activation	2'-fucosyllactose (purity >99%)	52, 164, 512, 1,600 or 5,000 µg/plate	The study author concluded that the test article was not mutagenic	Coulet *et al.*, 2014
Mouse lymphoma assay (OECD test guideline 476)	Mouse lymphoma L5178Y cells in the presence or absence of S9 activation	2'-fucosyllactose (purity >99%)	Short treatment 492, 878, 1,568, 2,800 or 5,000 µg/mL Long treatment 0, 1.7, 5.4, 17, 52, 164, 512, 1,600 or 5,000 µg/mL	The study author concluded that the test article was not mutagenic	Coulet *et al.*, 2014
Lacto-N-Neotetraose					
Bacterial reverse mutation assay (OECD Guideline Test No. 471)	*Salmonella typhimurium* histidine auxotrophic strains TA98, TA100, TA1535, TA1537 and TA102 in the presence or absence of S9 activation	Lacto-N-Neotetraose (98.9%)	52, 164, 512, 1,600 and 5000 µg /plate	The study author concluded that the test article was not mutagenic	Coulet *et al.*, 2014
Mouse lymphoma assay (OECD test guideline 476)	Mouse lymphoma L5178Y cells in the presence or absence of S9 activation	Lacto-N-Neotetraose (98.9%)	Short treatment 0, 418, 746, 1,333, 2,380 or 4,250 µg/mL Long treatment 0, 1.4, 4.5, 14, 44, 139, 435, 1,360 or 4,250 µg/mL	The study author concluded that the test article was not mutagenic	Coulet *et al.*, 2014

Table 6.10: Genotoxicity Studies using HMOs Mimics					
Assay (Quality system)	**Test System**	**Test Article**	**Concentration**	**Results**	**Reference**
In vivo					
3'-Sialyllactose					
Micronucleus test (OECD Guideline Test No. 473)	Mouse bone marrow cells	3'-sialyllactose sodium salt (purity 98.8%)	0, 500, 1,000, and 2,000 mg/kg bw	The study author concluded that the test article showed no evidence of mutagenic/clastogenic activity in this test	Kim *et al.*, 2018
6'Sialyllactose					
Micronucleus test (FDA Redbook 2000: chapter IV.C.1.d)	Mouse bone marrow cells	6'-sialyllactose sodium salt (purity >98%)	0, 500, 1,000, and 2,000 mg/kg bw	The study author concluded that the test article showed no evidence of mutagenic/clastogenic activity in this test	Gurung *et al.*, 2018

Abbreviations: 2'FL = 2'-fucosyllactose; 2'FL/DFL = 2'-fucosyllactose/difucosyllactose mixture; 3'FL = 3'-fucosyllactose; 3'SL = 3'-sialyllactose; 6'SL = 6'-sialyllactose; bw = body weight; DW = dry weight; HMO = human milk oligosaccharides; LNFP = lacto-N-fucopentaose; OECD = Organization for Economic Co-operation and Development;

[1] The aforementioned oligosaccharides were produced individually by fermentation, following the removal of the production strain and further purification and concentration processes.

6.7 Summary and Basis for the GRAS Conclusion

Gnubiotics intended to market **oligosaccharides-peptides complex** as a source free amino acids, peptides and glycopeptides for all life stages of cats and dogs at levels not to exceed 1.5% by weight in the diet. The primary application of **oligosaccharides-peptides complex** will be as an ingredient in complete cat and dog food, but the ingredient may also be incorporated into supplementary foods such as top-dressing sachets and treats.

The ingredient is manufactured from **protein hydrolysate of porcine intestinal mucosa** obtained from pigs fit for human consumption. The raw material is a recognized feed material in the EU and is marketed for use as a source of protein for pigs, poultry and fish.

One of the primary components of porcine intestinal mucosa are mucins, which are large extracellular glycoproteins responsible for the viscous, gel-like properties of mucus. They are highly glycosylated, comprising around 80% oligosaccharides which are linked by *O*-glycosidic bonds to the hydroxyl chains of serine and threonine in the protein core. The oligosaccharides are known as glycans, display moderate branching and exist as a diverse range of core structures which are common to mammalian mucins but also MMOs. (b) (6), (b) (4) The commercial manufacturing process will be conducted in accordance with cGMP, and a HACCP plan in place. The manufacturer will comply with the requirements for importing feed into the U.S. laid down by the FSMA including the FSVP, and the Bioterrorism Act (2002).

The resultant **oligosaccharides-peptides complex** comprises **free amino acids** (b) (4) well as **glycopeptides and peptides** (b) (4) Appropriate feed-grade specifications have been established for the product which sets well-defined ranges for the key compositional parameters, as well as criteria to control the levels of heavy metal contaminants and microorganisms. The results of analysis of 6 representatively lots of **oligosaccharides-peptides complex** demonstrate compliance with the proposed compositional and contaminant specifications.

A shelf-life of 20 months is proposed for **oligosaccharides-peptides complex** when stored unopened in the original packaging under cool and dry conditions. Stability data are provided for 3 representative batches of **oligosaccharides-peptides complex** under accelerated and real-time conditions which provide support for the proposed shelf-life. Considering that the moisture content will be controlled in the commercial batches and that the levels of microorganisms will meet acceptable criteria after manufacture, spoilage by growth of microorganisms is not expected over the proposed shelf-life of the product.

Analytical data on 6 representative batches of **oligosaccharides-peptides complex** indicate that in practice, glycopeptides and peptides, free amino acids and ash contents are around 42%, 40% and 13%,

respectively. Further compositional analysis indicates that the glycopeptides and peptides components represent around 20% and 21%, respectively of **oligosaccharides-peptides complex**.

Glycopeptides are comprised of glycans linked via *O*-glycosidic bonds to peptides. The glycans side chains of the glycopeptides component exist as a diverse range of structures and their steric properties render the peptides less susceptible to hydrolysis by proteolytic enzymes. Thus, the glycopeptides component is expected to remain largely intact during their passage through the GI tract.

Nutritionally, **oligosaccharides-peptides complex** provides a source of highly digestible **free amino acids** (*ca*. 40%) and **peptides** (*ca*. 21%), as well as a source of fermentable fiber in the form of **glycopeptides** (*ca*. 20%). The contribution of **oligosaccharides-peptides complex** to the nutrient recommendations laid down by AAFCO for cats and dogs of all life stages is estimated to be relatively low compared to the major protein and fermentable fiber sources in the diet. Thus, **oligosaccharides-peptides complex** will provide a supplementary source of nutrients alongside, rather than as a substitute for, other ingredients in the diet. *In vitro* modelling of the fermentation characteristics of the peptides and glycopeptides component of **oligosaccharides-peptides complex** confirms that the product acts as a source of fermentable fiber and is associated with SCFA production. Overall, under the conditions of intended use in cat and dog food, there are no anticipated nutritional disadvantages associated with **oligosaccharides-peptides complex**. Moreover, beyond the compositional value as a supplementary amino acid, peptides and oligosaccharides (as glycopeptides) source, the technical effect of **oligosaccharides-peptides complex** does not have any bearing on safety.

The safety of **oligosaccharides-peptides complex** is primarily based on (a) the known metabolic fate of free amino acids, glycopeptides and peptides by cats and dogs; (b) published feeding studies in adult cats and dogs using Gnubiotics' **oligosaccharides-peptides complex**; and (c) a basic battery of *in vitro* tests to evaluate the genotoxic potential of the ingredient. Taken together, these data are considered pivotal to the safety determination. Safety is further corroborated through studies in swine conducted on the raw material, **protein hydrolysate of porcine intestinal mucosa**, marketed as a spray-dried products that may be co-dried with a carrier. The common name for the ingredient in studies identified in the published literature is DPS, the nomenclature used to describe **protein hydrolysate of porcine intestinal mucosa** in Schedules IV, Part II of the Feed Regulations in Canada. Moreover, a battery of toxicity studies is available using individual glycans designed as MMO mimics and which have similar structures to the glycans side chains (prime constituent) of the glycopeptides component of **oligosaccharide-peptides complex**. These data in swine and rats provide additional information on juvenile and reproducing animals, and support the extrapolation of the body of evidence on safety to all life stages of cats and dogs.

Available published ADME data confirm that the free amino acids and peptides components of **oligosaccharides-peptides complex** will act as a readily available source of protein for cats and dogs. Studies on porcine intestinal mucins and related individual mimics of HMOs which are structurally related to the glycans side chains of glycopeptides, also corroborates that the glycopeptides component will remain intact on ingestion and act as a source of fermentable fiber in cats and dogs.

Conventional 2-day palatability studies in cats and dogs indicate that inclusion of 1% **oligosaccharides-peptides complex** in the diet was well-accepted by the animals. These studies were followed by 26-

week feeding studies in which adult cats and dogs were fed diets containing **oligosaccharides-peptides complex** at 0, 0.5, 1 and 1.5% by weight in the diet. The performance of the animals was acceptable for the duration of the study and the diets were reported to be highly digestible. There were no treatment-related effects on microbiota or on routine blood and hematology parameters over the 26-week period. In particular, no interaction was observed between **oligosaccharides-peptides complex** and thiamine; there were no clinical signs of thiamine deficiency over the 26-week feeding period and no differences in levels of the vitamin between the control and treatment diet containing 1.5% **oligosaccharides-peptides complex** after 18 months of storage. Therefore, it may be concluded that under the experimental conditions of the studies, oligosaccharide-peptides complex was well-tolerated by adult cats and dogs at levels of up to 1.5% in the diet. The results of *in vitro* genotoxicity testing confirm that **oligosaccharides-peptides complex** is not mutagenic and supports the lifetime feeding of the ingredient to cats and dogs.

Additionally, **protein hydrolysate of porcine intestinal mucosa**, the source material used in the manufacture of **oligosaccharides-peptides complex**, has a history of use as a protein source for animals in the EU and Canada. **Protein hydrolysate of porcine intestinal mucosa** is generally dried for ease of handling and mixing into feed, and may be co-dried with a carrier. In particular, **protein hydrolysate of porcine intestinal mucosa** of interest as an alternative source of highly digestible protein to spray-dried plasma products in the diets of weaned piglets and lactating sows. A number of studies were identified in the published literature in which swine were fed diets containing **protein hydrolysate of porcine intestinal mucosa** (often referred to by the alternative name, DPS). The results of the studies indicated that **protein hydrolysate of porcine intestinal mucosa** was a readily available form of protein and was not associated with any adverse effects on performance of growing, lactating and reproducing sows. The levels of inclusion of **protein hydrolysate of porcine intestinal mucosa** in the swine diets used in the studies varied but were generally between 1 to 6%. Assuming that **protein hydrolysate of porcine intestinal mucosa** is incorporated into the diet of swine at 3% DM, on a body weight basis the intakes were estimated to range from 1.8 to 2.6 g/kg bw/day for weaned piglets, grower/finisher pigs and lactating sows. By comparison, the estimated intakes by adult cats and dogs to **oligosaccharides-peptides complex** was estimated to be in the region of 0.2 and 0.3 g/kg bw/day which provides a margin of safety of around 10. The estimated intakes by kittens and puppies to **oligosaccharides-peptides complex** was estimated to be in the region of 0.8 and 0.6 g/kg bw/day which provides a margin of safety of around 3 to 4. Thus, studies conducted using **protein hydrolysate of porcine intestinal mucosa** in weaned piglets and sows support the tolerability of the higher purity **oligosaccharides-peptides complex** to cats and dogs of all life stages under the conditions of intended use.

The glycopeptides component comprises glycans side chains which are linked by *O*-glycosidic bonds to peptides. These glycans side chains (prime constituents) of the glycopeptides component of**oligosaccharides-peptides complex** are known to exhibit a diverse range of oligosaccharides. A battery of toxicology studies are available on individual oligosaccharides designed as HMO mimics and structurally similar to the glycans side chains (prime constituent) of glycopeptides in **oligosaccharides-peptides complex** which corroborates the safety of this group of compounds. NOAELs of 5,000 mg/kg bw/day, the highest dose tested, were derived for the group of compounds from a series of 90-day repeated dose oral toxicity studies in rats conducted using individual HMOs. By comparison, intakes of **oligosaccharides-peptides complex** by cats and dogs under the conditions of intended use was

estimated to lead to an exposure of 40 and 80 mg/kg bw/day of the glycopeptides component, respectively. Thus, the exposure by adult cat and dogs to the glycopeptides component is 125- and 83-fold lower than the NOAEL derived from the toxicity data in rats. Exposure by kittens and puppies to the glycopeptides component was estimated to be 31- and 50-fold lower than the NOAEL derived from the toxicity data in rats. Notably, a study in Beagle dogs was conducted in which the highest use level of 2,000 mg/kg bw/day was well-tolerated when delivered by gavage. The results of *in vitro* genotoxicity testing on these individual HMOs also confirms that these structural units are not associated with any mutagenicity.

Following critical evaluation of the data and information summarized above, it can be concluded that **oligosaccharides-peptides complex** produced by Gnubiotics using suitable feed-grade materials in accordance with cGMP and meeting appropriate feed-grade specifications, is safe and suitable for use as a source of free amino acids, peptides and glycopeptides in food for all life stages of cats and dogs at levels not to exceed 1.5% by weight in the diet. It is further concluded that **oligosaccharides-peptides complex** is GRAS for use in cat and dog food based on scientific procedures.

PART 7. §570.255. LIST OF SUPPORTING DATA AND INFORMATION

7.1 List of Appendices

Appendices 001 to 014, and 016 to 033 are CONFIDENTIAL as indicated in the list below.

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Appendix 015B	Ph. Eur. 2.5.32 Method (Moisture)
Appendix 015C	Ph. Eur. 2.4.14 Method (Ash)
Appendix 015D	Ph. Eur. 2.2.56 Method (Free AAs)
Appendix 015E	Method Gnubiotics 5 (Glycopeptides and Peptides)
Appendix 015F	prEN 15763:2008 (IPC-MS)
Appendix 015G	ISO 4833-1:2003 (Total Aerobic Plate Count)
Appendix 015H	Ph. Eur. 2.6.12 Method (Total Aerobic Plate Count)
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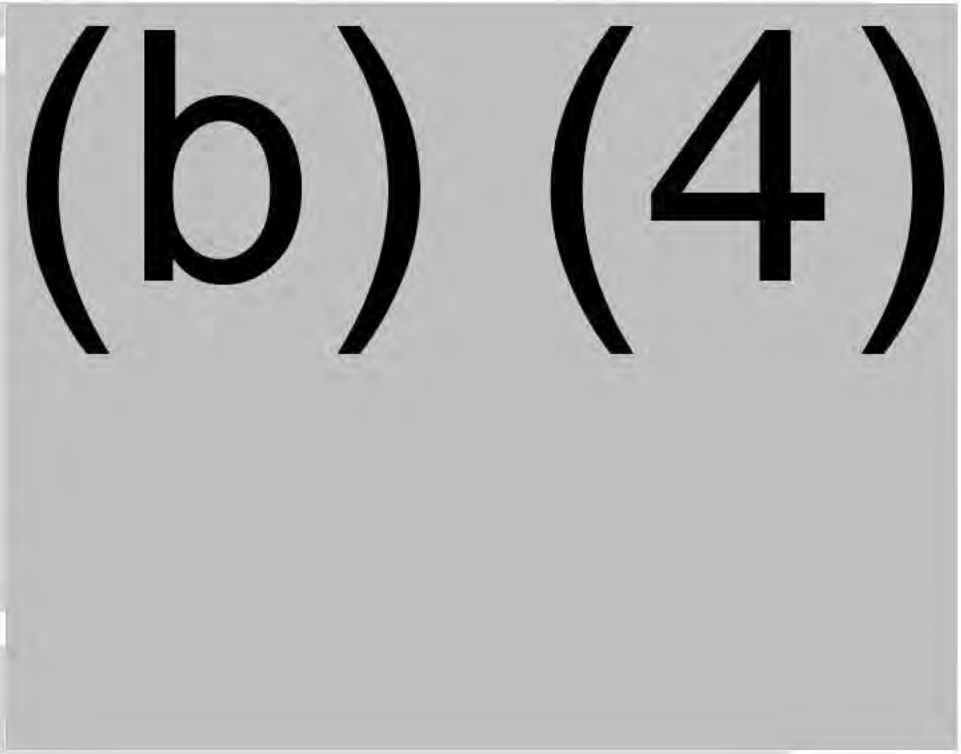

7.2 List of Abbreviations

AA	Amino Acid
AAFCO	Association of American Feed Control Officials
ADFI	Average Daily Feed Intake
ADG	Average Daily Gain
ADME	Absorption, Distribution, Metabolism, Excretion
AID	Apparent Ileal Digestibility
ASFV	African Swine Fever Virus
ATP	Adenosine Triphosphate
ATTD	Apparent Total Tract Digestibility
BBMs	Brush Border Membranes
BBMV	Brush Border Membrane Vesicles
BCFAs	Branched-Chain Fatty Acids
BCS	Body Condition Score
bw	Body Weight
cGMP	current Good Manufacturing Practices
CFIA	Canadian Food Inspection Agency
CFU	Colony Forming Units
CMO	Contract Manufacturing Organization
CP	Crude Protein
CPS	Condensed Porcine Solubles
CRP	C-Reactive Protein
Da	Daltons
DE	Digestible Energy
DFB	Dried Fermentation Biomass
DM	Dry Matter
DPS	Dried Porcine Solubles
DW	Dry Weight
EC	European Commission
EU	European Union
FCR	Feed Conversion Ratio
FDA	Food and Drug Administration
FEDIAF	European Pet Food Manufacturers Association
FFDCA	Federal Food, Drug and Cosmetic Act
FI	Feed Intake
FL	Fucosyllactose
FOS	Fructooligosaccharides
FSMA	Food Safety Modernization Act
Fuc	Fucose
Gal	Galactose
GalNAc	N-acetylgalactosamine
GE	Gross Energy
G:F	Gain-to-Feed Ratio
GI	Gastrointestinal
Glc	Glucose

GlcNAc	N-acetylglucosamine
GLP	Good Laboratory Practice
GMP	Good Manufacturing Practice
GRAS	Generally Recognized As Safe
HACCP	Hazard Analysis and Critical Control Point
HexNAc	N-acetylhexosamine
HMOs	Human Milk Oligosaccharides
HPAEC-PAD	High Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection
ICP-MS	Inductively Coupled Plasma-Mass Spectrometry
ID	Ileal Digestibility
IgA	Immunoglobulin A
IgE	Immunoglobulin E
IL-6	Interleukin-6
ISO	International Standardization Organization
IW	Initial Weight
LC-MS/MS	Liquid Chromatography-Tandem Mass Spectrometry
Man	Mannose
ME	Metabolizable Energy
MMOs	Mammalian Milk Oligosaccharides
MOs	Milk Oligosaccharides
ND	Not Detected
NEN	Royal Netherlands Standardization Institute
NOAELs	No-Observed-Adverse-Effect-Levels
NR	Not Reported
O-GalNAcylation	*O*-glycosylation between the protein/peptide core and N-acetylgalactosamine
OECD	Organization for Economic Co-operation and Development
OP	Official Publication
PCA	Principal Component Analysis
PCBs	Polychlorinated Biphenyls
PCDD	Polychlorinated-P-Dioxins
PCDF	Polychlorinated Dibenzofurans
PD	Population Doubling
Ph. Eur.	European Pharmacopoeia
PM	Porcine stomach Mucin
prEN	Provisional European Standard
RH	Relative Humidity
SBM	Soybean Meal
SCFAs	Short-Chain Fatty Acids
SD	Standard Deviation
SDAP	Spray Dried Animal Plasma
SDBC	Spray-Dried Blood Cells
SDP	Spray-Dried Plasma
SDPP	Spray-Dried Plasma Protein
SEM	Standard Error of the Mean
Sia	Sialic Acid

SID	Standardized Ileal Digestibility
SMFM	Select Menhaden Fish Meal
SNFGs	Symbol Nomenclature For Glycans
STTD	Standardized Total Tract Digestibility
TEQ	Toxic Equivalence
TNFα	Tumor Necrosis Factor-Alpha
TVB-N	Total Volatile Base Nitrogen
TSE	Transmissible Spongiform Encephalopathies
U.S.	United States
VNTR	Variable Number Tandem Repeat
WHO	World Health Organization

Note: Every abbreviation in the text is defined in full the first time and the abbreviation provided in parenthesis. From then onwards, only the abbreviation is given in the text.

7.3 References

AAFCO, 2021a. Association of American Feed Control Officials (AAFCO) Official Publication (OP). Ingredient definition 9.68: Animal digest. *Chapter Six – Feed Terms and Ingredient Definitions*.

AAFCO, 2021b. Association of American Feed Control Officials (AAFCO) Official Publication (OP). Official guidelines for contaminants levels permitted in mineral feed ingredients. *Chapter Five – AAFCO Model Guidance Documents*.

AAFCO, 2021c. Association of American Feed Control Officials (AAFCO) Official Publication (OP). AAFCO cat and dog food nutrient profiles. *Chapter Four – Model Bill and Regulations*.

(b) (4)

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(b) (4)

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Bansil, R. and Turner, B.S., 2006. Mucin structure, aggregation, physiological functions and biomedical applications. *Current Opinion in Colloid & Interface Science*, *11*(2-3), pp.164-170.

Barry, K.A., Wojcicki, B.J., Middelbos, I.S., Vester, B.M., Swanson, K.S. and Fahey Jr, G.C., 2010. Dietary cellulose, fructooligosaccharides, and pectin modify fecal protein catabolites and microbial populations in adult cats. *Journal of Animal Science*, *88*(9), pp.2978-2987.

(b) (4)

Biagi, G., Cipollini, I., Grandi, M. and Zaghini, G., 2010. Influence of some potential prebiotics and fibre-rich foodstuffs on composition and activity of canine intestinal microbiota. *Animal Feed Science and Technology*, *159*(1-2), pp.50-58.

(b) (4)

Bregendahl, K. and Zimmerman, D.R., 2001. Comparison of hydrolyzed intestinal by-products. *Animal Industry Report*, *1*(1).

Bregendahl, K., Sparks, J.C., Bassaganya, J. and Zimmerman, D.R., 1999. Effect of spray-dried plasma and dried porcine solubles on the growth performance of weanling pigs raised in different health-status environments. *Animal Industry Report*, *1*(1).

Brockhausen, I. and Stanley, P., 2017. O-GalNAc Glycans. Chapter 10. *Essentials of Glycobiology*. 3rd edition. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press; 2015-2017.

CFIA, 2022. Canadian Food Inspection Agency (CFIA). Schedule IV, part II, entry 5.6 Porcine solubles, dried (or dried porcine solubles). *Administrative Schedules IV and V of Feed Regulations (1983) in*

Canada [Updated version obtained from CFIA in February 2022]. Enforcing regulation: https://laws-lois.justice.gc.ca/eng/regulations/sor-83-593/page-1.html

Chang, Y.P., Chiu, P.Y., Lin, C.T., Liu, I.H. and Liu, C.H., 2017. Outbreak of thiamine deficiency in cats associated with the feeding of defective dry food. *Journal of Feline Medicine and Surgery*, *19*(4), pp.336-343.

Cho, J.H., Lindemann, M.D., Monegue, H.J. and Cromwell, G.L., 2010. Feeding value of dried porcine solubles for weanling pigs. *The Professional Animal Scientist*, *26*(4), pp.425-434.

Cho, S.M., Park, S.W., Kim, N.H., Park, J.A., Yi, H., Cho, H.J., Park, K.H., Hwang, I. and Shin, H.C., 2013. Expression of intestinal transporter genes in beagle dogs. *Experimental and Therapeutic Medicine*, *5*(1), pp.308-314.

Choi, S.S., Baldwin, N., Wagner III, V.O., Roy, S., Rose, J., Thorsrud, B.A., Phothirath, P. and Röhrig, C.H., 2014. Safety evaluation of the human-identical milk monosaccharide sialic acid (N-acetyl-d-neuraminic acid) in Sprague-Dawley rats. *Regulatory Toxicology and Pharmacology*, *70*(2), pp.482-491.

Coulet, M., Phothirath, P., Constable, A., Marsden, E. and Schilter, B., 2013. Pre-clinical safety assessment of the synthetic human milk, nature-identical, oligosaccharide Lacto-N-neotetraose (LNnT). *Food and Chemical Toxicology*, *62*, pp.528-537.

Coulet, M., Phothirath, P., Allais, L. and Schilter, B., 2014. Pre-clinical safety evaluation of the synthetic human milk, nature-identical, oligosaccharide 2'-*O*-Fucosyllactose (2'FL). *Regulatory Toxicology and Pharmacology, 68*, pp.59-69

Darula, Z. and Medzihradszky, K.F., 2018. Analysis of mammalian O-glycopeptides—we have made a good start, but there is a long way to go. *Molecular & Cellular Proteomics*, *17*(1), pp.2-17.

(b) (4)

Dwivedi, B.K. and Arnold, R.G., 1973. Chemistry of thiamine degradation on food products and model systems. Review. *Journal of Agricultural and Food Chemistry*, *21*(1), pp.54-60.

EC, 2002. European Commission (EC). Directive 2002/32/EC of the European Parliament and of the Council of 7 May 2002 on undesirable substances in animal feed. *Official Journal of the European Union,* L 140, p.10. Available at: https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A02002L0032-20191128&qid=1611745582421

EC, 2005. European Commission (EC). Regulation (EC) No 183/2005 of the European Parliament and of the Council of 12 January 2005 laying down requirements for feed hygiene. *Official Journal of the European Union,* L35, p.1. Available at: https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A02005R0183-20190726&qid=1611588006046

EC, 2009a. European Commission (EC). Regulation (EC) No 1069/2009 of the European Parliament and of the Council of 21 October 2009 laying down health rules as regards animal by-products and derived products not intended for human consumption and repealing Regulation (EC) No 1774/2002 (Animal by-

products Regulation. *Official Journal of the European Union*, L300, p.1. Available at: https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A02009R1069-20191214&qid=1608628175619

EC, 2009b. Commission Regulation (EC) No 152/2009 of 27 January 2009 laying down the methods of sampling and analysis for the official control of feed. *Official Journal of the European Union*, L54, p.1. Available at: https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A02009R0152-20201116&qid=1617111432177

EC, 2010. European Commission (EC). EU guidelines to good manufacturing practice medicinal products for human and veterinary use. *EudraLex: The Rules Governing Products in the European Union, 4*. Available at: https://ec.europa.eu/health/documents/eudralex/vol-4_en

EC, 2011. Commission Regulation (EU) No 142/2011 of 25 February 2011 implementing Regulation (EC) No 1069/2009 of the European Parliament and of the Council laying down health rules as regards animal by-products and derived products not intended for human consumption and implementing Council Directive 97/78/EC as regards certain samples and items exempt from veterinary checks at the border under that Directive. *Official Journal of the European Union*, L54, p.1. Available at: https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A02011R0142-20201208&qid=1617111633539

EC, 2013. European Commission (EC). Commission Regulation (EU) No 68/2013 of 16 January 2013 on the Catalogue of feed materials. *Official Journal of the European* Union, L29, p.1. Available at: https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A02013R0068-20200701&qid=1608632975519

EFSA, 2011a. European Food Safety Authority (EFSA) Panel on Biological Hazards (BIOHAZ). Scientific opinion on risk based control of biogenic amine formation in fermented foods. *EFSA Journal, 9*(10), p.2393. Available at: https://www.efsa.europa.eu/en/efsajournal/pub/2393

EFSA, 2011b. EFSA Scientific Committee. Guidance on conducting repeated-dose 90-day oral toxicity study in rodents on whole food/feed. *EFSA Journal, 9*(12), p. 2438. Available at: https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efsa.2011.2438

EFSA, 2015a. EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA). Safety of 2'-*O*-fucosyllactose as a novel food pursuant to Regulation (EC) No 258/97. *EFSA Journal, 13*(7), p. 4184. Available at: https://www.efsa.europa.eu/en/efsajournal/pub/4184

EFSA, 2015b. EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA). Safety of lacto-*N*-neotetraose and 2'-*O*-fucosyllactose as a novel food pursuant to Regulation (EC) No 258/97. *EFSA Journal, 15*(13), p. 4299. Available at: https://www.efsa.europa.eu/en/efsajournal/pub/4184

Engfer, M.B., Stahl, B., Finke, B., Sawatzki, G. and Daniel, H., 2000. Human milk oligosaccharides are resistant to enzymatic hydrolysis in the upper gastrointestinal tract. *The American Journal of Clinical Nutrition*, *71*(6), pp.1589-1596.

EU Feed Materials Register, 2022. Search terms: Hydrolyzed protein from porcine intestinal mucosa and oligosaccharides-peptides complex. Managed by the EU Feed Chain Task Force. Available at: (Accessed March, 2022) https://feedmaterialsregister.eu/register

Faber, T.A., Hopkins, A.C., Middelbos, I.S., Price, N.P. and Fahey Jr, G.C., 2011. Galactoglucomannan oligosaccharide supplementation affects nutrient digestibility, fermentation end-product production, and large bowel microbiota of the dog. *Journal of Animal Science*, *89*(1), pp.103-112.

FEDIAF, 2020. Nutritional Guidelines for Complete and Complementary Pet Food for Cats and Dogs. Available at: https://fediaf.org/images/FEDIAF_Nutritional_Guidelines_2020_20200917.pdf

Felig, P., 1973. The glucose-alanine cycle. *Metabolism*, *22*(2), pp.179-207.

Figueroa, J., Solà-Oriol, D., Guzmán-Pino, S.A., Chetrit, C., Borda, E. and Pérez, J.F., 2016. The use of porcine digestible peptides and their continuity effect in nursery pigs. *Journal of Animal Science*, *94*(4), pp.1531-1540. [Abstract only]

(b) (4)

.

Gilbert, E.R., Wong, E.A. and Webb Jr, K.E., 2008. Board-invited review: peptide absorption and utilization: implications for animal nutrition and health. *Journal of Animal Science*, *86*(9), pp.2135-2155.

Gitler, C., 1964. Protein digestion and absorption in nonruminants. *Mammalian Protein Metabolism,* pp. 35-69.

Gnoth, M.J., Kunz, C., Kinne-Saffran, E. and Rudloff, S., 2000. Human milk oligosaccharides are minimally digested in vitro. *The Journal of Nutrition*, *130*(12), pp.3014-3020.

González-Solé, F., Criado-Mesas, L., Villodre, C., García, W.C., Farré, M., Borda, E., Pérez-Cano, F.J., Folch, J.M., Solà-Oriol, D. and Pérez, J.F., 2020. Porcine digestible peptides (PDP) in weanling diets improves gut barrier function, immune response and nutrient transport in nursery pigs. *Research Square, 1*.

Gou, J., Choi, K.P., He, X. and Ahn, J., 2010. Dimethylamine, Trimethylamine, and Biogenic Amine Formation in High-Pressure Processed Semidried Squid (*Todarodes pacificius*) during Refrigerated Storage. *Journal of Food Science*, *75*(7), pp.M489-M495.

Gurung, R.B., Kim, D.H., Kim, L., Lee, A.W., Wang, Z. and Gao, Y., 2018. Toxicological evaluation of 6'-sialyllactose (6'-SL) sodium salt. *Regulatory Toxicology and Pharmacology*, *95*, pp.182-189.

Hino, S., Mizushima, T., Kaneko, K., Kawai, E., Kondo, T., Genda, T., Yamada, T., Hase, K., Nishimura, N. and Morita, T., 2020. Mucin-derived o-glycans act as endogenous fiber and sustain mucosal immune homeostasis via short-chain fatty acid production in rat cecum. *The Journal of Nutrition, 150*(10), pp.2656-2665.

Houston, D.M. and Hulland, T.J., 1988. Thiamine deficiency in a team of sled dogs. *The Canadian Veterinary Journal*, *29*(4), p.383.

Hussein, H.S., Flickinger, E.A. and Fahey Jr, G.C., 1999. Petfood applications of inulin and oligofructose. *The Journal of Nutrition*, *129*(7), pp.1454S-1456S.

(b) (4)

INRAE-CIRAD-AFZ Feed Tables, 2021. INRAE CIRAD AFZ. Feed Tables. Composition and nutritive values of feed for cattle, sheep, goats, pigs, poultry, rabbits, horses and salmonids. Search term within feed tables: processed animal protein, pigs. 2017-2021. France. Available at: https://feedtables.com (Accessed 15 June, 2021).

Johnston, L.J., Pettigrew, J.E., Baidoo, S.K., Shurson, G.C. and Walker, R.D., 2003. Efficacy of sucrose and milk chocolate product or dried porcine solubles to increase feed intake and improve performance of lactating sows. *Journal of Animal Science*, *81*(10), pp.2475-2481.

Jones, C.K., DeRouchey, J.M., Nelssen, J.L., Tokach, M.D., Goodband, R.D. and Dritz, S.S., 2008. Effects of pepsoygen and dried porcine solubles 50 in nursery pig diets. *Swine Day Conference*. Kansas State University, Manhattan, KS. Kansas State University. Agricultural Experiment Station and Cooperative Extension Service, pp.52-61.

Jones, C.K., DeRouchey, J.M., Nelssen, J.L., Tokach, M.D., Dritz, S.S. and Goodband, R.D., 2010. Effects of fermented soybean meal and specialty animal protein sources on nursery pig performance. *Journal of Animal Science*, *88*(5), pp.1725-1732.

Kanakupt, K., Vester Boler, B.M., Dunsford, B.R. and Fahey Jr, G.C., 2011. Effects of short-chain fructooligosaccharides and galactooligosaccharides, individually and in combination, on nutrient digestibility, fecal fermentative metabolite concentrations, and large bowel microbial ecology of healthy adults cats. *Journal of Animal Science*, *89*(5), pp.1376-1384.

Karlsson, K.G., Nordman, H., Karlsson, H., Carlstedt, I. and Hansson, G.C., 1997. Glycosylation differences between pig gastric mucin populations: a comparative study of the neutral oligosaccharides using mass spectrometry. *Biochemical Journal, 326*(3), pp.911-917.

Kim, J.H., Chae, B.J. and Kim, Y.G., 2000. Effects of replacing spray dried plasma protein with spray dried porcine intestine hydrolysate on ileal digestibility of amino acids and growth performance in early-weaned pigs. *Asian-Australasian Journal of Animal Sciences*, *13*(12), pp.1738-1742.

Kim, J.D., Hyun, Y., Sohn, K.S., Kim, T.J., Woo, H.J. and Han, I.K., 2001. Optimal dietary ratio of spray dried plasma protein (SDPP) and dried porcine solubles (DPS) in improving growth performance and immune status in pigs weaned at 21 days of age. *Asian-Australasian Journal of Animal Sciences*, *14*(3), pp.338-345.

Kim, D., Gurung, R.B., Seo, W., Lee, A.W. and Woo, J., 2018. Toxicological evaluation of 3'-sialyllactose sodium salt. *Regulatory Toxicology and Pharmacology*, *94*, pp.83-90.

Kunz, C., Rudloff, S., Baier, W., Klein, N. and Strobel, S., 2000. Oligosaccharides in human milk: structural, functional, and metabolic aspects. *Annual Review of Nutrition*, *20*(1), pp.699-722.

[redacted] (b) (4)

[redacted] (b) (4)

Mantle, M. and Allen, A., 1981. Isolation and characterization of the native glycoprotein from pig small-intestinal mucus. *Biochemical Journal*, *195*(1), pp.267-275.

Matthews, D.M., 1975. Intestinal absorption of peptides. *Physiological Reviews*, *55*(4), pp.537-608.

Middelbos, I.S., Fastinger, N.D. and Fahey Jr, G.C., 2007. Evaluation of fermentable oligosaccharides in diets fed to dogs in comparison to fiber standards. *Journal of Animal Science*, *85*(11), pp.3033-3044.

MidWest, 2021. Biogenic amines. *Midwest Laboratories*. Customer information sheet.

Moon, T.W., 1988. Adaptation, constraint, and the function of the gluconeogenic pathway. *Canadian Journal of Zoology*, *66*(5), pp.1059-1068.

Morin, P., Gorman, A. and Lambrakis, L., 2021. A literature review on vitamin retention during the extrusion of dry pet food. *Animal Feed Science and Technology*, p.114975.

Morris, J.G., 2001. Unique nutrient requirements of cats appear to be diet-induced evolutionary adaptations. *Recent Advances in Animal Nutrition in Australia*, *13*, pp.187-194.

[redacted] (b) (4)

Myers, A.J., Moline, J., Xu, G., Ratliff, B.W., McKilligan, D.M., Tokach, M.D., Goodband, R.D., DeRouchey, J.M., Nelssen, J.L. and Dritz, S.S., 2010. An evaluation of peptone products and fish meal on nursery pig performance. *Kansas Agricultural Experimaental Station Research Report*, *0*(10), pp.35-43

Myers, A.J., Steidinger, M.U., Ratliff, B.W., McKilligan, D.M., Tokach, M.D., Goodband, R.D., DeRouchey, J.M., Nelssen, J.L. and Dritz, S.S., 2011. Evaluation of heparin production by-products in nursery pig diets. *Kansas Agricultural Experimaental Station Research Report*, *0*(10), pp.81-89

Myers, A.J., Goodband, R.D., Tokach, M.D., Dritz, S.S., DeRouchey, J.M. and Nelssen, J.L., 2014. The effects of porcine intestinal mucosa protein sources on nursery pig growth performance. *Journal of Animal Science*, *92*(2), pp.783-792.

Naila, A., Flint, S., Fletcher, G., Bremer, P. and Meerdink, G., 2010. Control of biogenic amines in food—existing and emerging approaches. *Journal of Food Science*, *75*(7), pp.R139-R150.

NRC, 2012. National Research Council (NRC). *Nutrient requirements of swine*. 11th revised edition. Washington, DC: National Academies Press.

(b) (4)

OIE, 2019. Section 5: Trade measures, import/export procedures and veterinary certification. *Terrestrial Animal Health Code, 1*. Available at: https://www.oie.int/index.php?id=169&L=0&htmfile=titre_1.5.htm

Parschat, K., Oehme, A., Leuschner, J., Jennewein, S. and Parkot, J., 2020. A safety evaluation of mixed human milk oligosaccharides in rats. *Food and Chemical Toxicology*, *136*, p.111118.

Phipps, K.R., Baldwin, N., Lynch, B., Flaxmer, J., Šoltésová, A., Gilby, B., Mikš, M.H. and Röhrig, C.H., 2018. Safety evaluation of a mixture of the human-identical milk oligosaccharides 2'-fucosyllactose and difucosyllactose. *Food and Chemical Toxicology*, *120*, pp.552-565.

Phipps, K.R., Lynch, B., Stannard, D.R., Gilby, B., Baldwin, N., Mikš, M.H., Lau, A. and Röhrig, C.H., 2020. Genotoxicity and neonatal subchronic toxicity assessment of a novel mixture of the human-identical milk oligosaccharides lacto-N-fucopentaose I and 2'-fucosyllactose. *Journal of Applied Toxicology, pp.1-8*

Pinna, C. and Biagi, G., 2014. The utilisation of prebiotics and synbiotics in dogs. *Italian Journal of Animal Science*, *13*(1), p.3107.

Pinna, C., Vecchiato, C.G., Bolduan, C., Grandi, M., Stefanelli, C., Windisch, W., Zaghini, G. and Biagi, G., 2018. Influence of dietary protein and fructooligosaccharides on fecal fermentative end-products, fecal bacterial populations and apparent total tract digestibility in dogs. *BMC Veterinary Research*, *14*(1), pp.1-10.

Prieto, P.A., 2005. *In vitro* and clinical experiences with a human milk oligosaccharide, lacto-N-*neo*Tetraose, and fructooligosaccharides. *Foods and Food Ingredients Journal of Japan, 210*(11), pp.1018-1030.

Propst, E.L., Flickinger, E.A., Bauer, L.L., Merchen, N.R. and Fahey Jr, G.C., 2003. A dose-response experiment evaluating the effects of oligofructose and inulin on nutrient digestibility, stool quality, and fecal protein catabolites in healthy adult dogs. *Journal of Animal Science*, *81*(12), pp.3057-3066.

Pruss, K.M., Marcobal, A., Southwick, A.M., Dahan, D., Smits, S.A., Ferreyra, J.A., Higginbottom, S.K., Sonnenburg, E.D., Kashyap, P.C., Choudhury, B. and Bode, L., 2020. Mucin-derived O-glycans supplemented to diet mitigate diverse microbiota perturbations. *The ISME Journal*, *15*(2), pp.577-591.

Purina, 2021. Myth: Animal Digest is a low quality ingredient. Available at: (Accessed March, 2021). https://www.purinaproclub.com/resources/dog-articles/nutrition/animal-digest#:~:text=Animal

Rochus, K., Janssens, G.P., Van de Velde, H., Verbrugghe, A., Wuyts, B., Vanhaecke, L. and Hesta, M., 2013. Highly viscous guar gum shifts dietary amino acids from metabolic use to fermentation substrate in domestic cats. *British Journal of Nutrition*, *109*(6), pp.1022-1030.

Rogers, Q.R., Morris, J.G. and Freeland, R.A., 1977. Lack of hepatic enzymatic adaptation to low and high levels of dietary protein in the adult cat. *Enzyme*, *22*, pp.348-356.

Rostami, S.M., Bénet, T., Spears, J., Reynolds, A., Satyaraj, E., Sprenger, N. and Austin, S., 2014. Milk oligosaccharides over time of lactation from different dog breeds. *PloS One*, *9*(6), p.e99824.

(b) (4)

.

Senda, A., Hatakeyama, E., Kobayashi, R., Fukuda, K., Uemura, Y., Saito, T., Packer, C., Oftedal, O.T. and Urashima, T., 2010. Chemical characterization of milk oligosaccharides of an African lion (Panthera leo) and a clouded leopard (Neofelis nebulosa). *Animal Science Journal*, *81*(6), pp.687-693.

Singh, M., Thompson, M., Sullivan, N. and Child, G., 2005. Thiamine deficiency in dogs due to the feeding of sulphite preserved meat. *Australian Veterinary Journal*, *83*(7), pp.412-417.

Sparkes, A.H., Papasouliotis, K., Sunvold, G., Werrett, G., Gruffydd-Jones, E.A., Egan, K., Gruffydd-Jones, T.J. and Reinhart, G., 1998. Effect of dietary supplementation with fructo-oligosaccharides on fecal flora of healthy cats. *American Journal of Veterinary Research*, *59*(4), pp.436-440. [Abstract only]

Steel, R.J.S., 1997. Thiamine deficiency in a cat associated with the preservation of pet meat with sulphur dioxide. *Australian Veterinary Journal*, *75*(10), pp.719-721.

Studdert, V.P. and Labuc, R.H., 1991. Thiamin deficiency in cats and dogs associated with feeding meat preserved with sulphur dioxide. *Australian Veterinary Hournal*, *68*(2), pp.54-57.

Sulabo, R.C., Mathai, J.K., Usry, J.L., Ratliff, B.W., McKilligan, D.M., Moline, J.D., Xu, G. and Stein, H.H., 2013. Nutritional value of dried fermentation biomass, hydrolyzed porcine intestinal mucosa products, and fish meal fed to weanling pigs. *Journal of Animal Science*, *91*(6), pp.2802-2811.

Swanson, K.S., Grieshop, C.M., Flickinger, E.A., Bauer, L.L., Healy, H.P., Dawson, K.A., Merchen, N.R. and Fahey Jr, G.C., 2002a. Supplemental fructooligosaccharides and mannanoligosaccharides influence immune function, ileal and total tract nutrient digestibilities, microbial populations and concentrations of protein catabolites in the large bowel of dogs. *The Journal of Nutrition*, *132*(5), pp.980-989.

Swanson, K.S., Grieshop, C.M., Flickinger, E.A., Bauer, L.L., Chow, J., Wolf, B.W., Garleb, K.A. and Fahey Jr, G.C., 2002b. Fructooligosaccharides and *Lactobacillus acidophilus* modify gut microbial populations, total tract nutrient digestibilities and fecal protein catabolite concentrations in healthy adult dogs. *The Journal of Nutrition*, *132*(12), pp.3721-3731.

Thompson, C., Morley, P., Kirkland, D. and Proudlock, R., 2005. Modified bacterial mutation test procedures for evaluation of peptides and amino acid-containing material. *Mutagenesis*, *20*(5), pp.345-350.

U.S. FDA, 2015a. Generally Recognized As Safe (GRAS) Notice for 2'-*O*-Fucosyllactose. GRN No. 546. Glycom A/S. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/?set=GRASNotices&id=546&sort=GRN_No&order=DESC&startrow=1&type=basic&search=fucosyllactose

U.S. FDA, 2015b. Generally Recognized As Safe (GRAS) Notice for 2'-*O*-Fucosyllactose. GRN No. 571. Jennewein Biotechnologies, GmgH. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/?set=GRASNotices&id=571&sort=GRN_No&order=DESC&startrow=1&type=basic&search=fucosyllactose

U.S. FDA, 2019. Generally Recognized As Safe (GRAS) Notice for 2'-*O*-Fucosyllactose and difucosyllactose. GRN No. 815. Glycom A/S. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/?set=GRASNotices&id=815&sort=GRN_No&order=DESC&startrow=1&type=basic&search=fucosyllactose

U.S. FDA, 2020a. United States Food and Drug Administration (U.S. FDA). Title 21 Code of Federal Regulations Part 582, Subpart D – Chemical Preservatives. Section 582.3739. Sodium bisulfite. Available at: https://www.ecfr.gov/cgi-bin/text-idx?rgn=div8&mc=true&node=se21.6.582_13739

U.S. FDA, 2020b. United States Food and Drug Administration (U.S. FDA). Title 21 Code of Federal Regulations Part 570 Subpart E – Generally Recognized as Safe (GRAS) Notice. Section 570.230. Part 2 of a GRAS notice: Identity, method of manufacture, specifications, and physical or technical effect. In: *U.S. Code of Federal Regulations (CFR). Title 21: Food and Drugs (U.S. Food and Drug Administration)*. Washington (DC): U.S. Food and Drug Administration (U.S. FDA), U.S. Government Printing Office (GPO). Available at: https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=570.230&SearchTerm=part%202%20of%20a%20gras%20notice

(b) (4)

.

Verbrugghe, A., Hesta, M., Daminet, S. and Janssens, G.P., 2012. Nutritional modulation of insulin resistance in the true carnivorous cat: a review. *Critical Reviews in Food Science and Nutrition*, *52*(2), pp.172-182.

Washabau, 2013. Chapter 58: Large intestine. *Canine & Feline Gastroenterology*, pp.729-777

Webb Jr, K.E., 1990. Intestinal absorption of protein hydrolysis products: a review. *Journal of Animal Science*, *68*(9), pp.3011-3022.

Wrigglesworth, D.J., Goonatilleke, E., Haydock, R., Hughes, K.R., Lebrilla, C.B., Swanson, K.S., Jones, P. and Watson, P., 2020. High-throughput glycomic analyses reveal unique oligosaccharide profiles of canine and feline milk samples. *PloS One*, *15*(12), p.e0243323.

Yamada, T., Hino, S., Iijima, H., Genda, T., Aoki, R., Nagata, R., Han, K.H., Hirota, M., Kinashi, Y., Oguchi, H. and Suda, W., 2019. Mucin O-glycans facilitate symbiosynthesis to maintain gut immune homeostasis. *EBioMedicine*, *48*, pp.513-525.

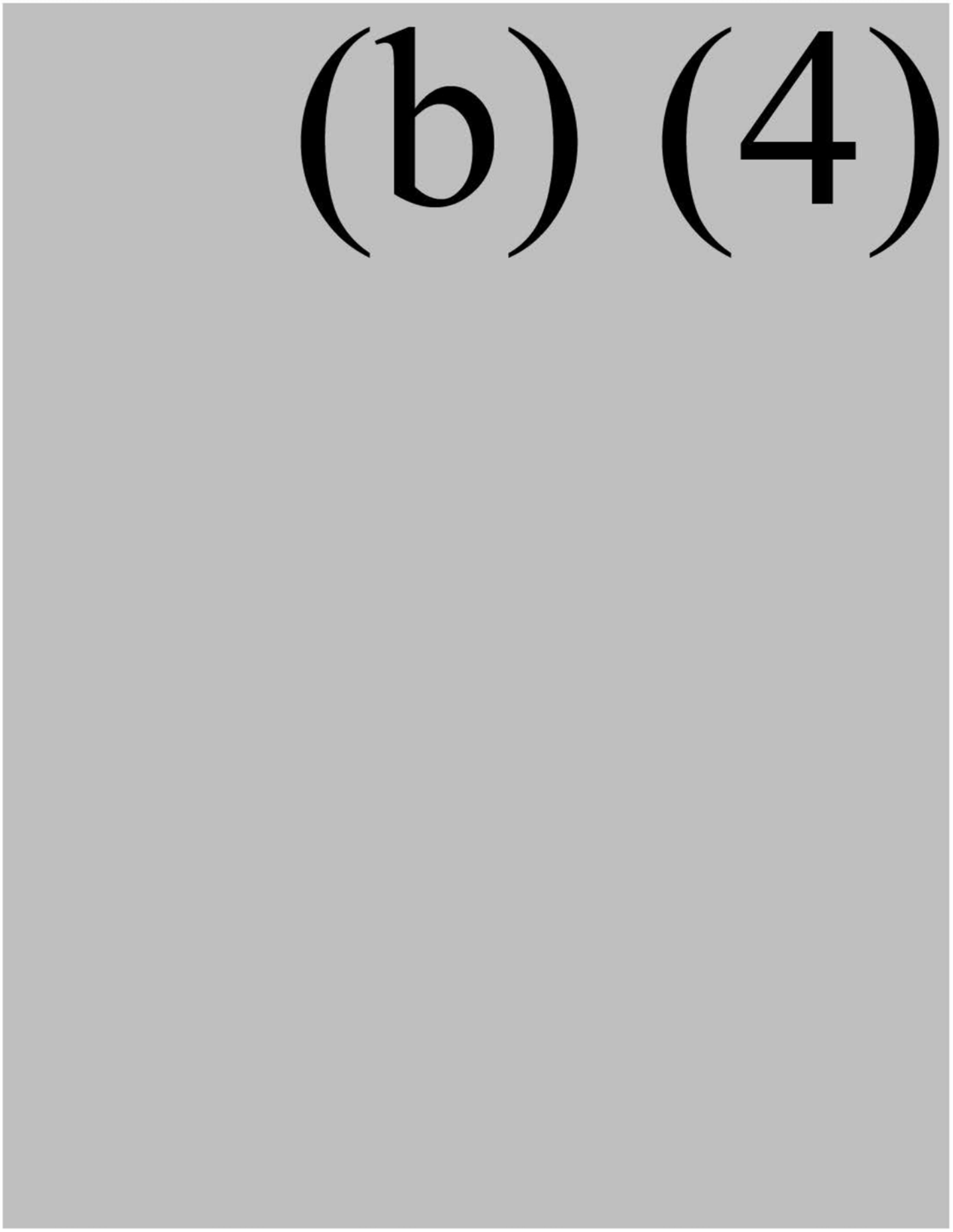
Yen, J.T., 2004. Digestion and absorption of nutrients. *Encyclopedia of Animal Science*, pp.285-287. Available at: https://www.google.co.uk/books/edition/Encyclopedia_of_Animal_Science_Print/1SQl7Ao3mHoC?hl=en&gbpv=1&pg=PA1&printsec=frontcover

Zentek, J., Marquart, B. and Pietrzak, T., 2002. Intestinal effects of mannanoligosaccharides, transgalactooligosaccharides, lactose and lactulose in dogs. *The Journal of Nutrition*, *132*(6), pp.1682S-1684S.

(b) (4)

.

Zimmerman, D.R. and Sparks, C., 1997. Evaluation of a byproduct from hydrolyzed porcine small intestines as an ingredient in pig starters. *Animal Industry Report*, *1*(1).



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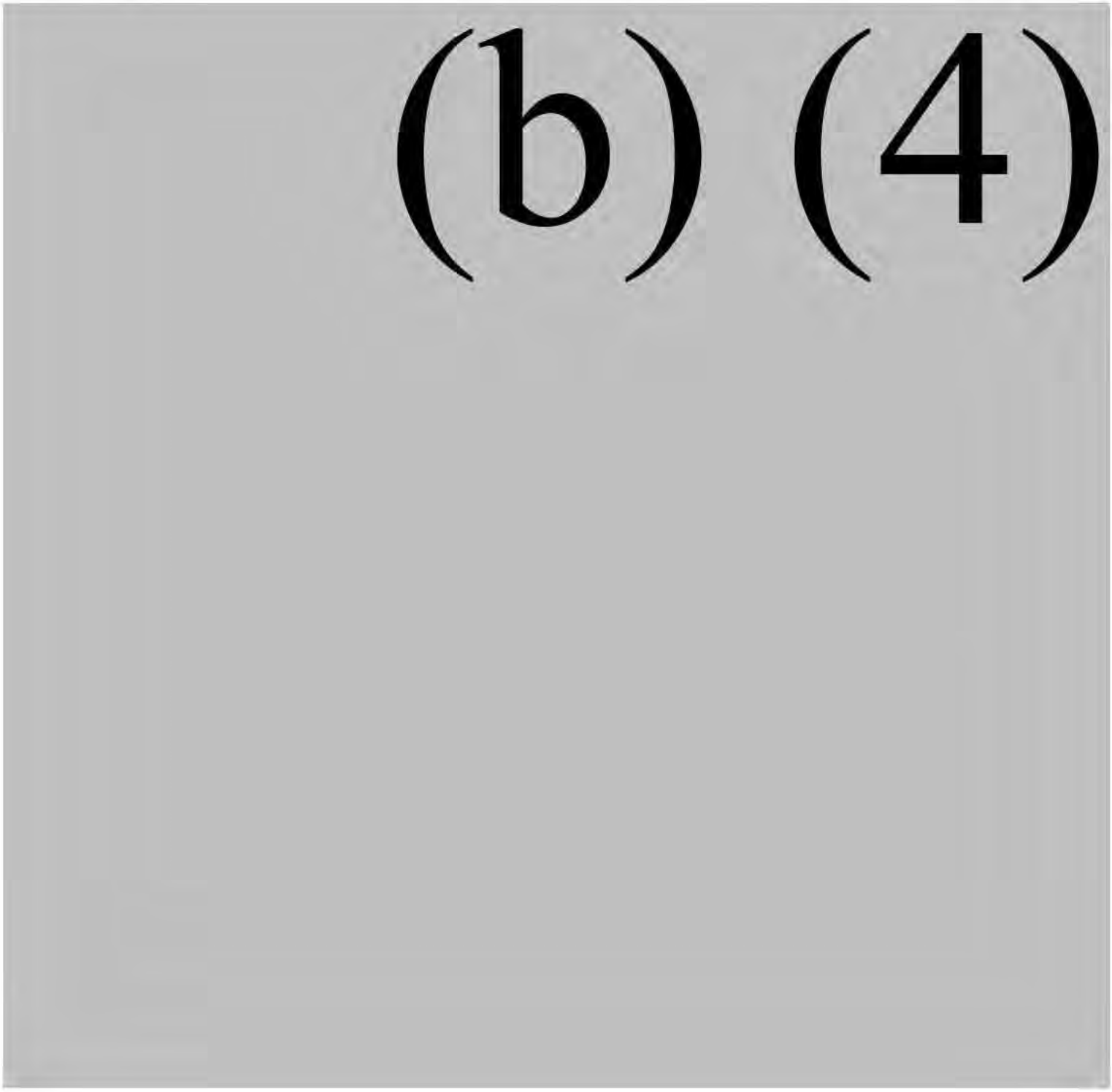
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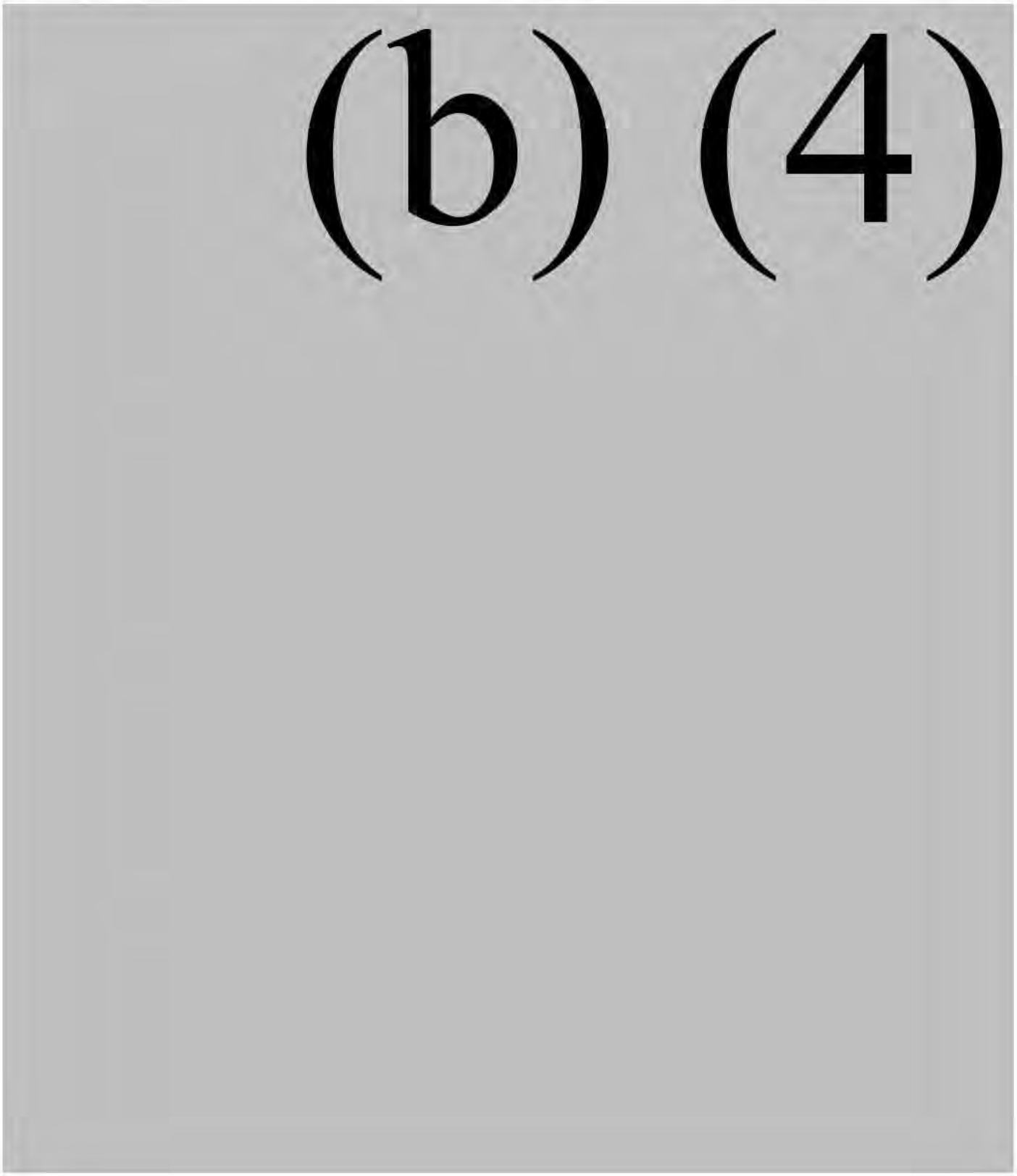
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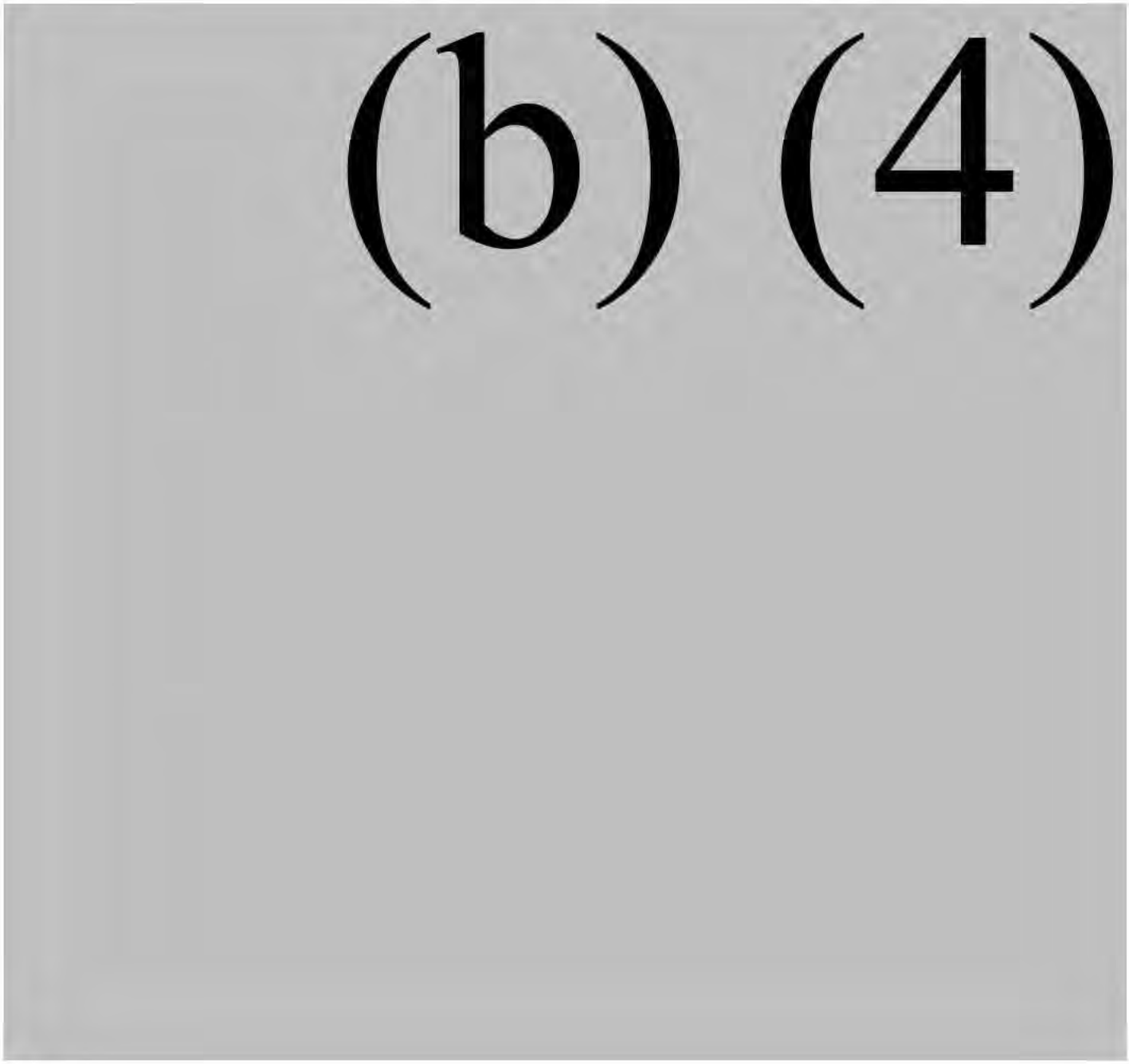
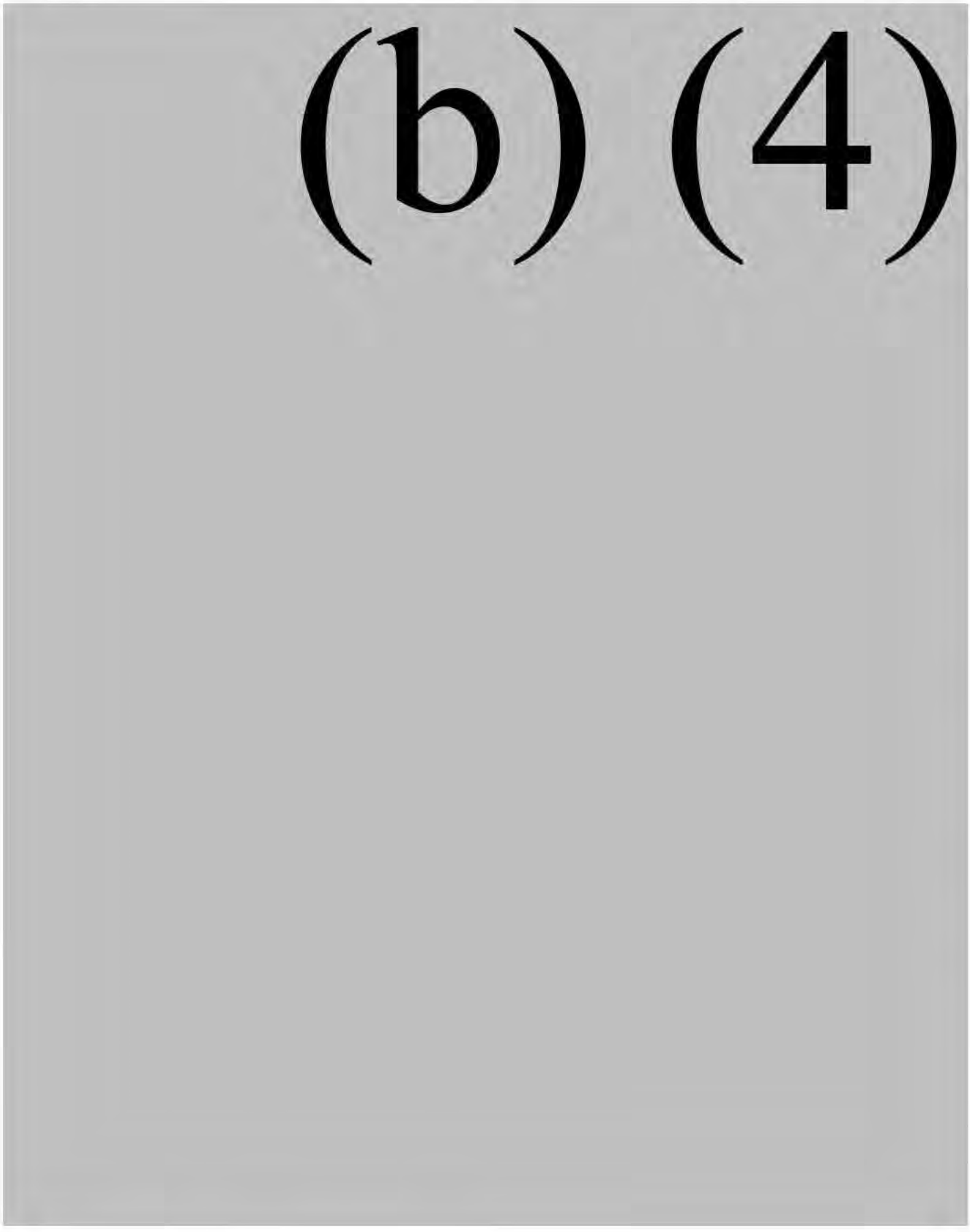
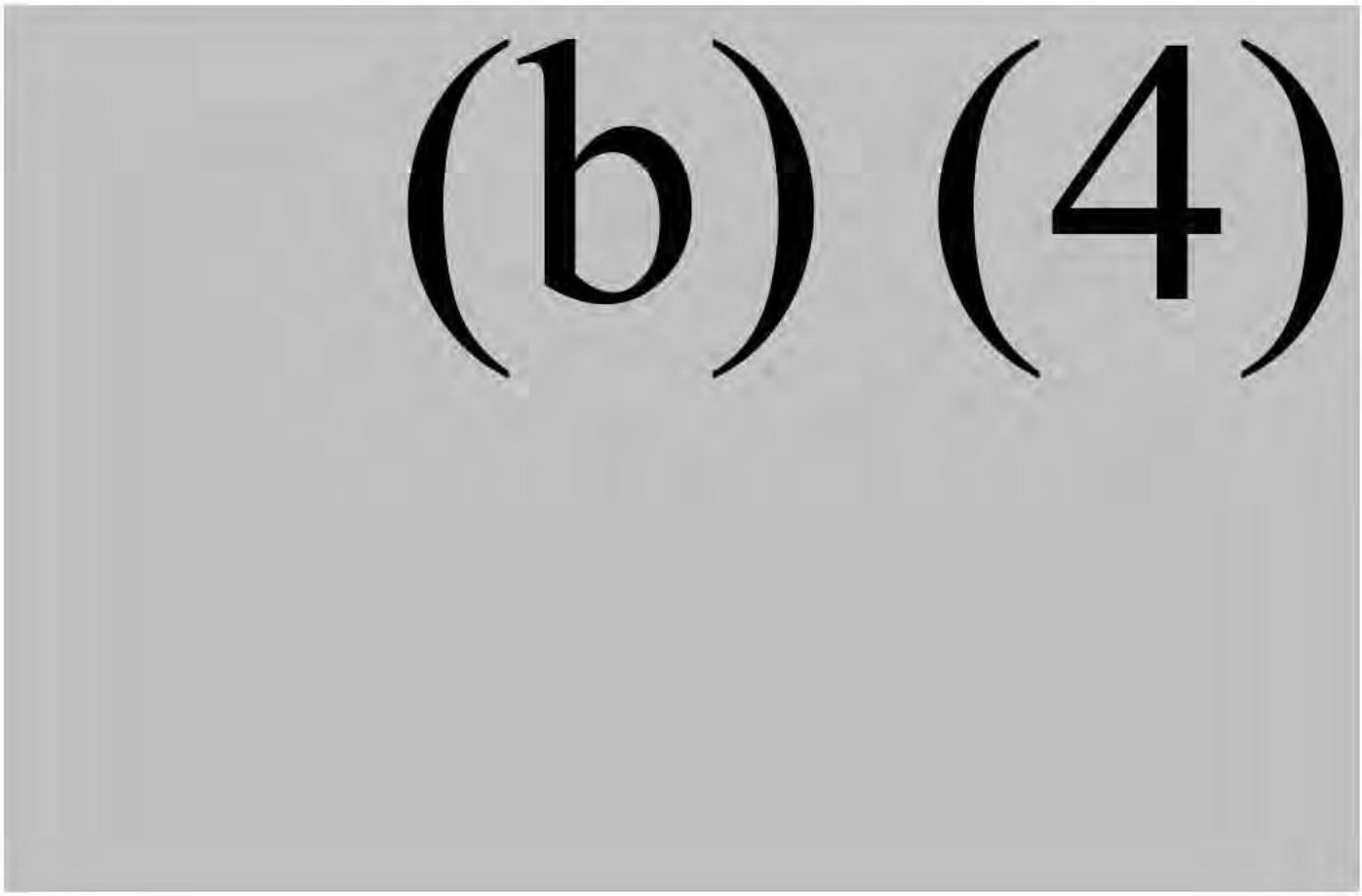

(b) (4)


(b) (4)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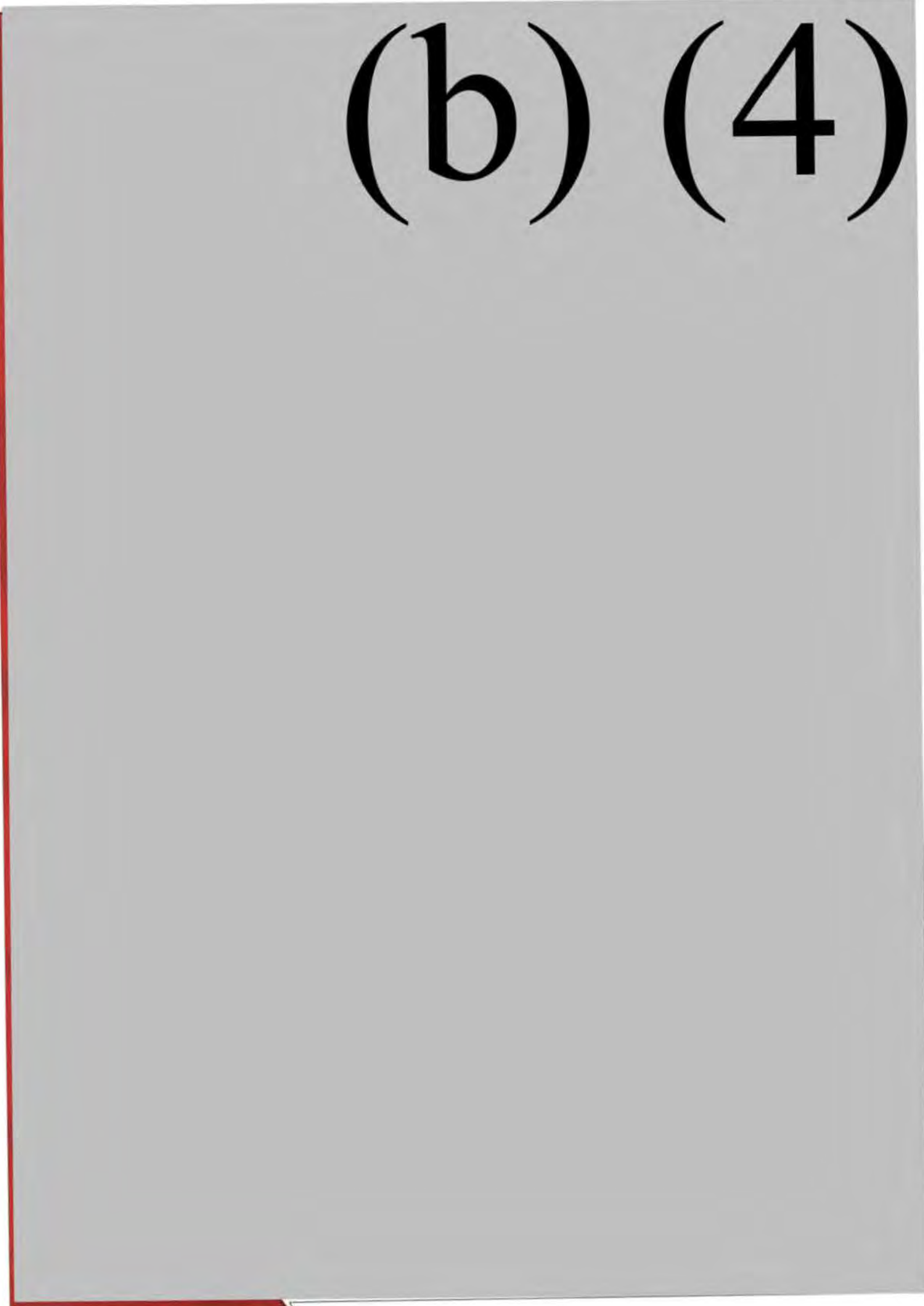
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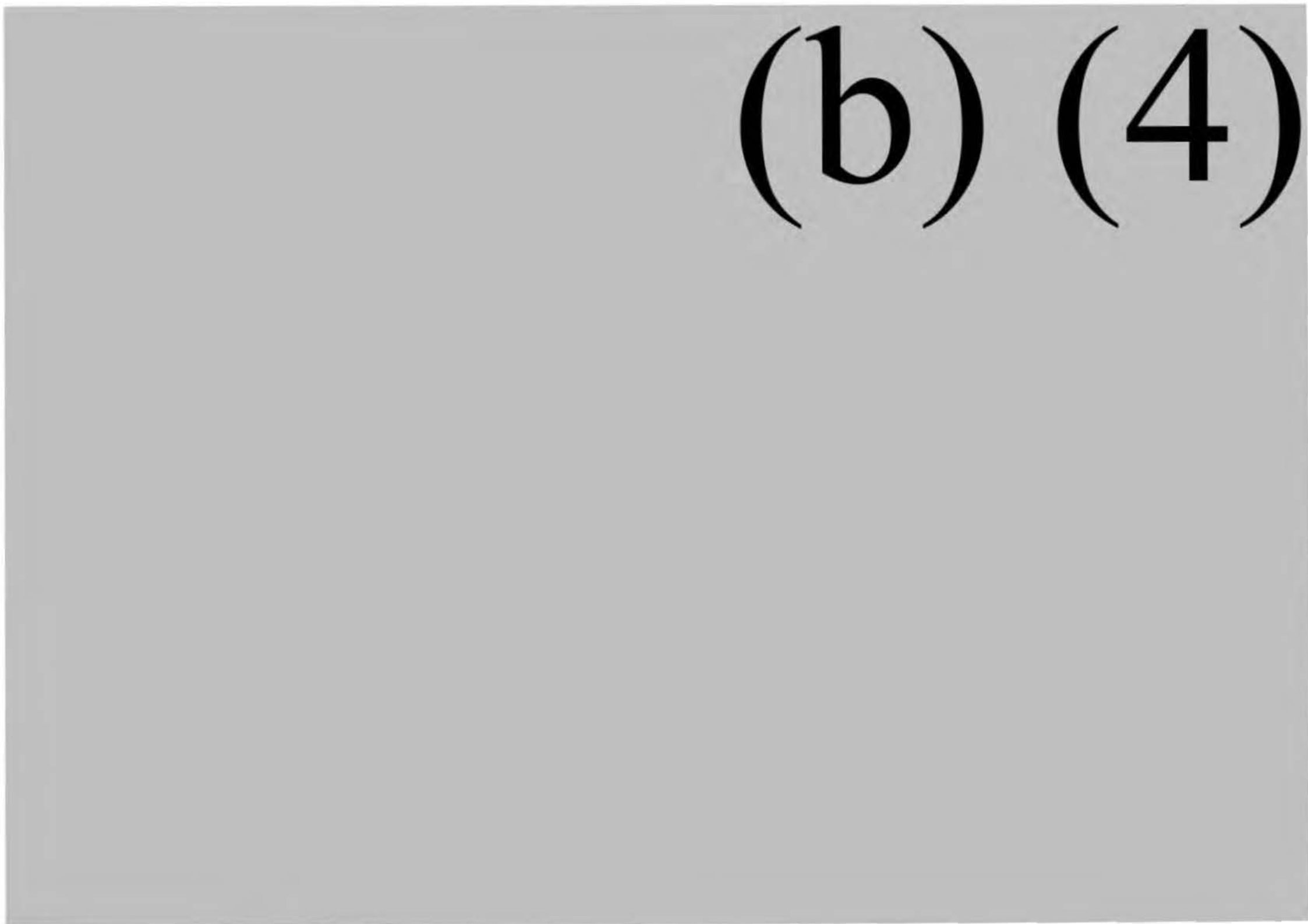
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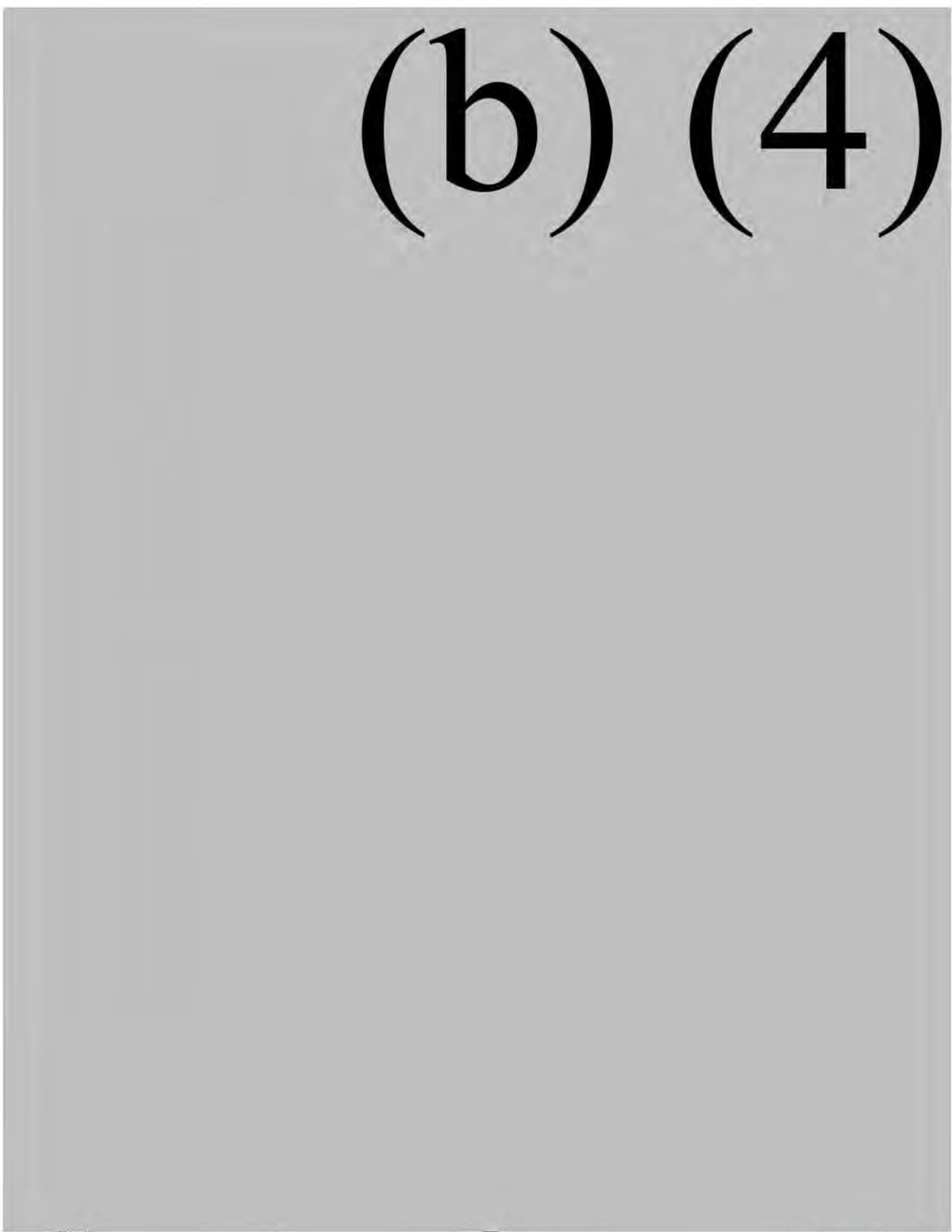
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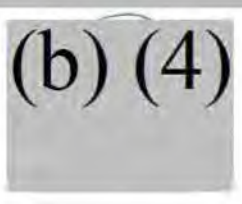
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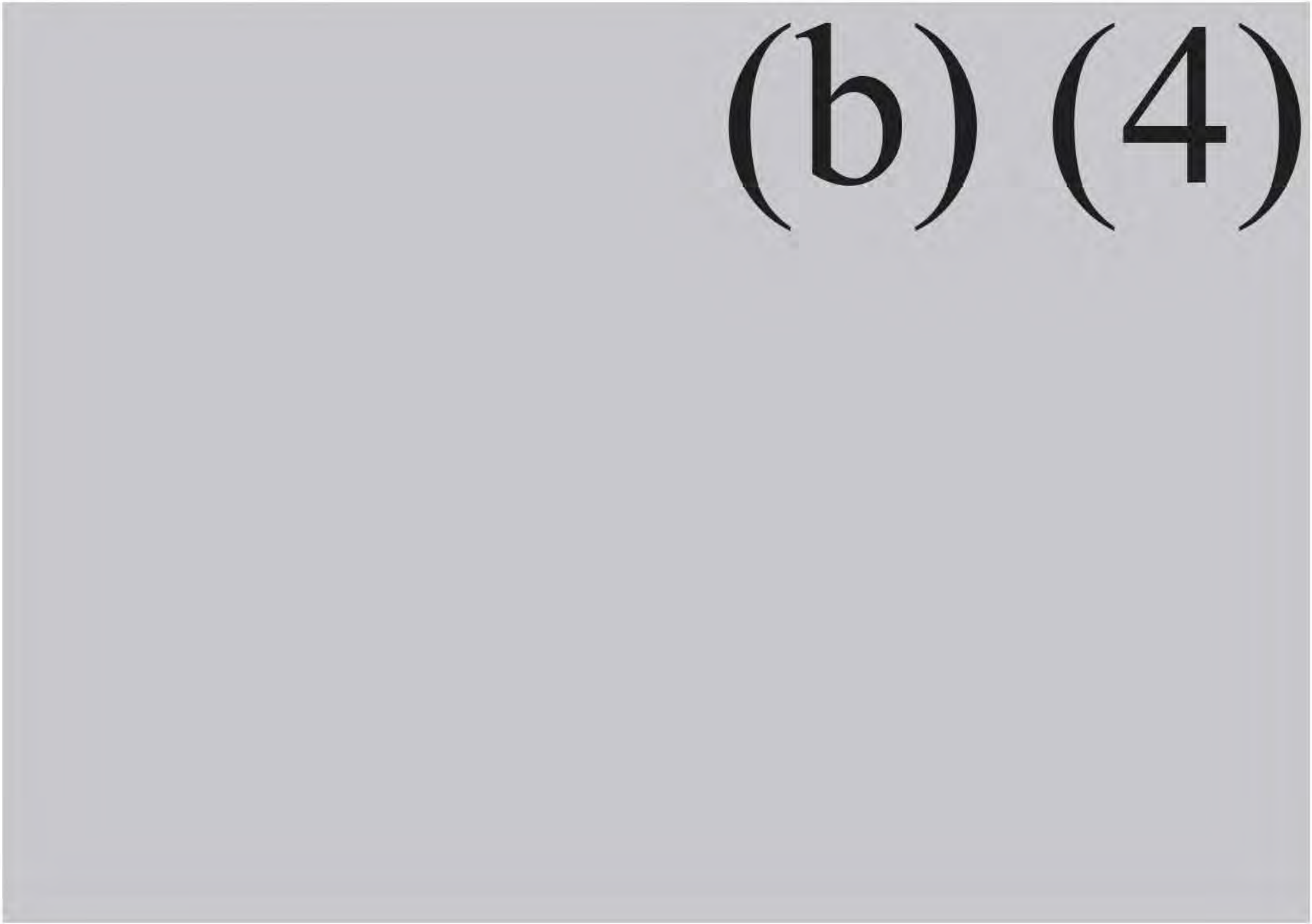
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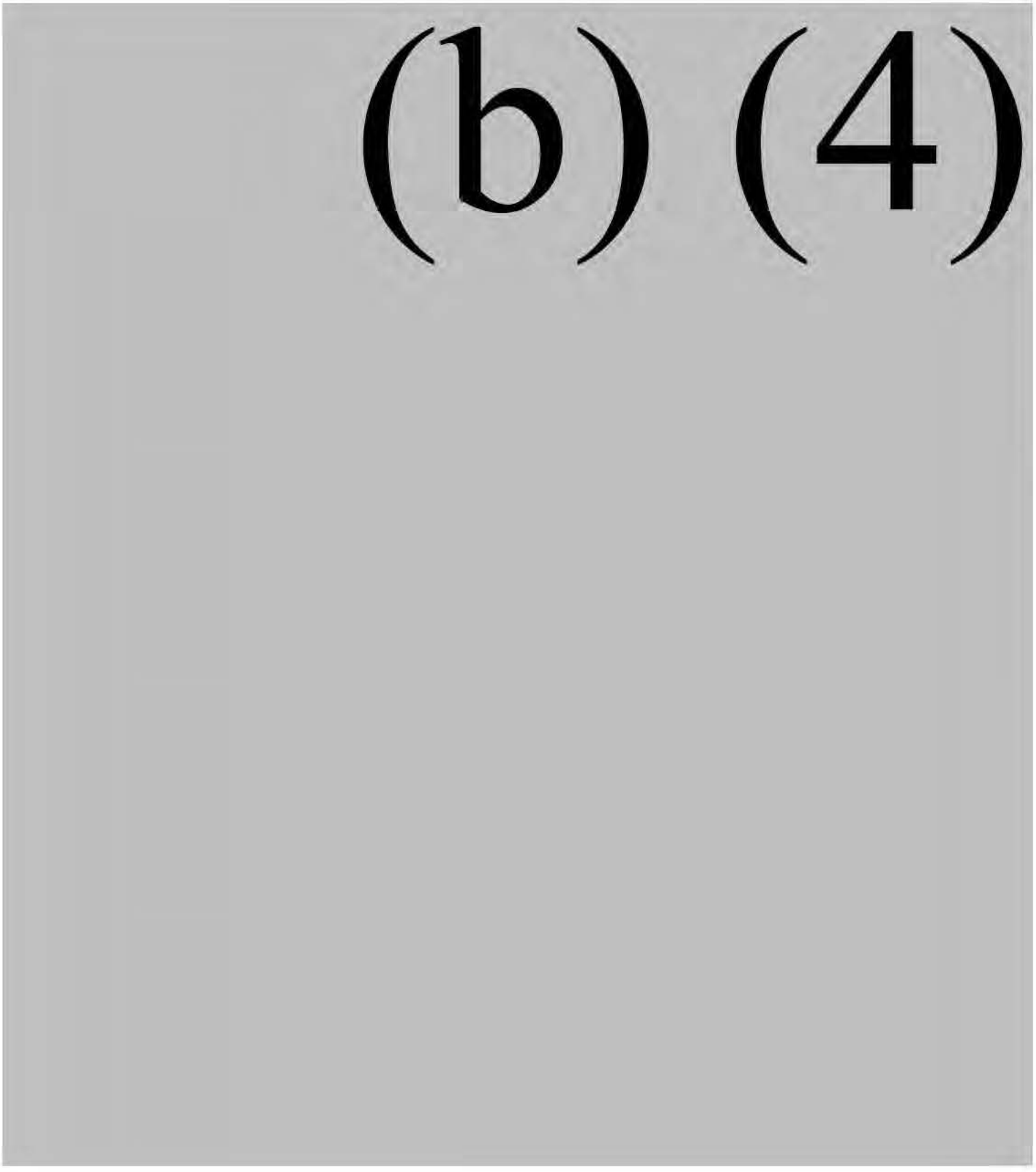
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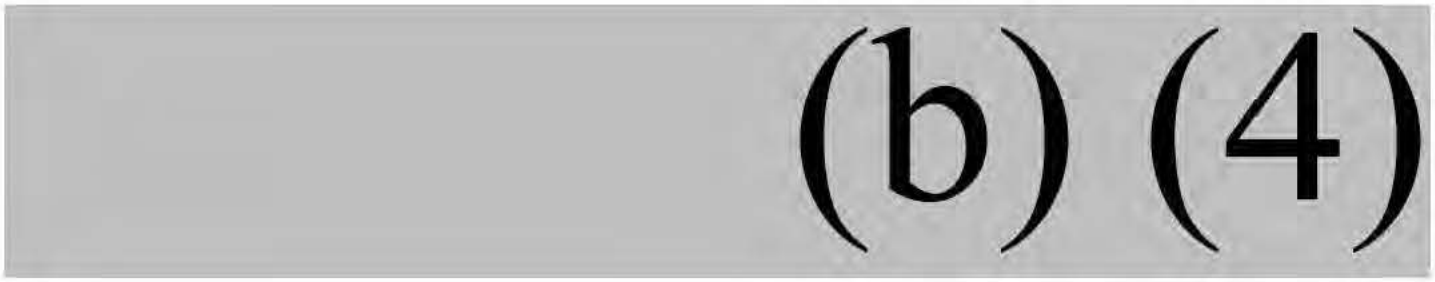
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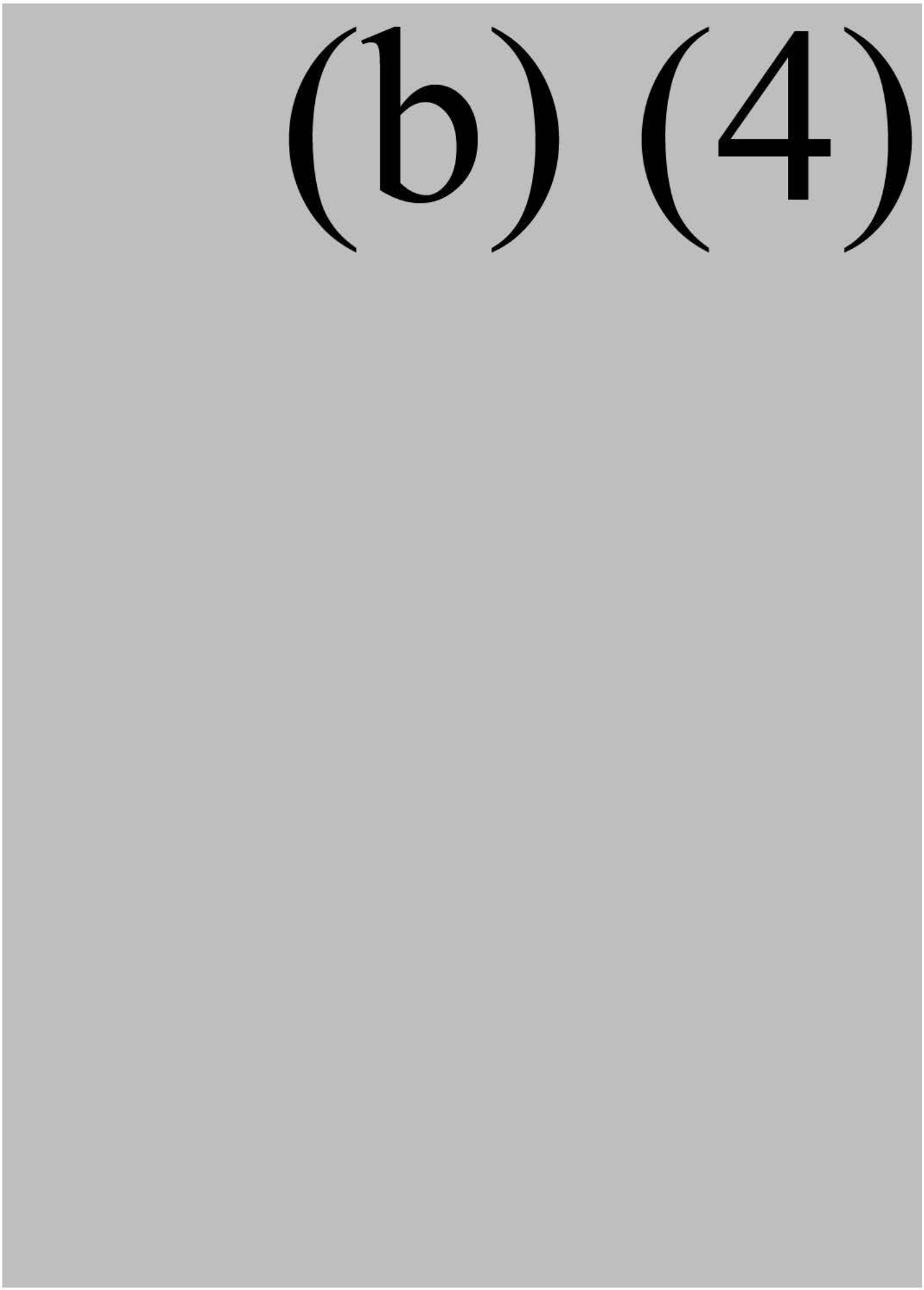
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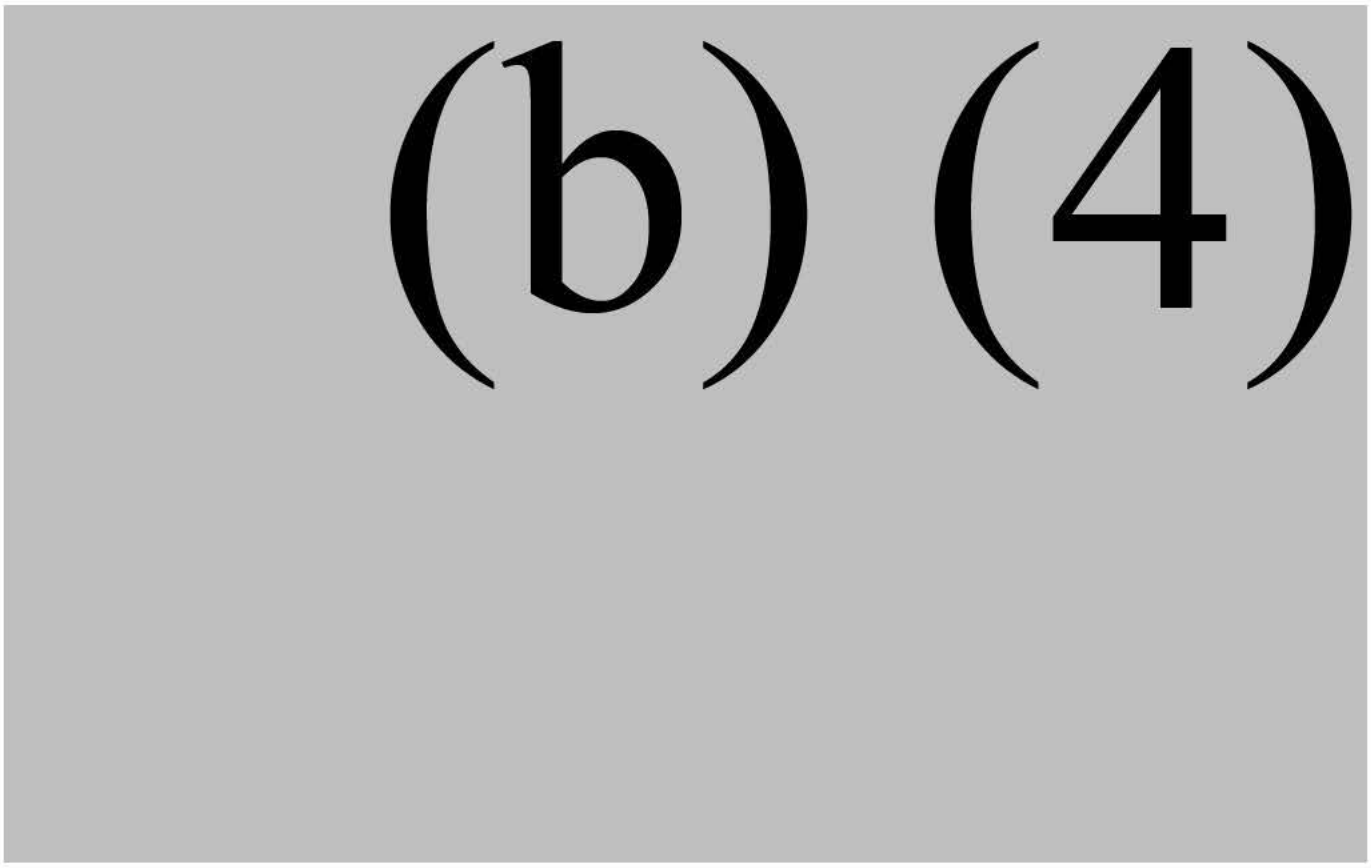
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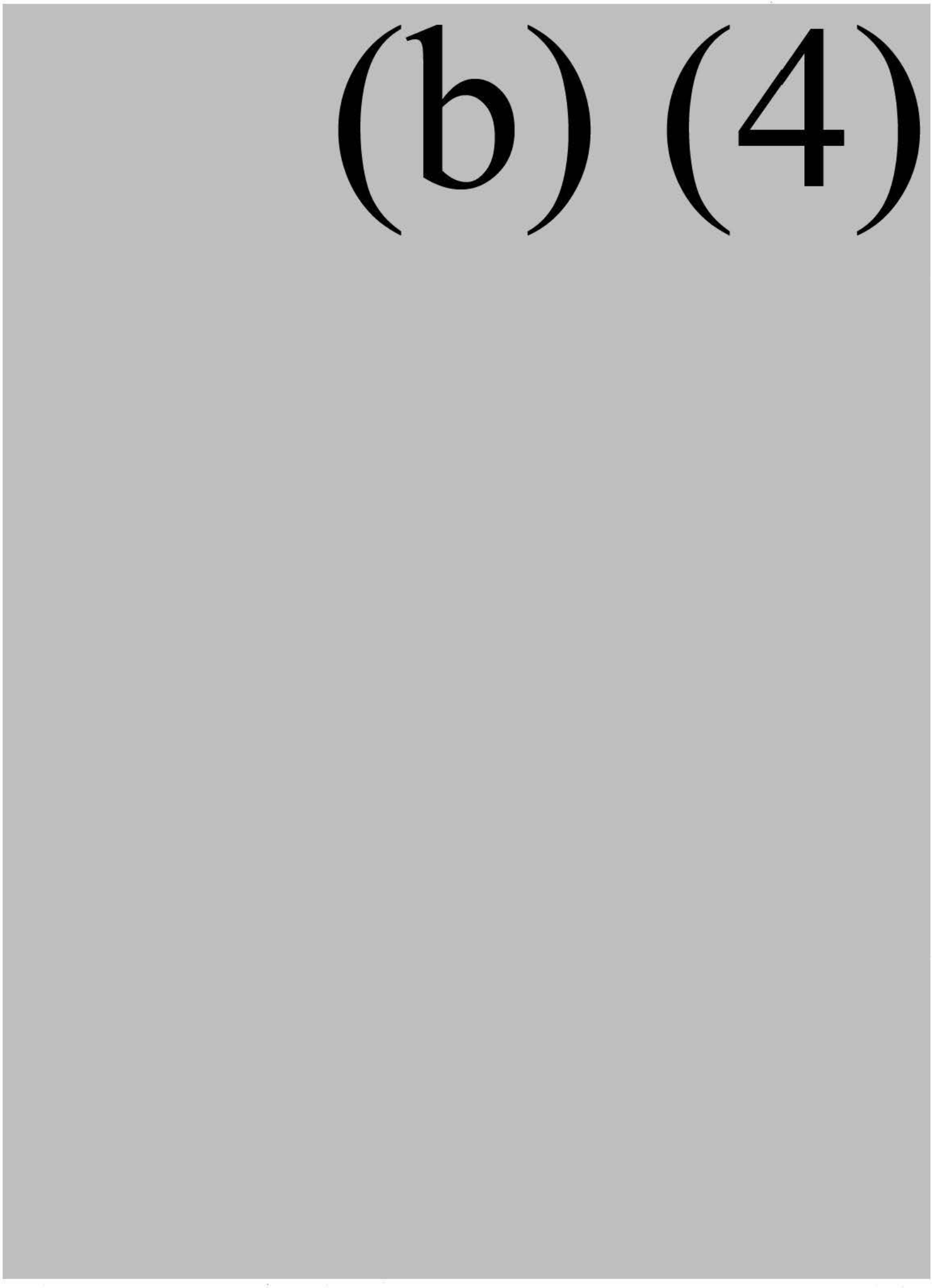
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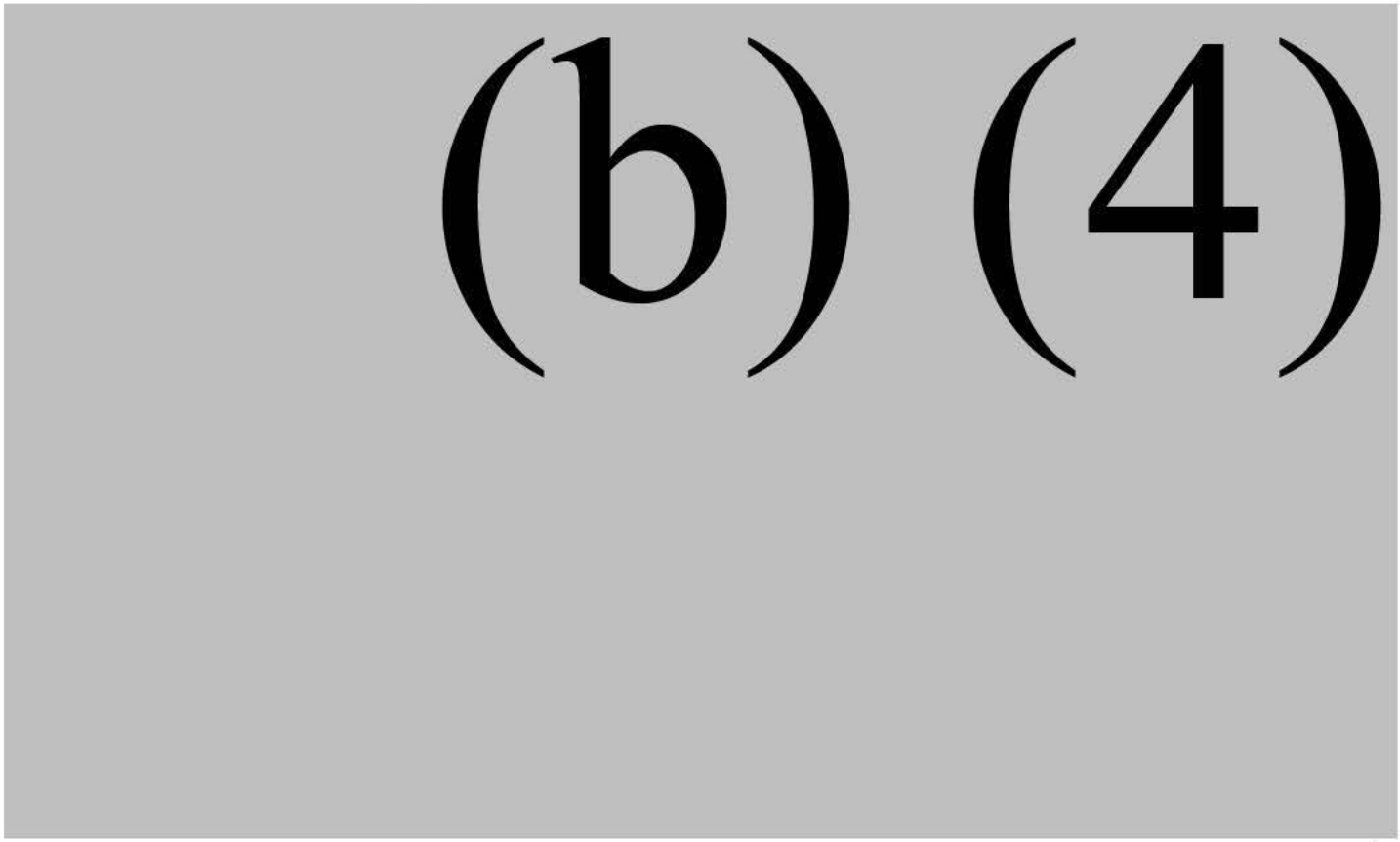
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Equipment & Materials

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Procedure

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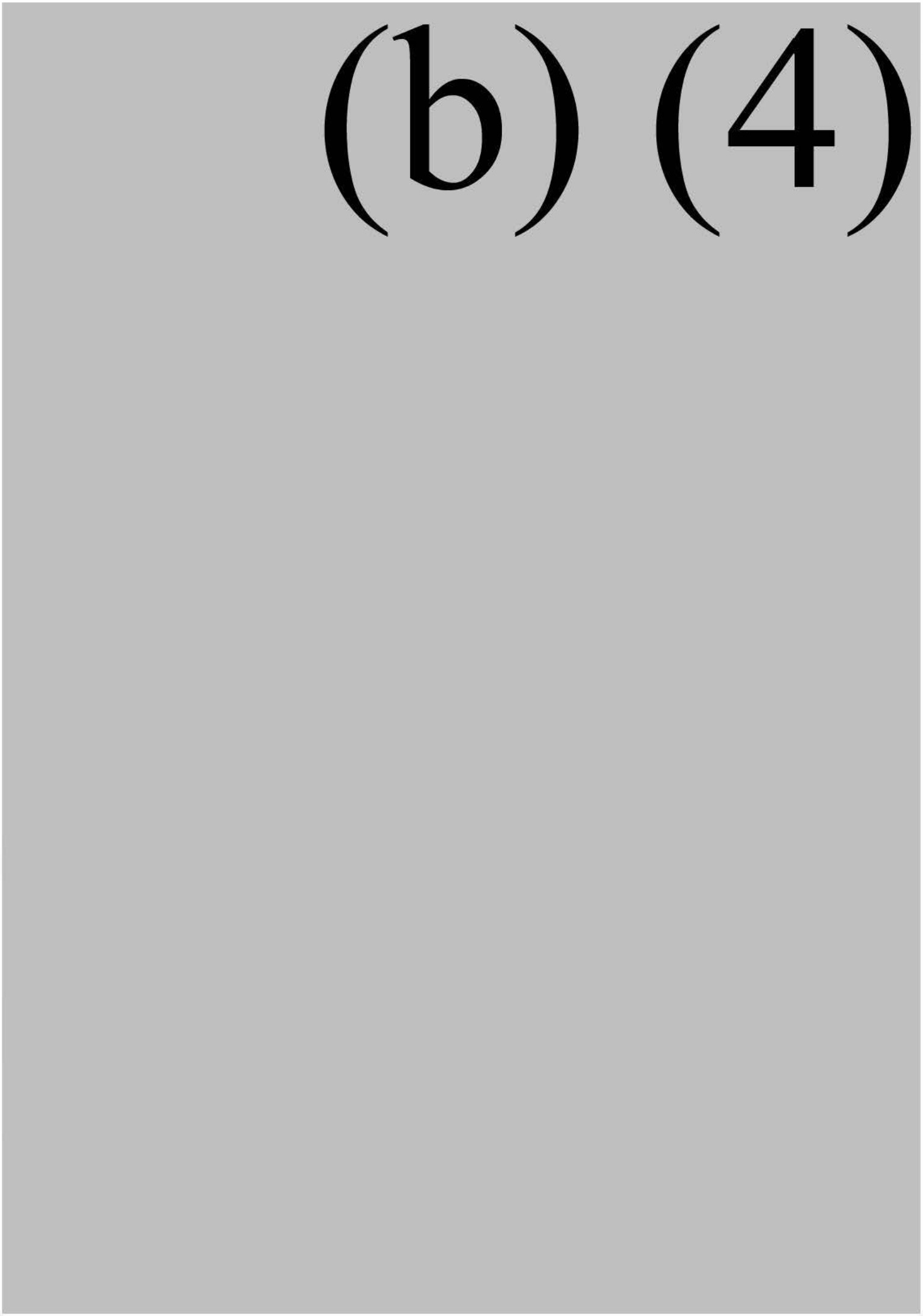
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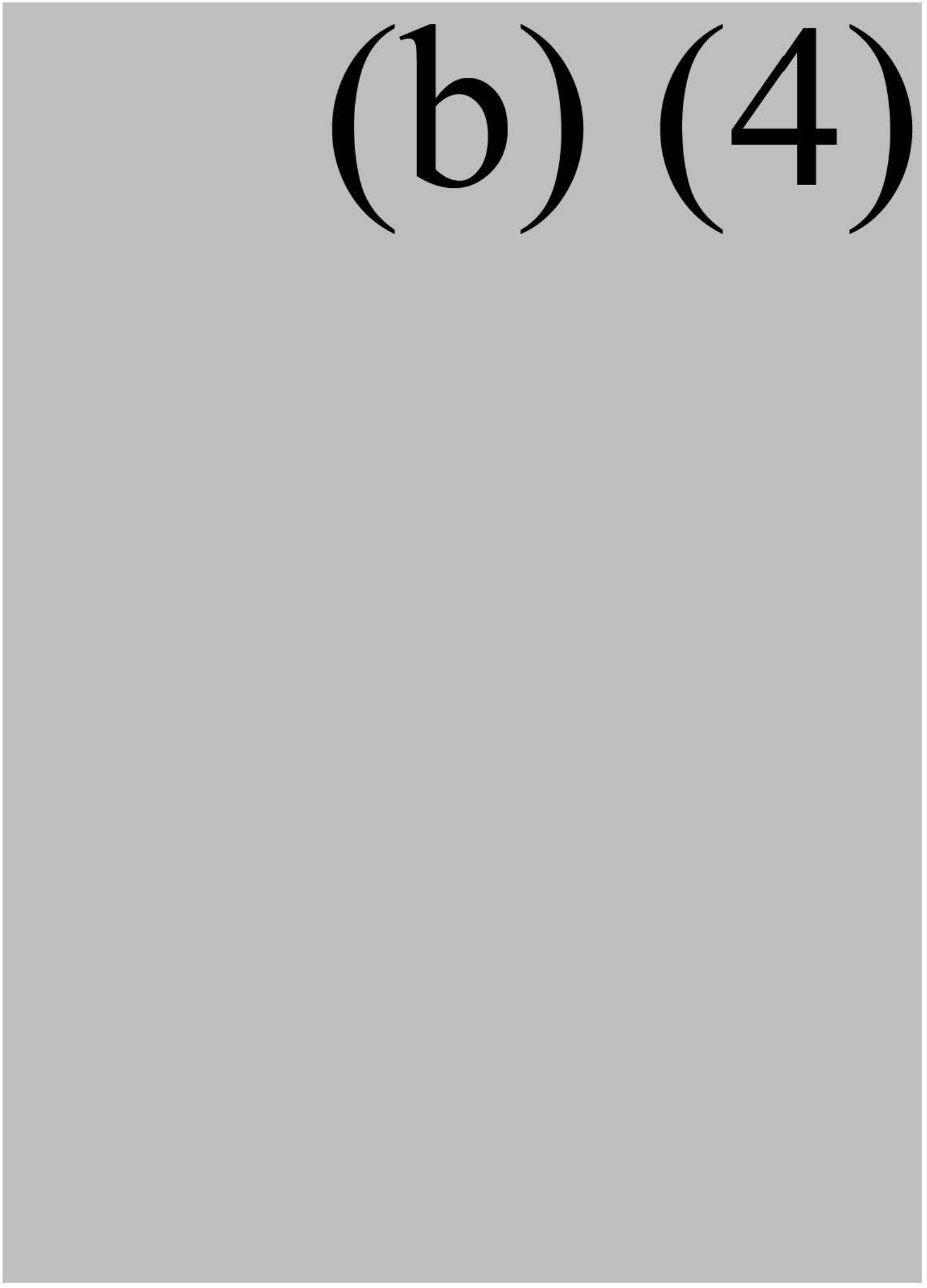
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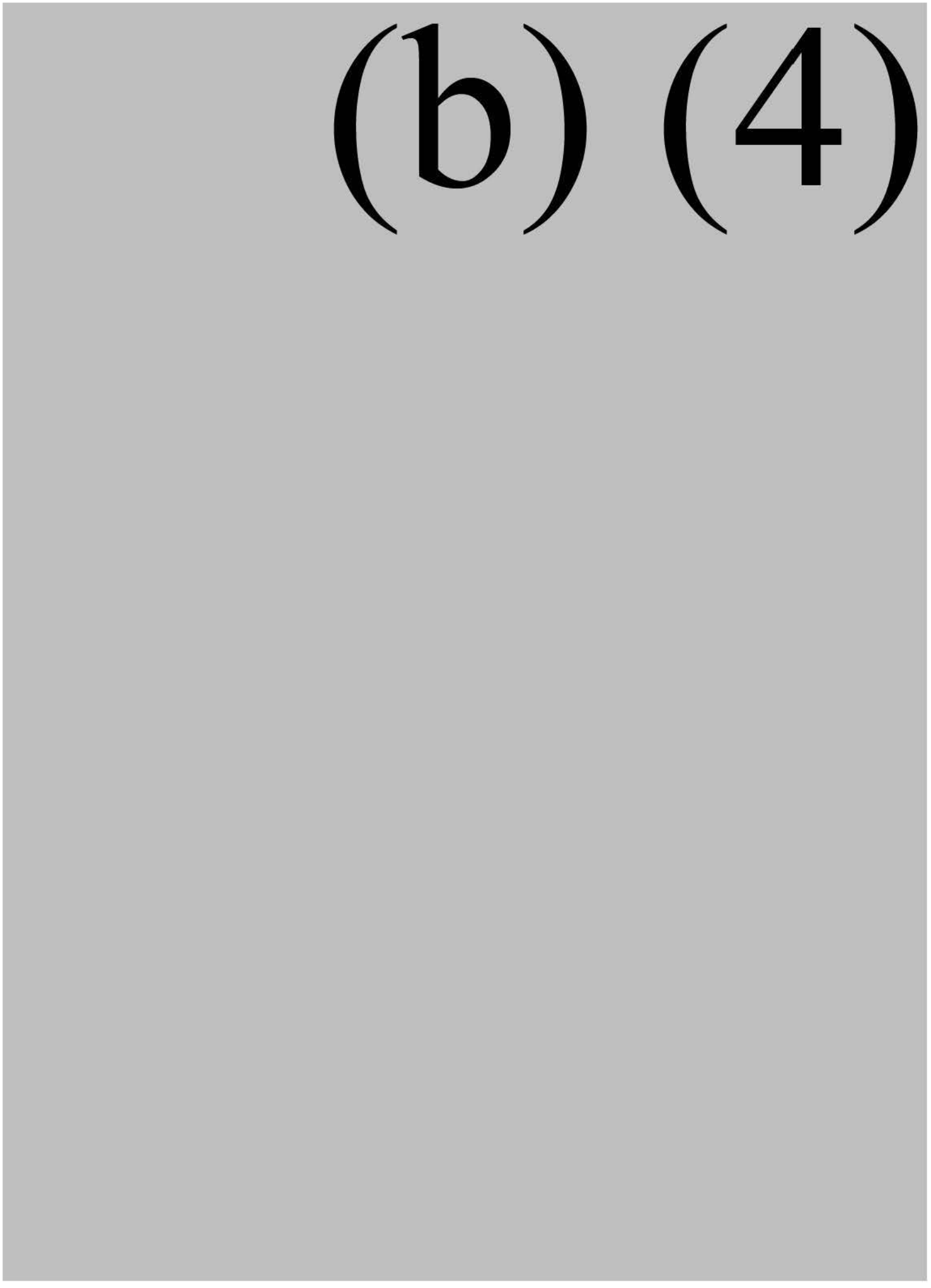
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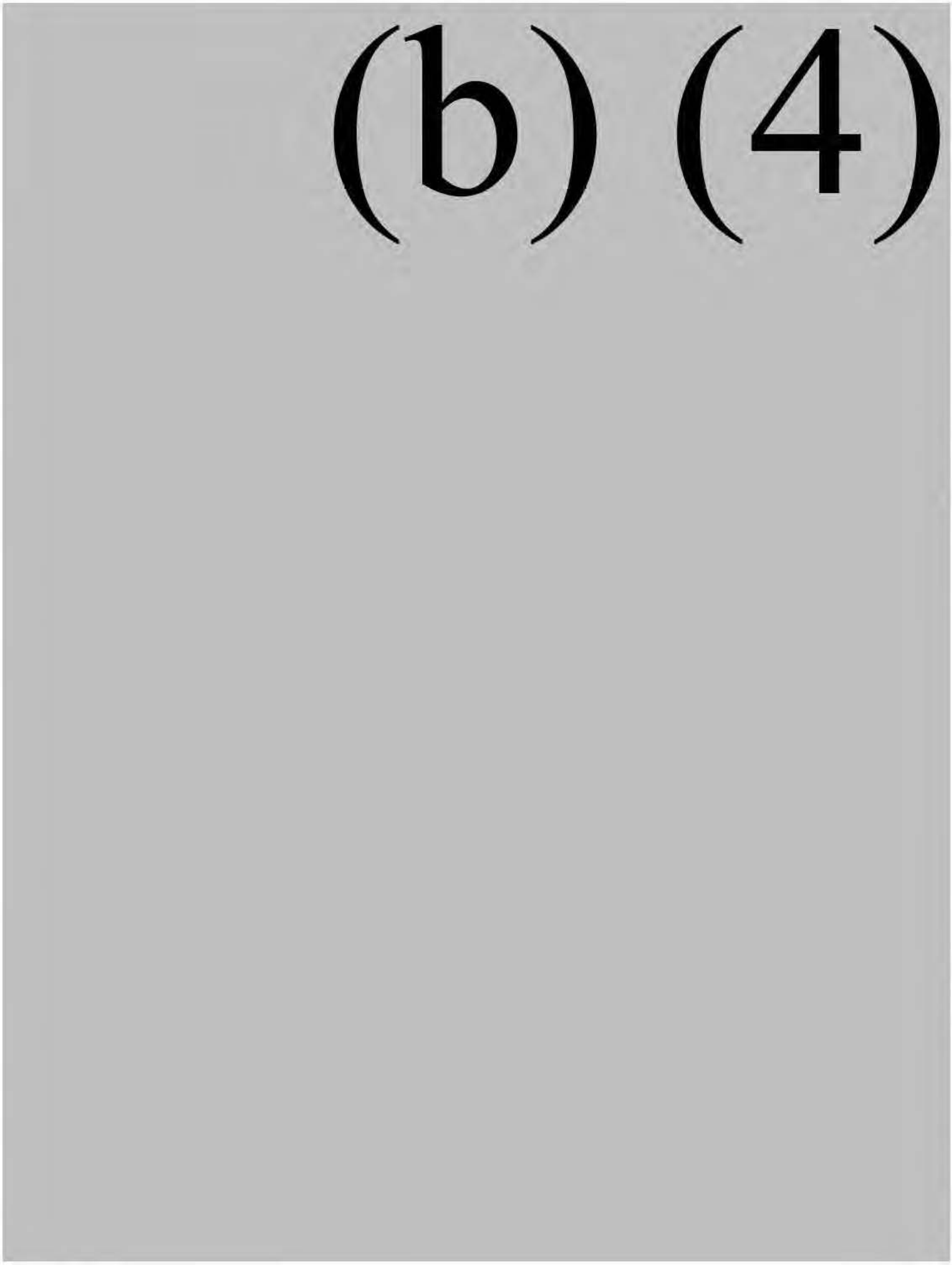
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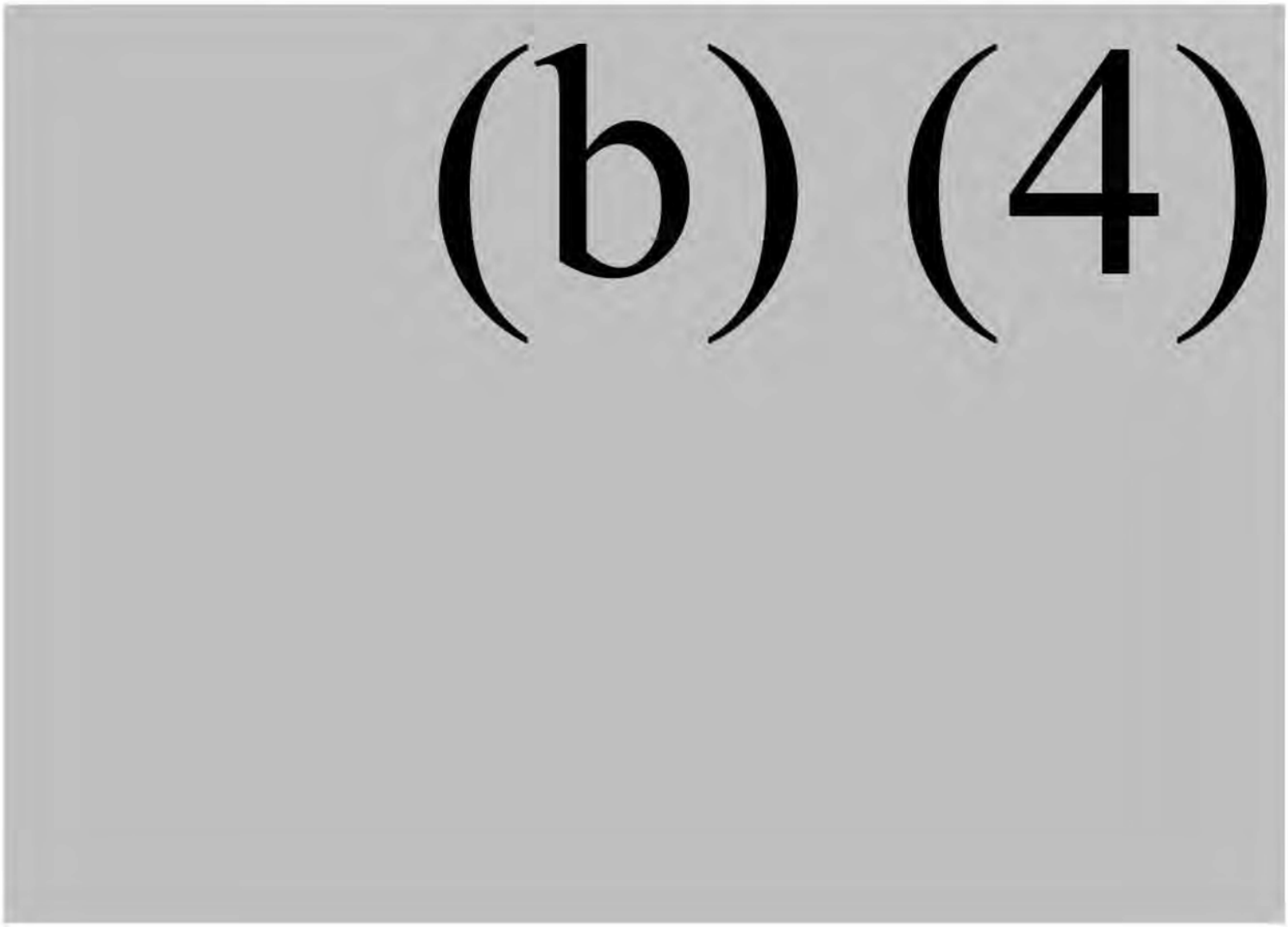
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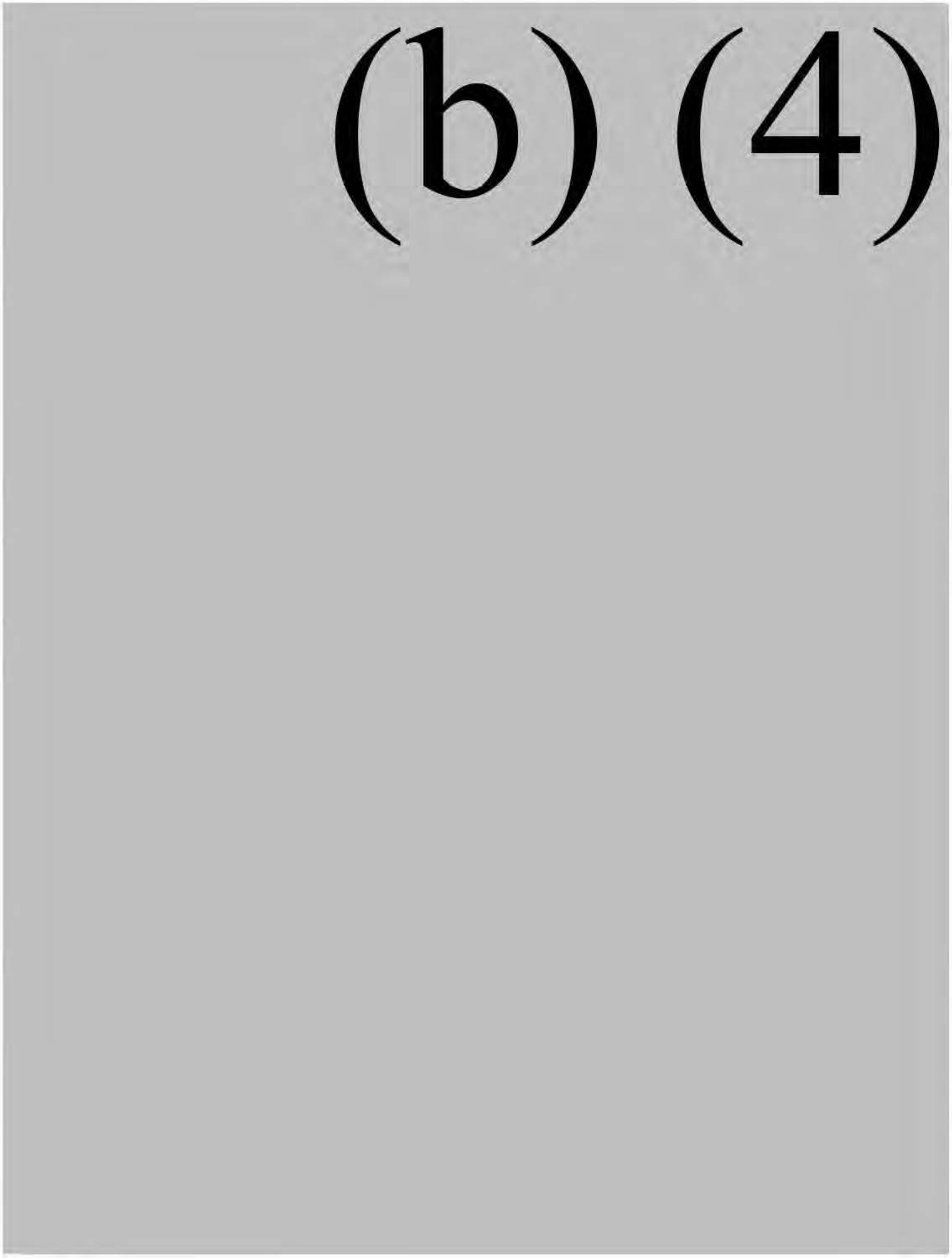
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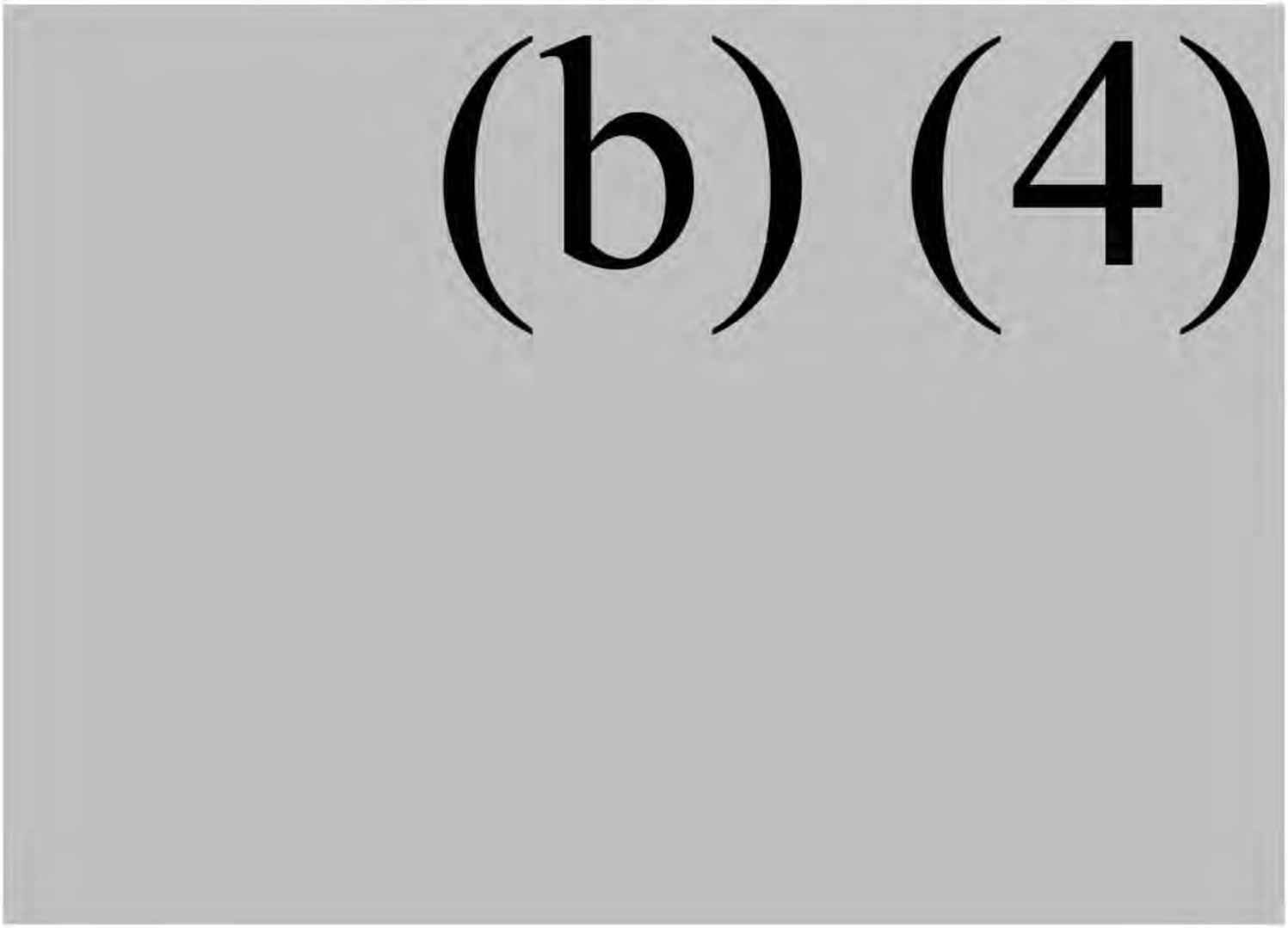
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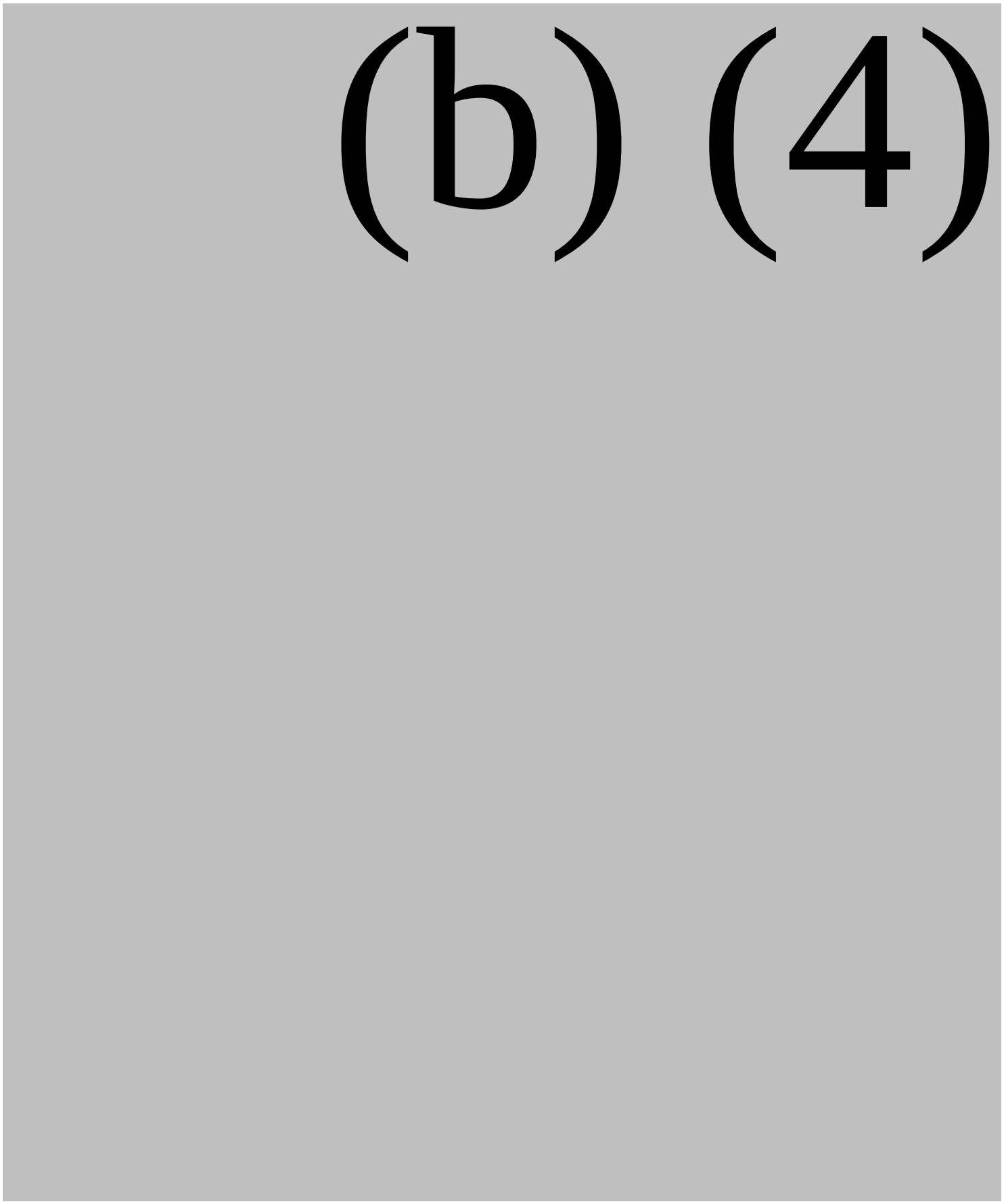

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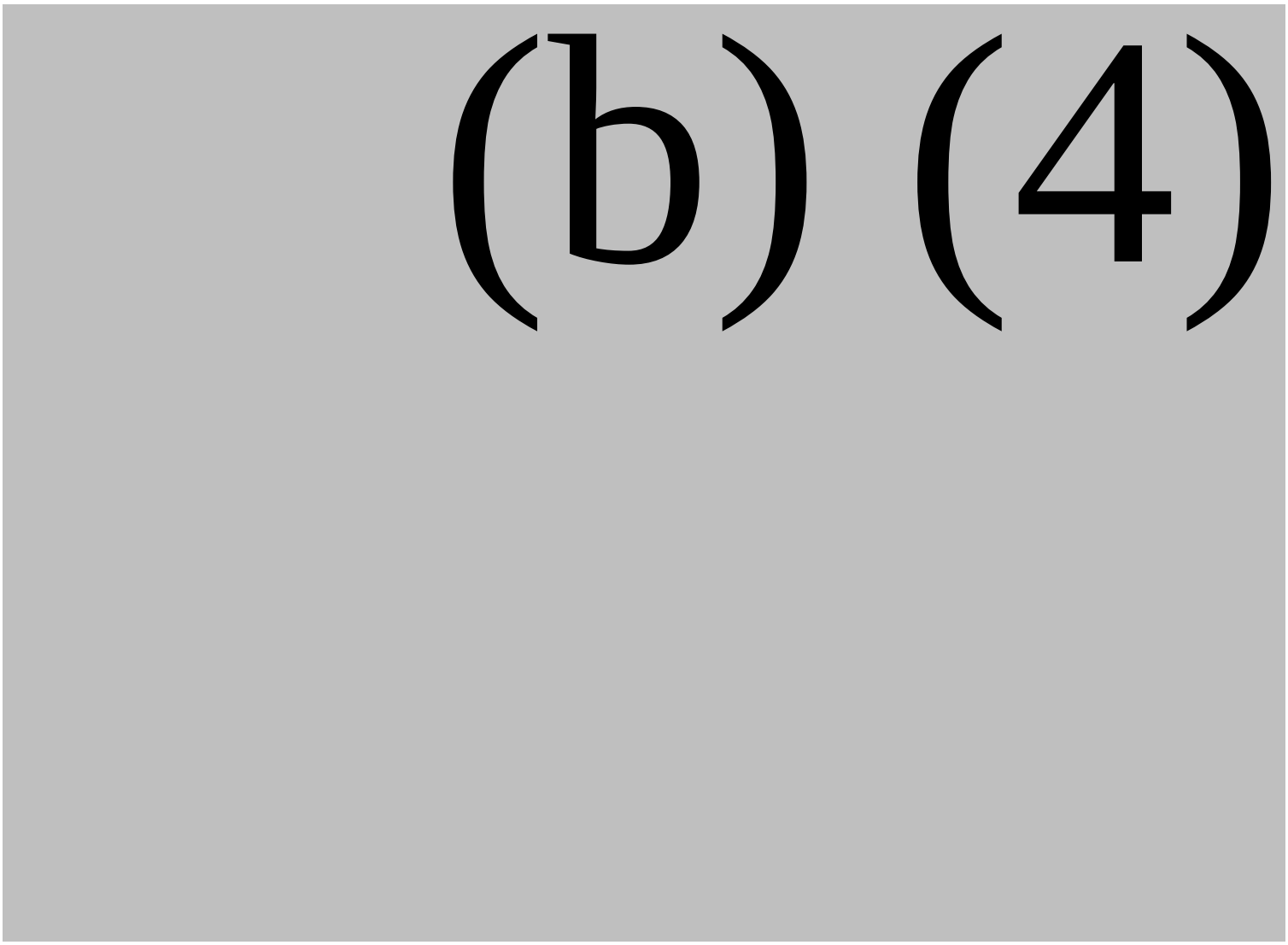

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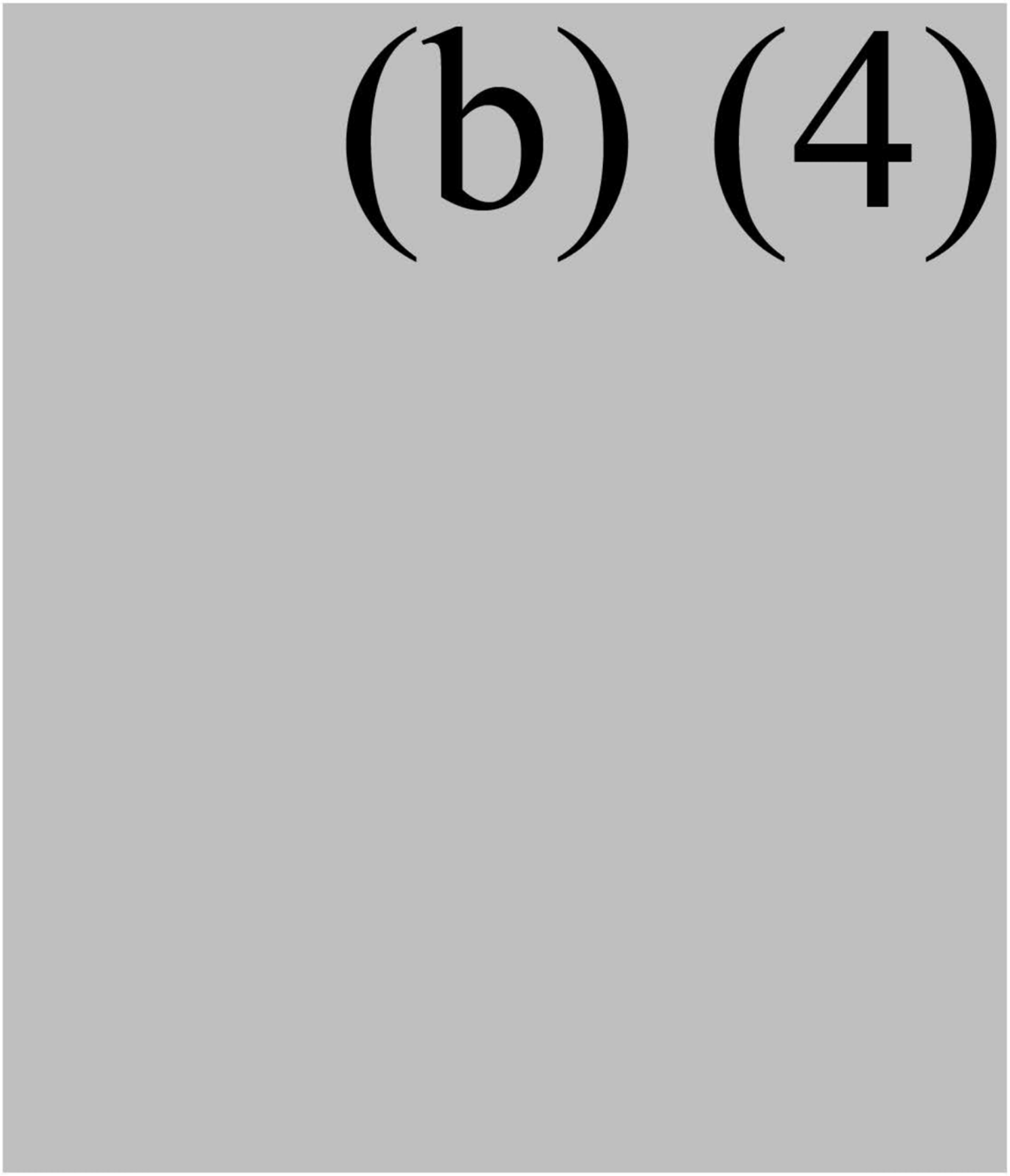
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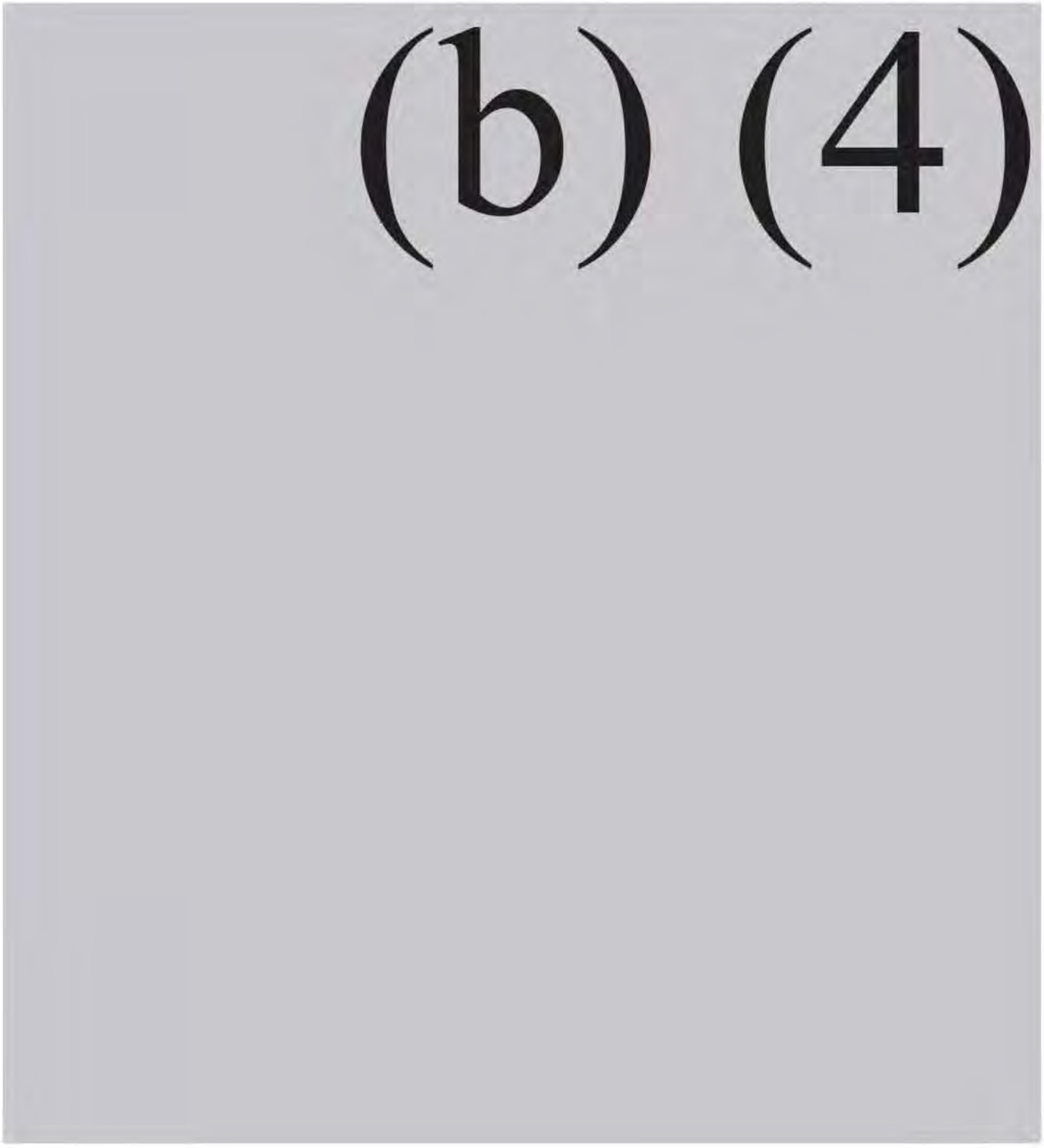
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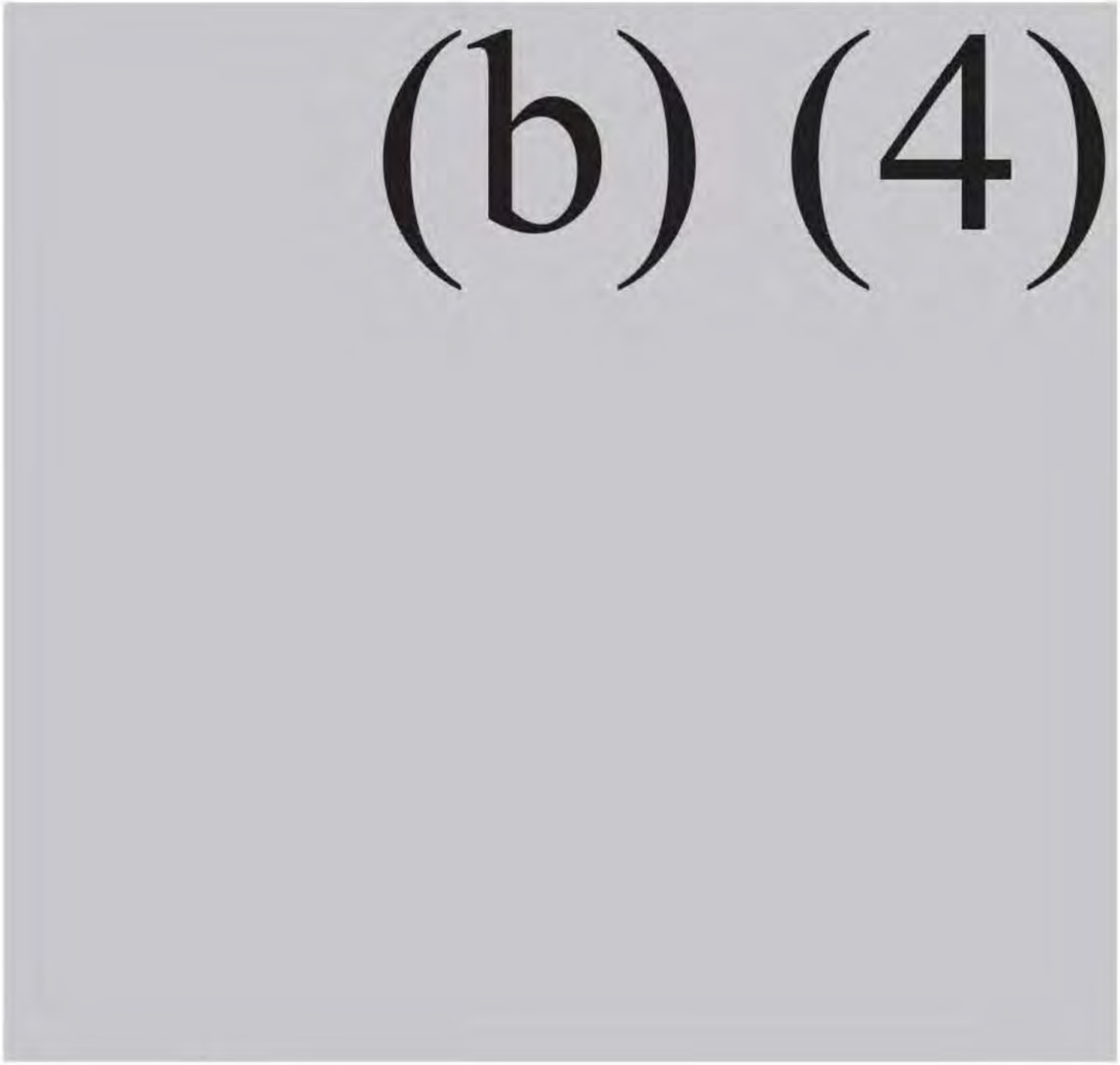
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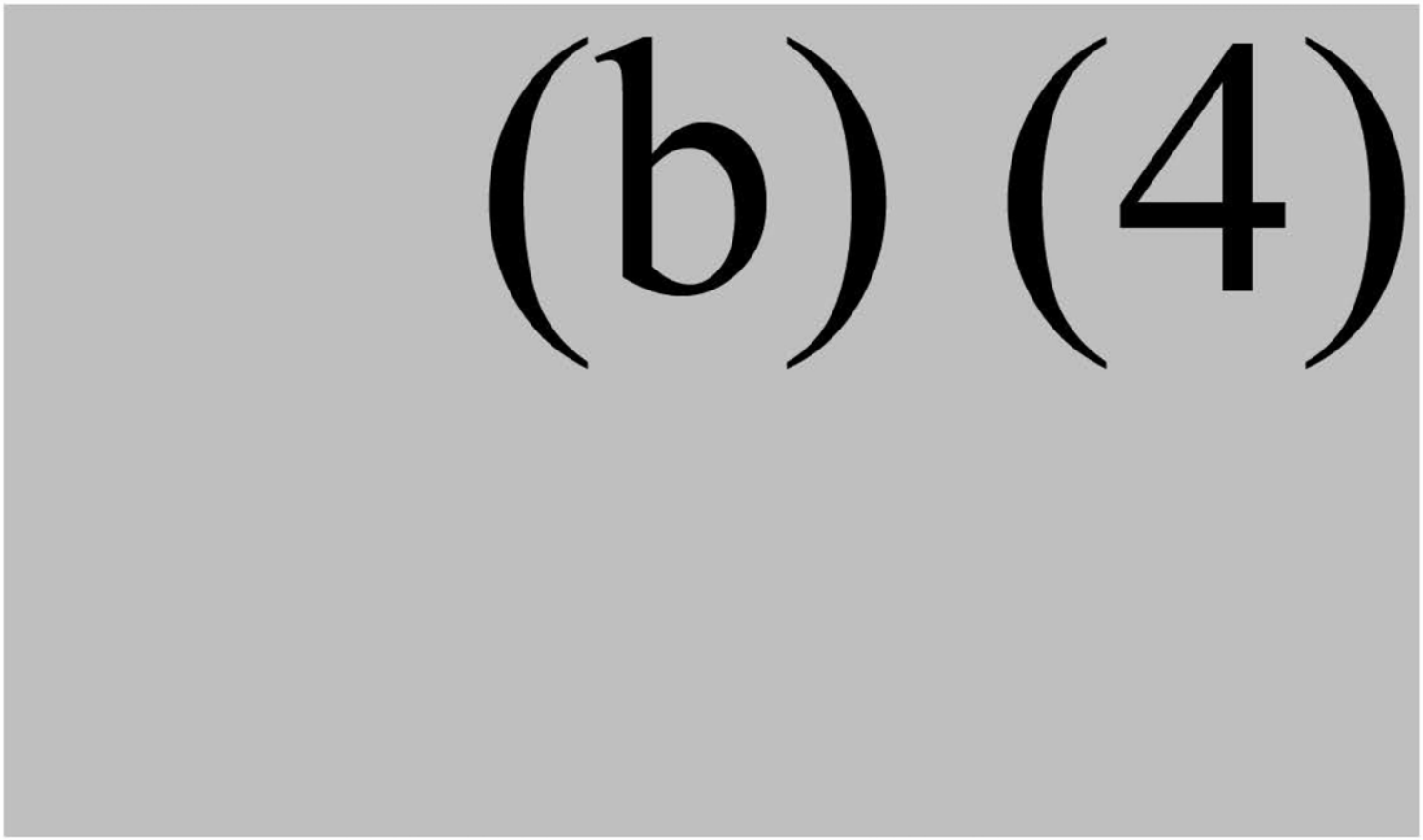
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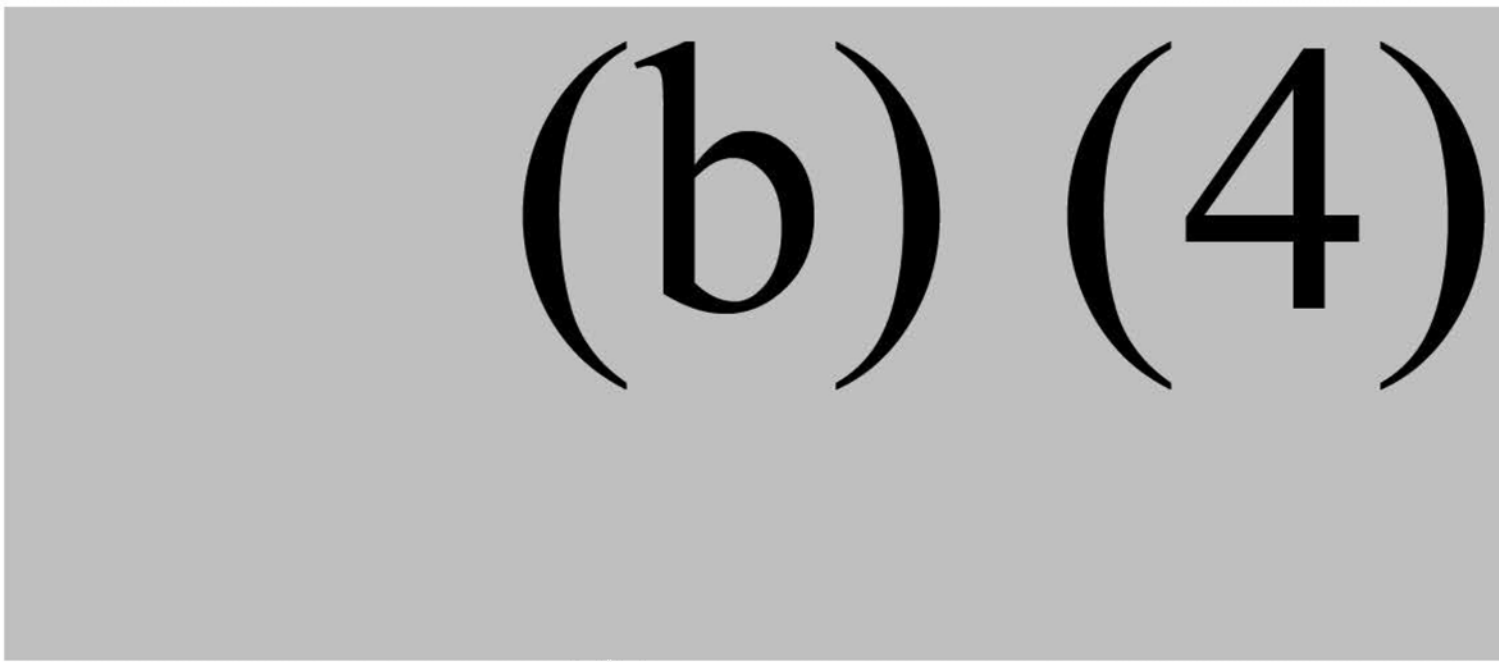
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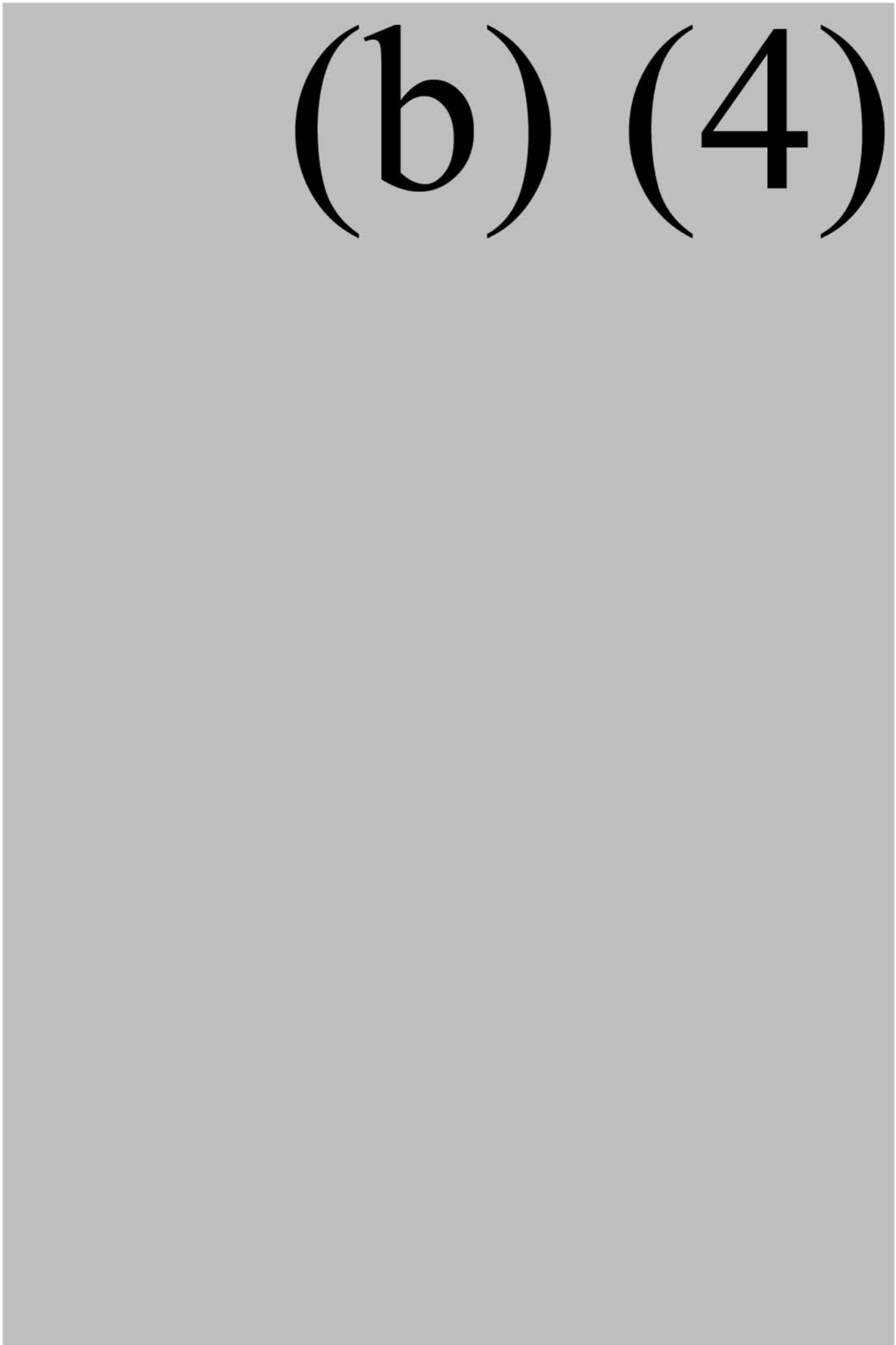
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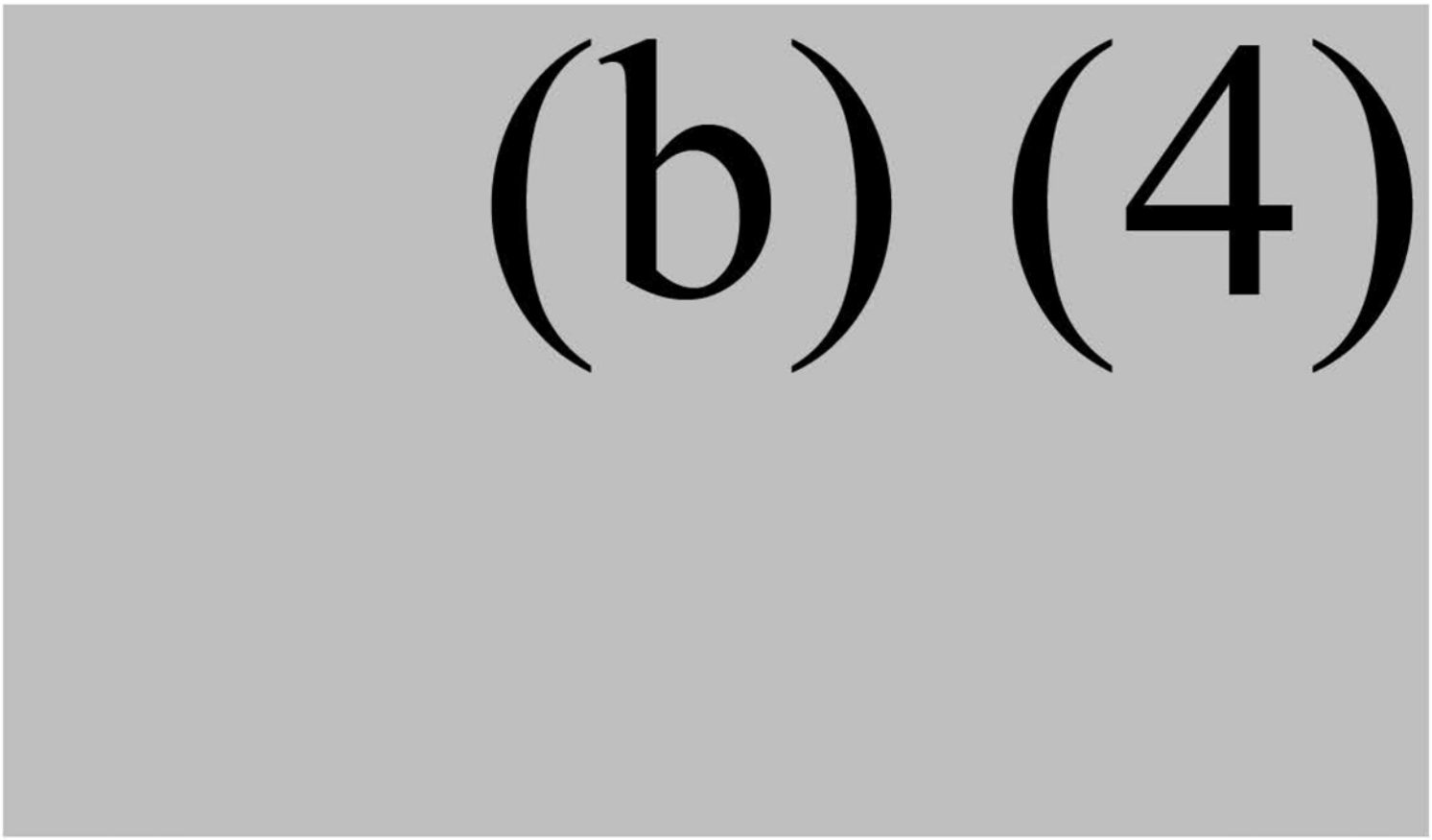
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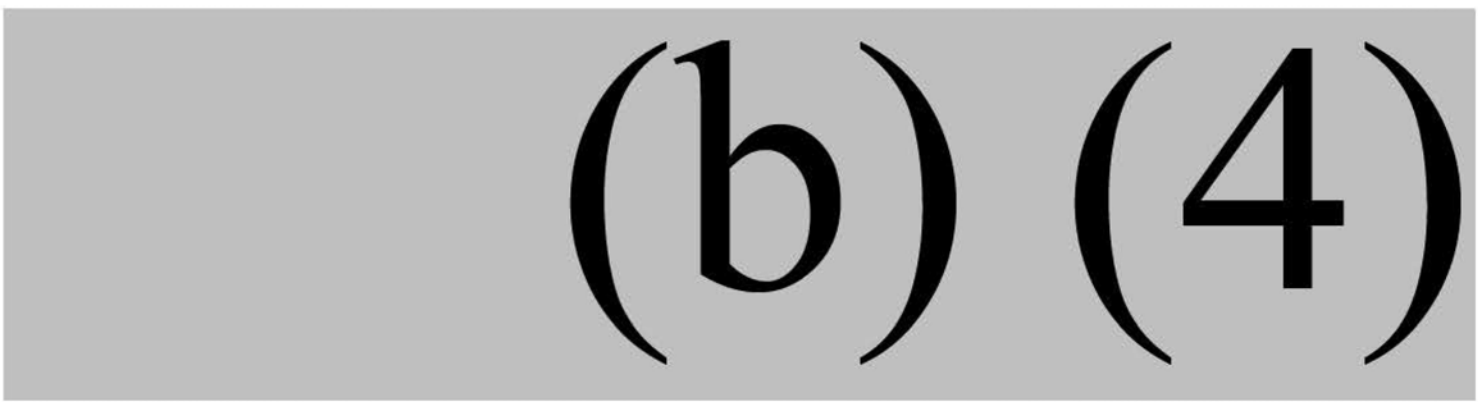
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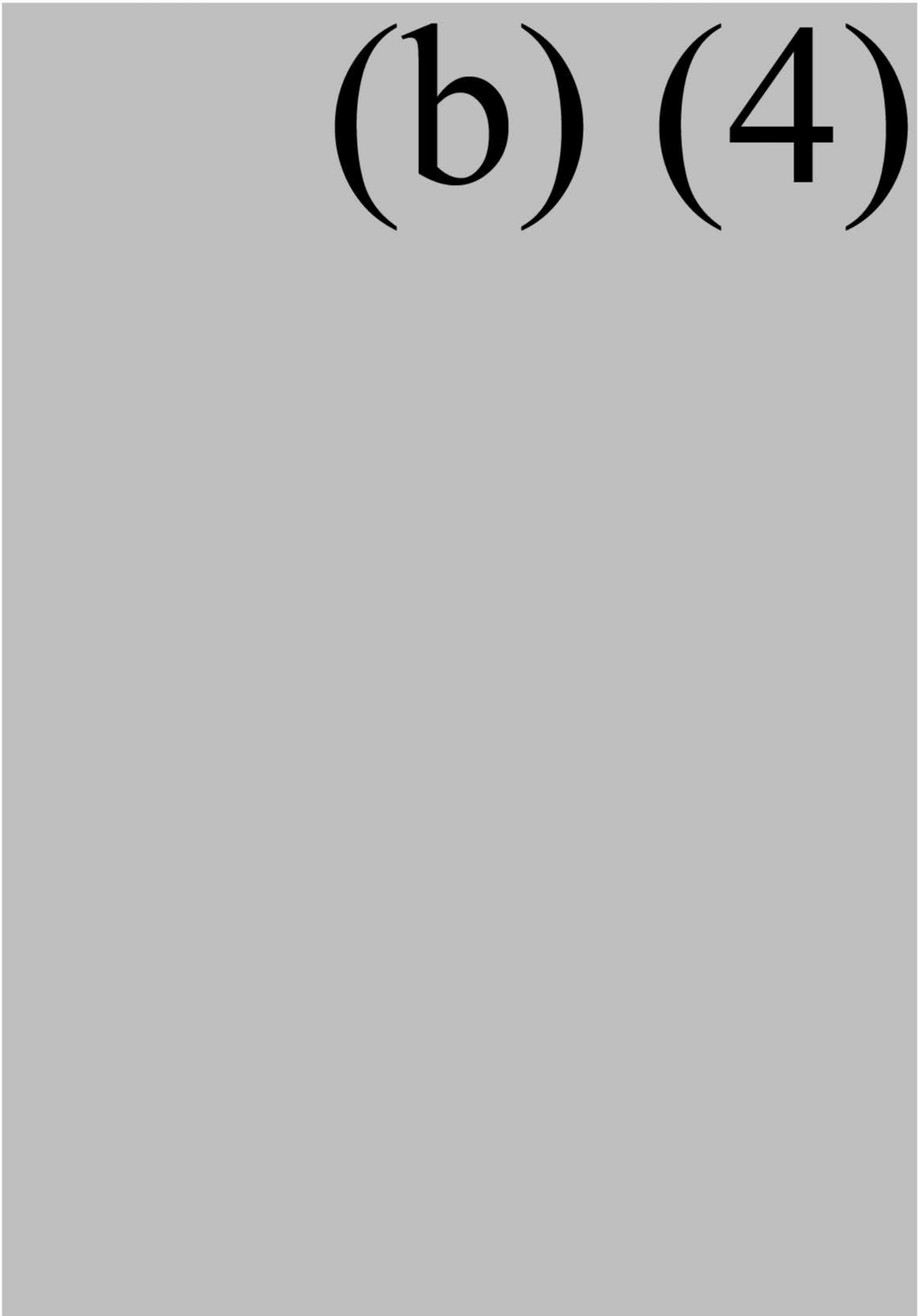
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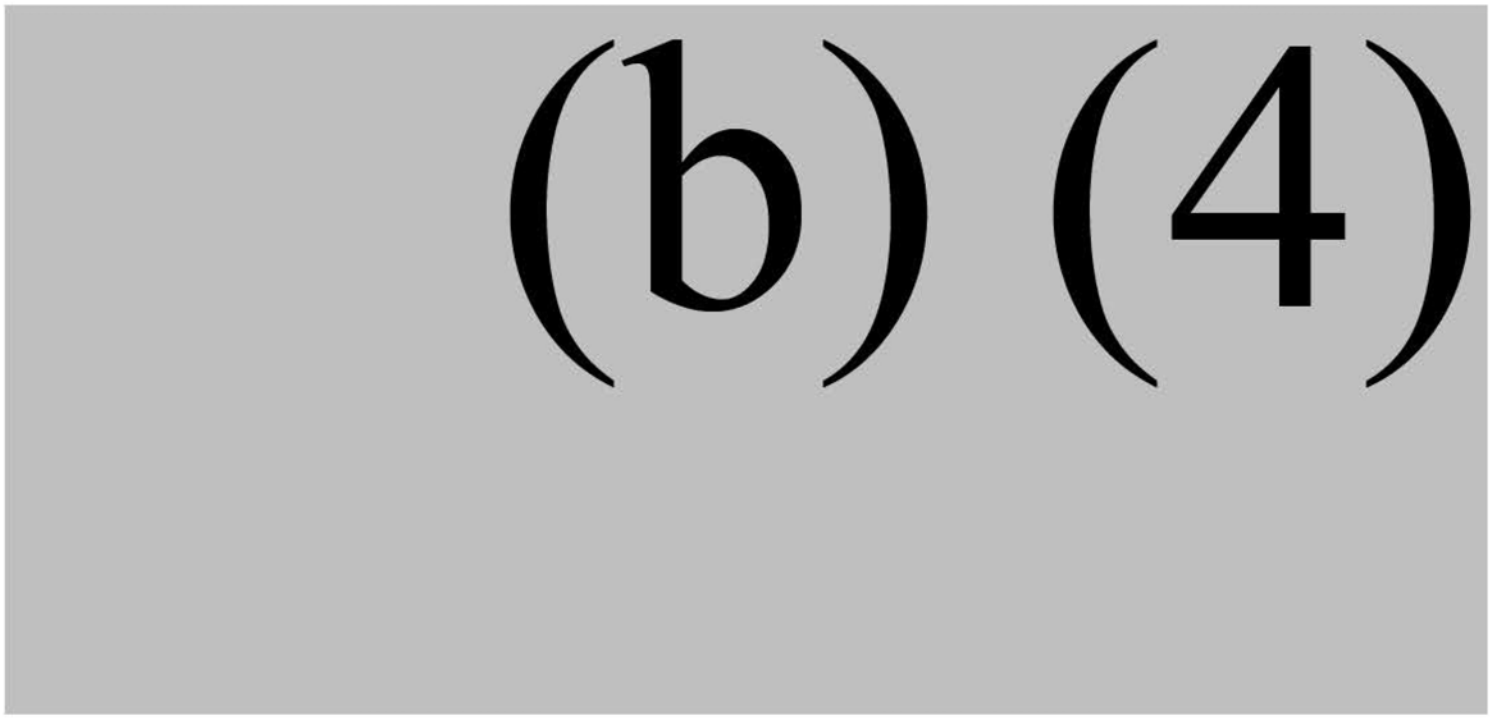
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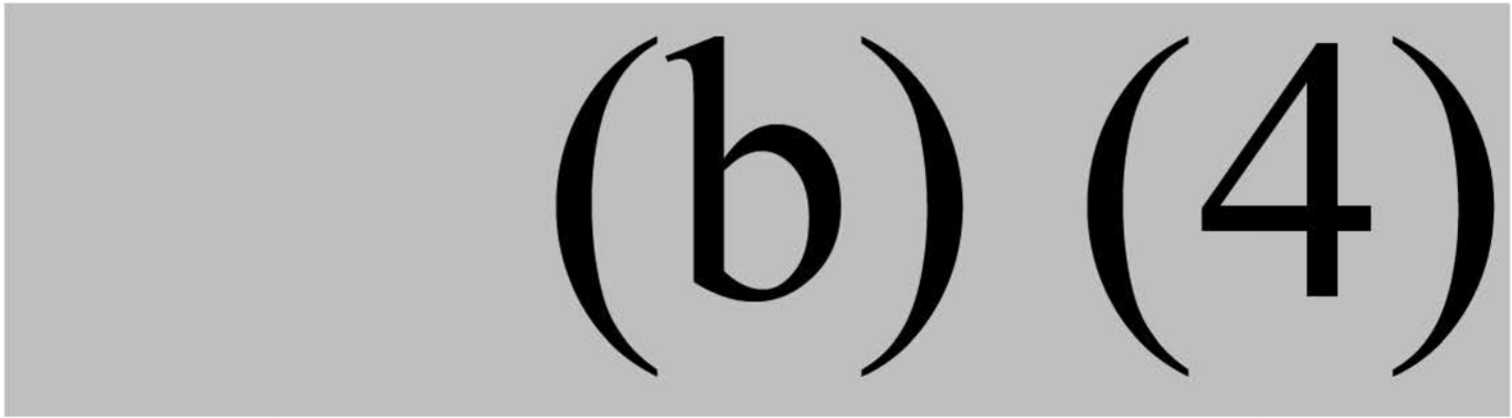
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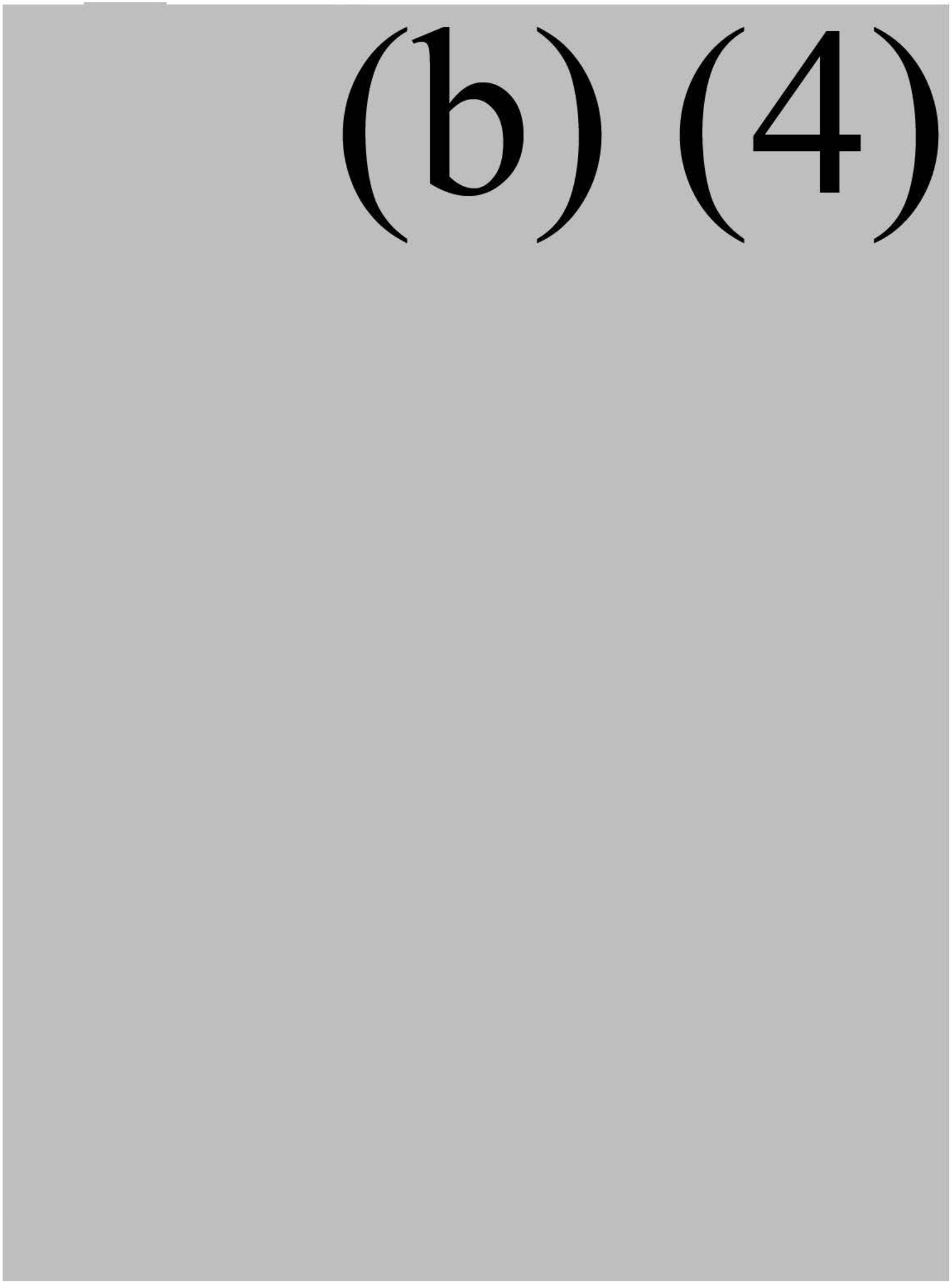
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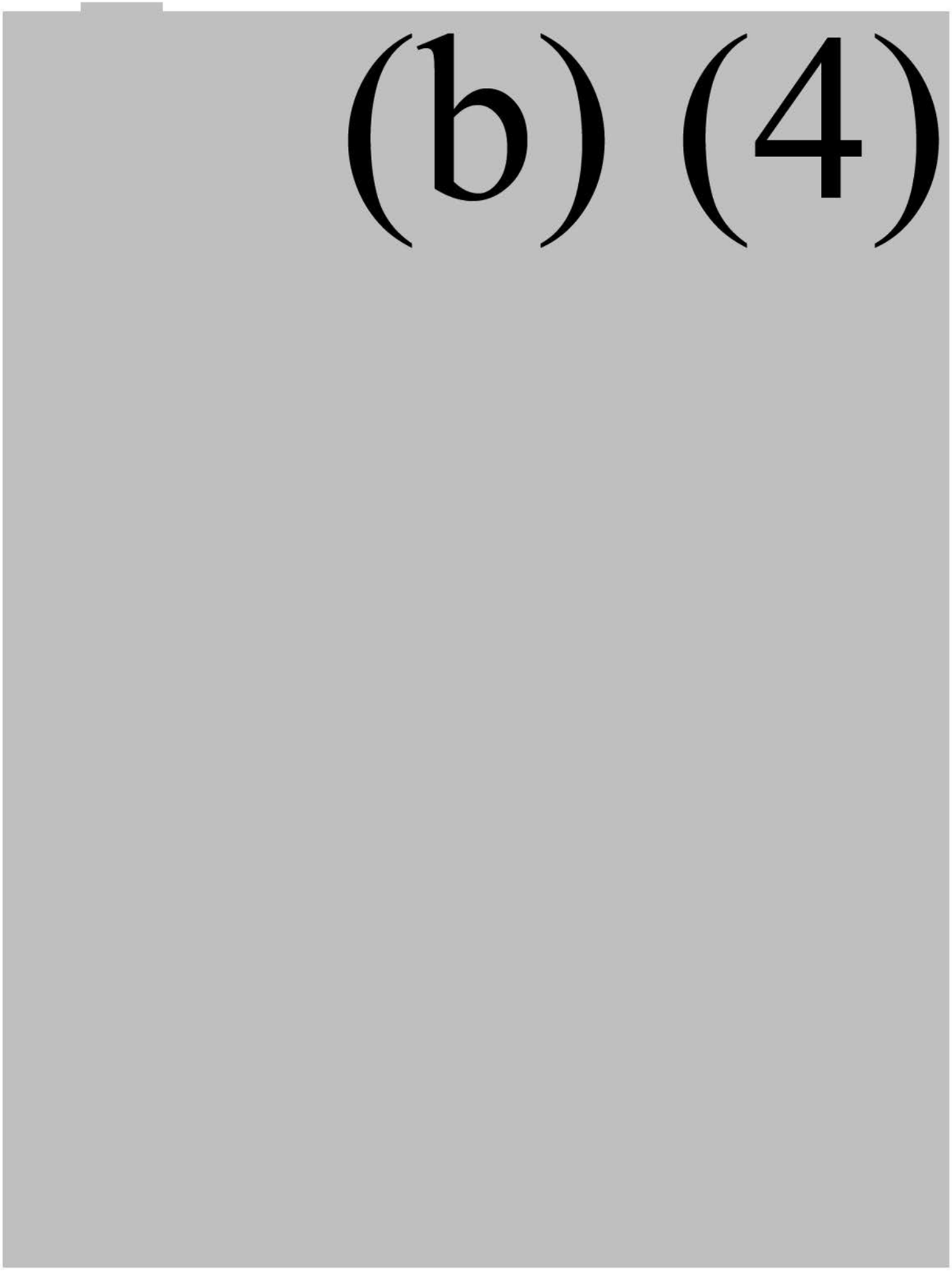
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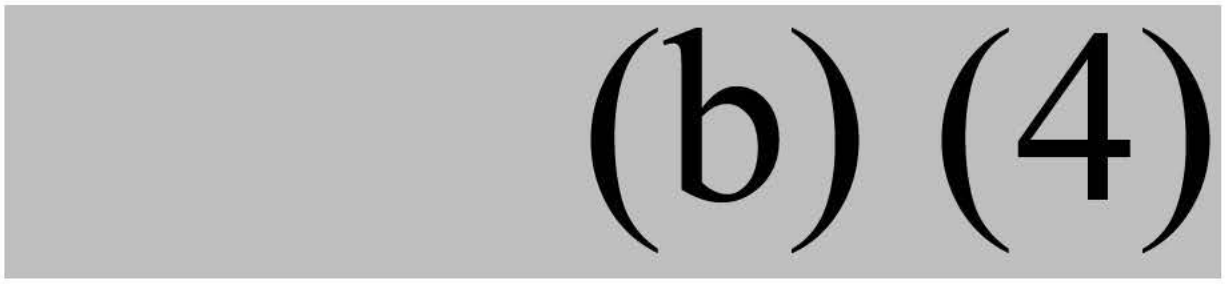
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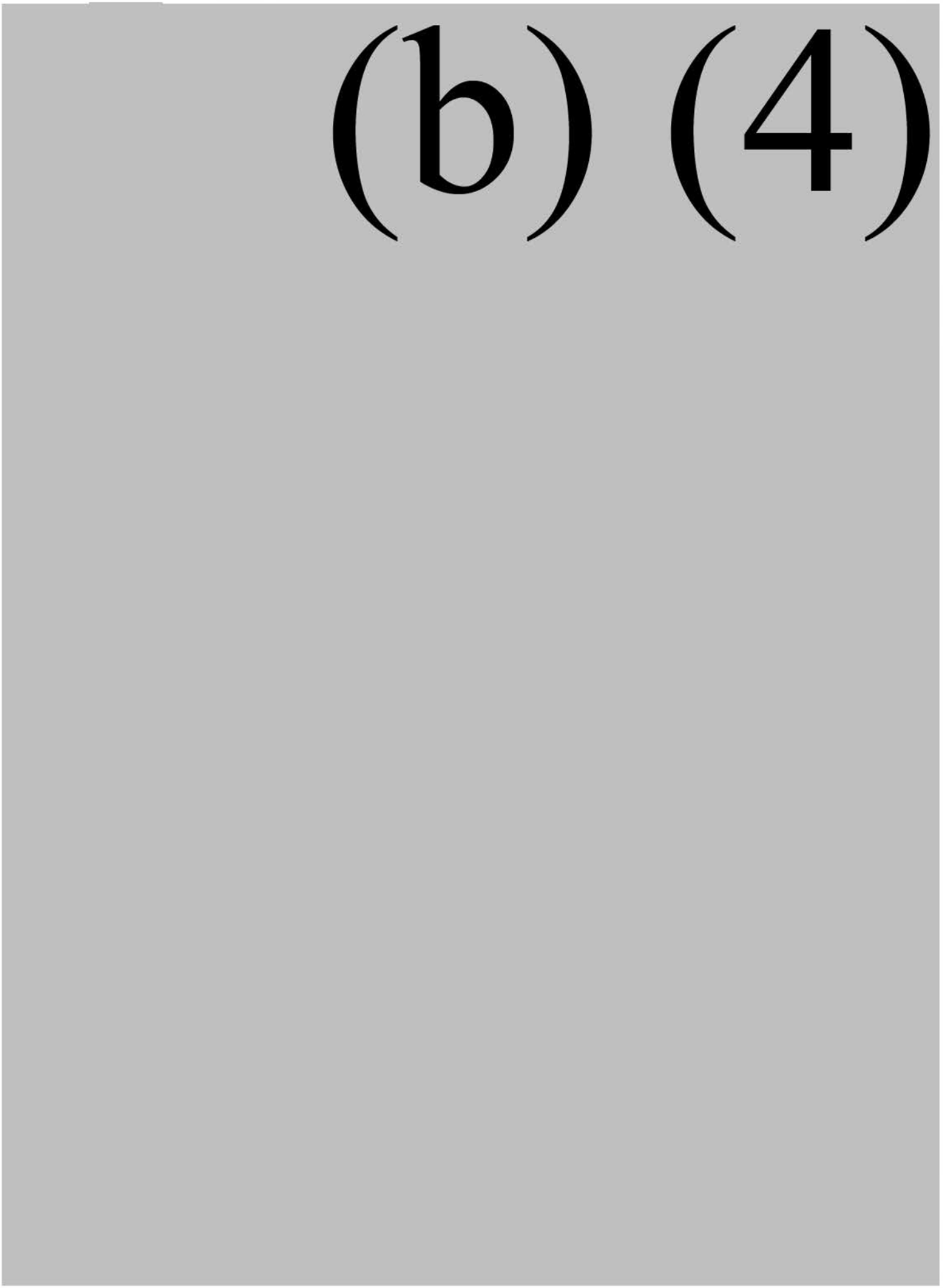
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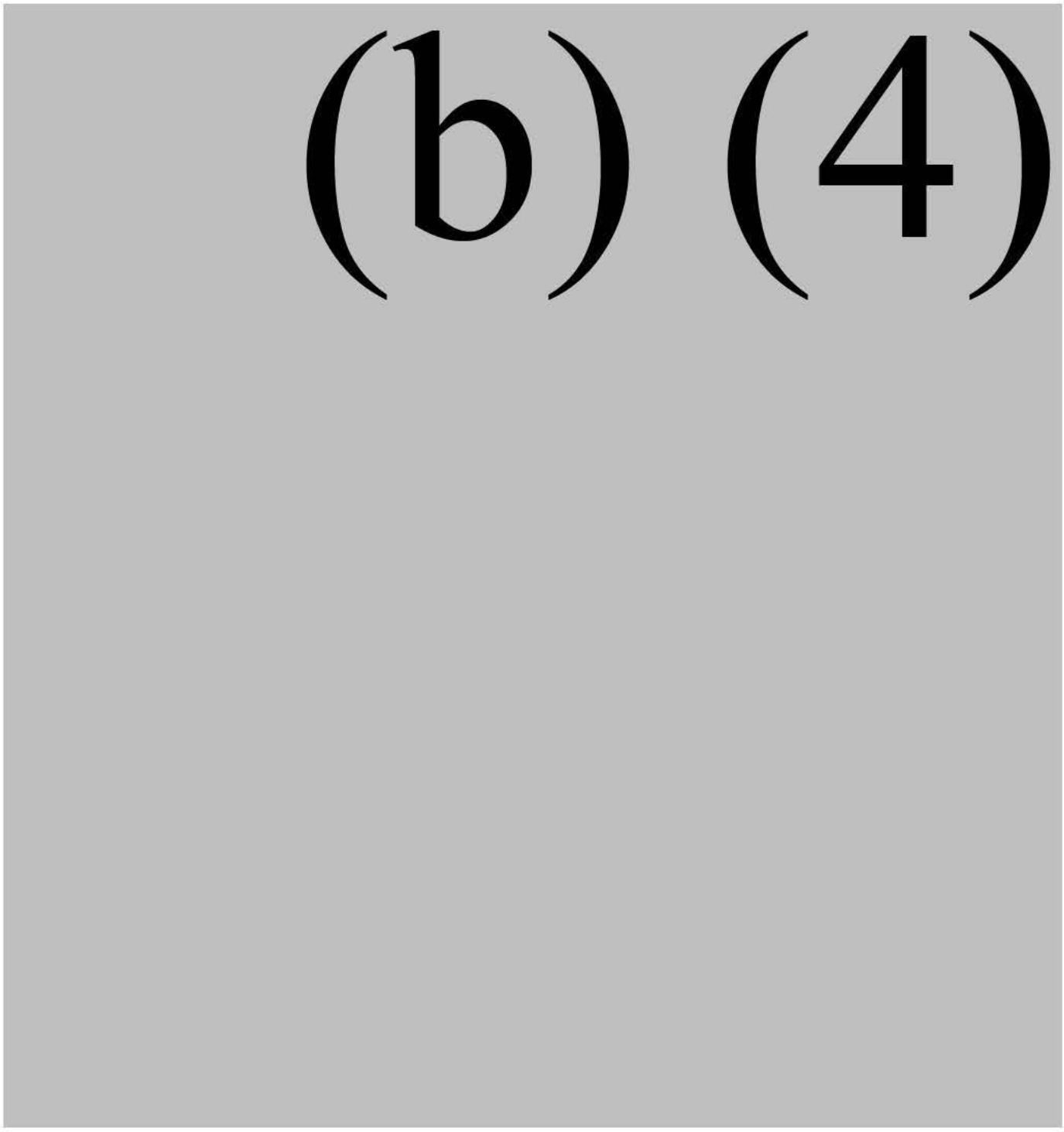
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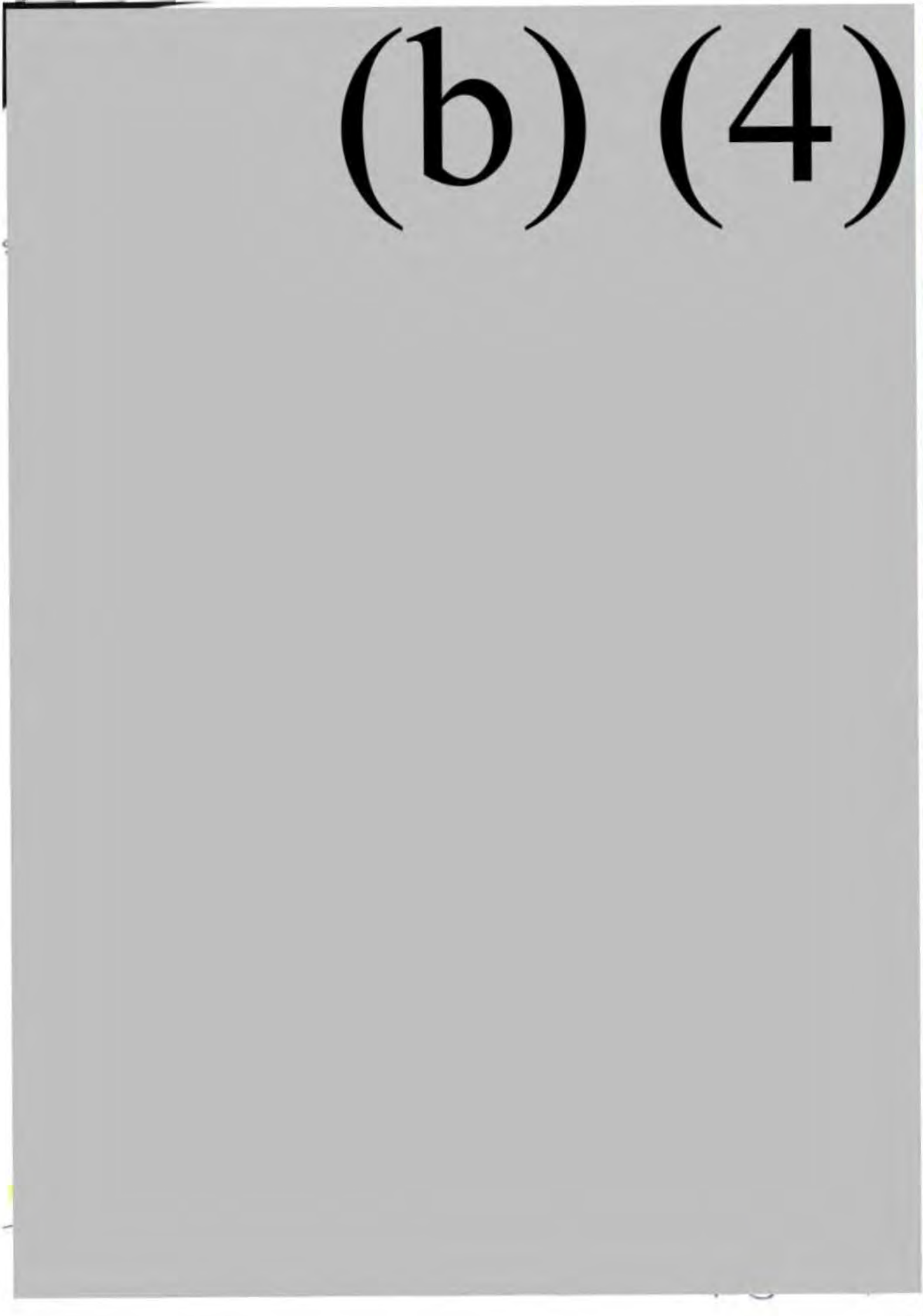
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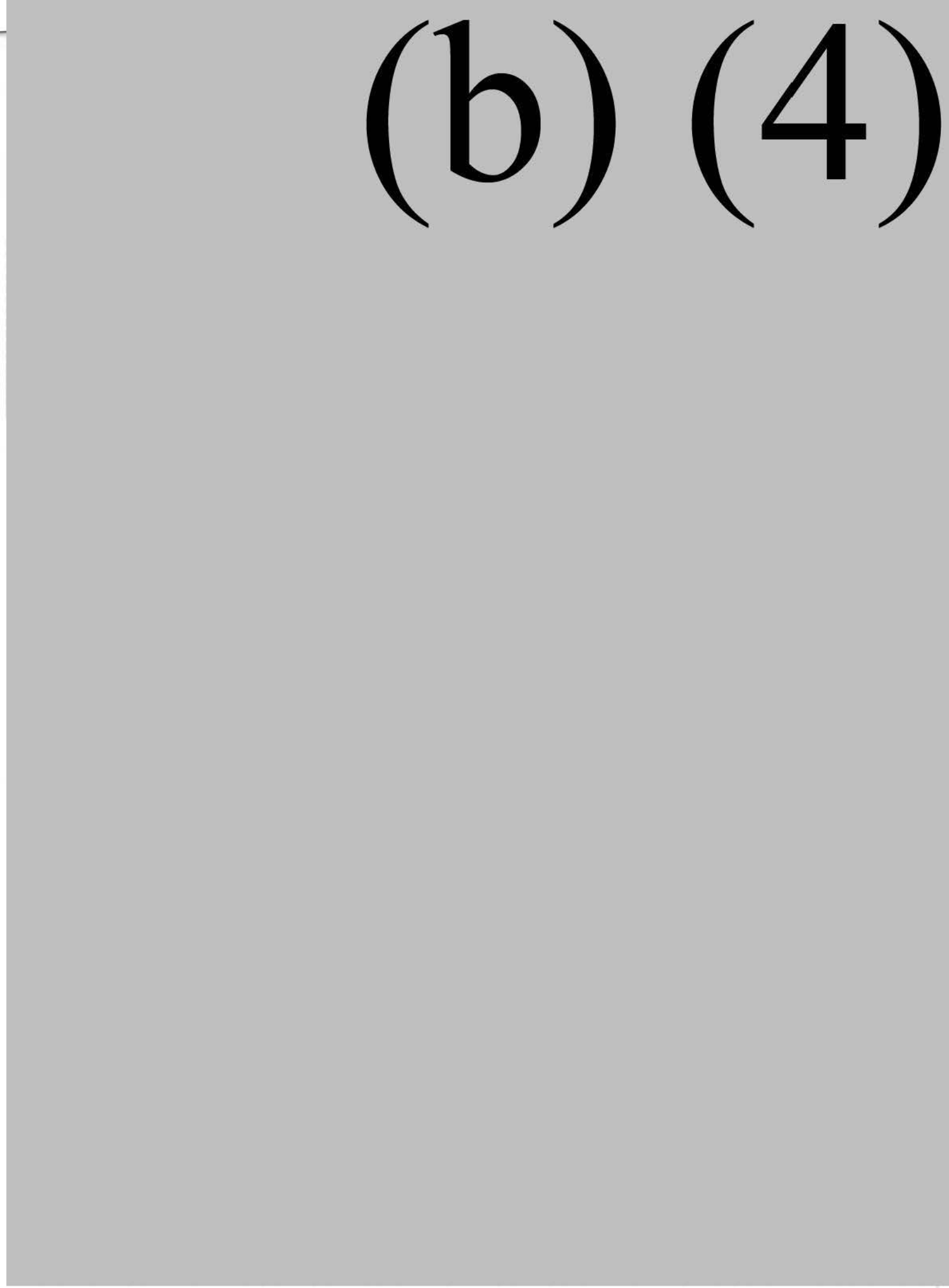
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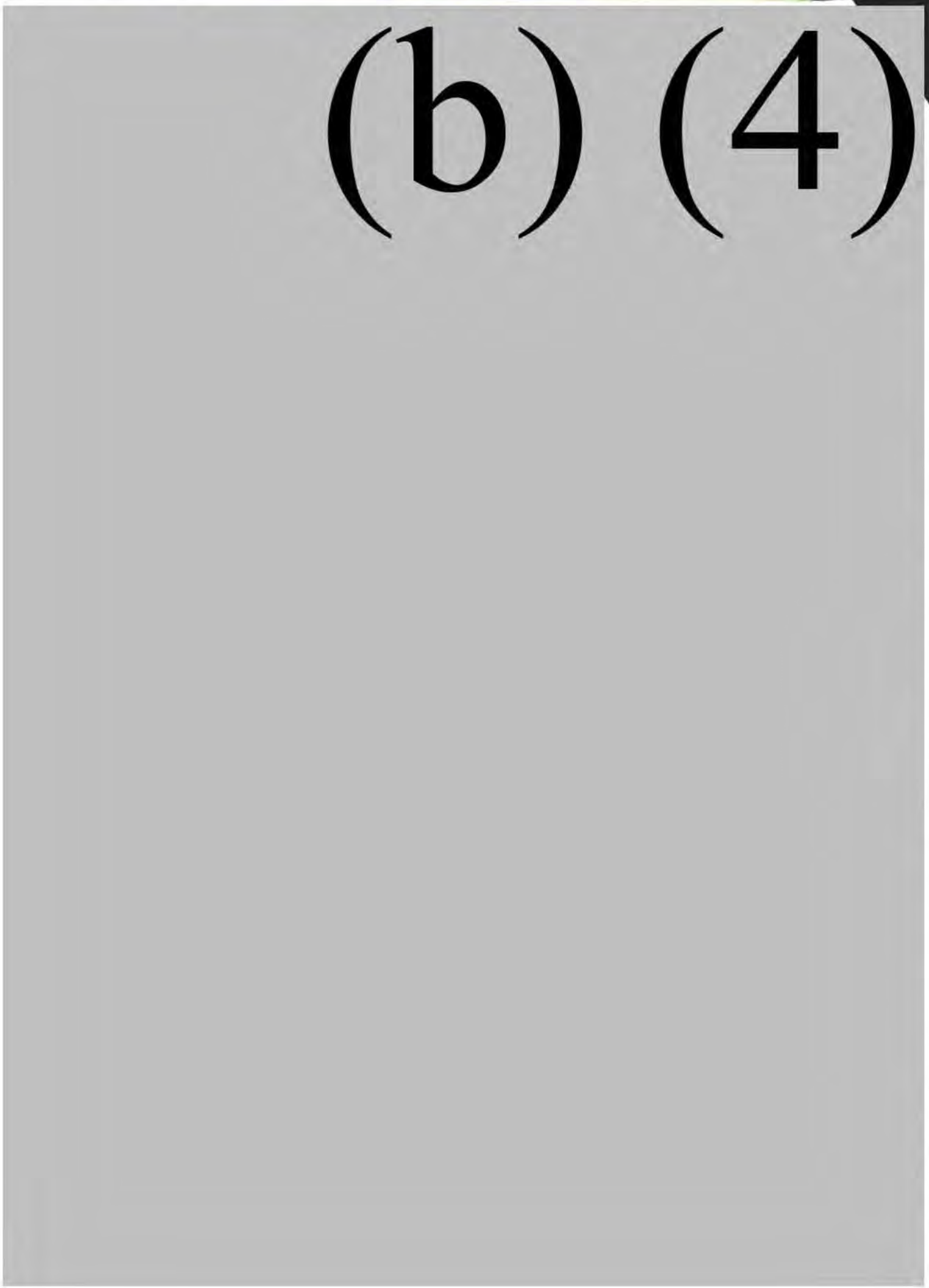
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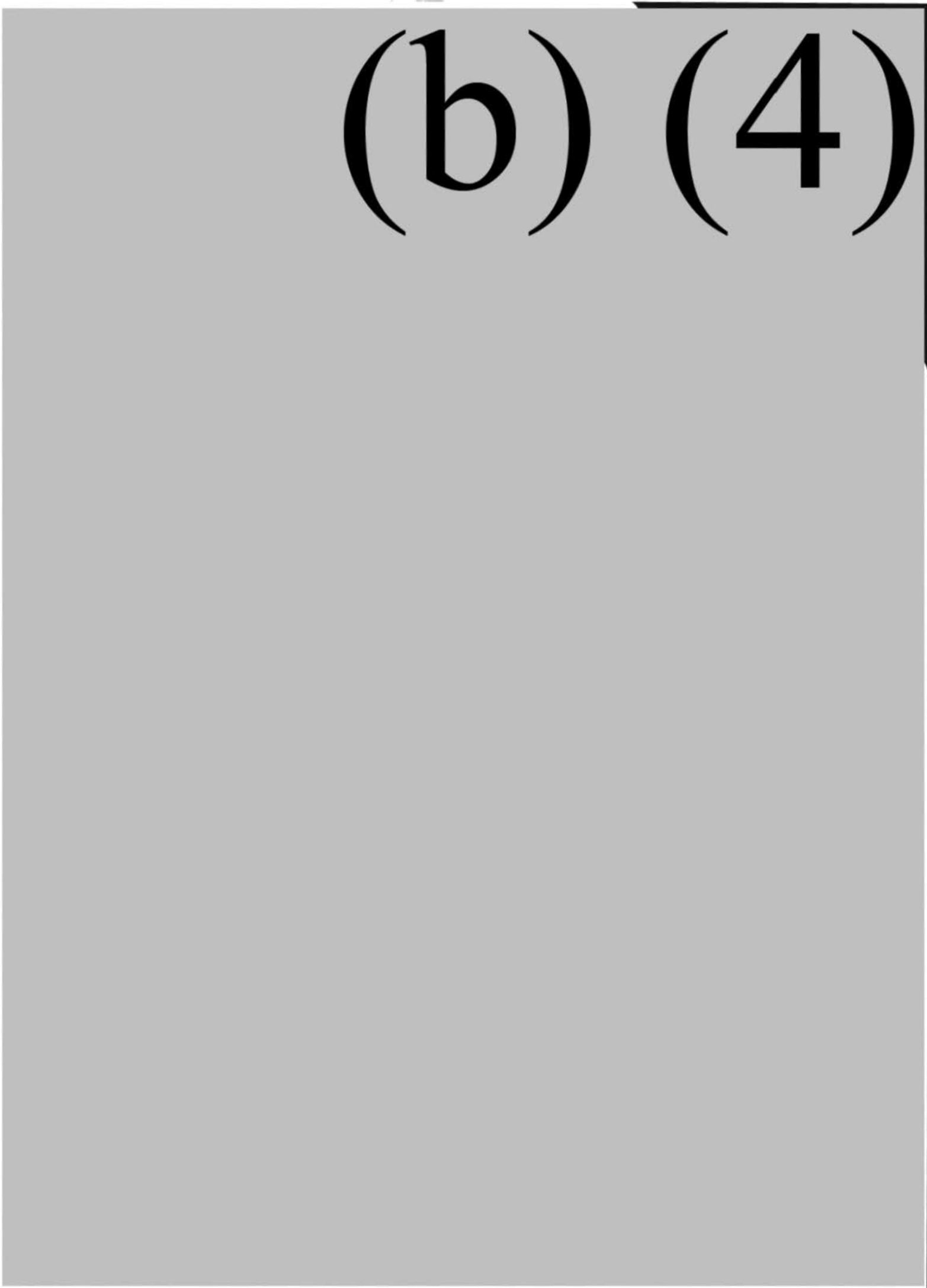
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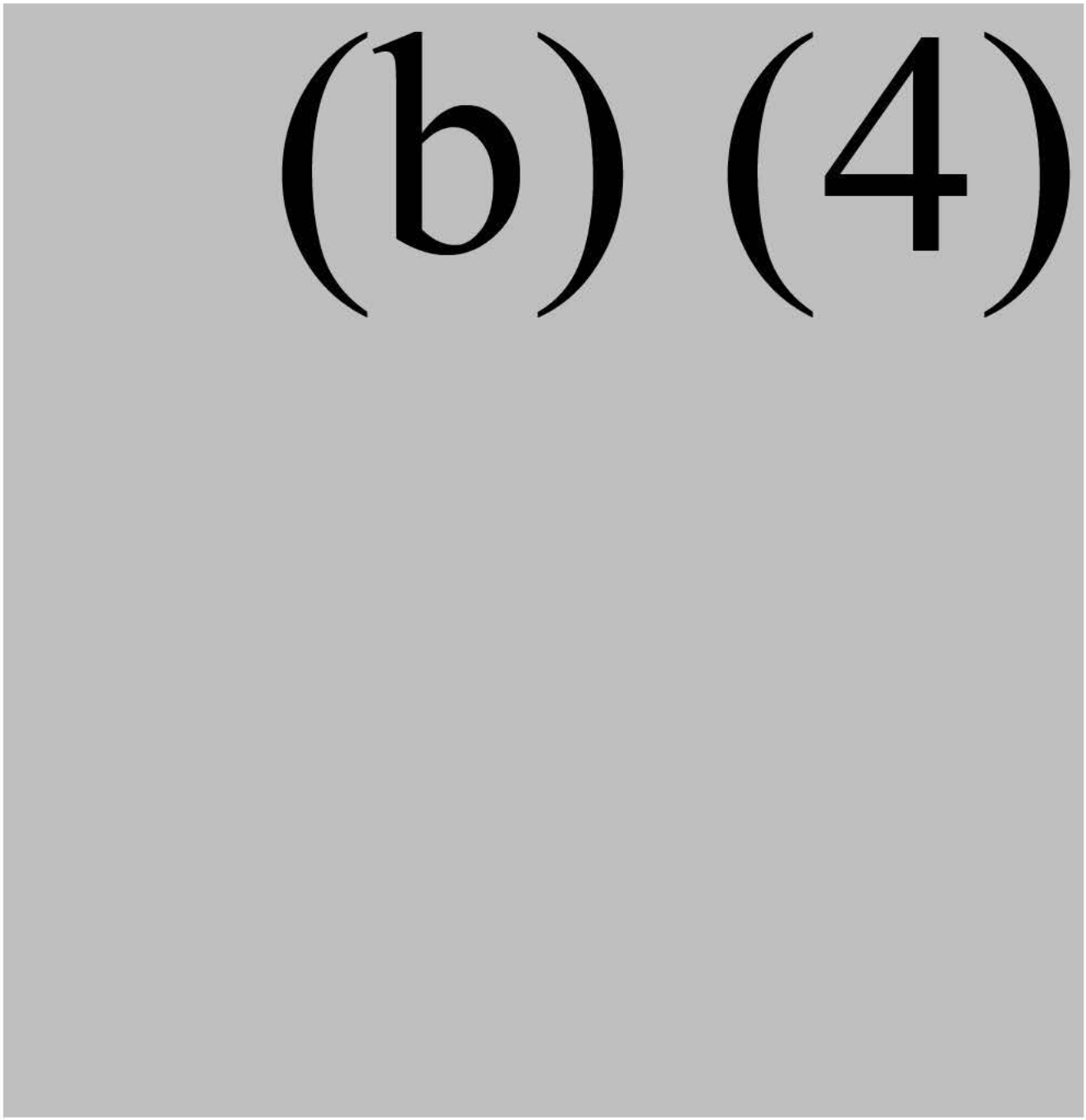
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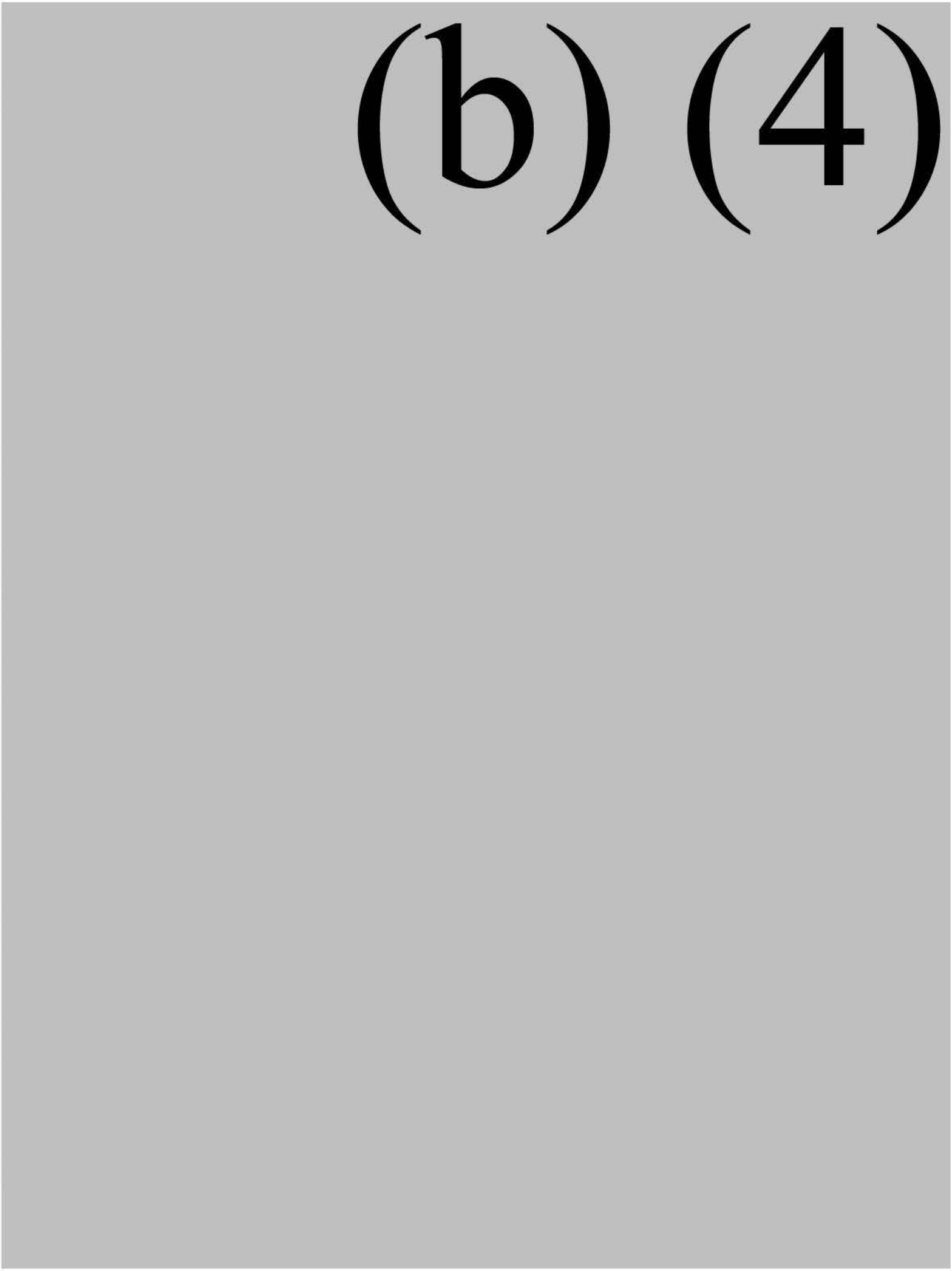
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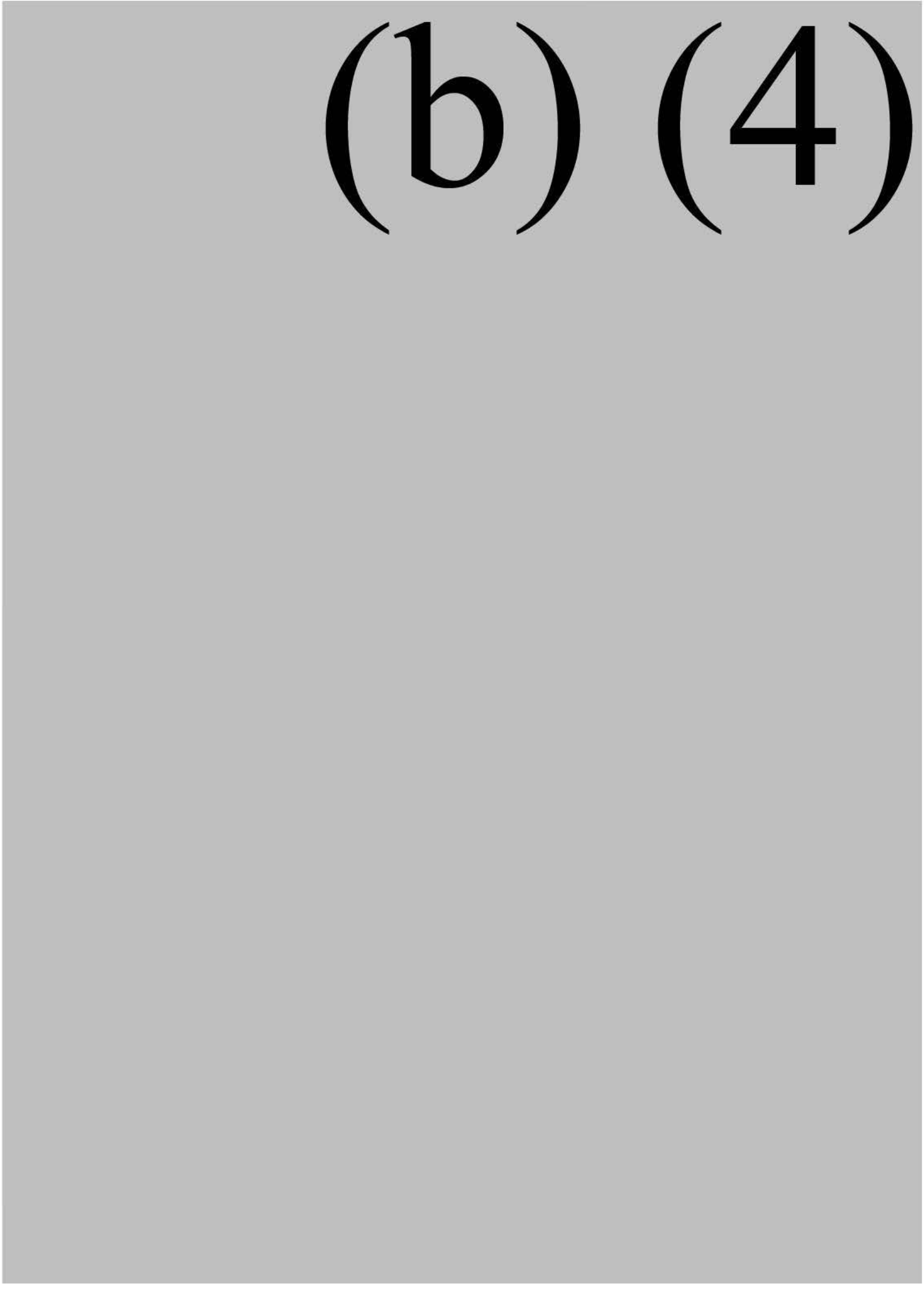
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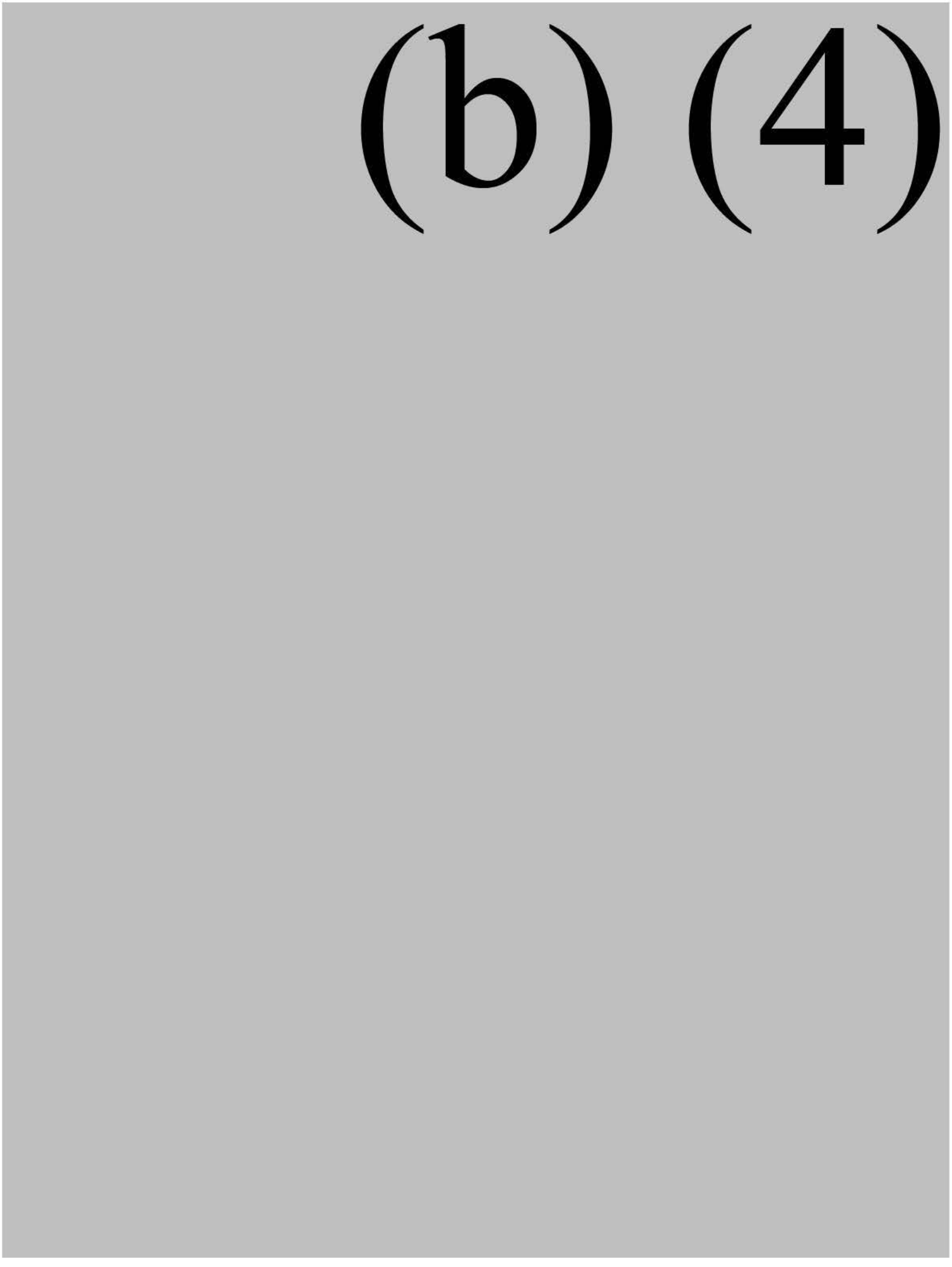
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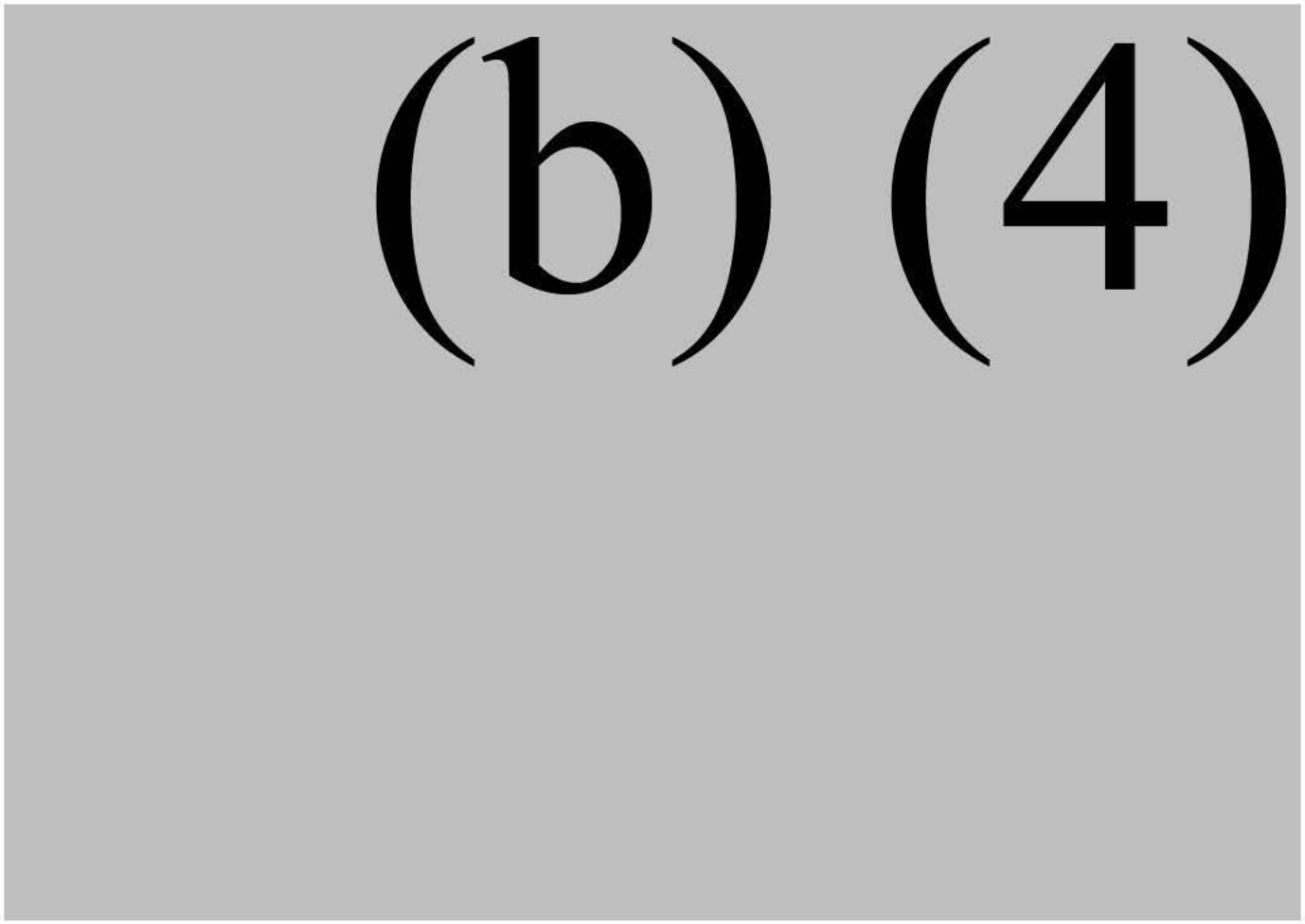
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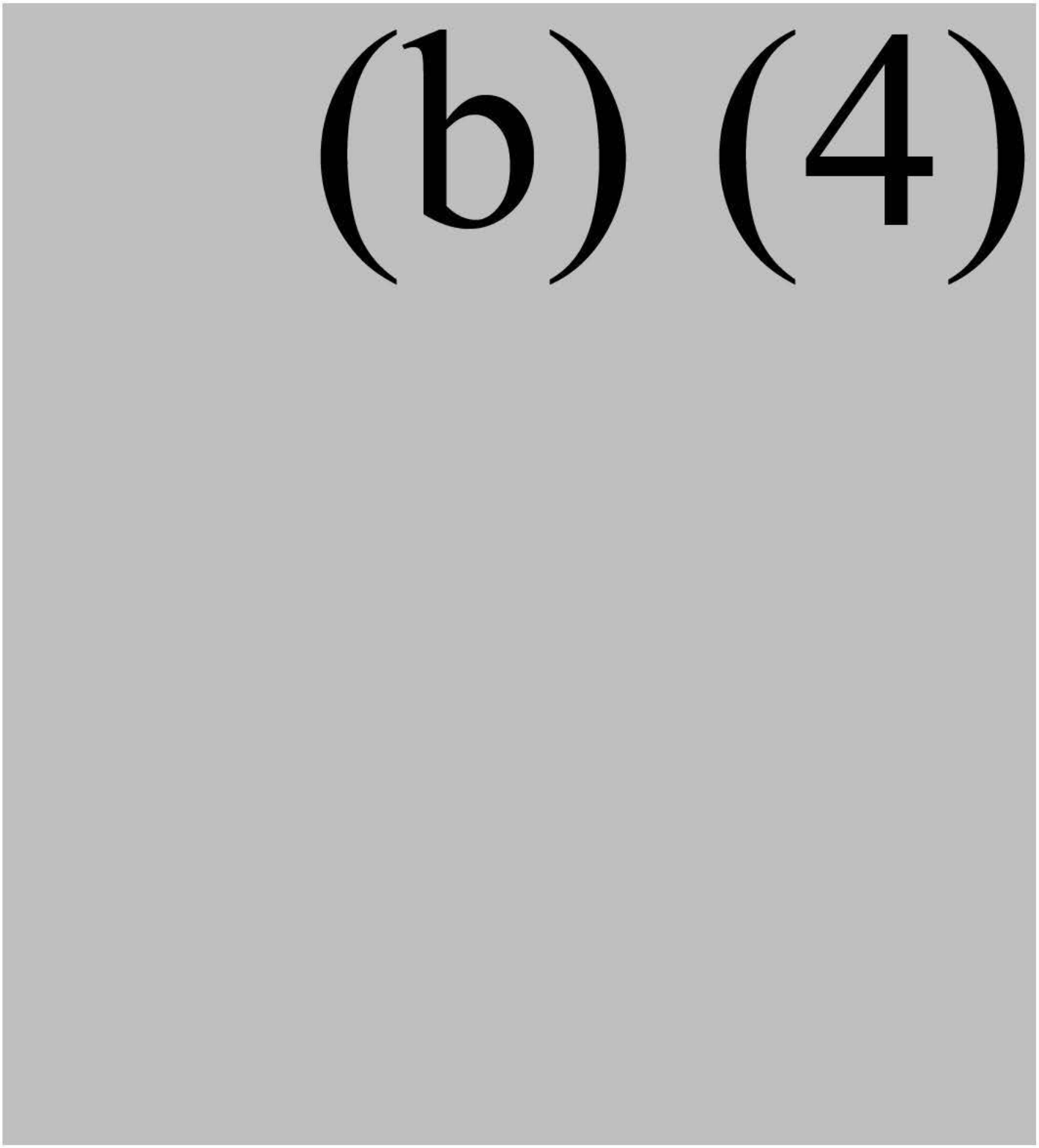
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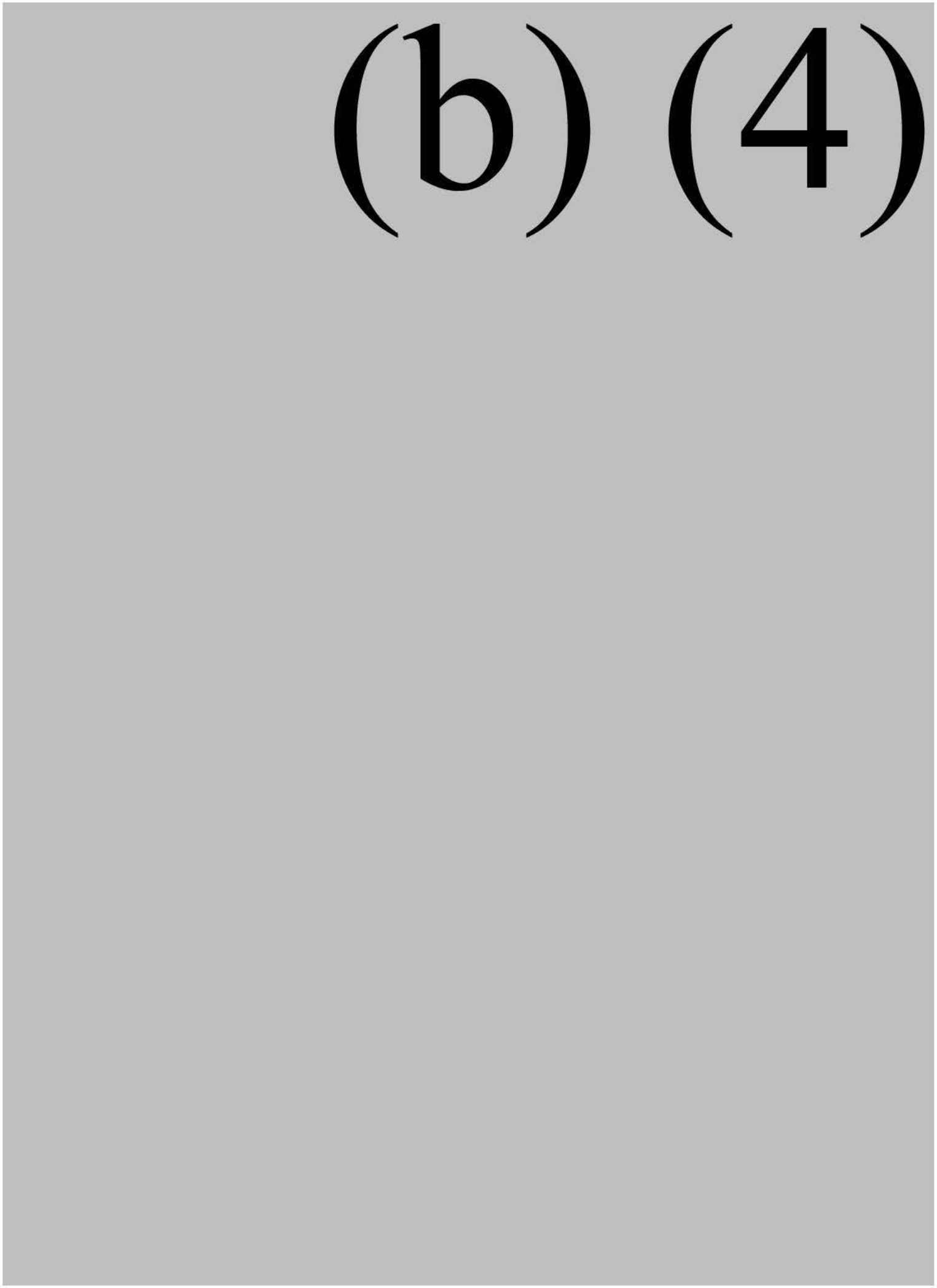
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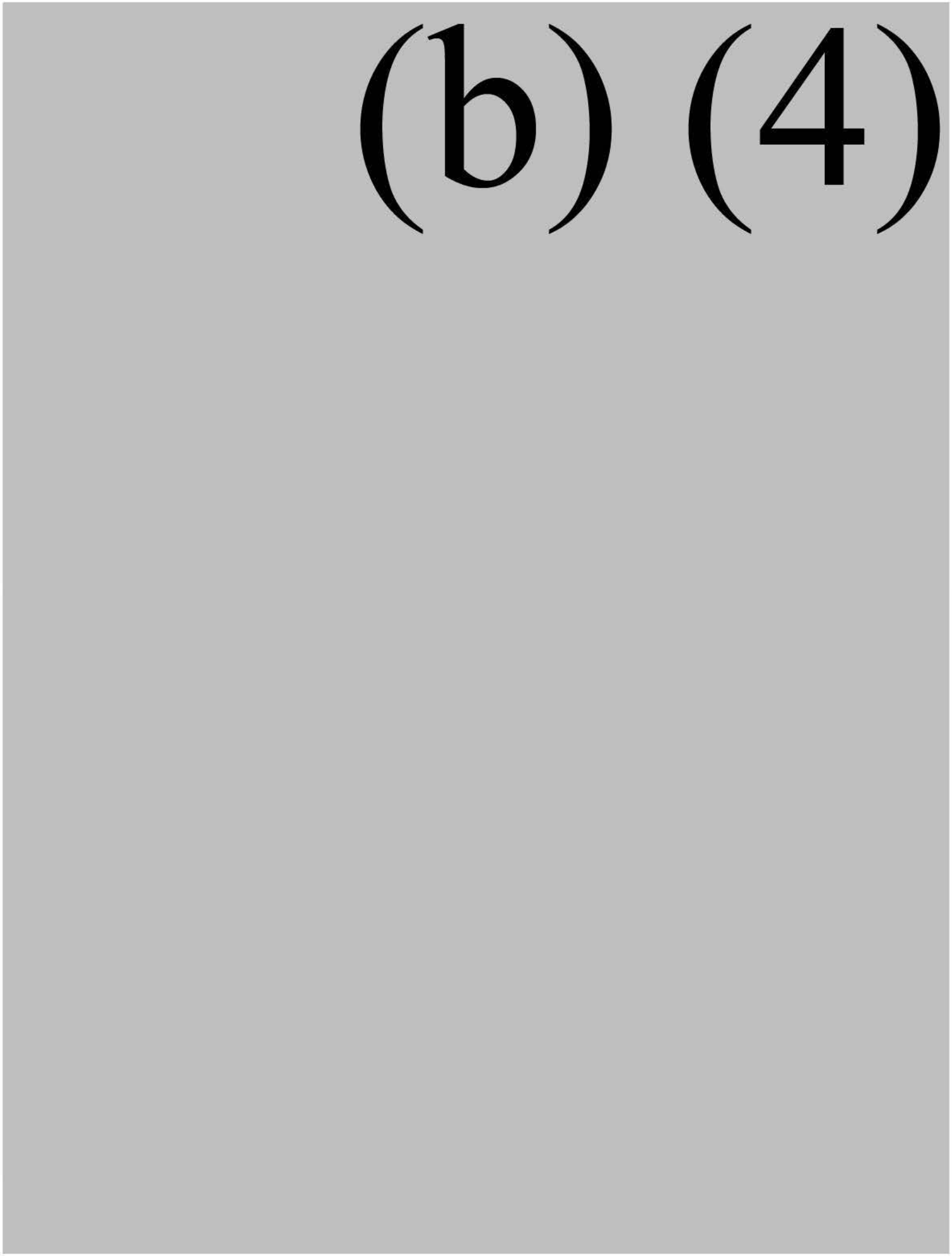
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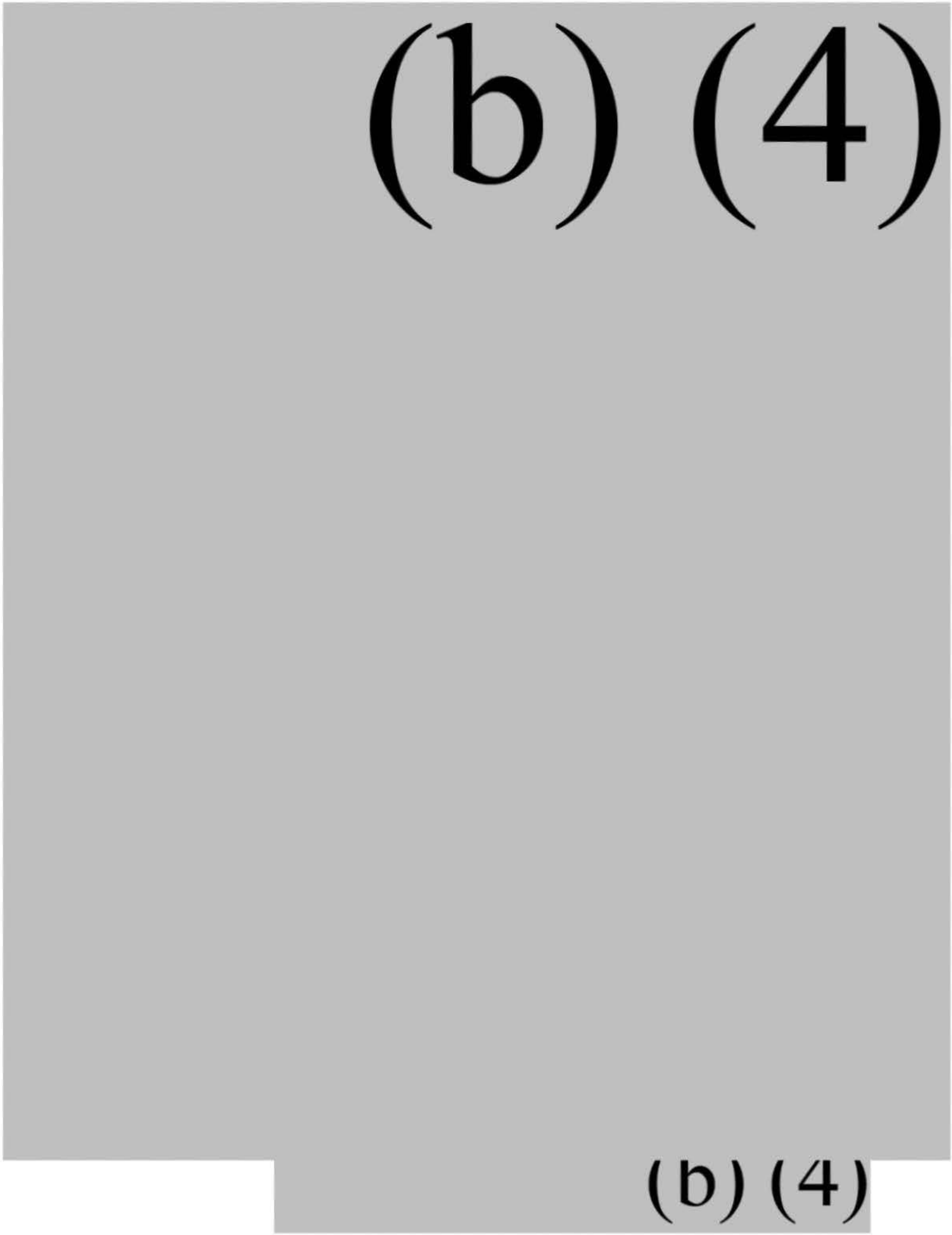
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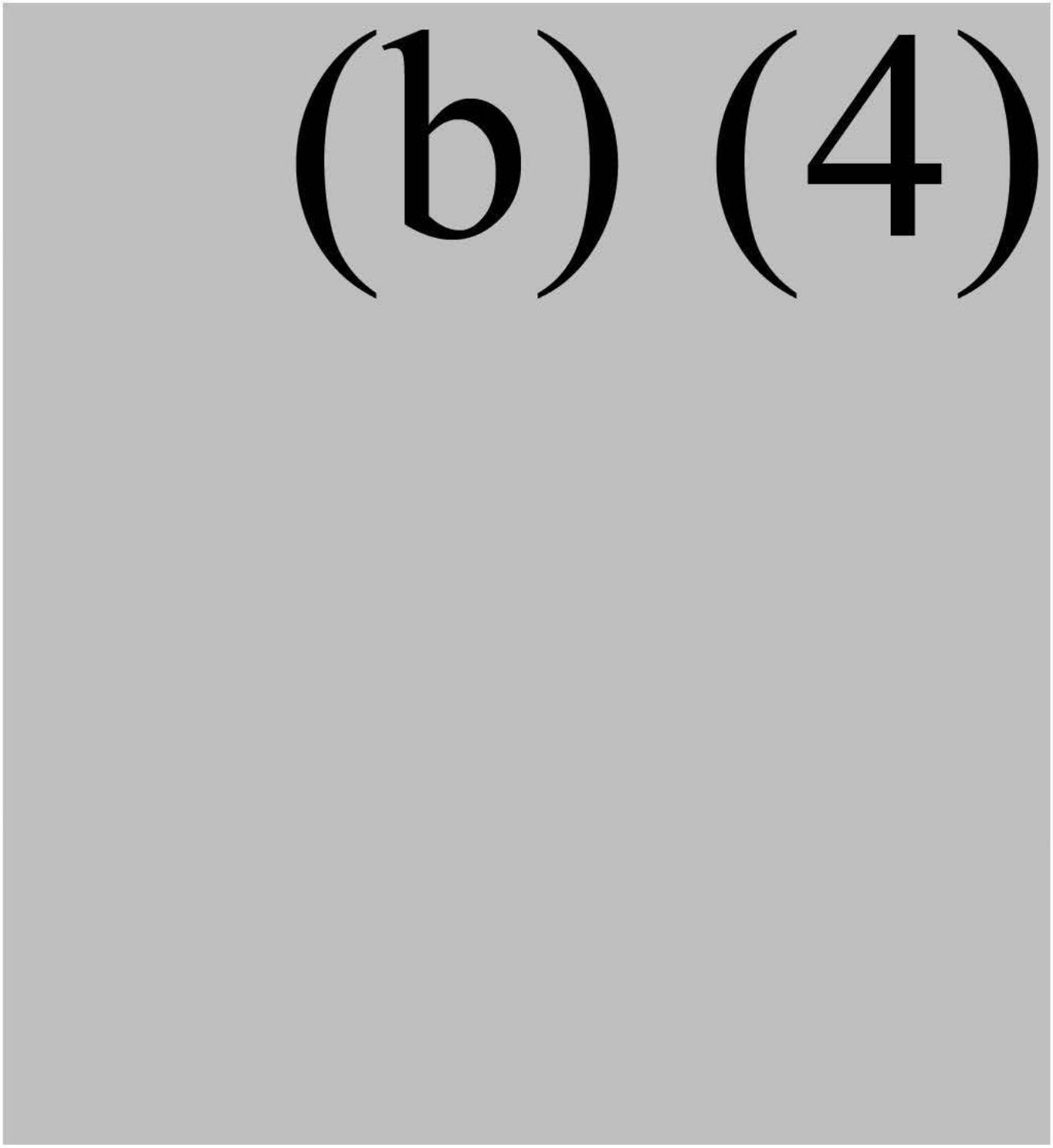
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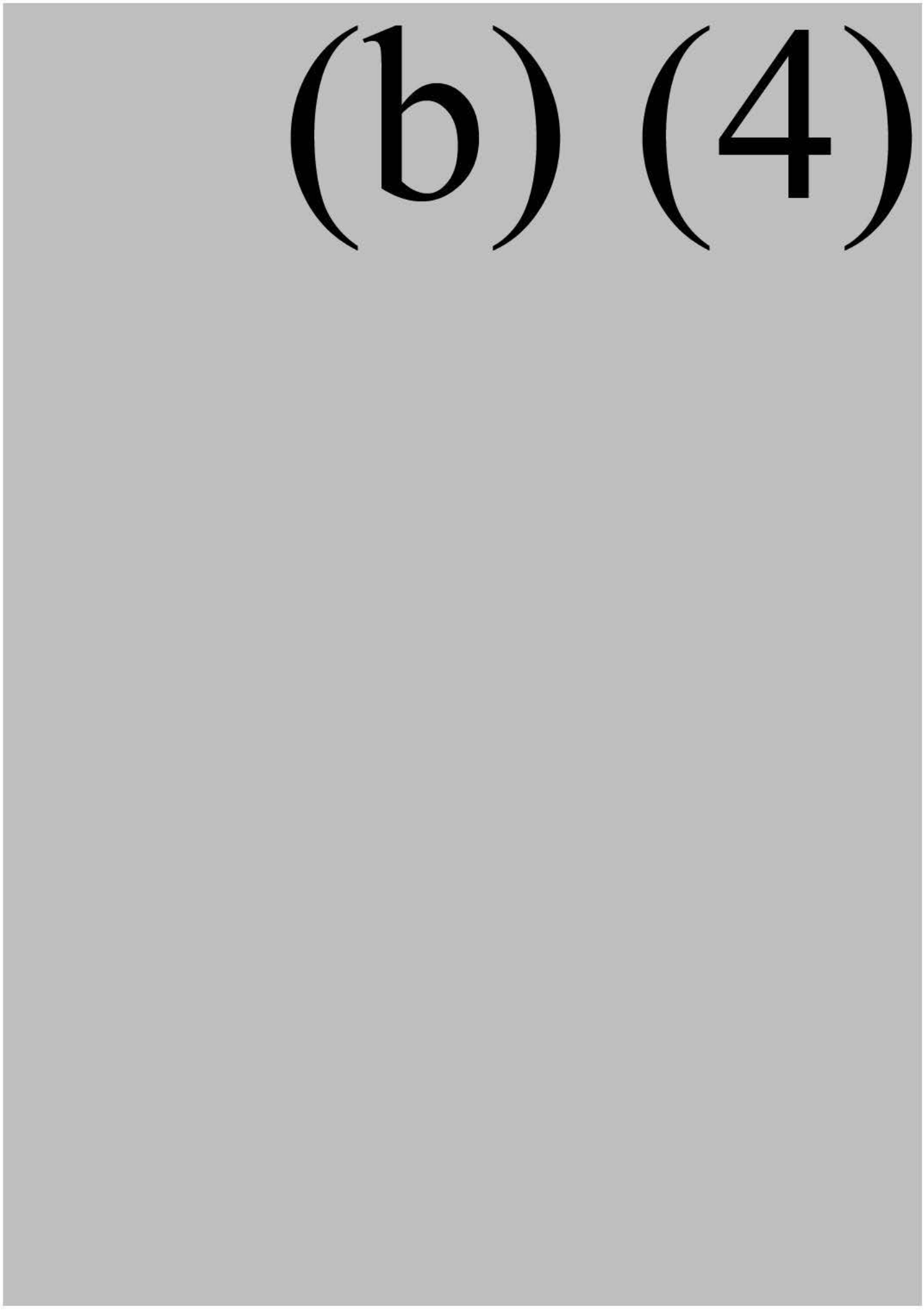
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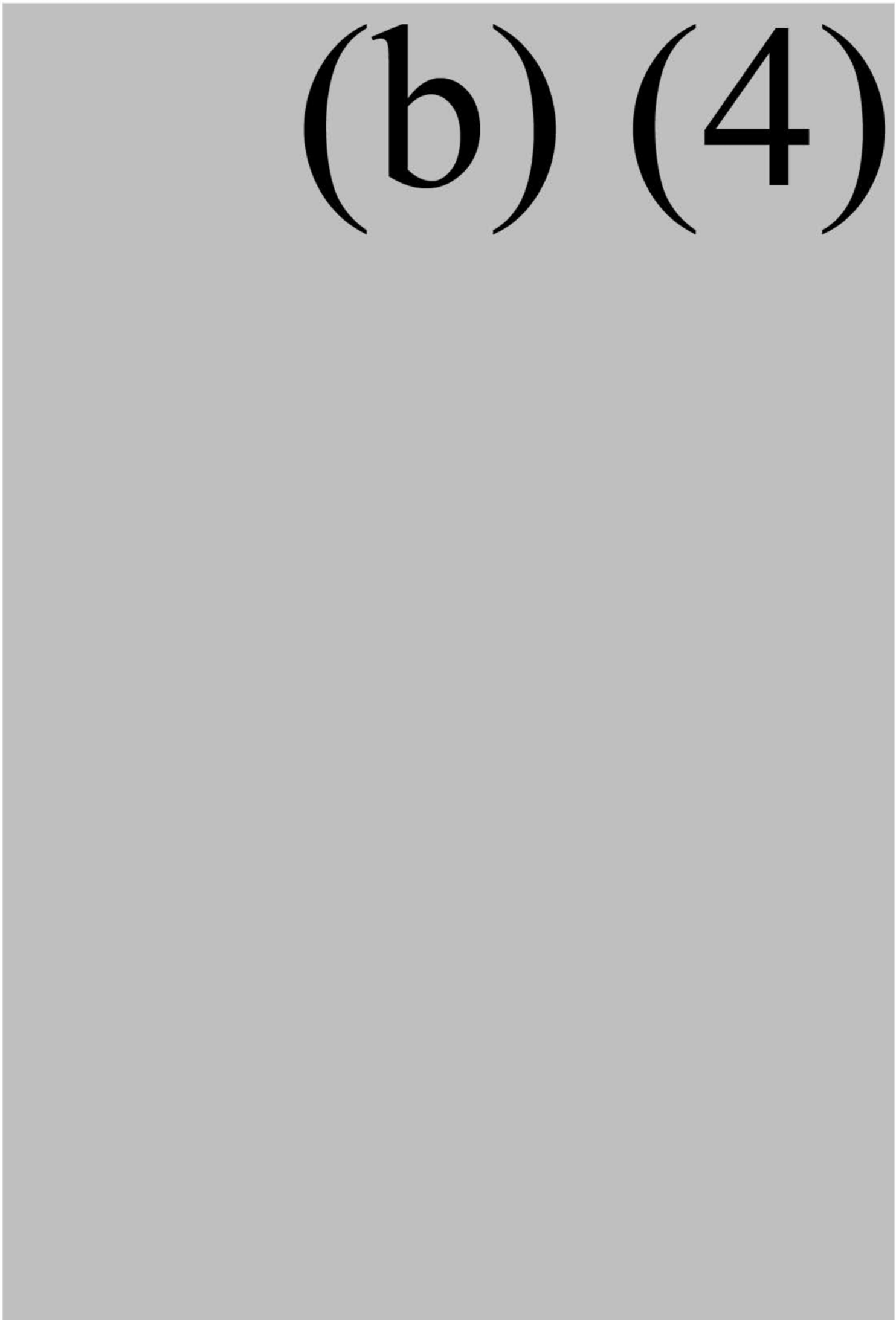
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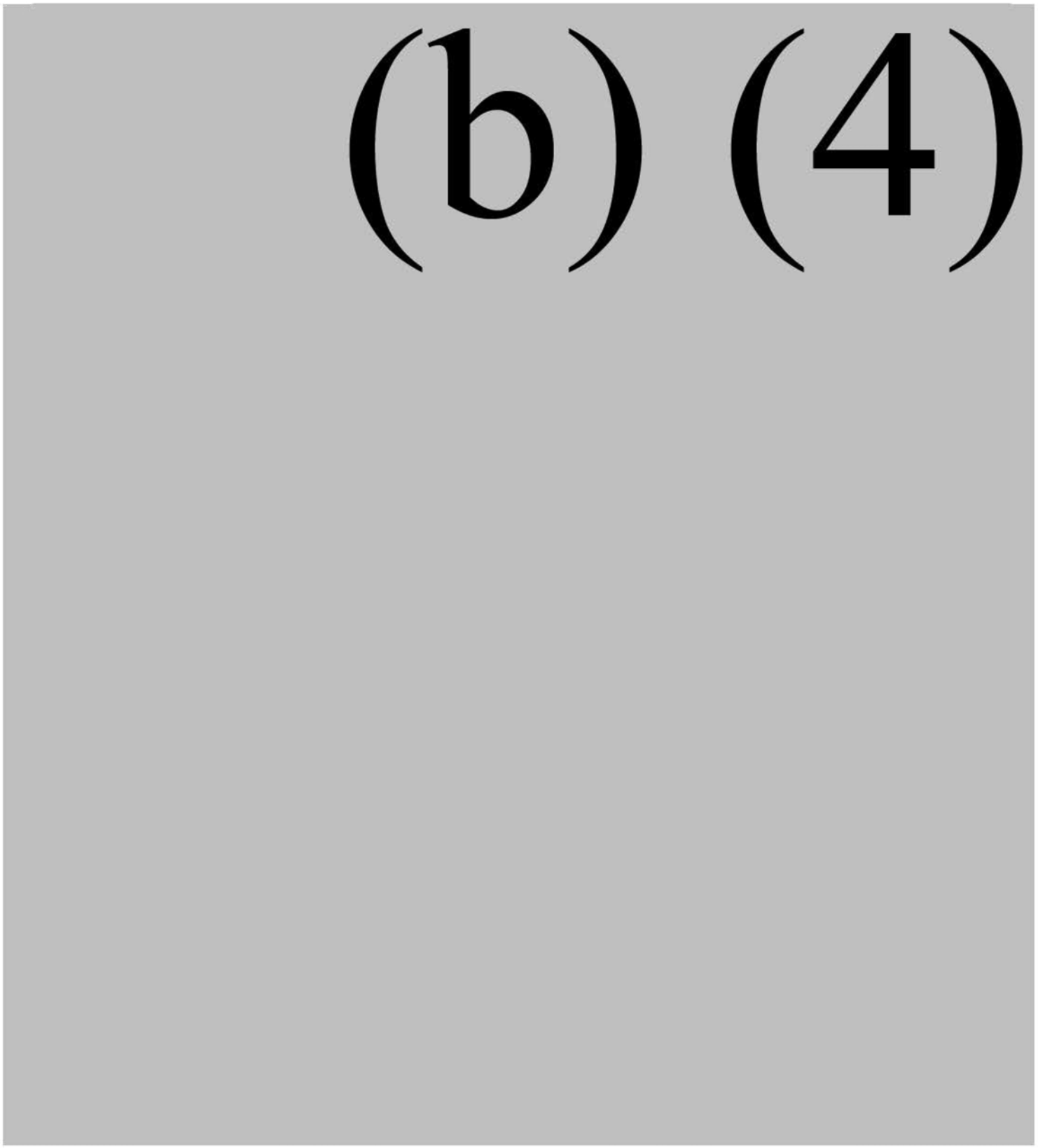
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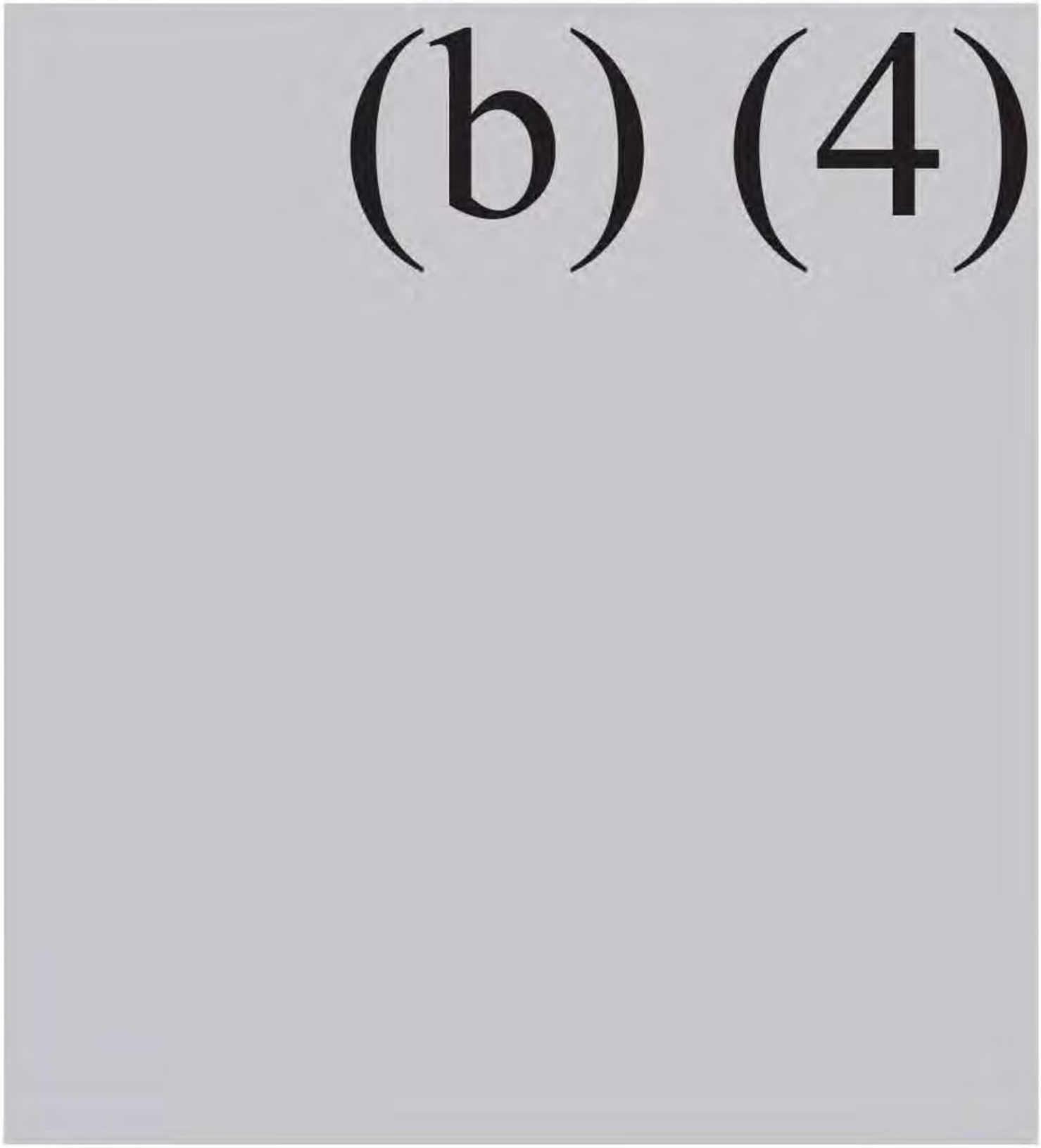
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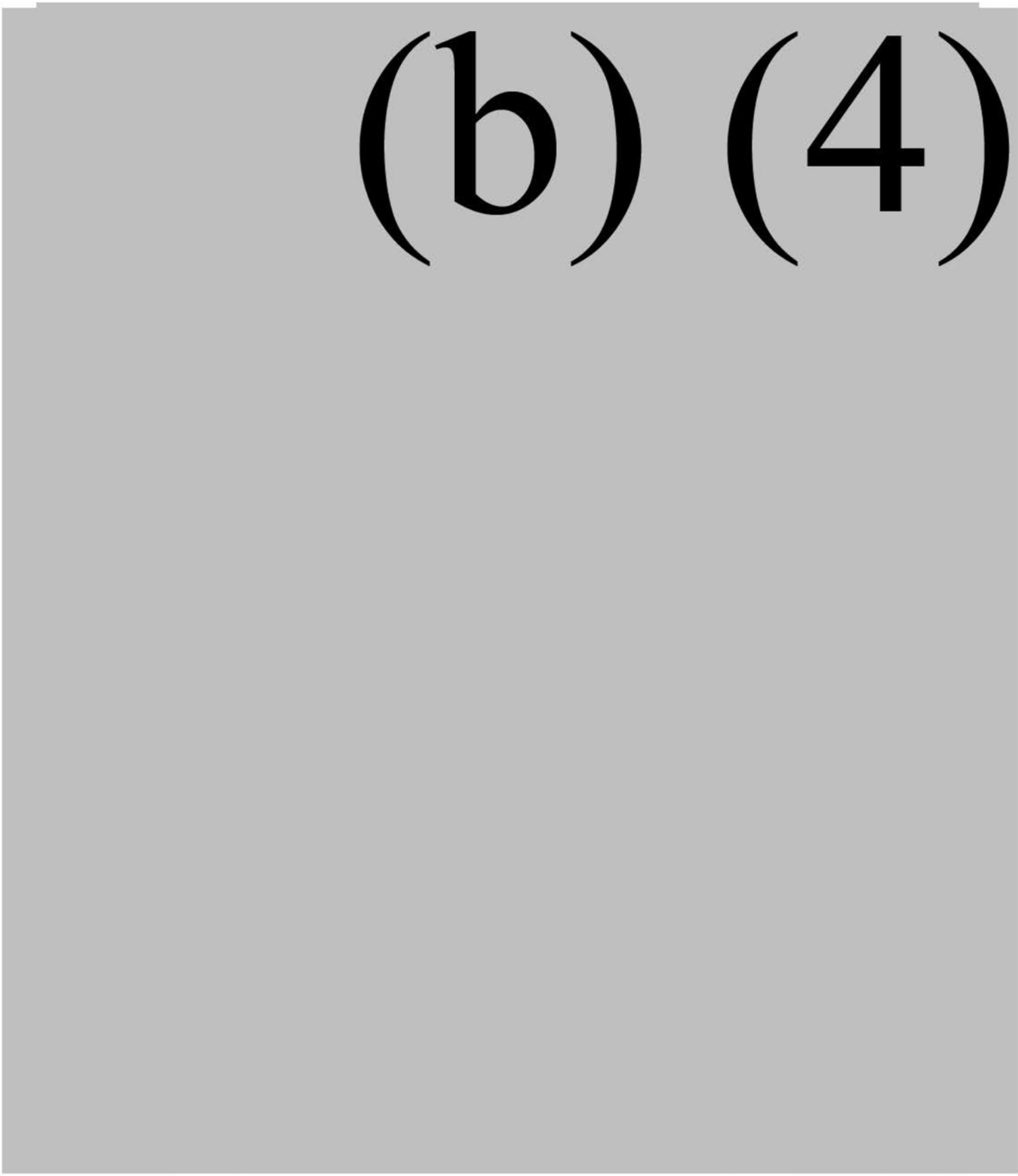
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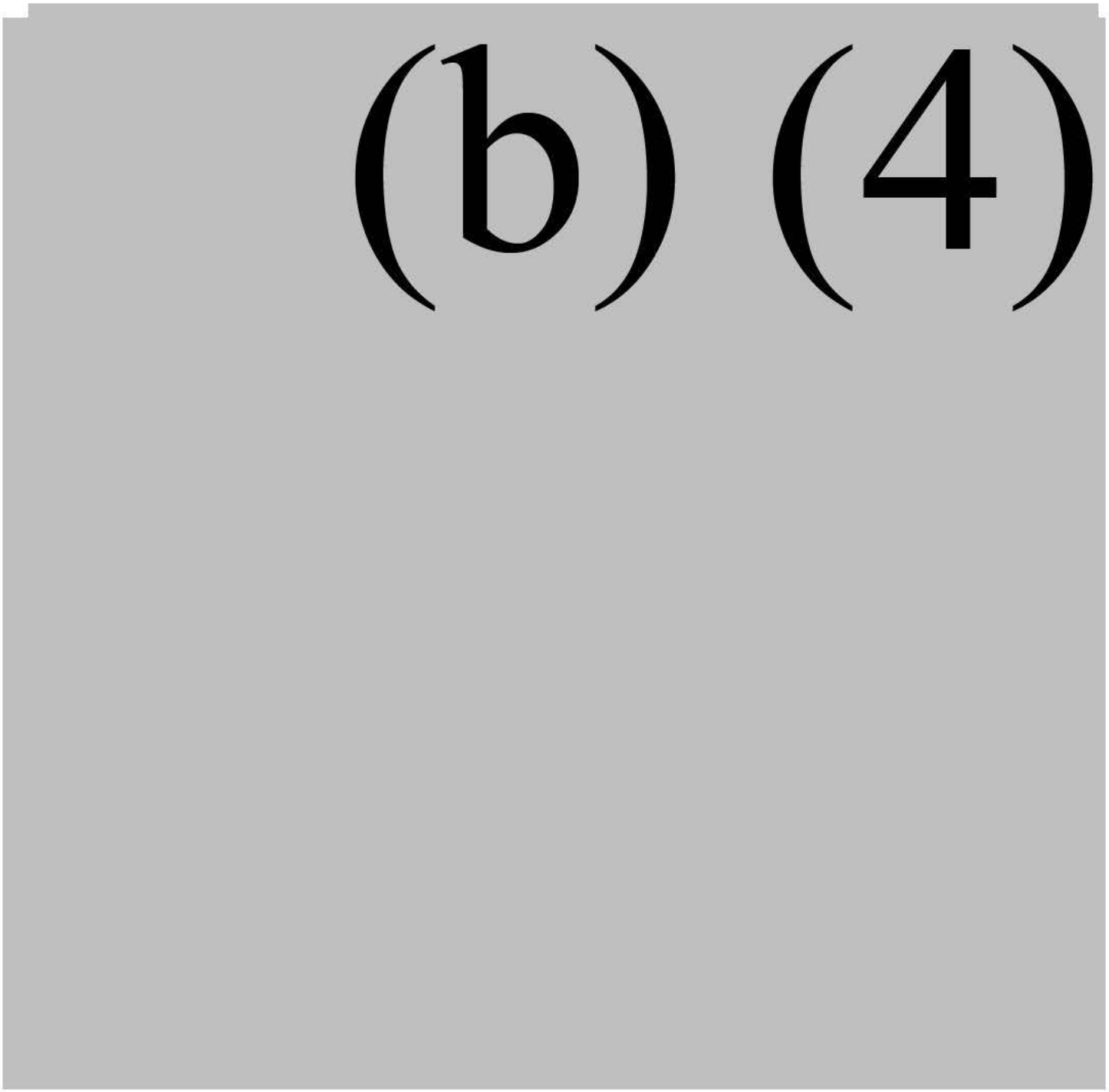
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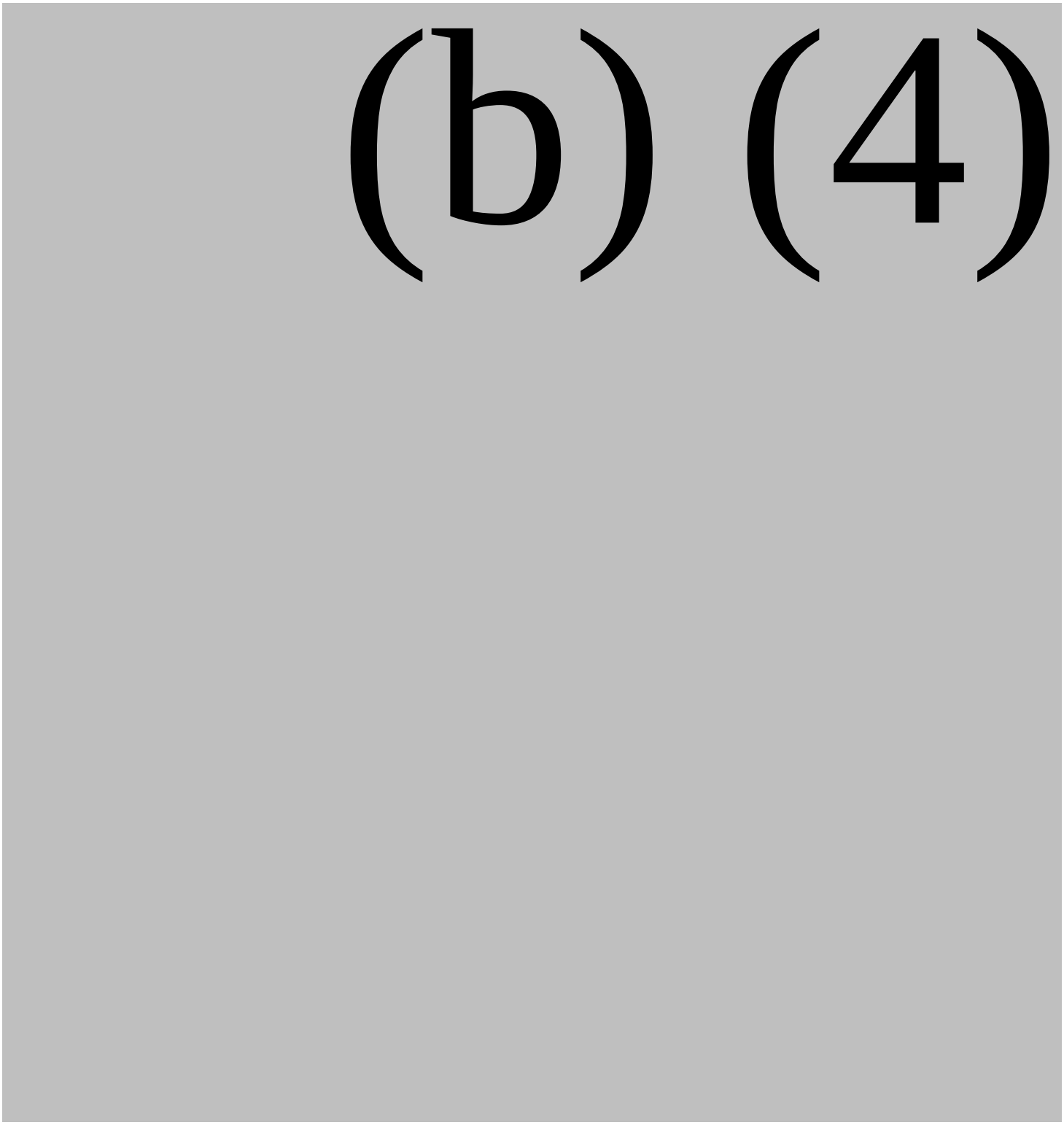

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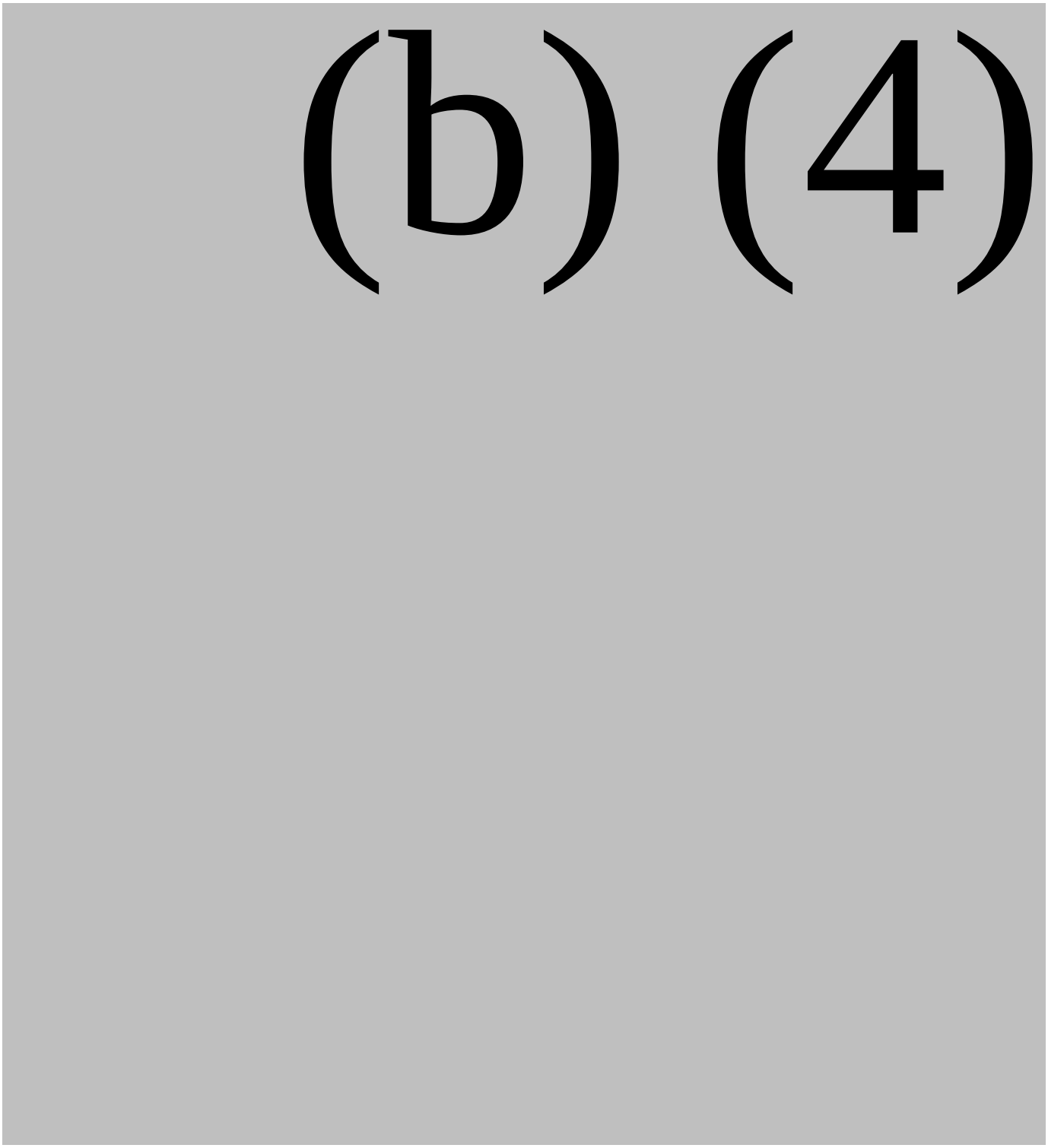

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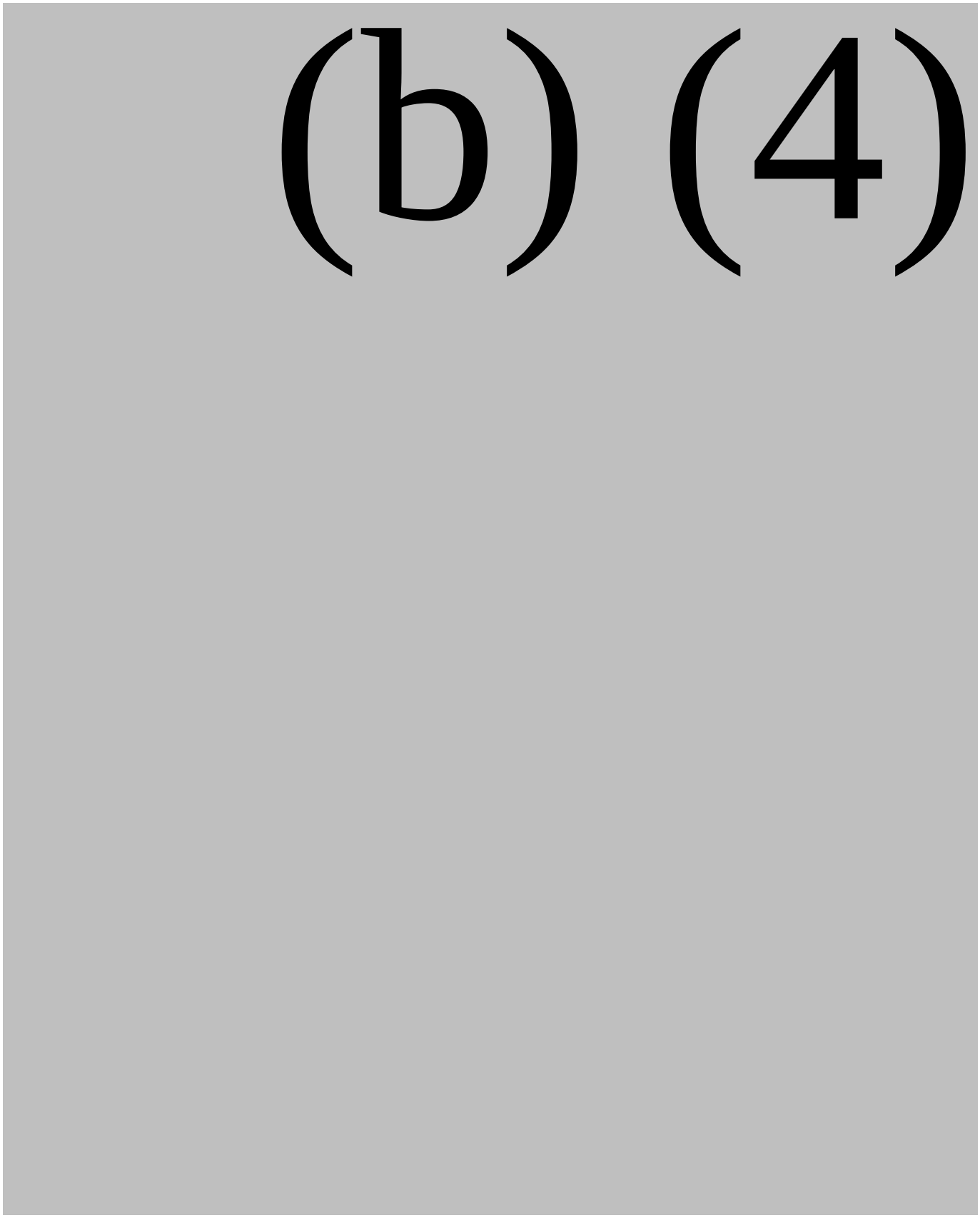

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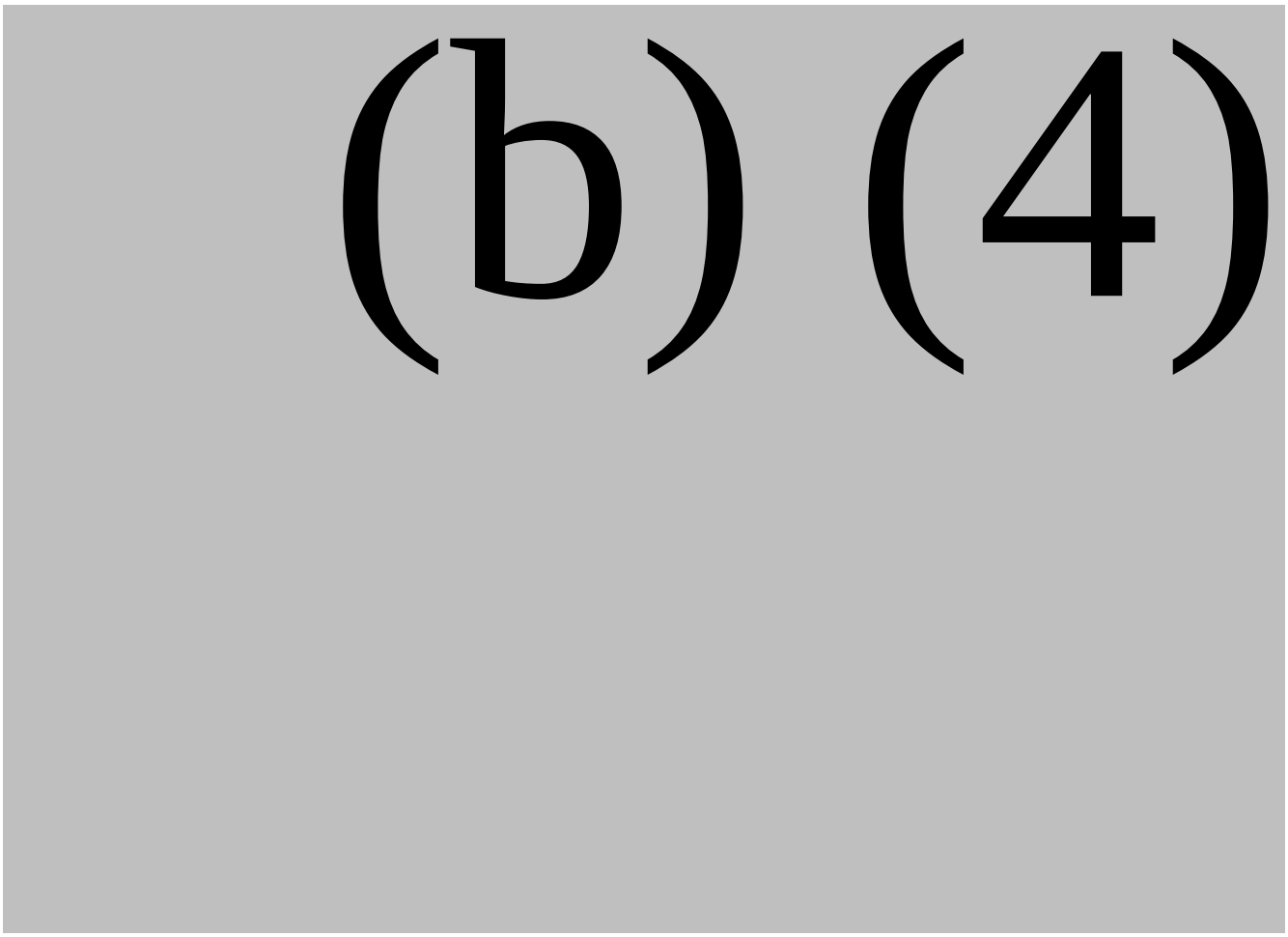

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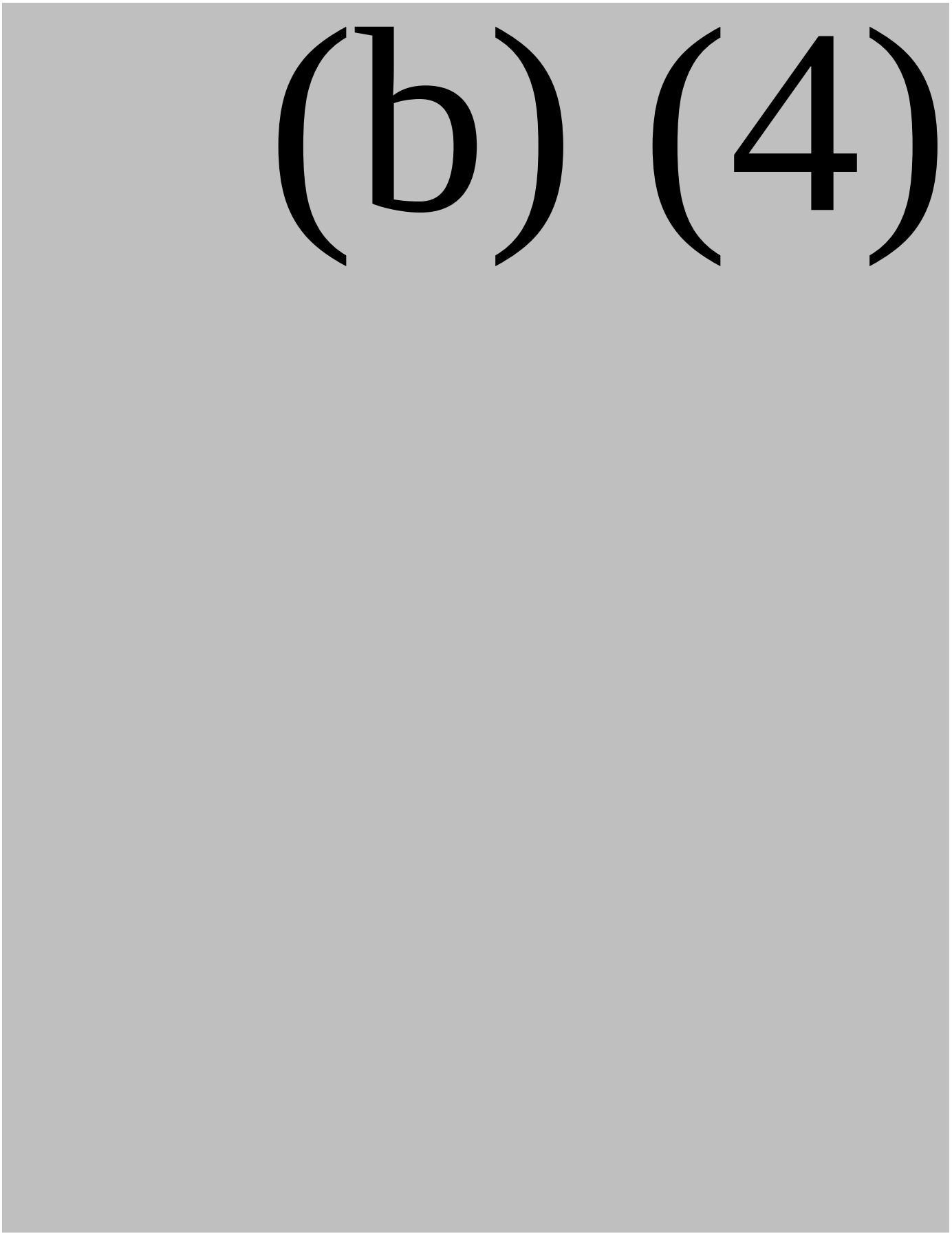

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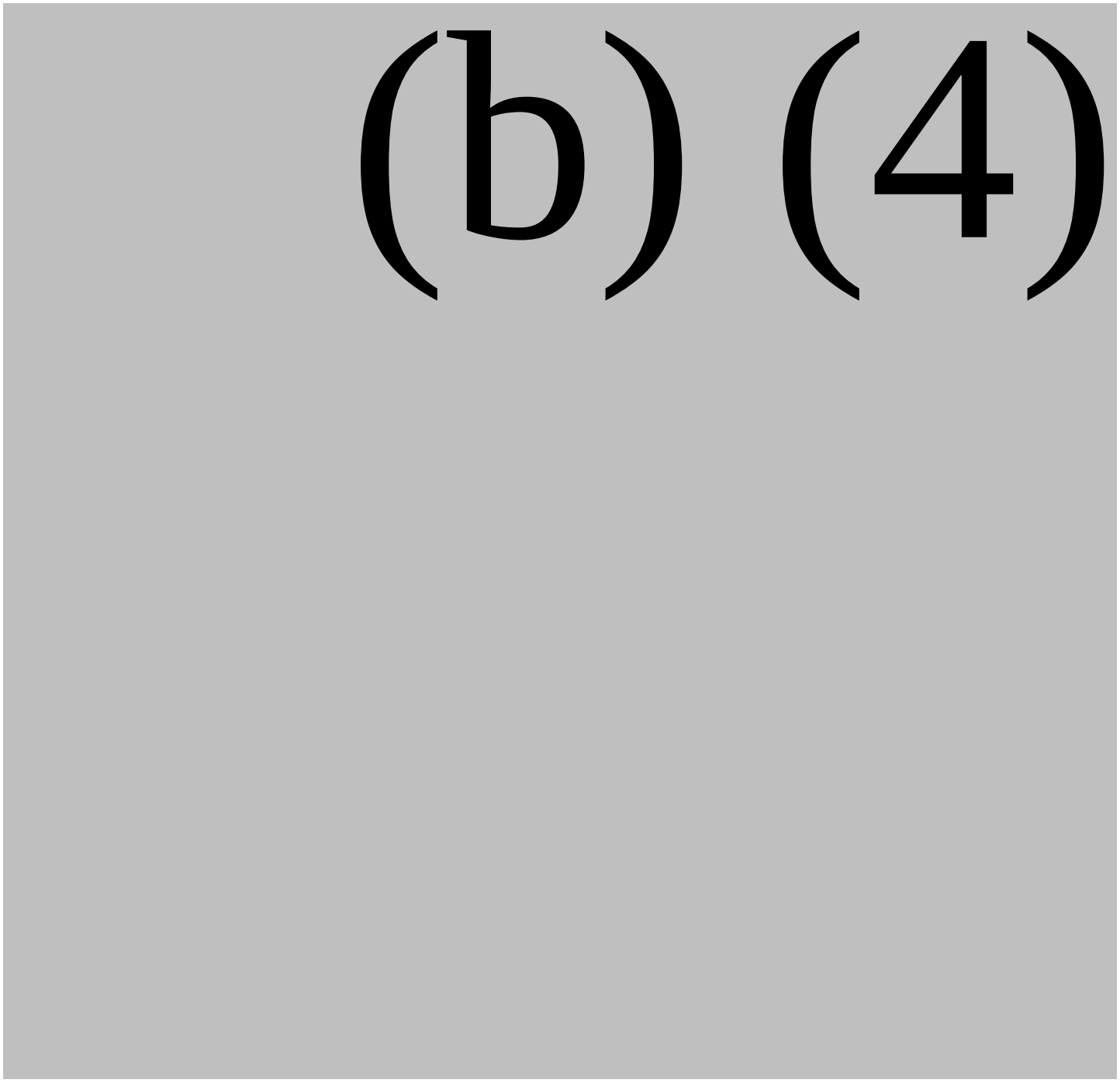

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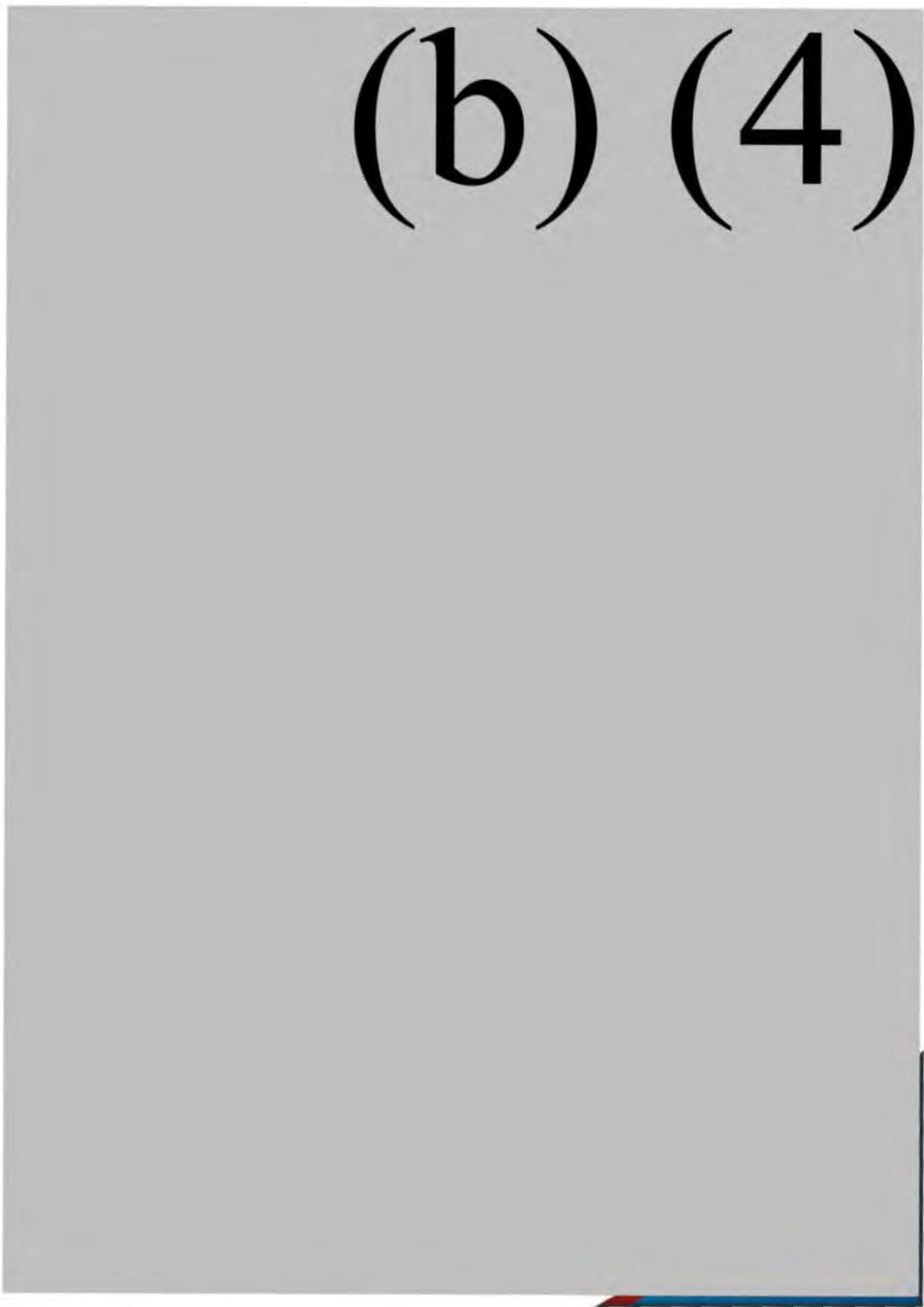
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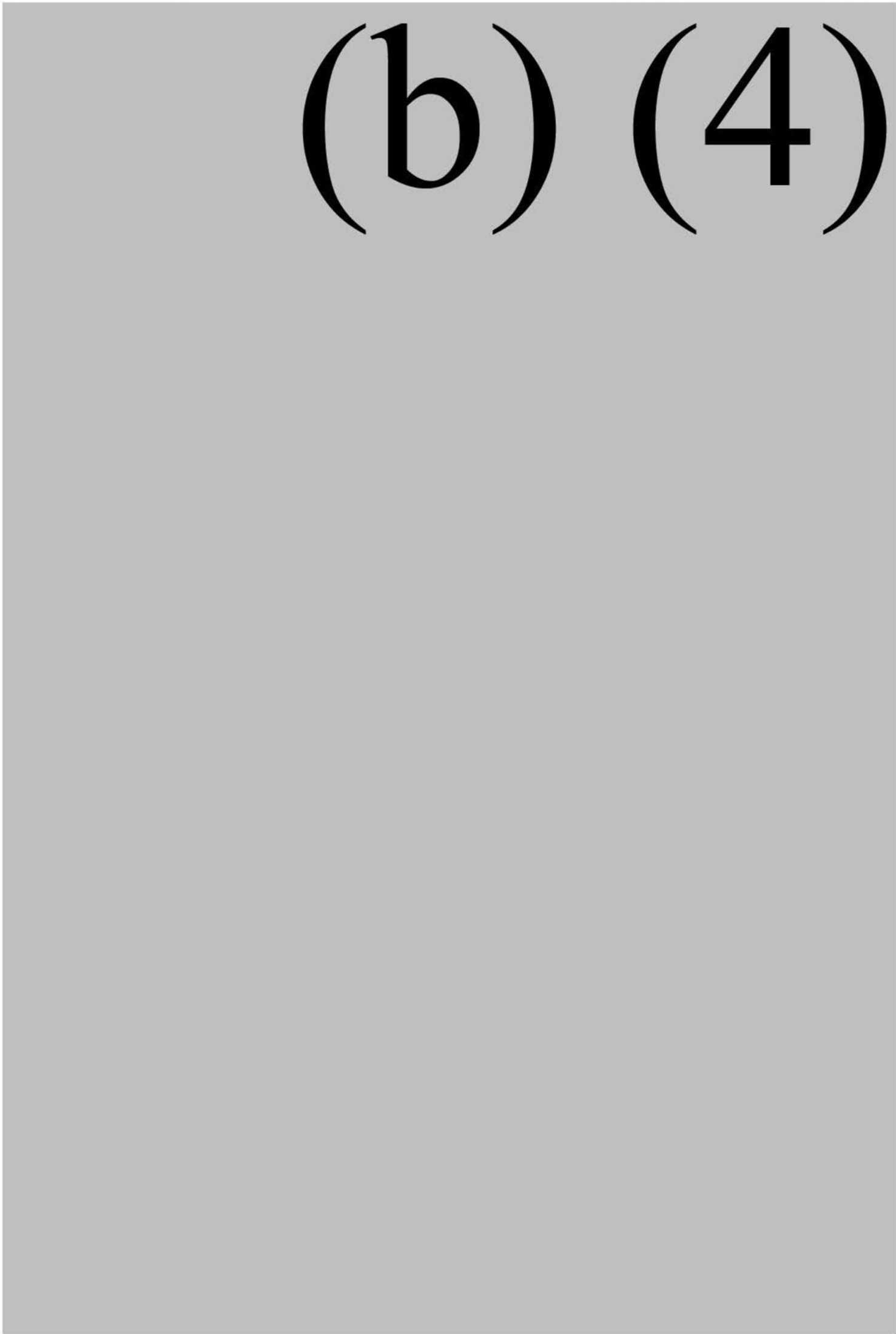
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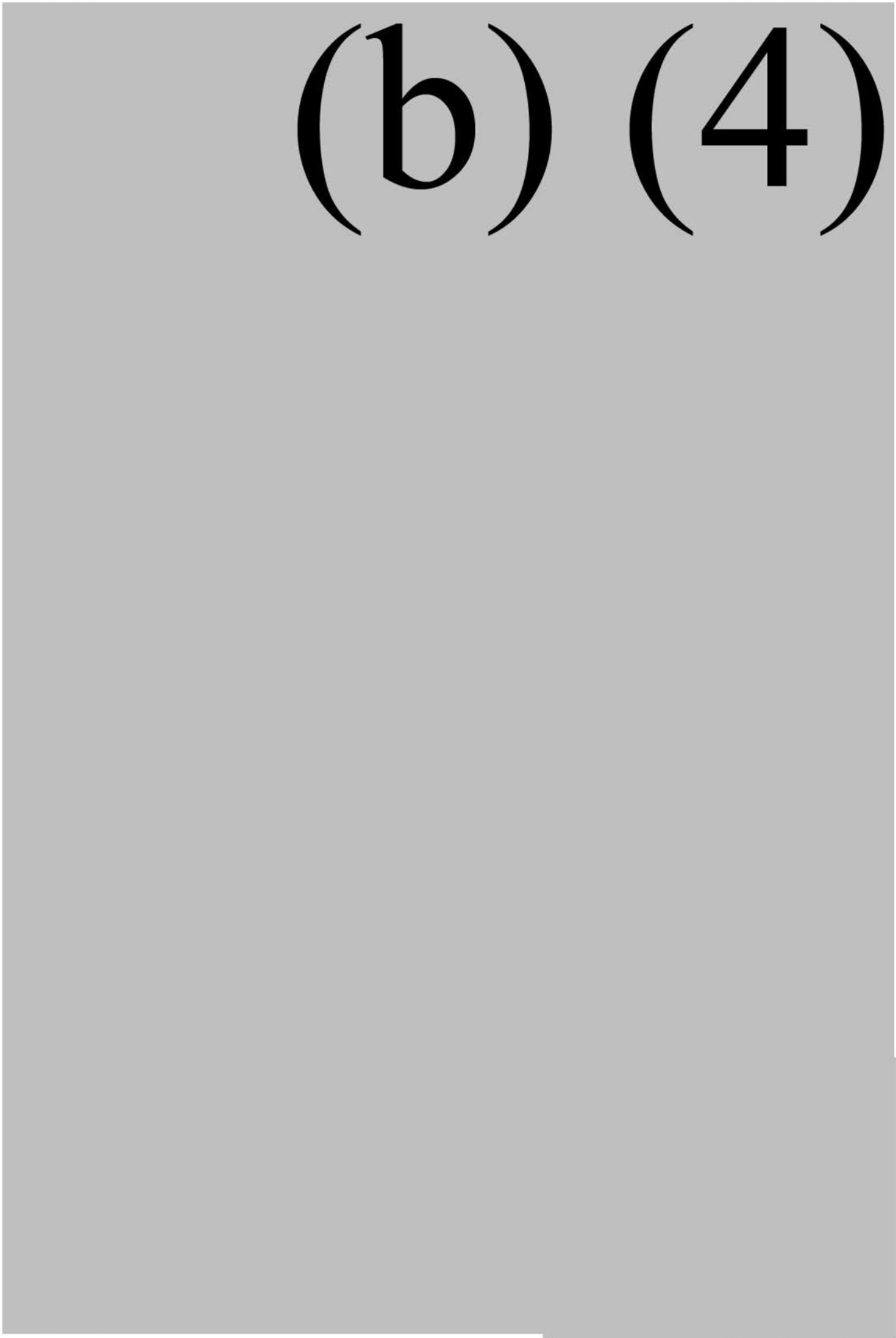
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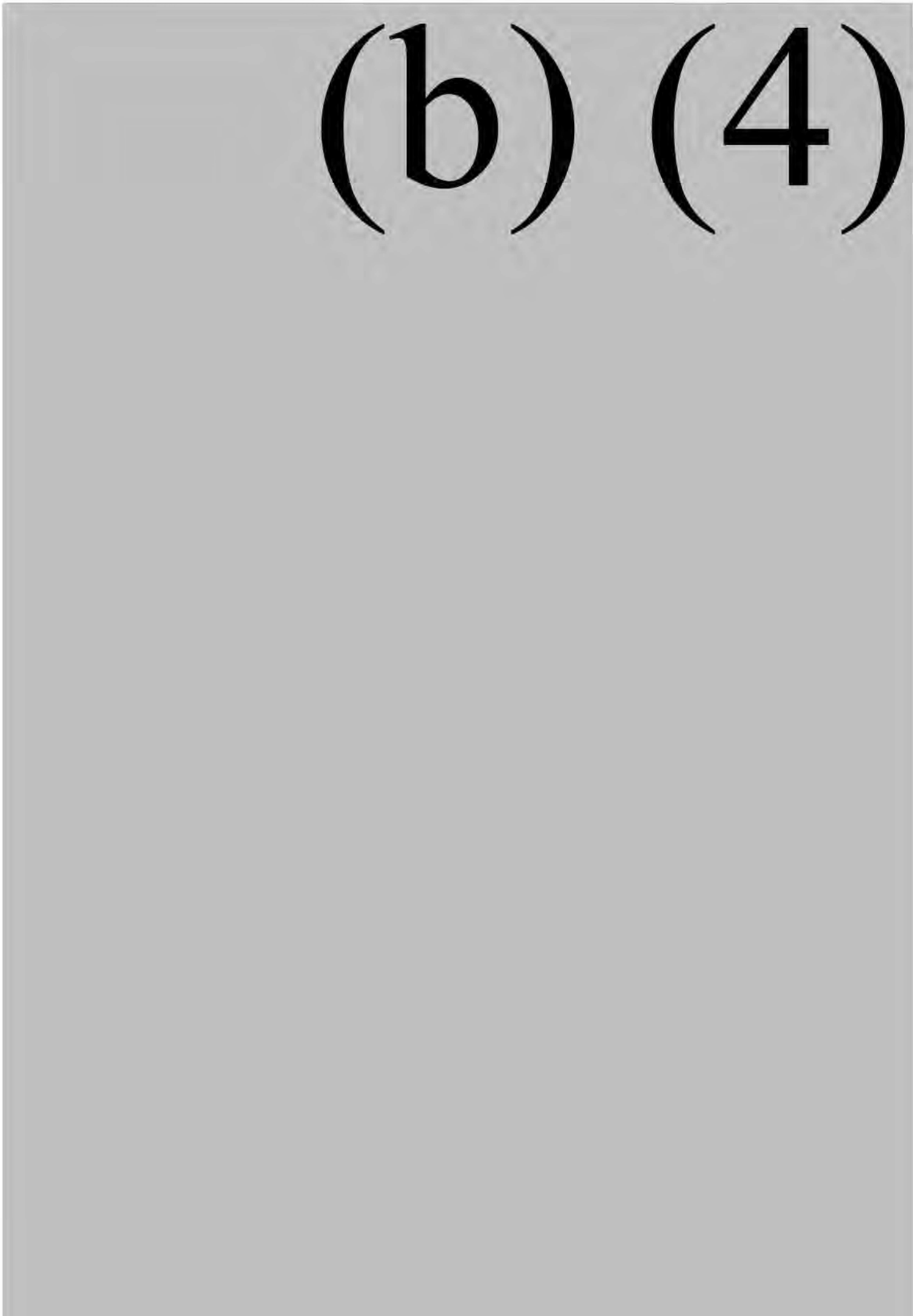
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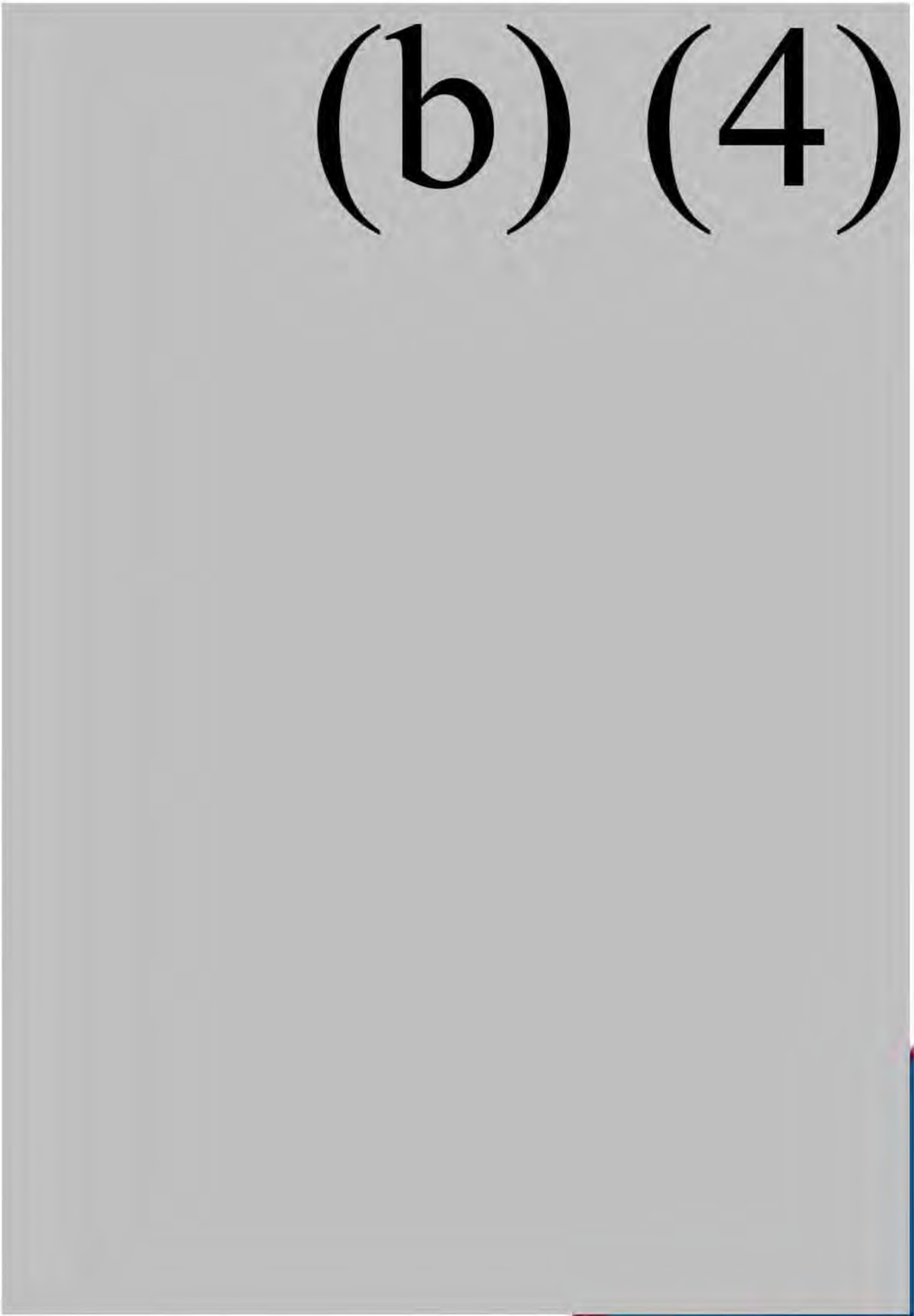
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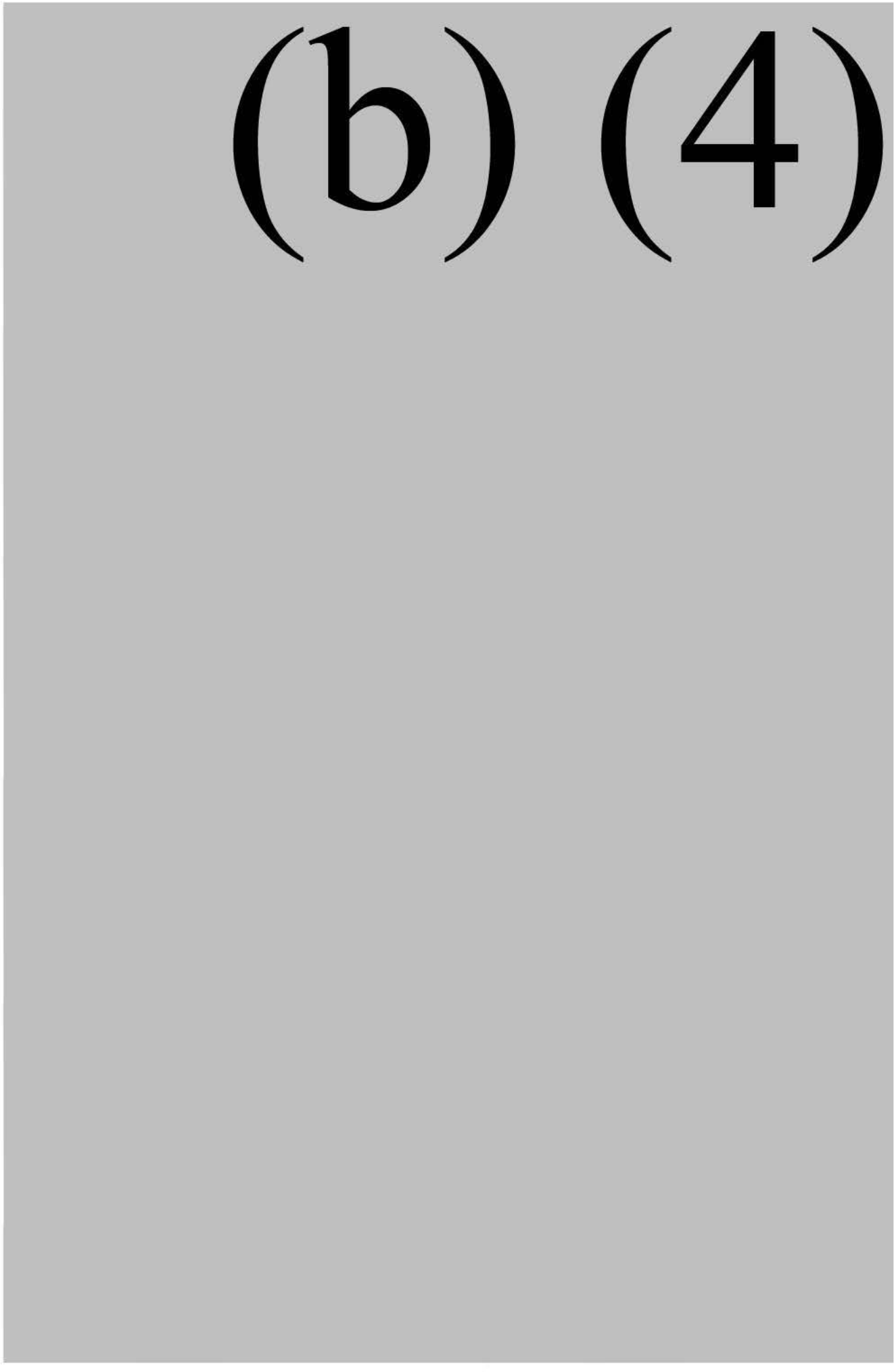
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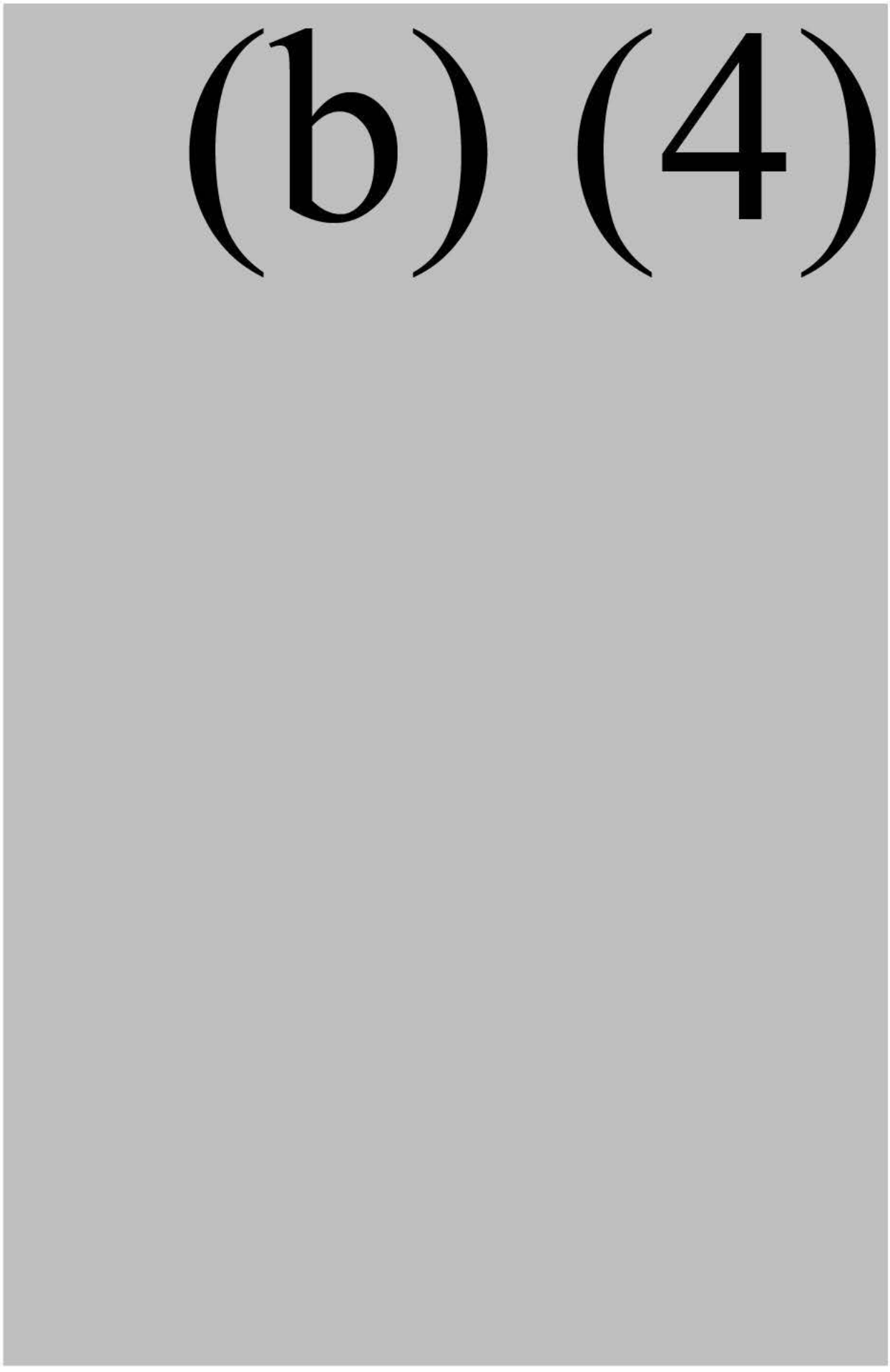
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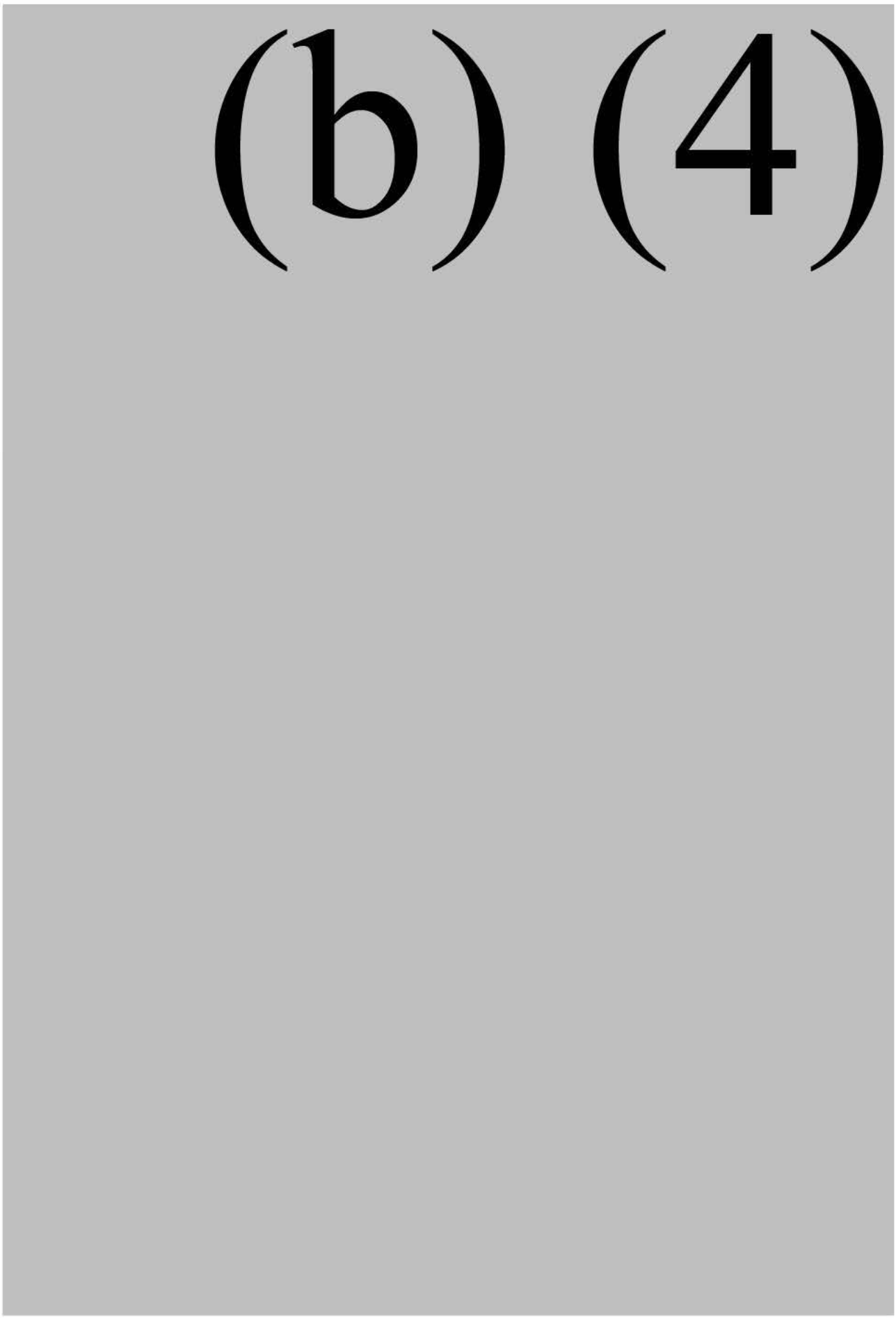
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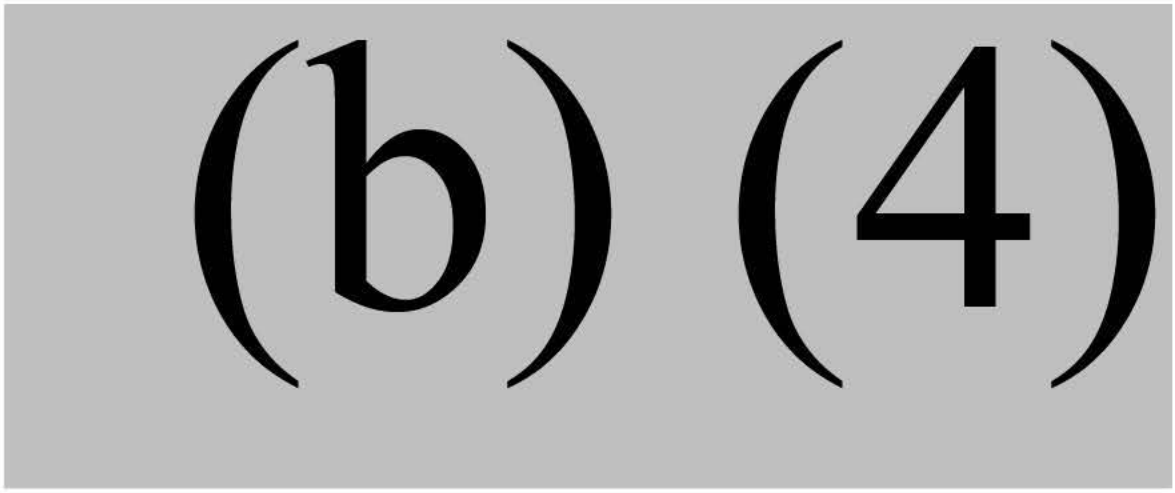
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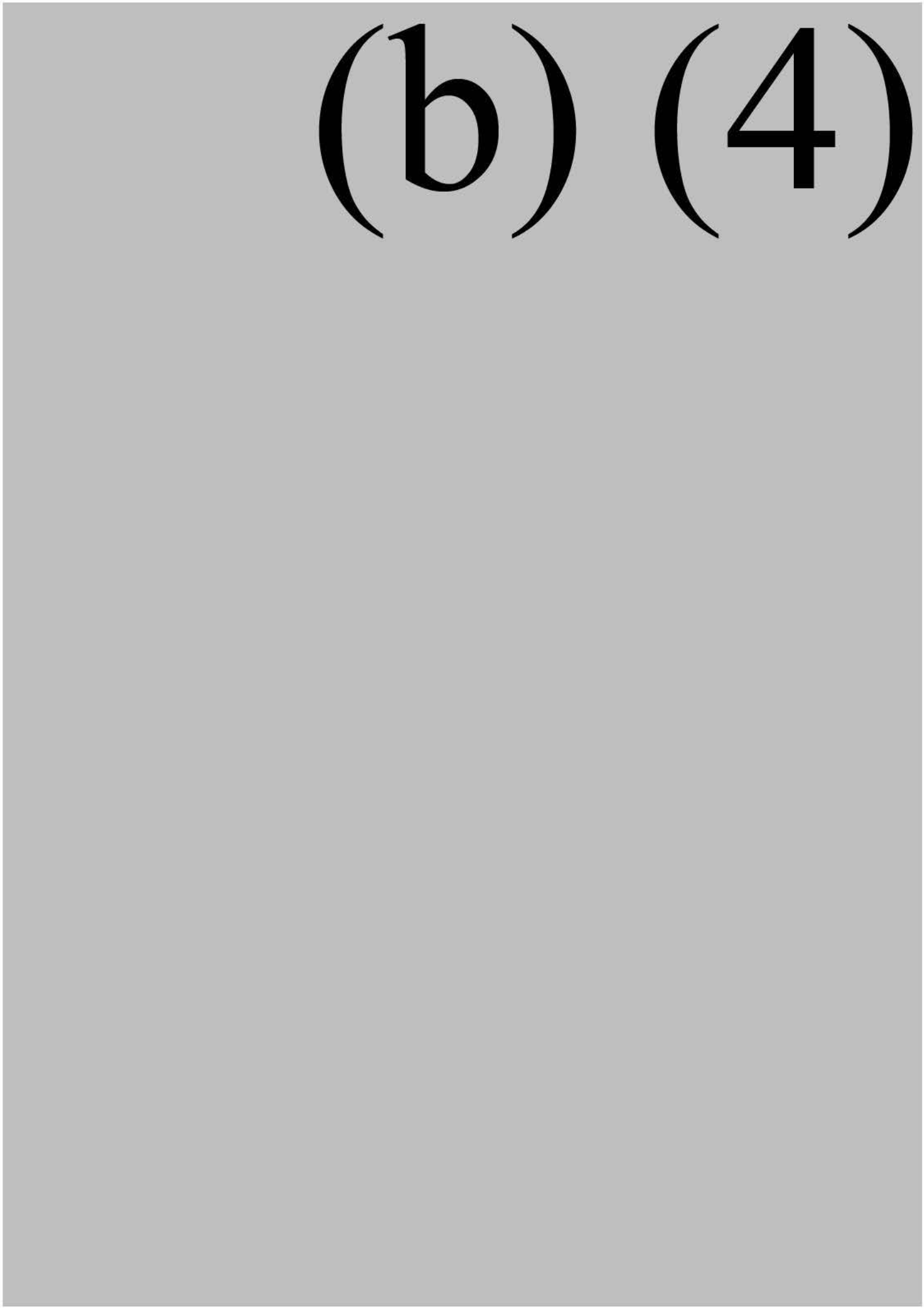
APPENDIX 1

(b) (4)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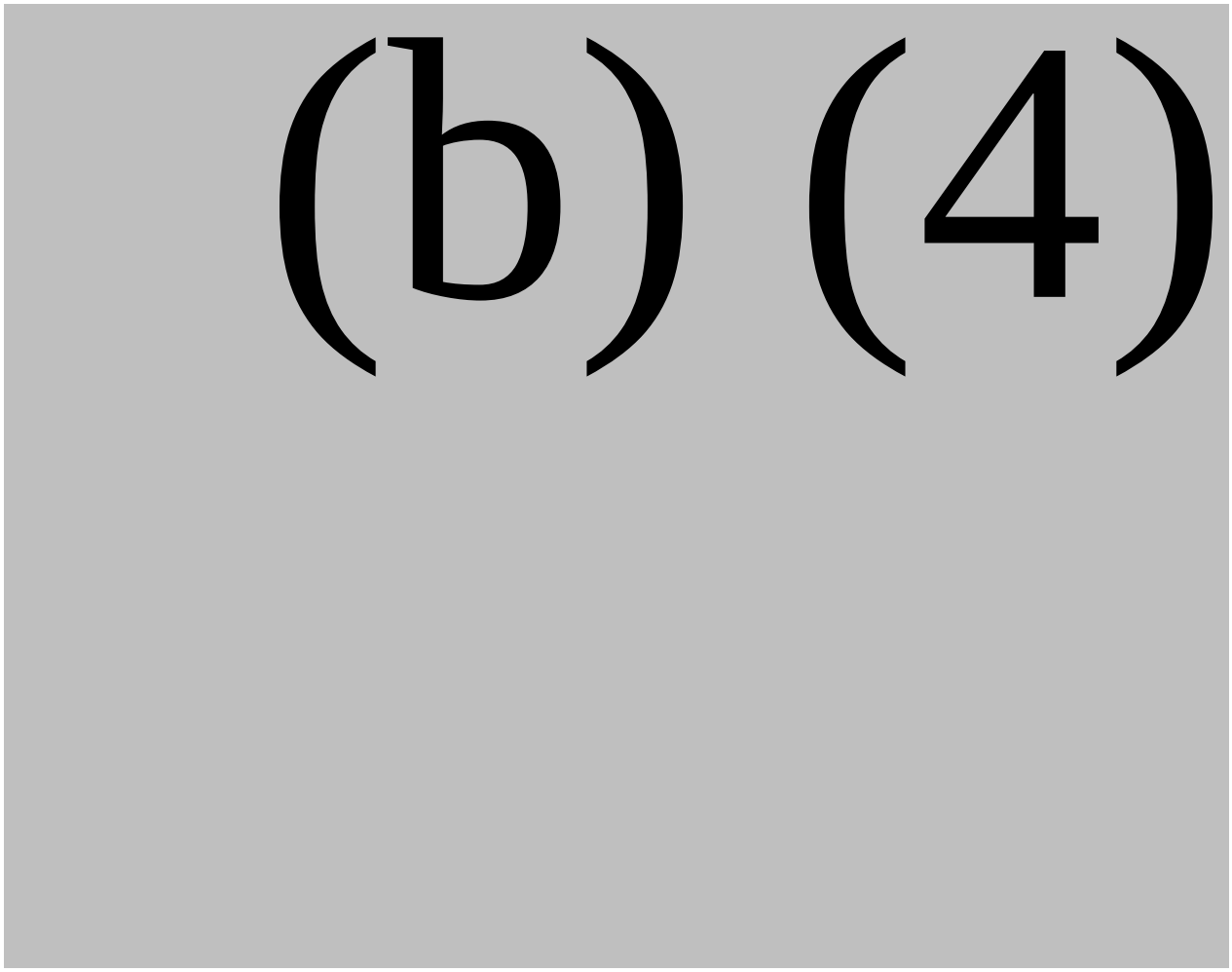
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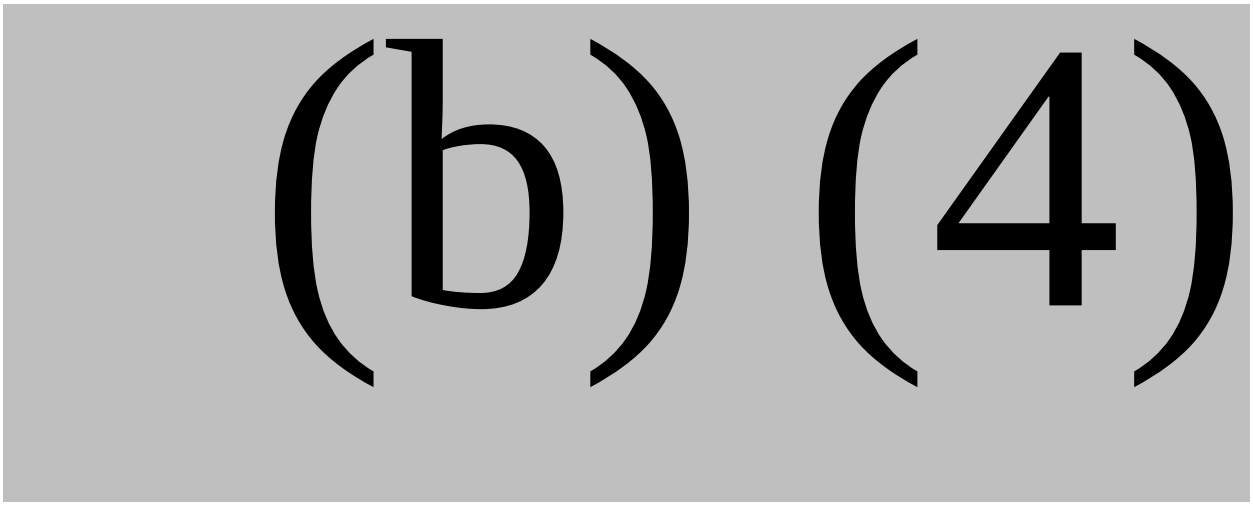
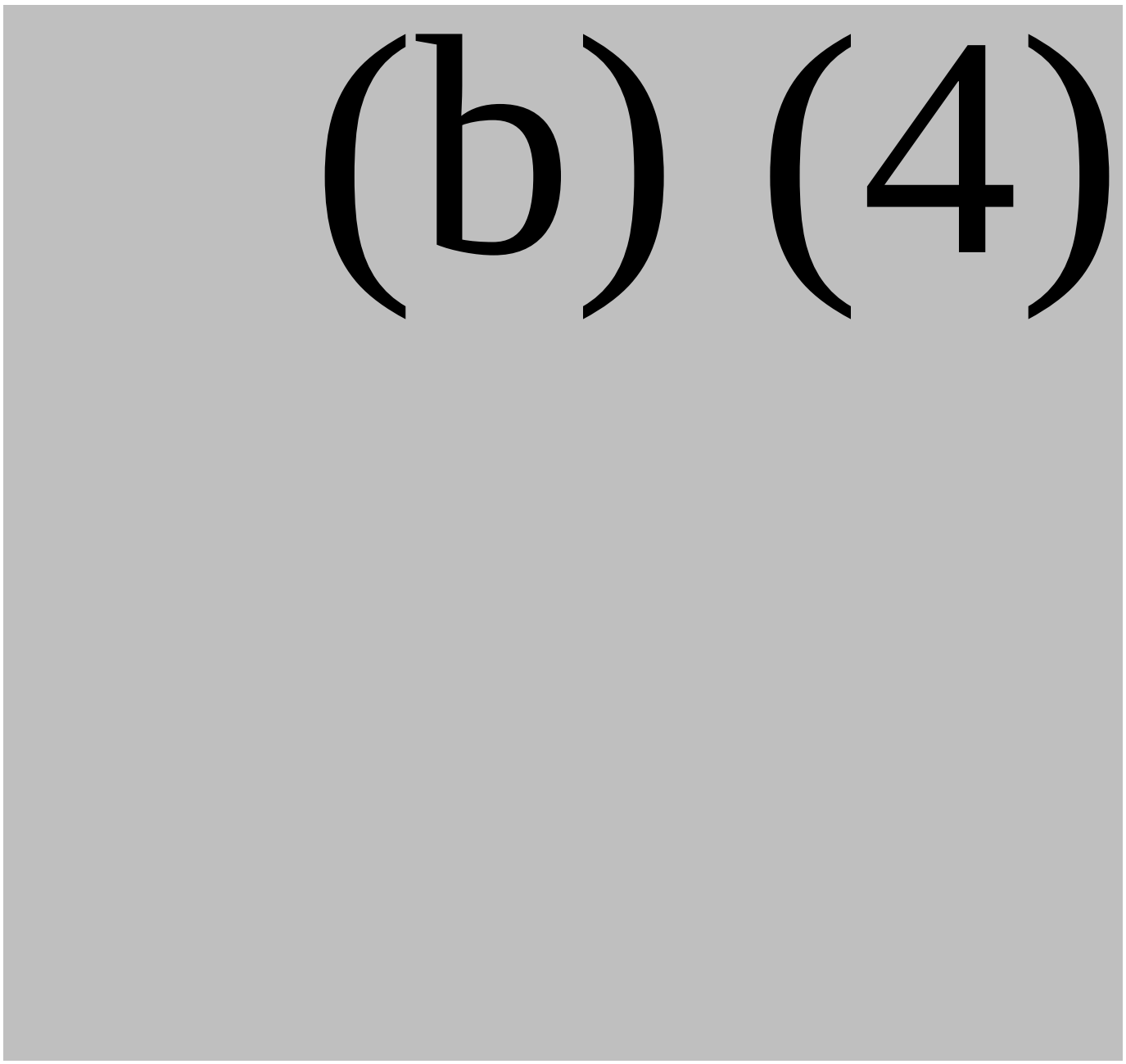
APPENDIX 2

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APPENDIX 3

(b) (4)

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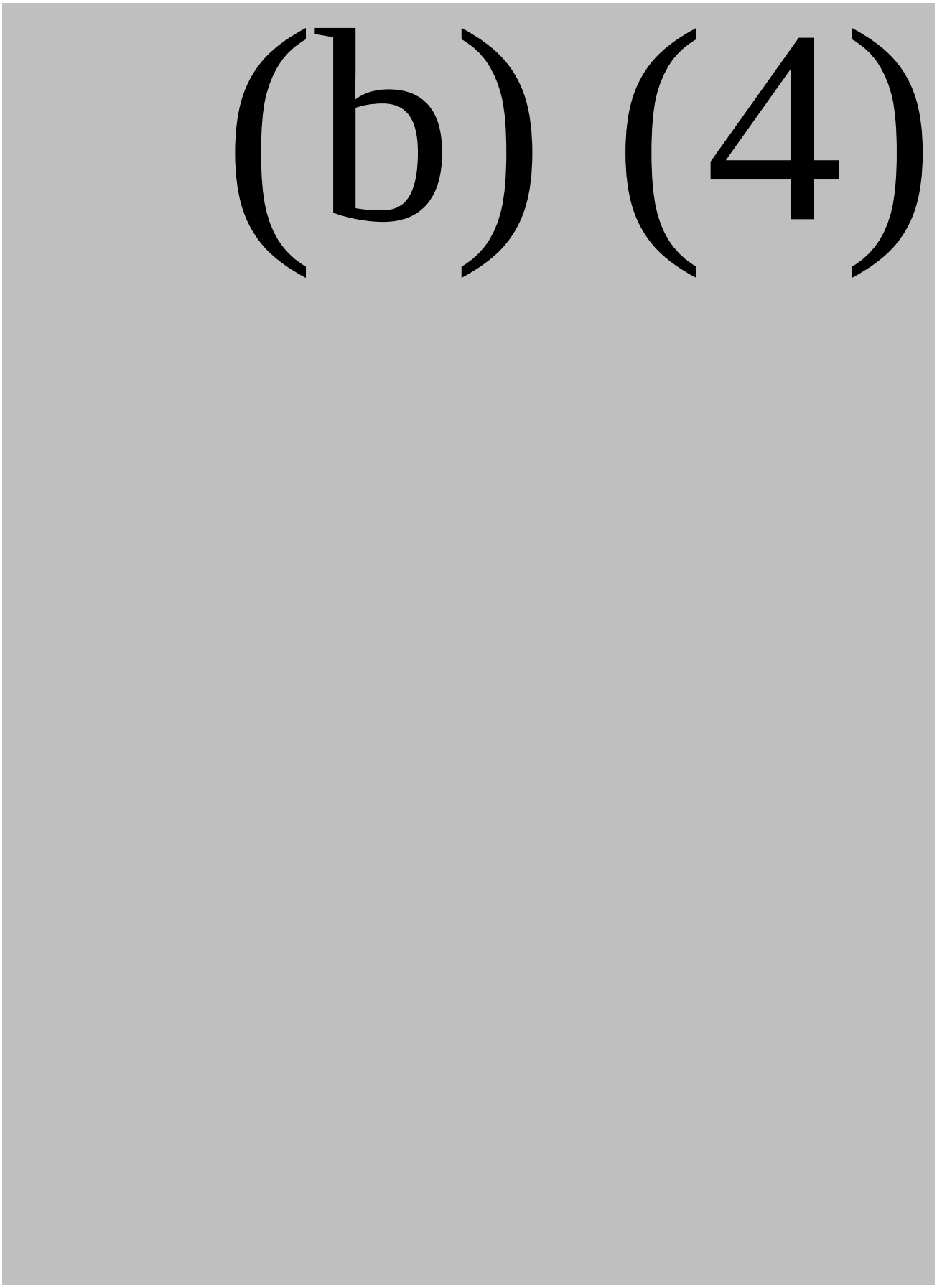
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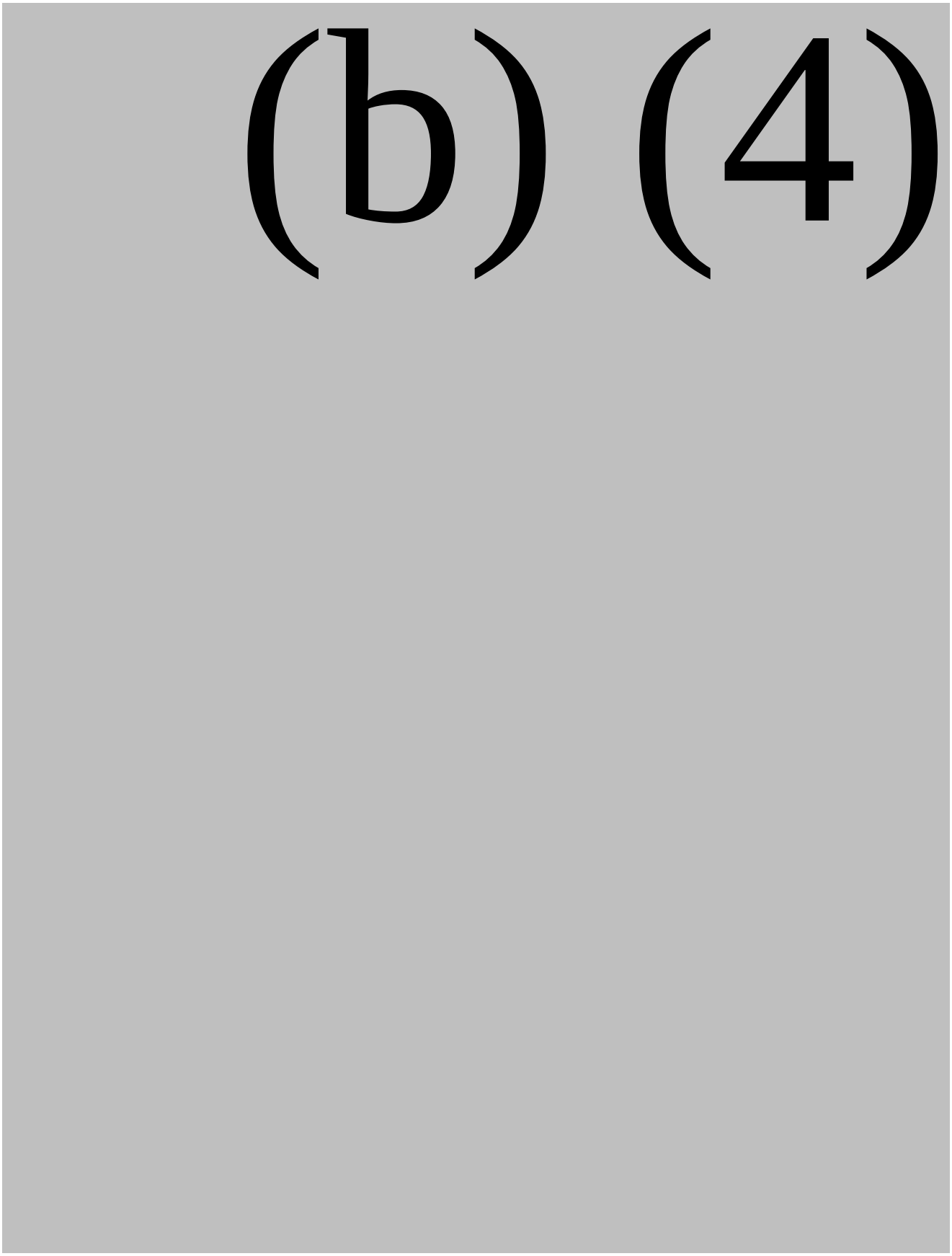
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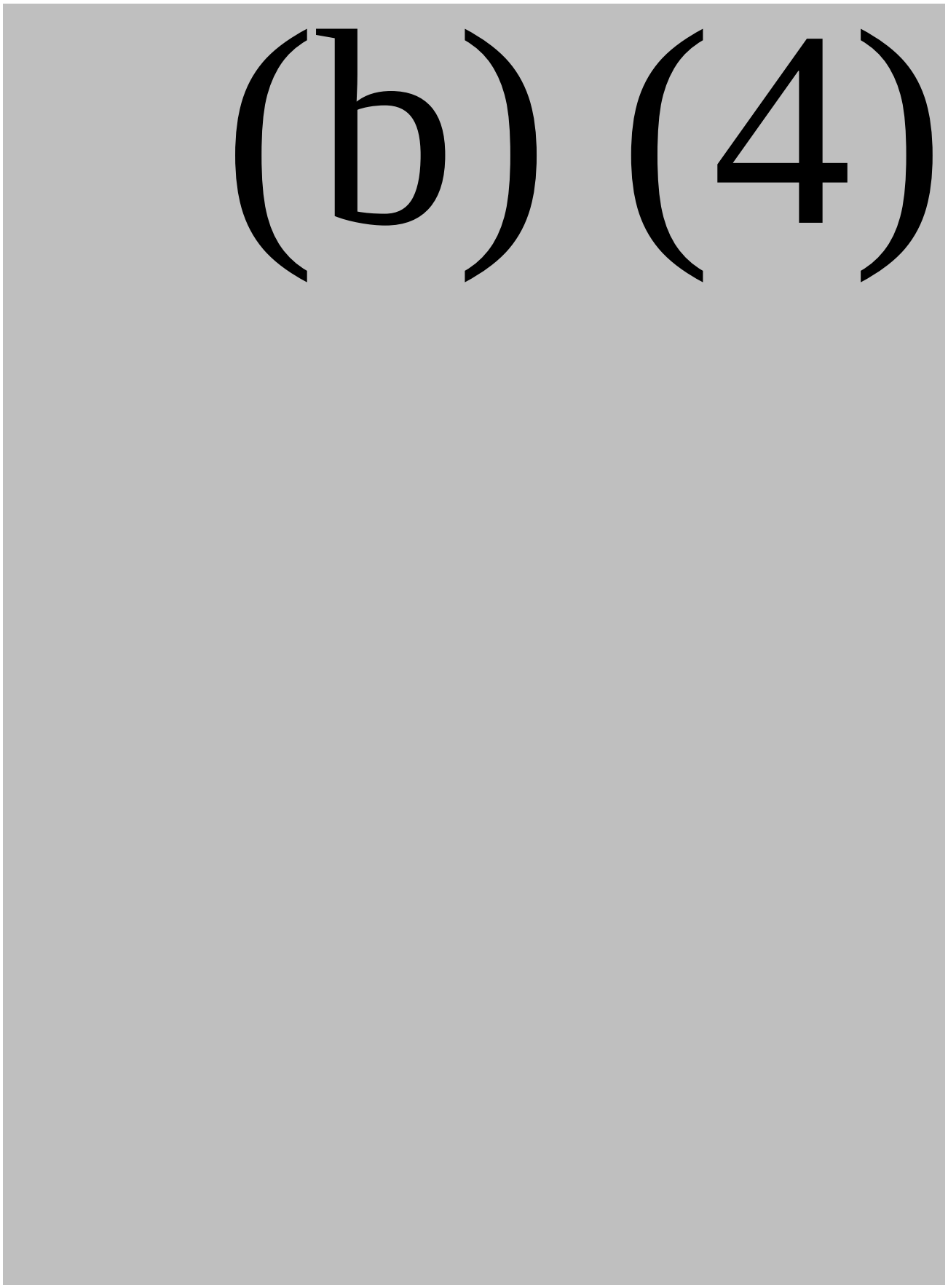
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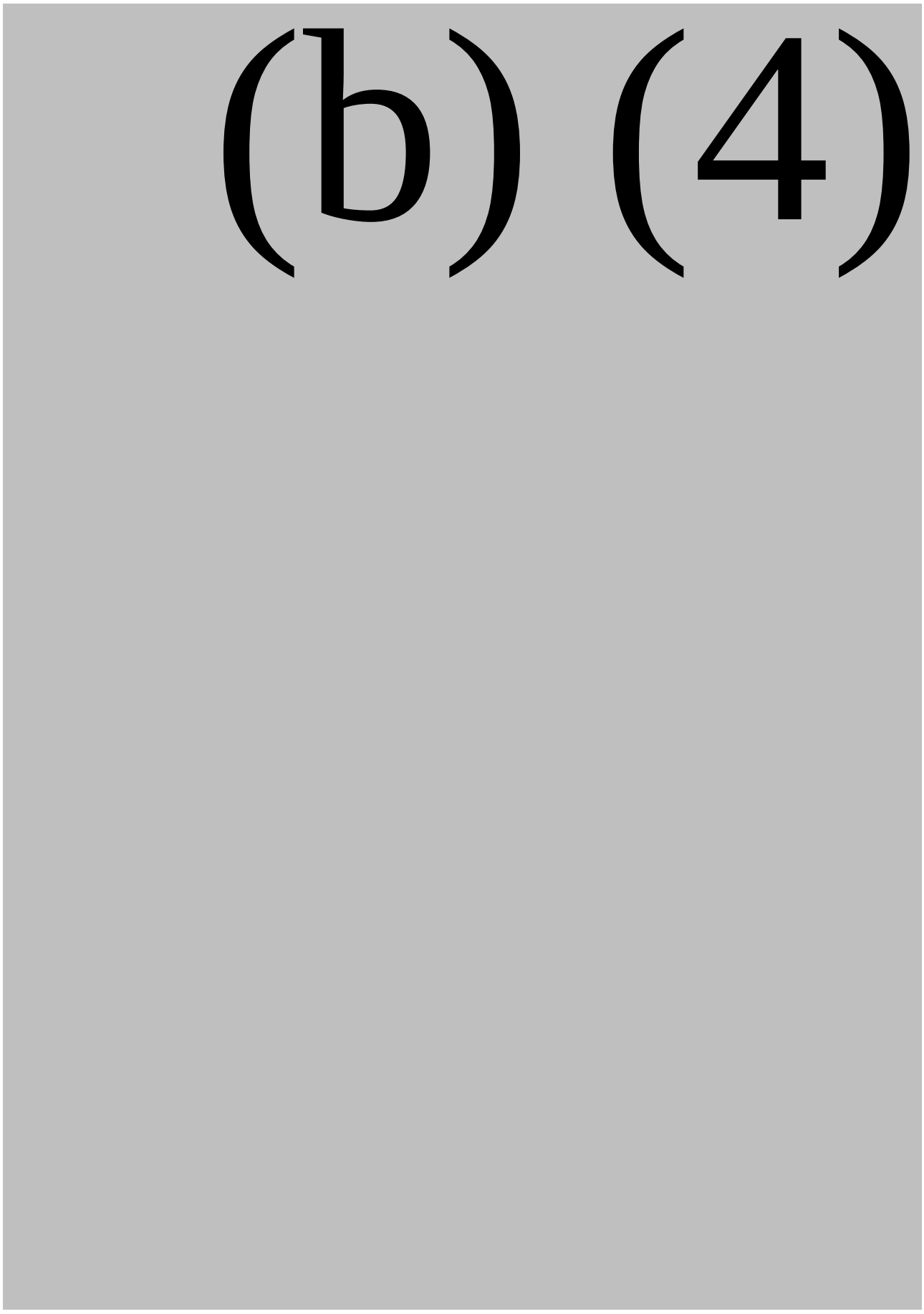
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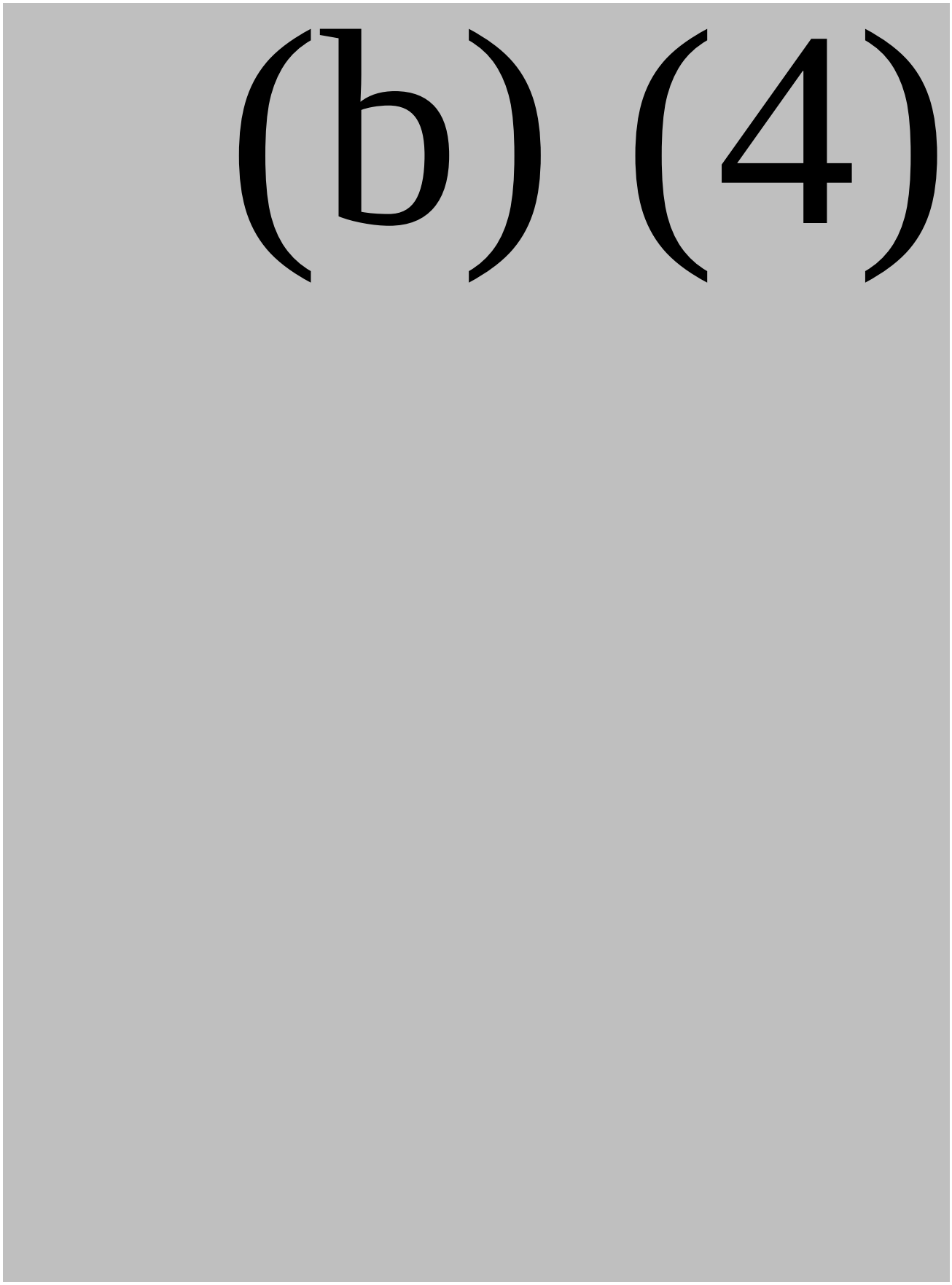
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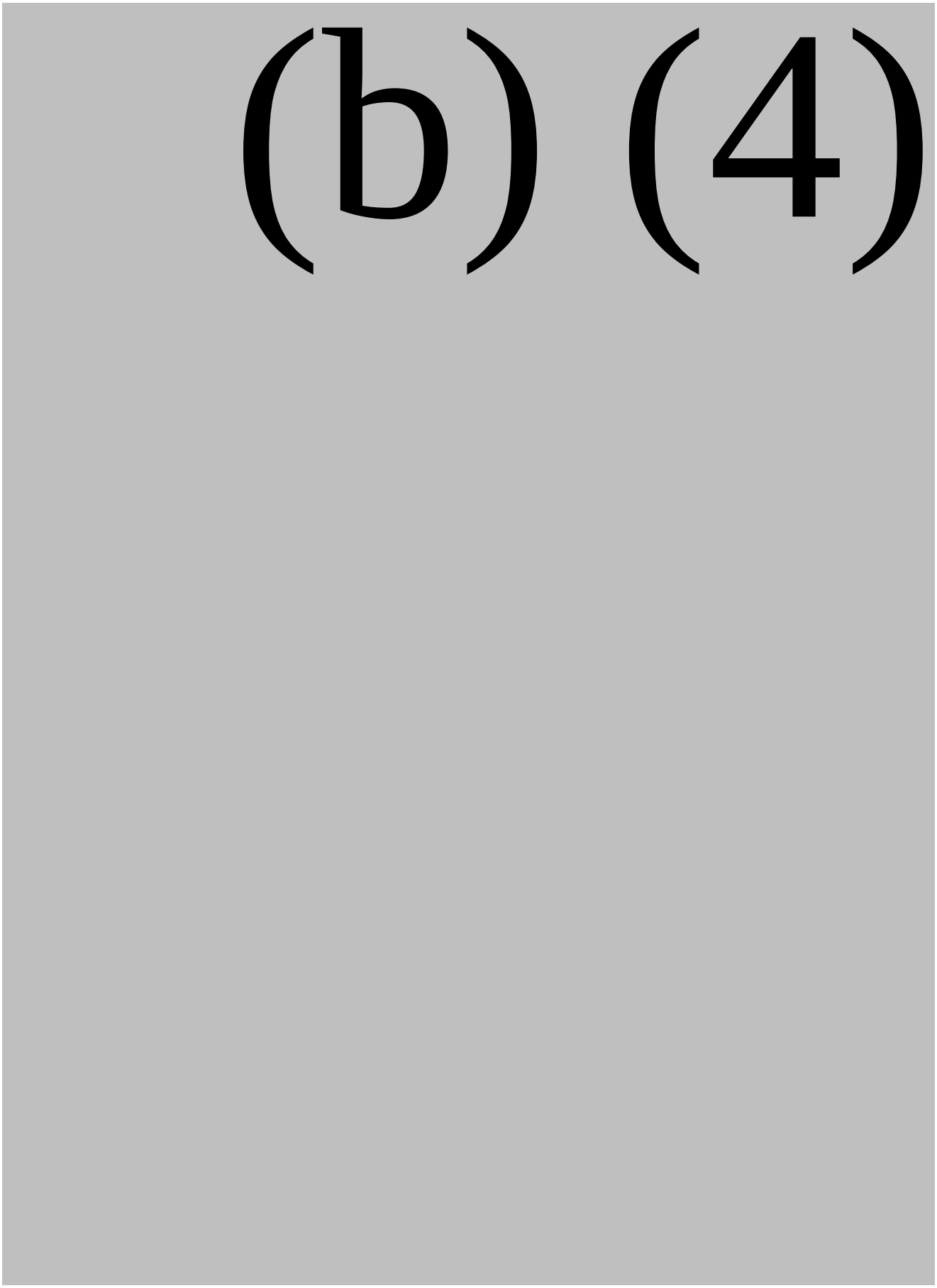
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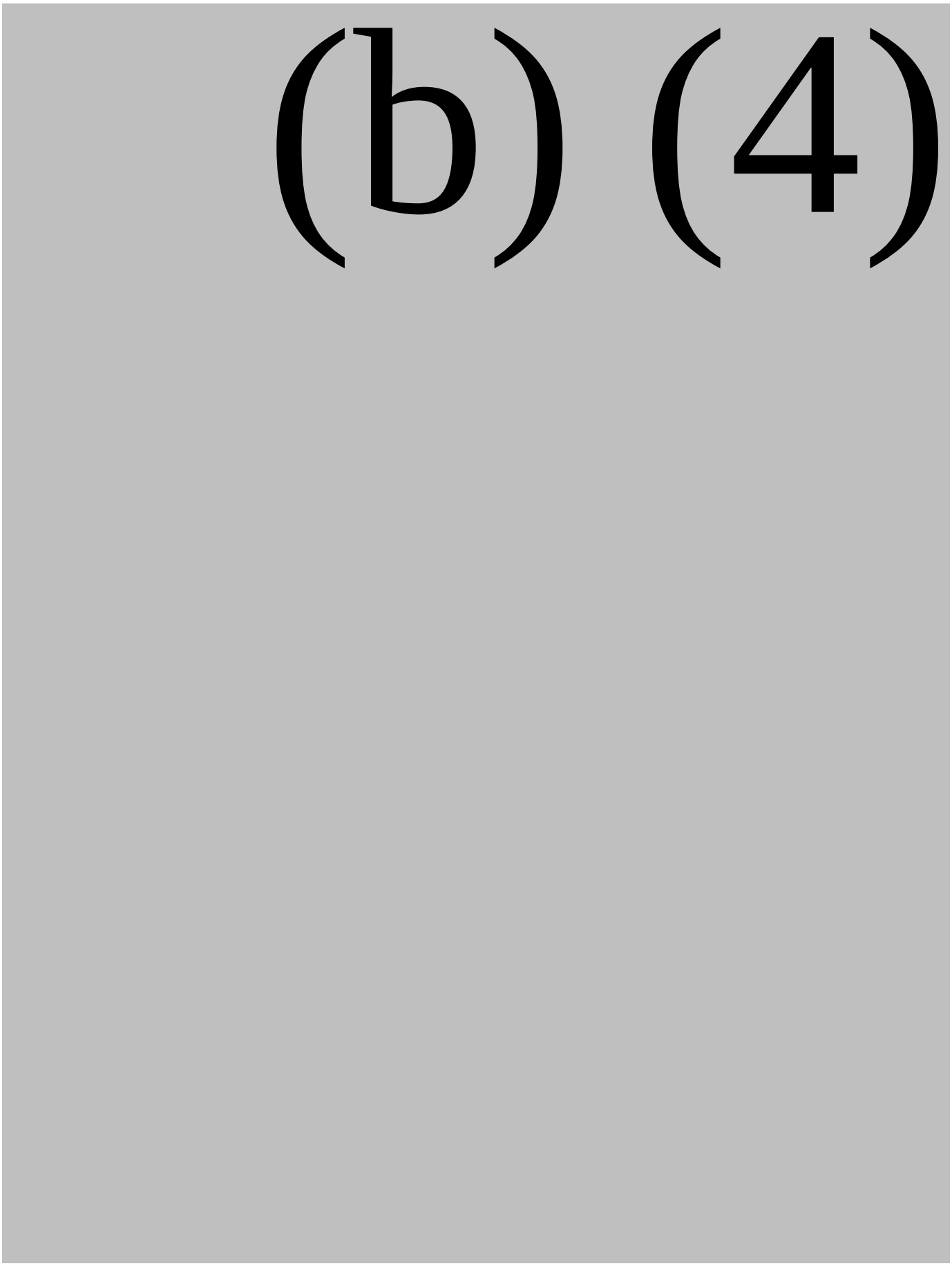
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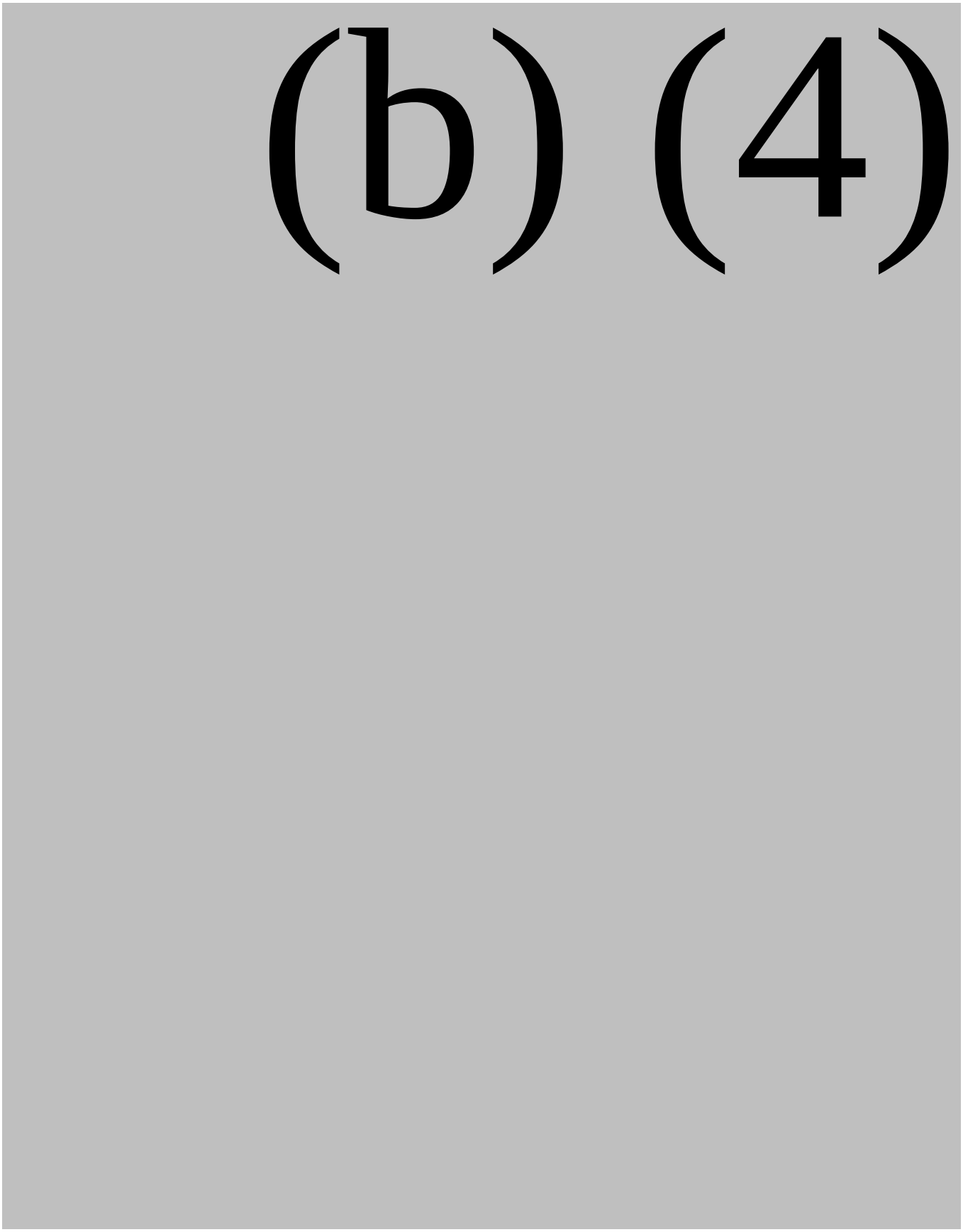
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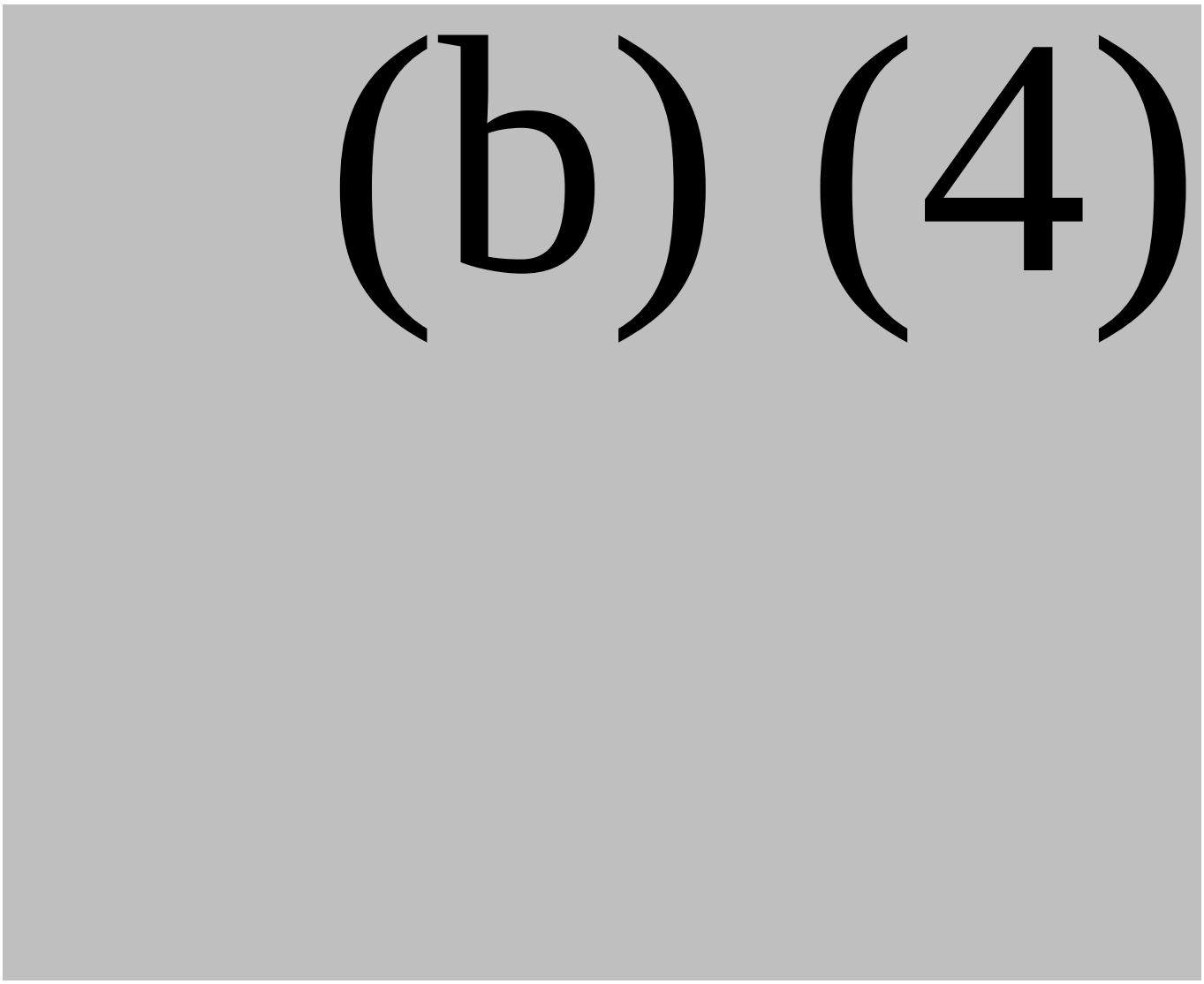
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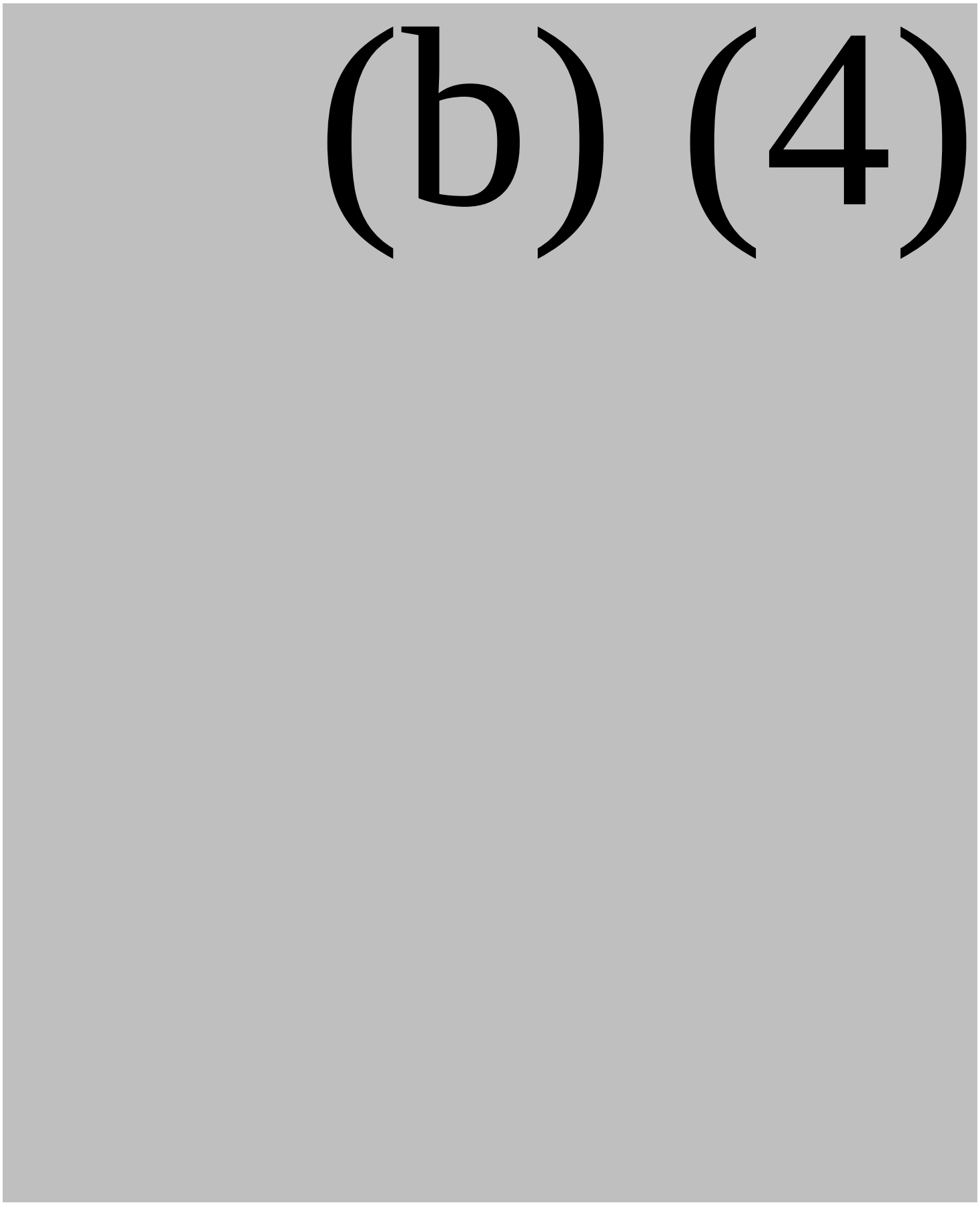
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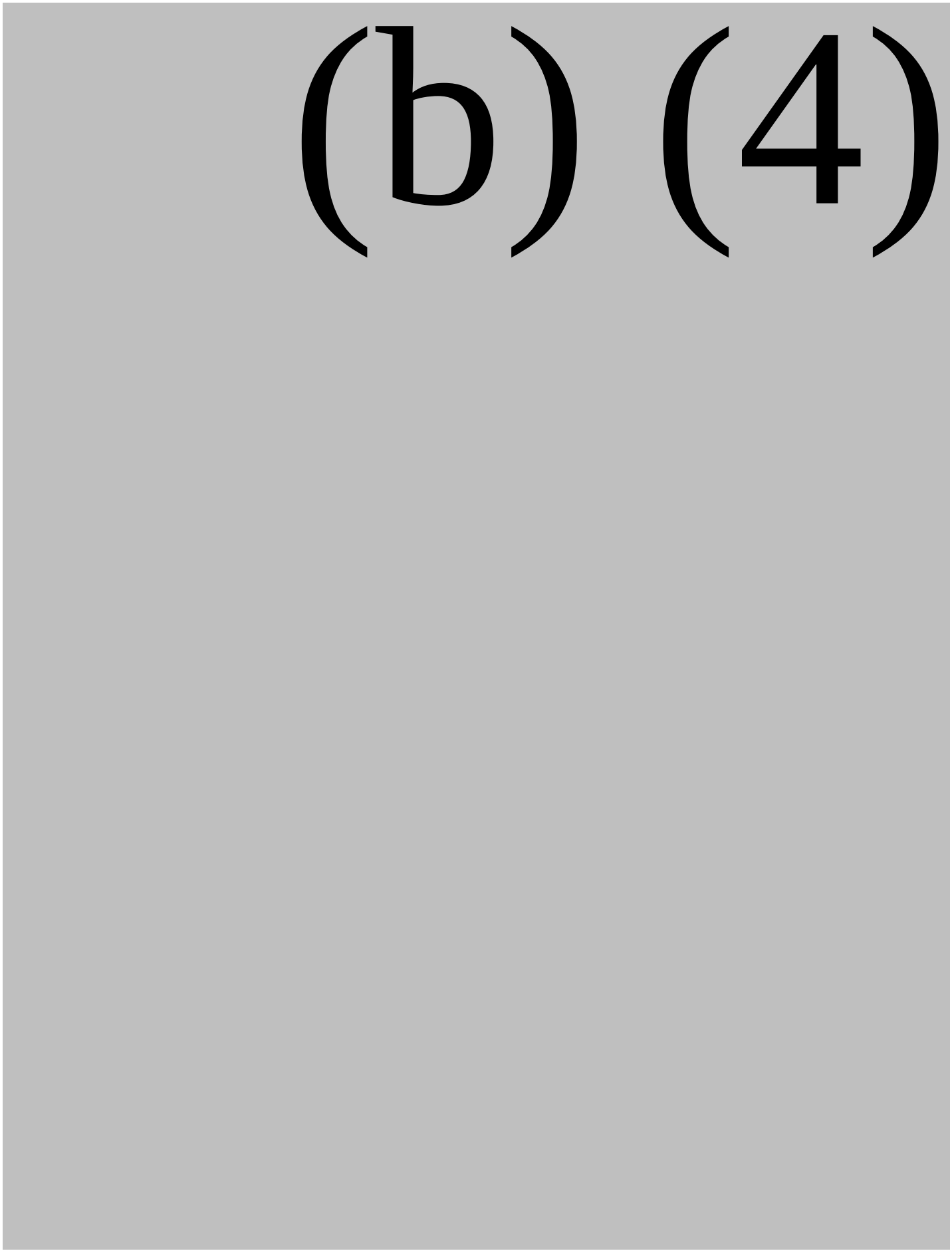
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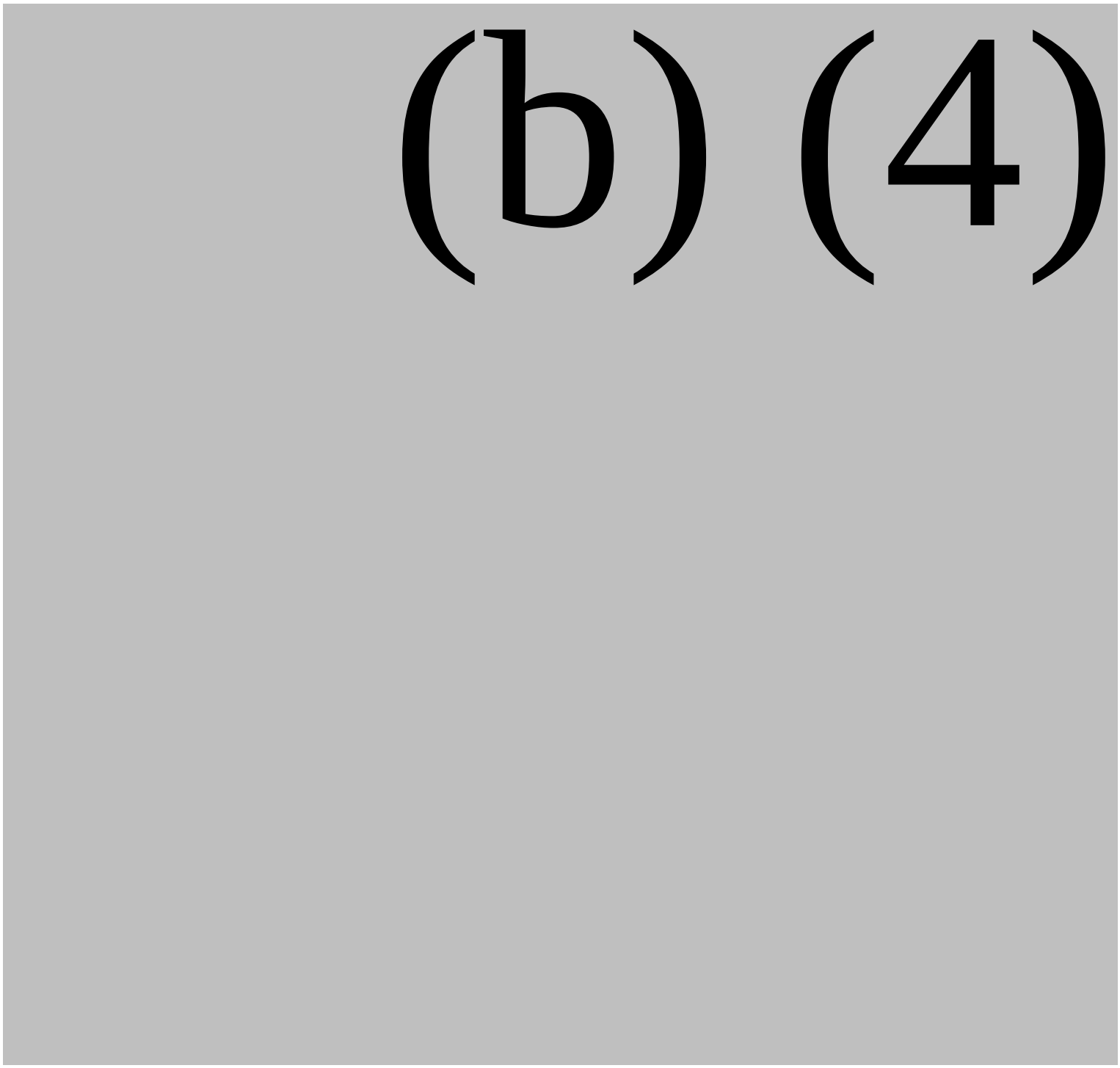
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APPENDIX 4

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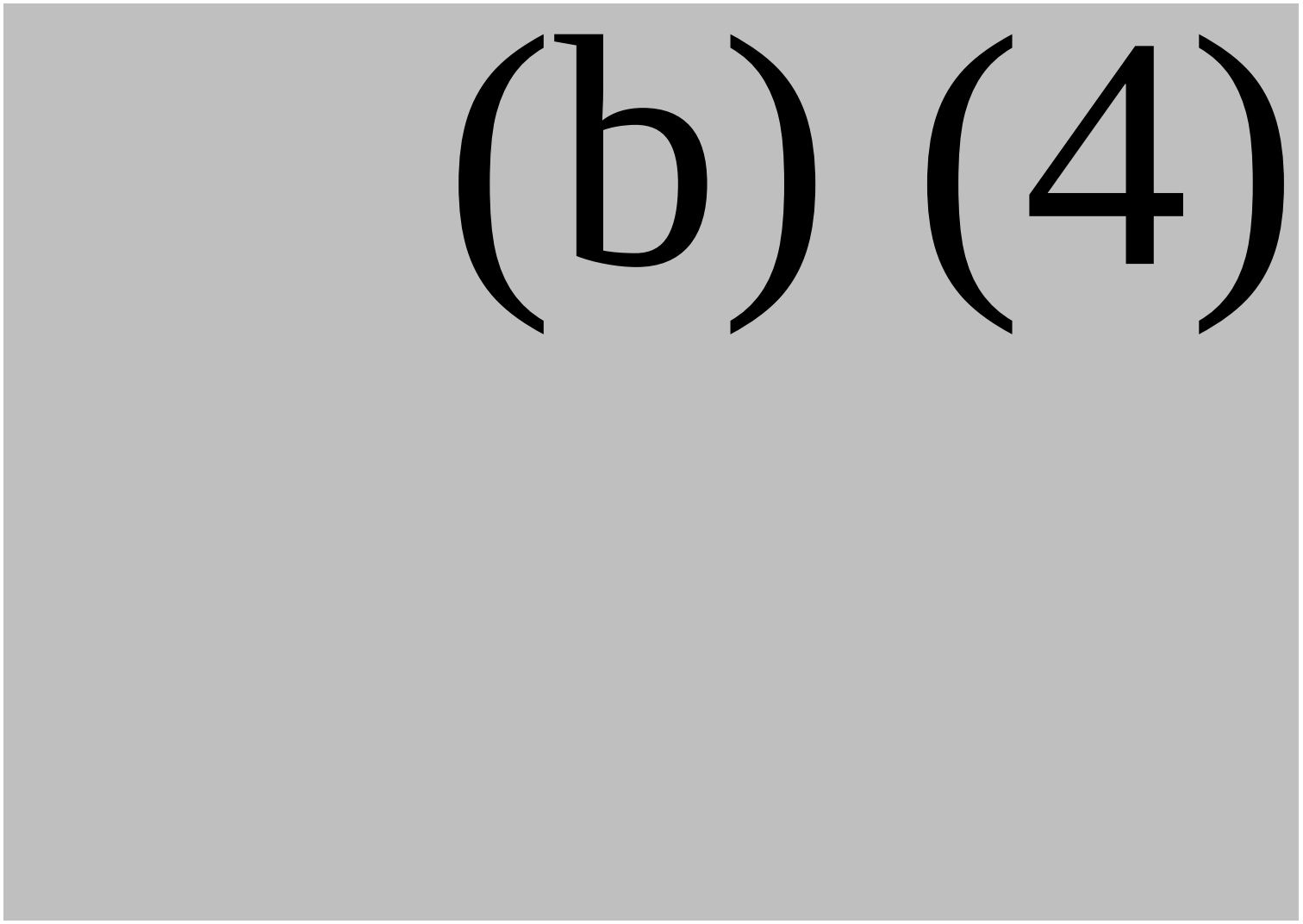
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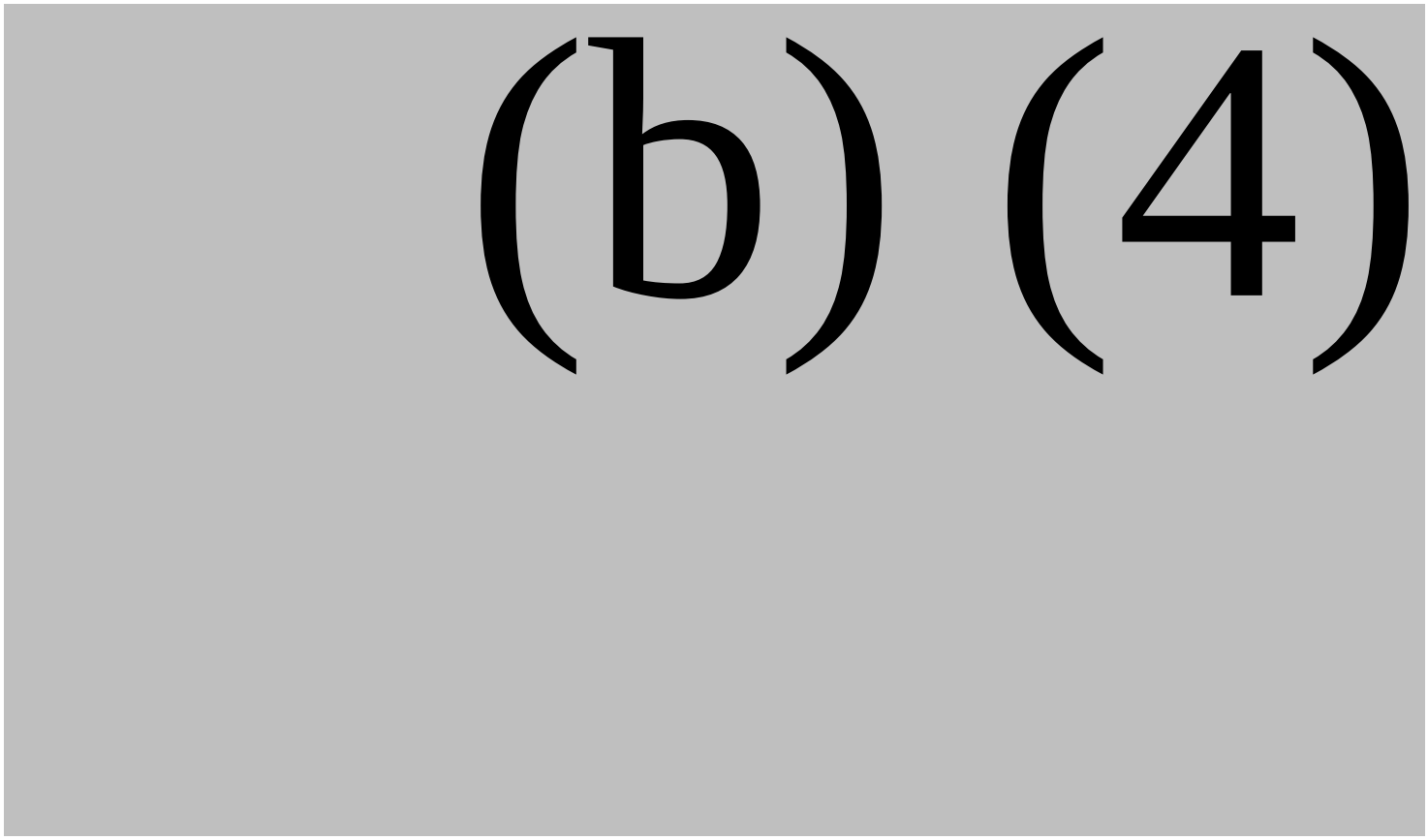
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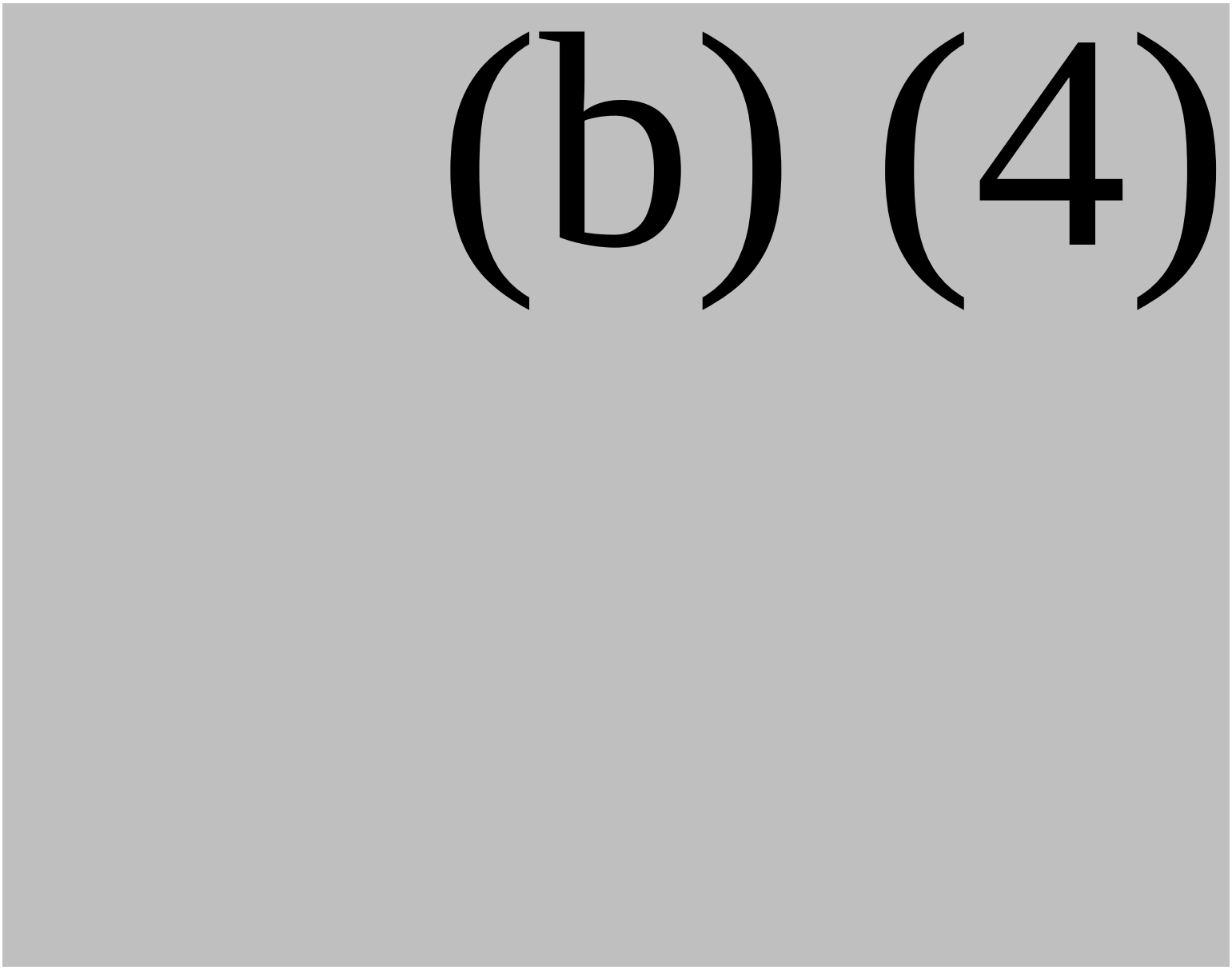
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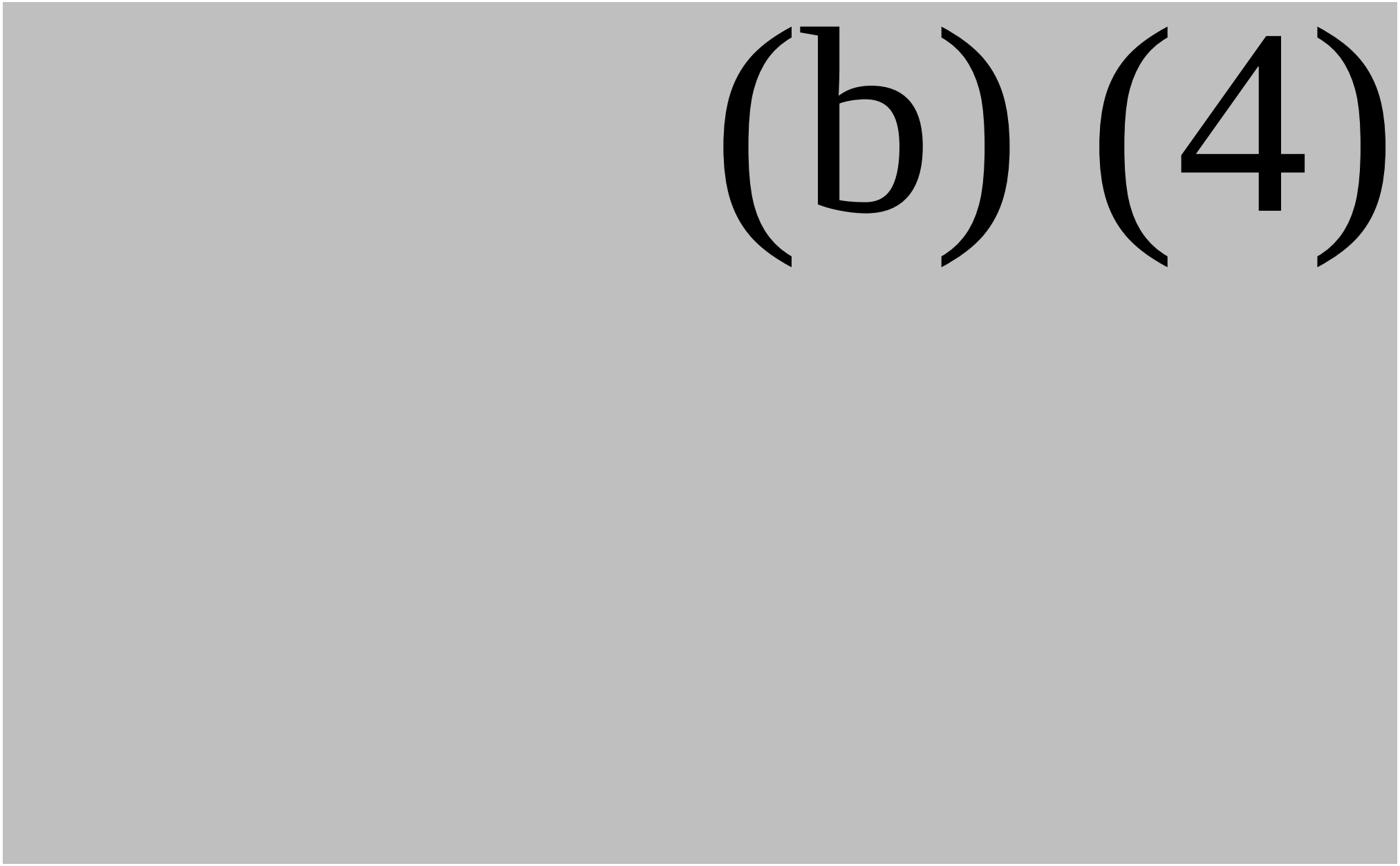
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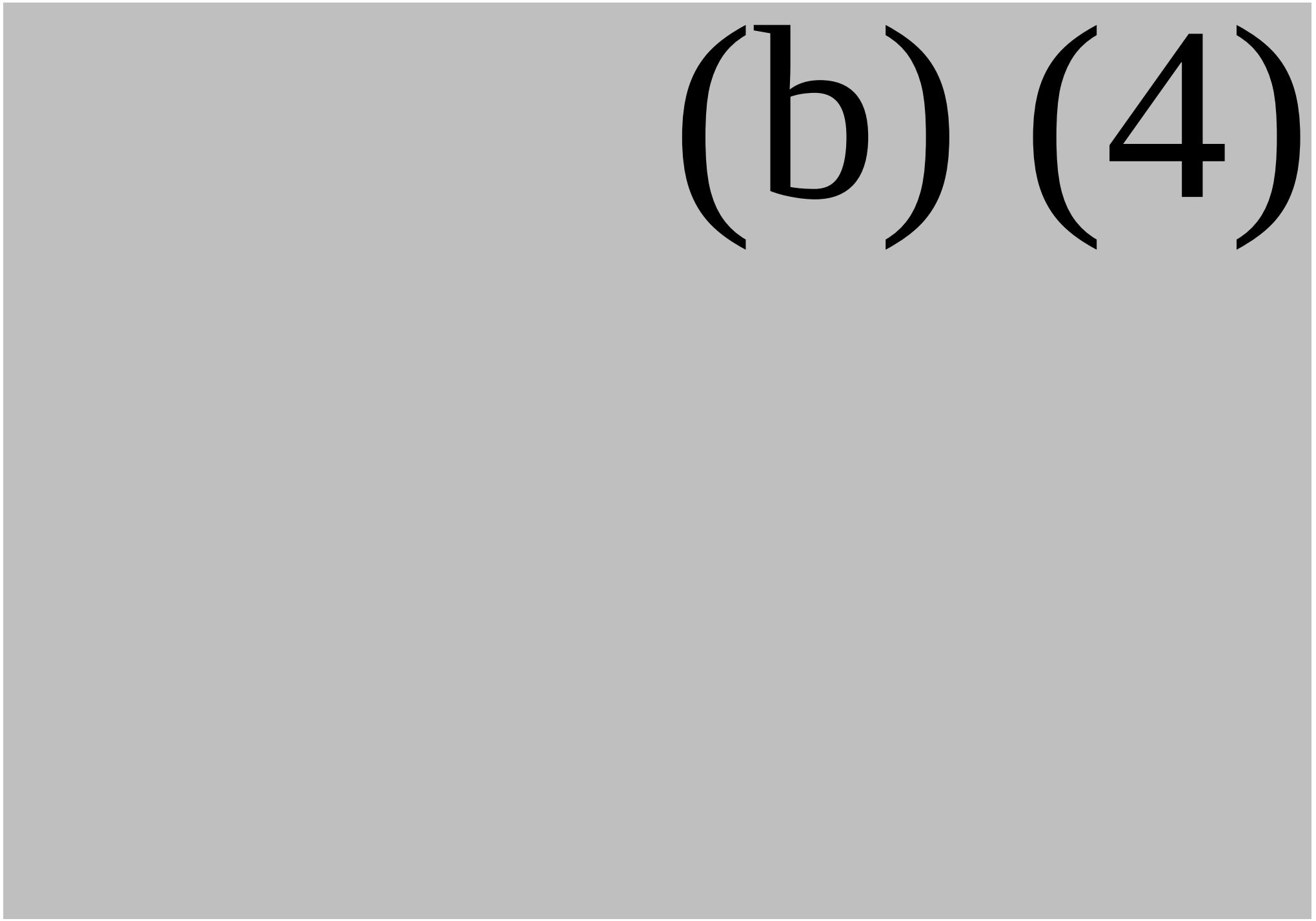
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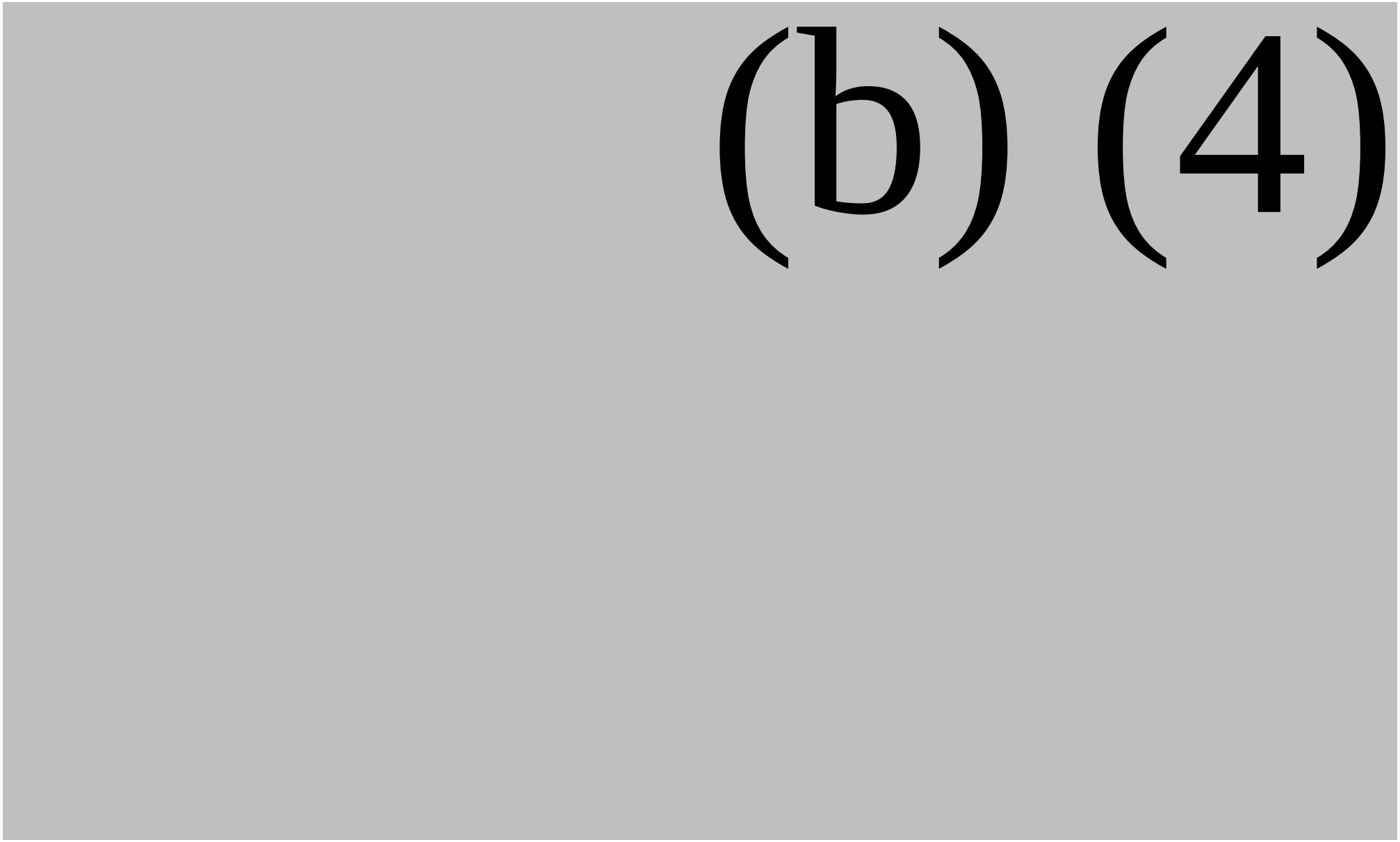
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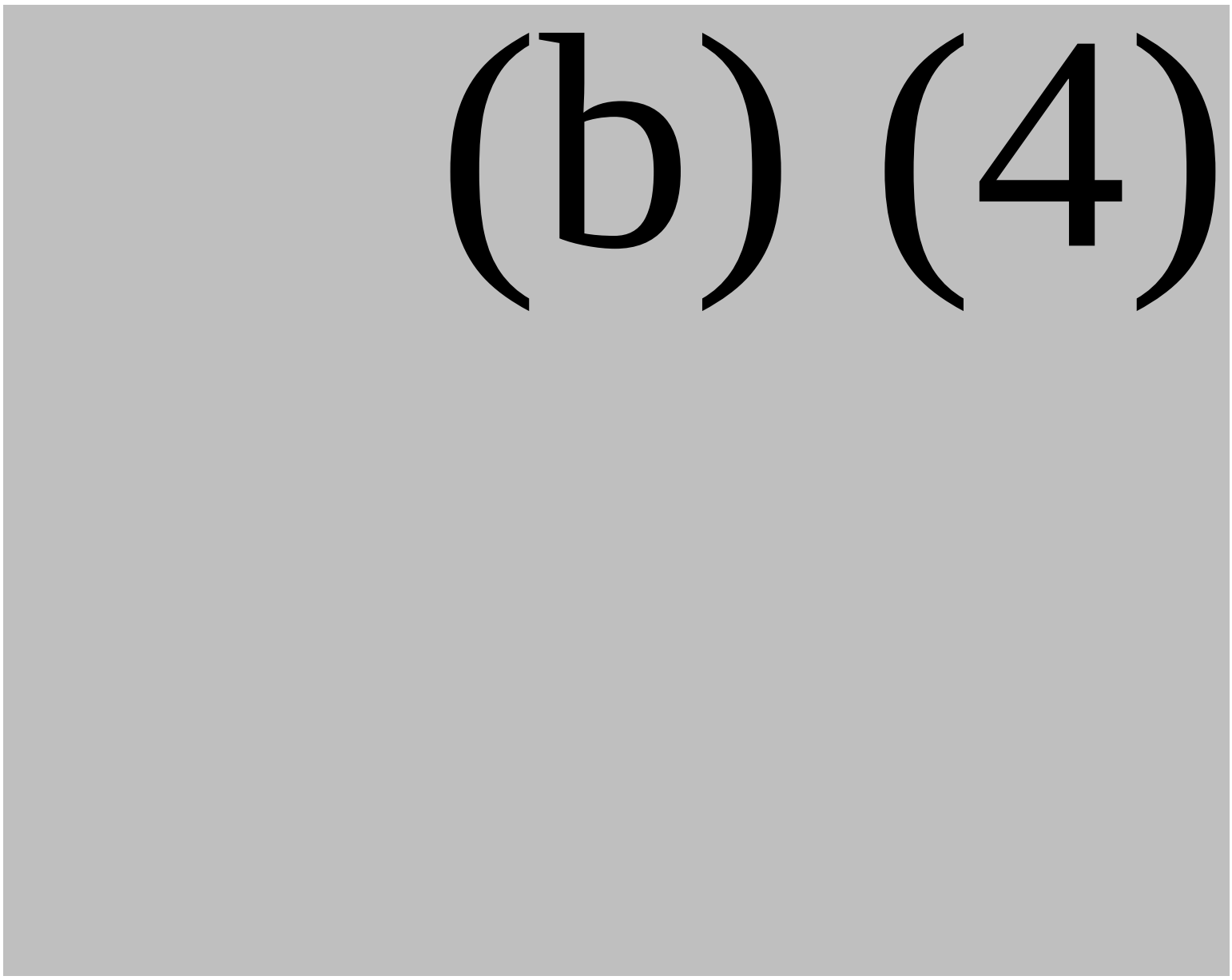
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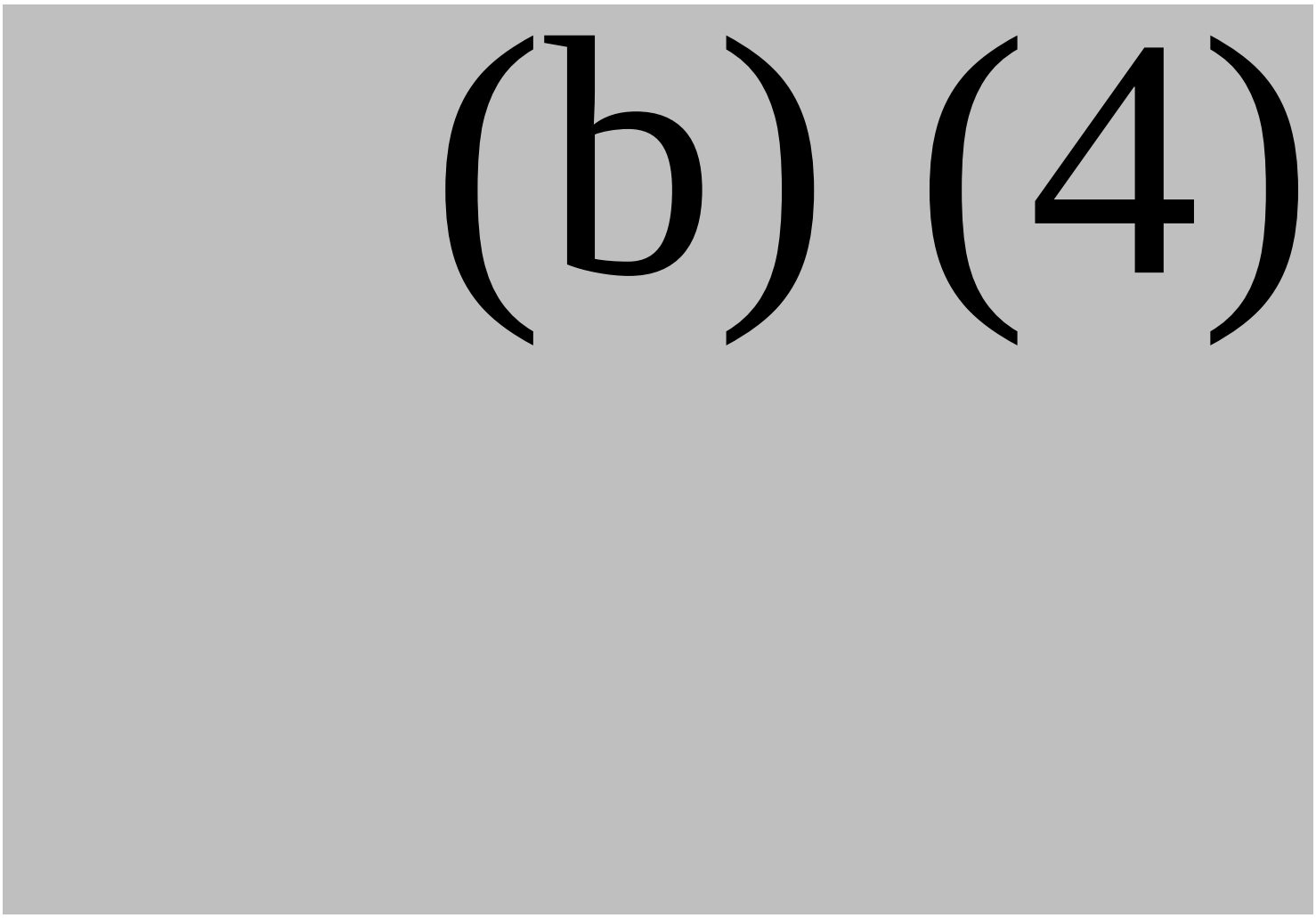
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APPENDIX 5

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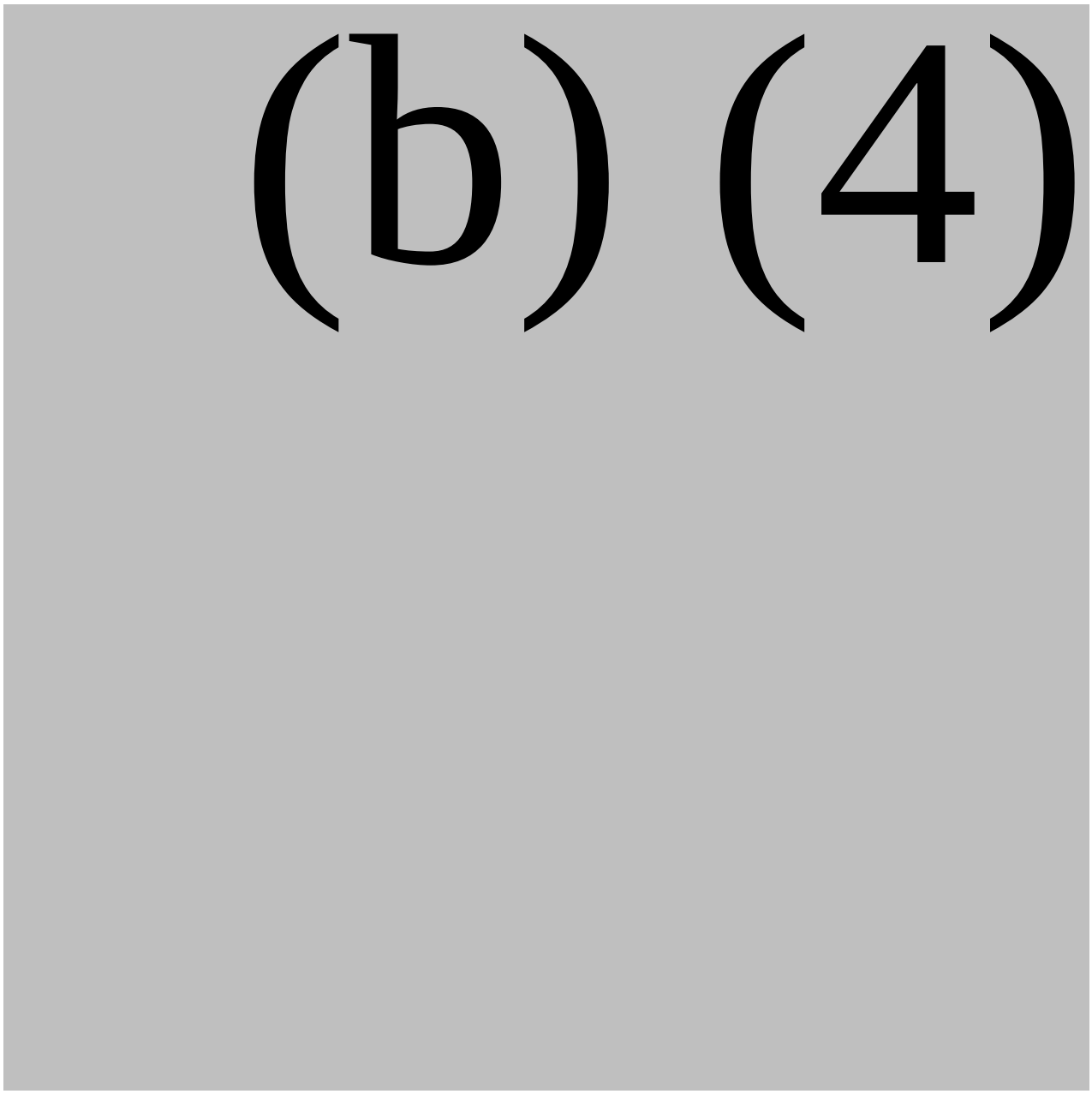
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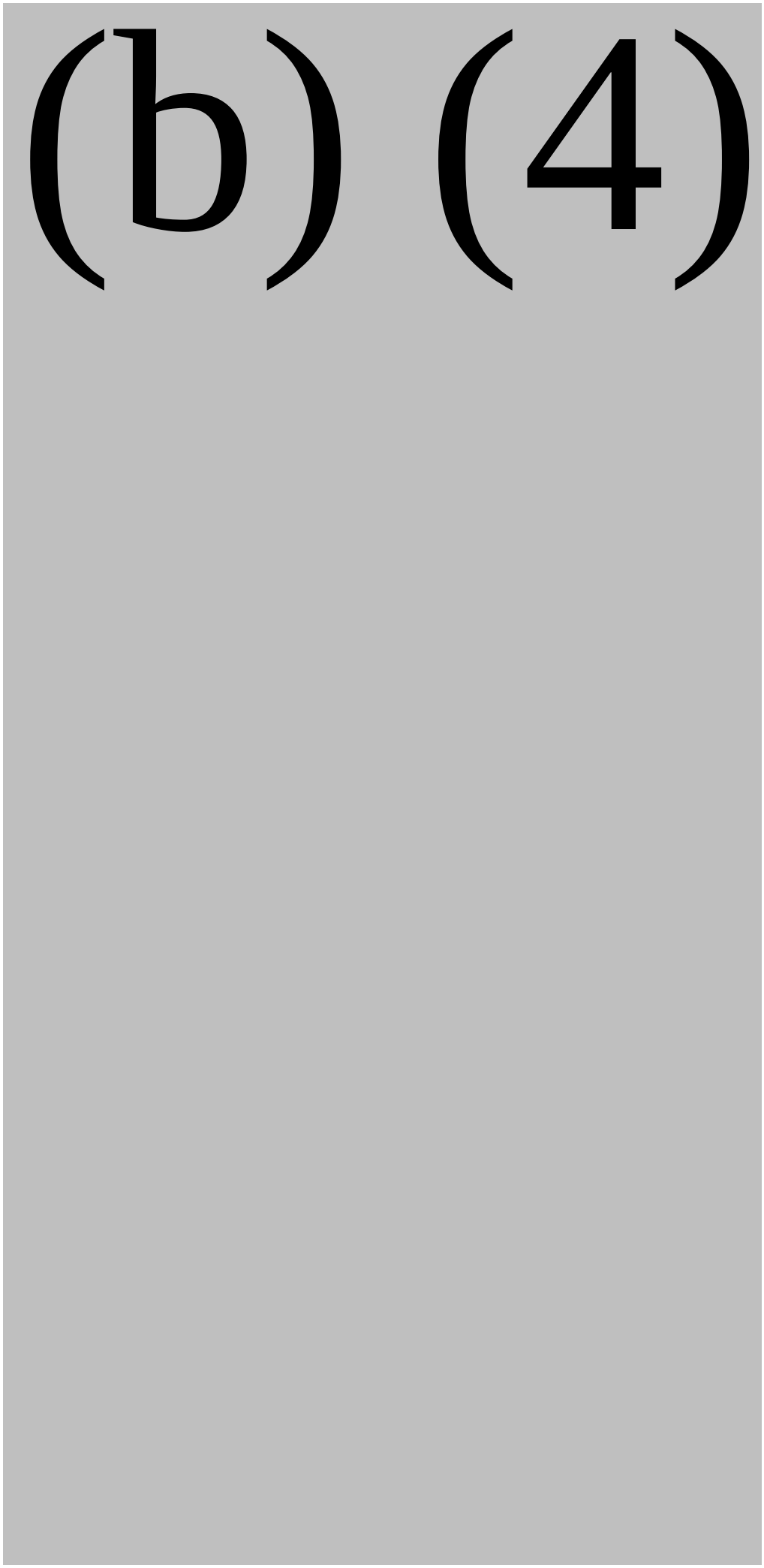
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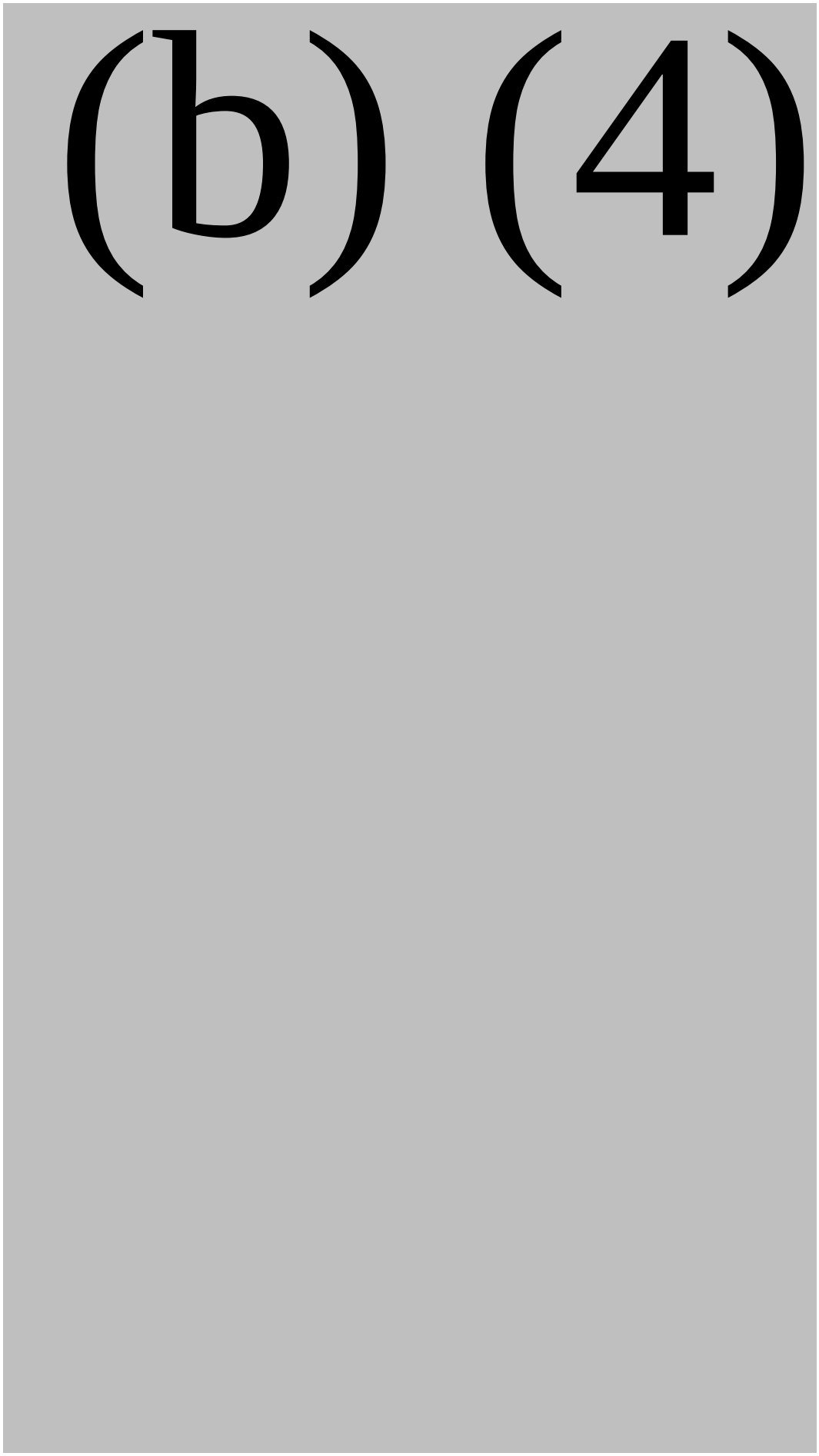
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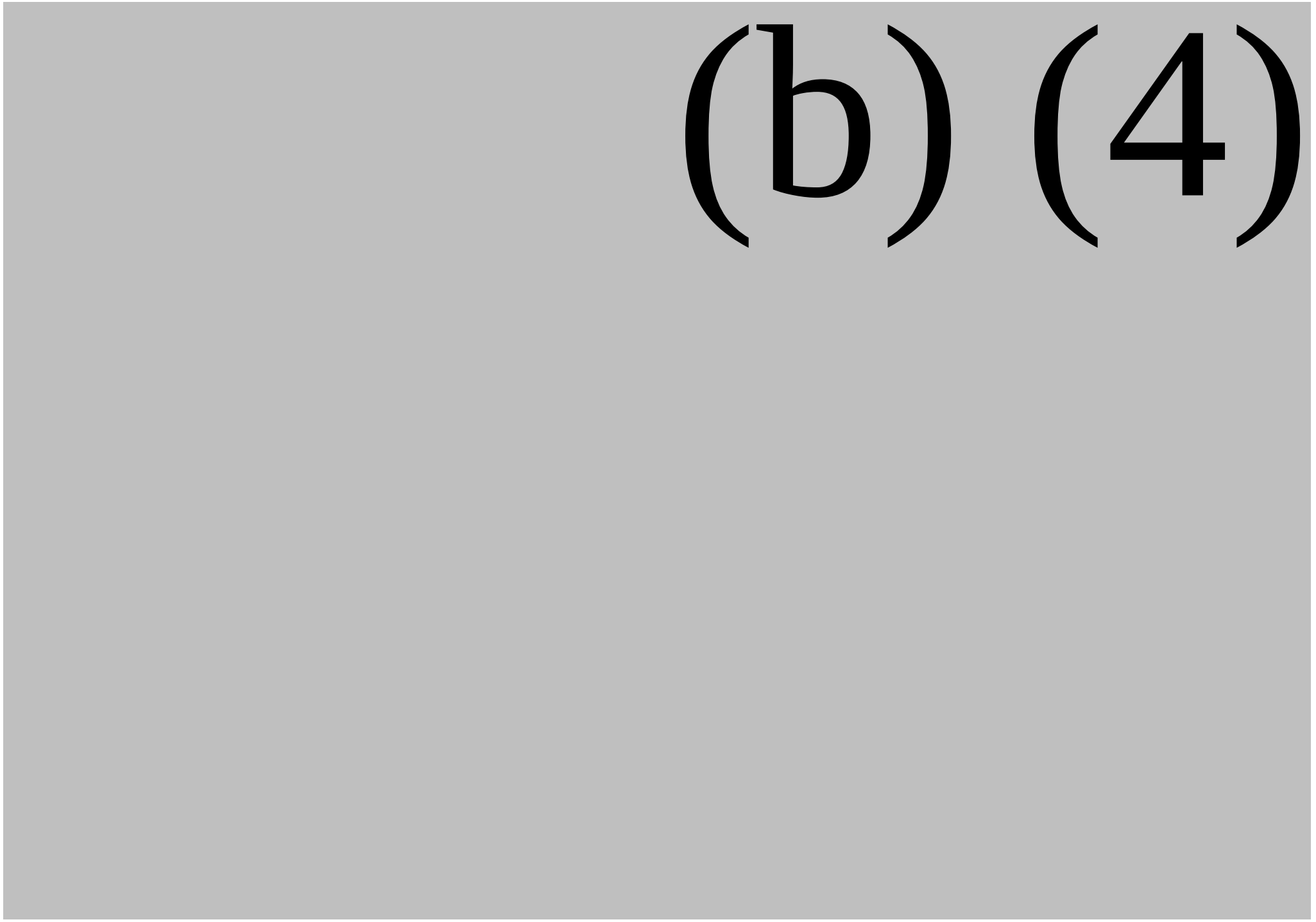
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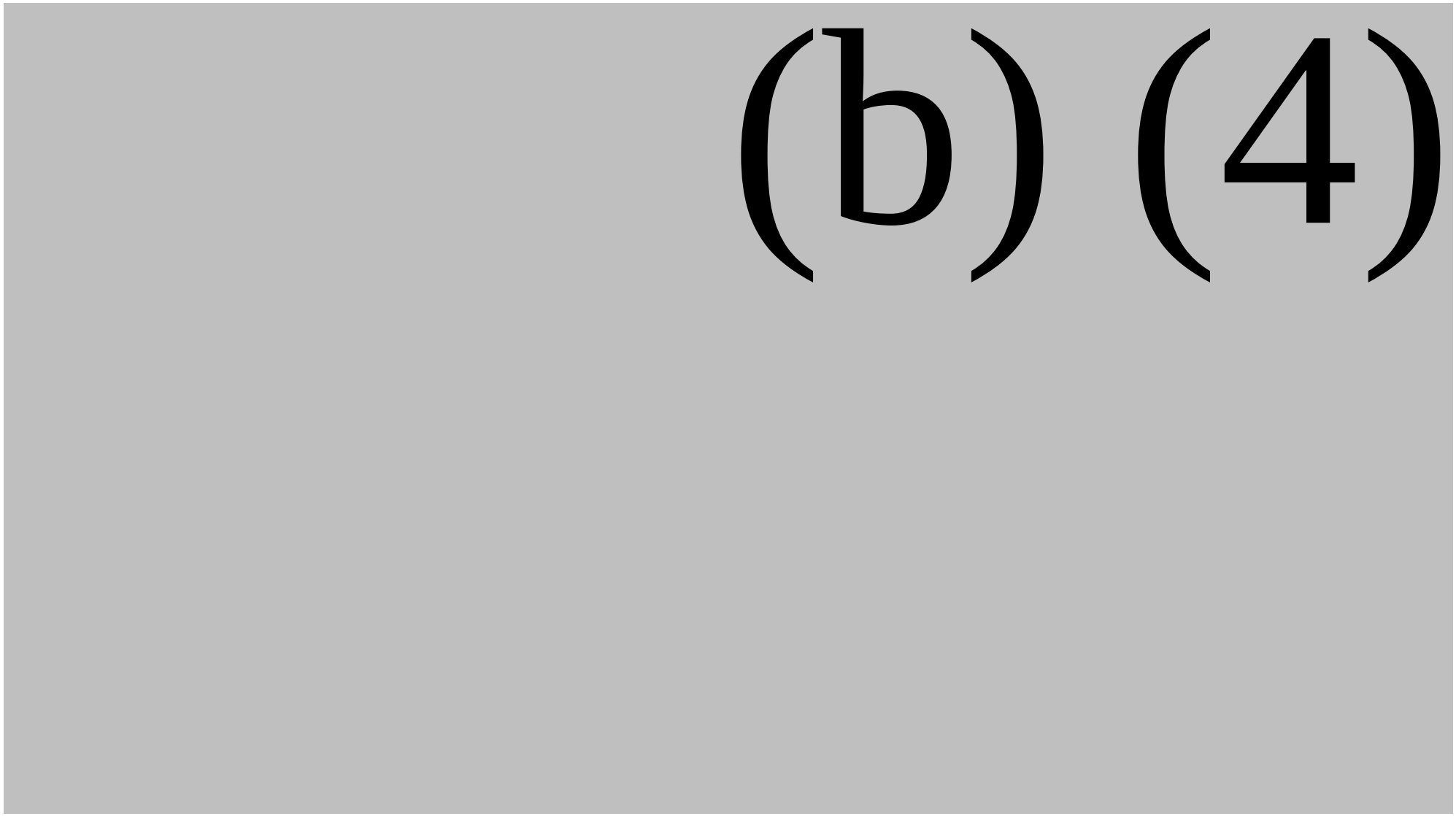
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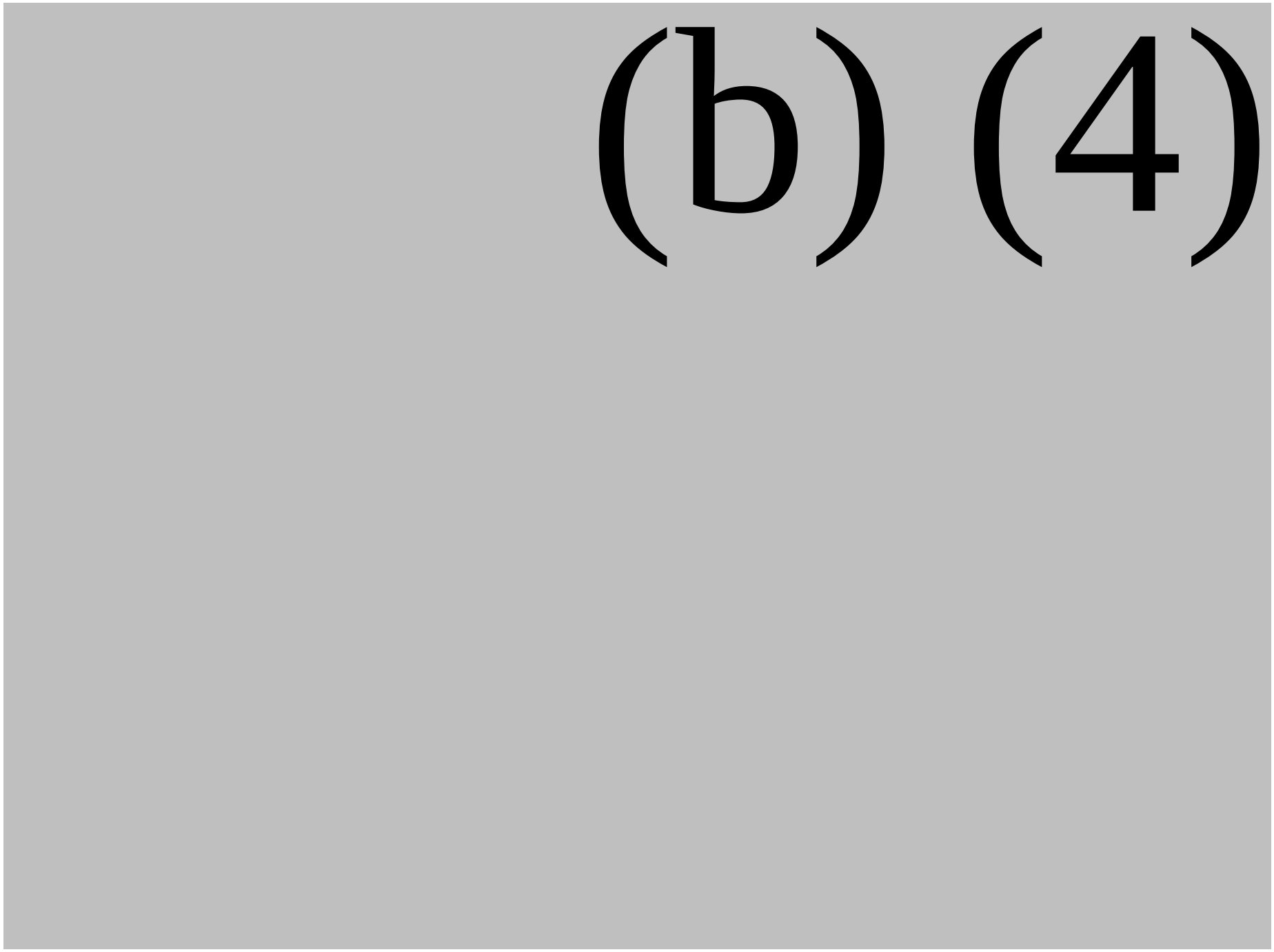
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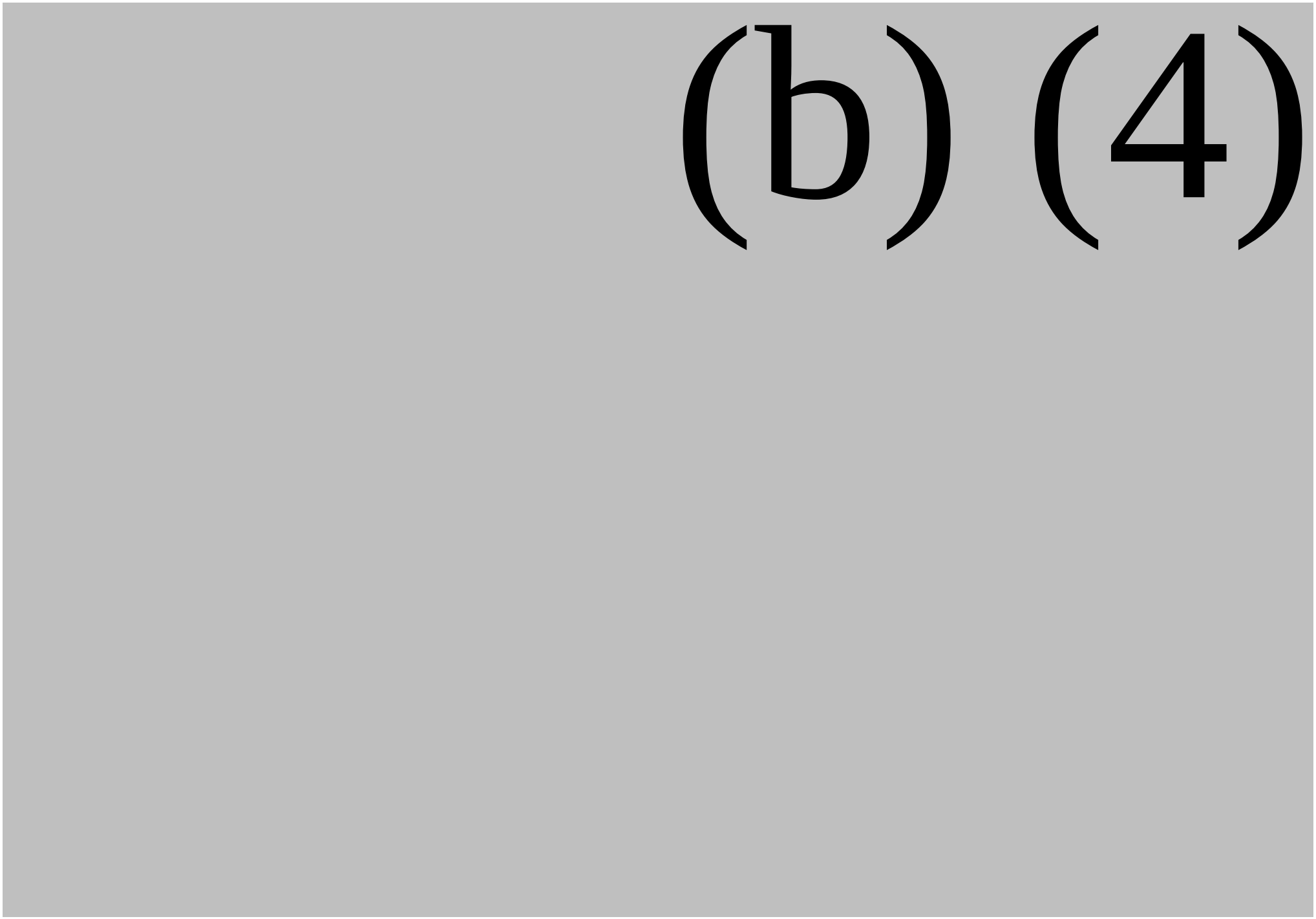
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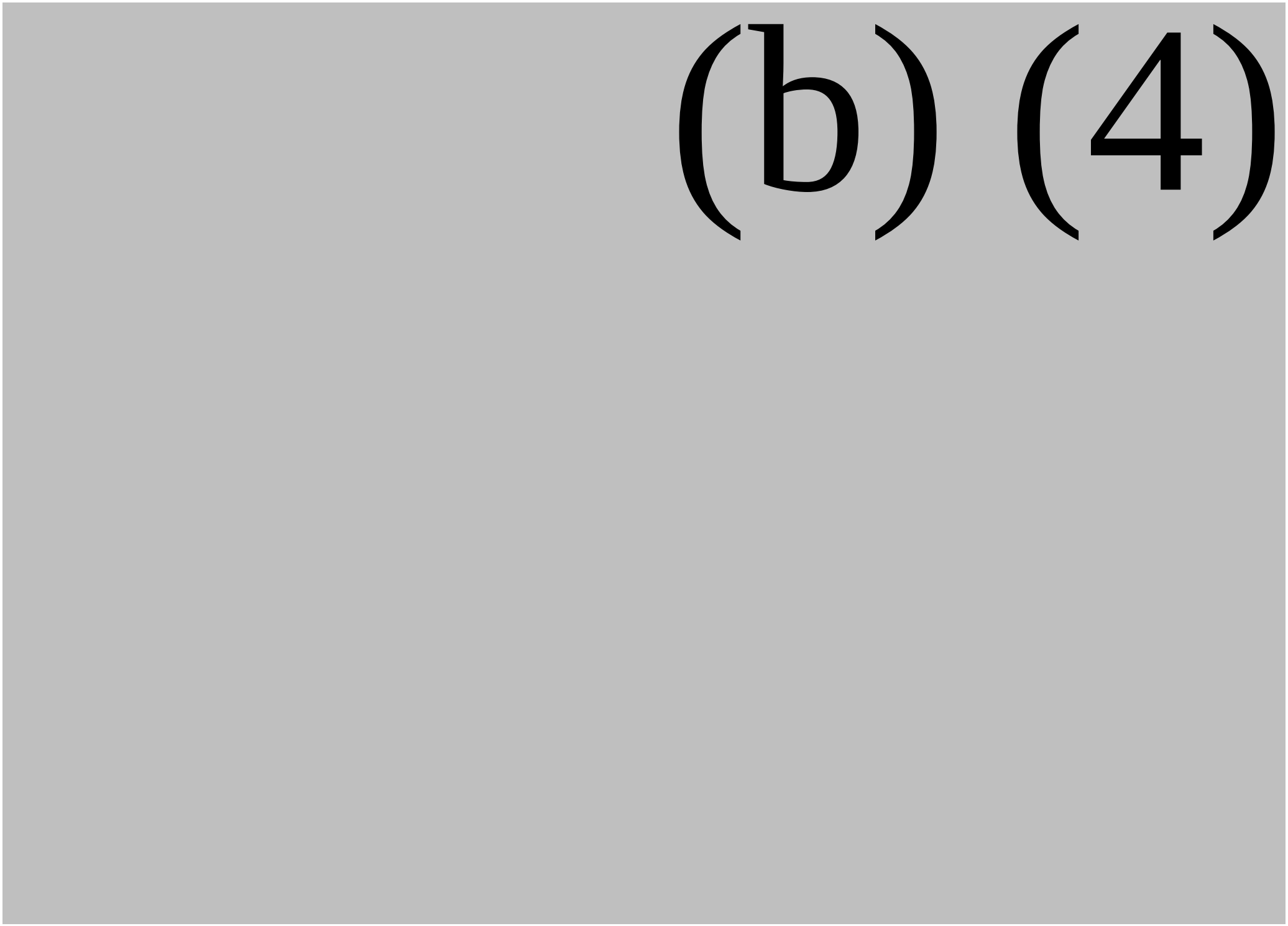
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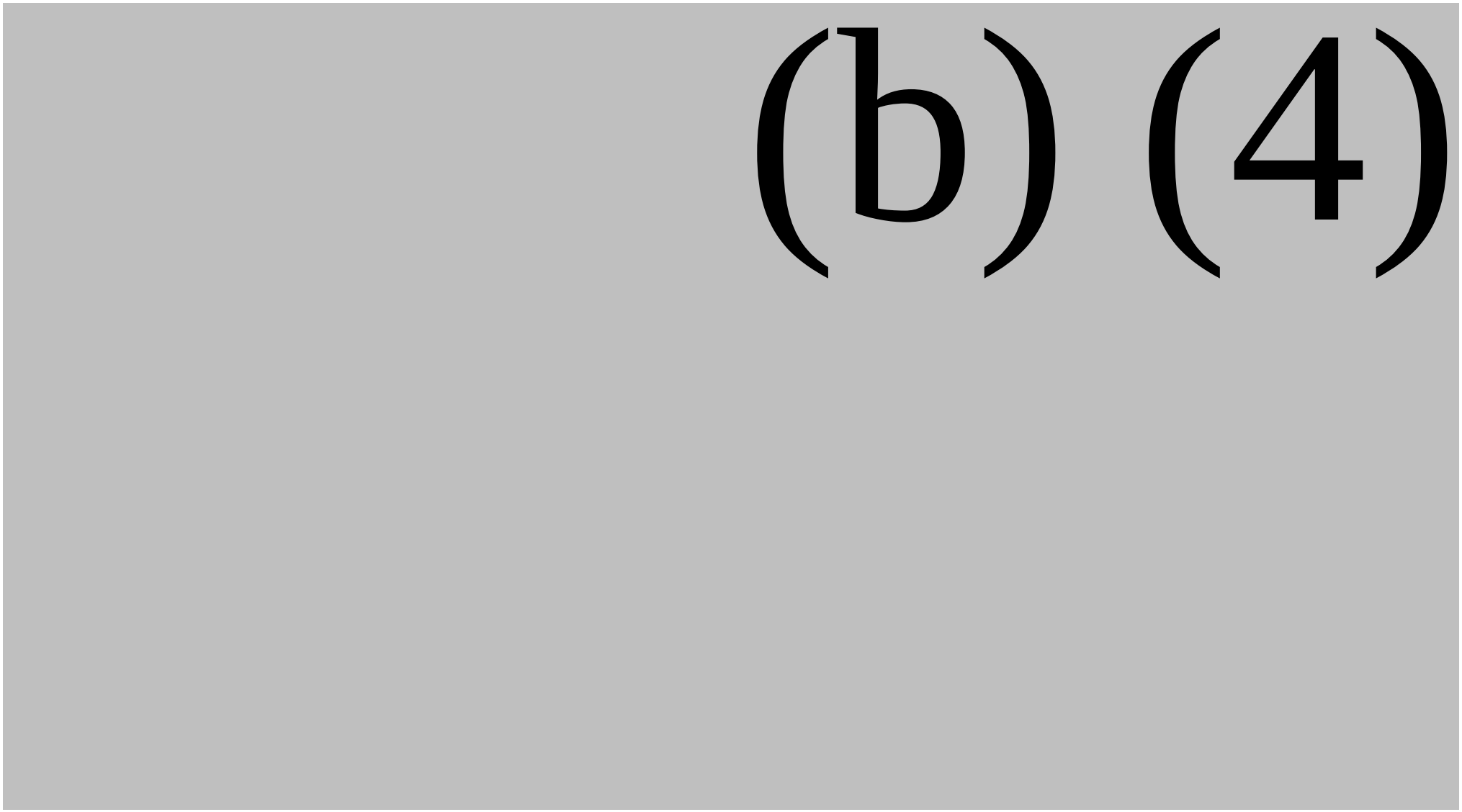
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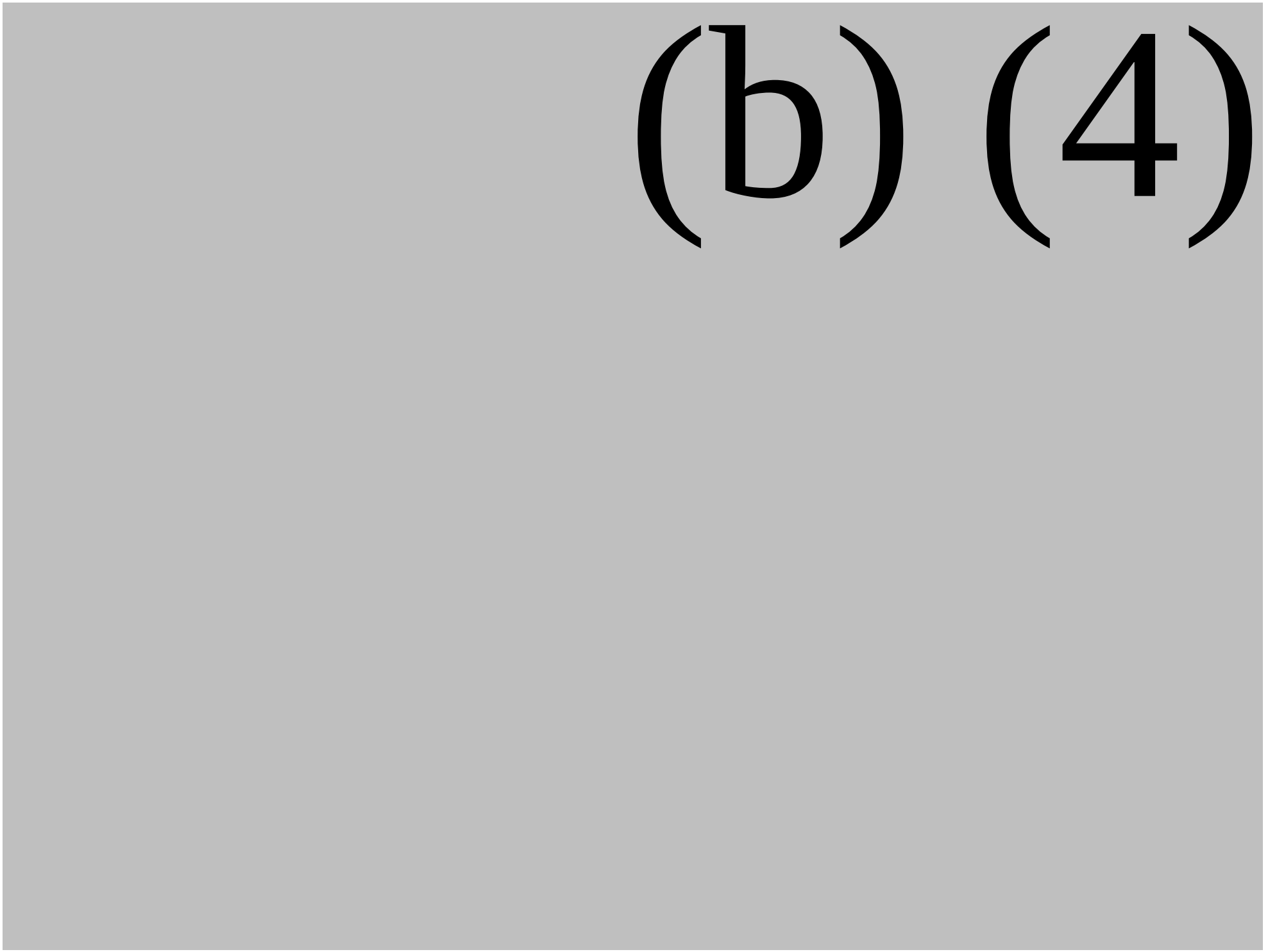
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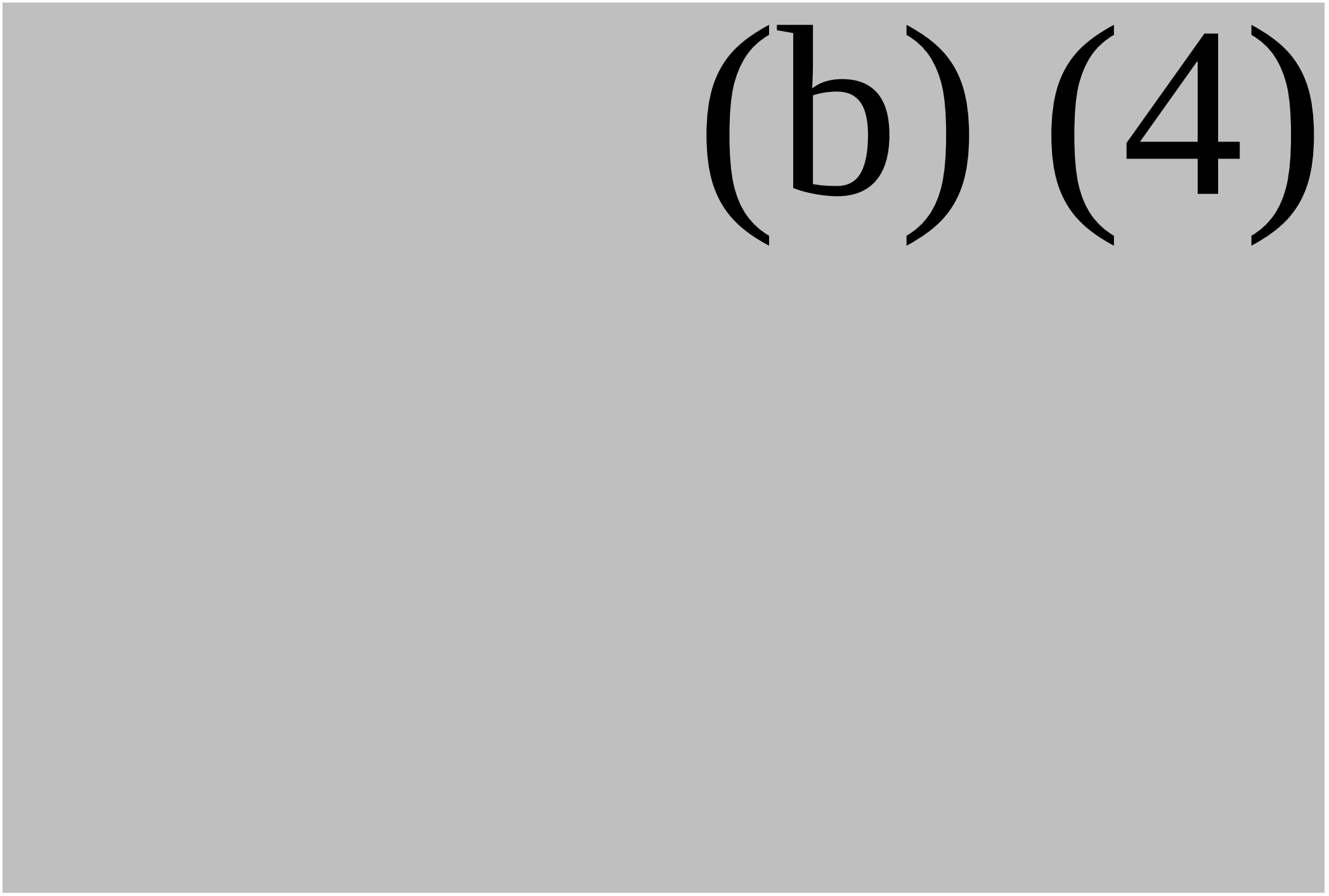
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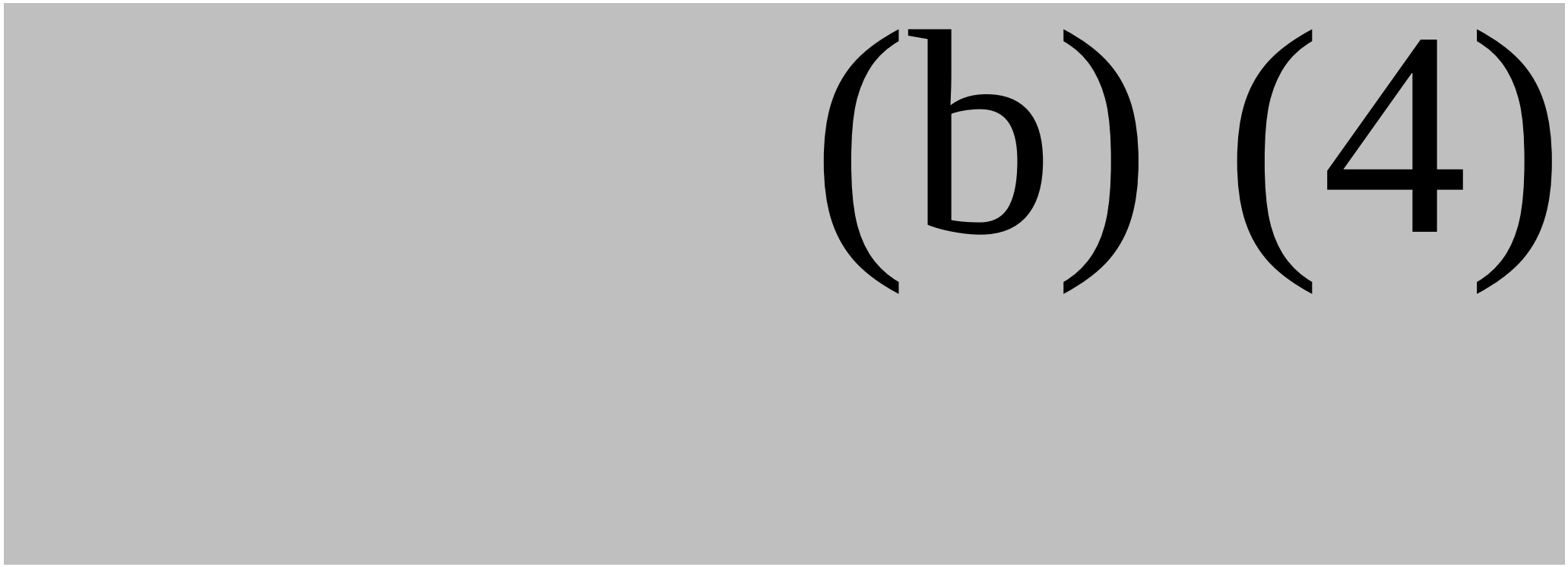
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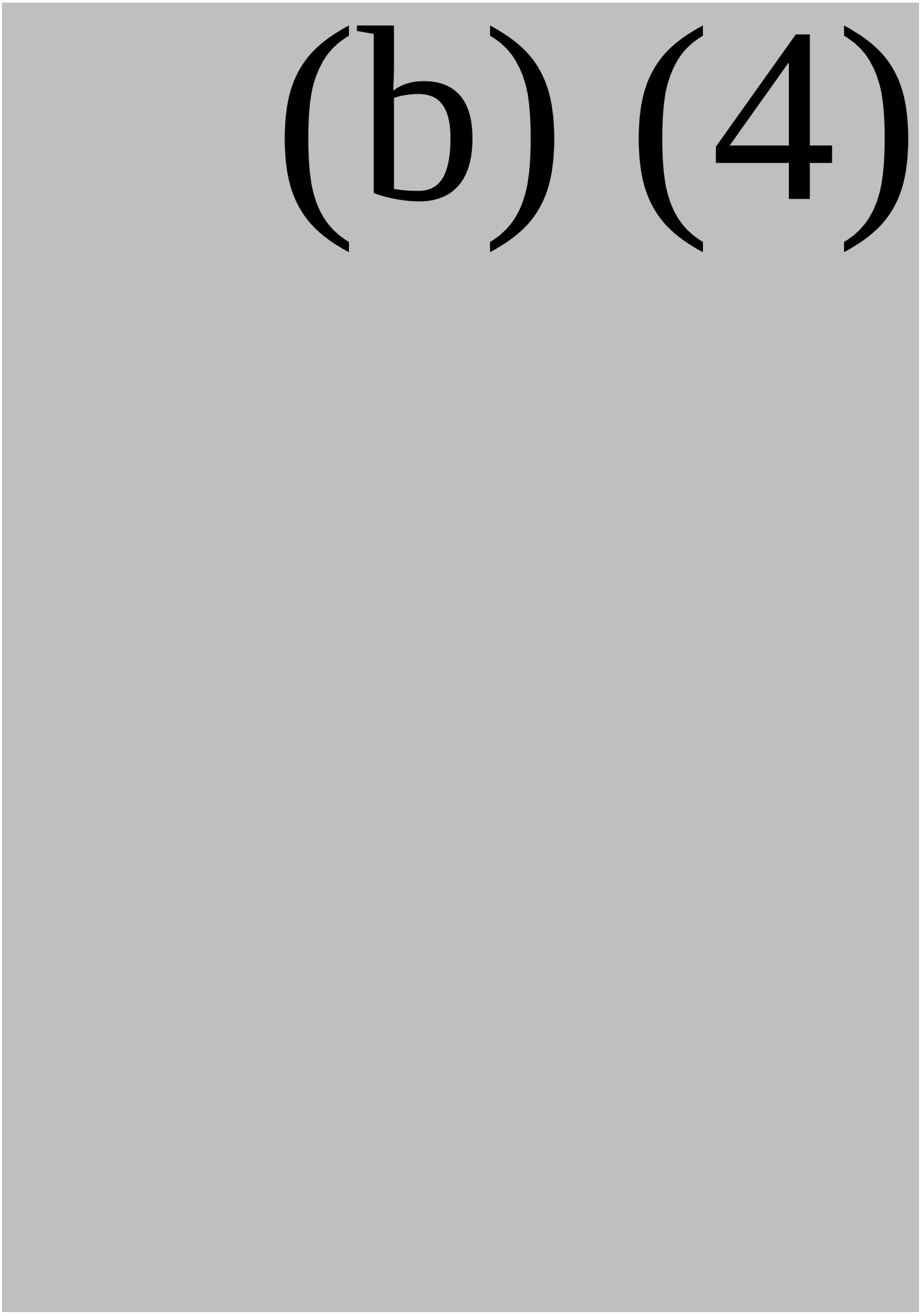
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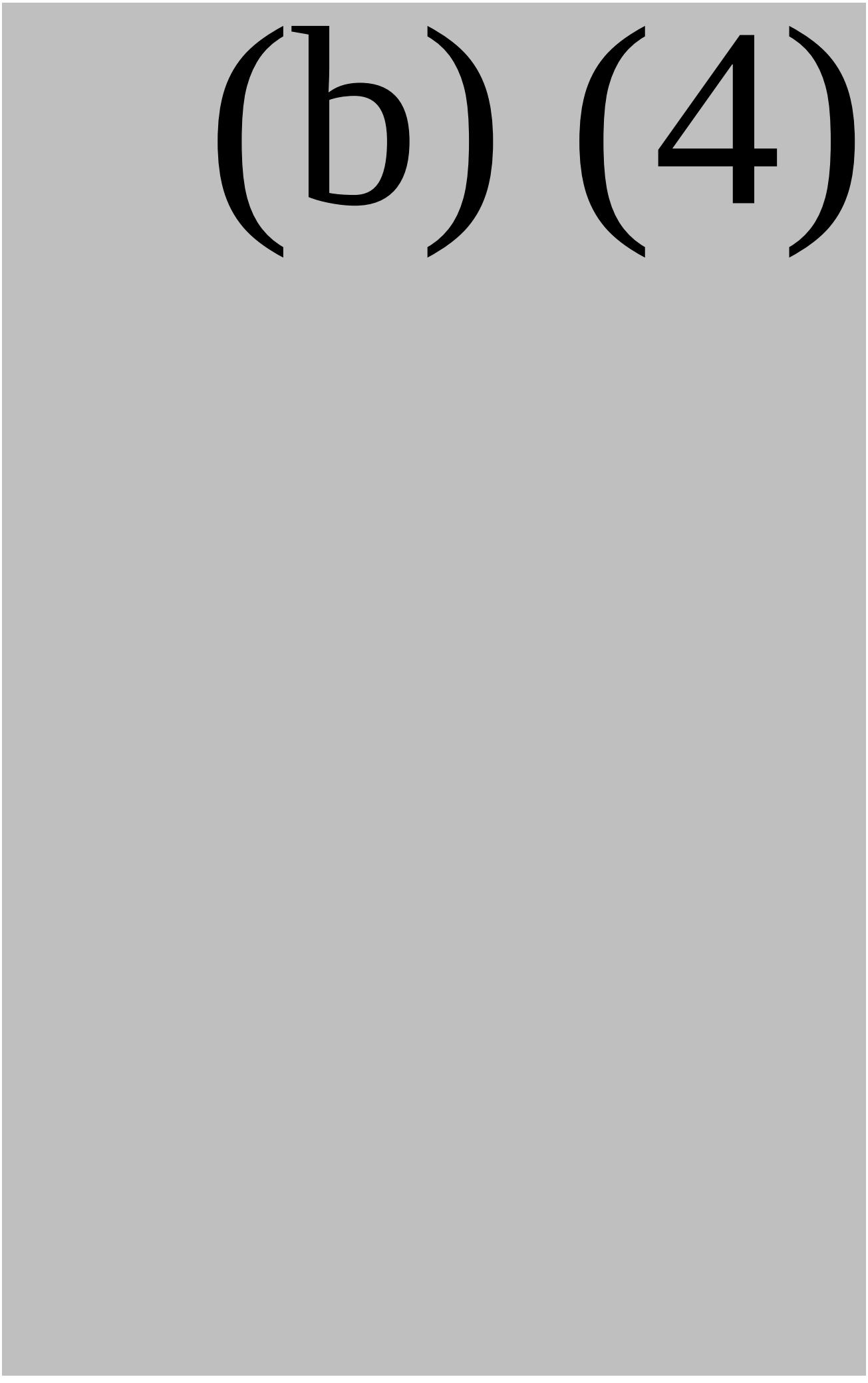
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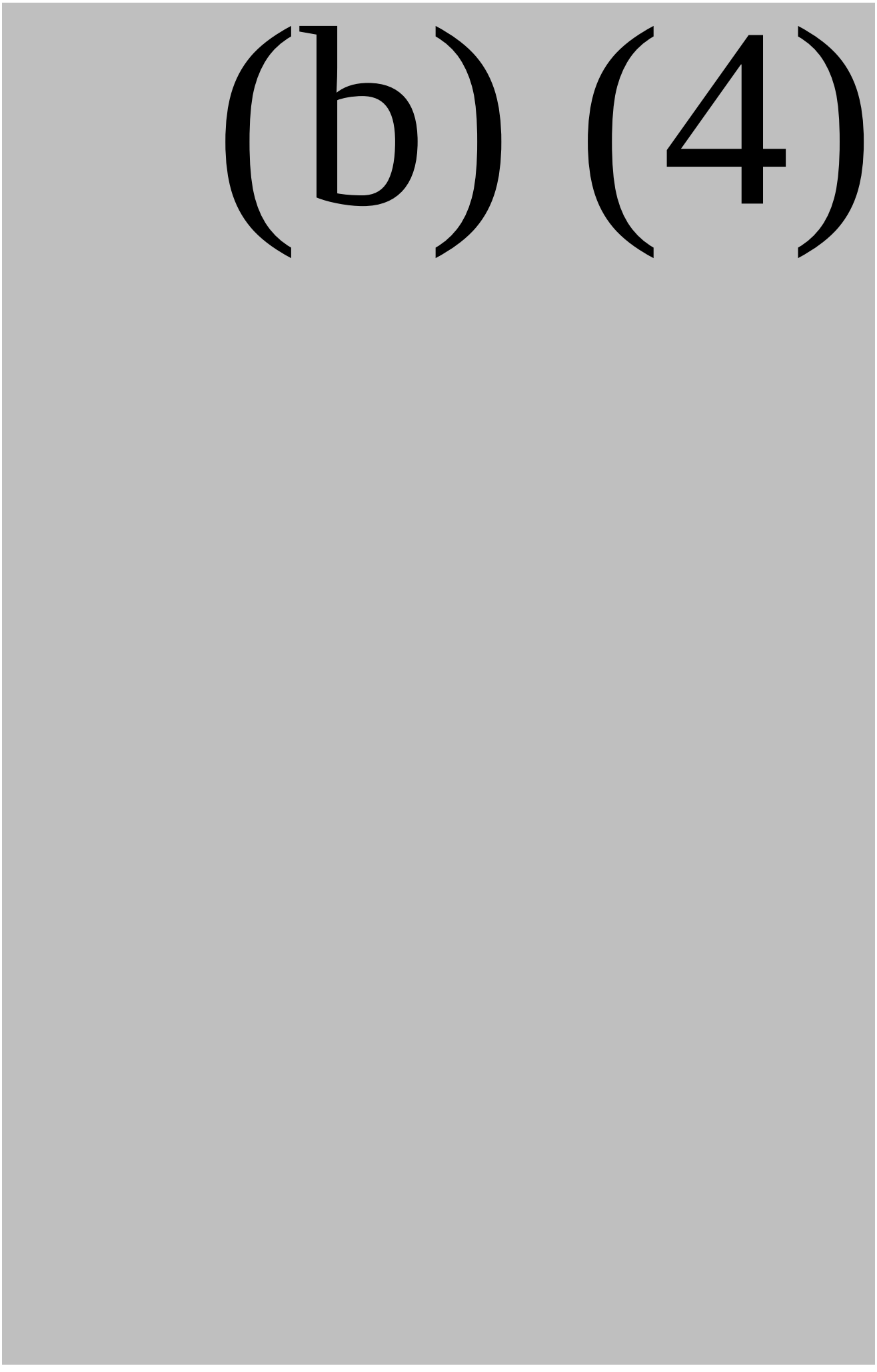
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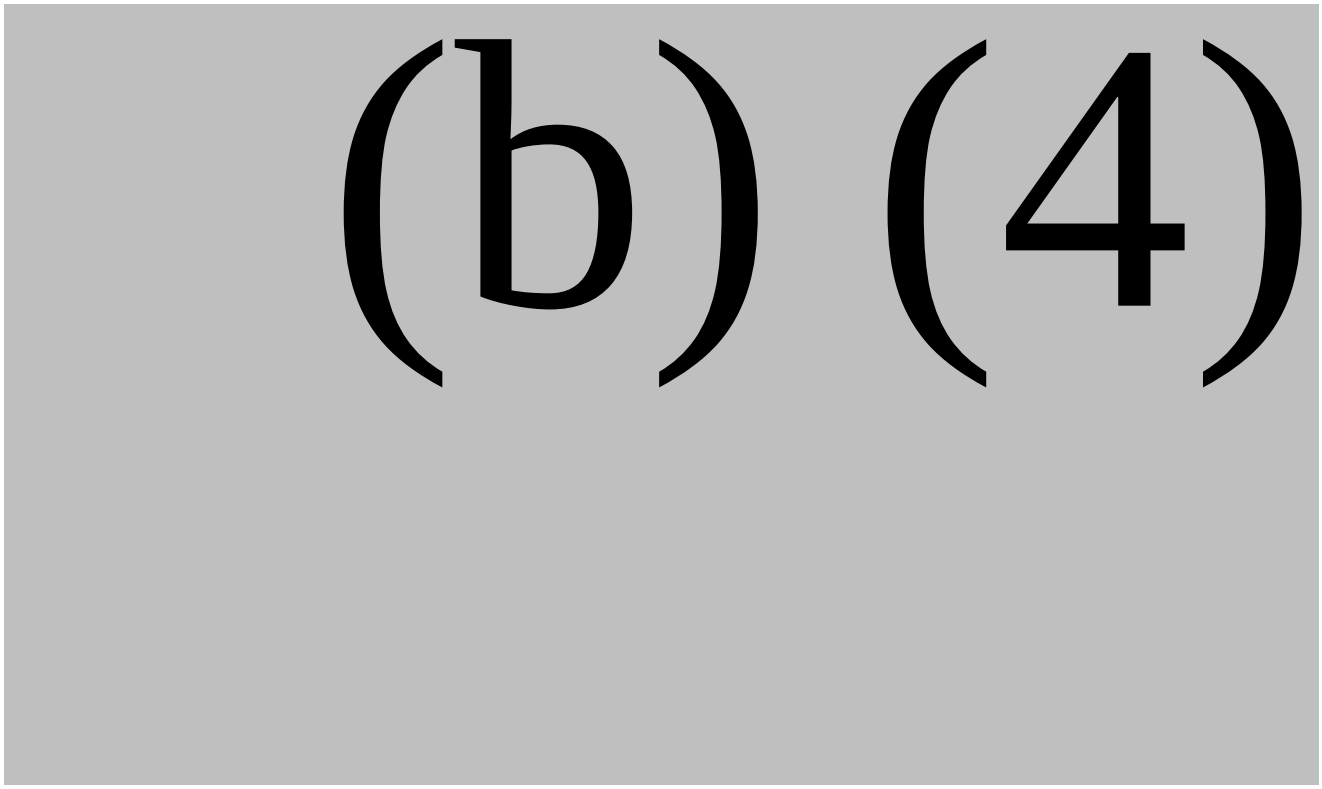
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APPENDIX 6

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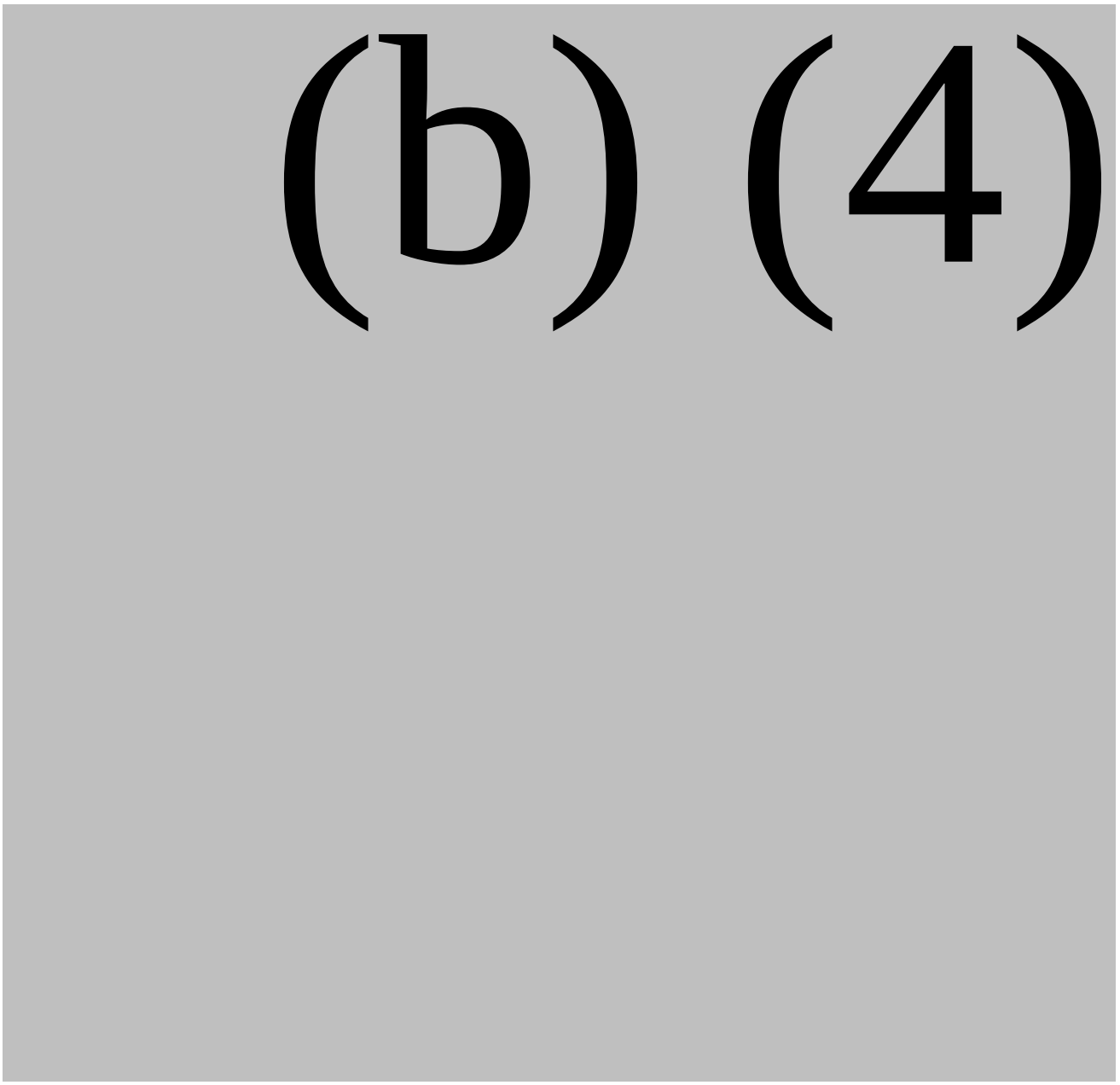

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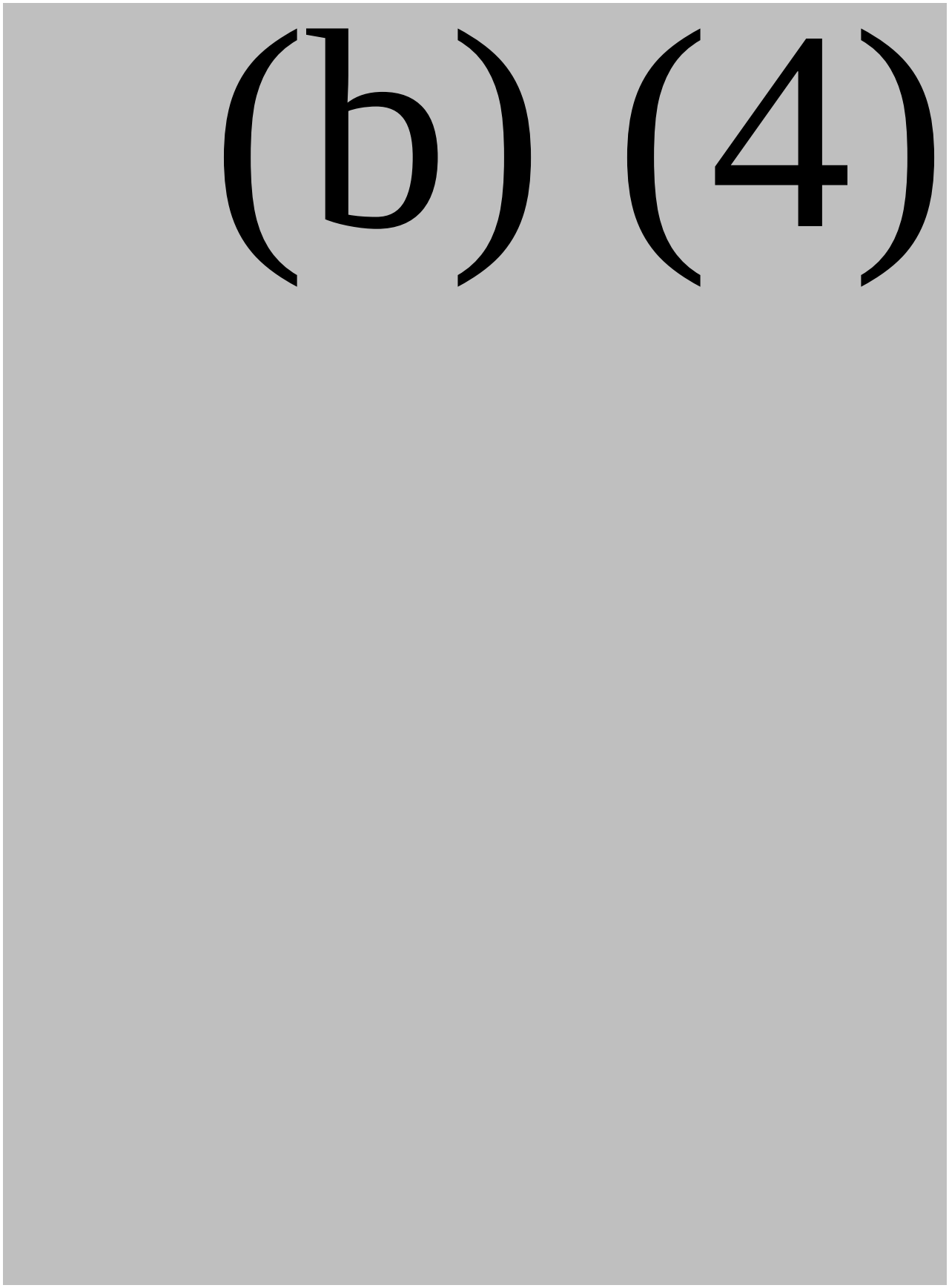

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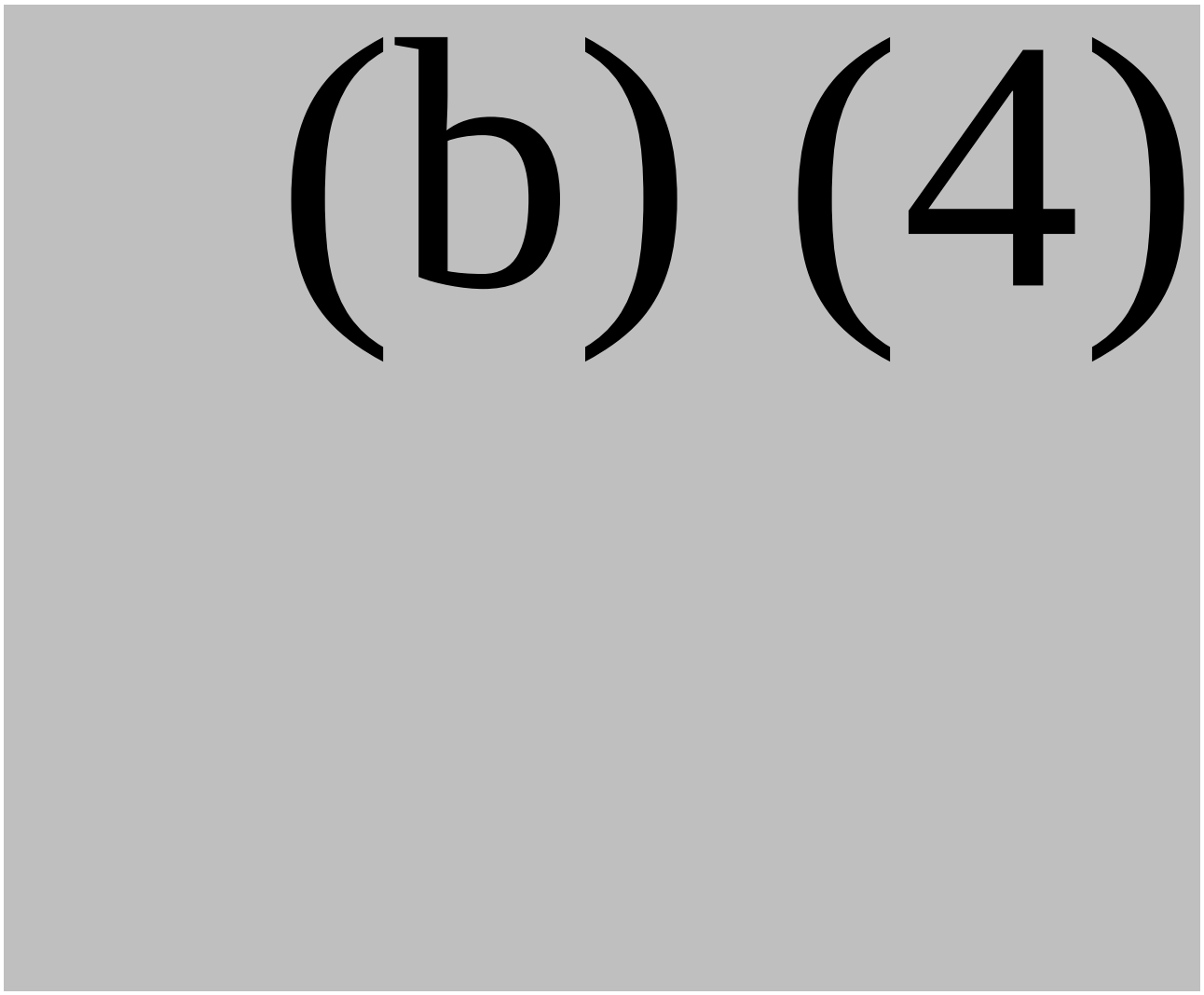

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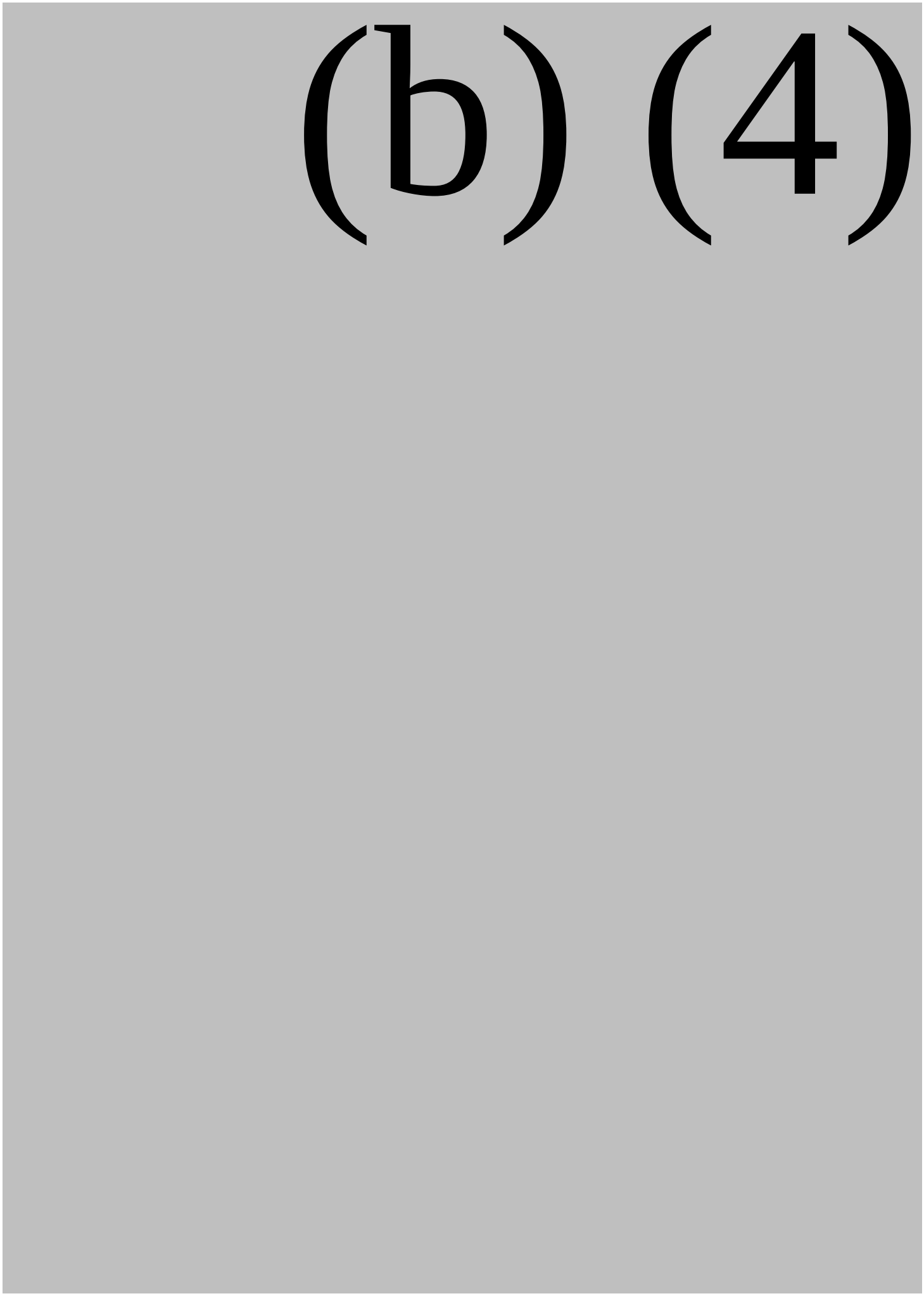
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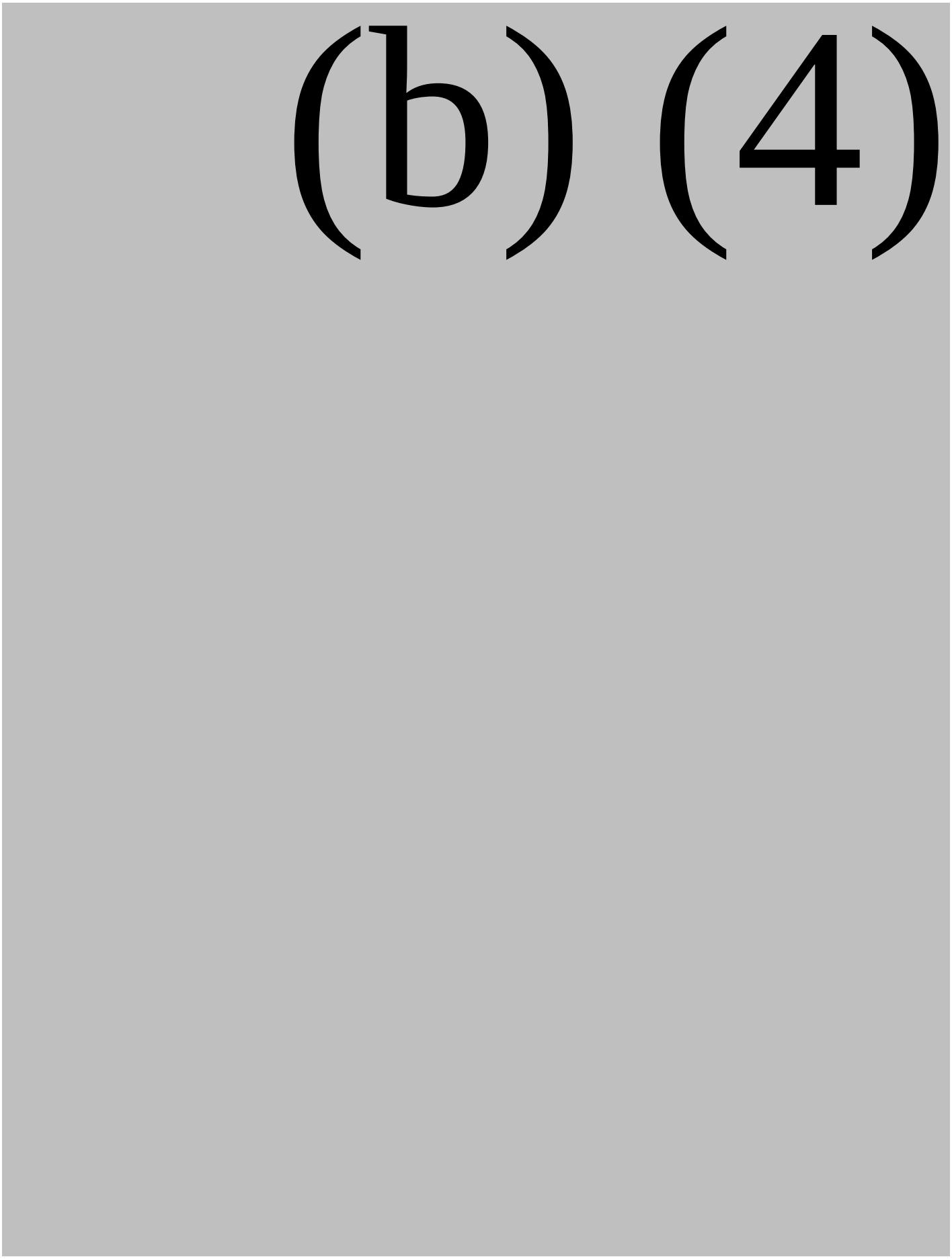
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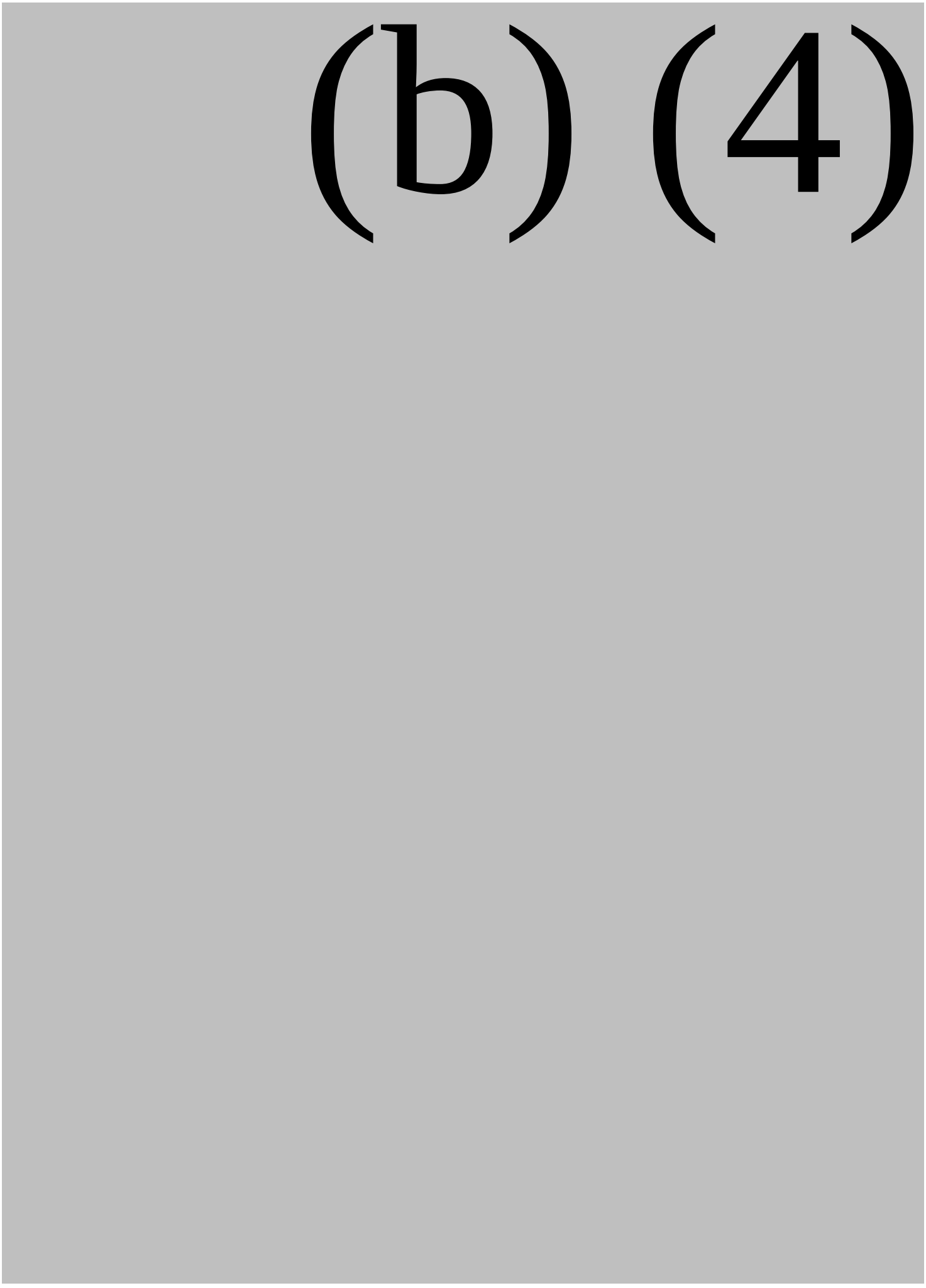
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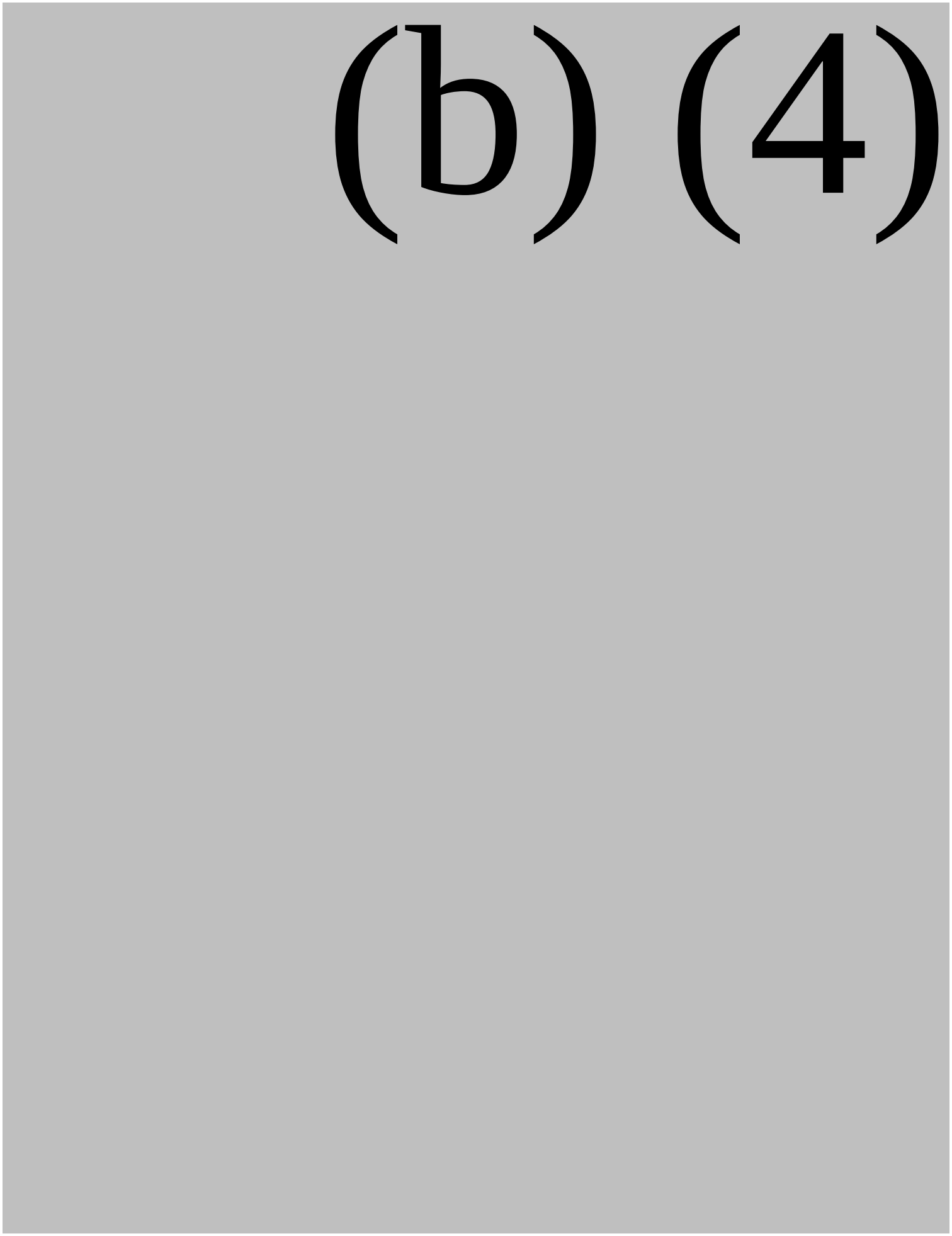
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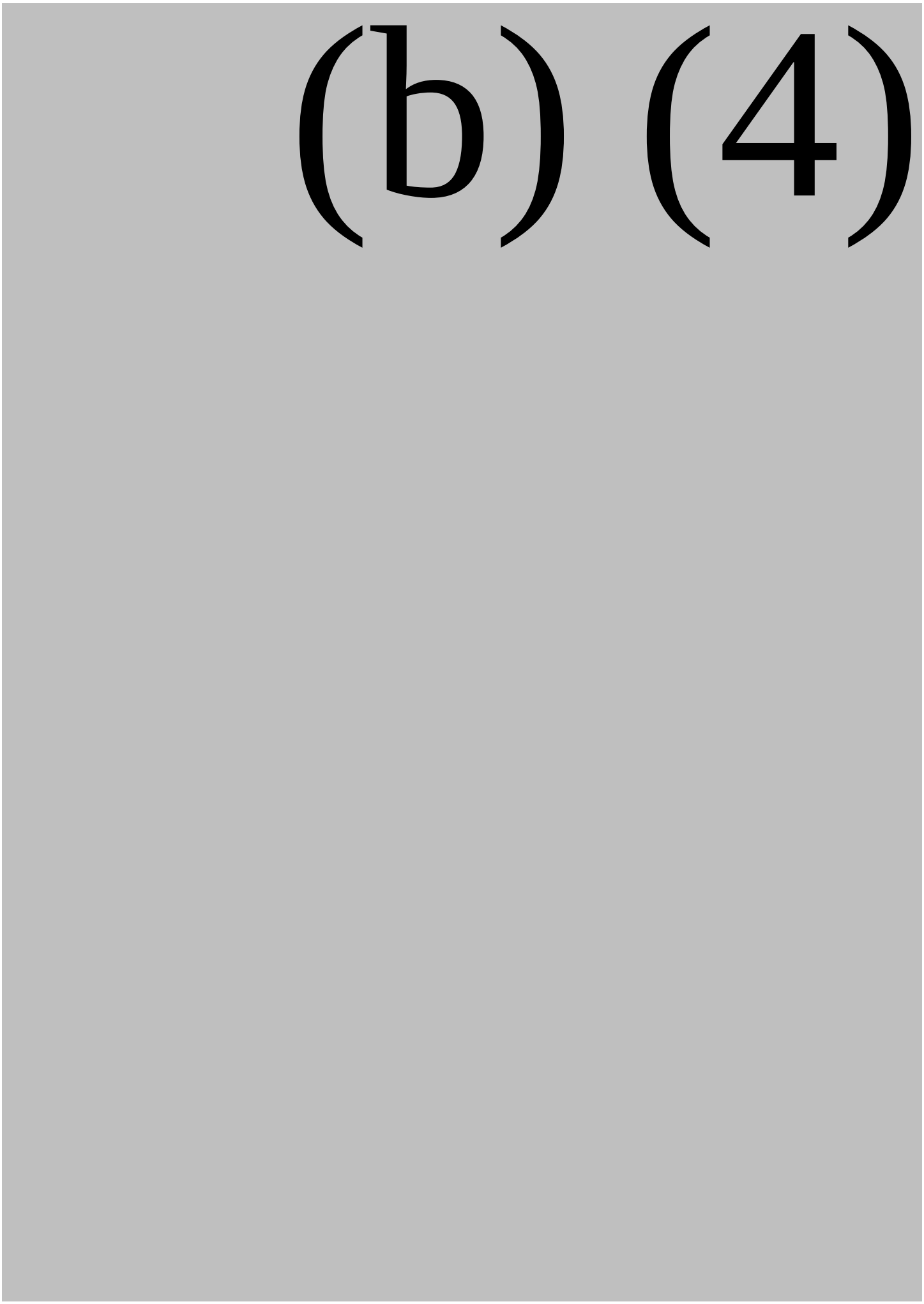
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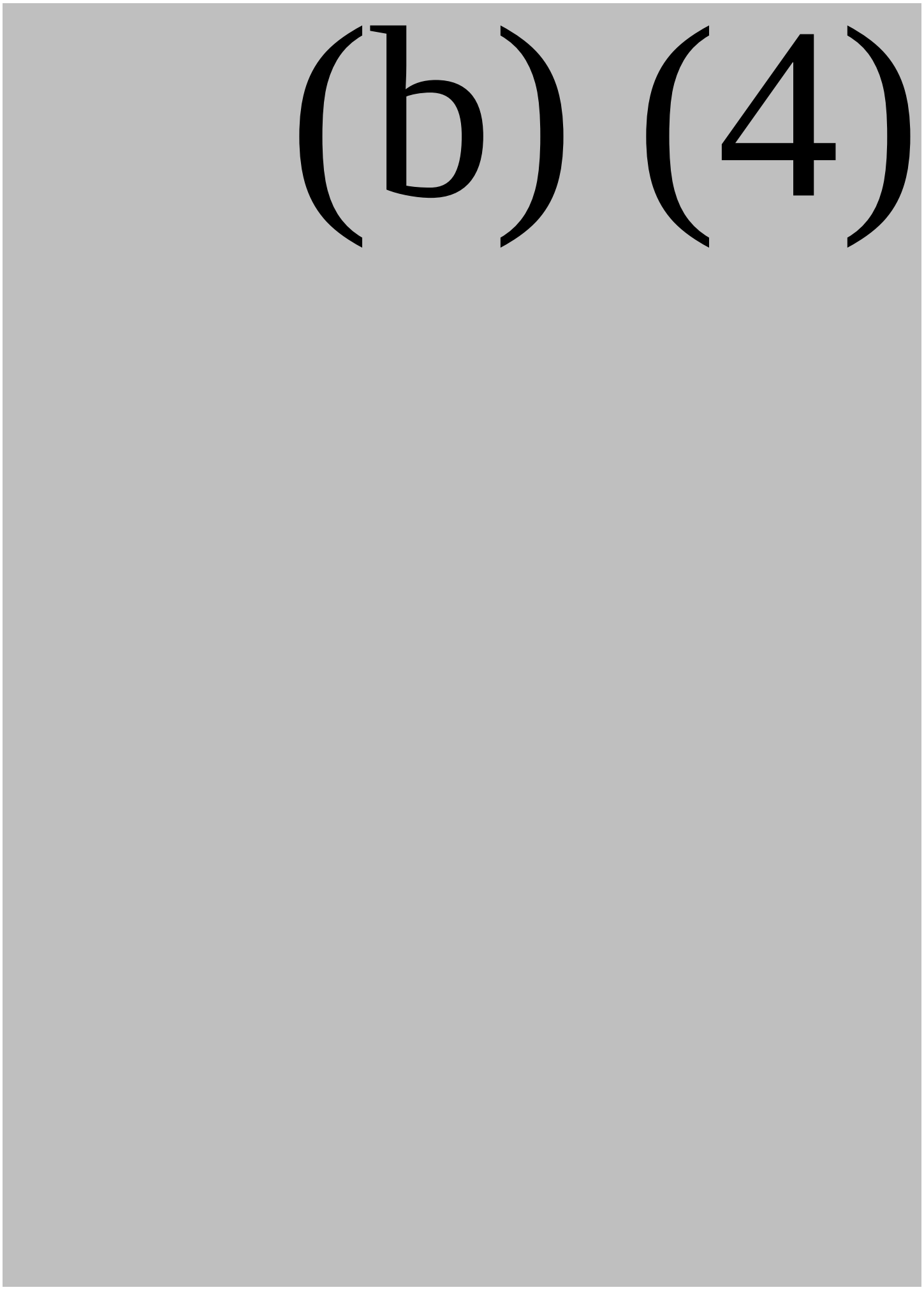
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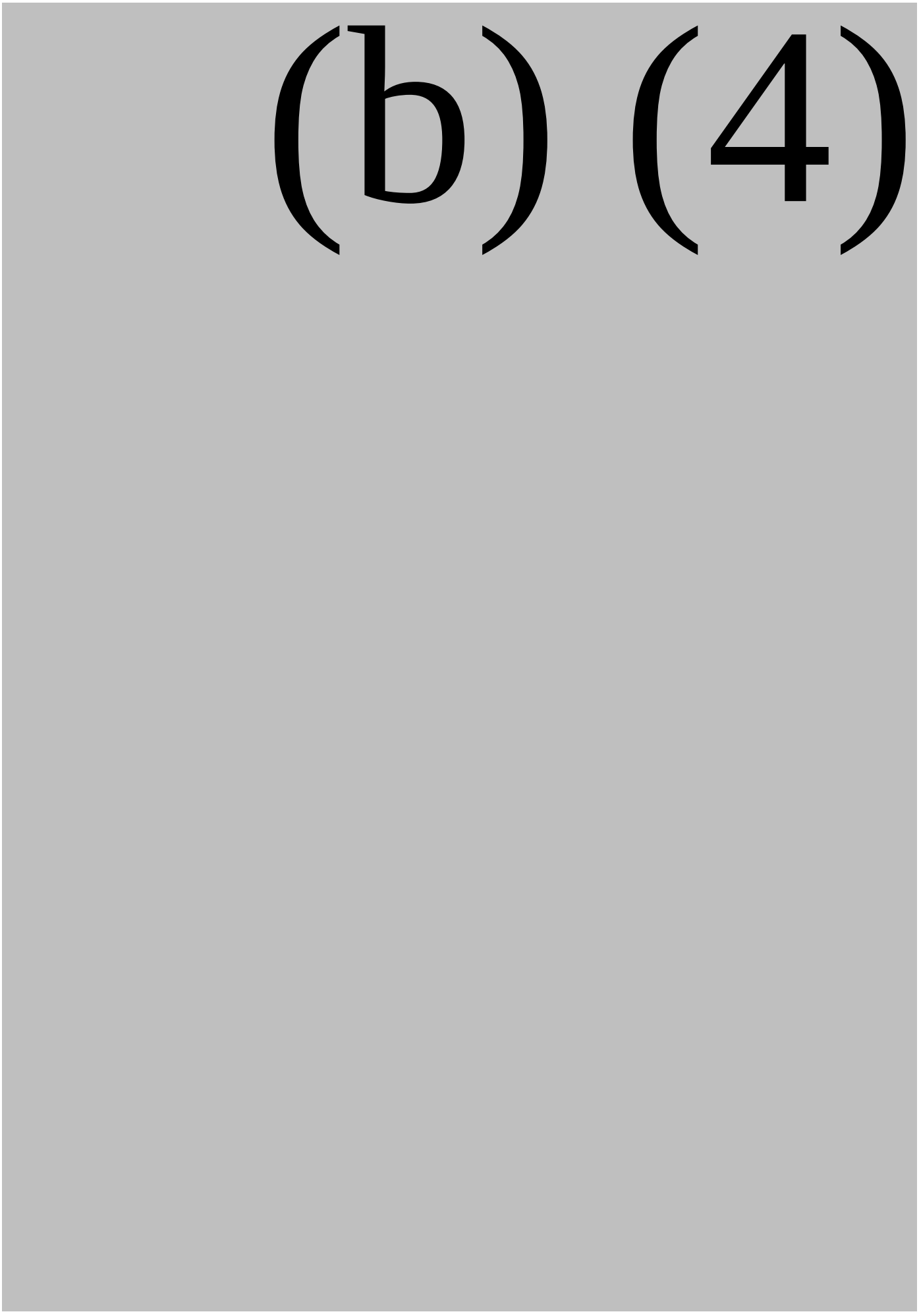
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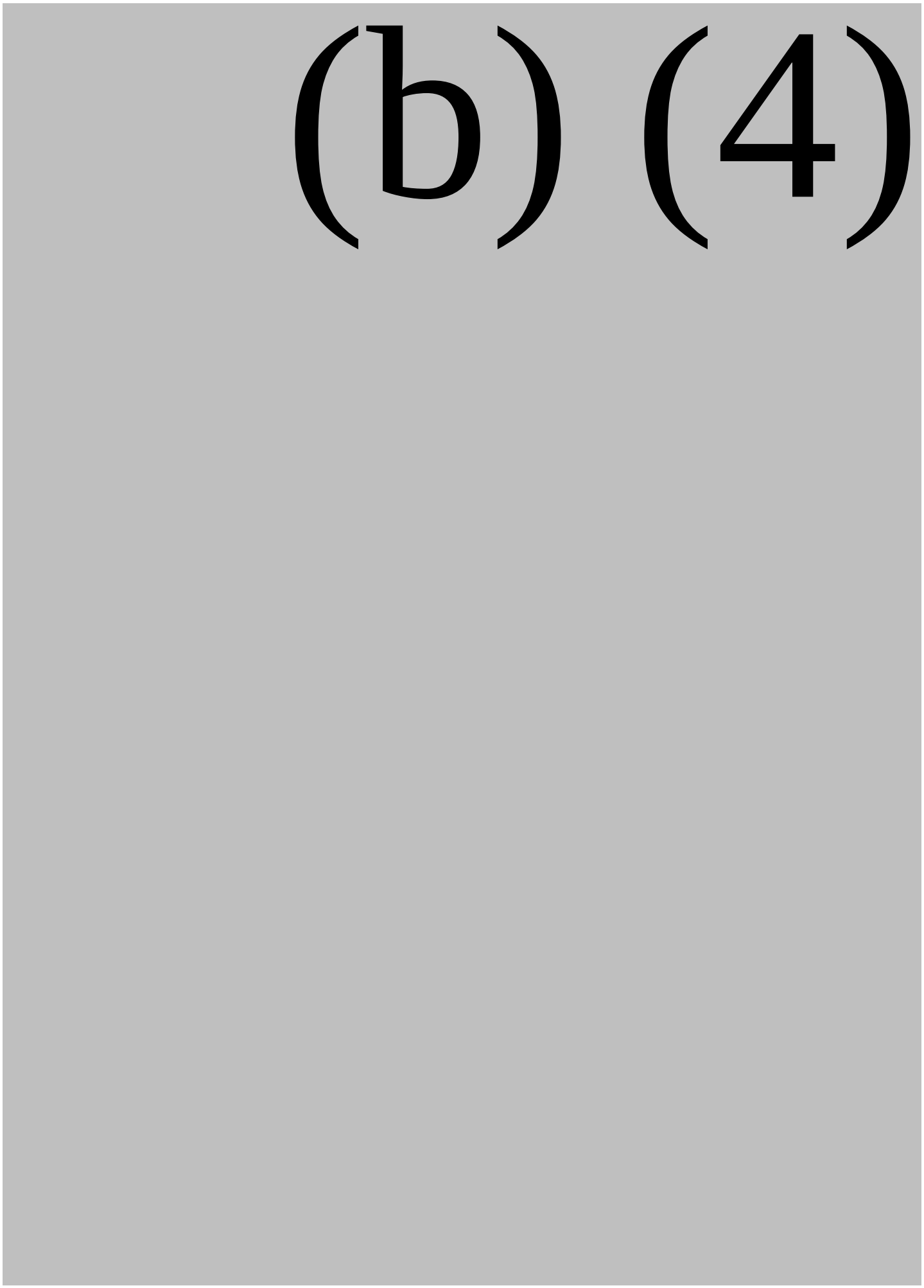
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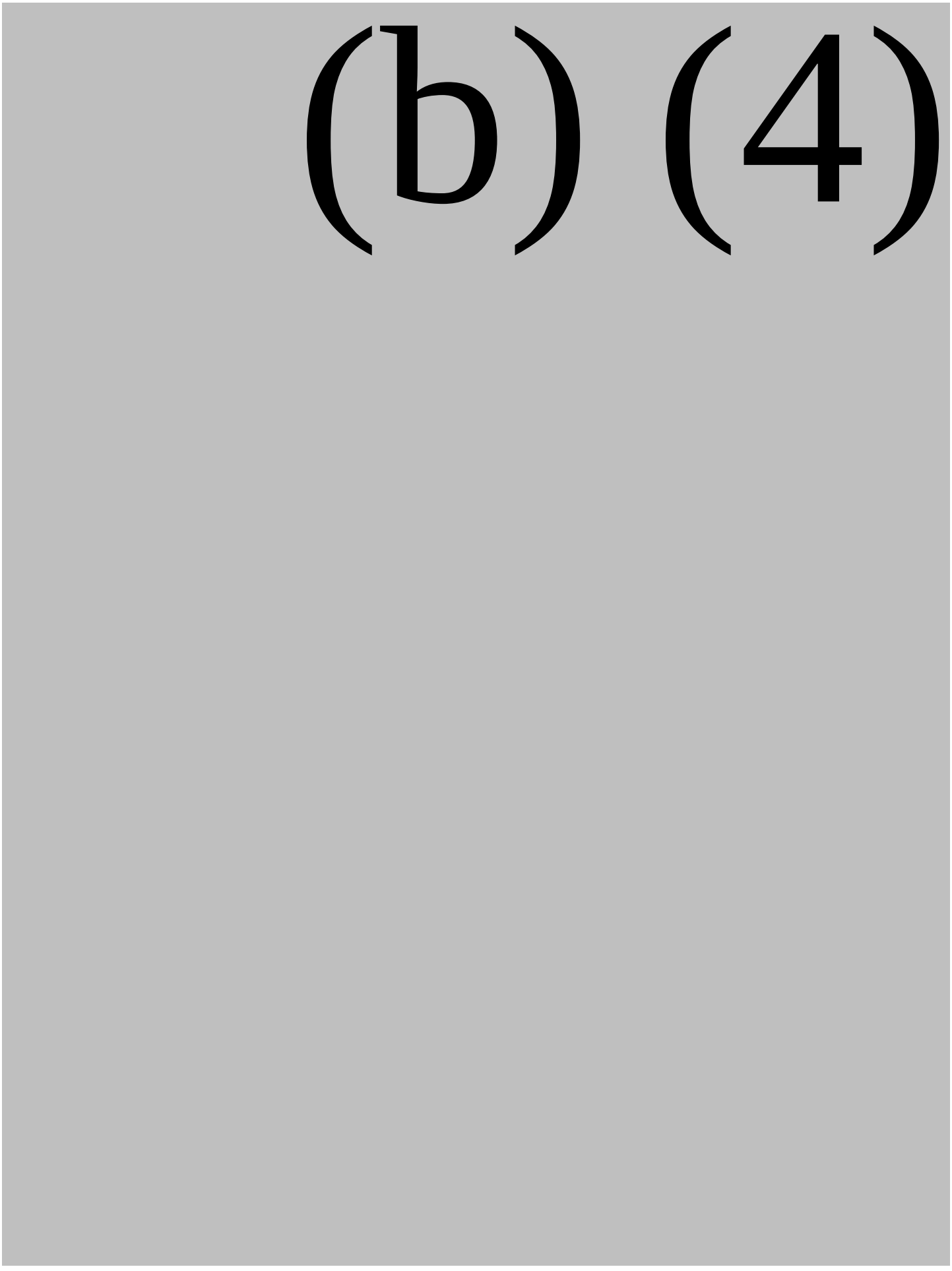
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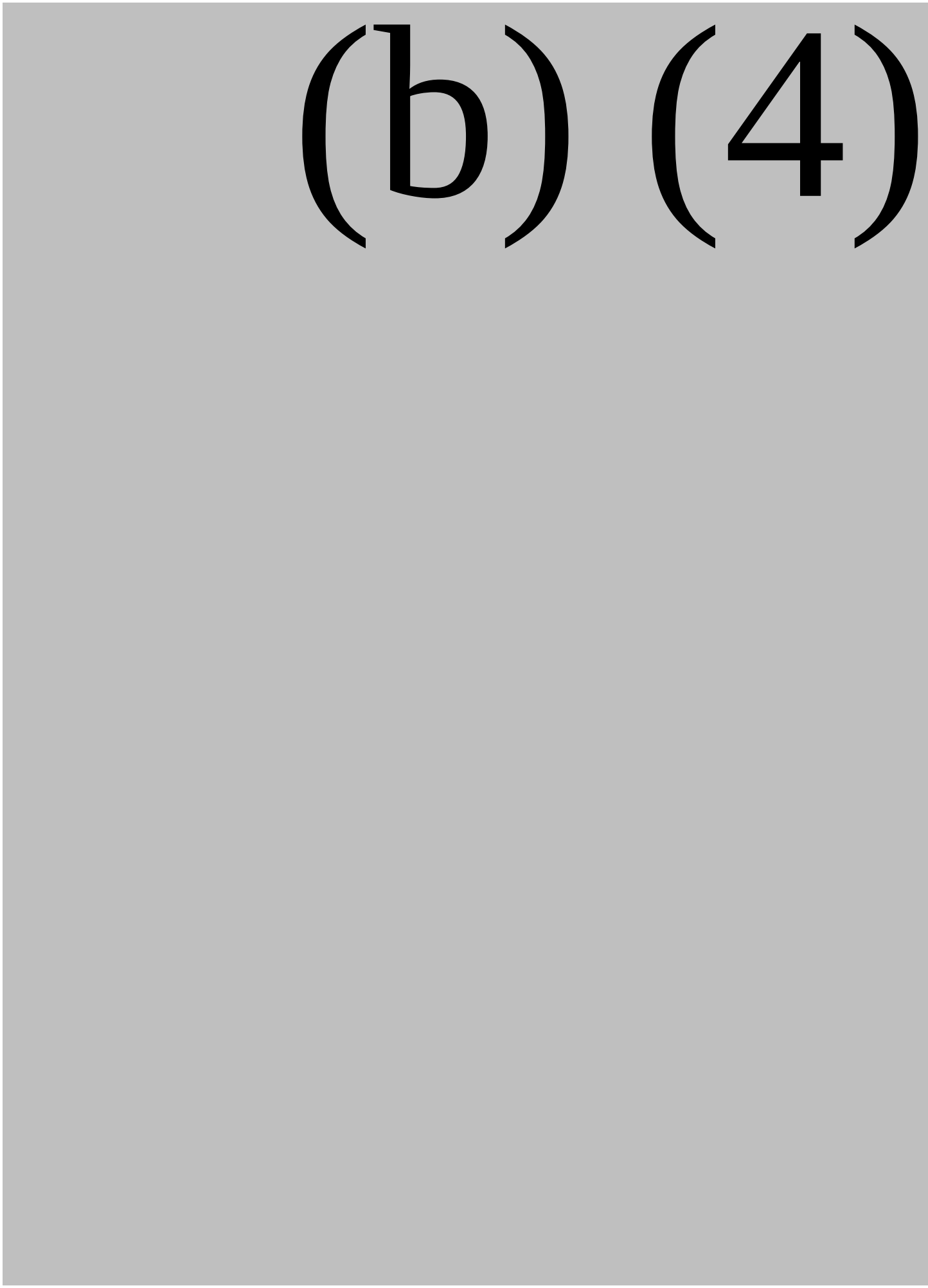
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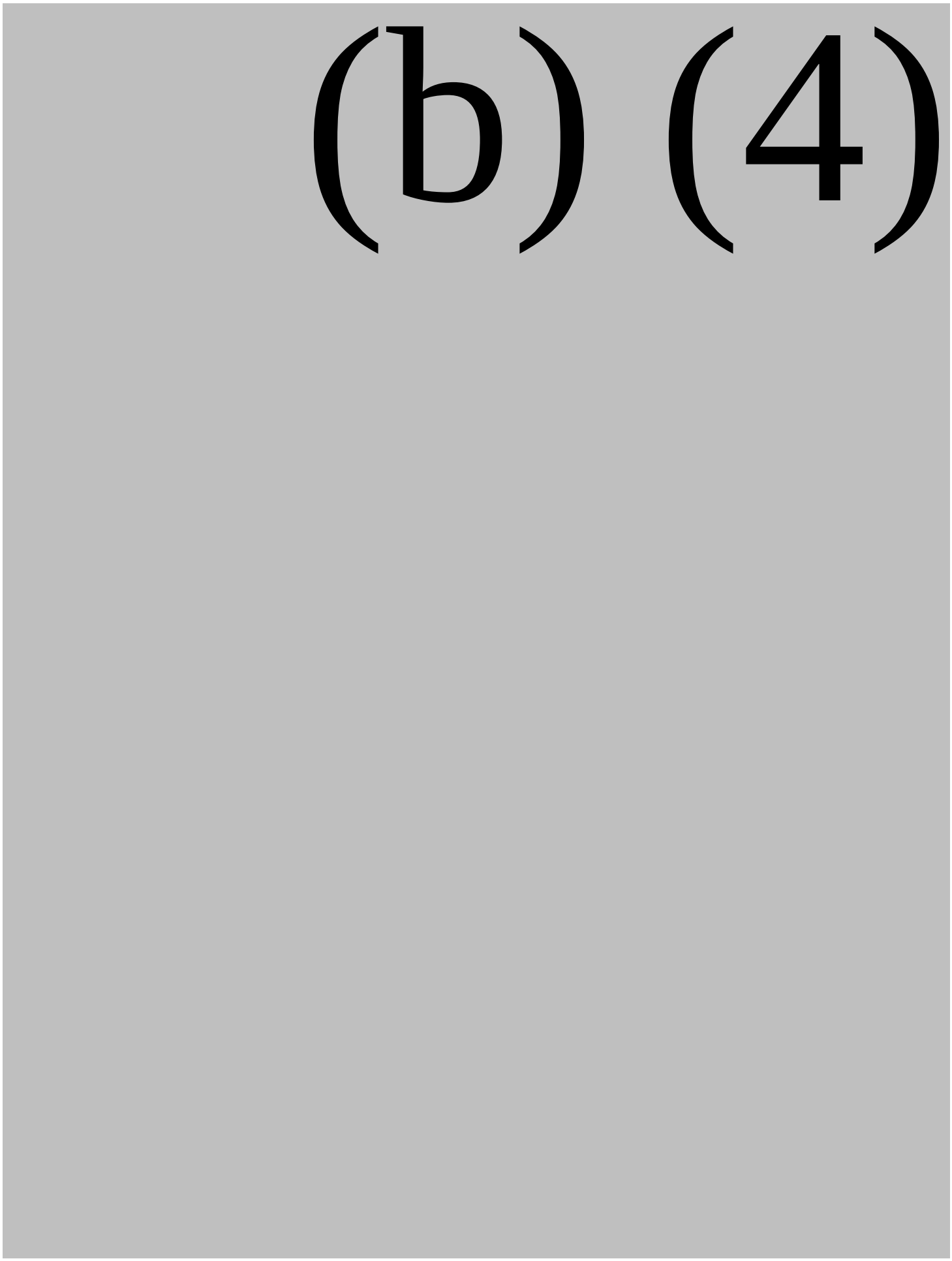
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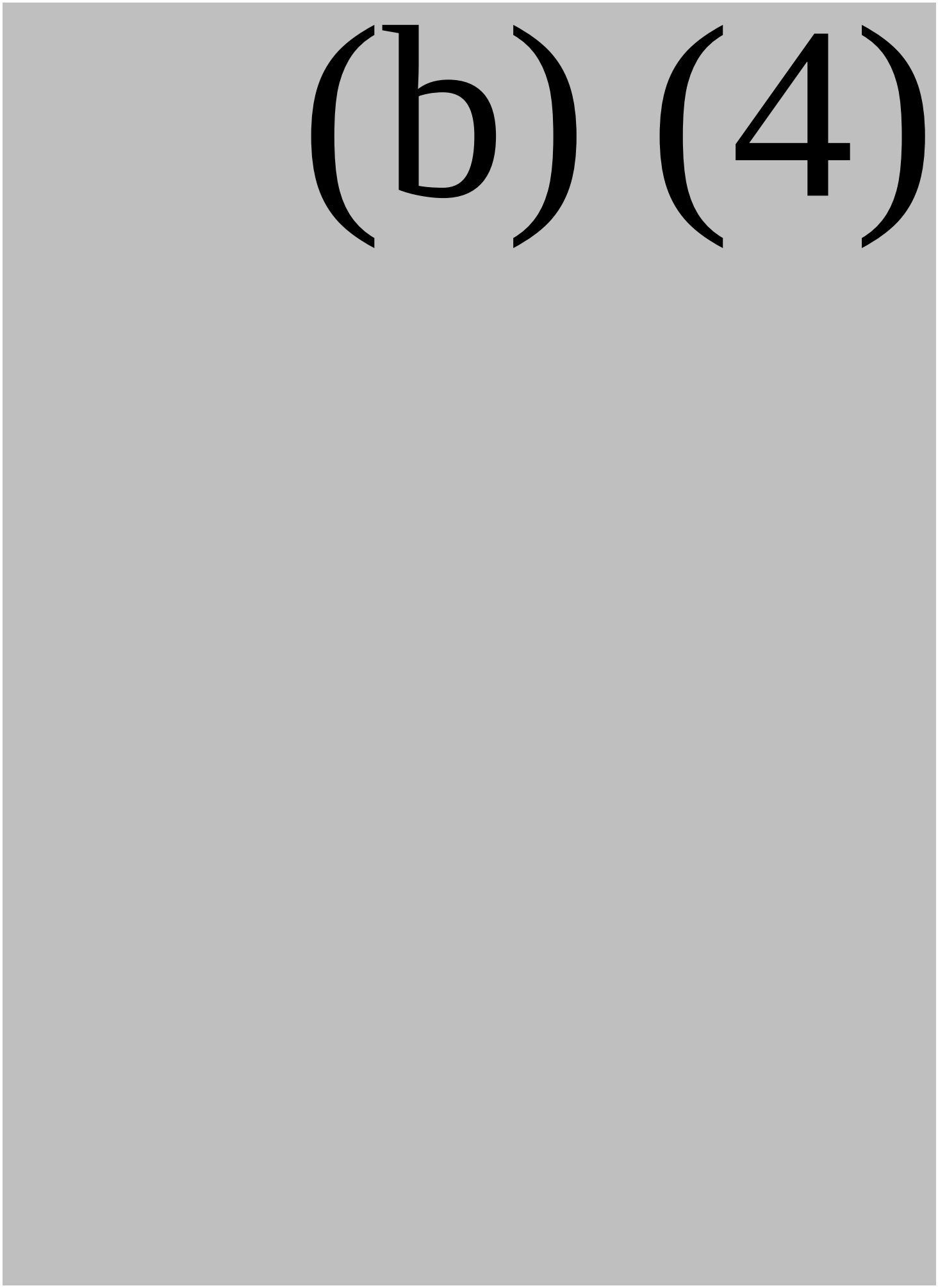
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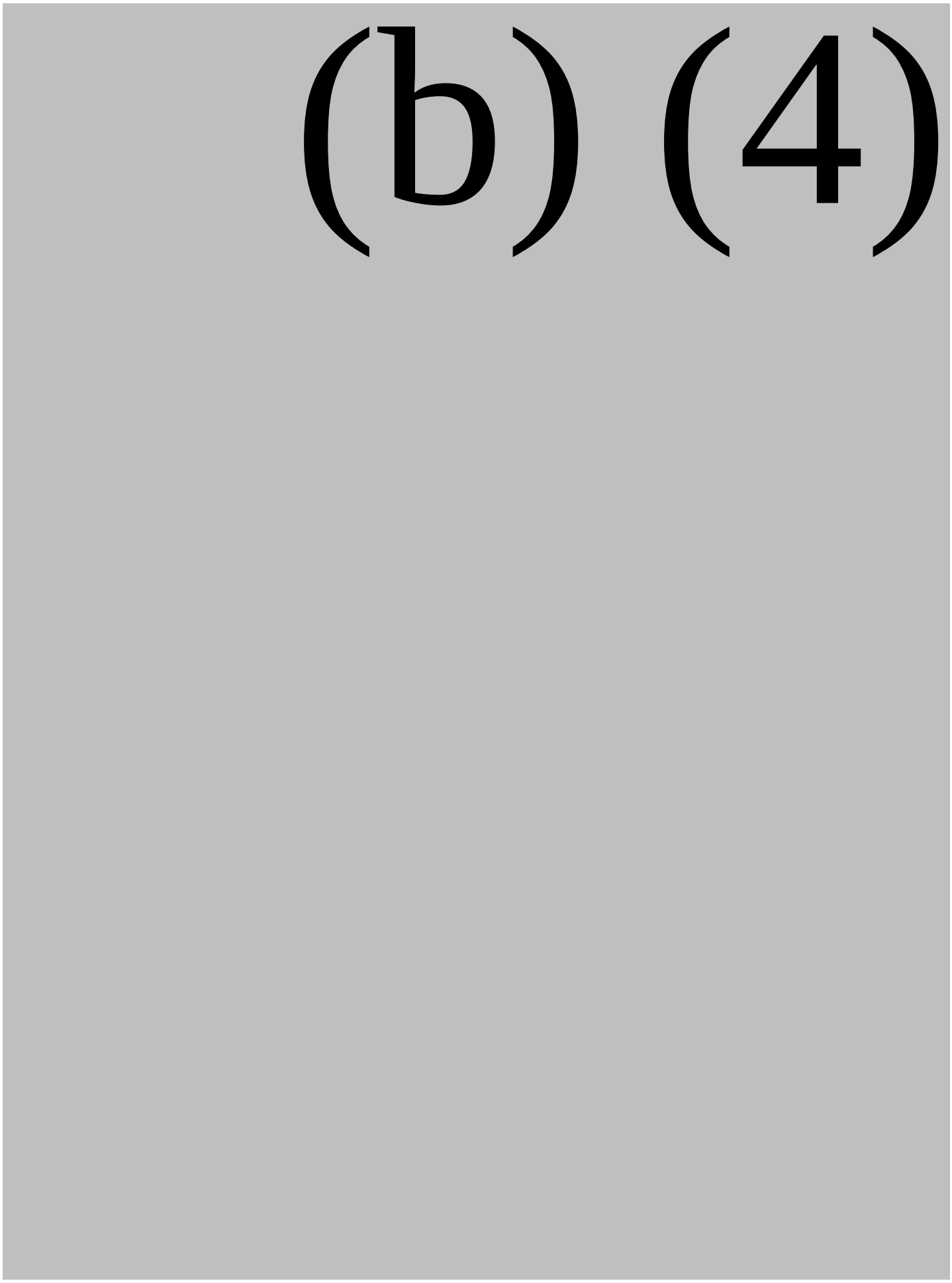
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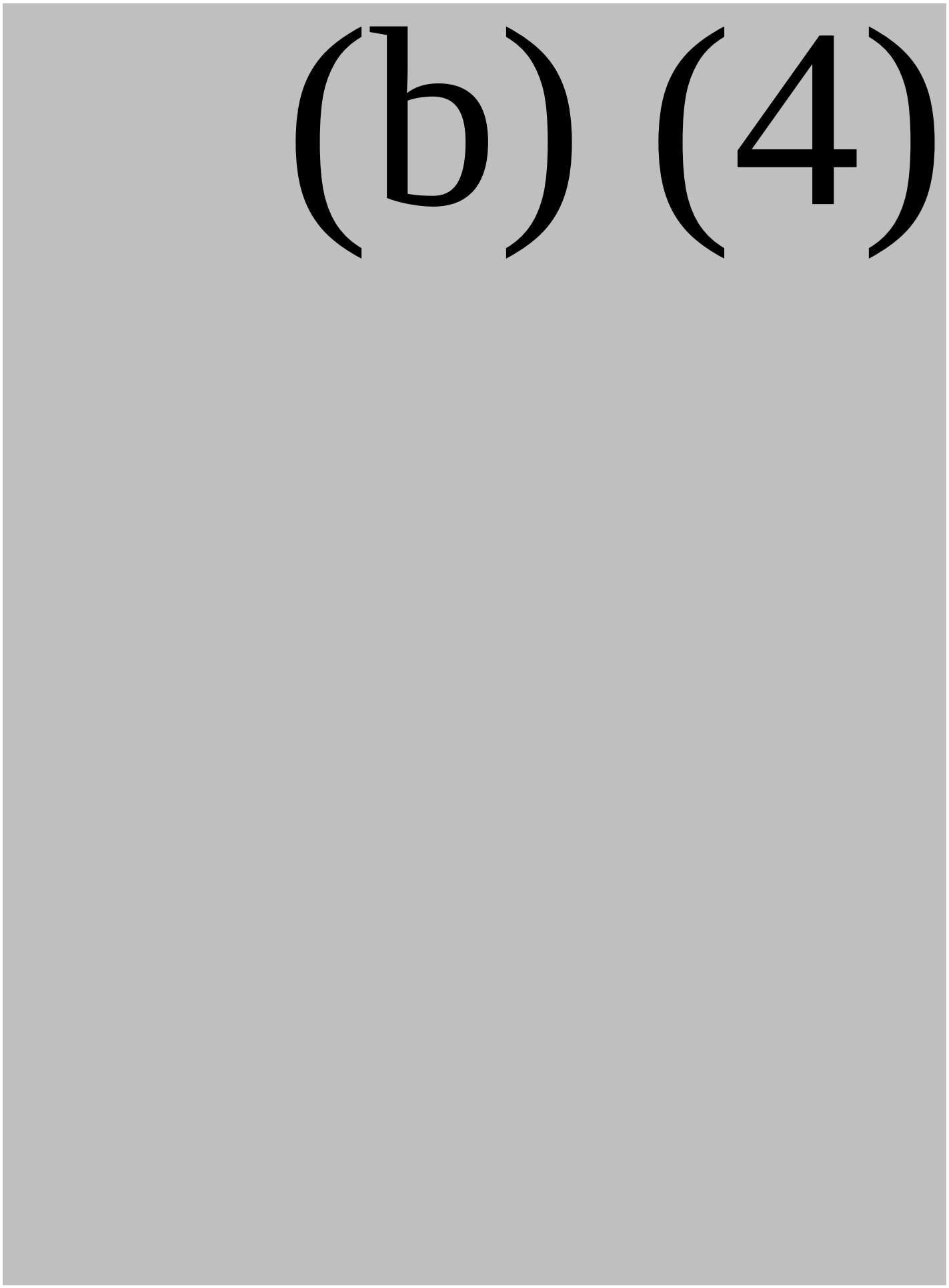
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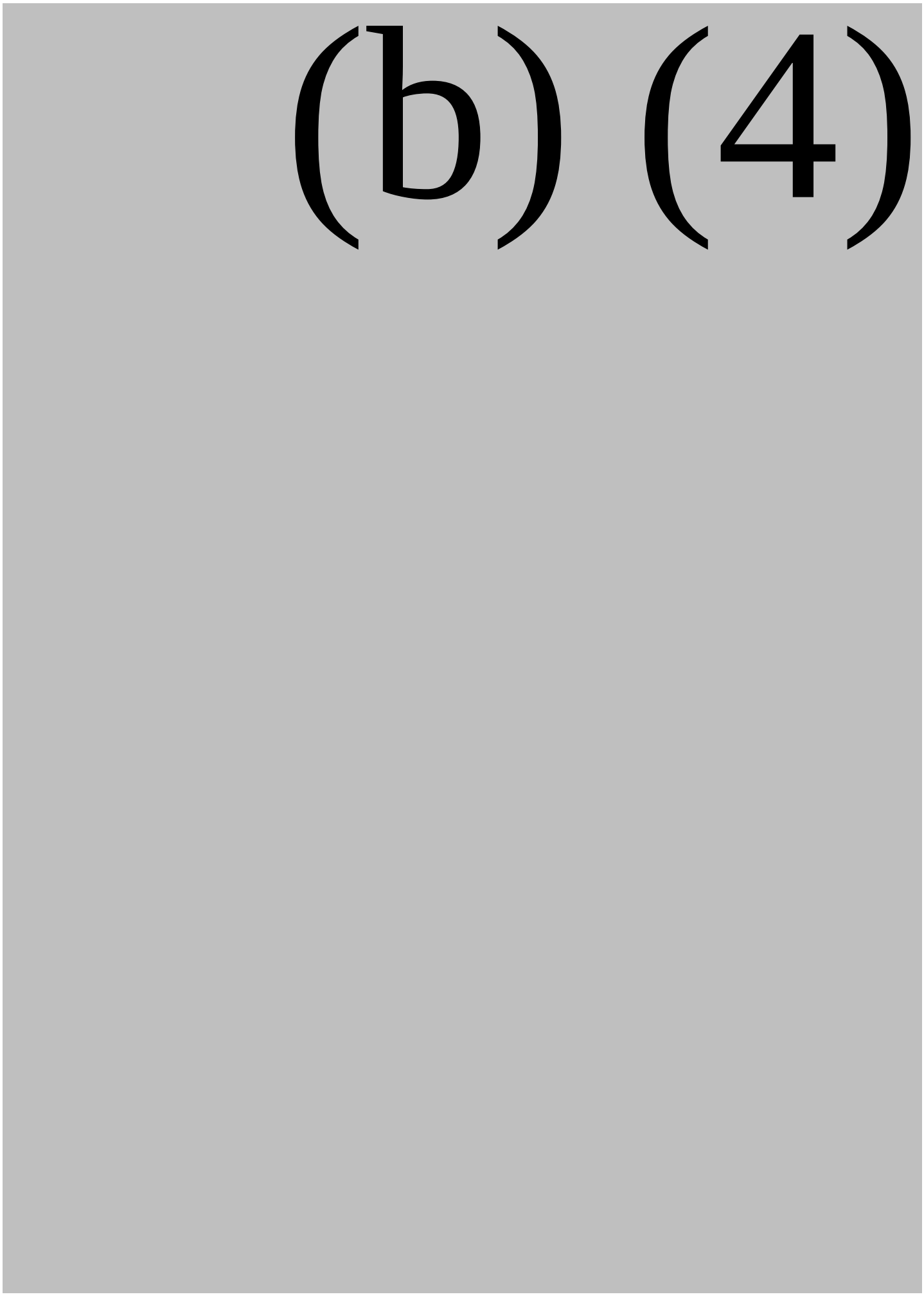
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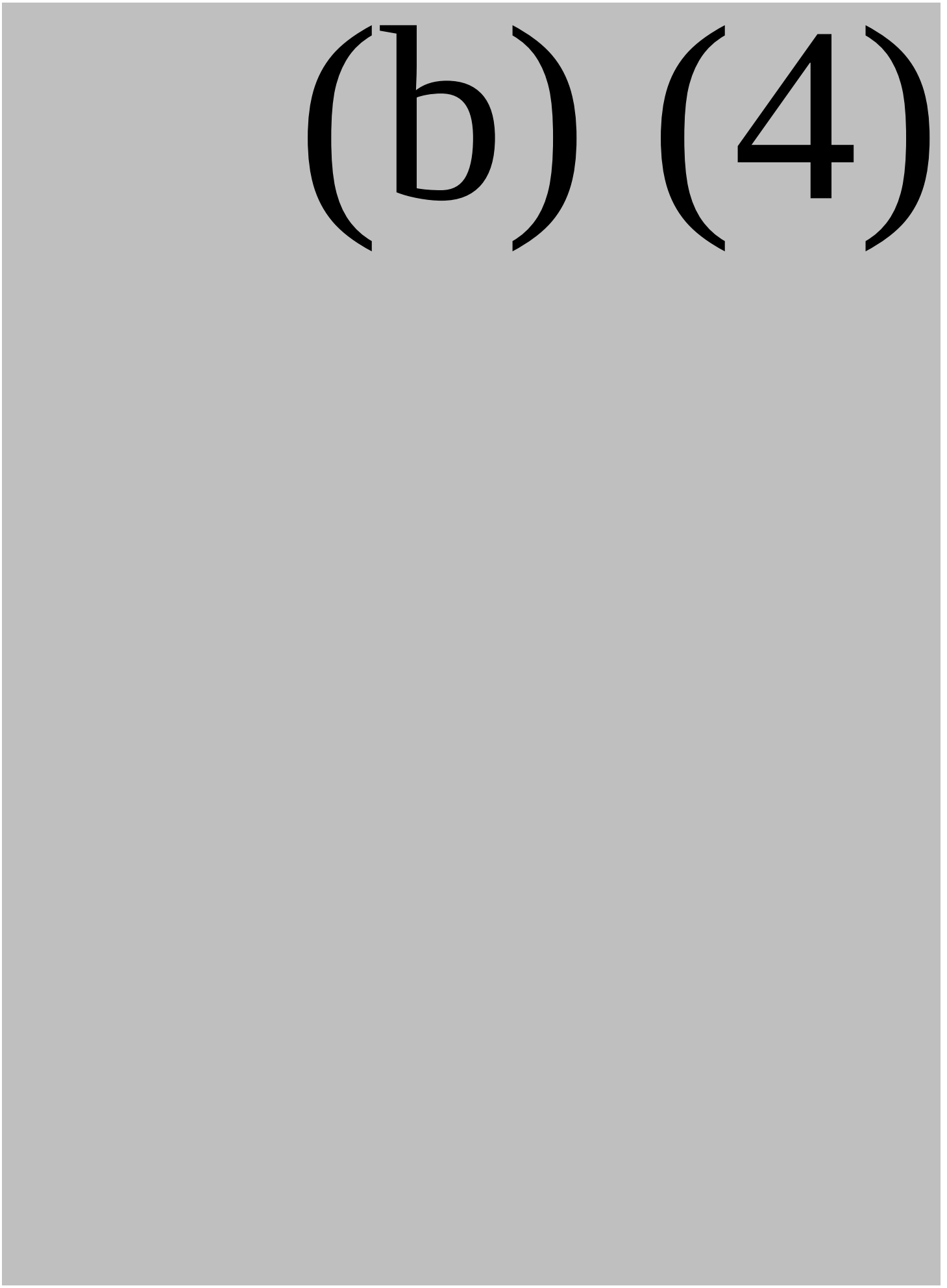
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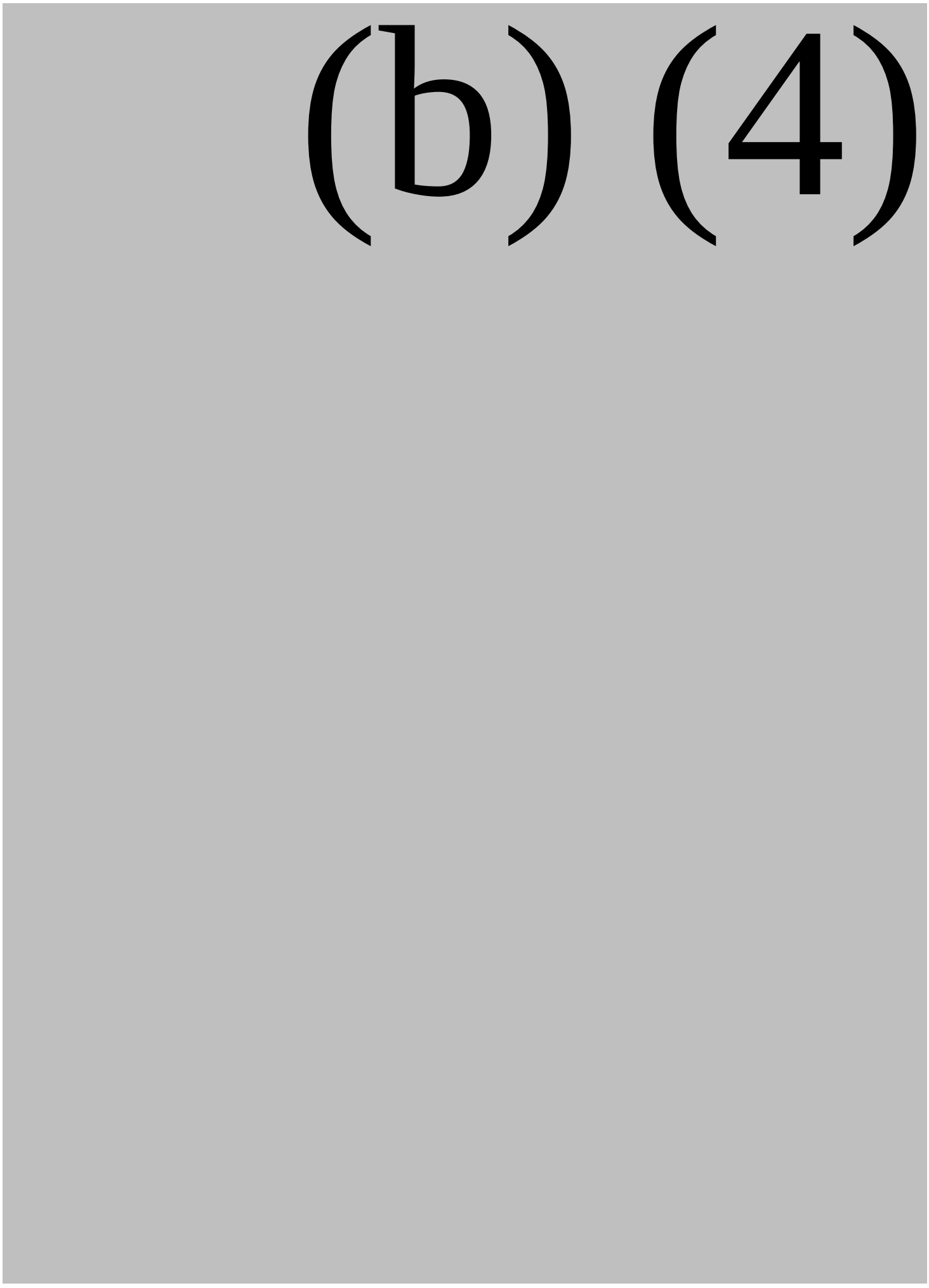
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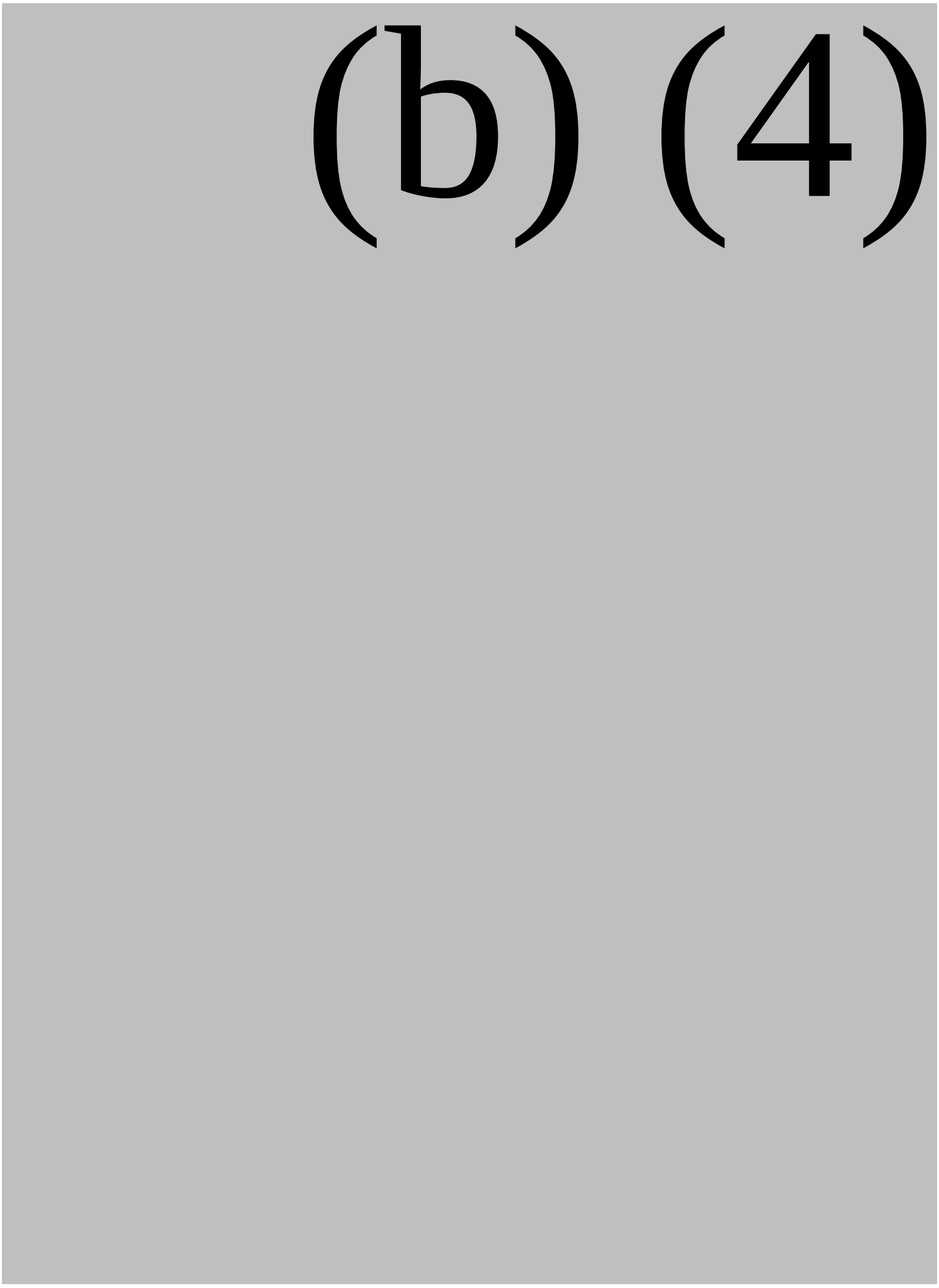
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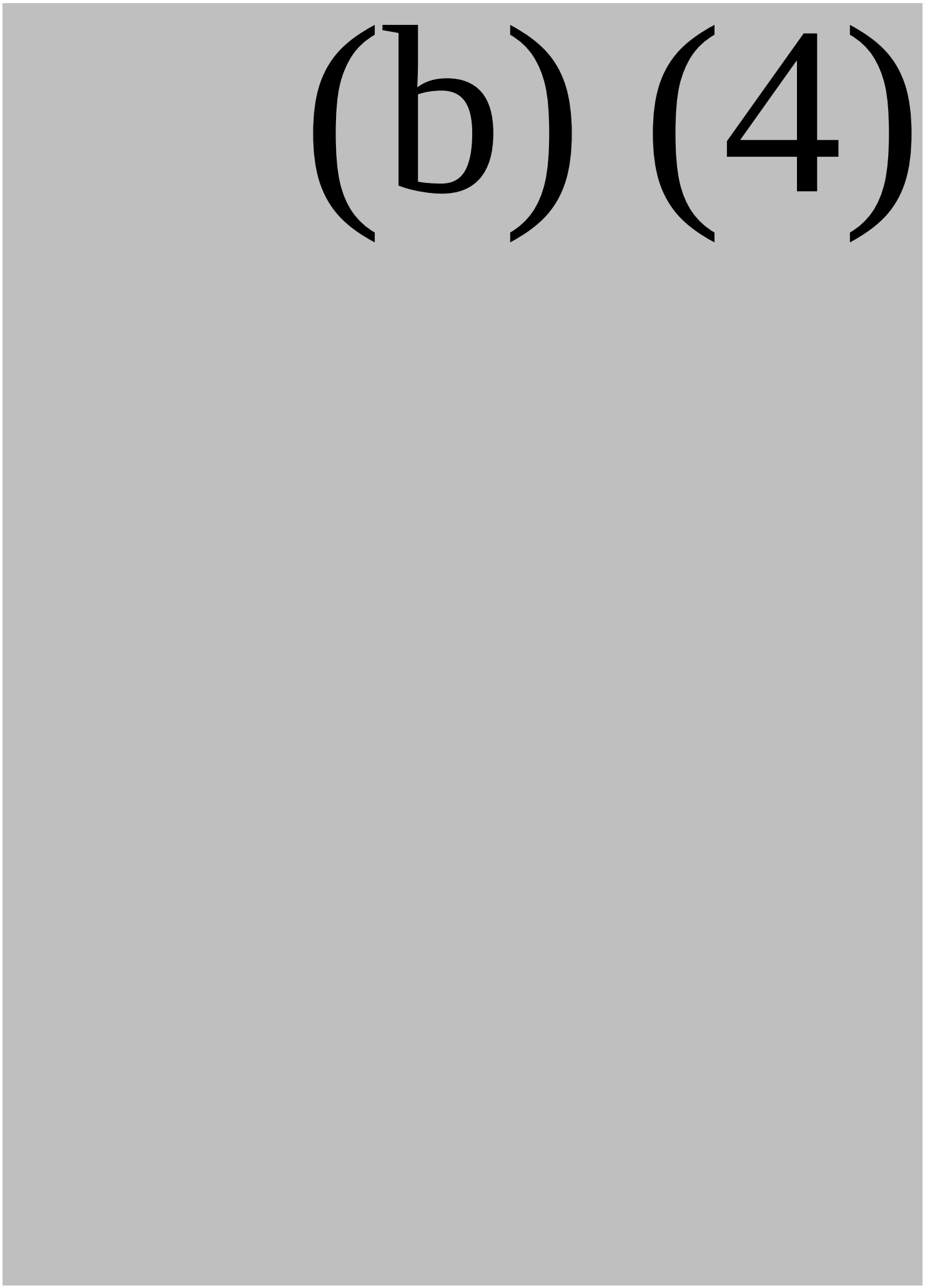
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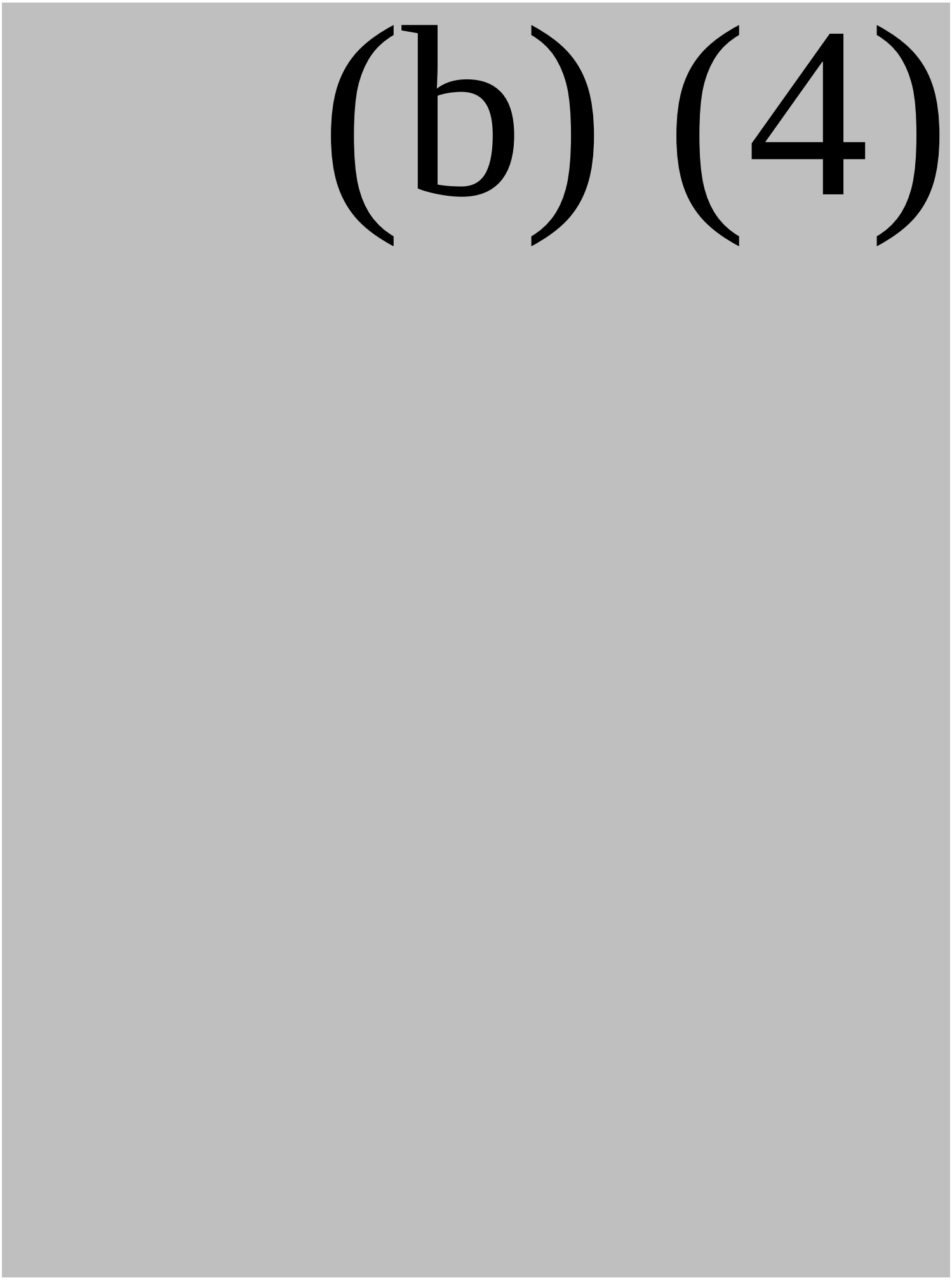
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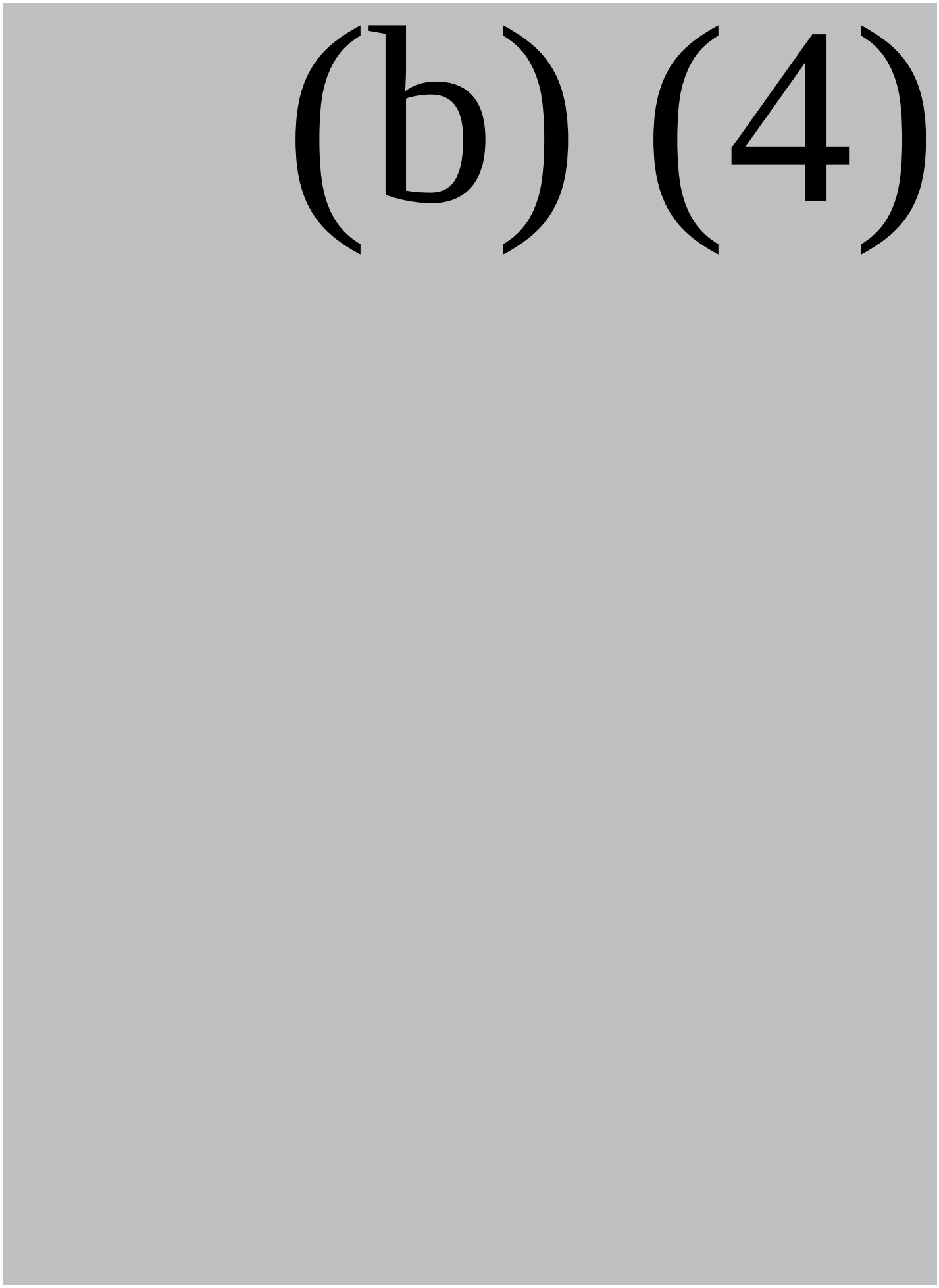
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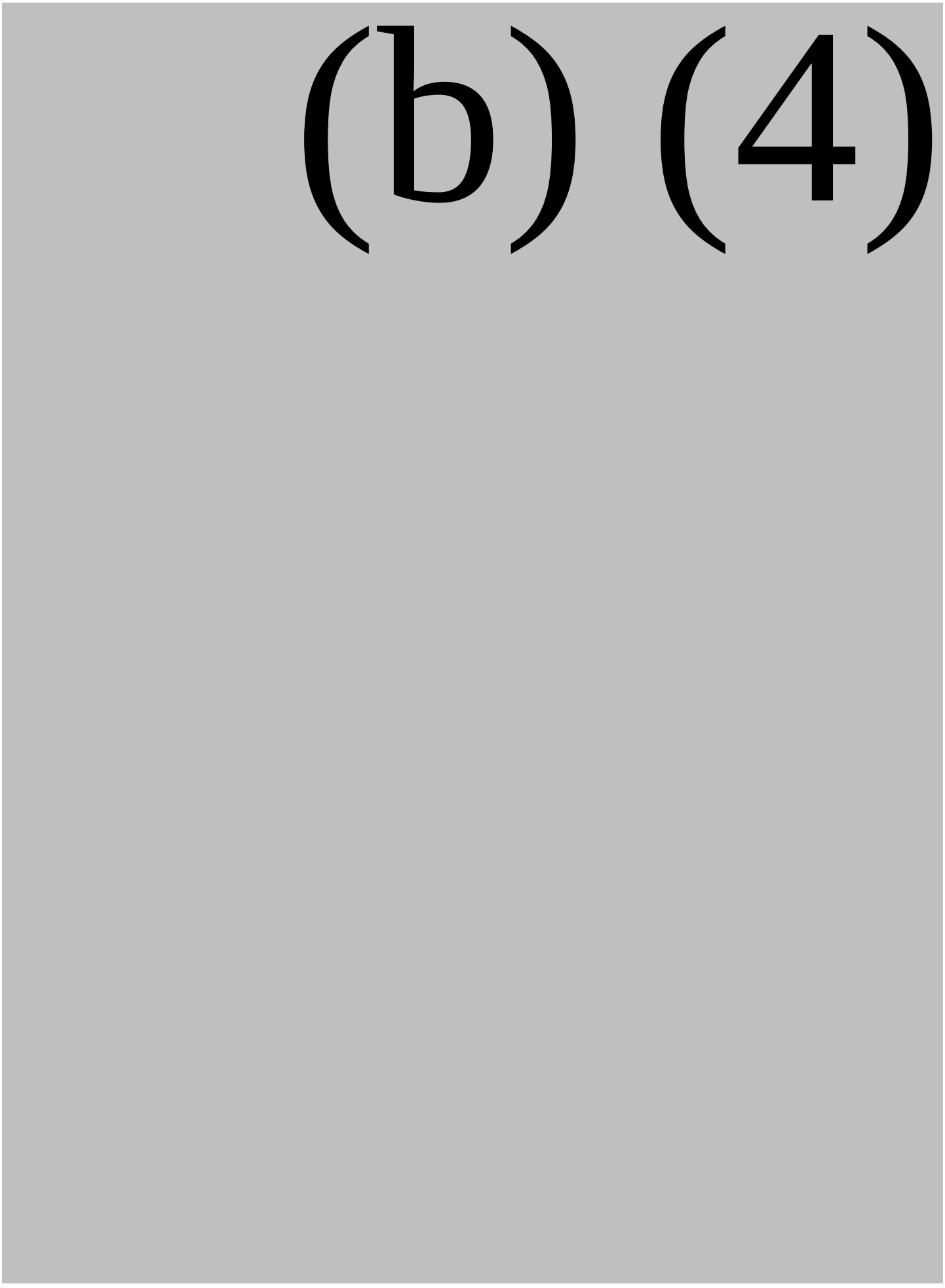
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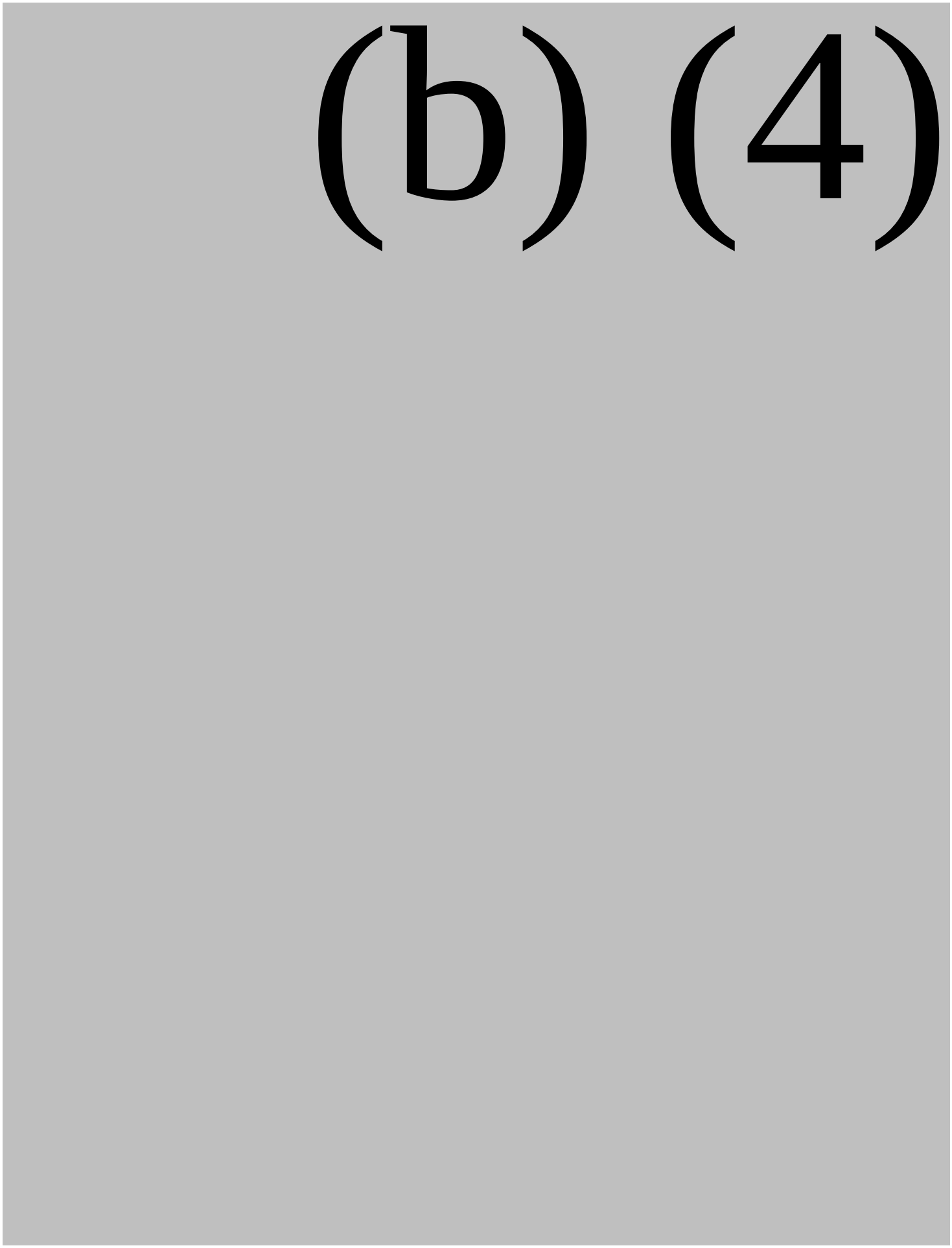
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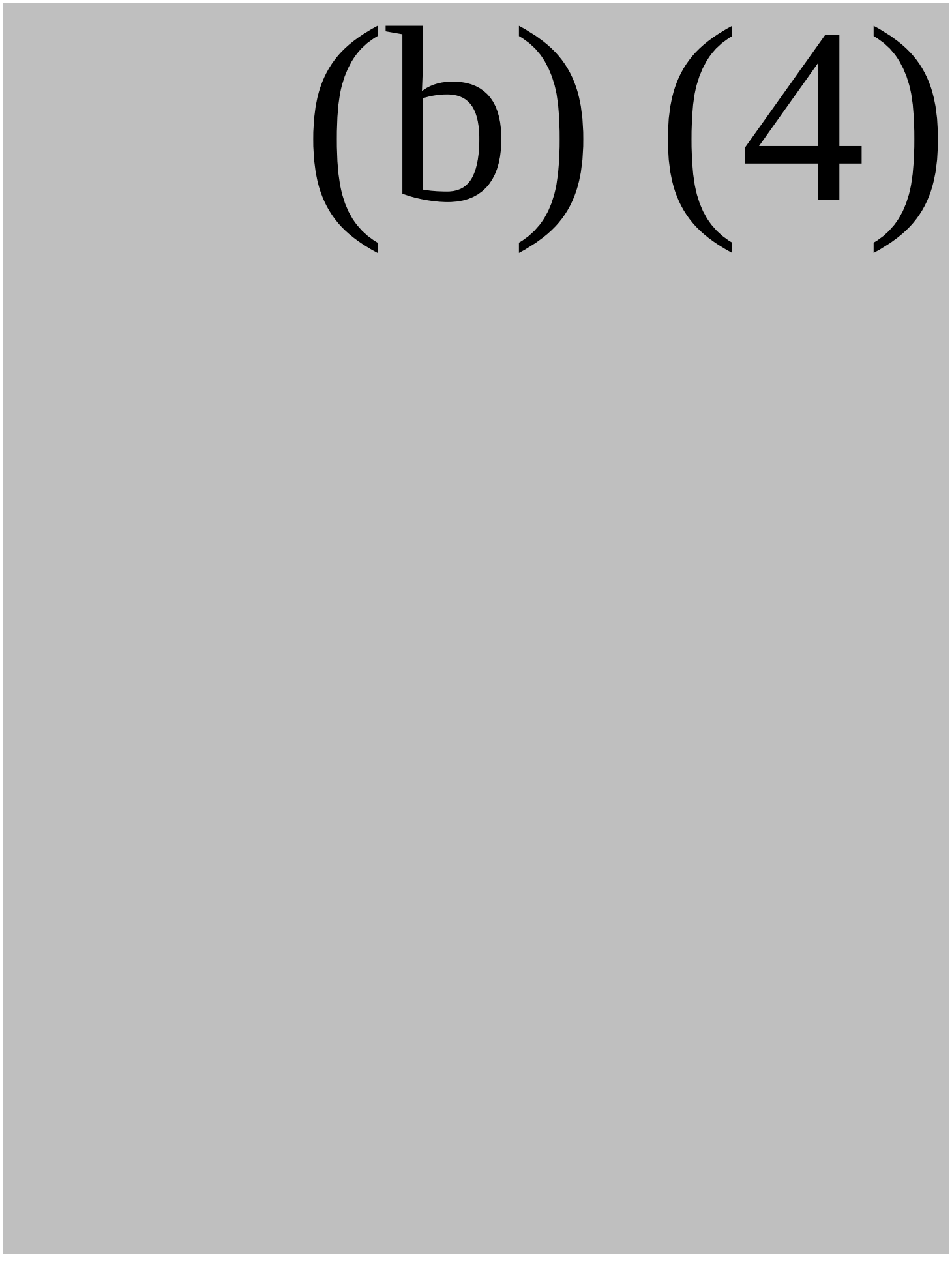
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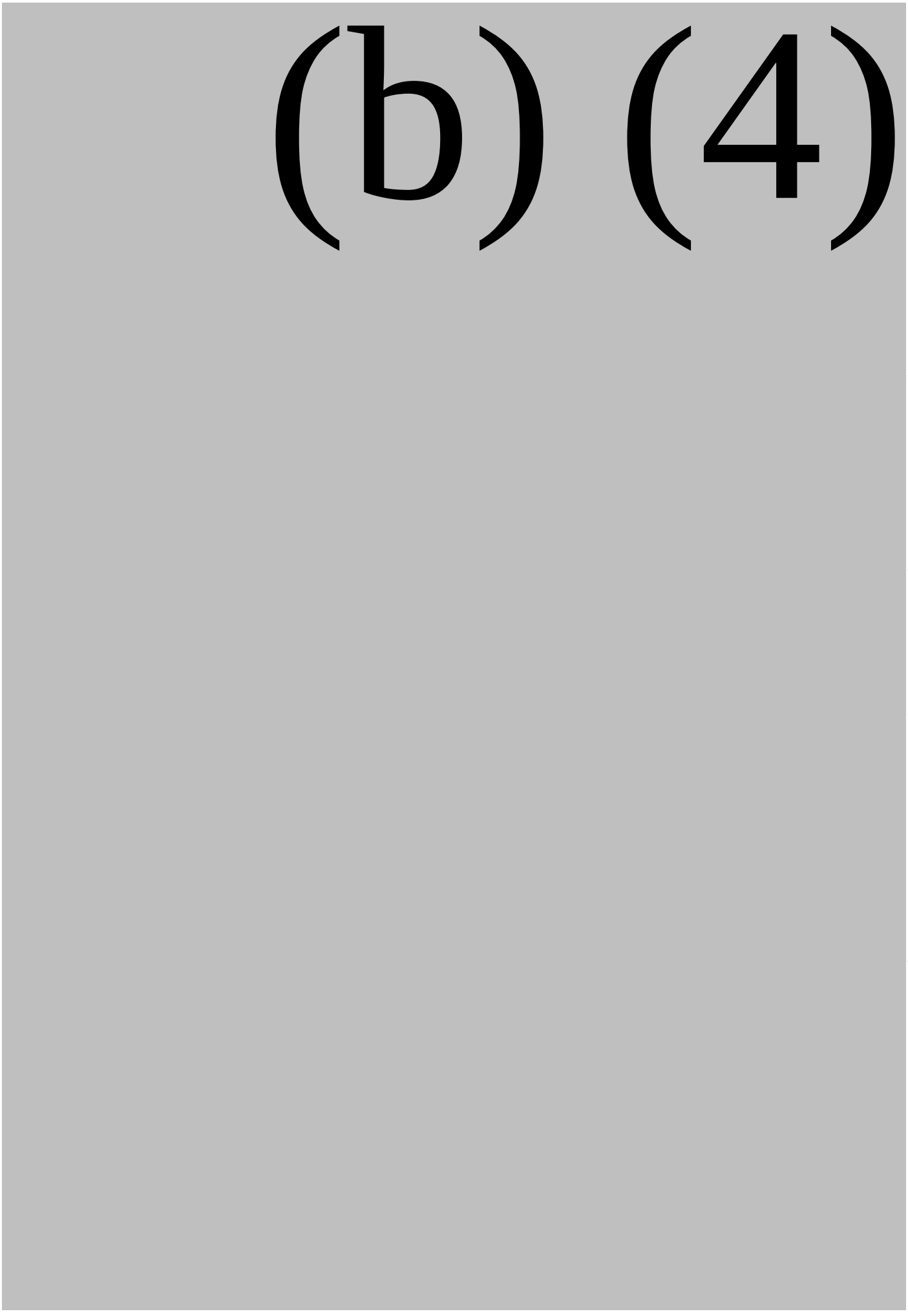

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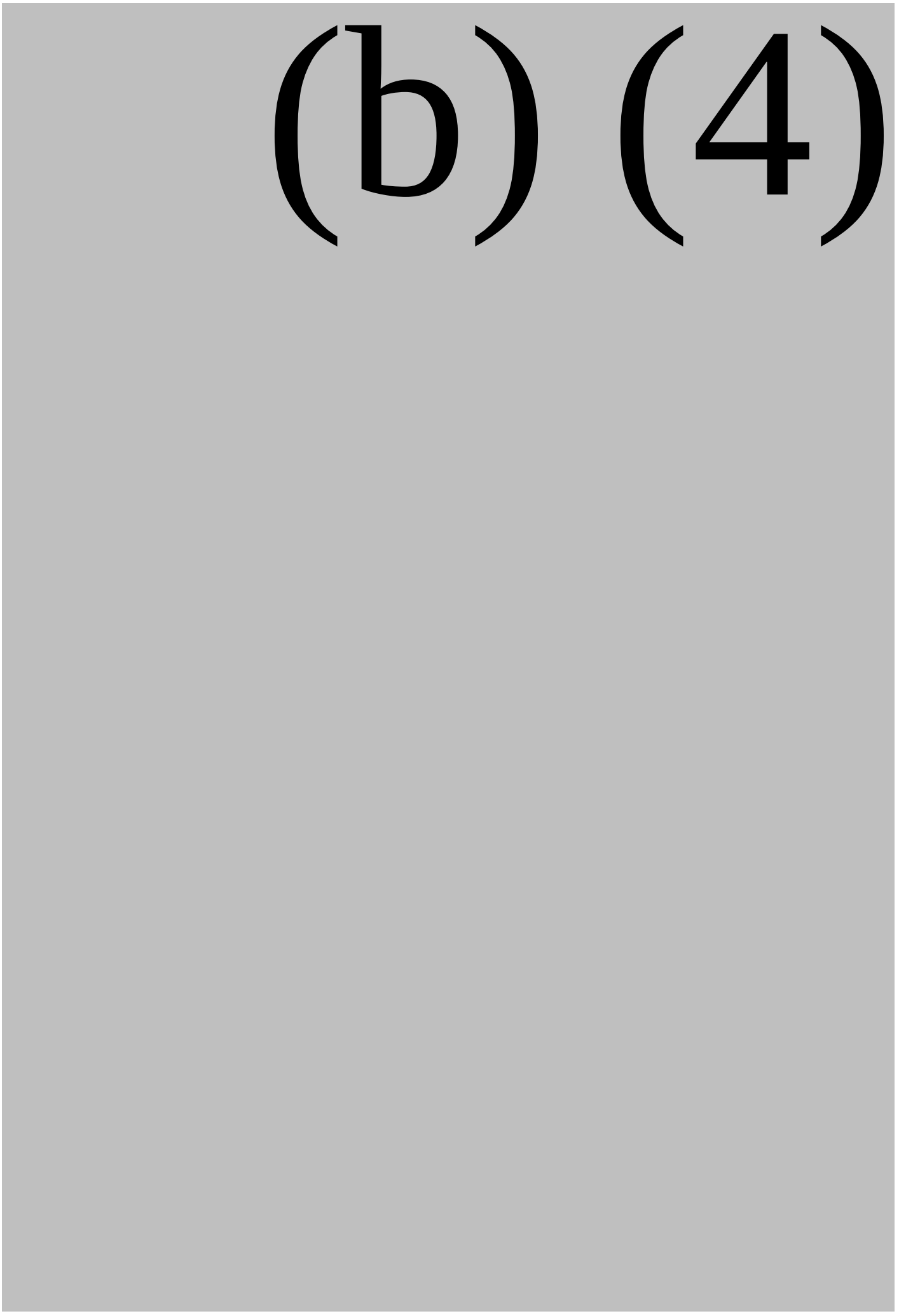
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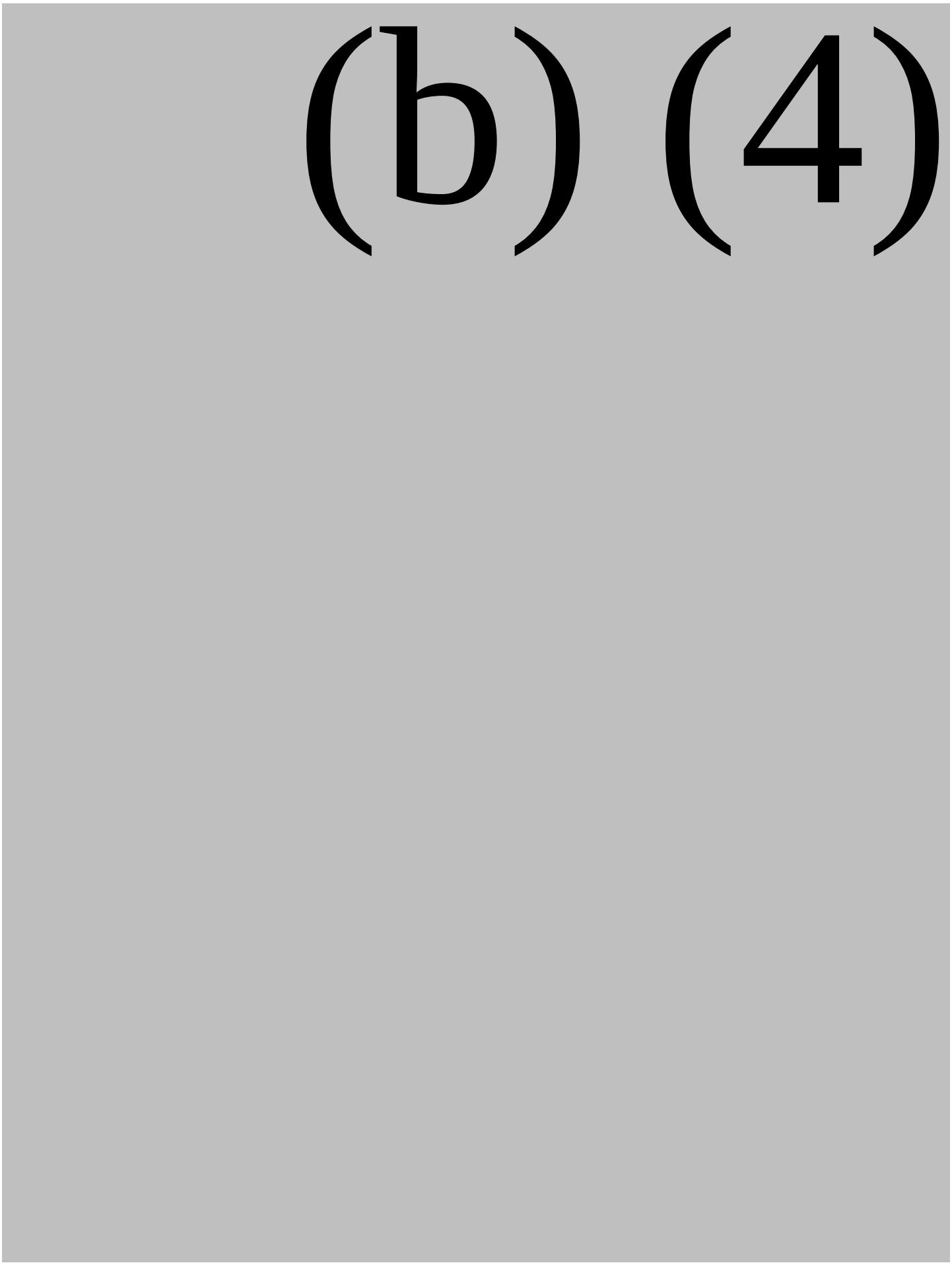
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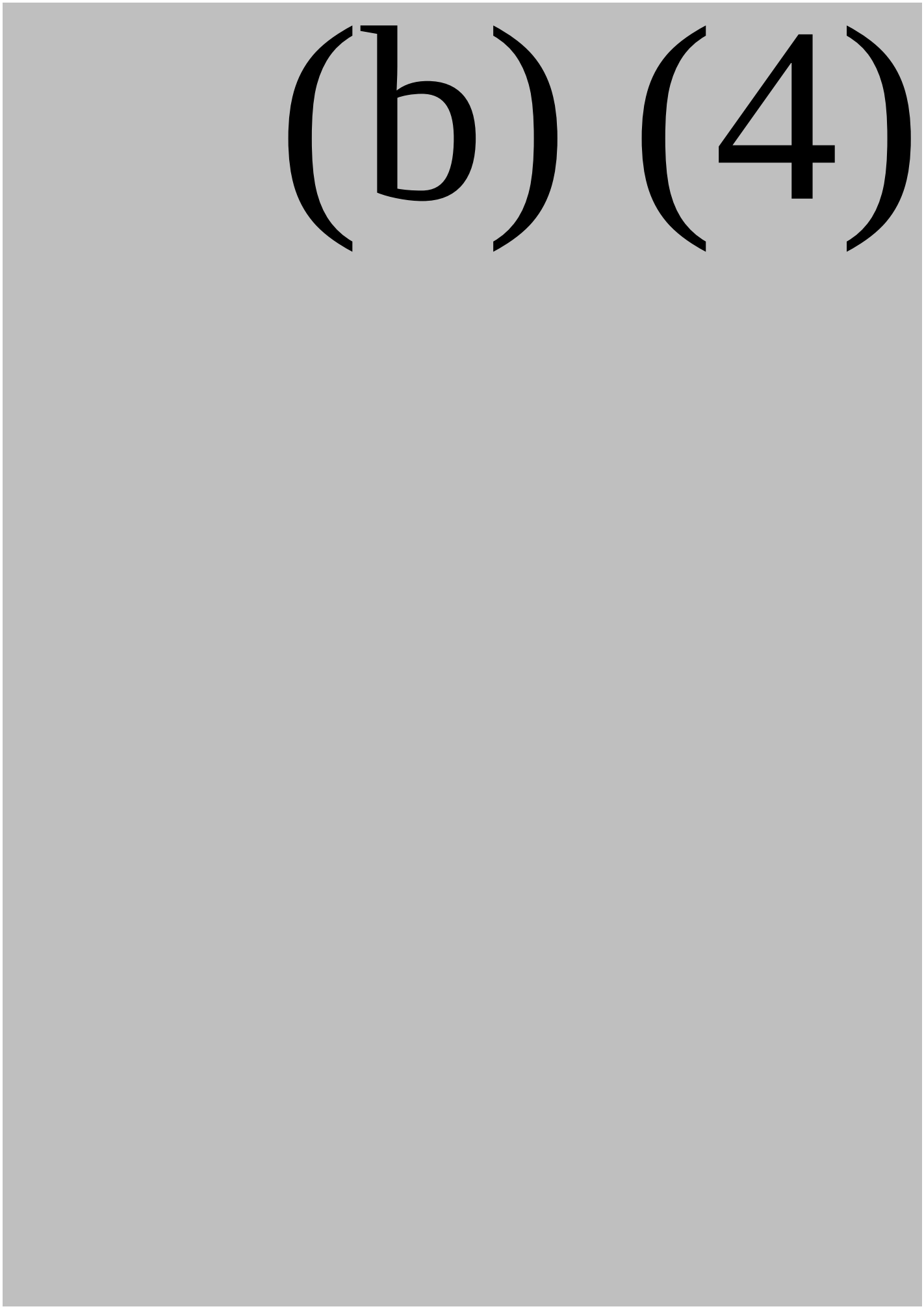
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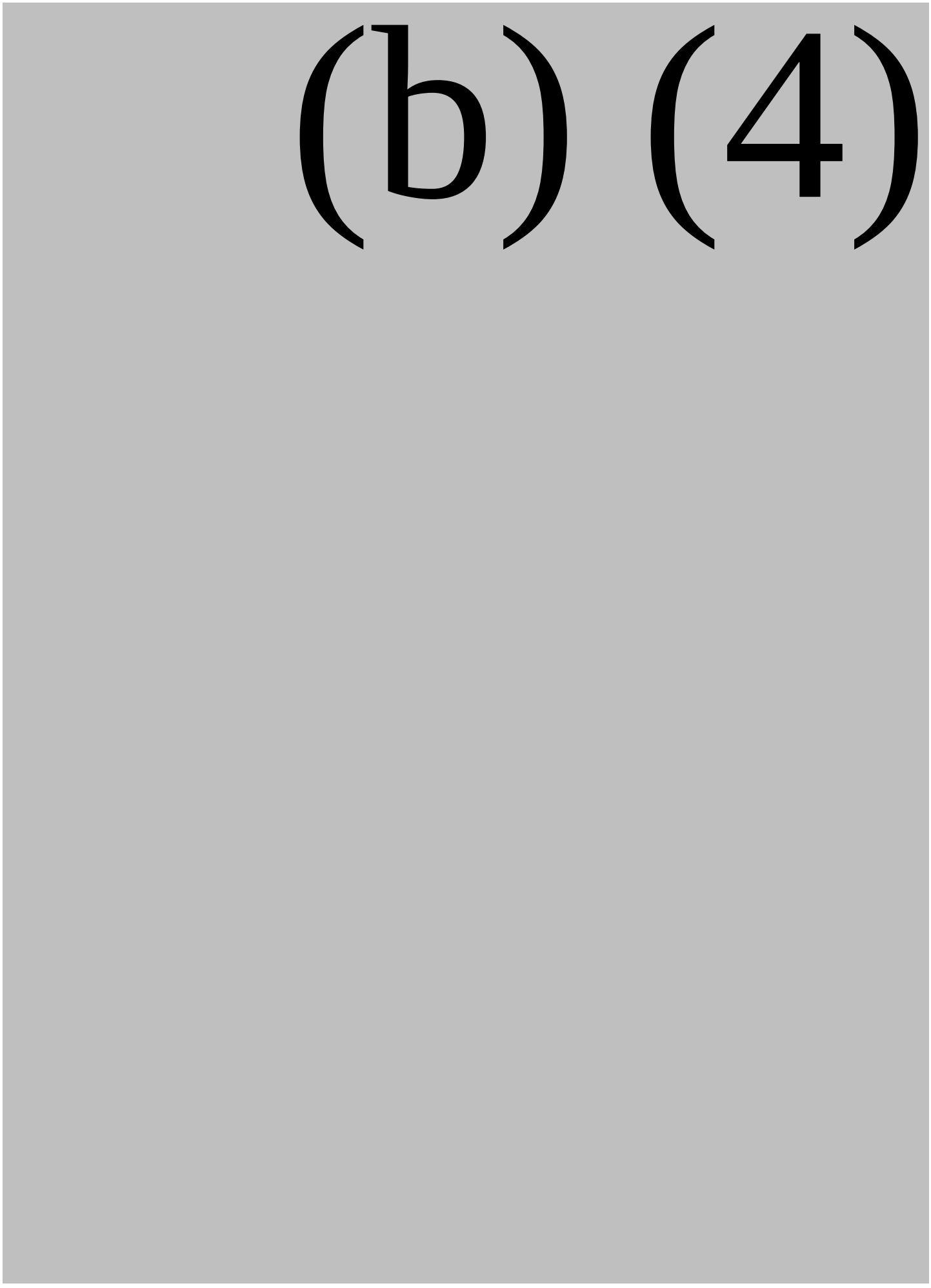
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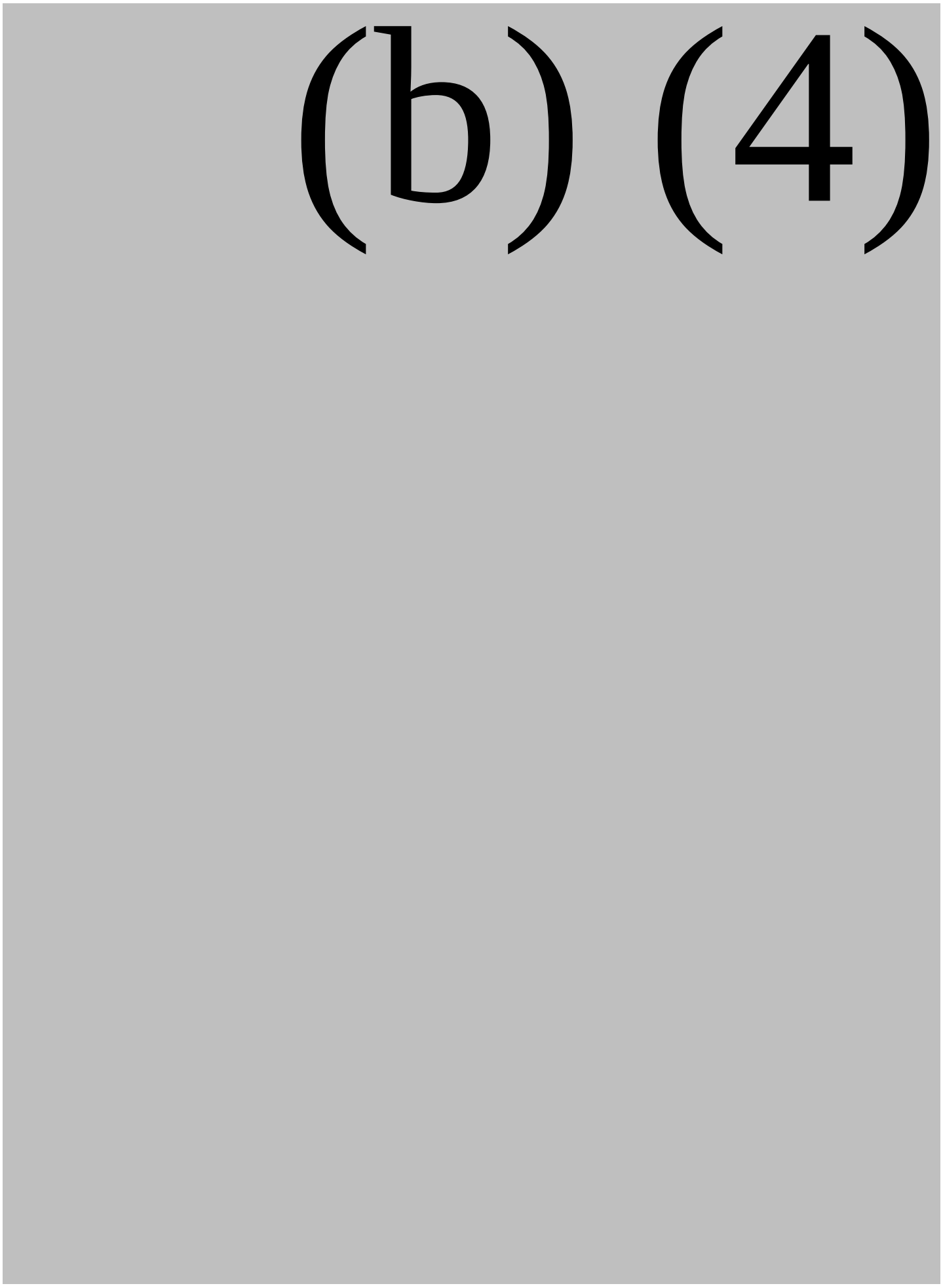
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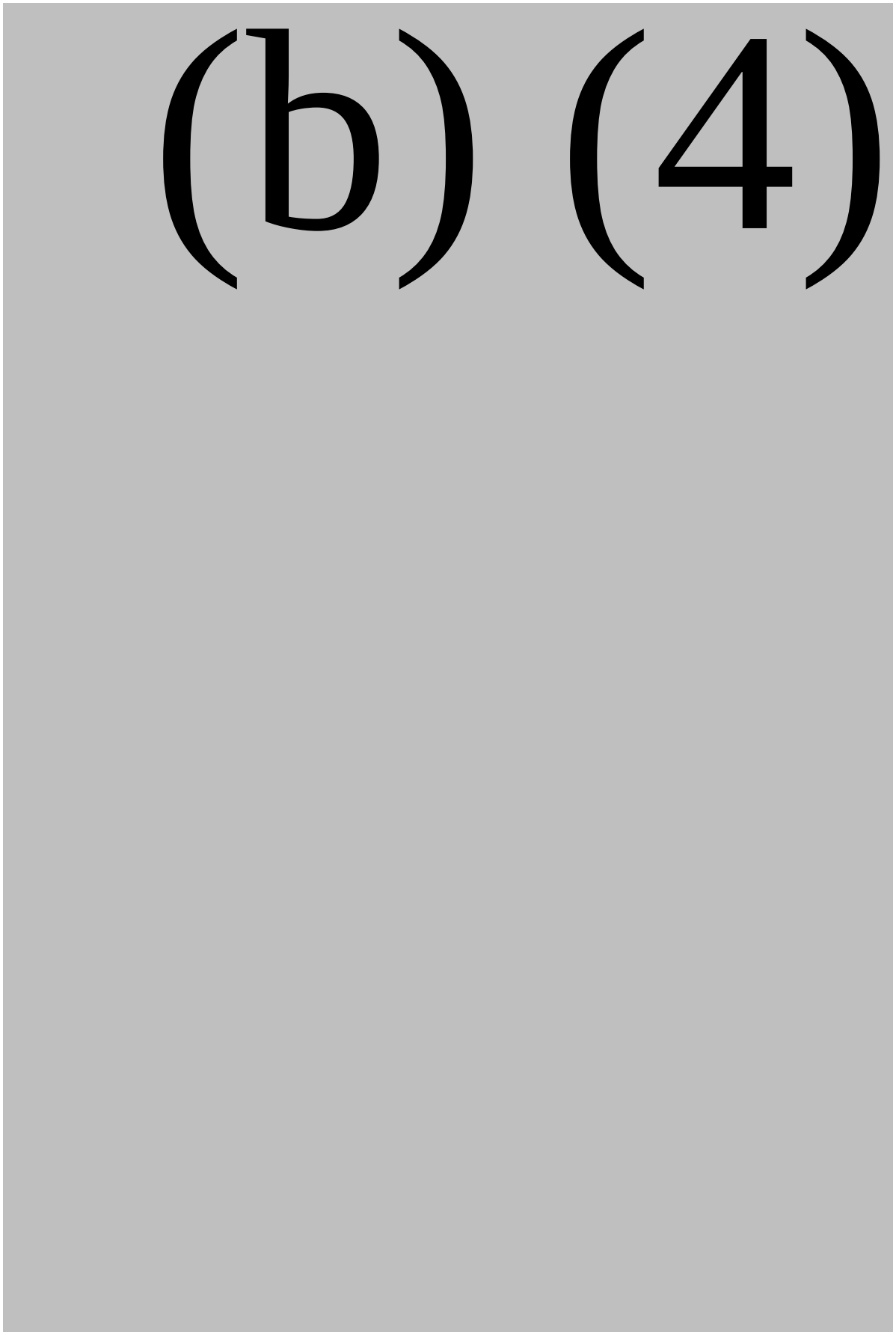

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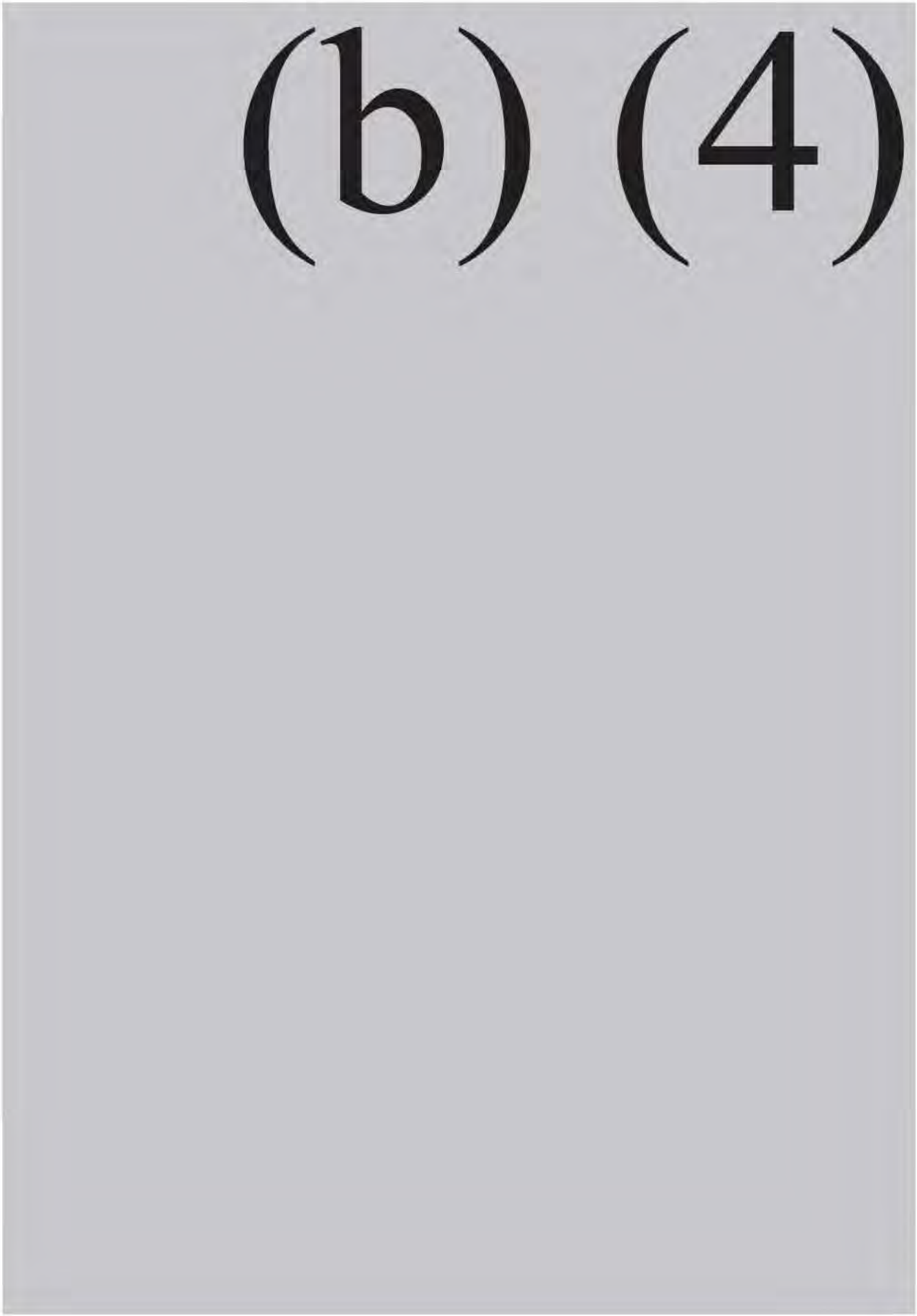
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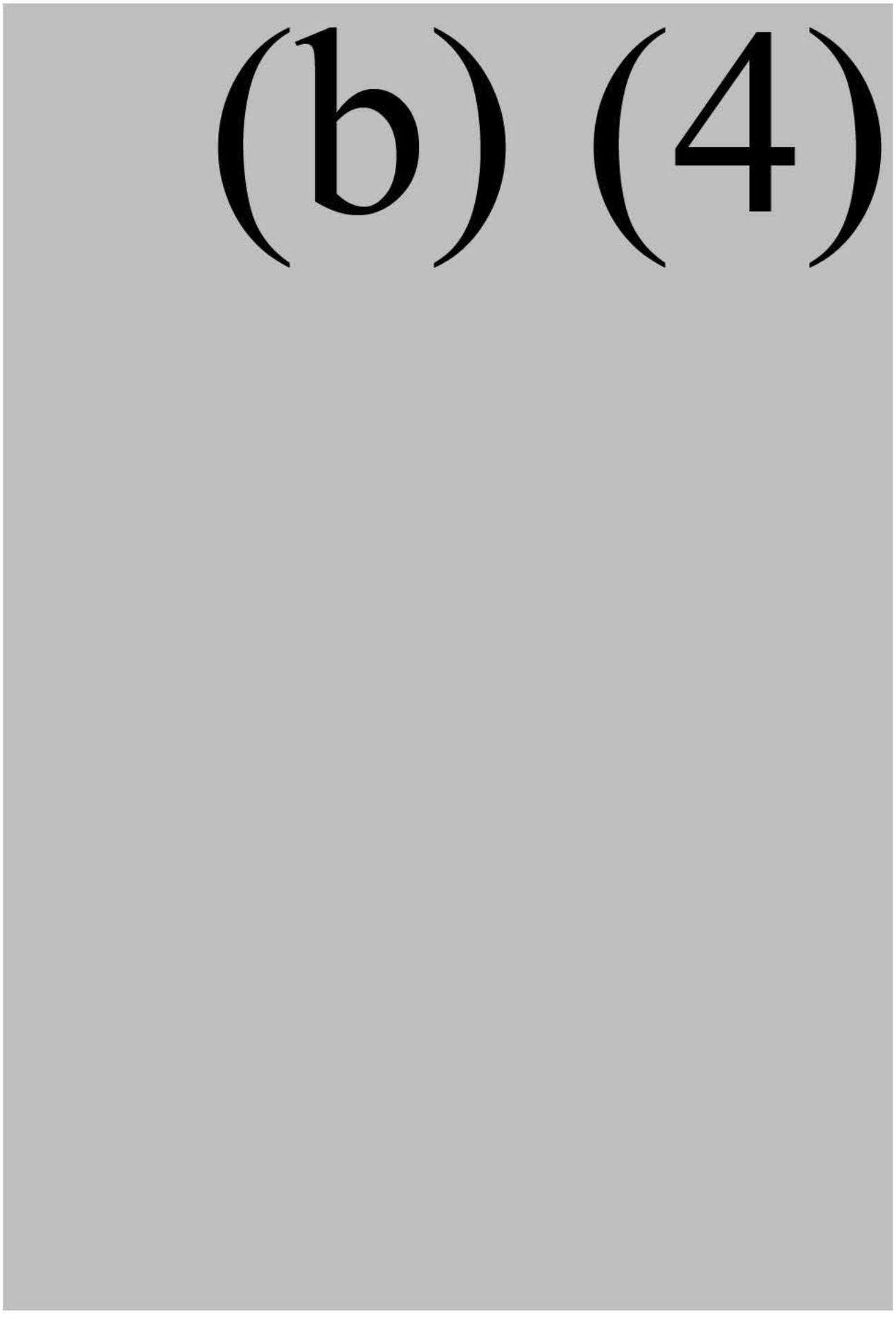
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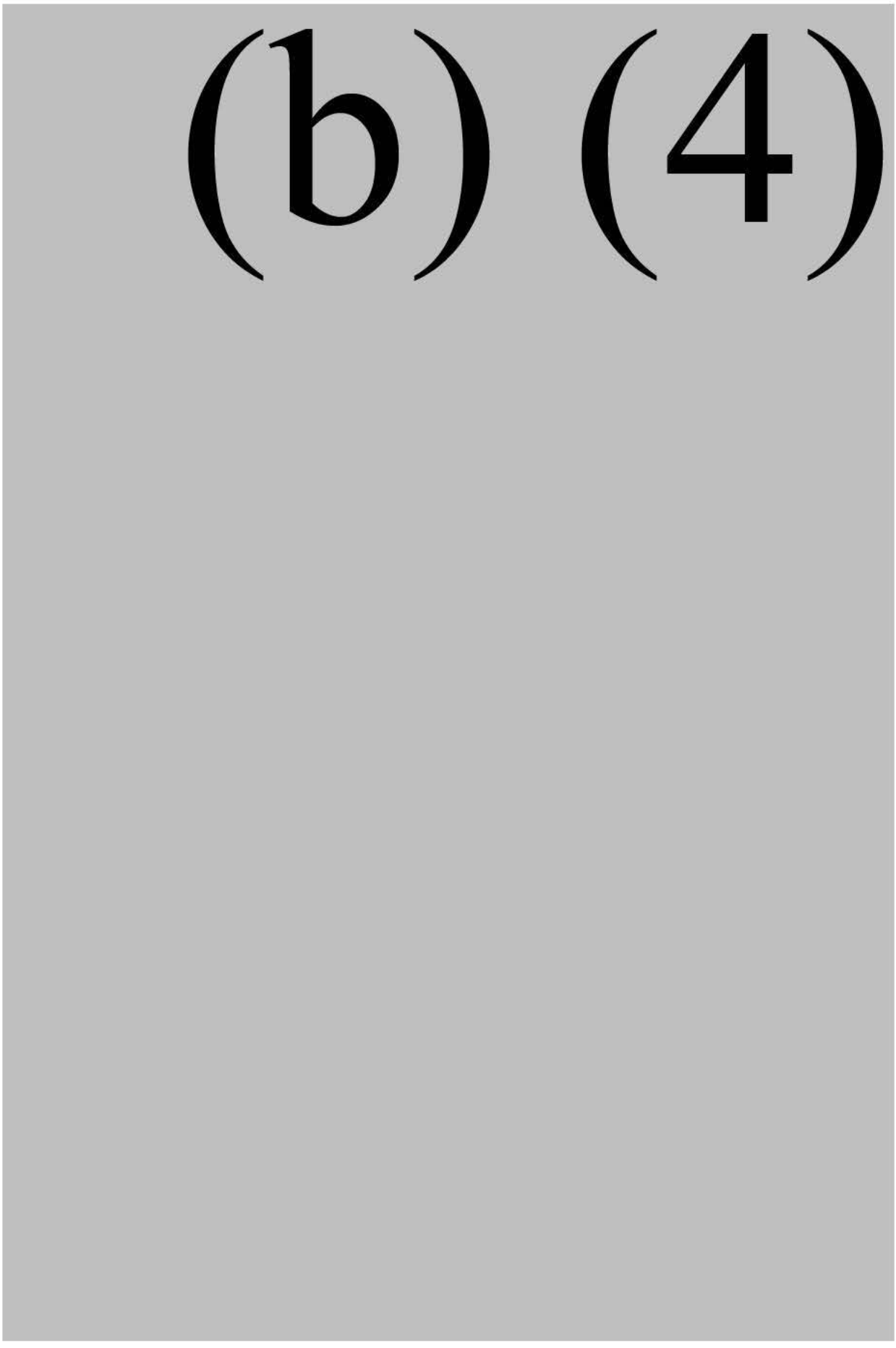
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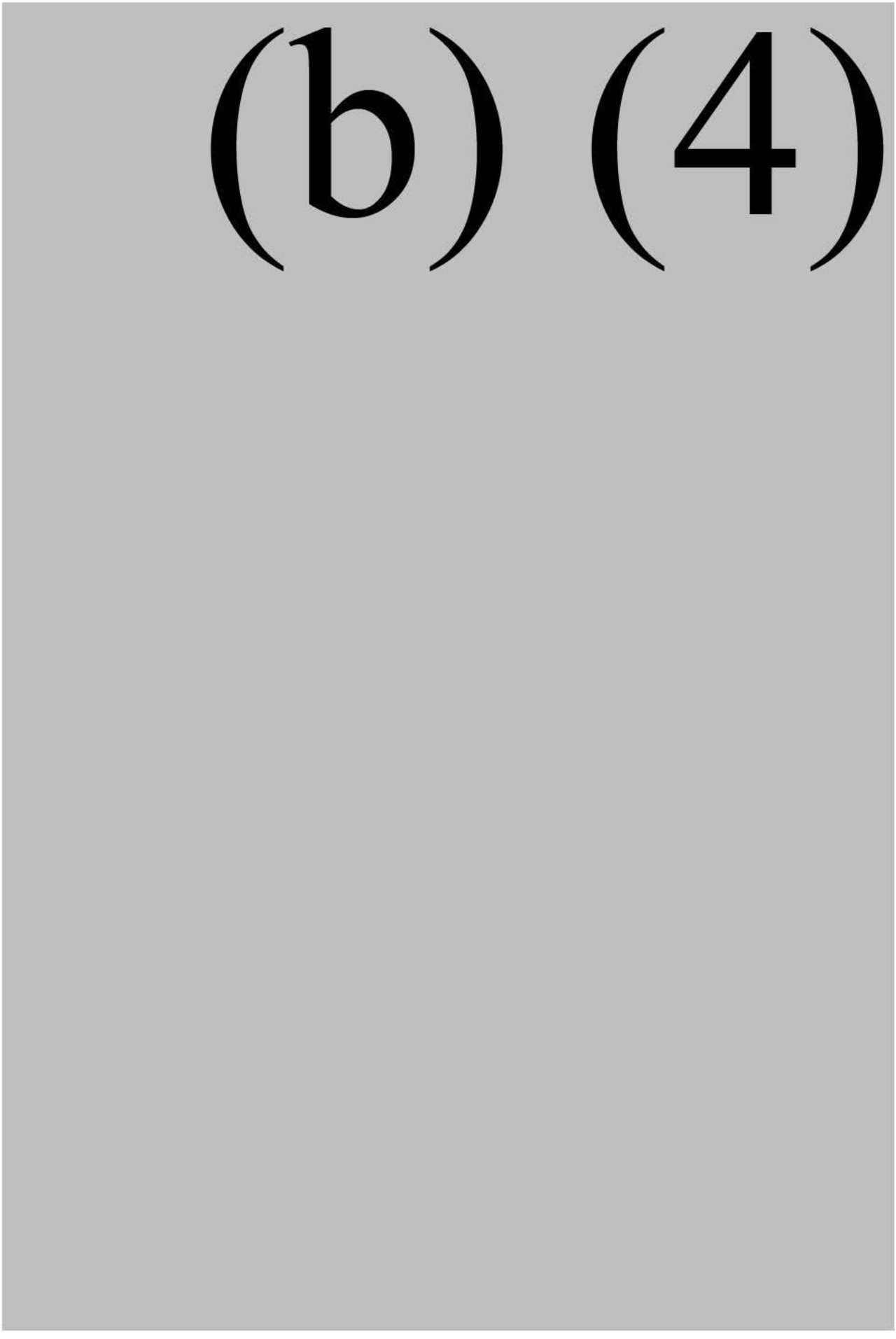
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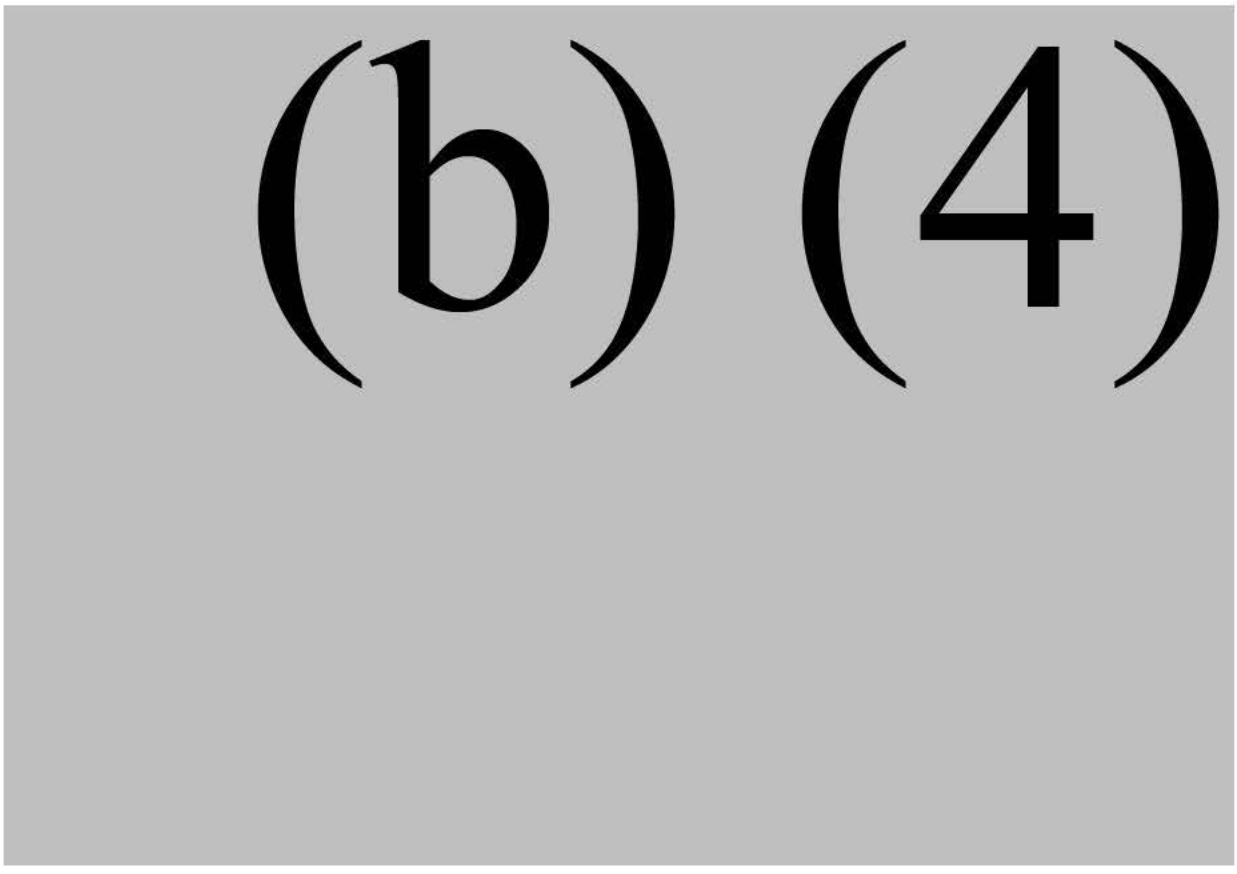
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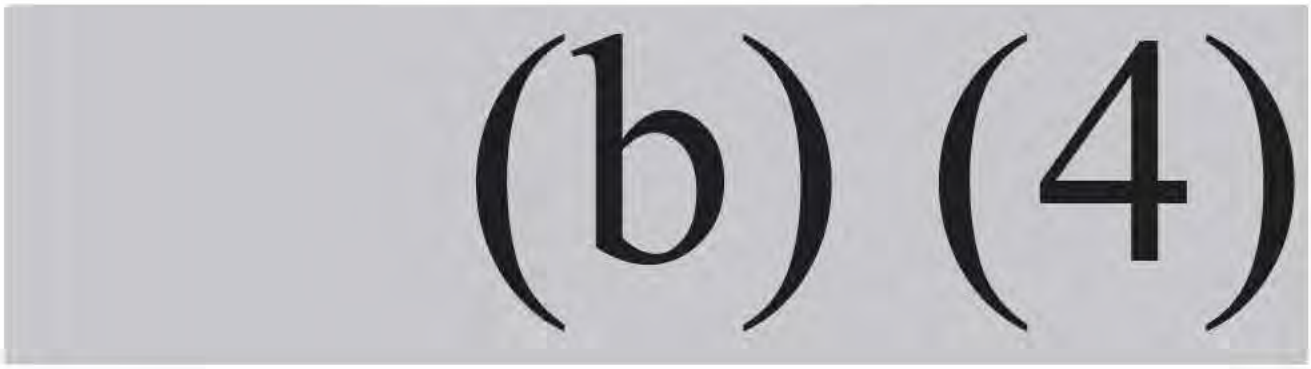
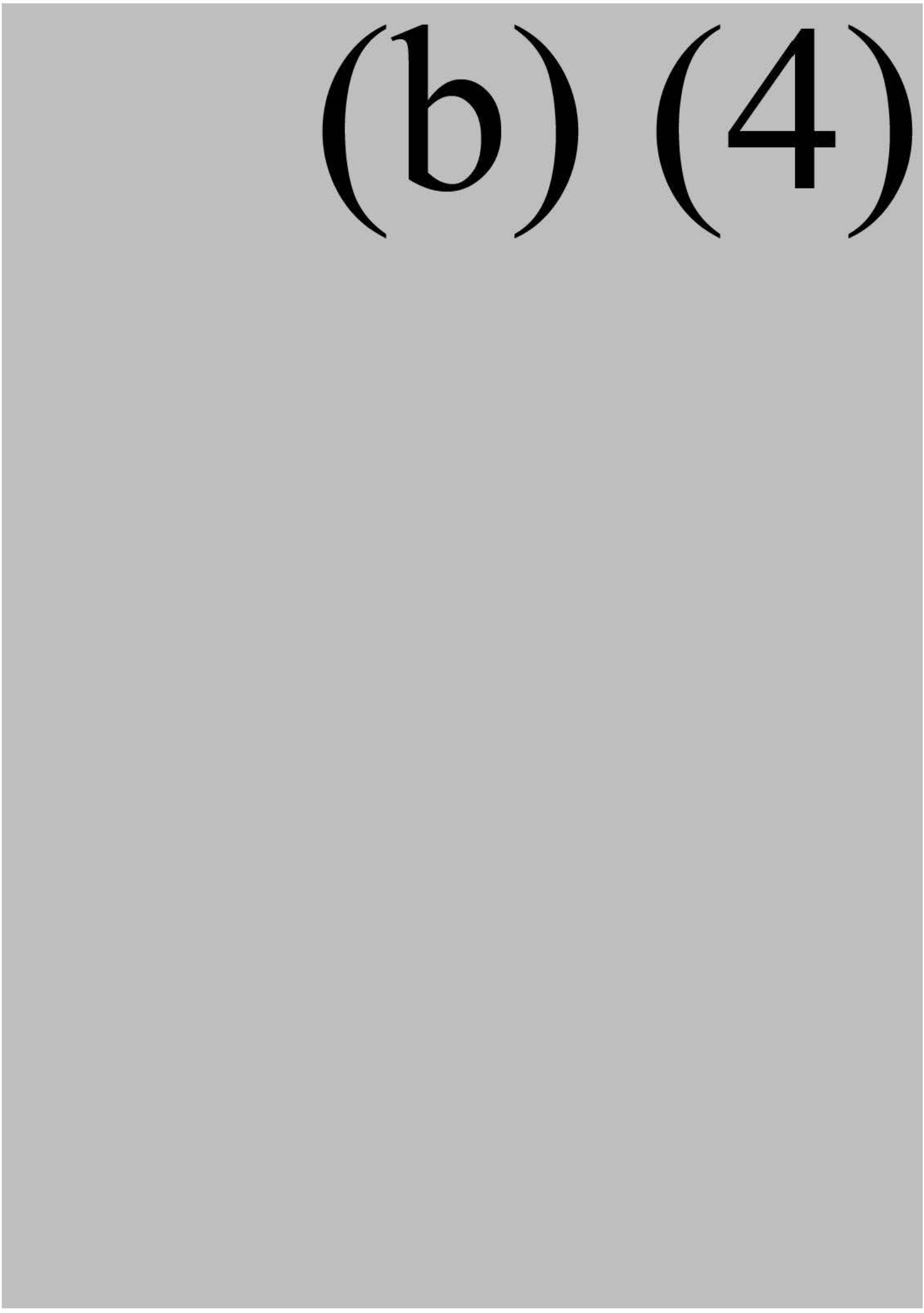
APPENDIX 1

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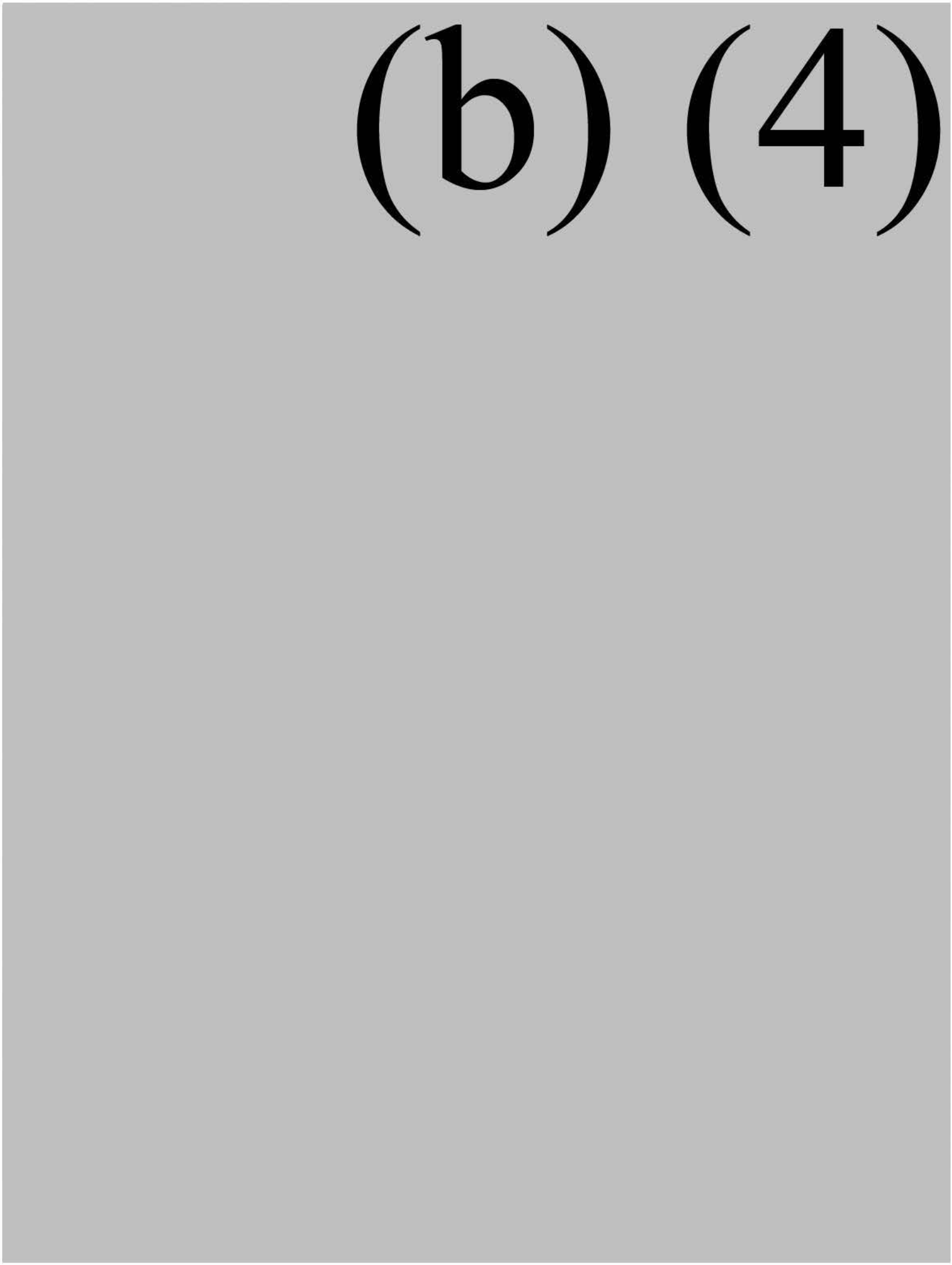
APPENDIX 2

(b) (4)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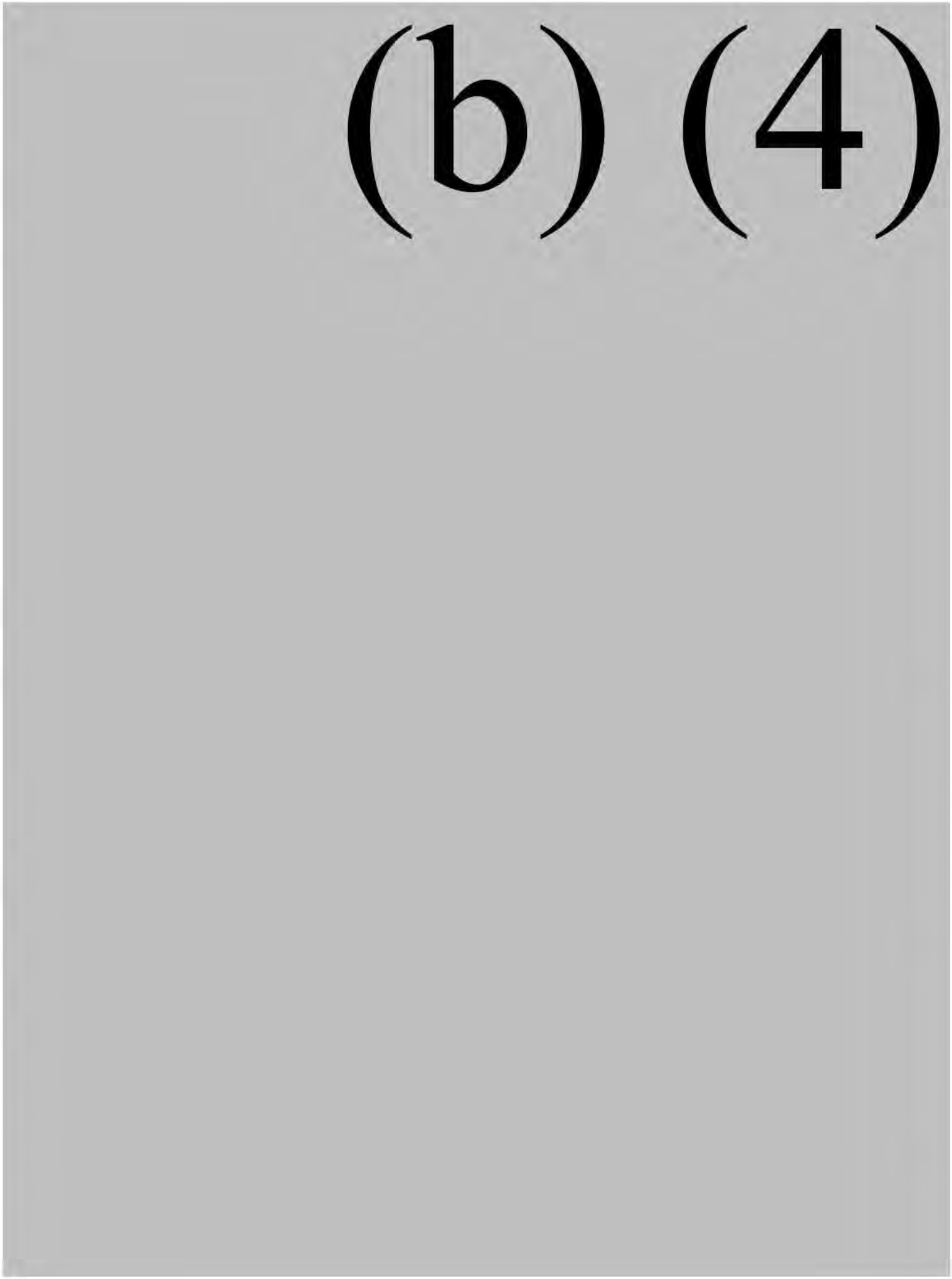
(b) (4)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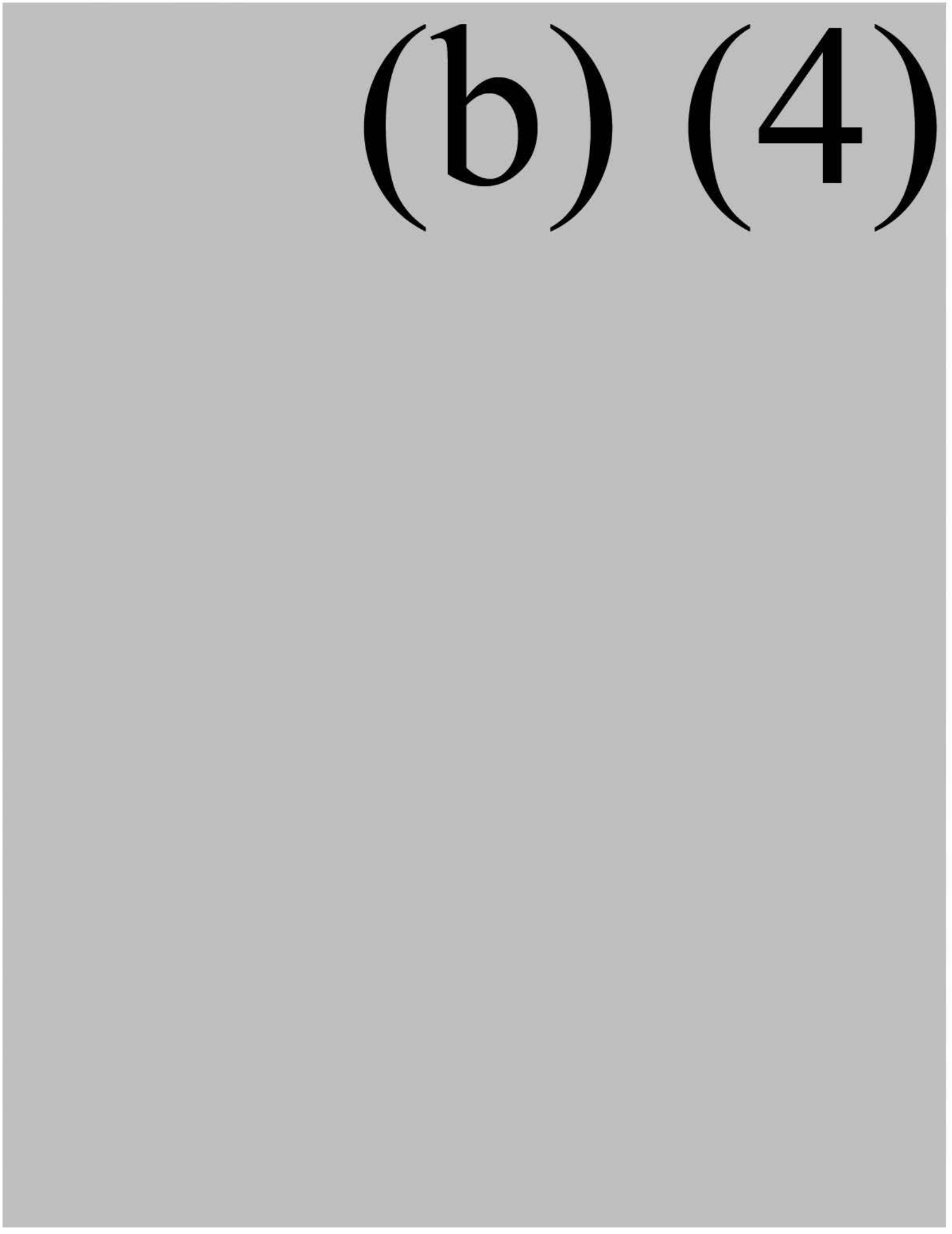
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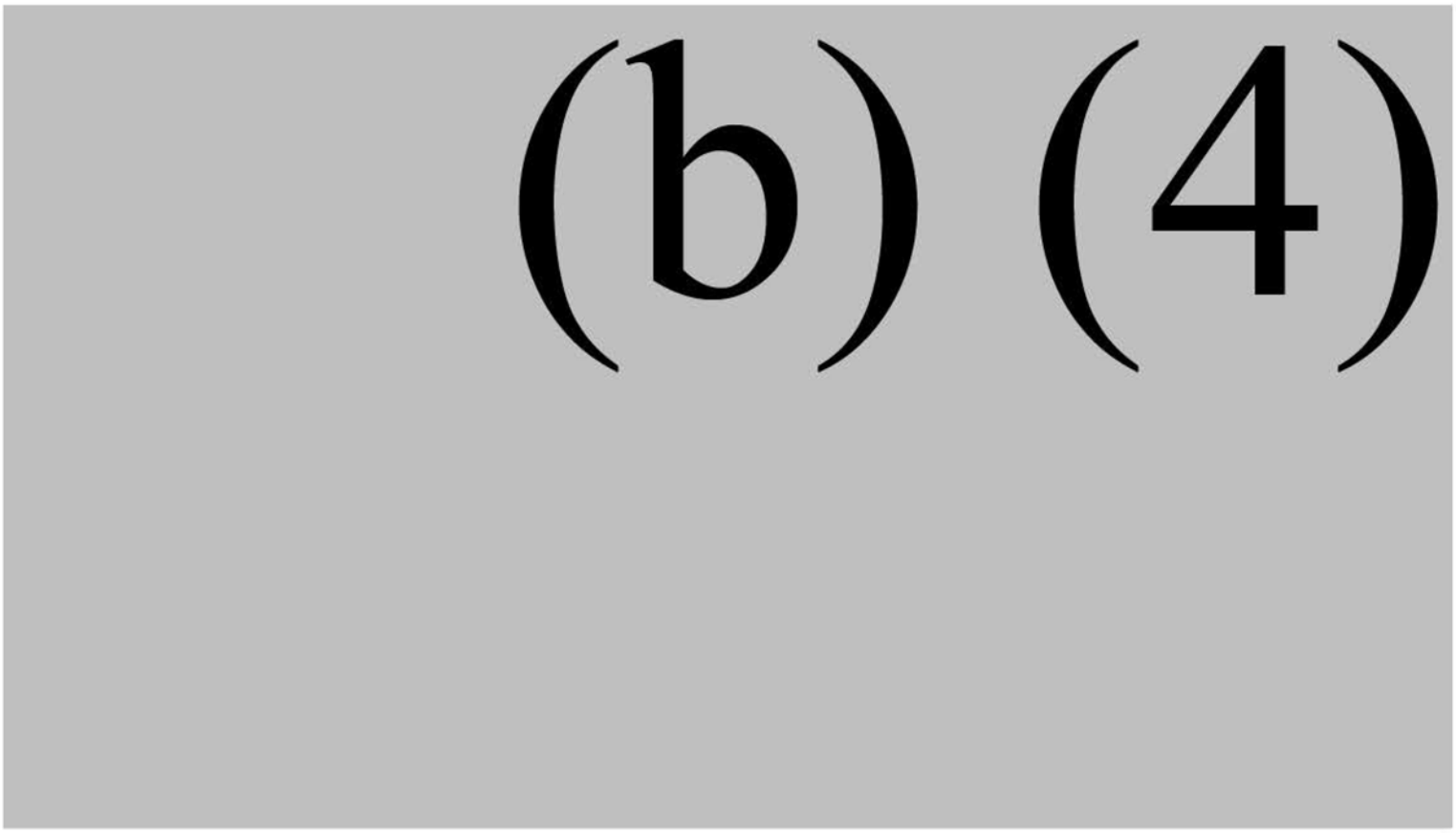
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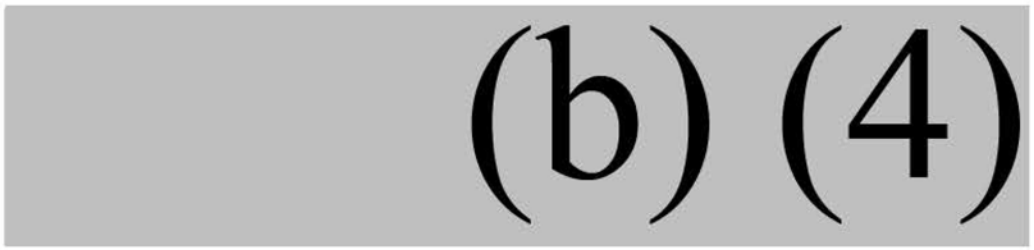
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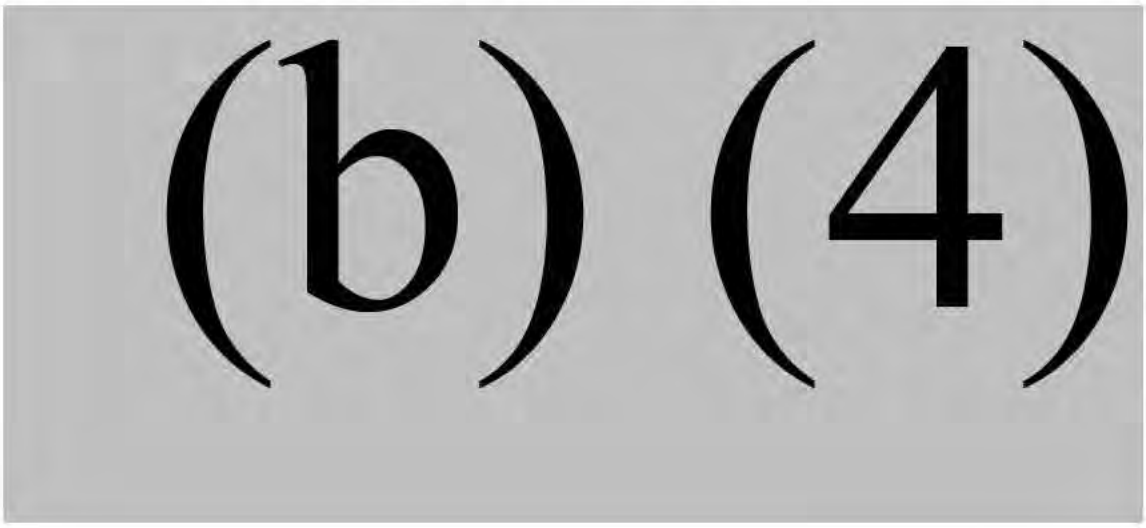
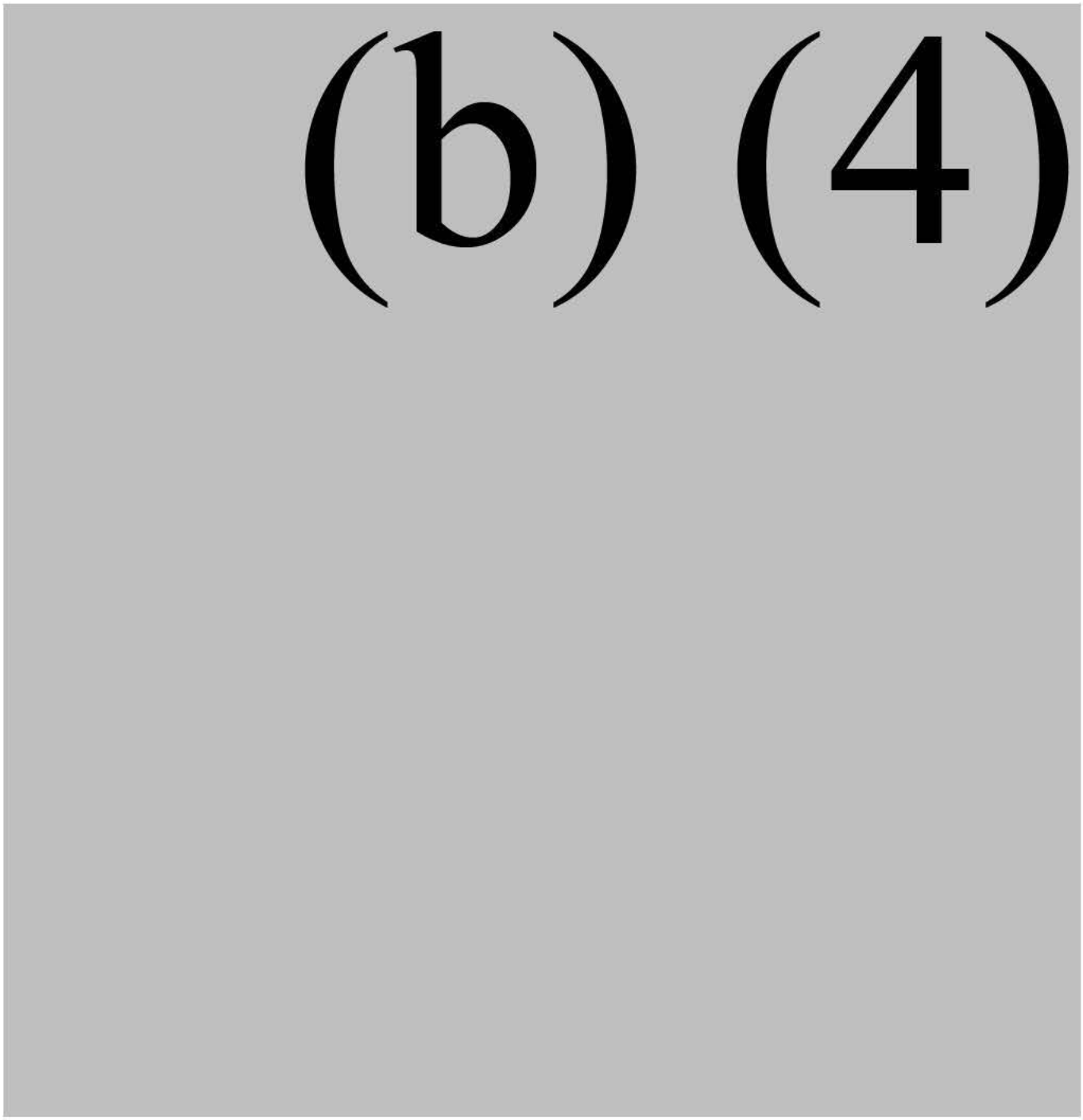
APPENDIX 2

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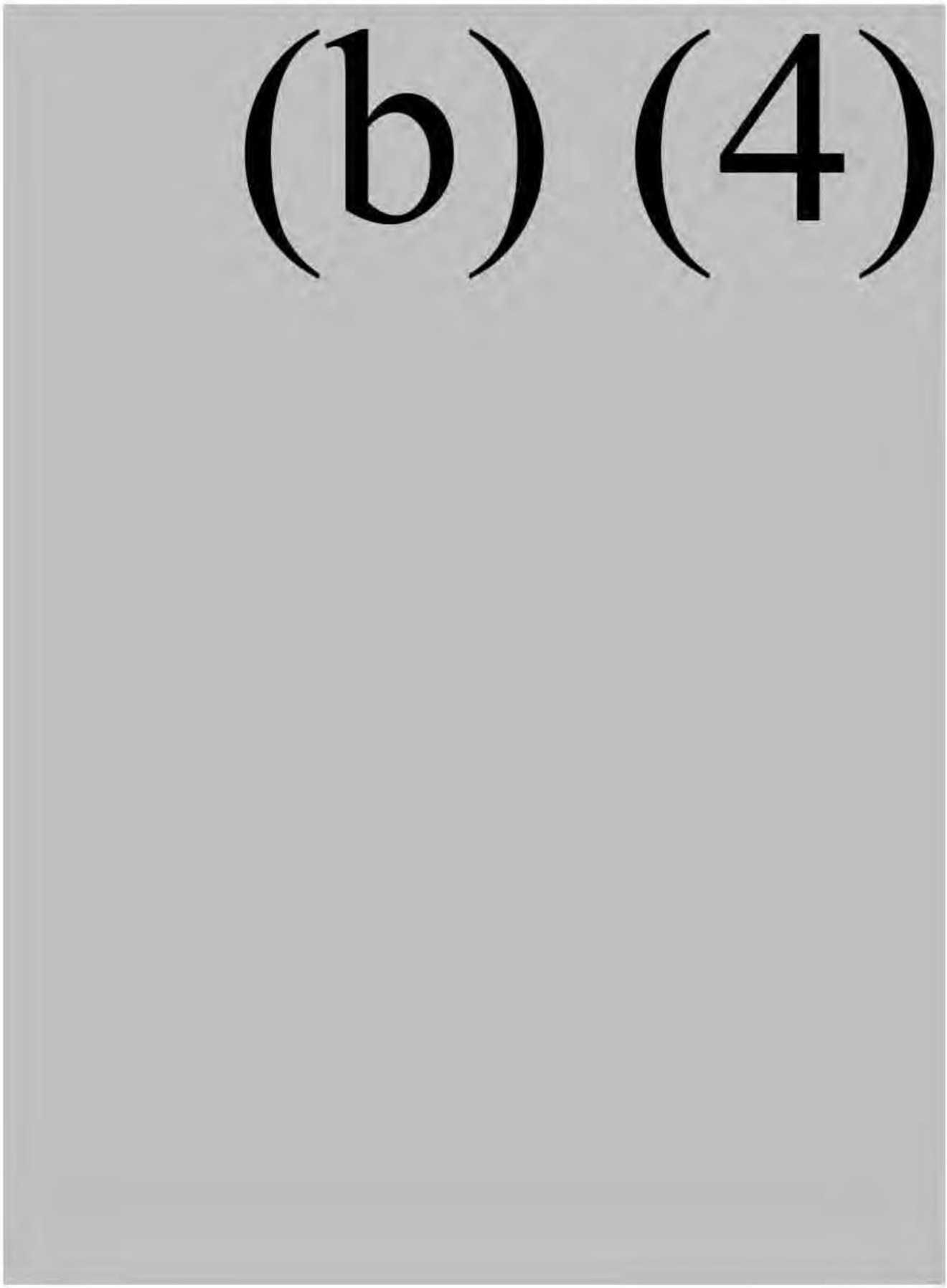
APPENDIX 3

(b) (4)



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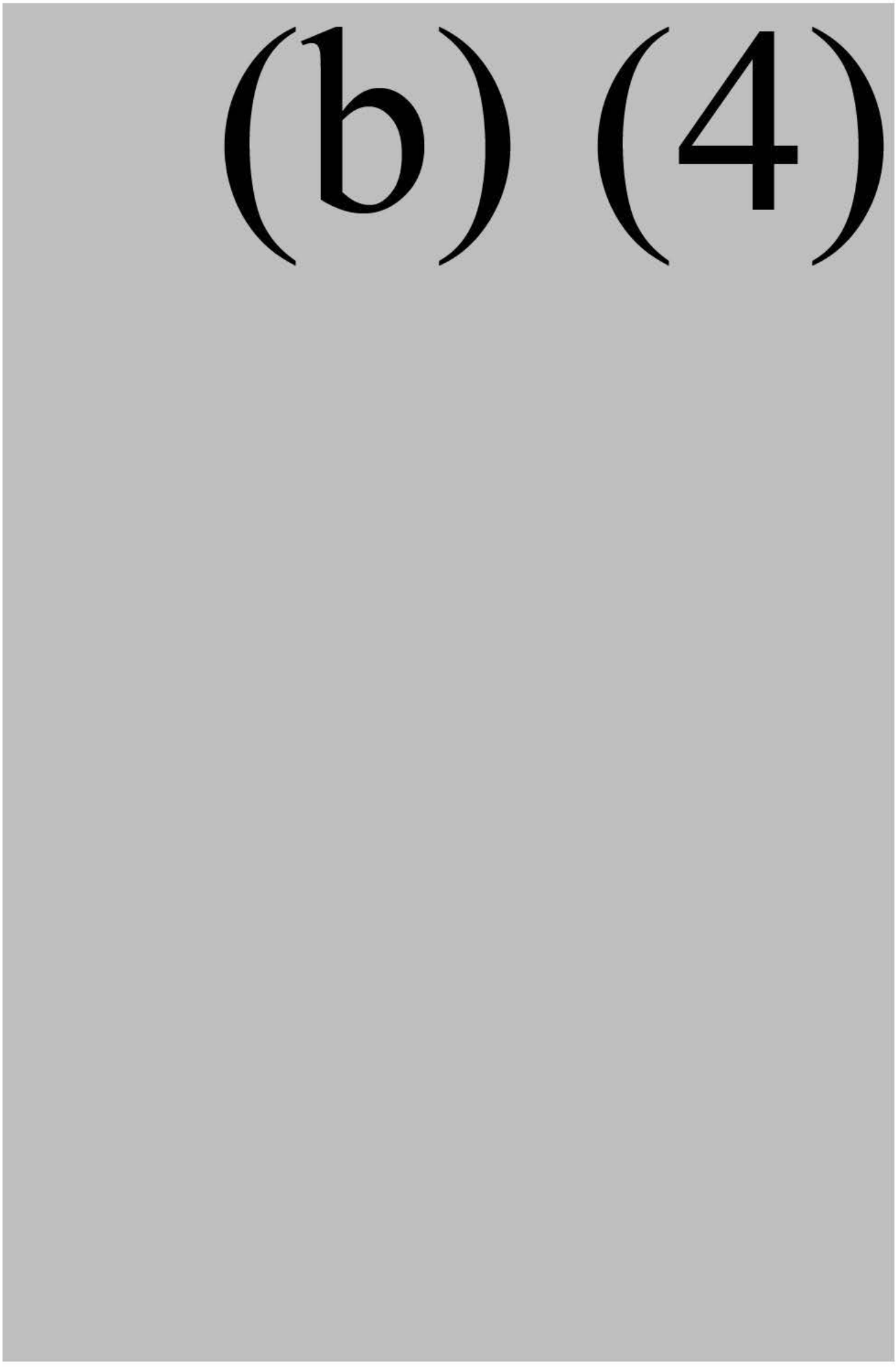
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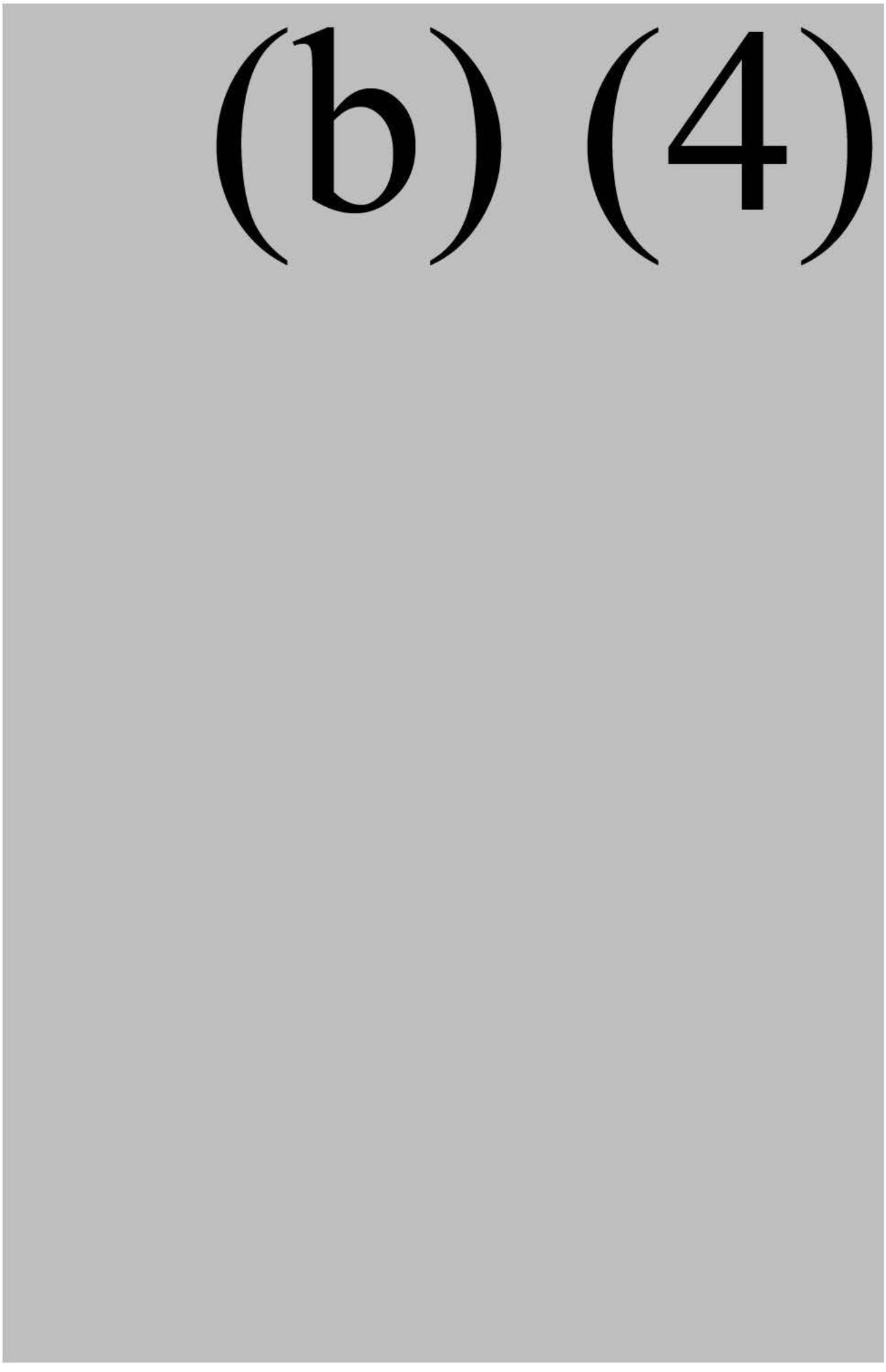
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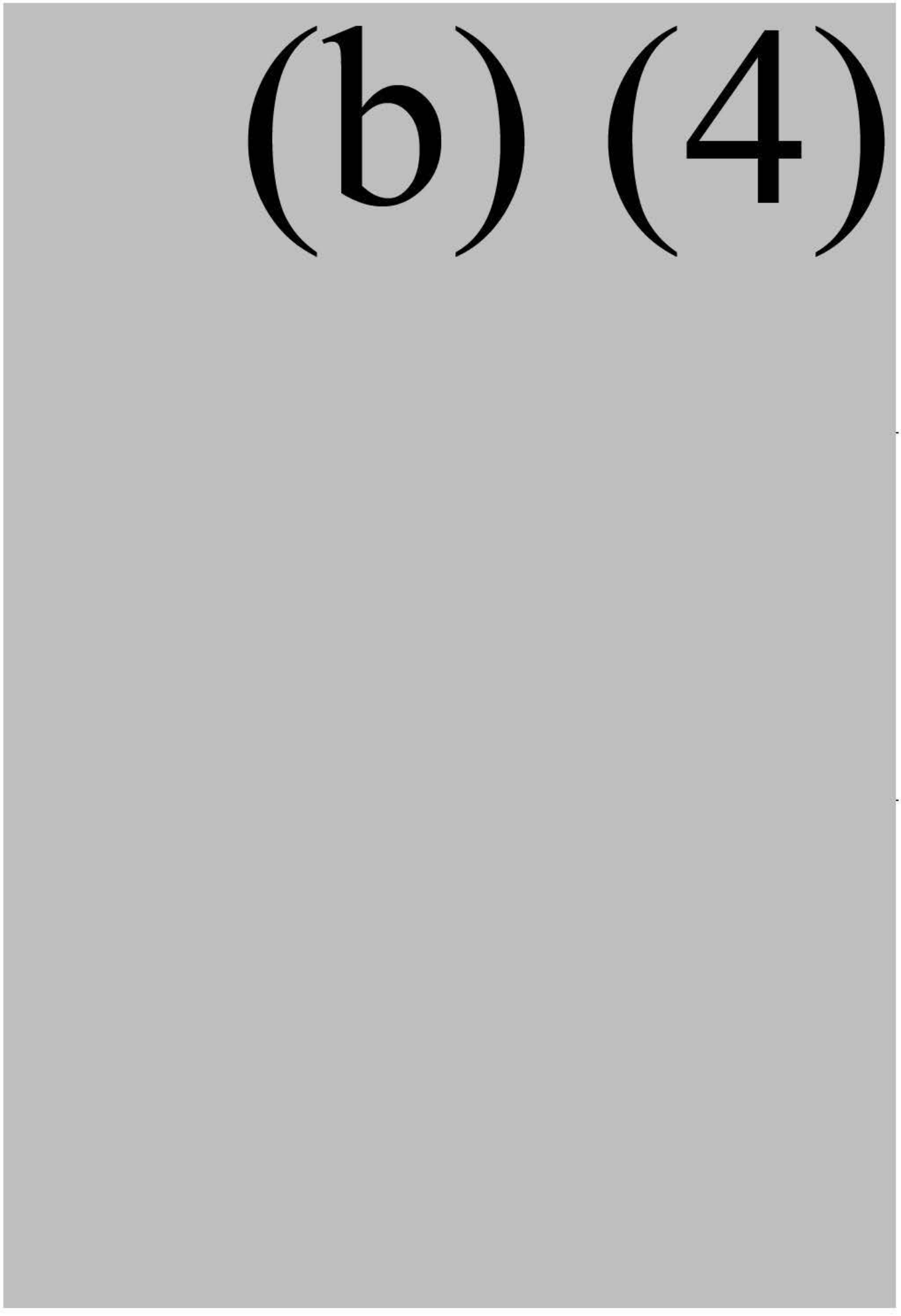
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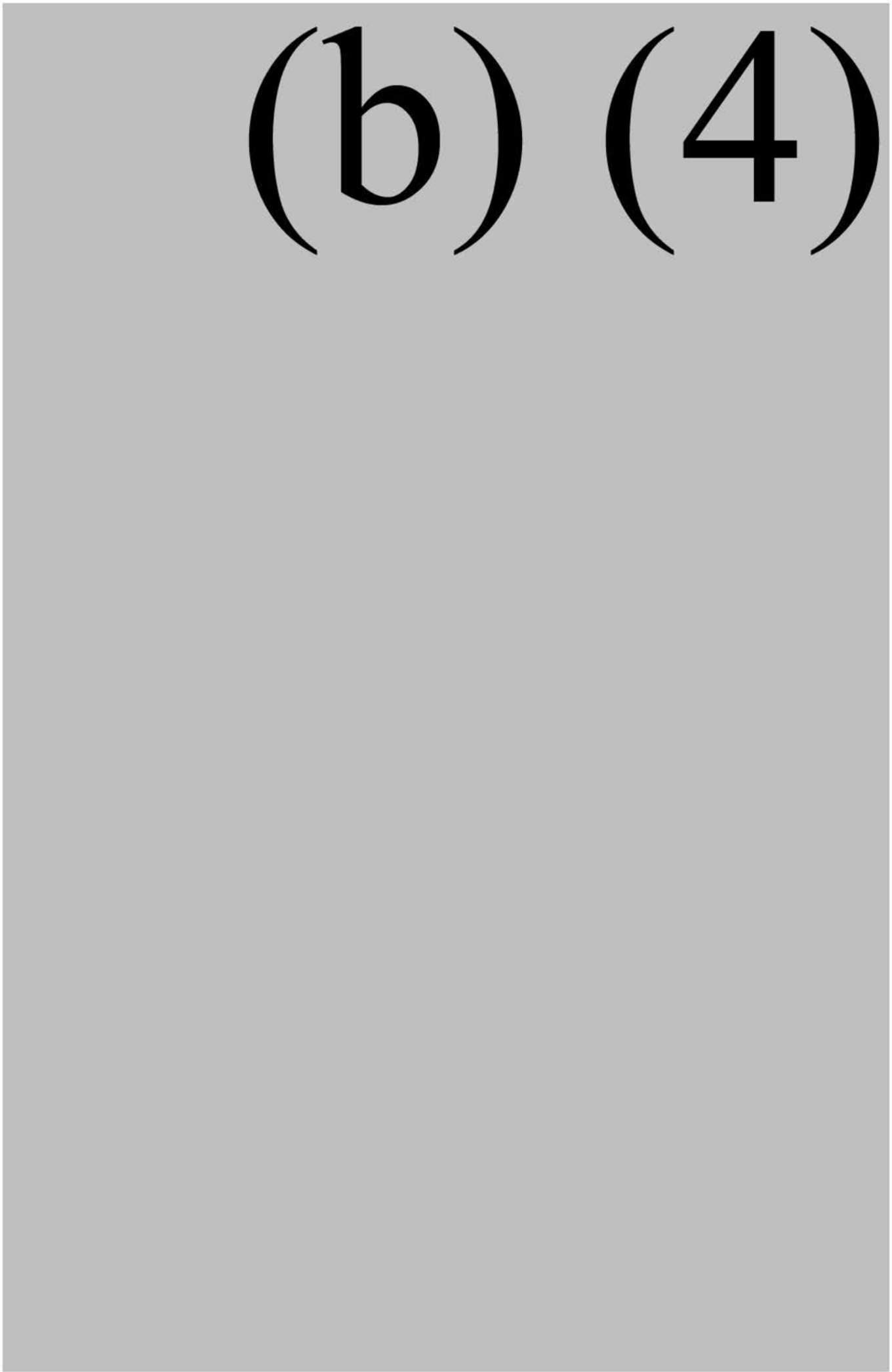
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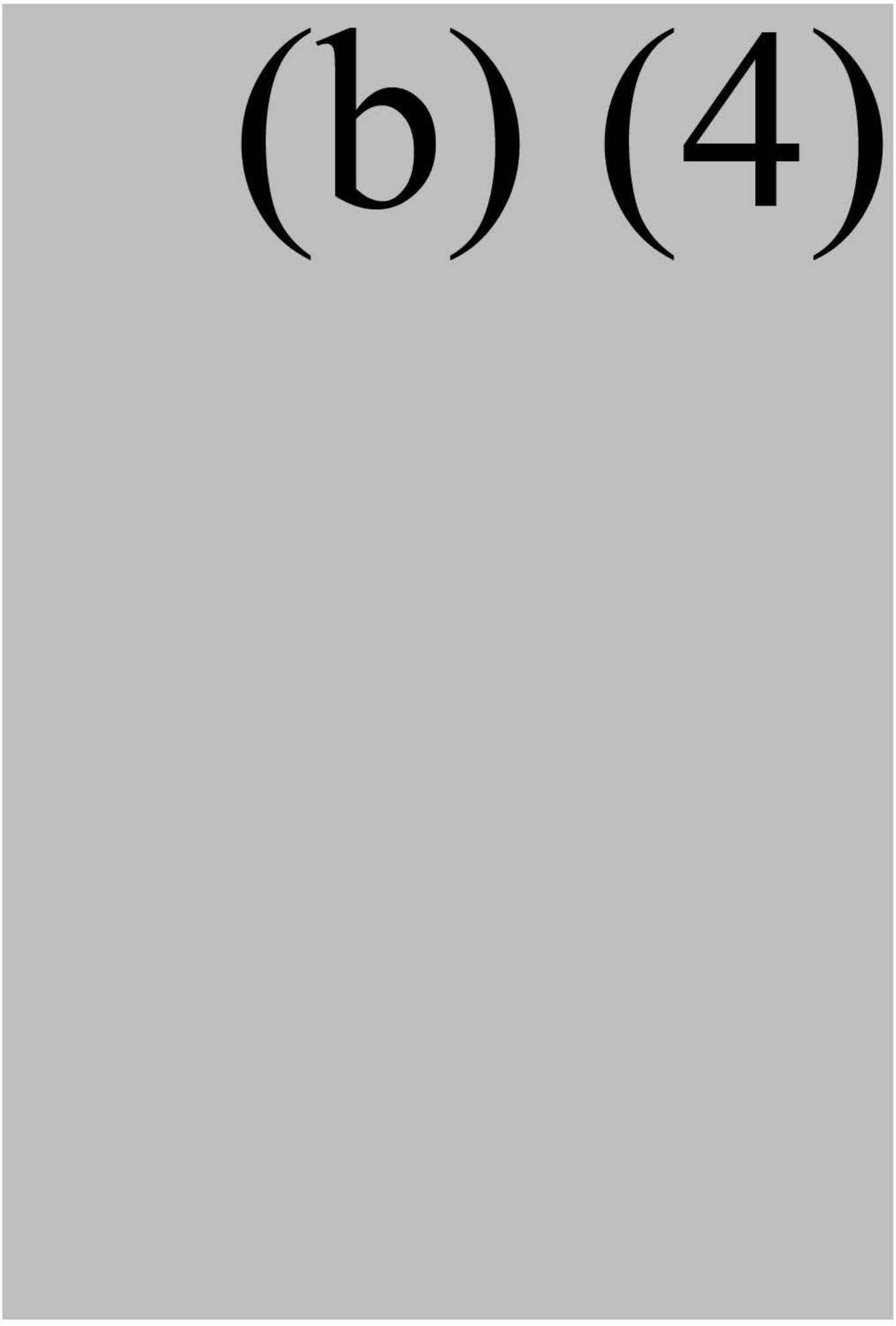
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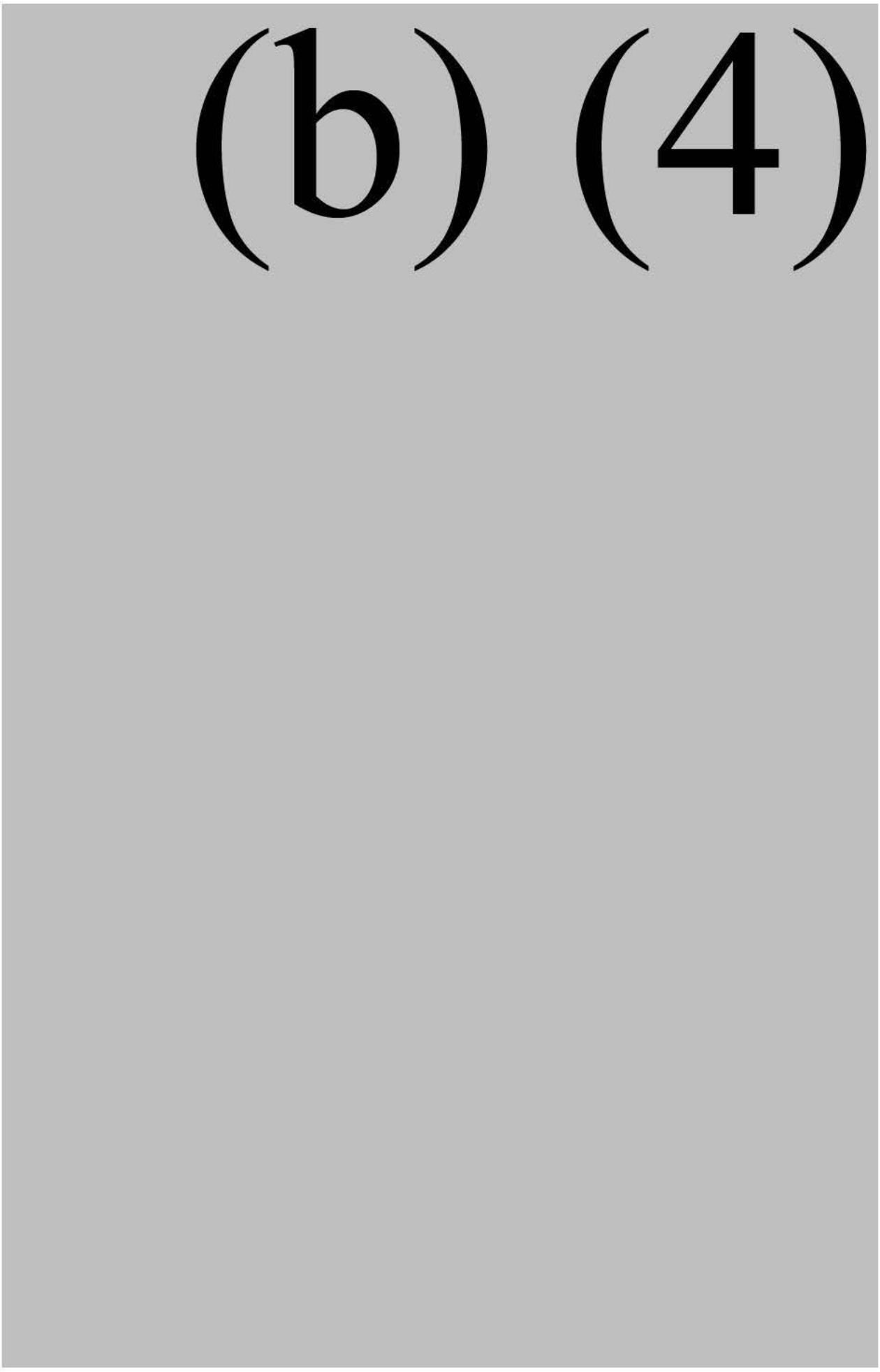
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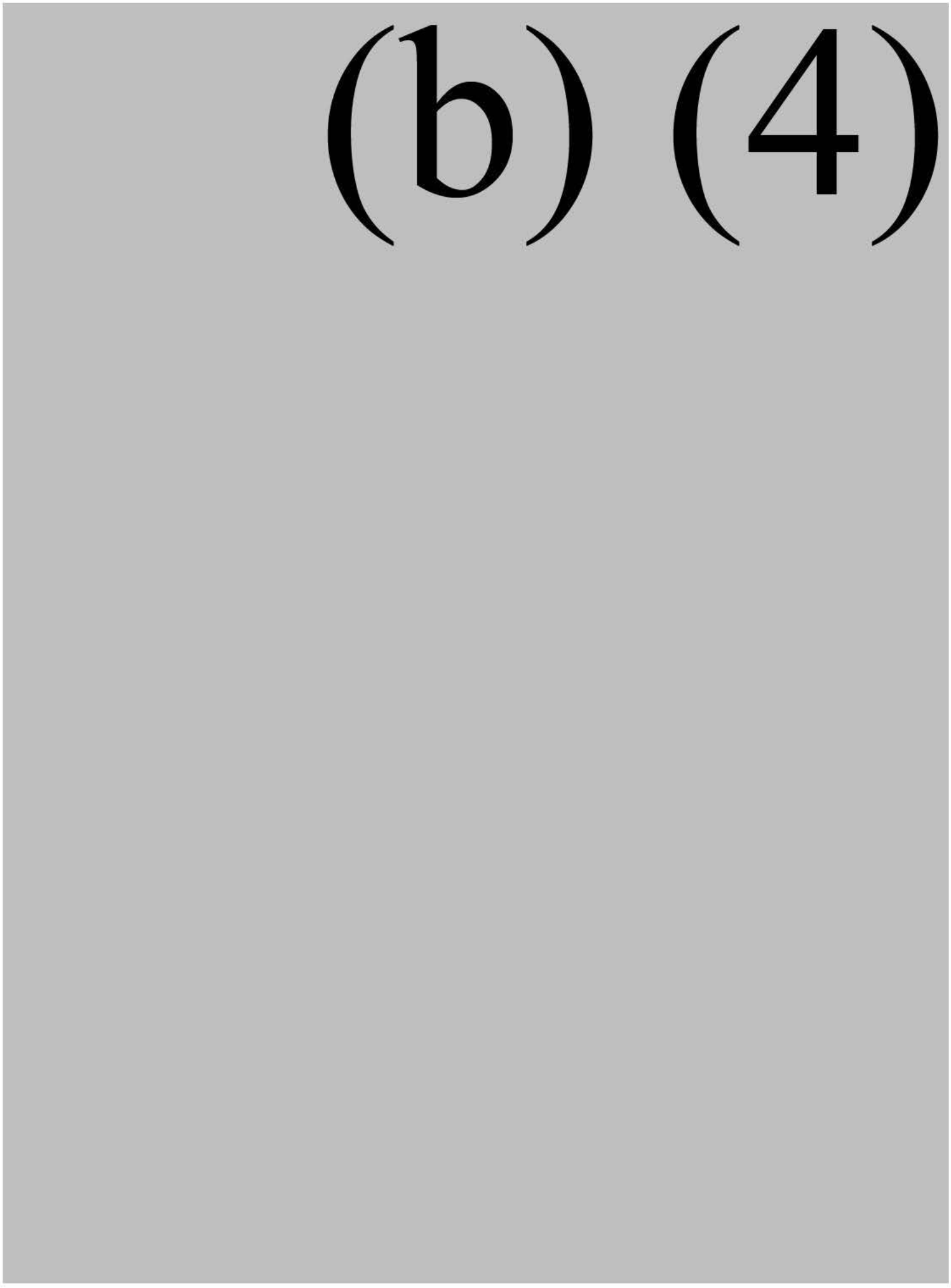
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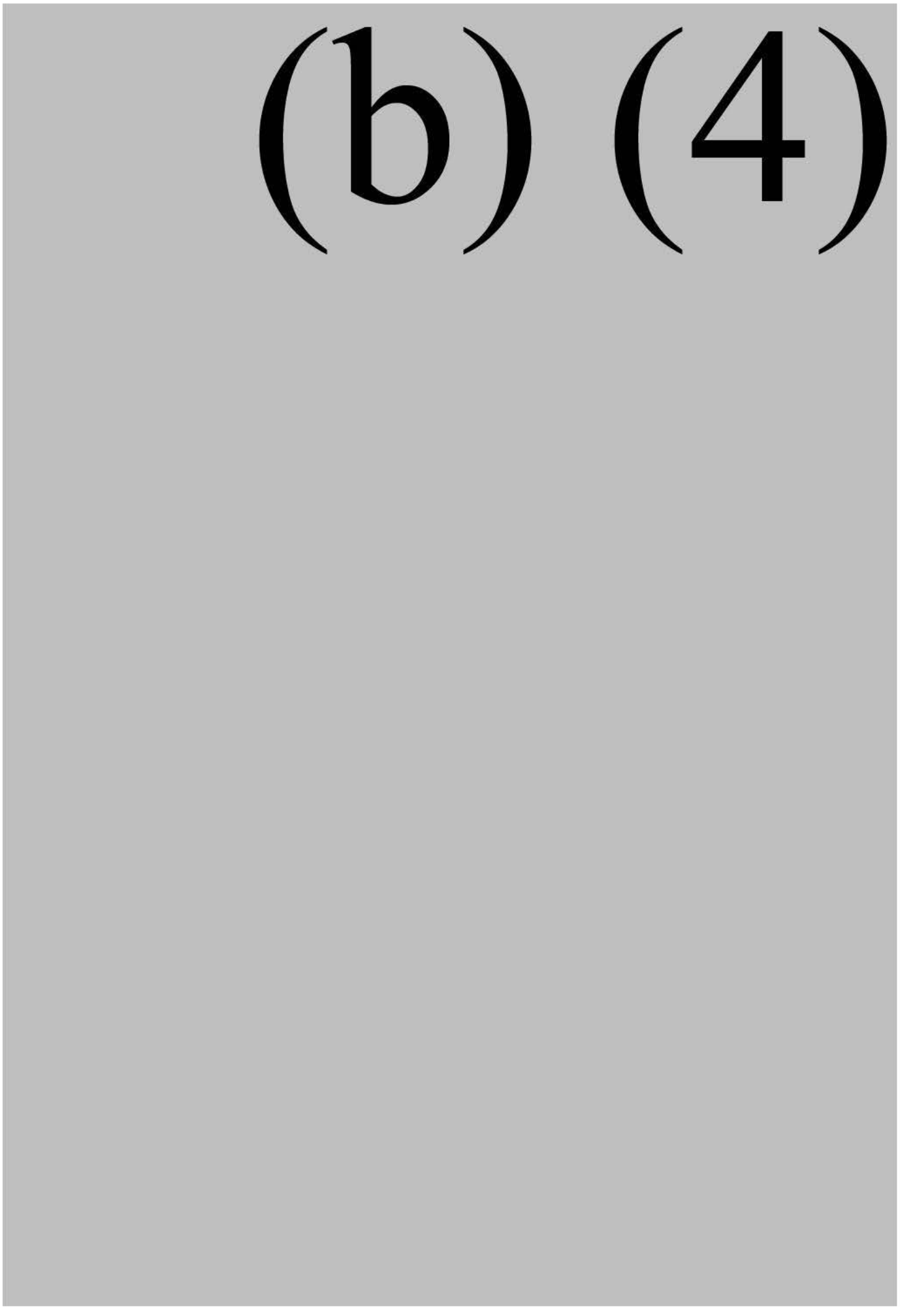
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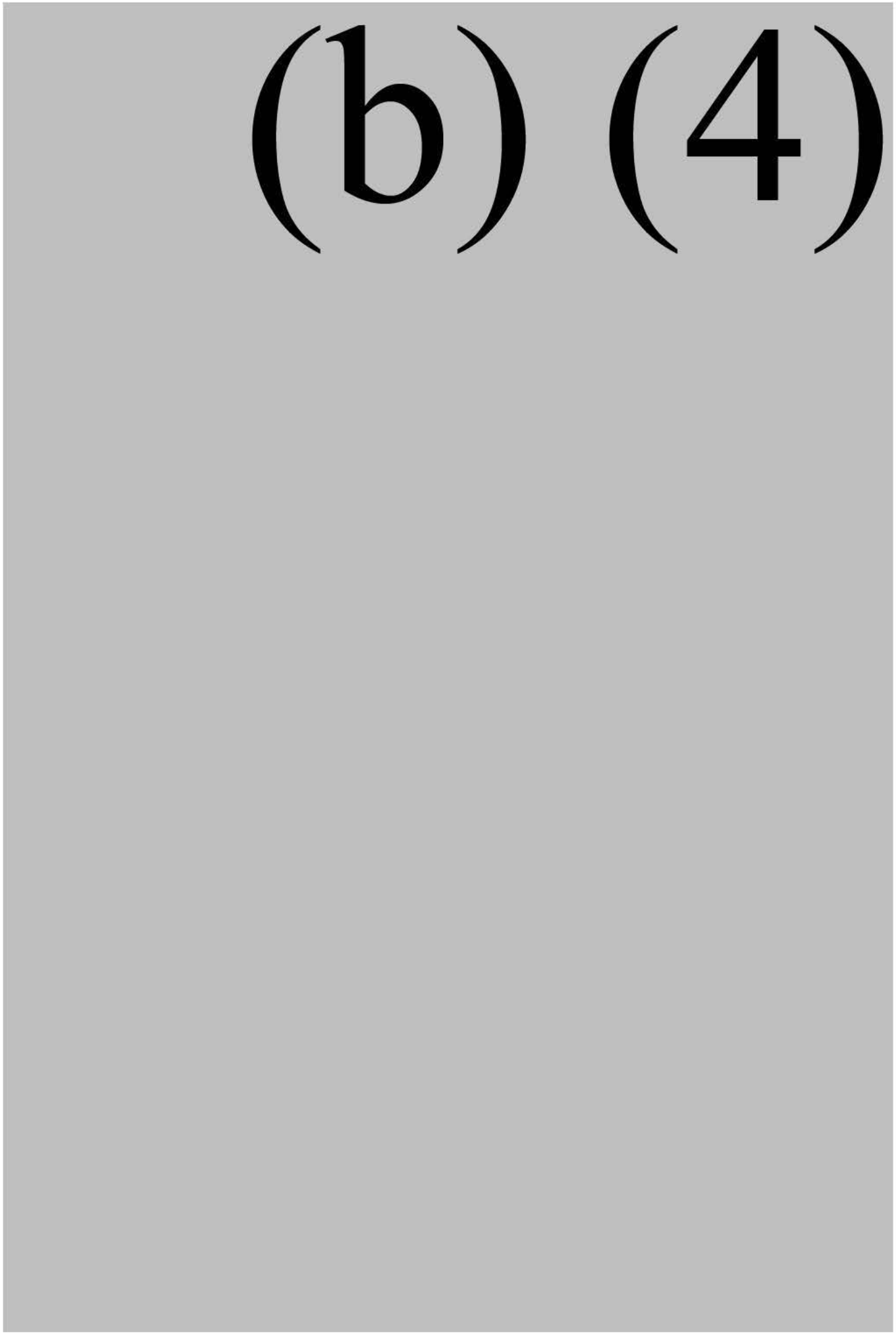
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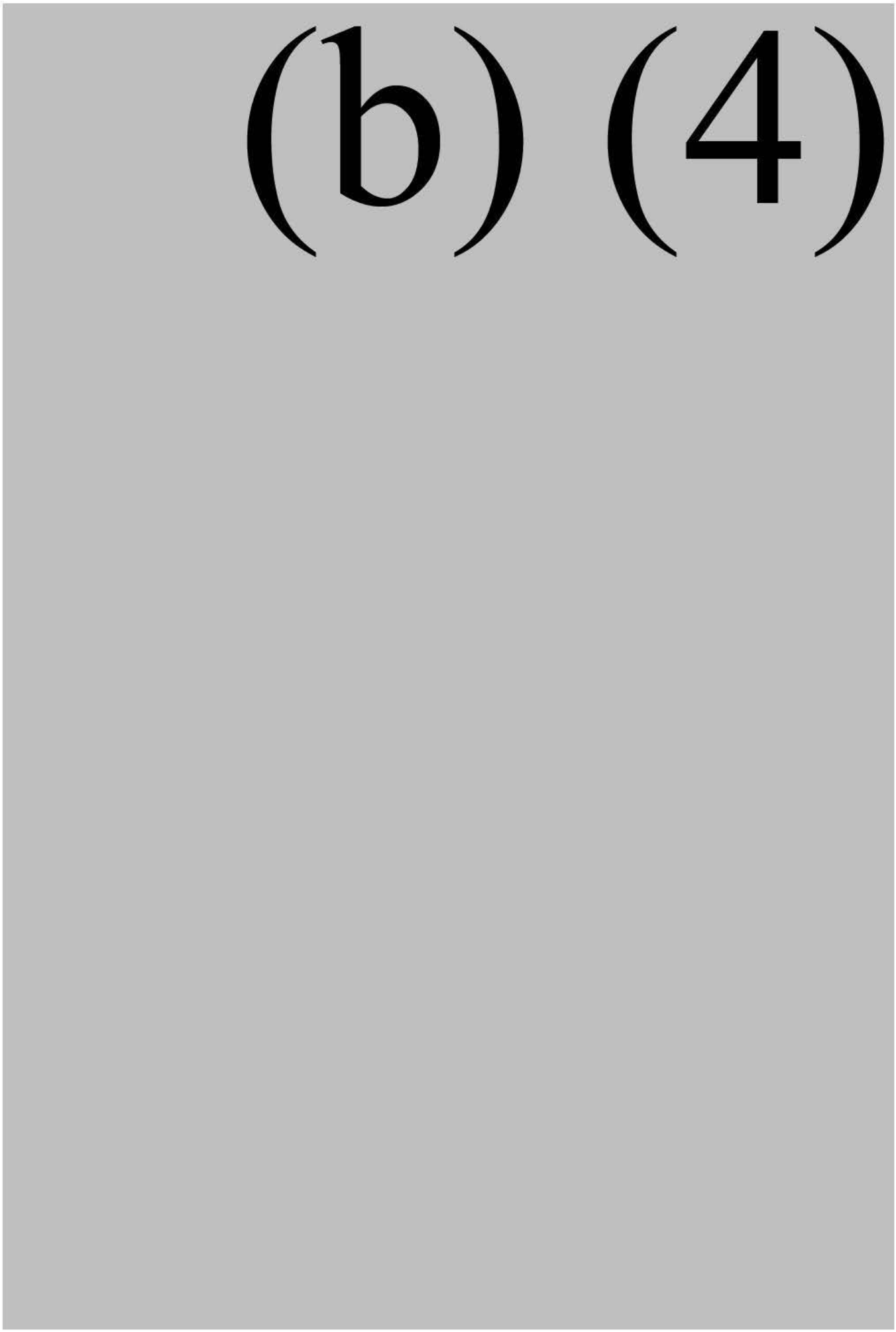
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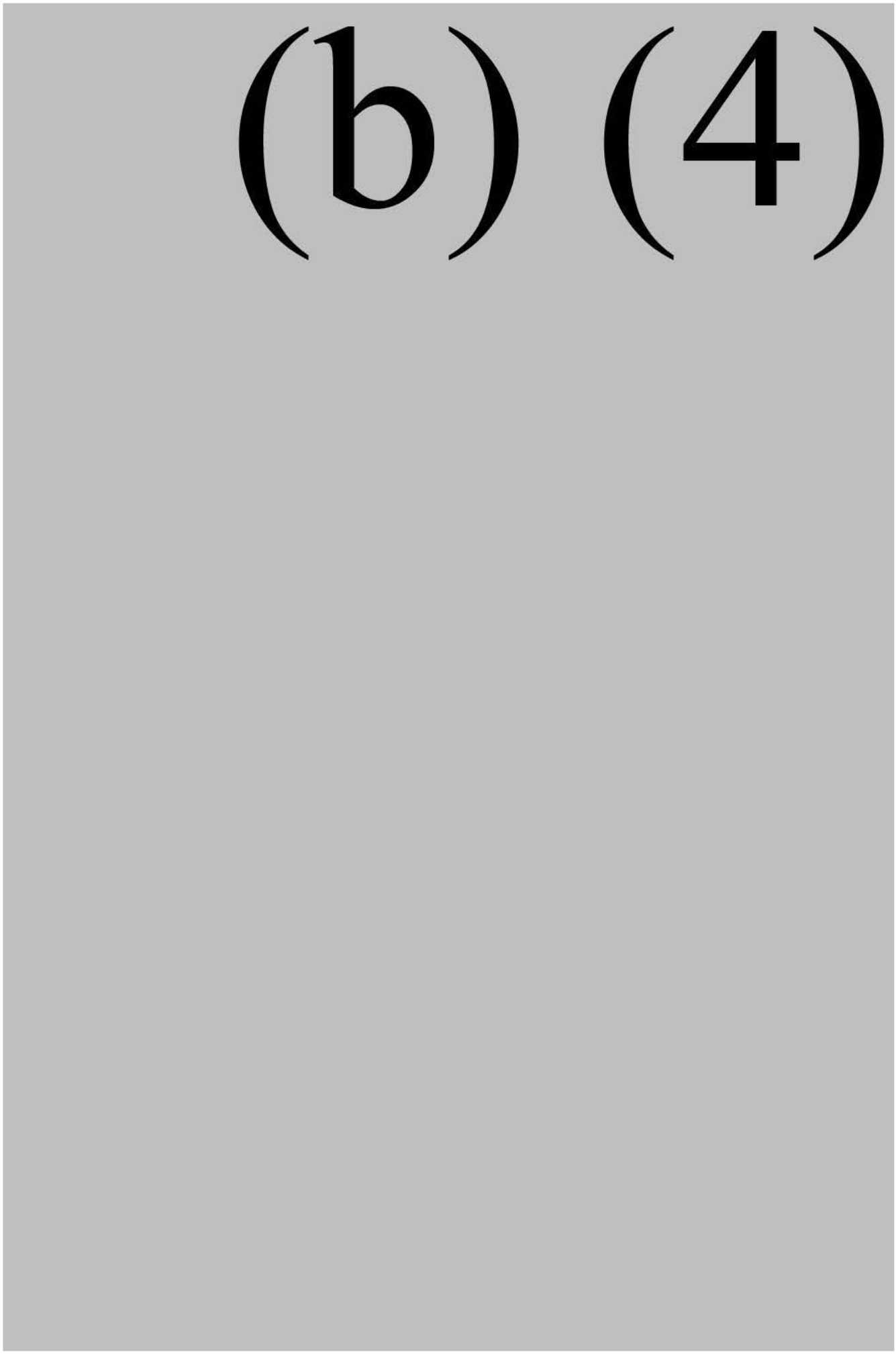
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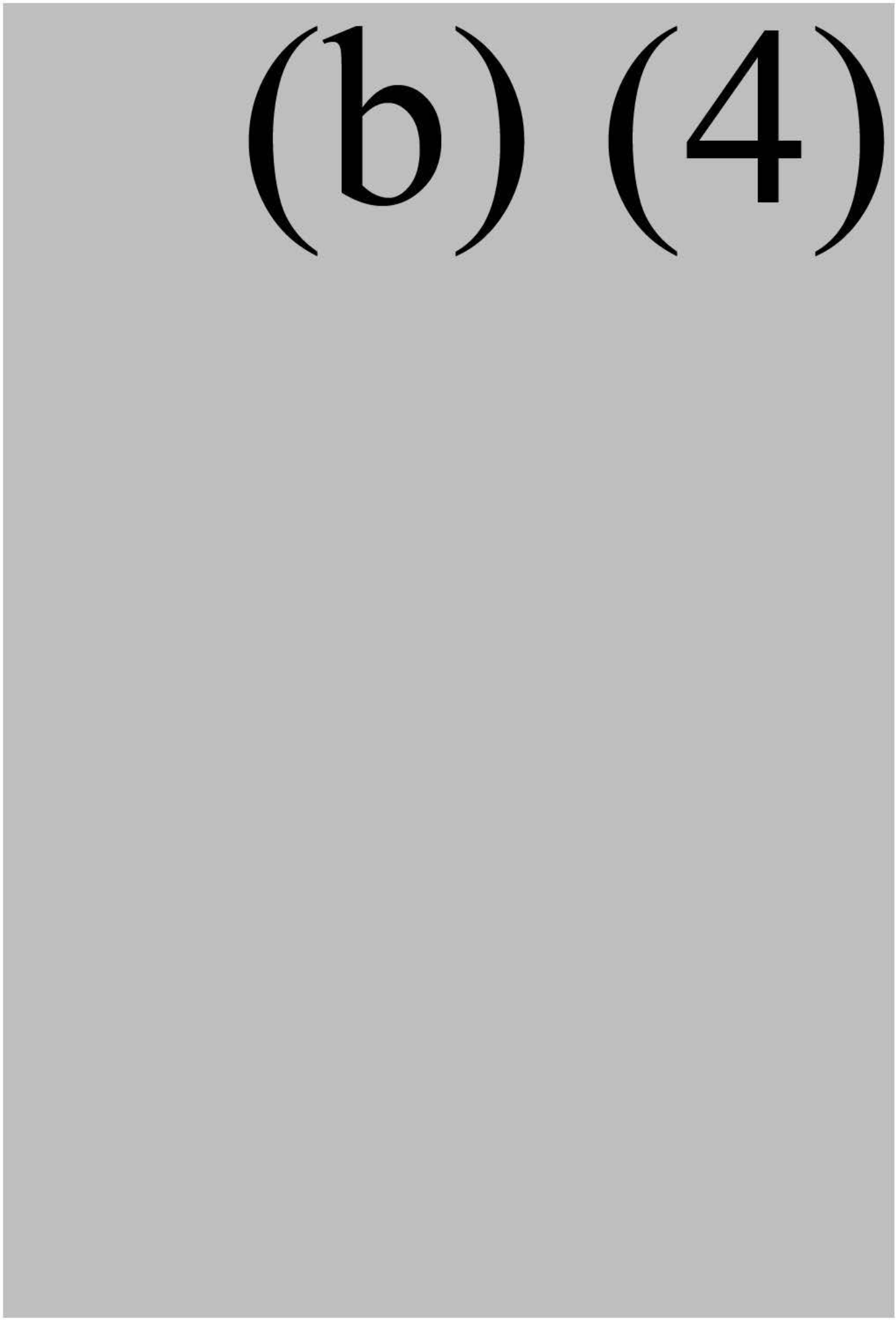
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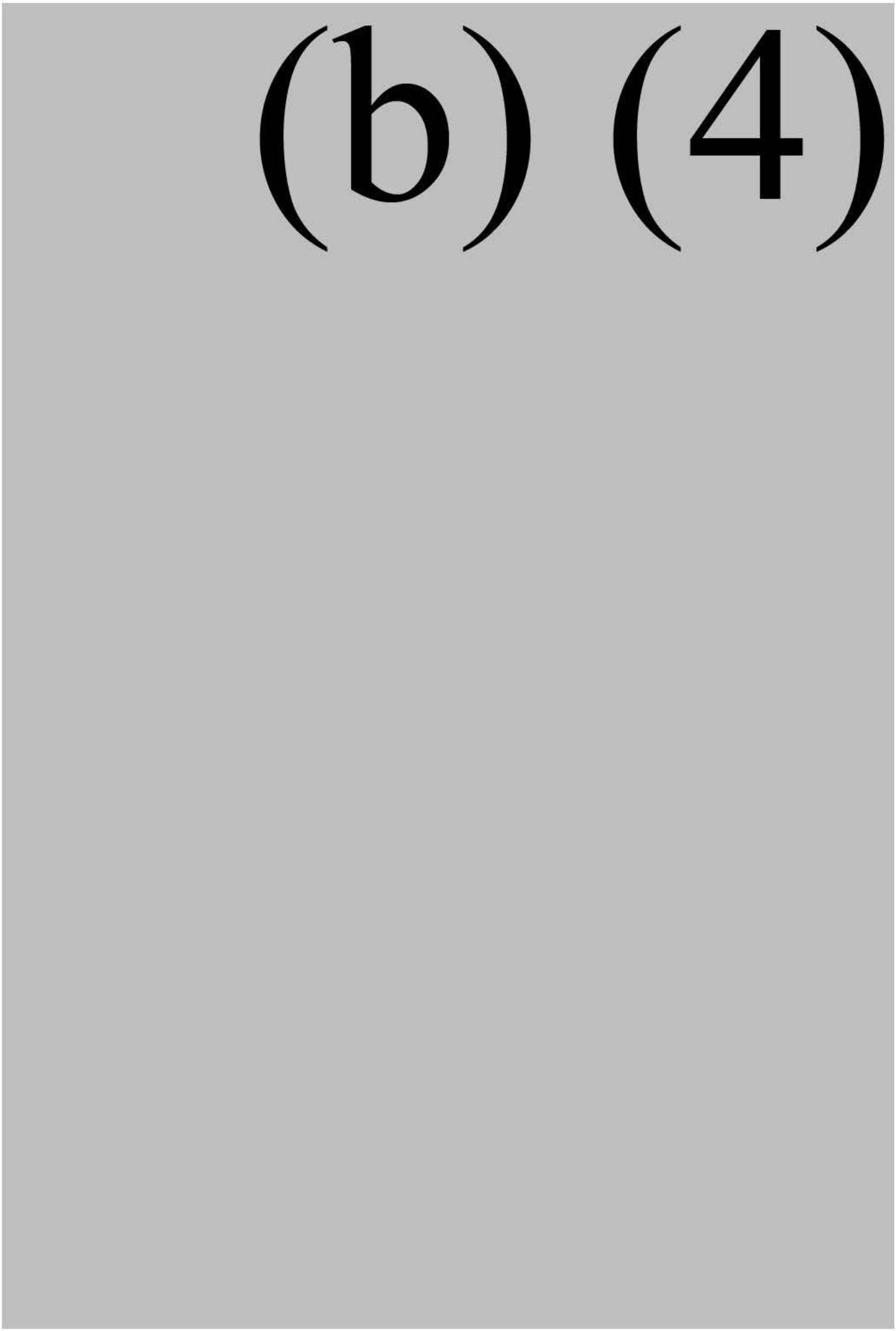
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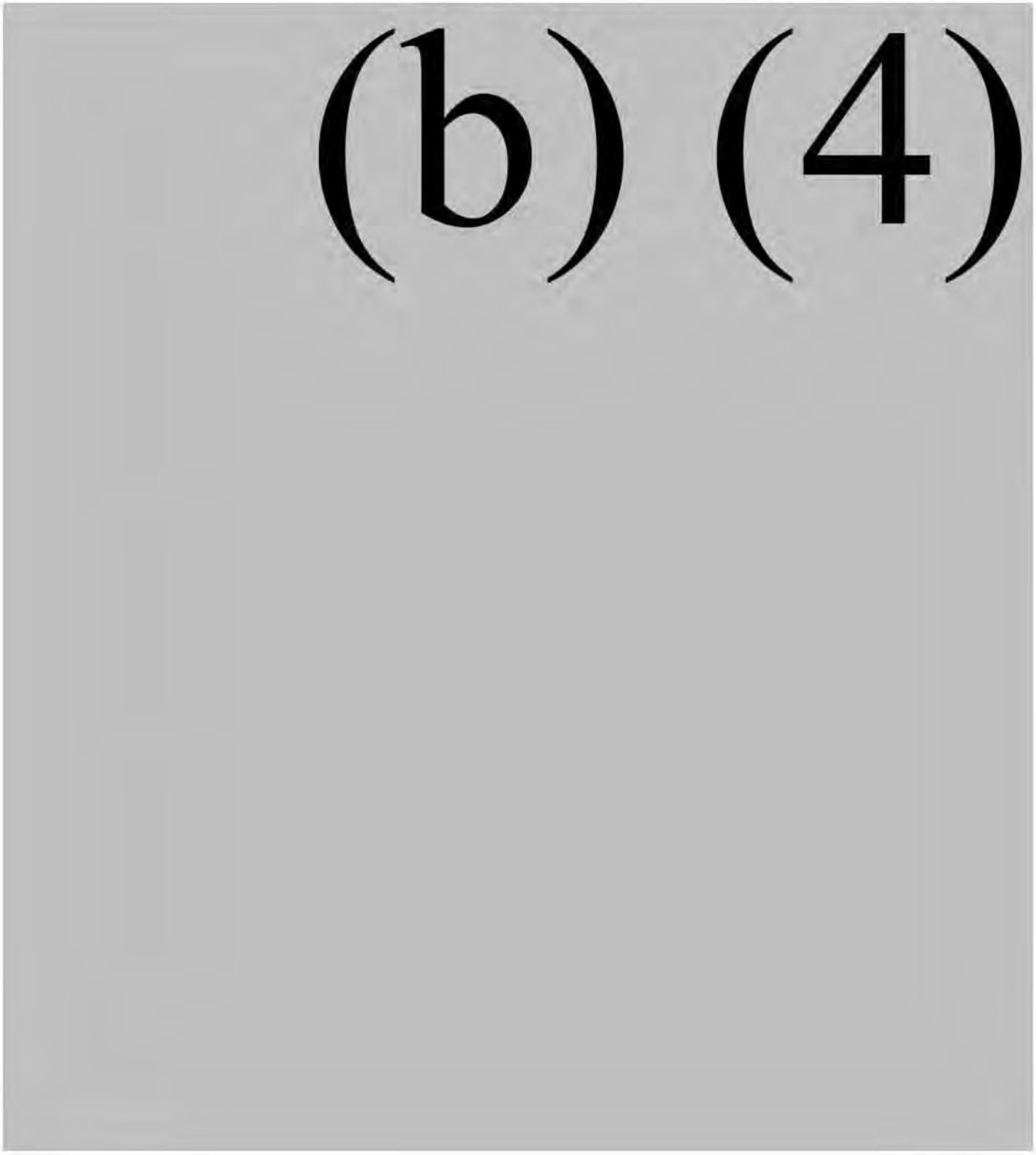

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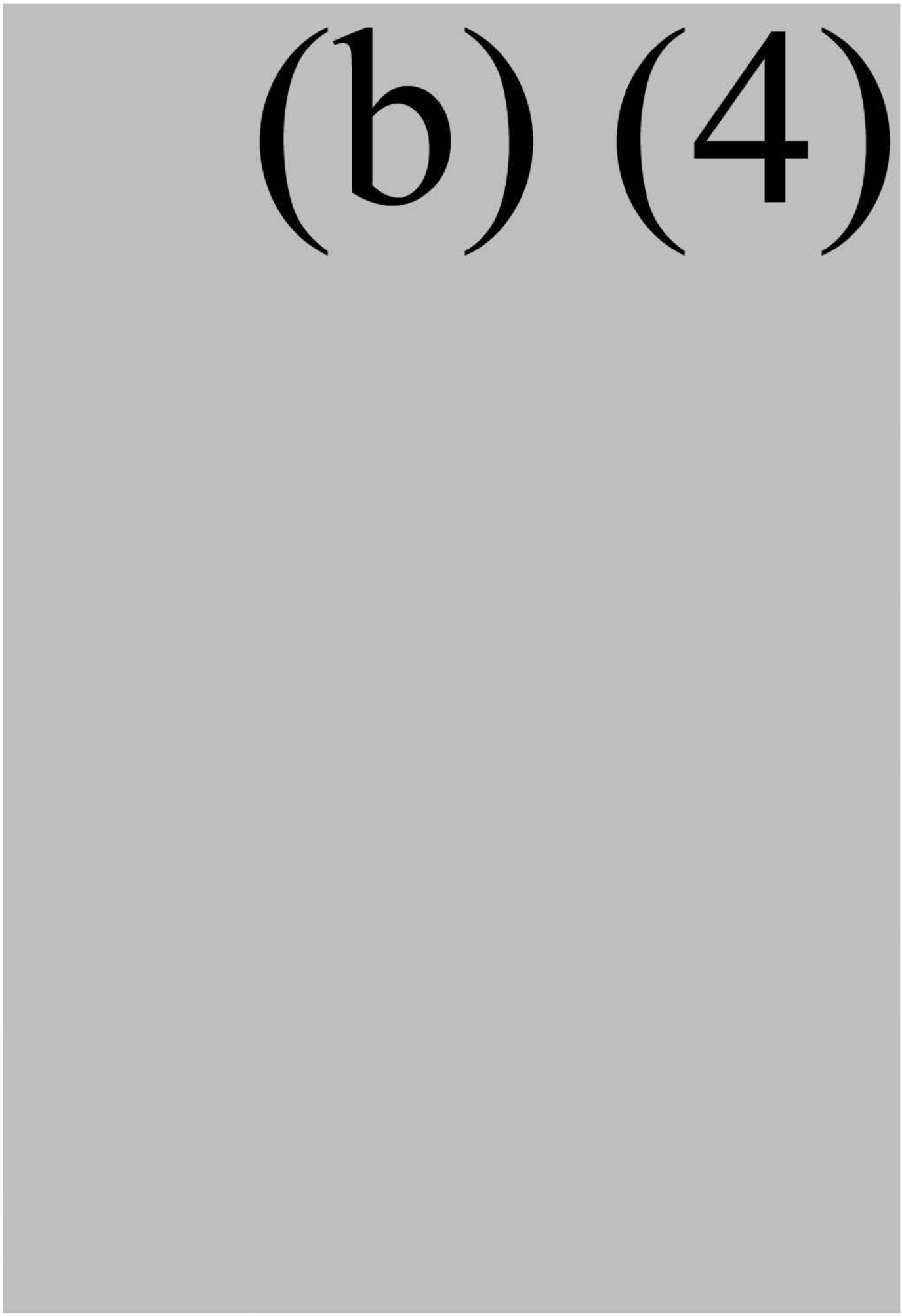
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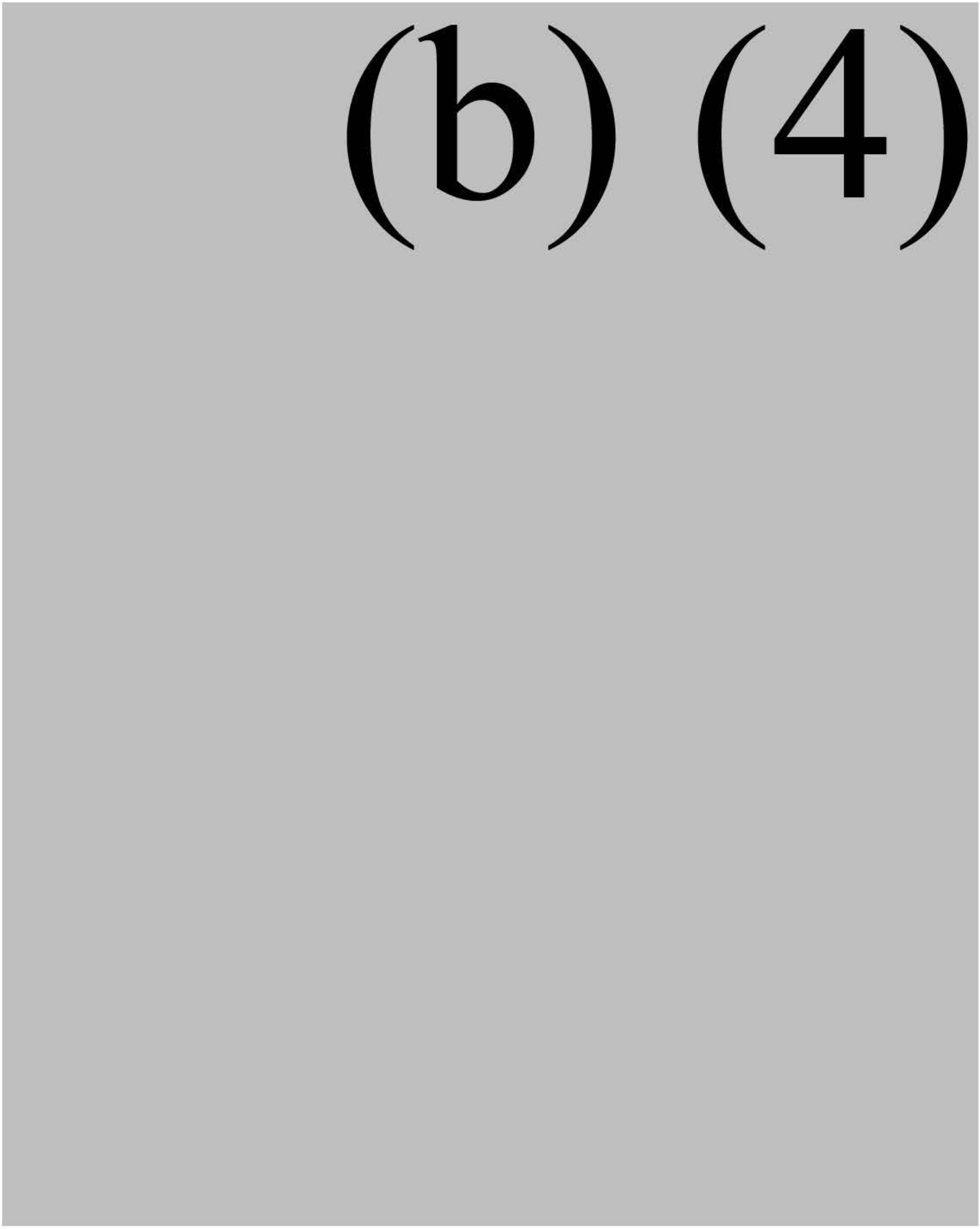
APPENDIX 4

(b) (4)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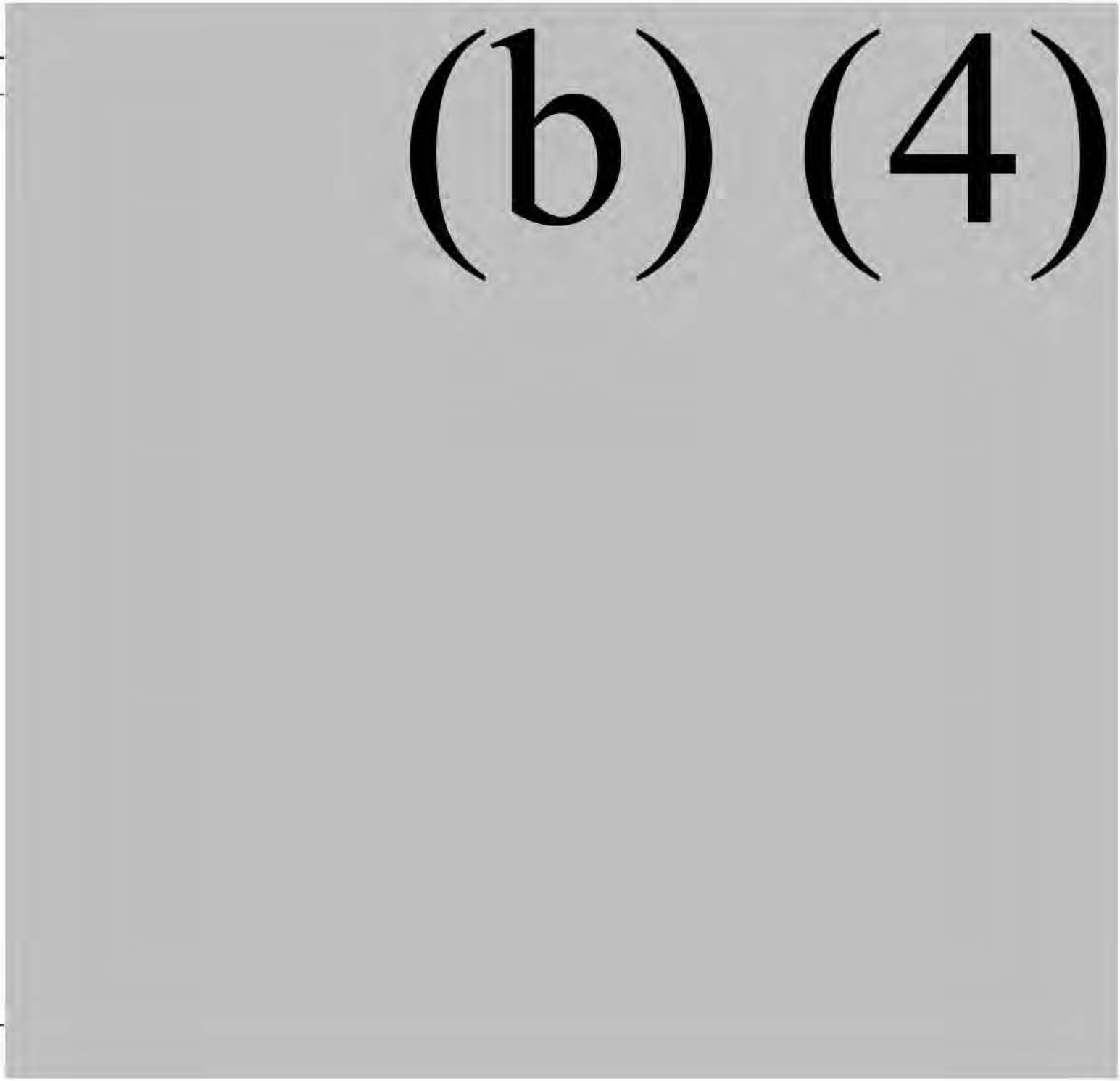
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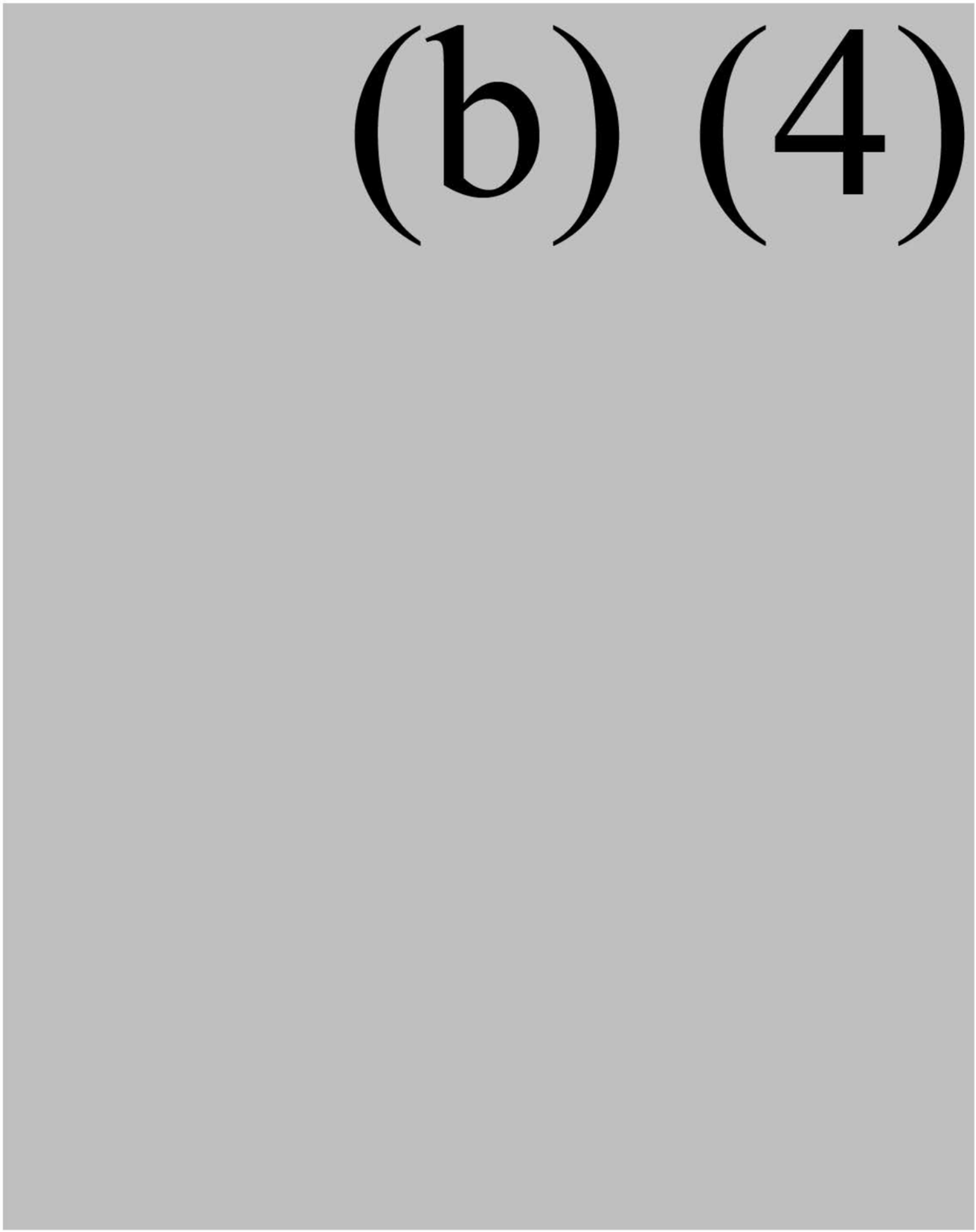
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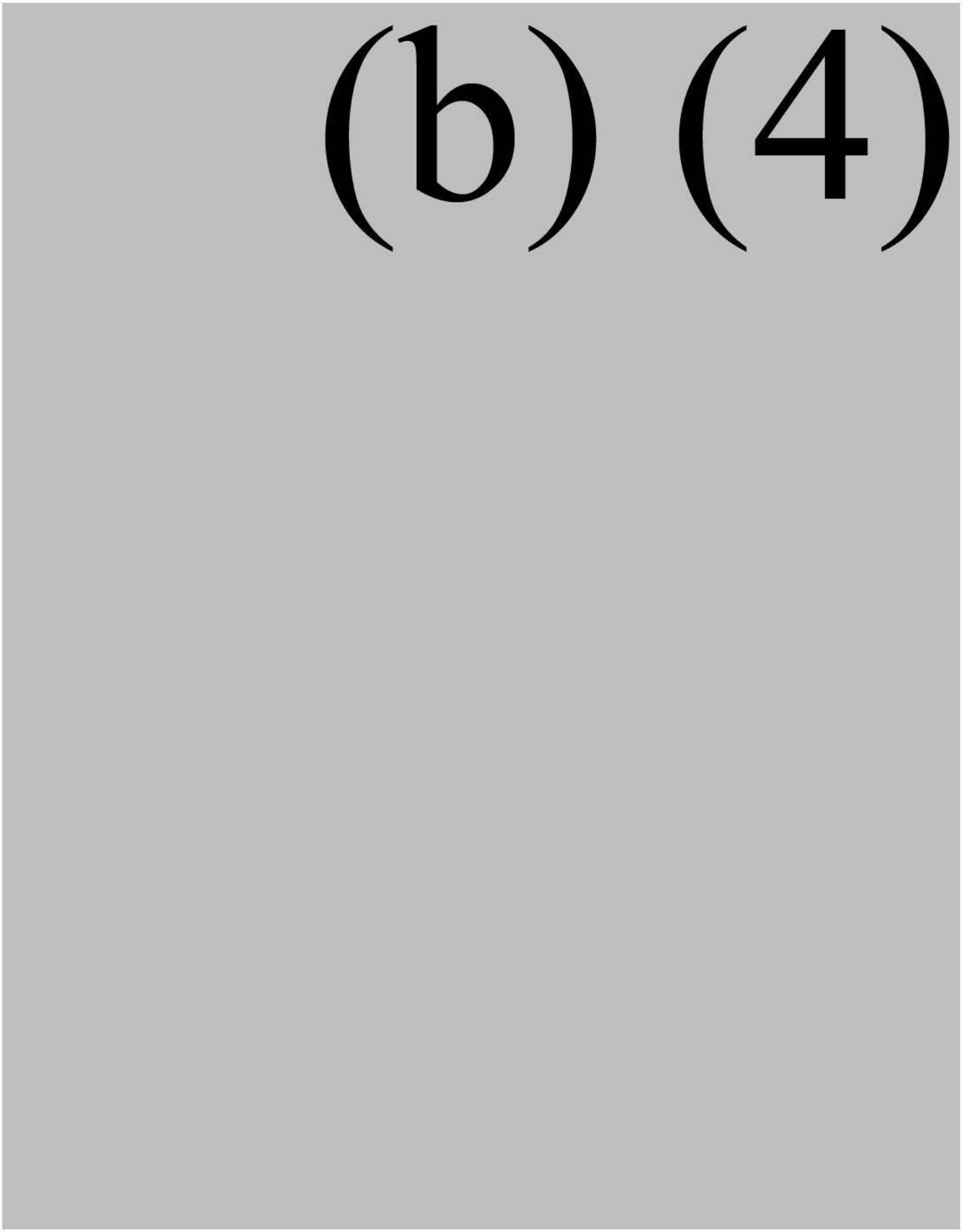
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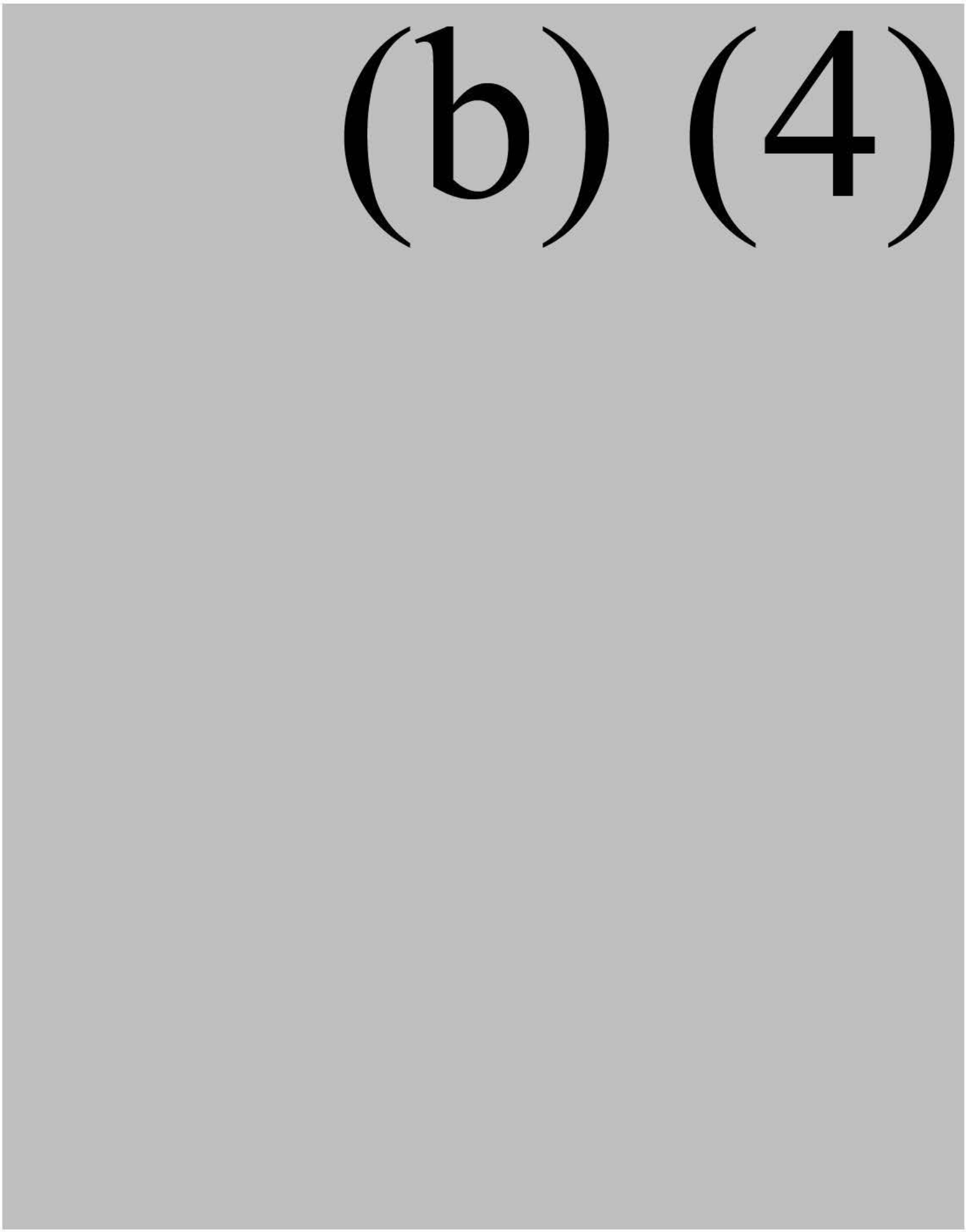
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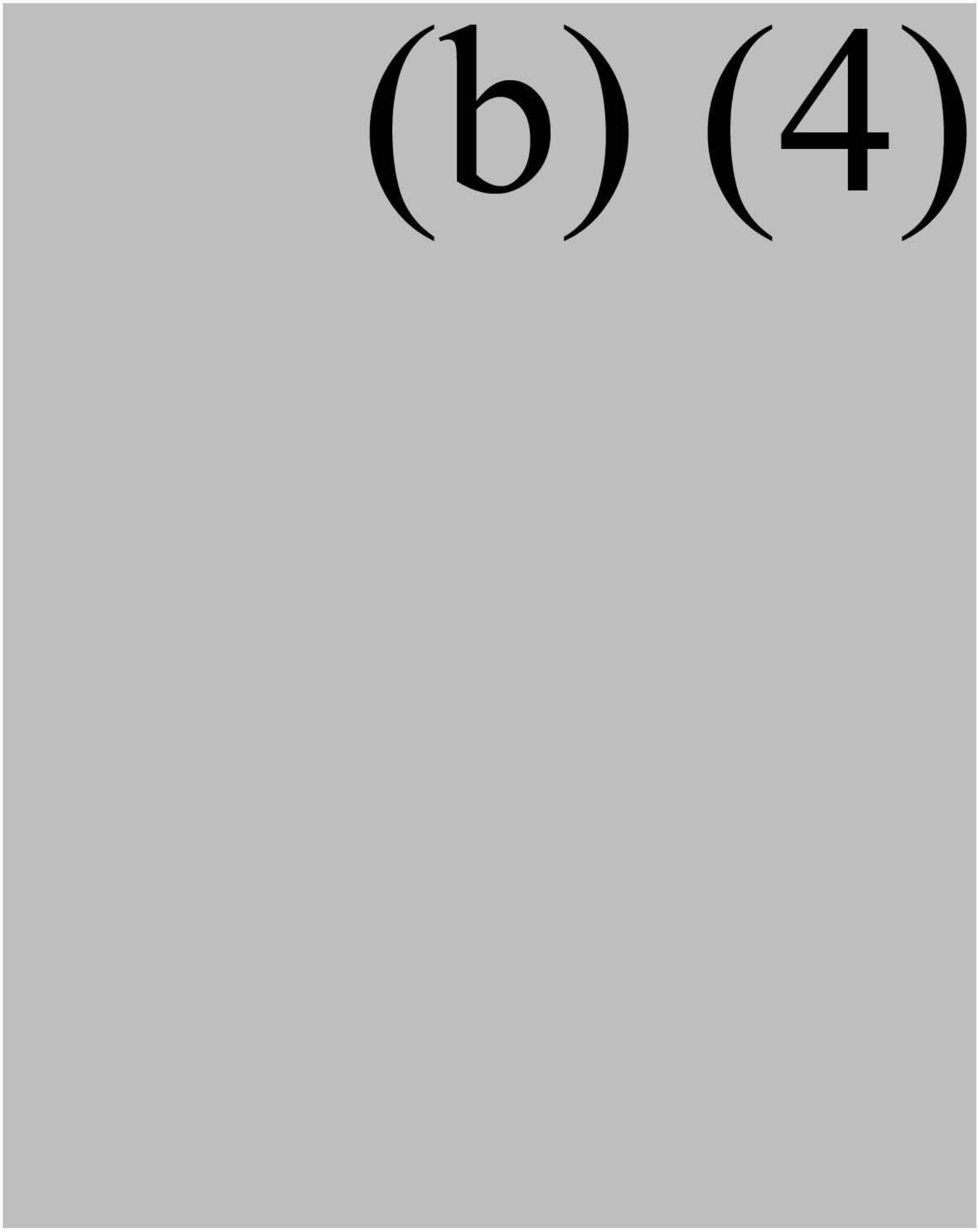
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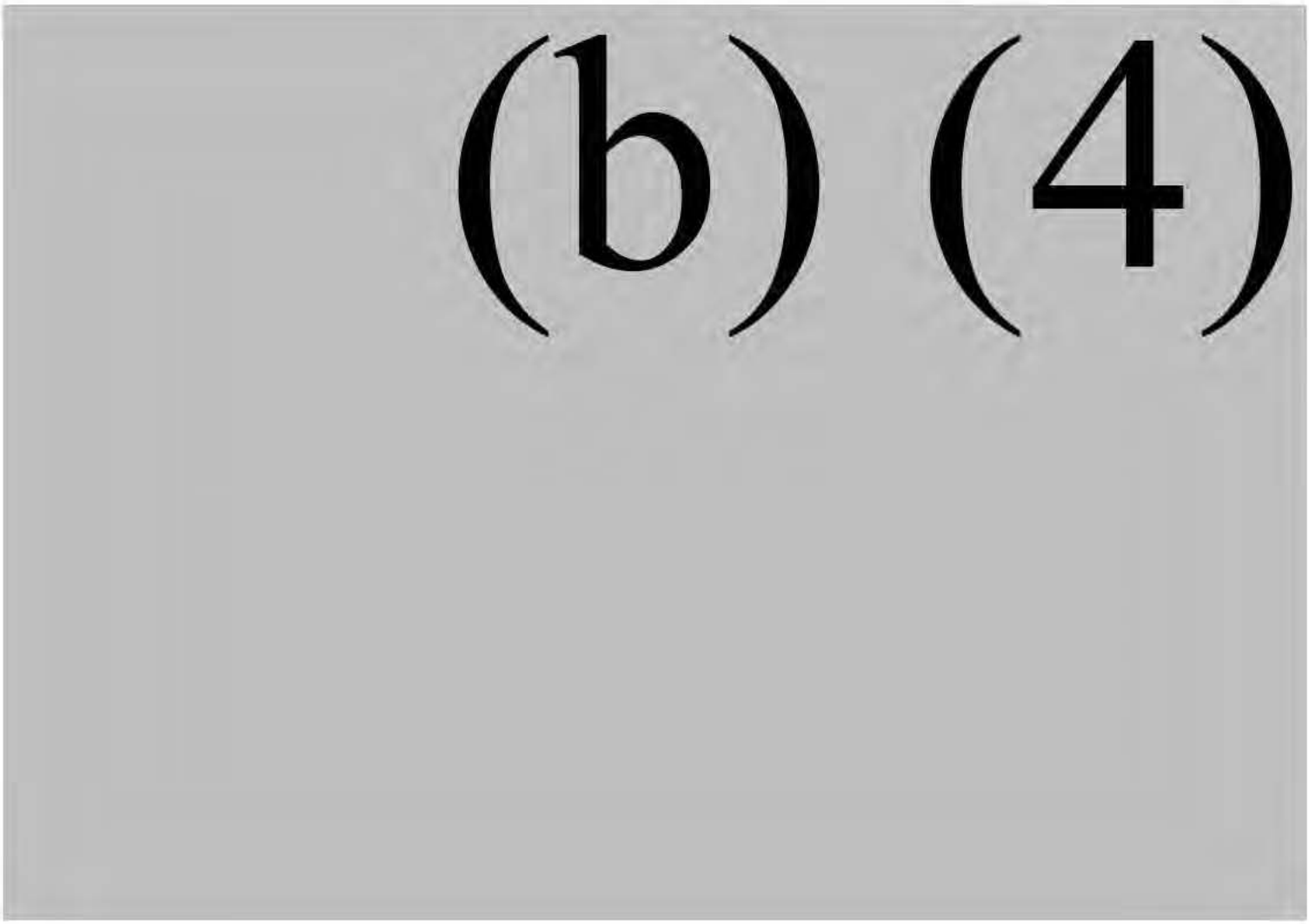
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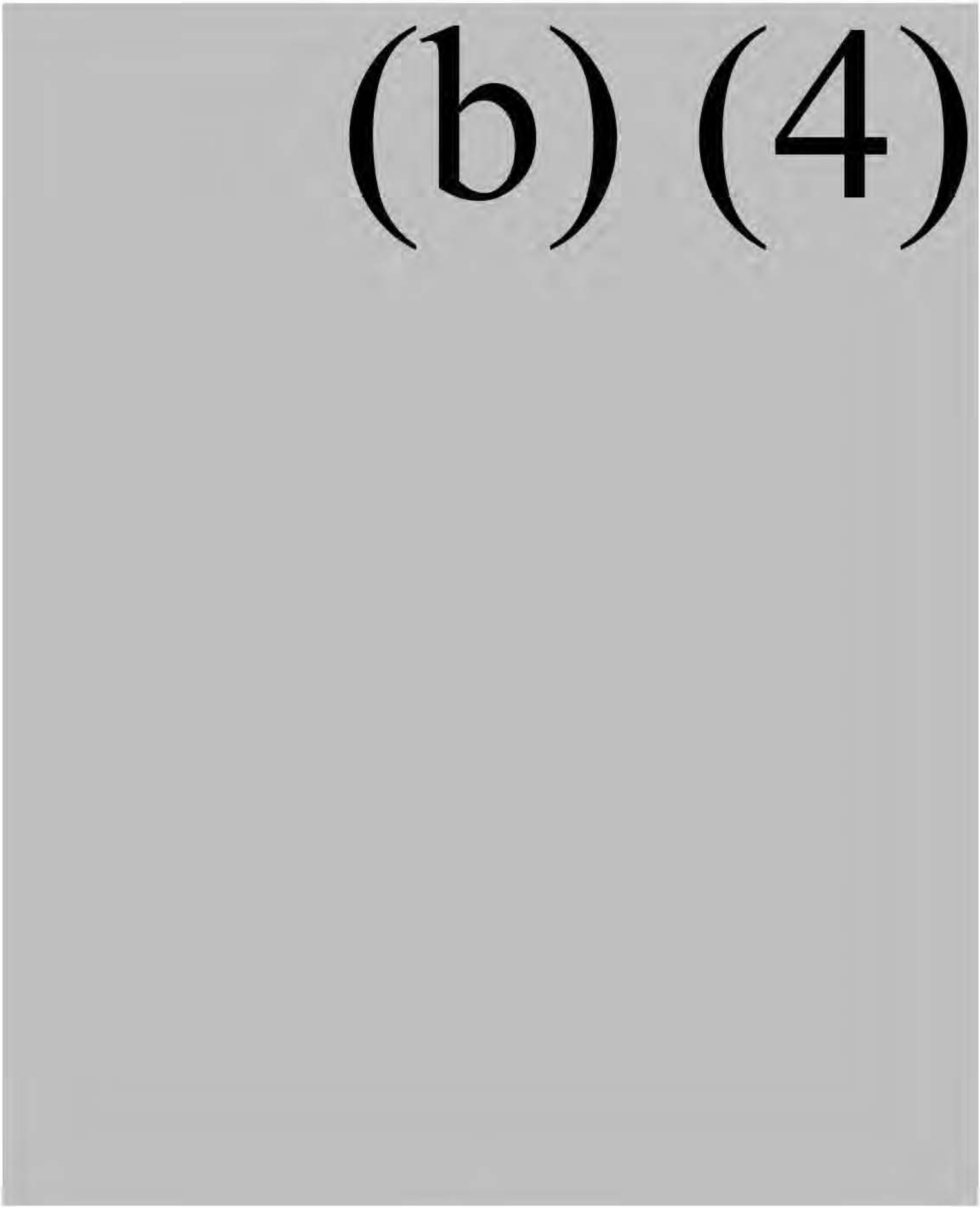
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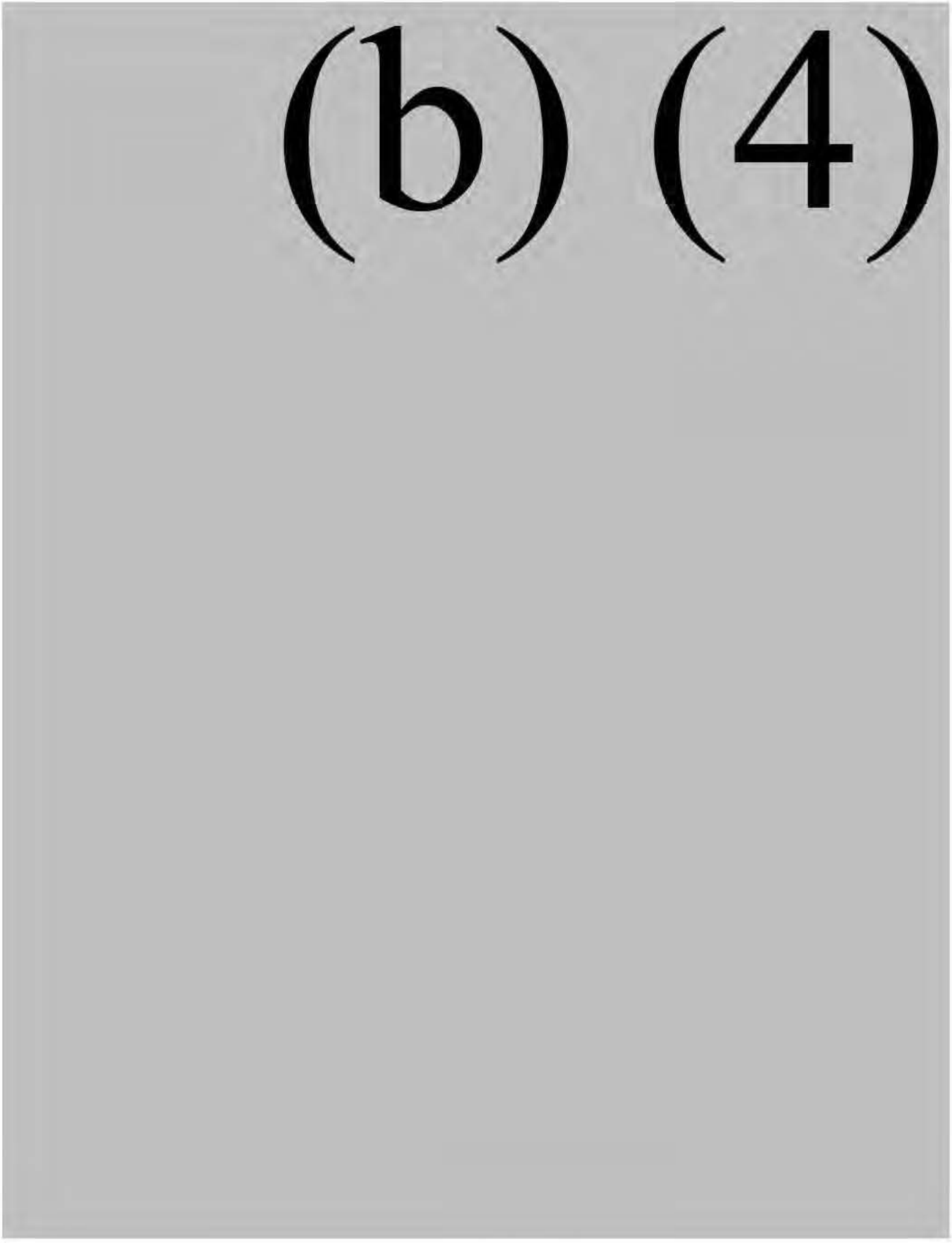
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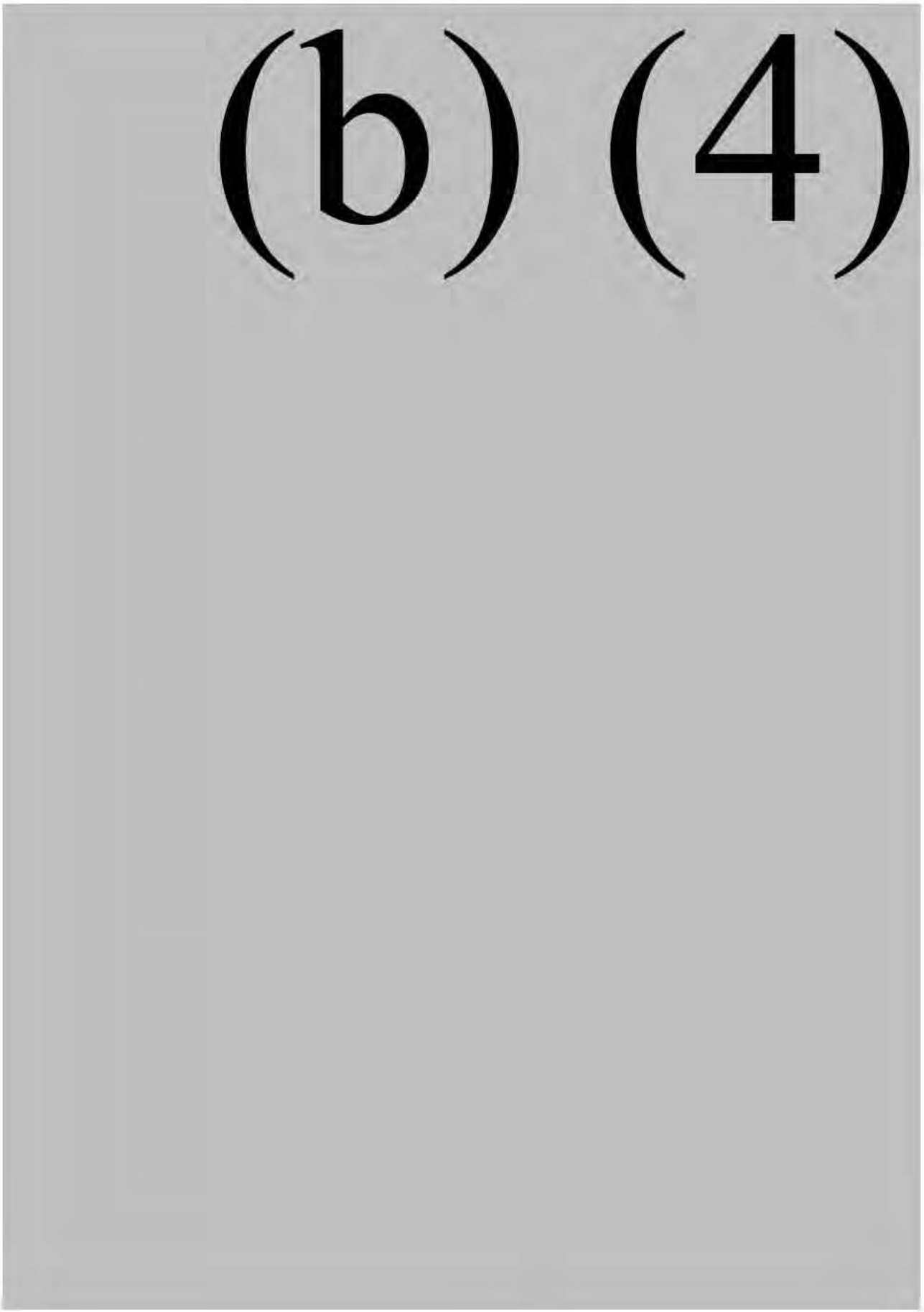
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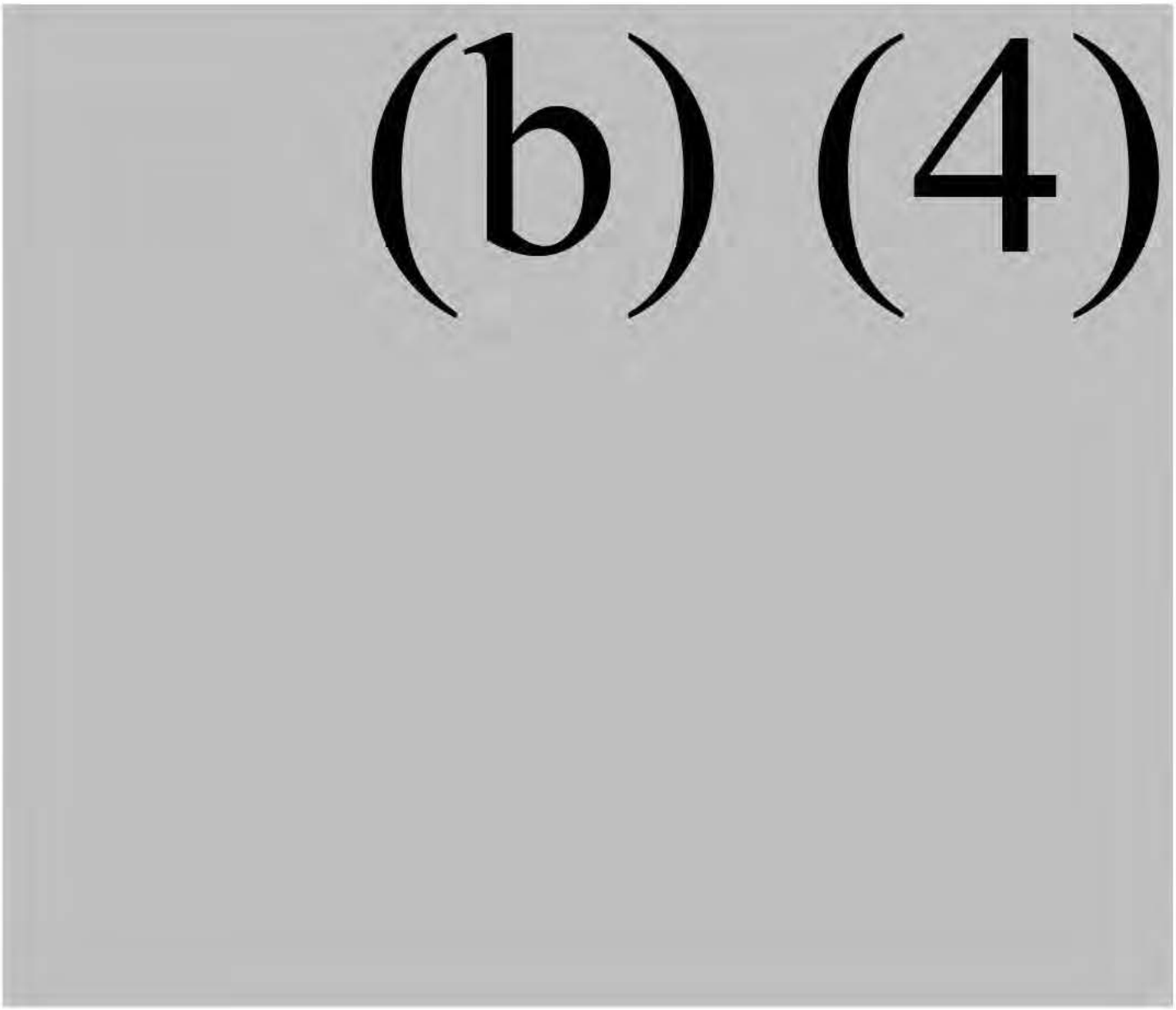
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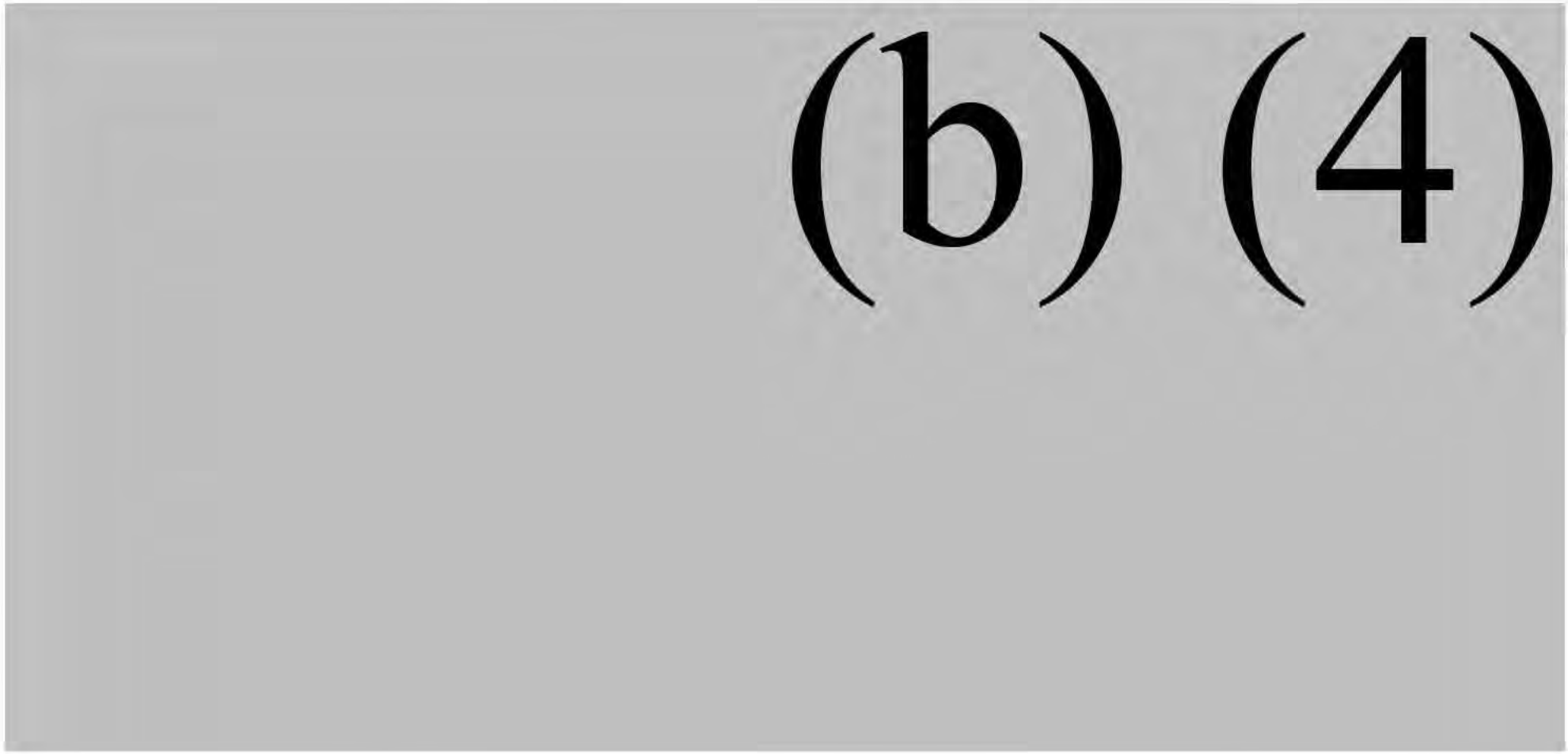
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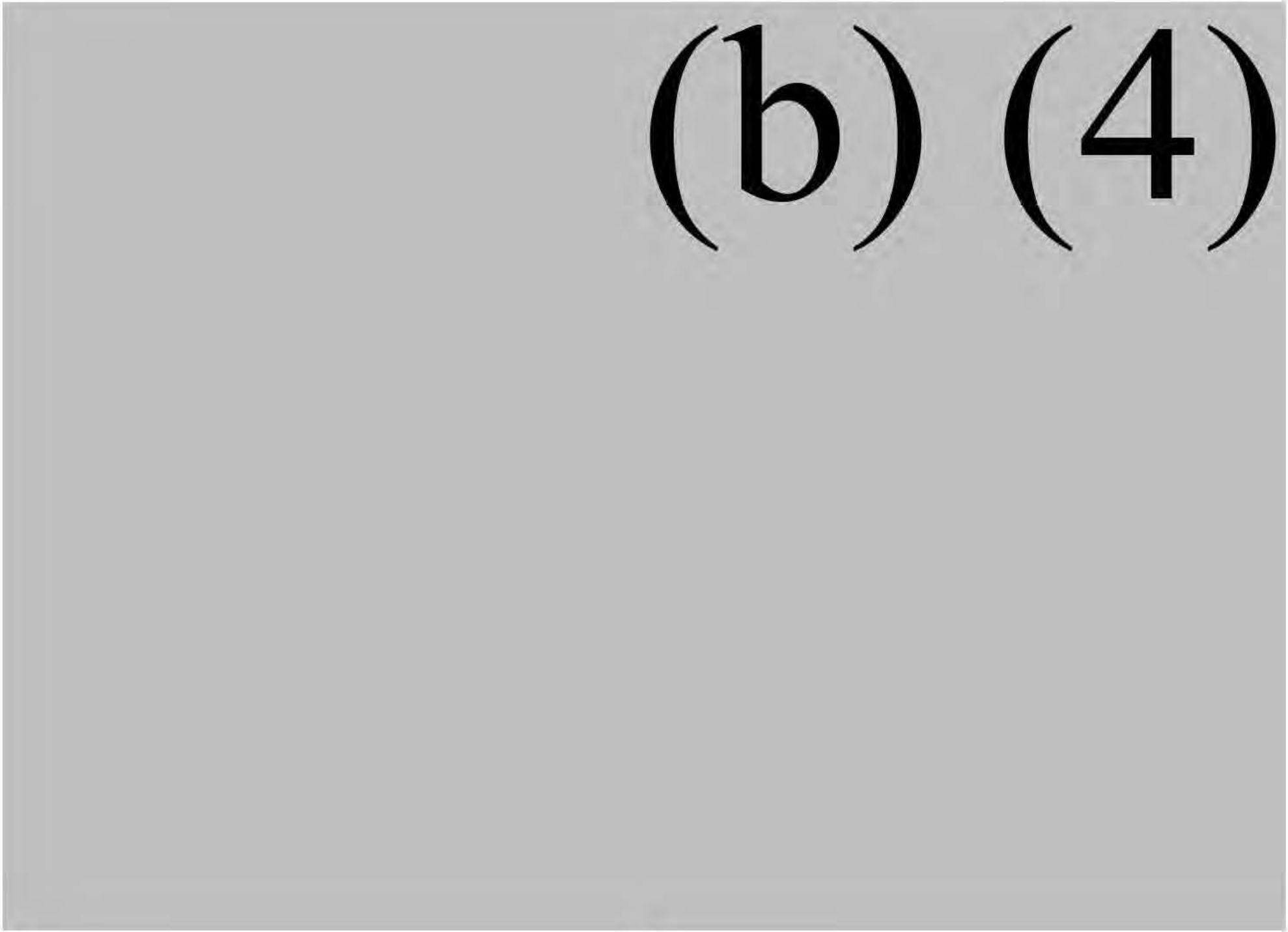
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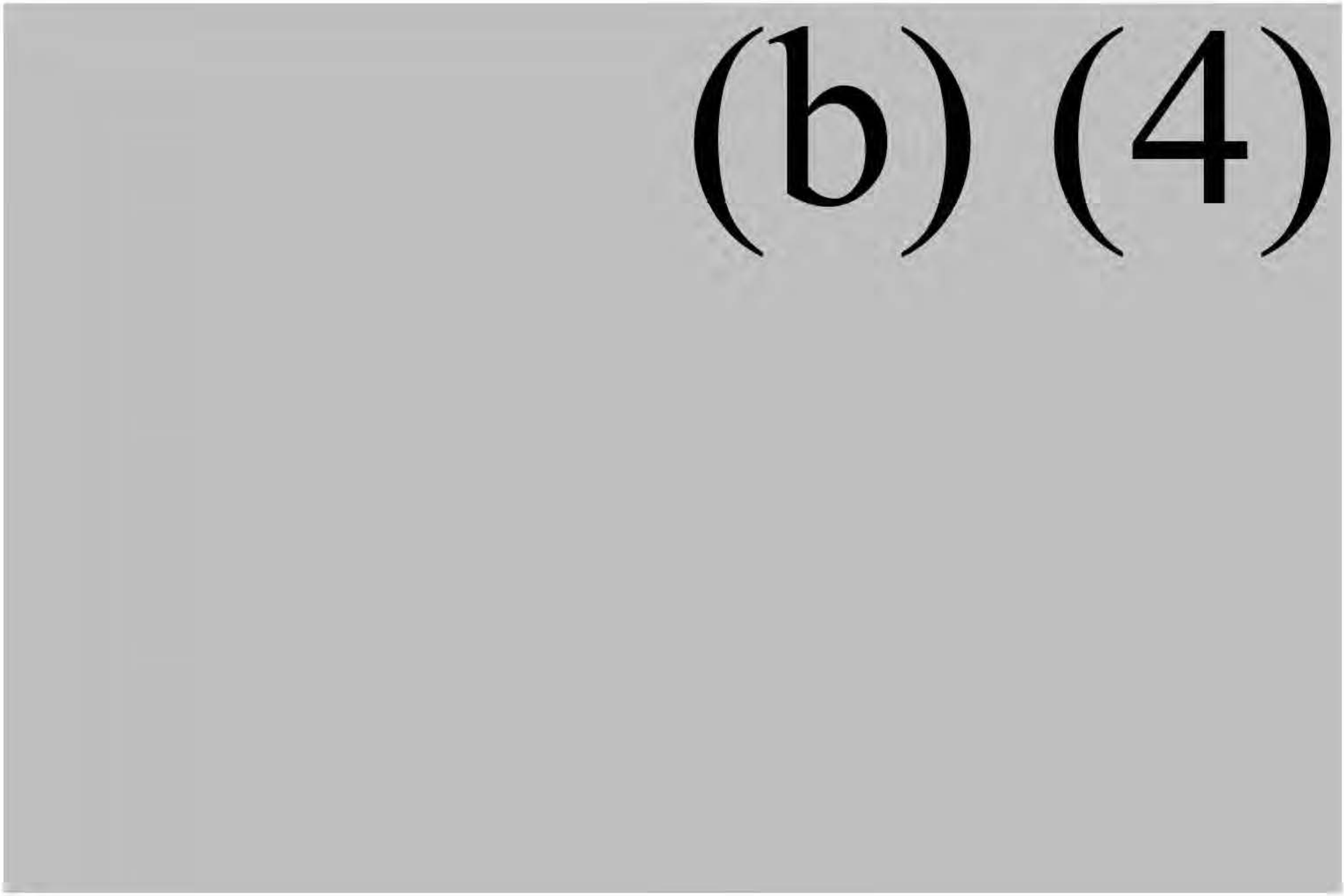
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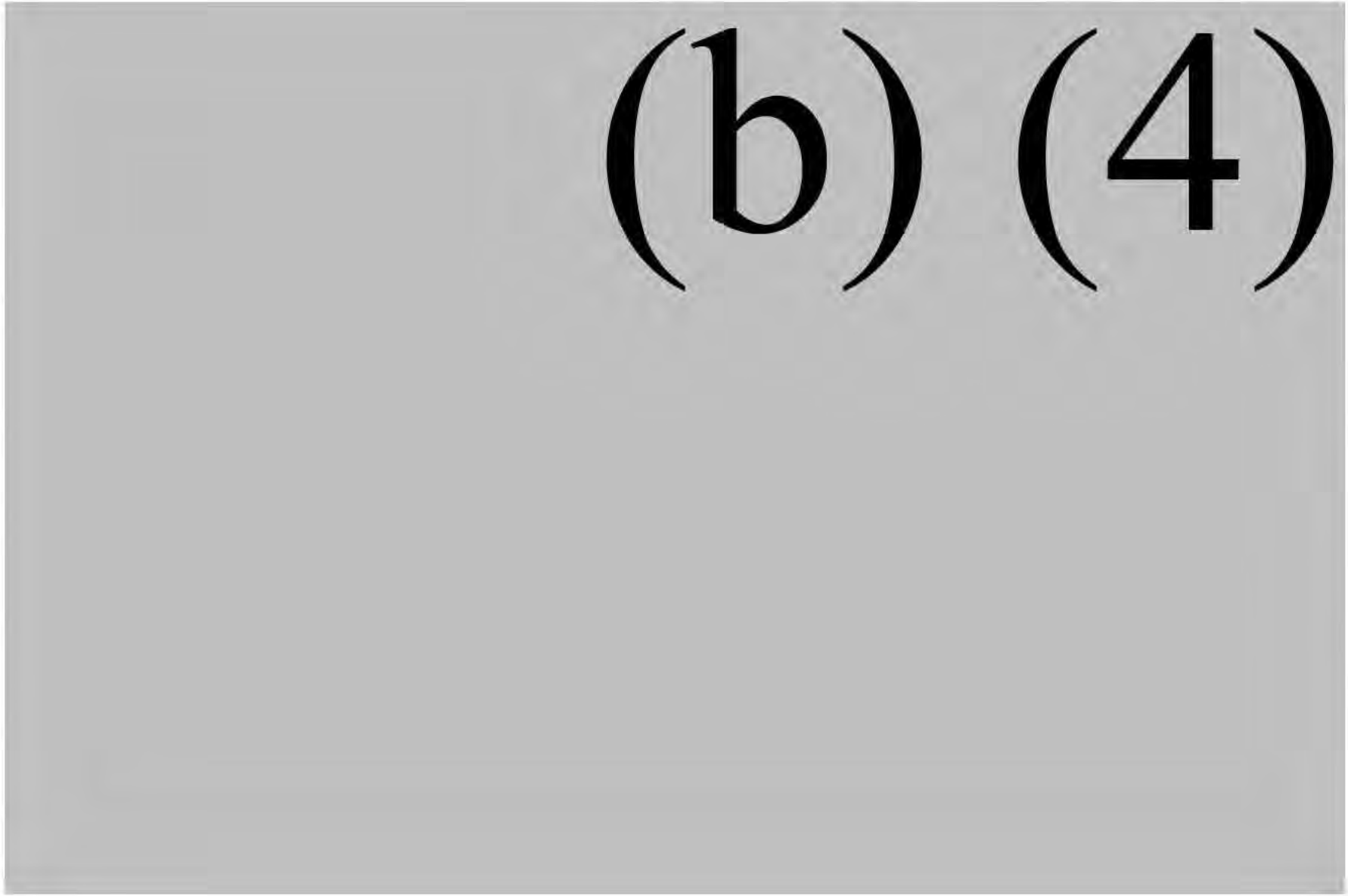
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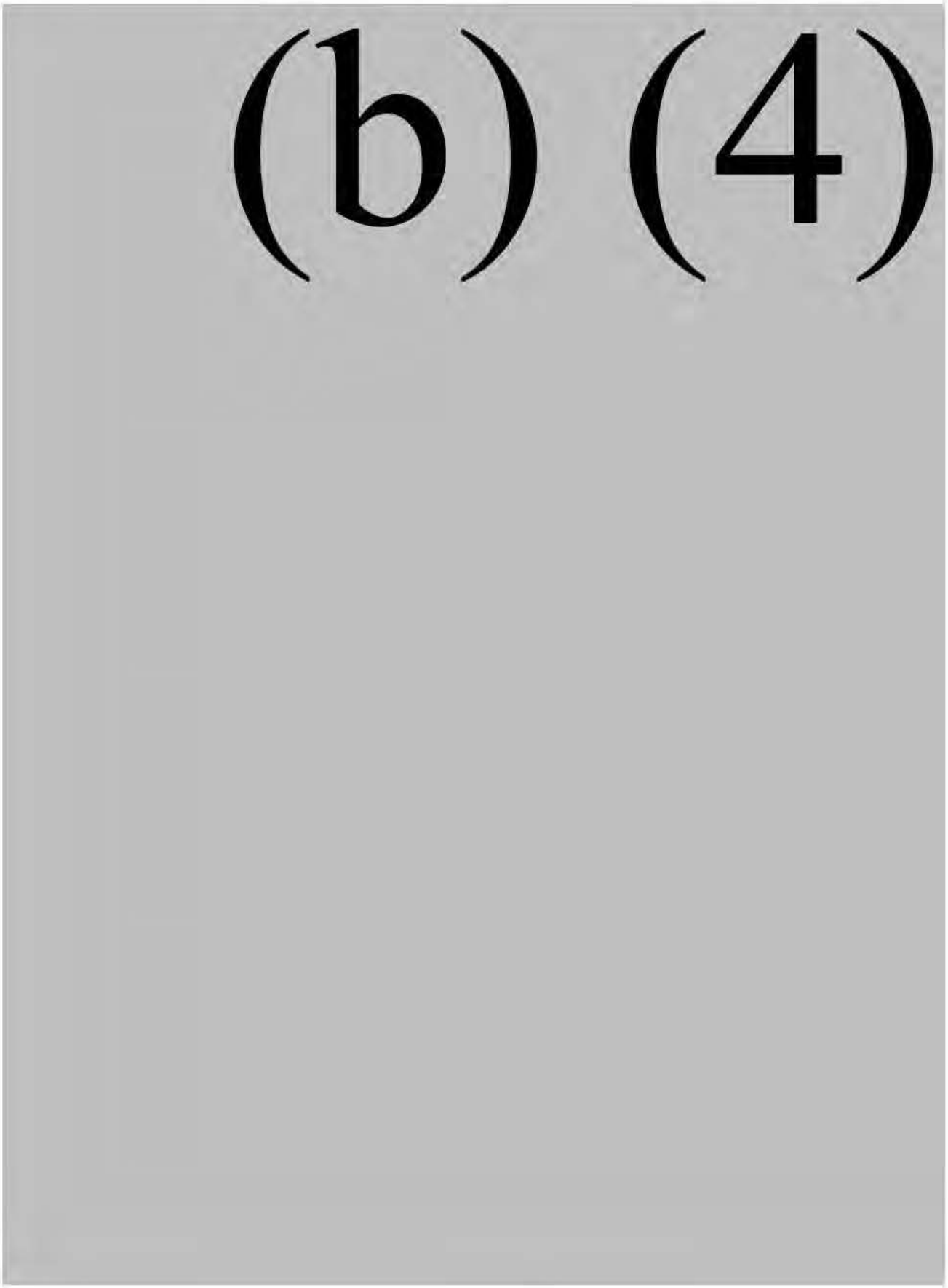
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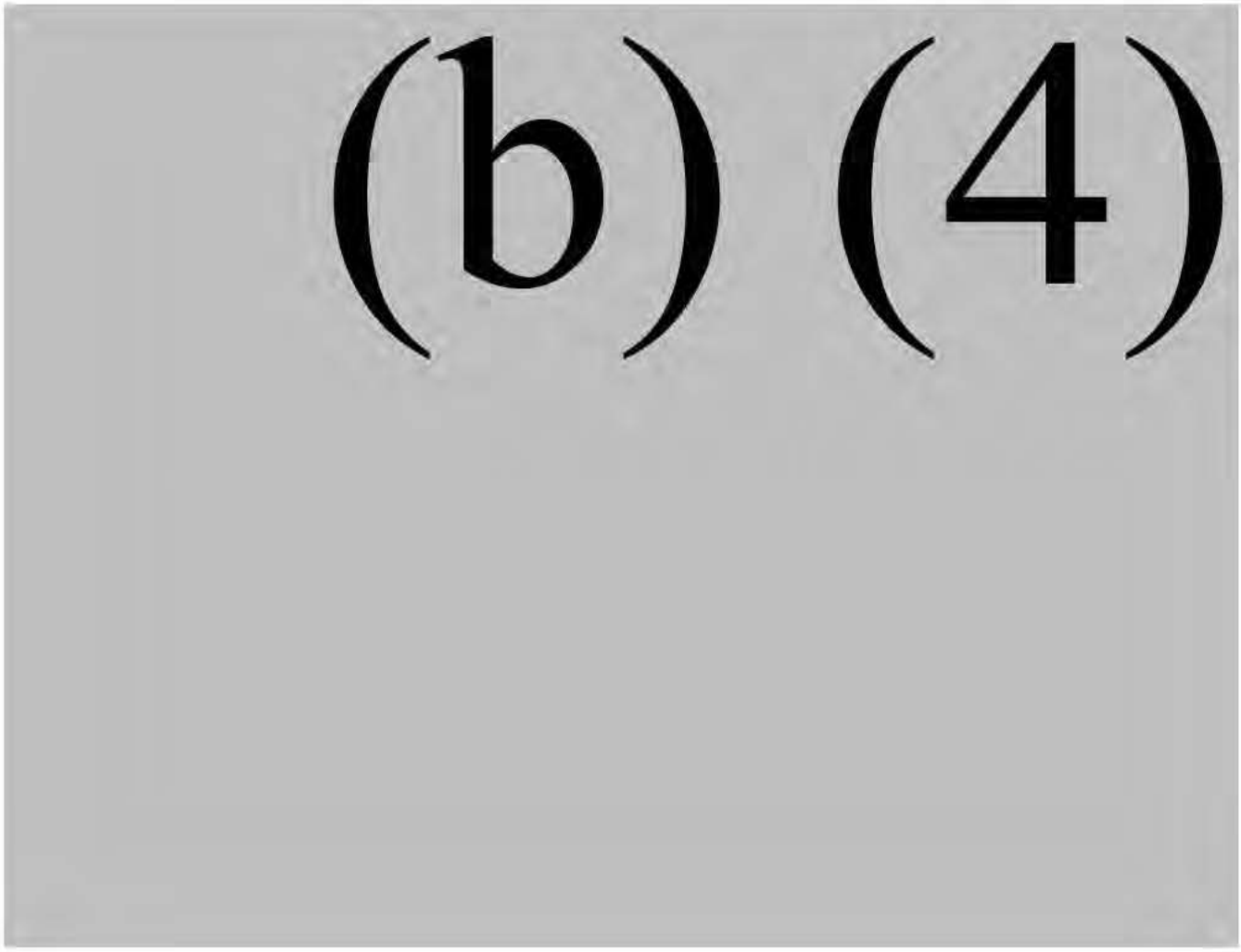
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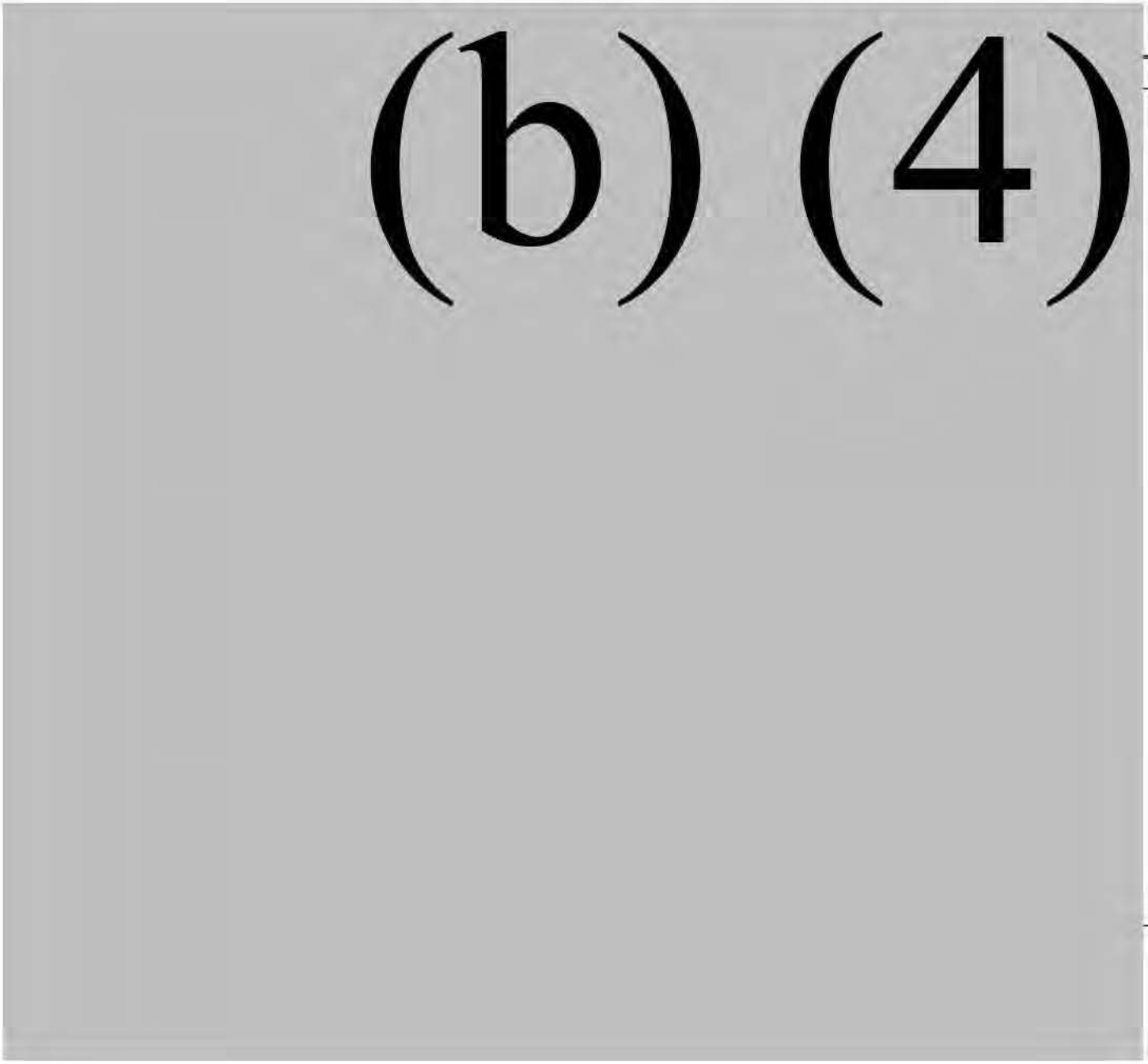
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APPENDIX 5

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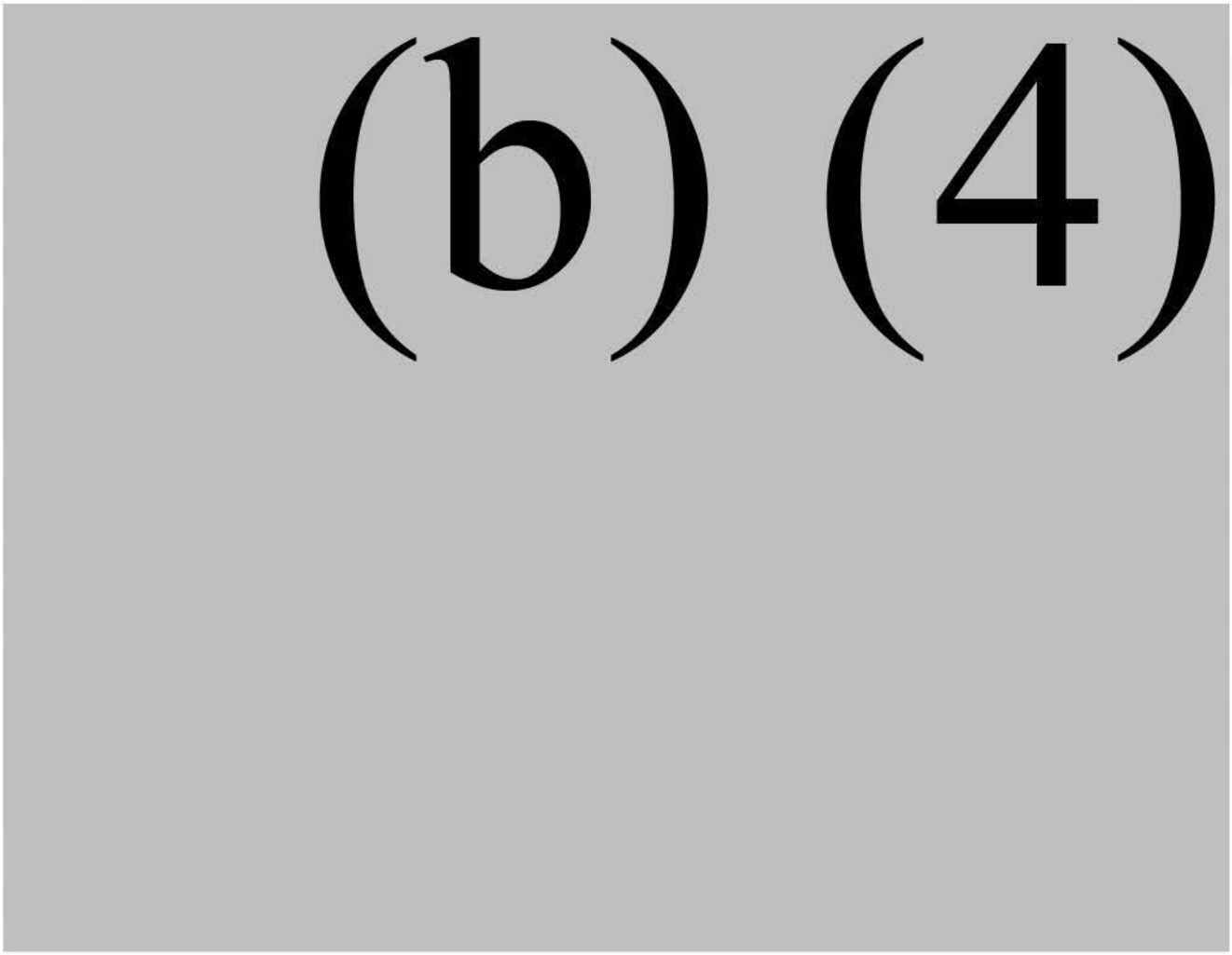
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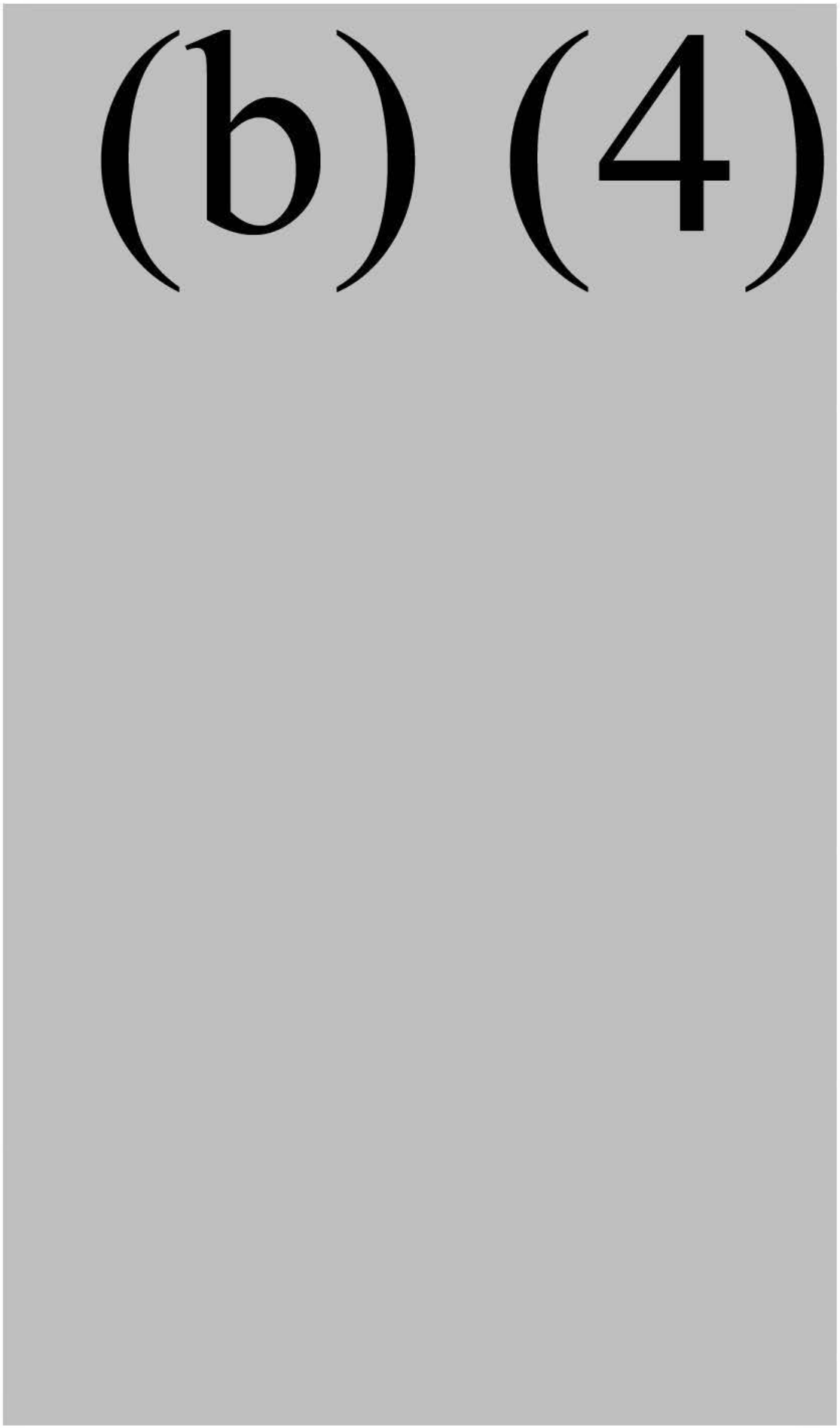
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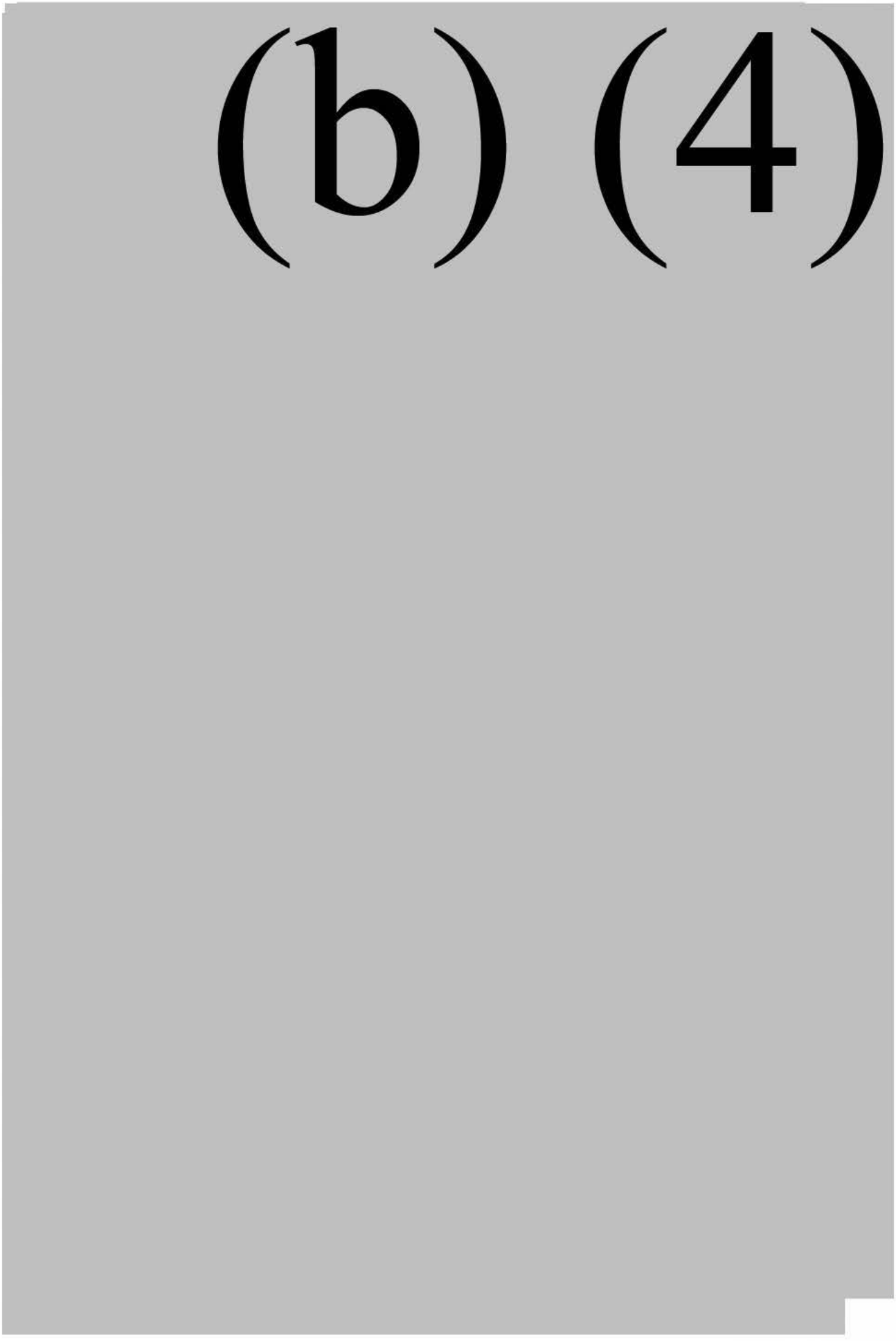
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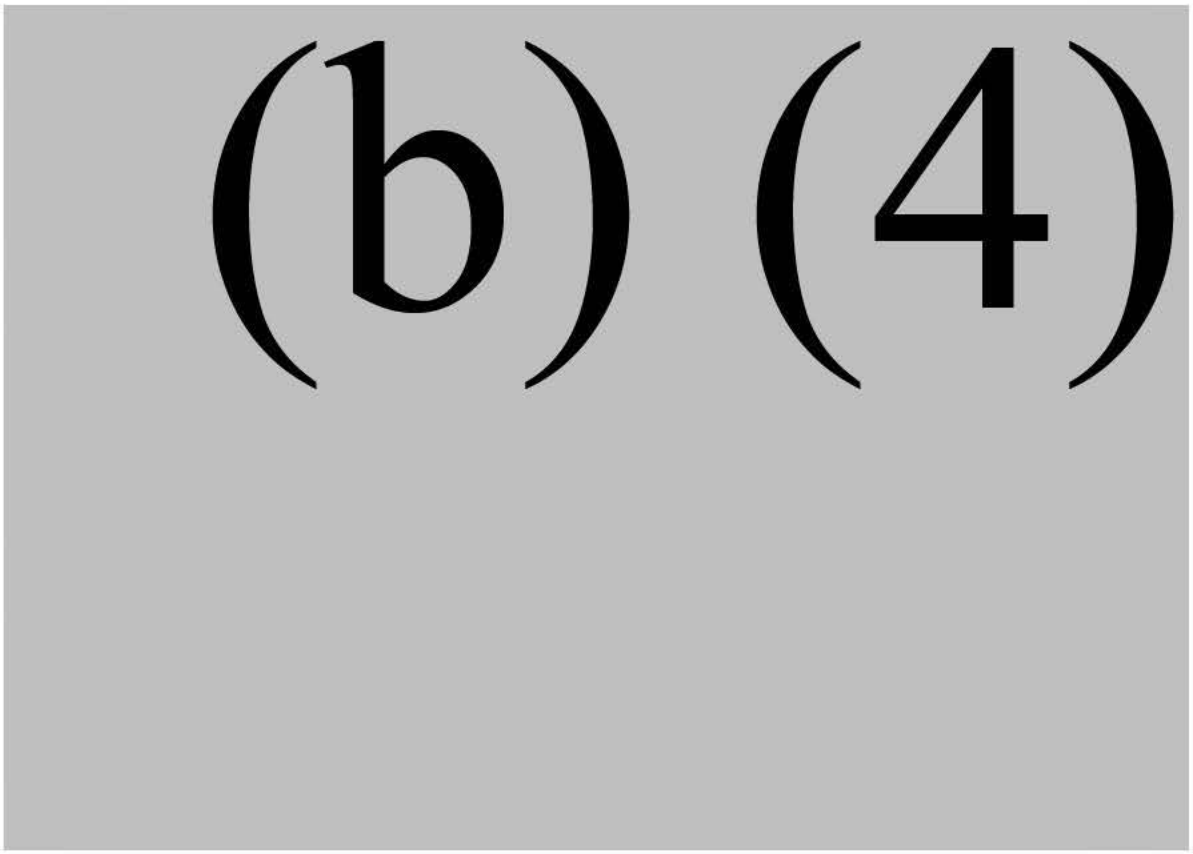
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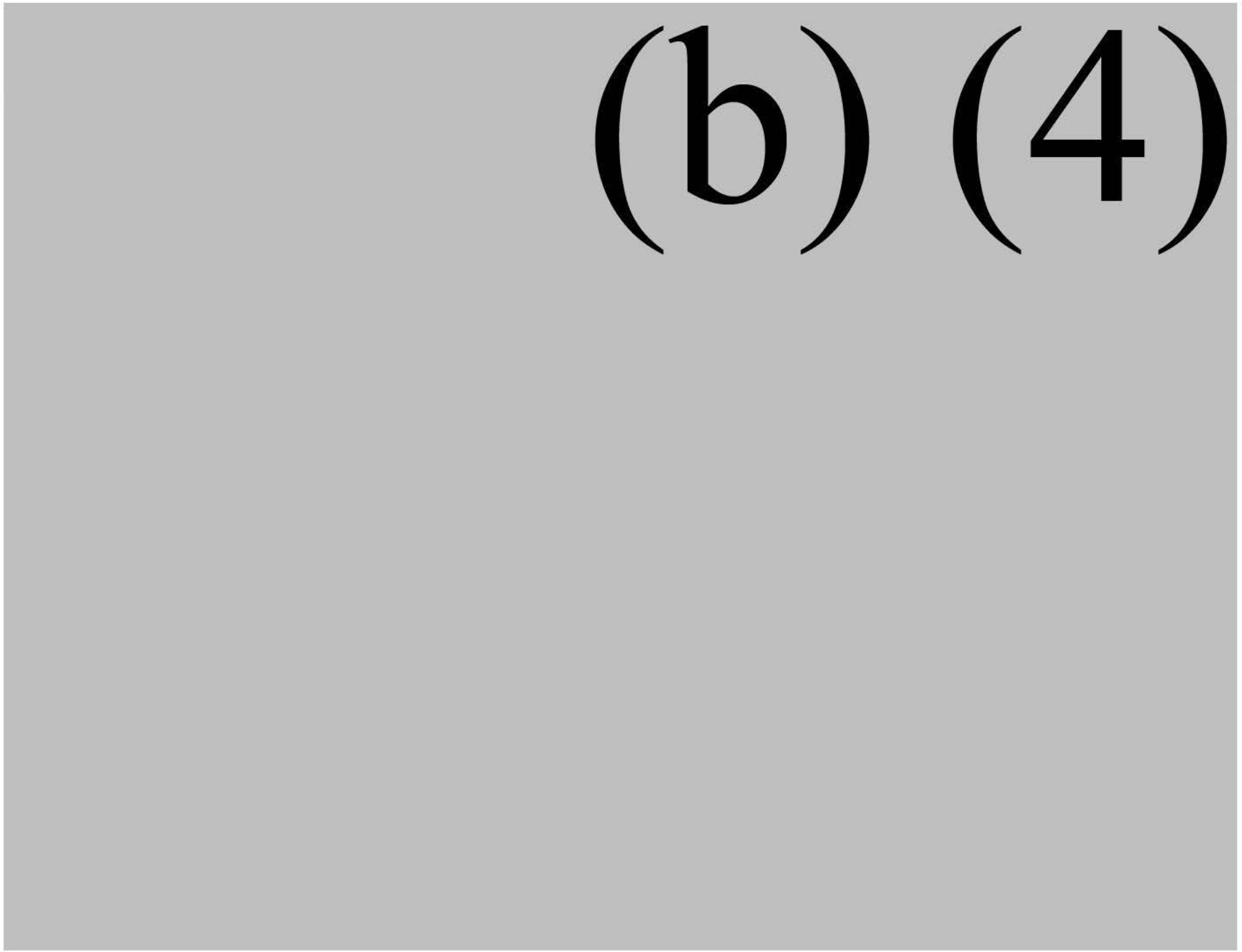
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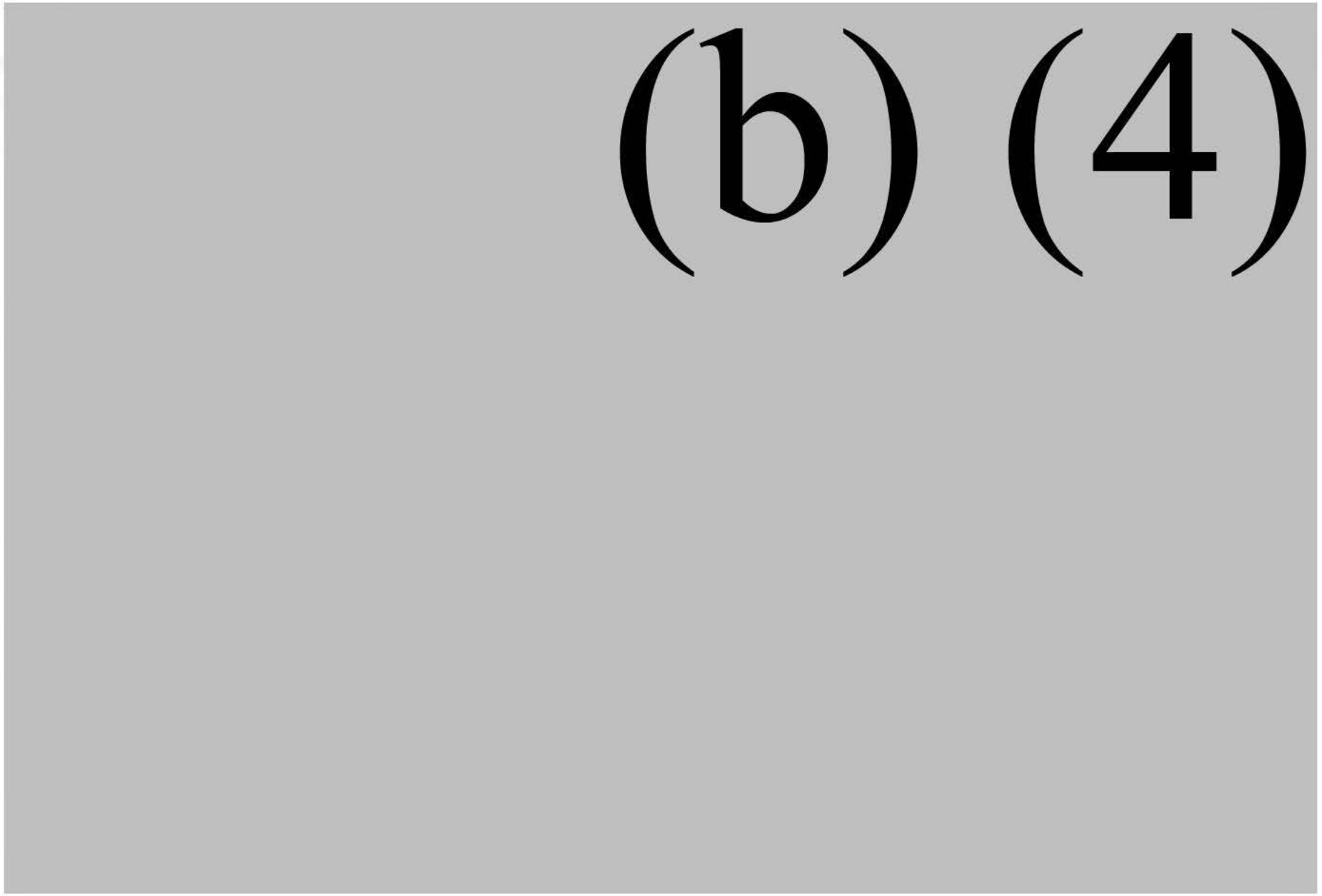
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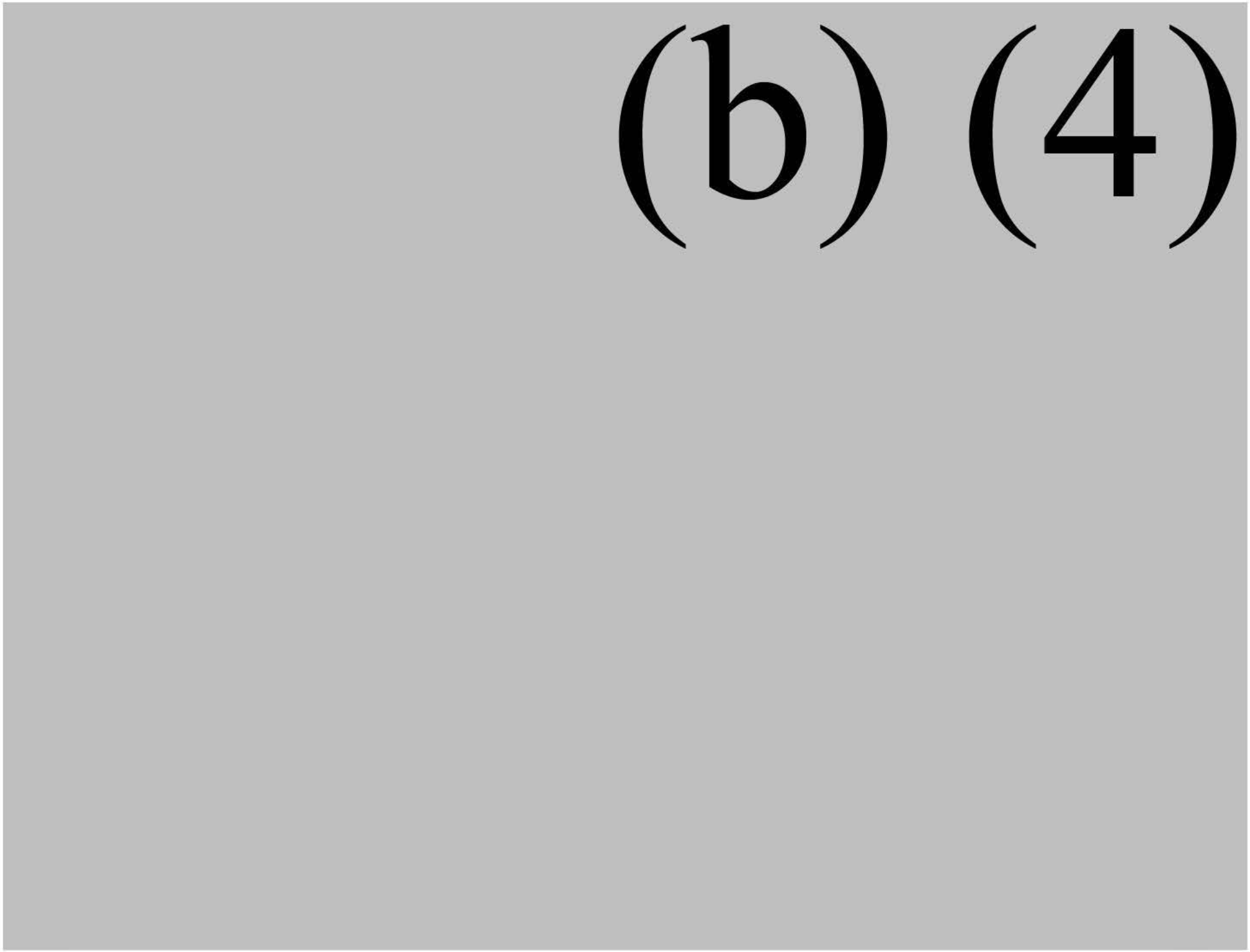
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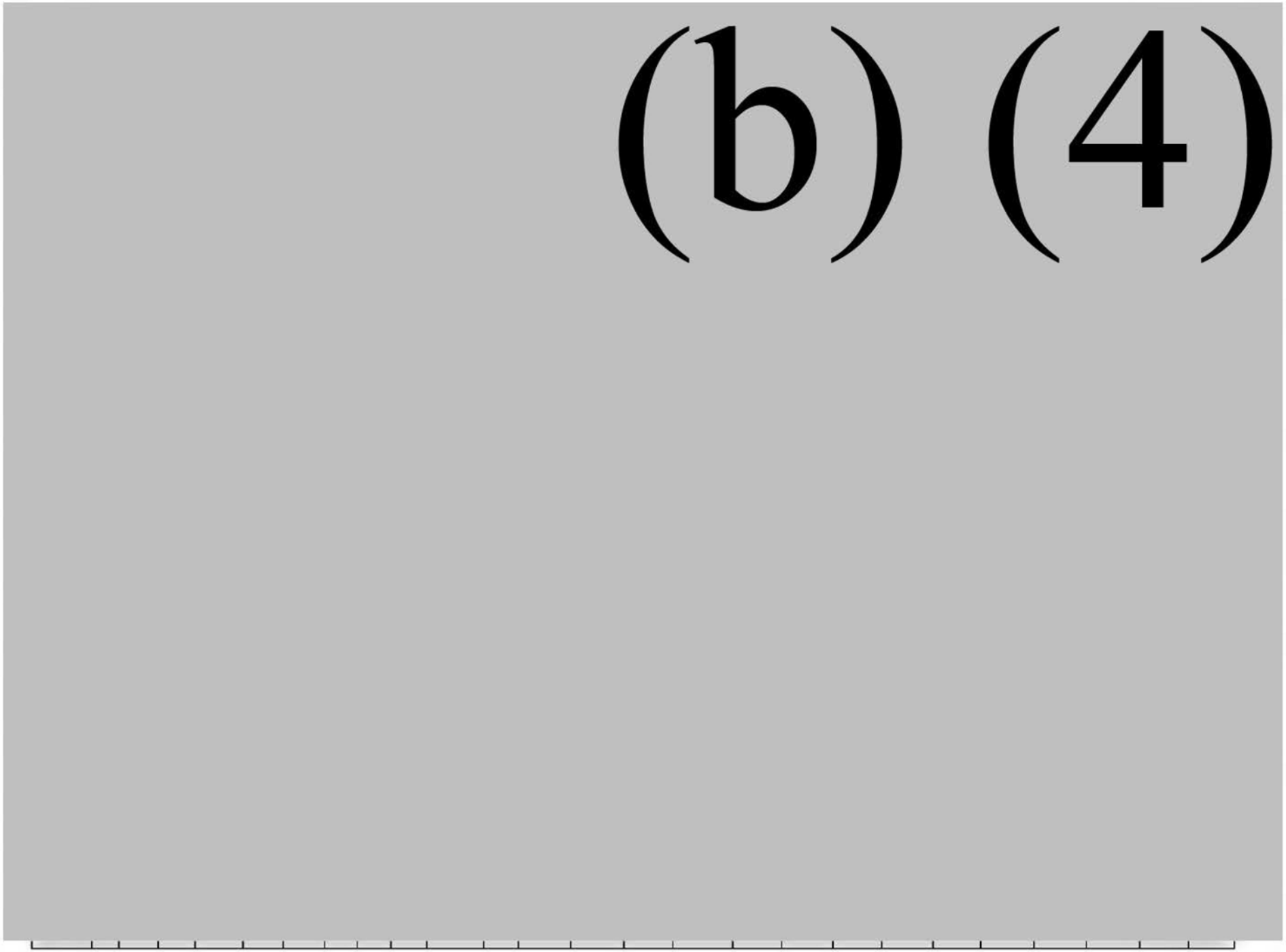
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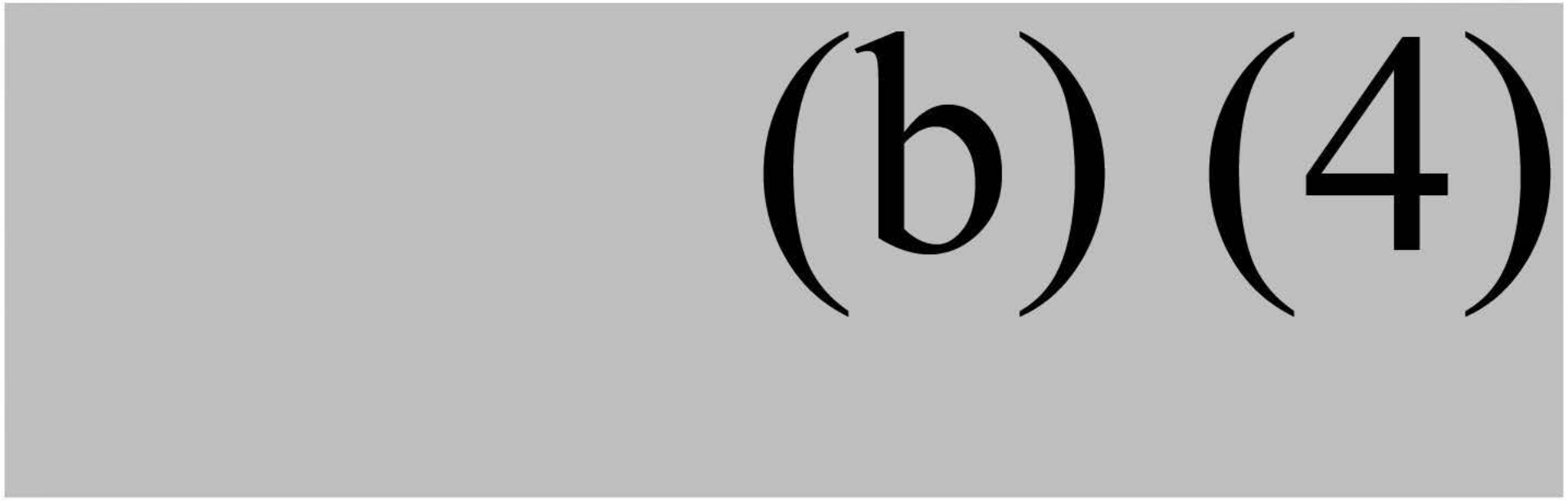
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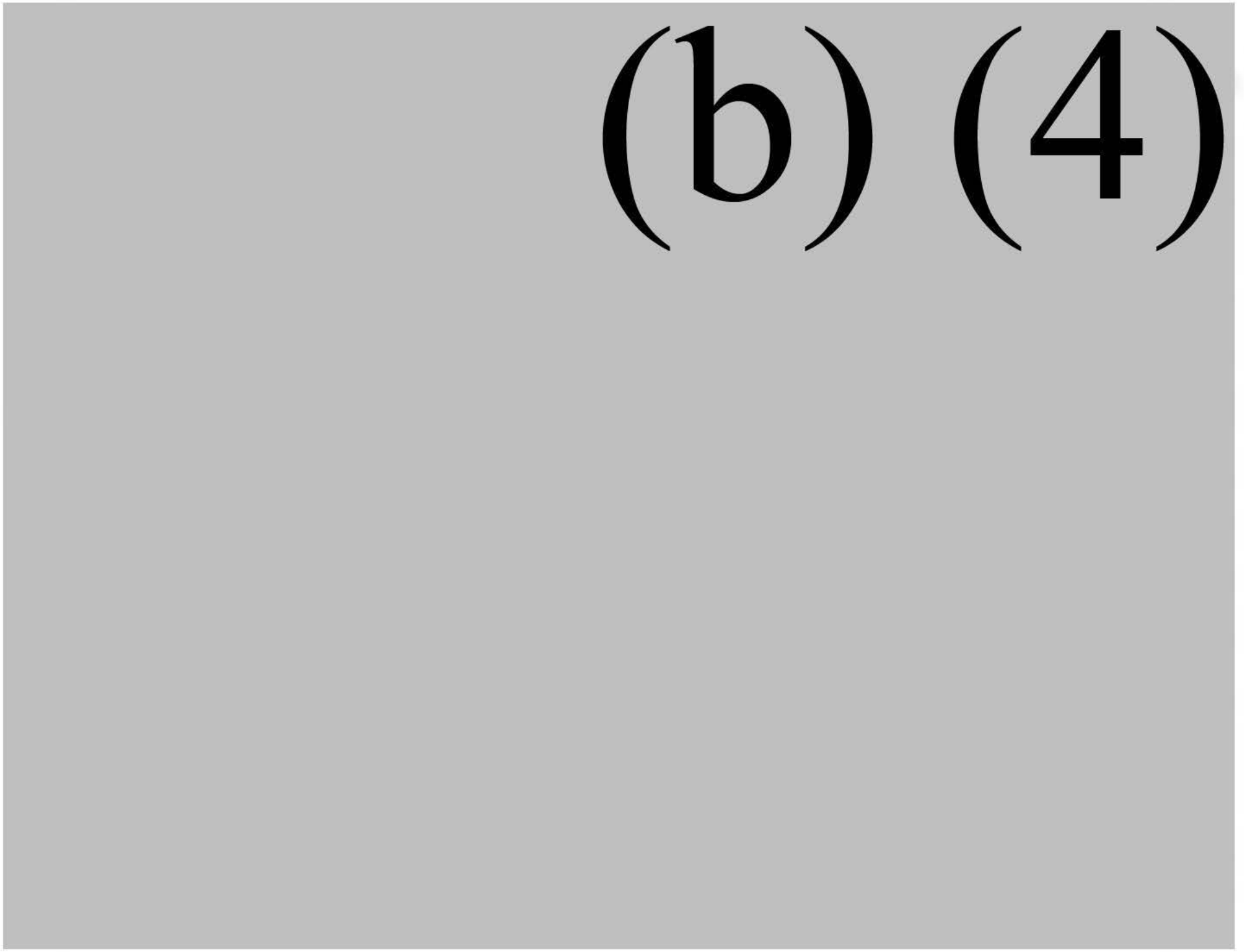
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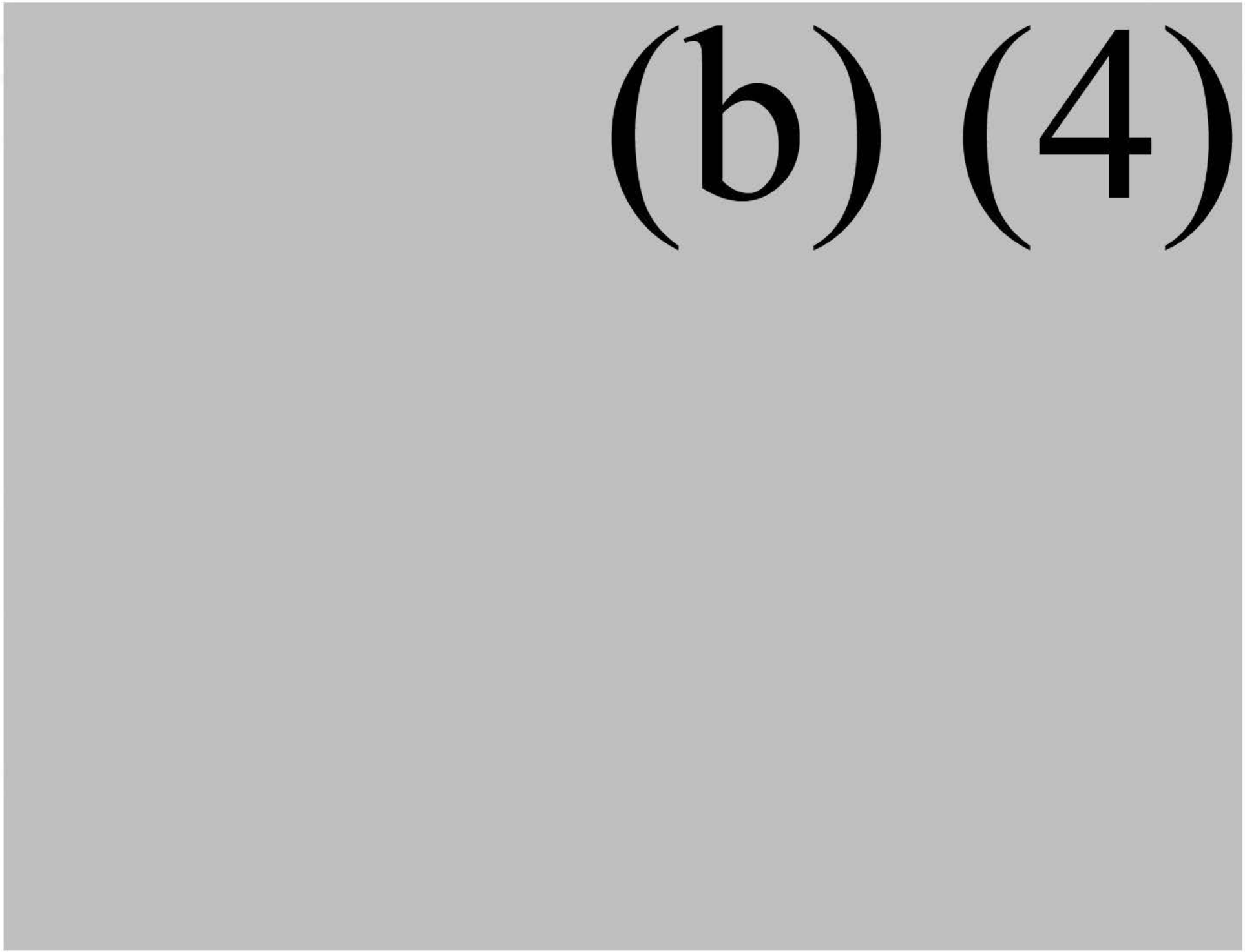
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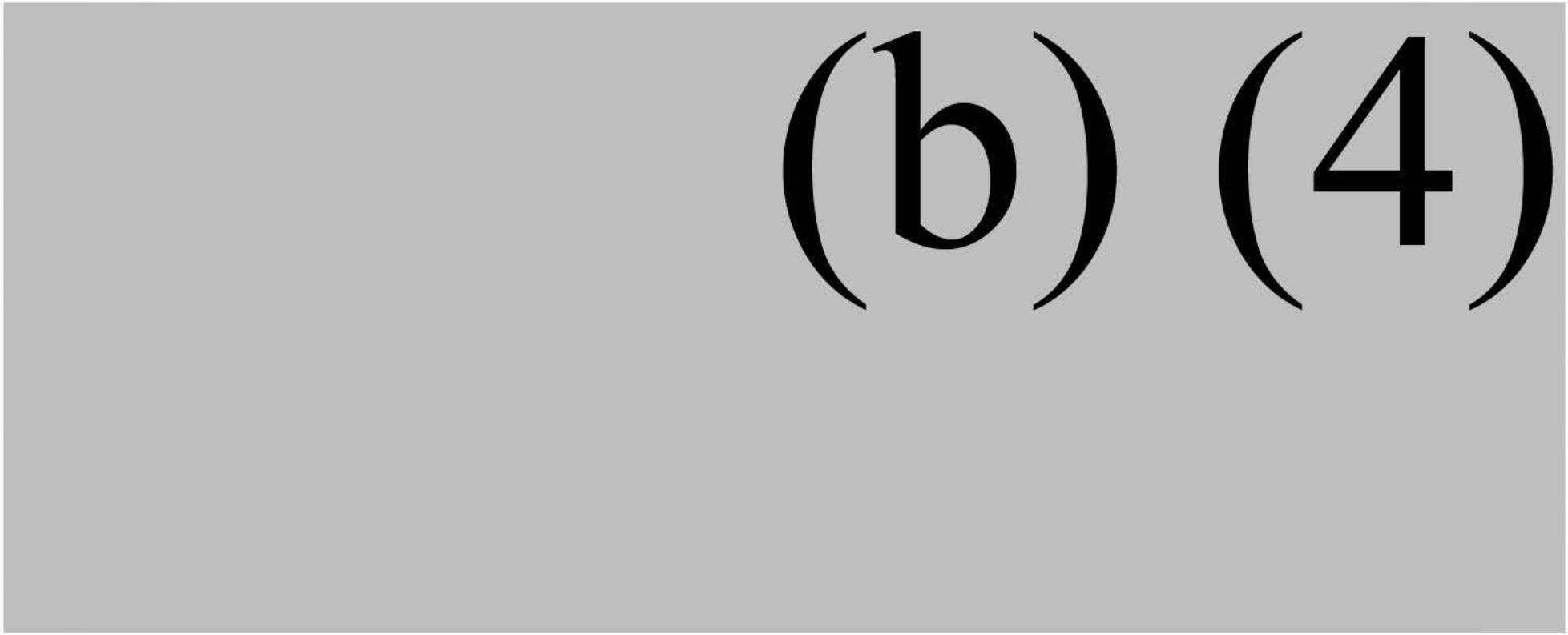
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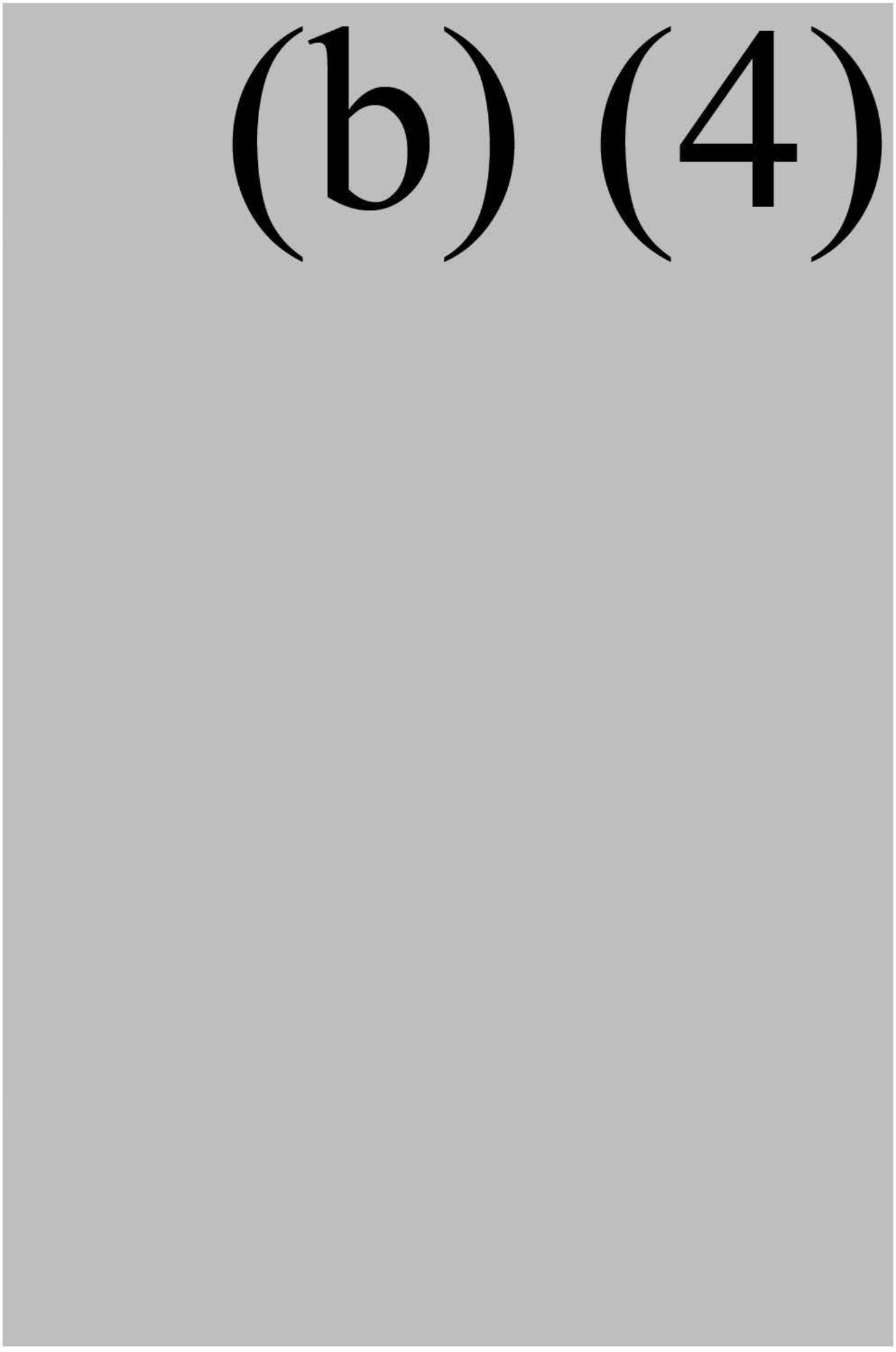
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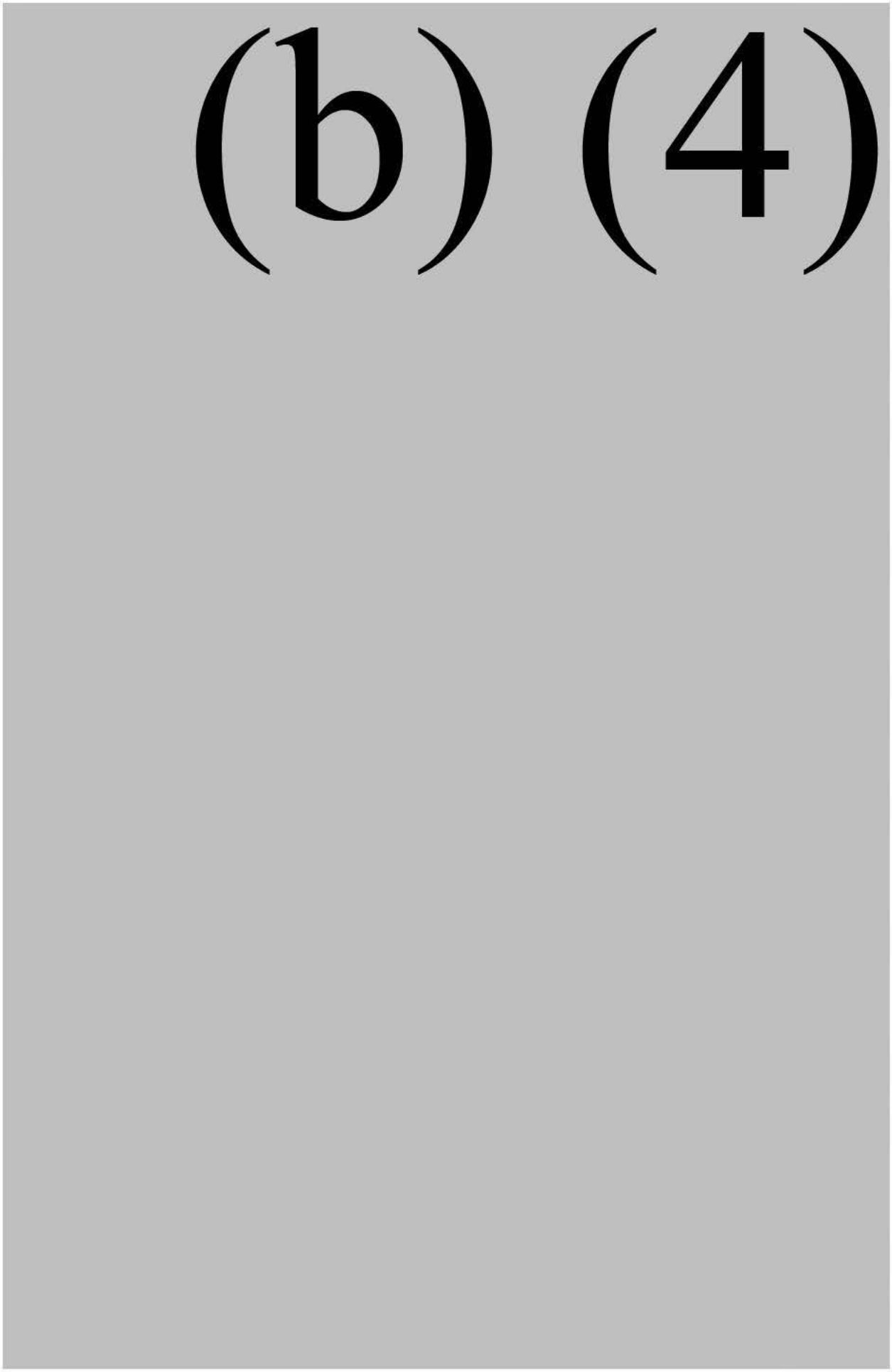
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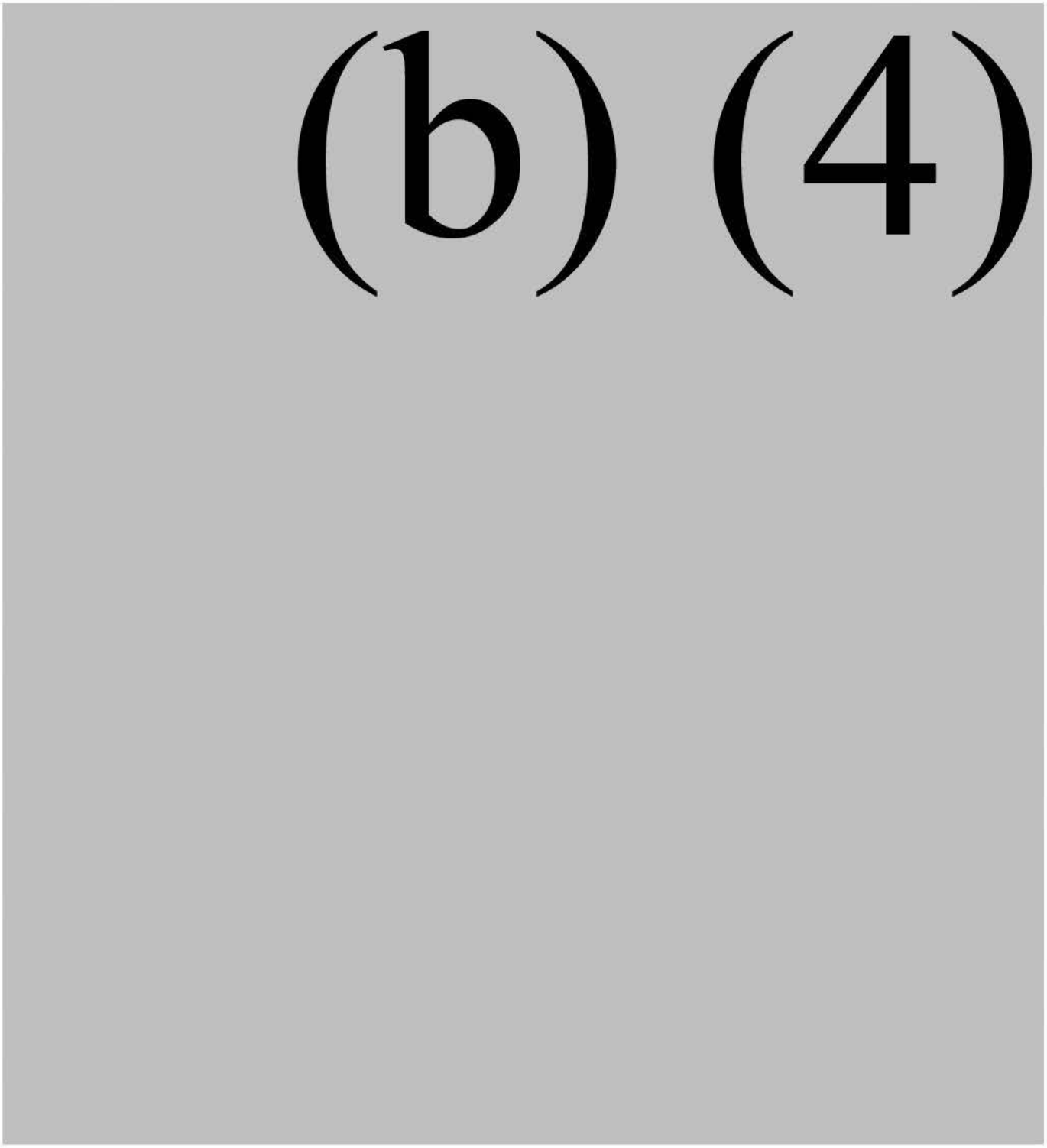
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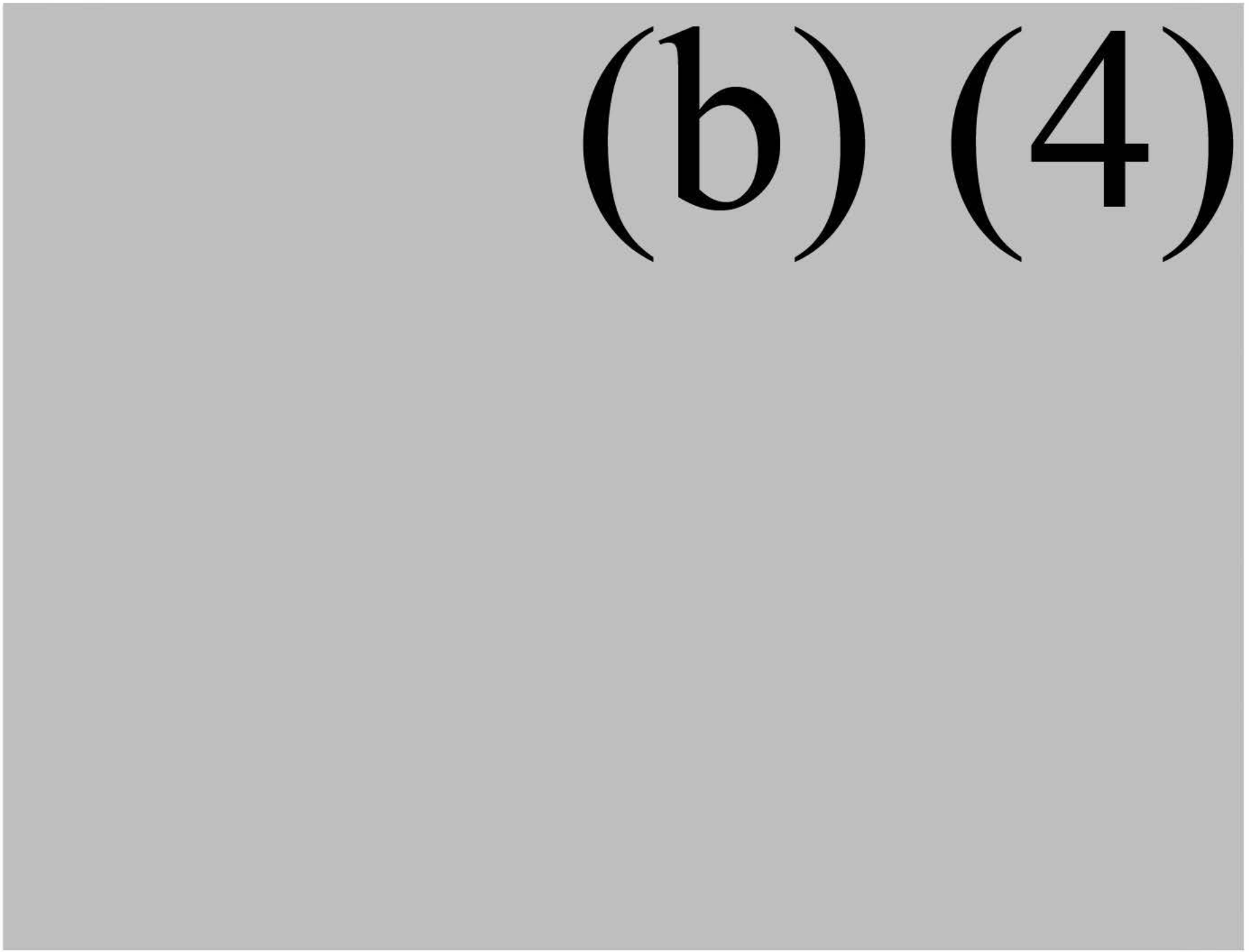
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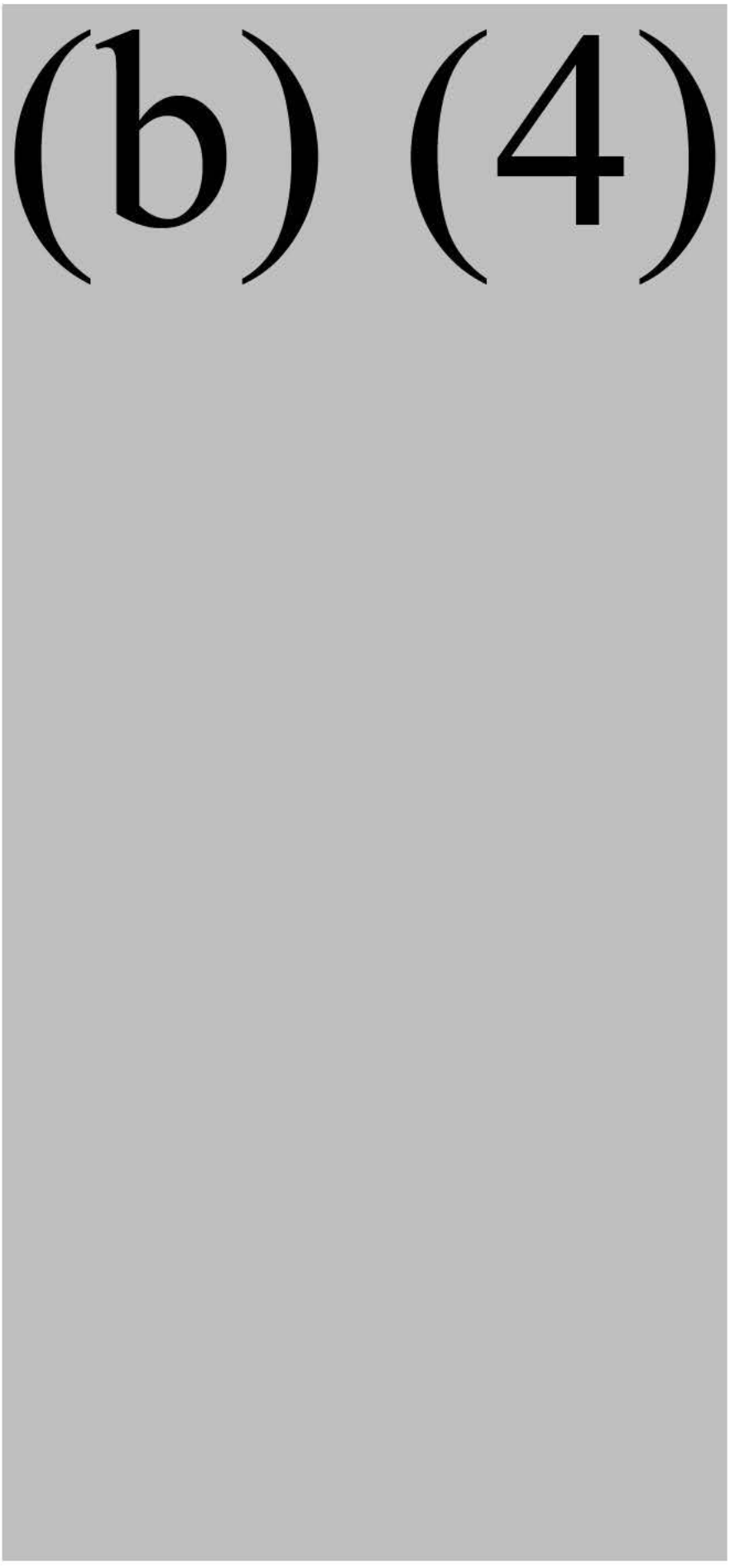
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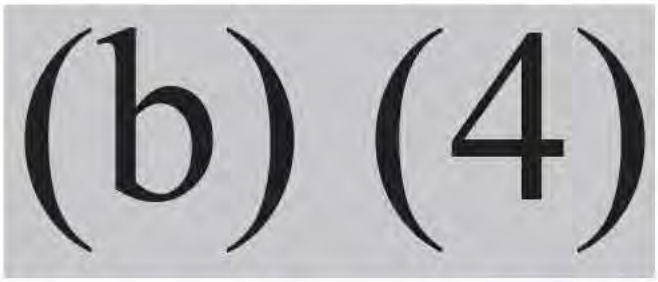
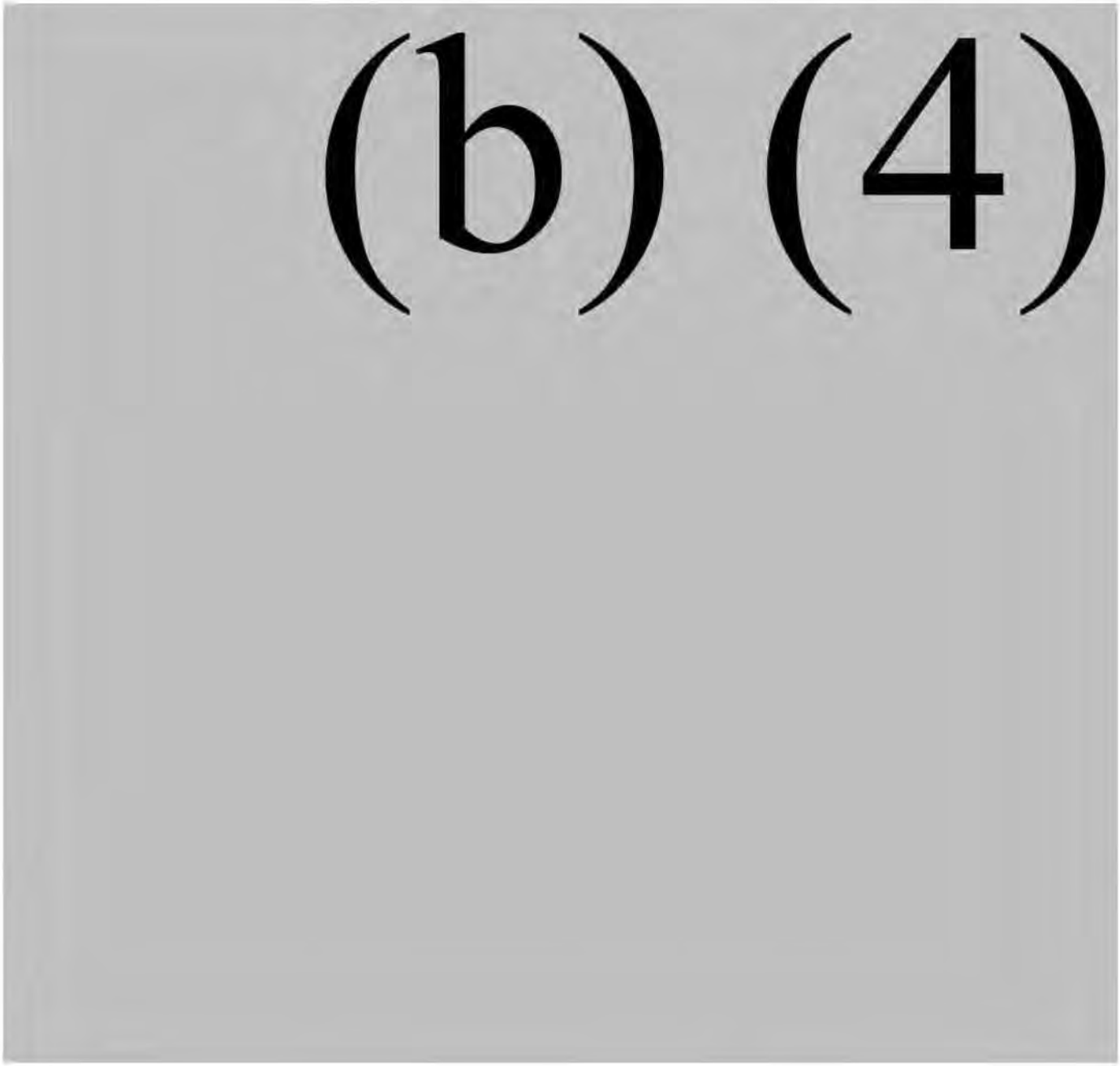
APPENDIX 6





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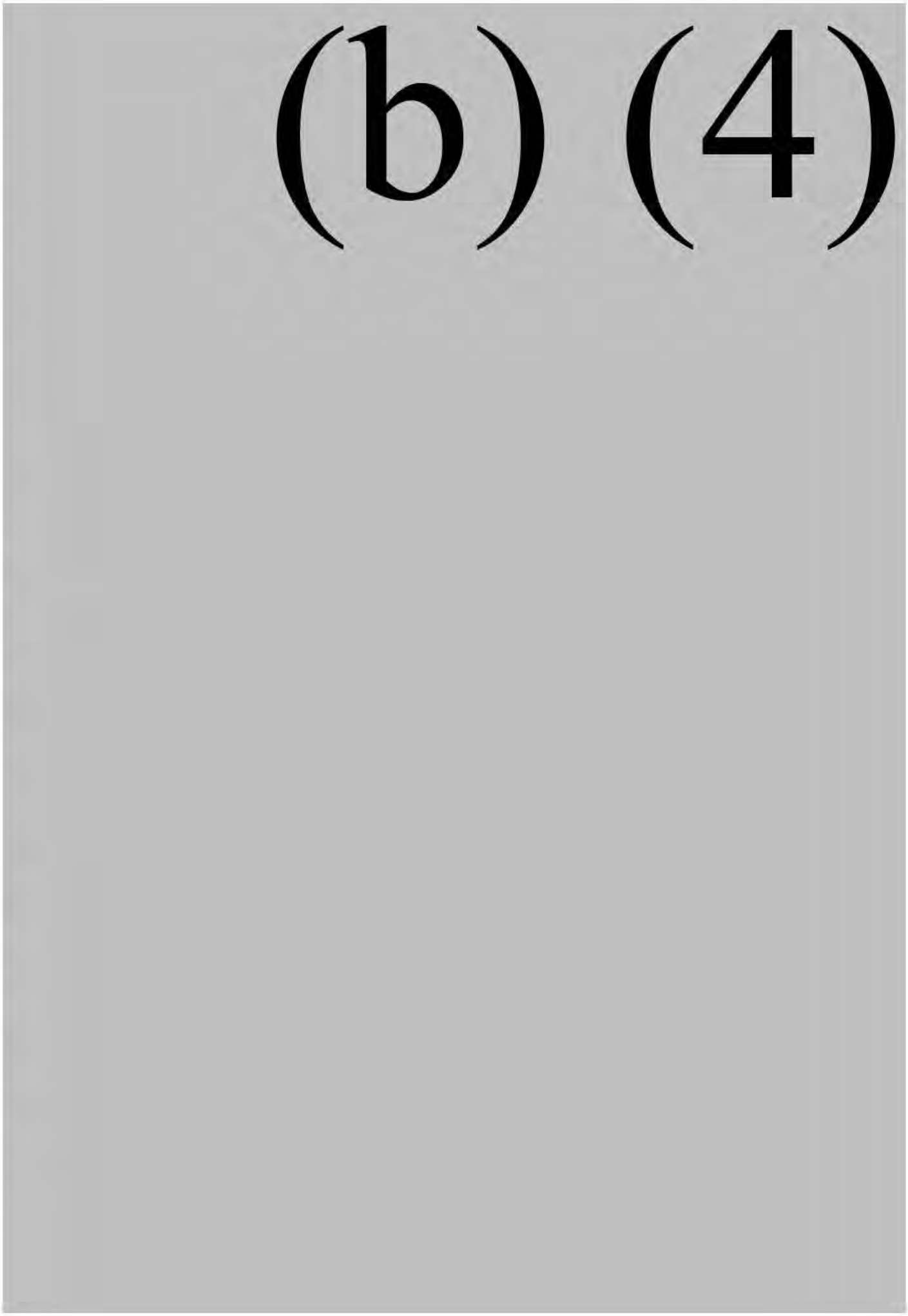
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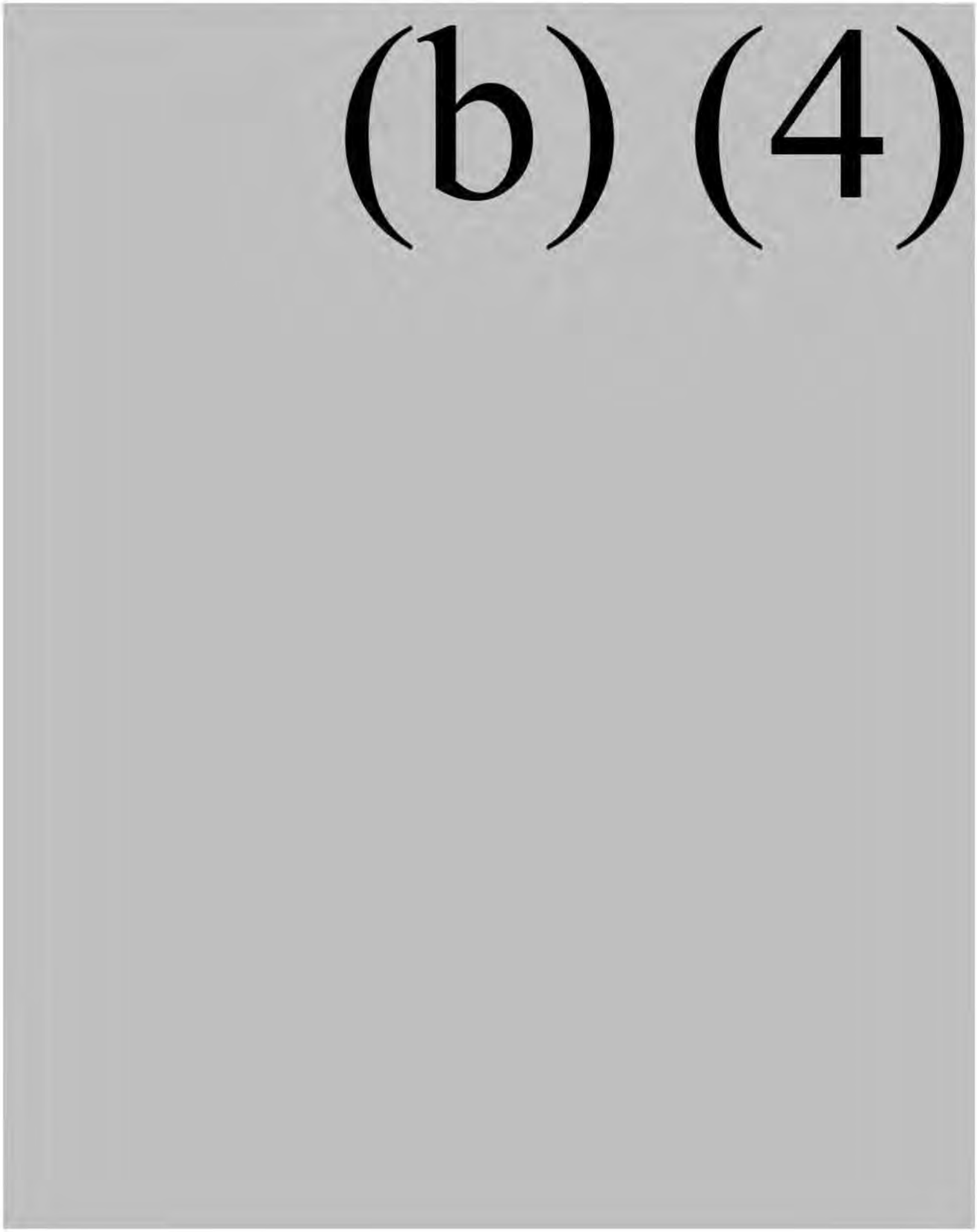

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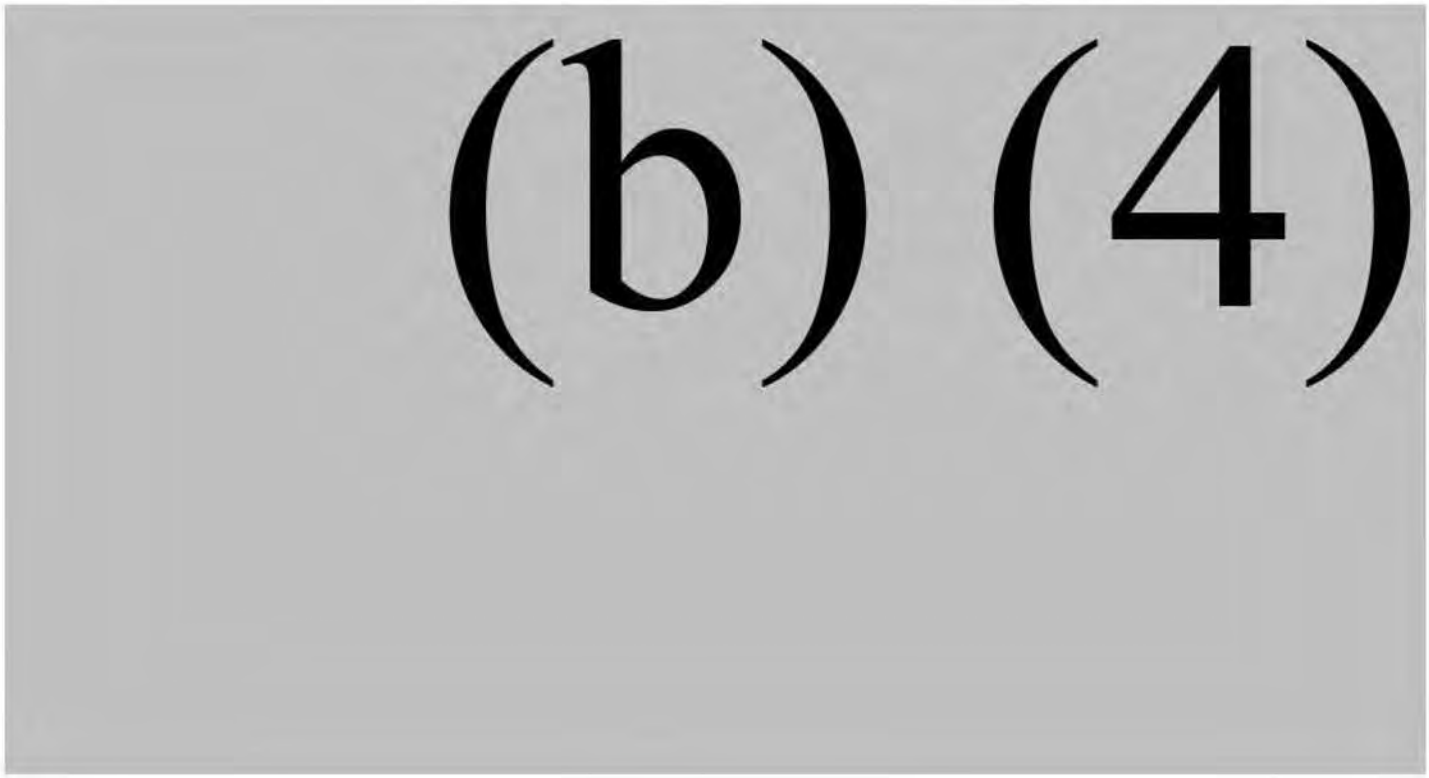

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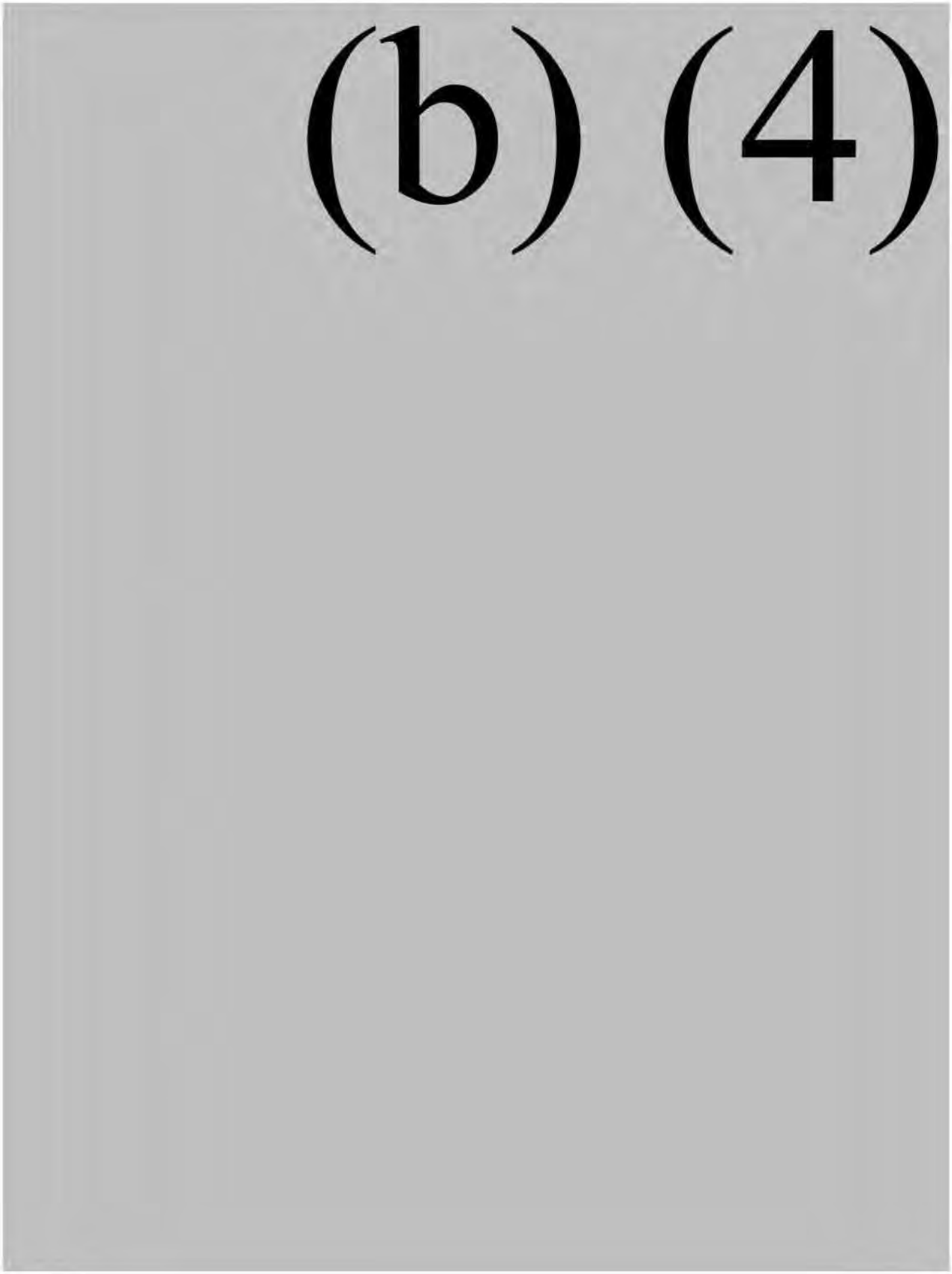

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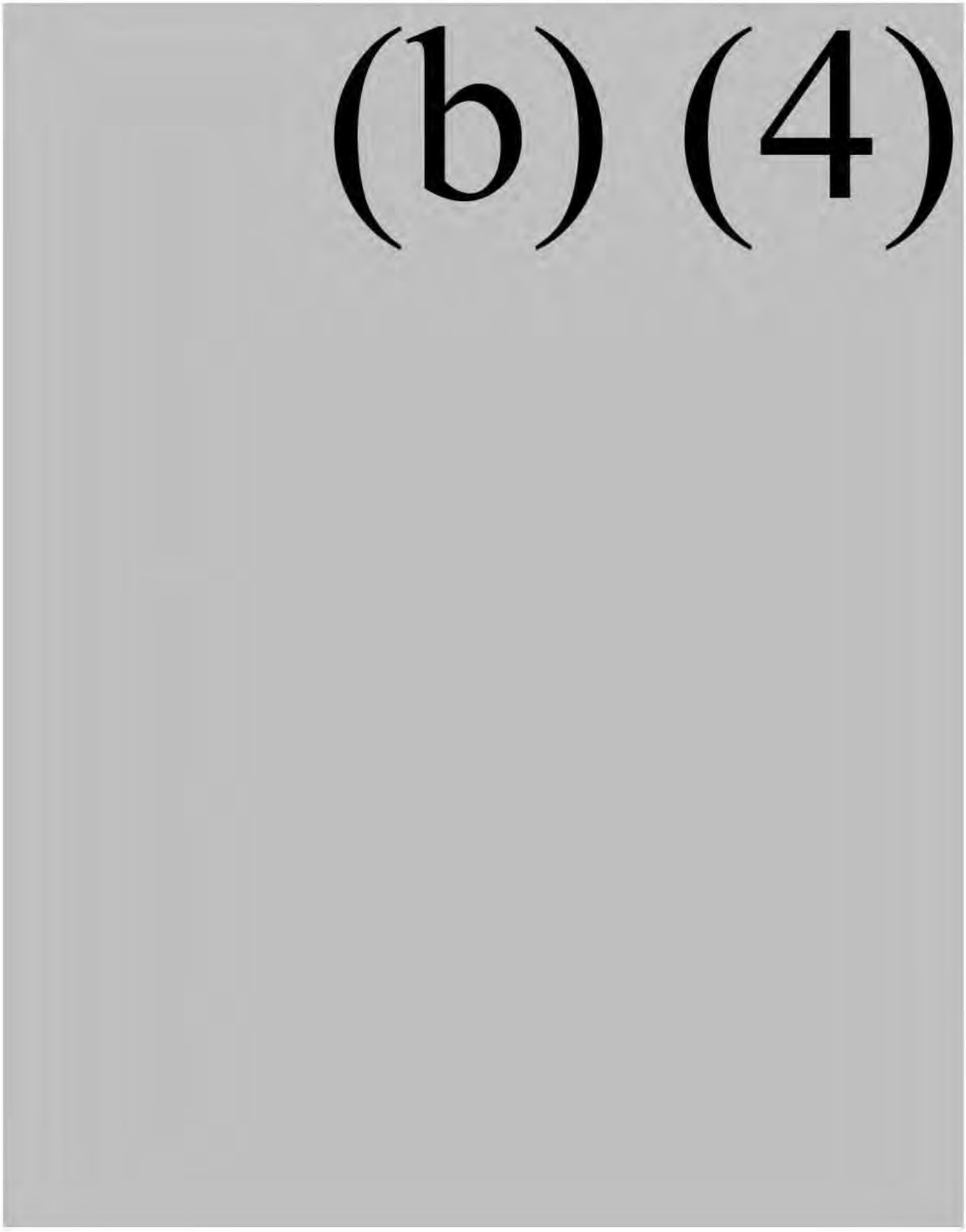

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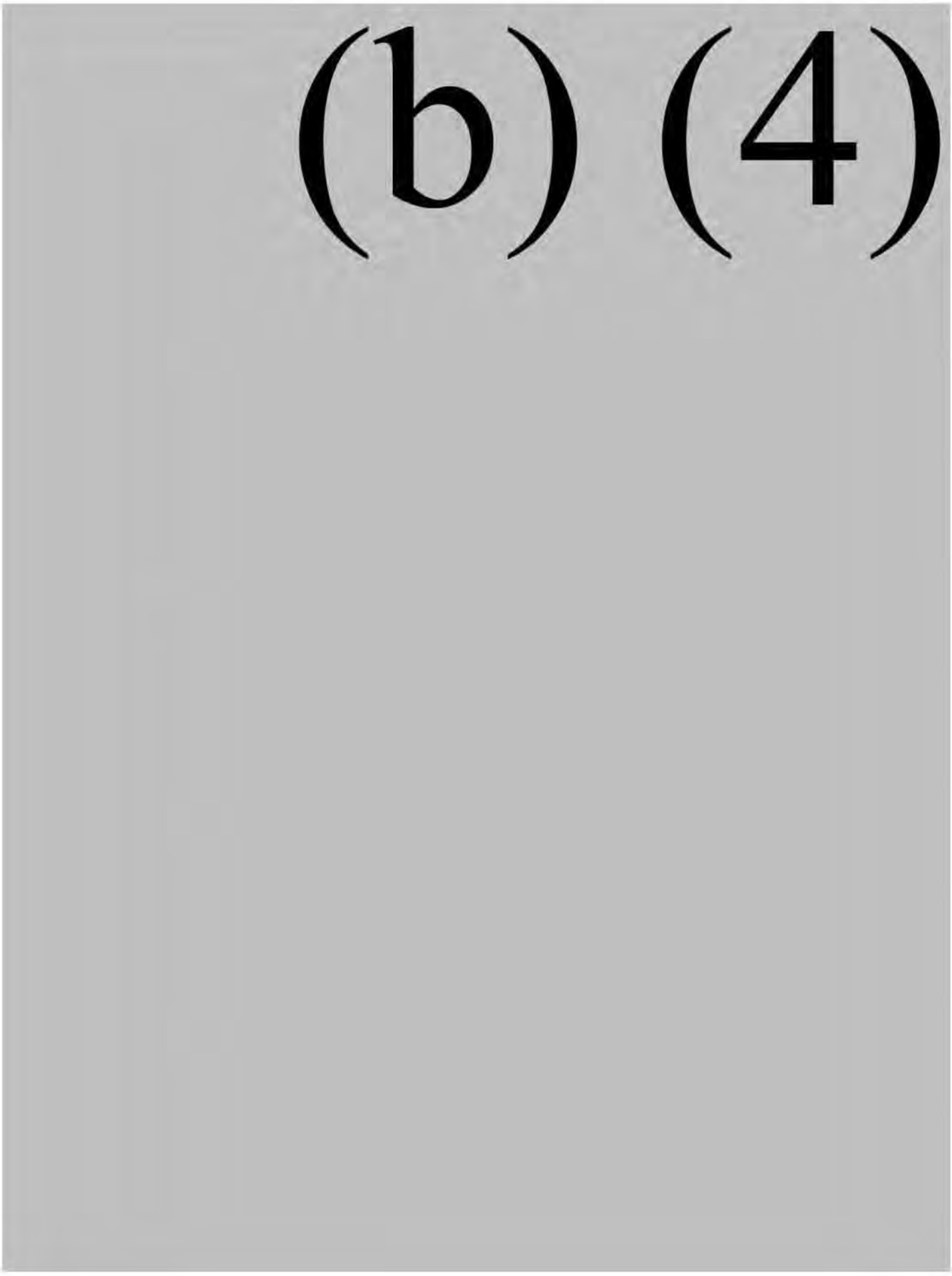

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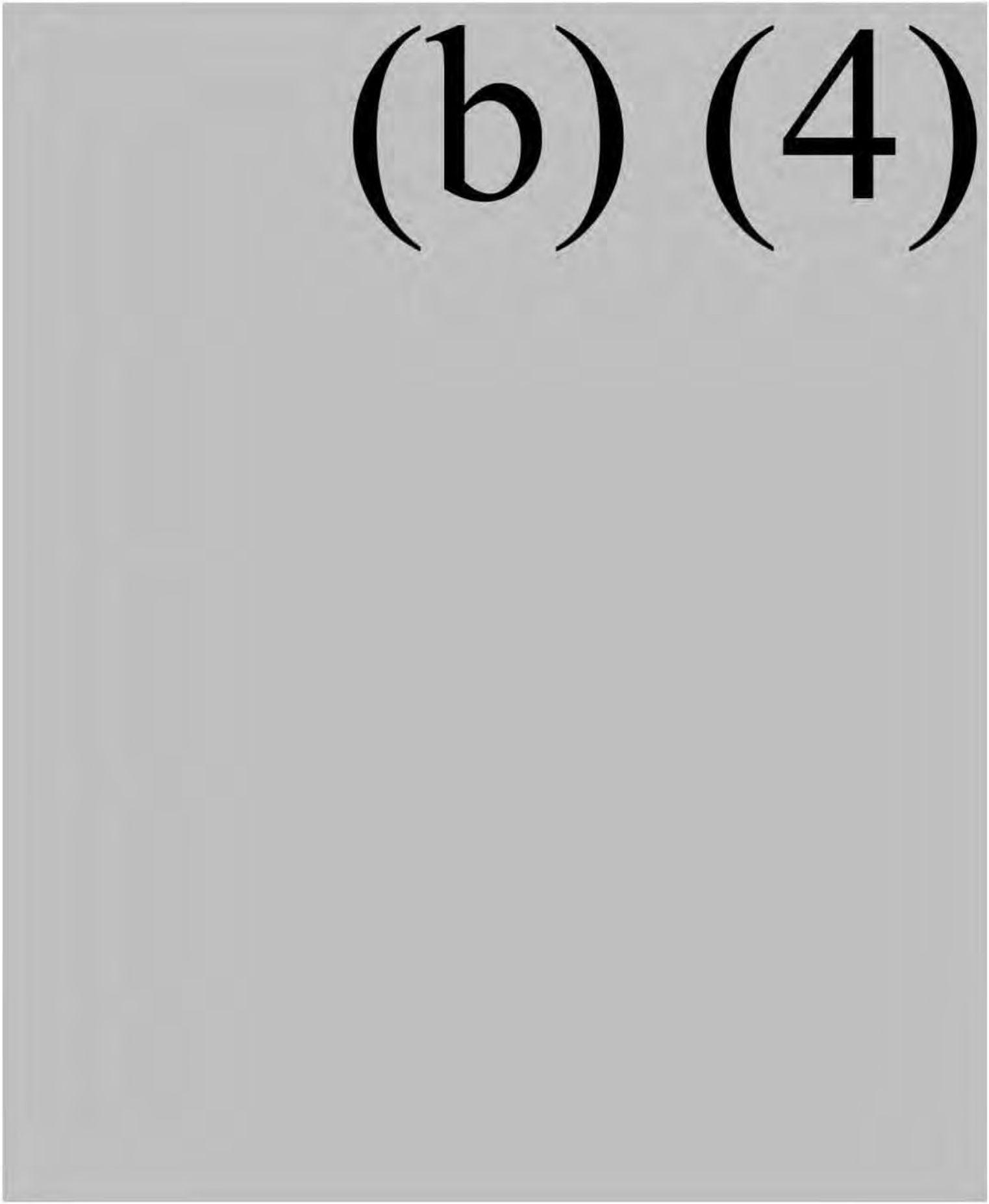

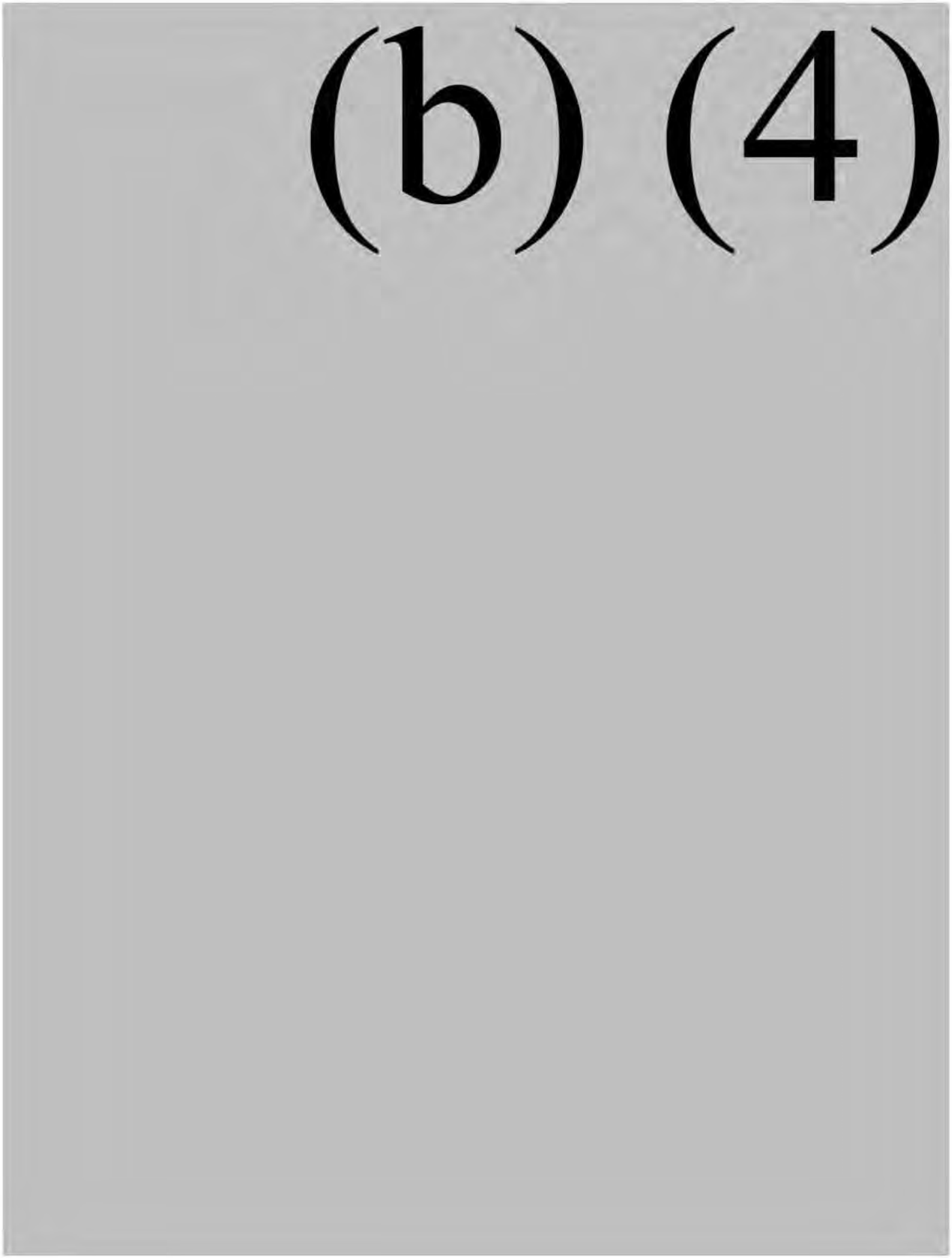
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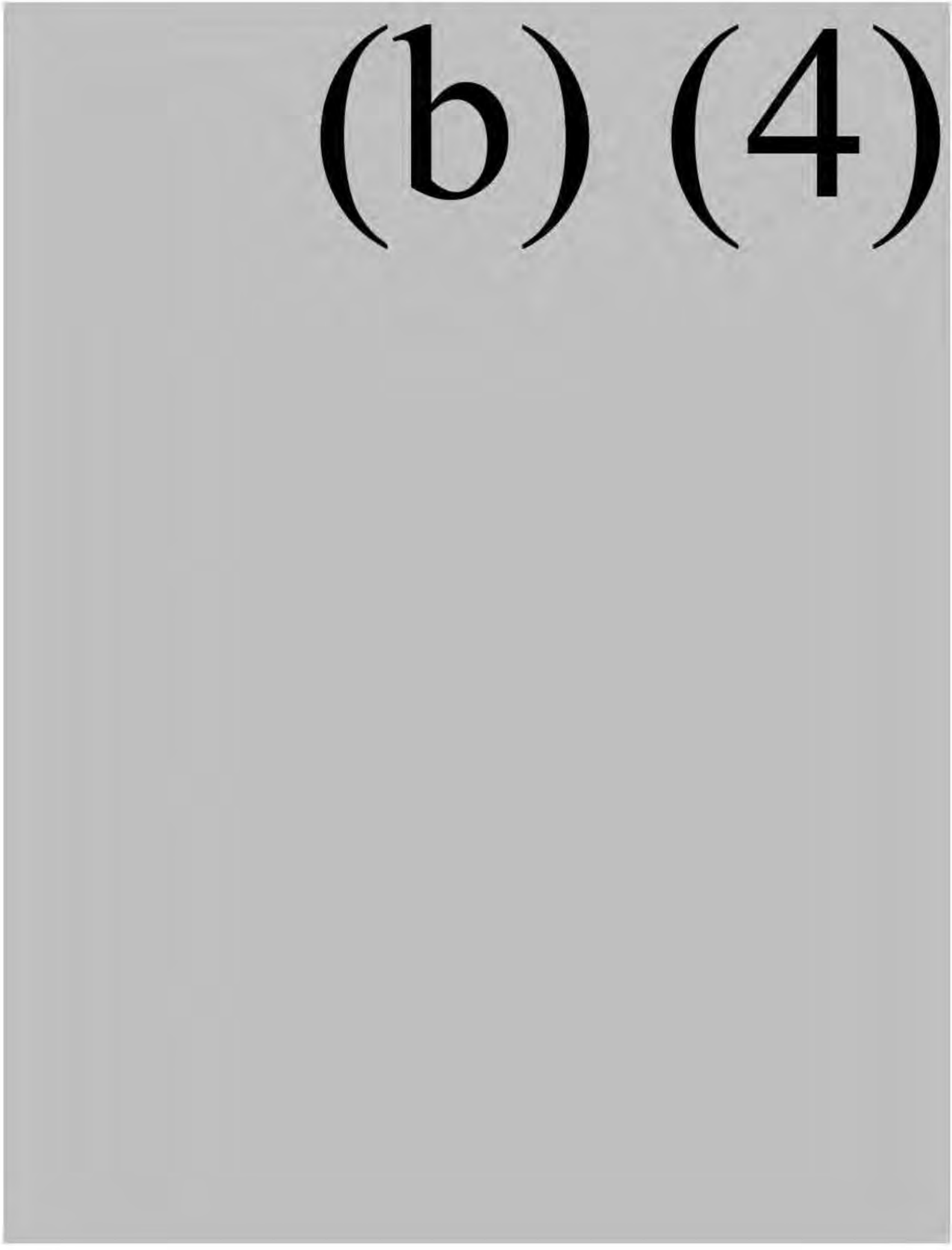
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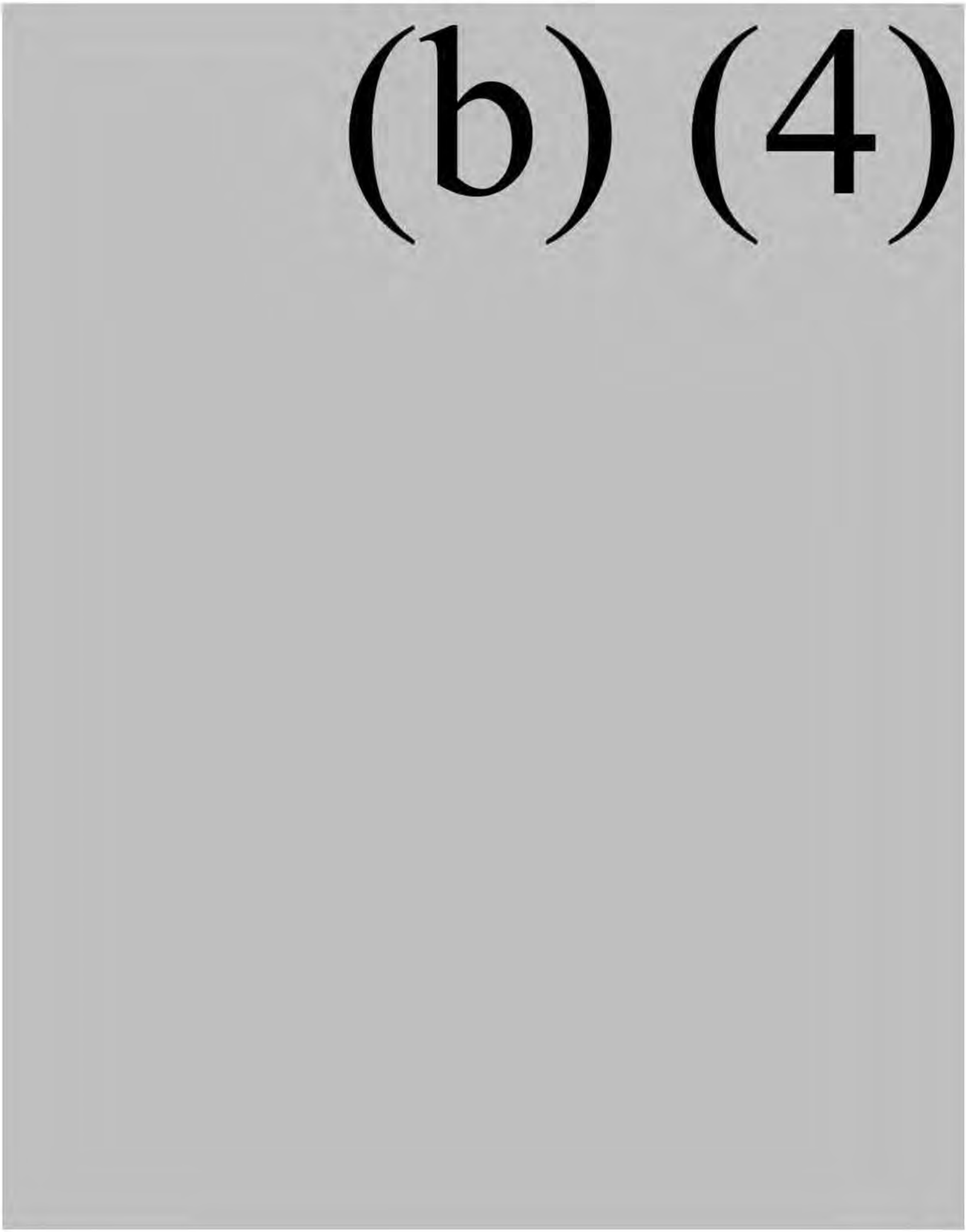
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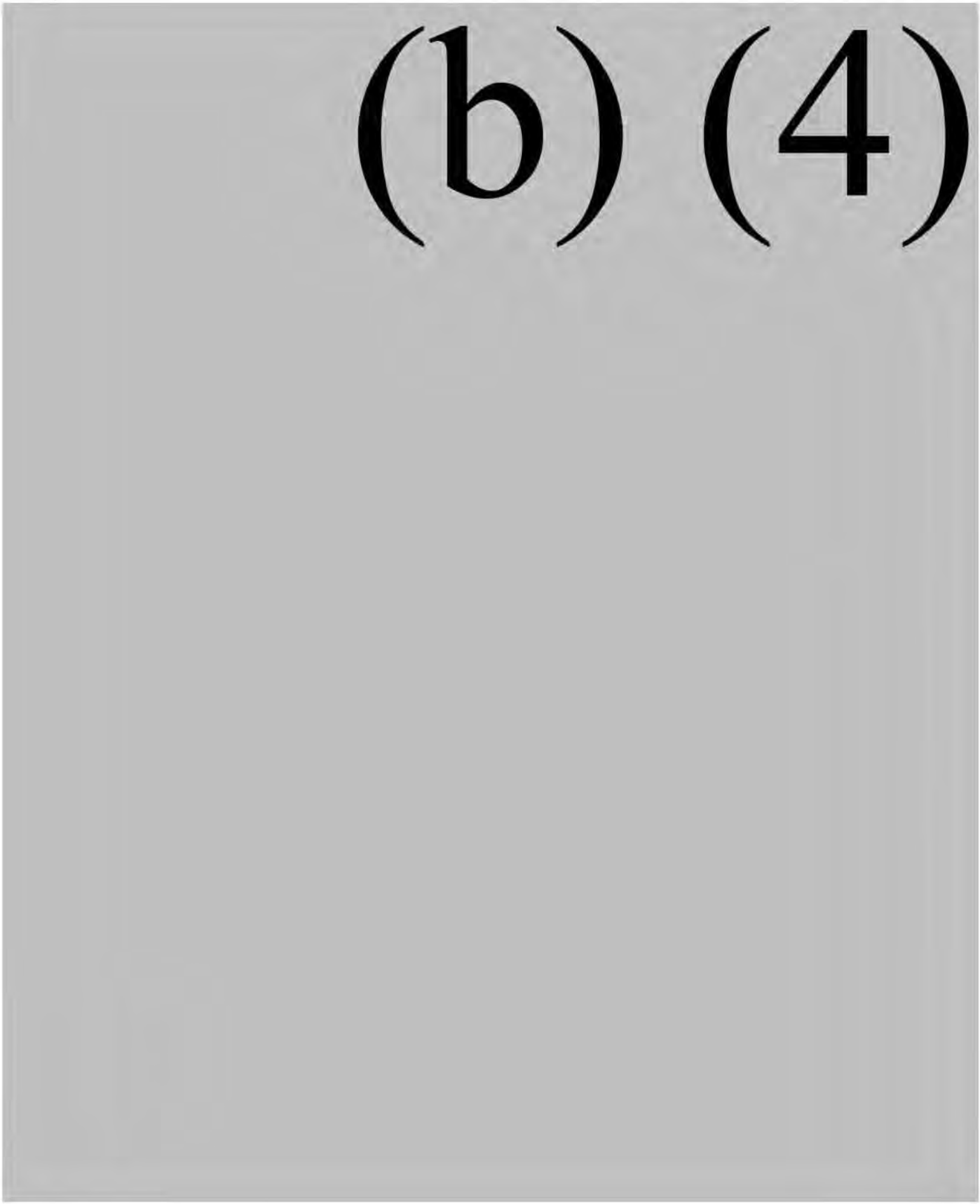
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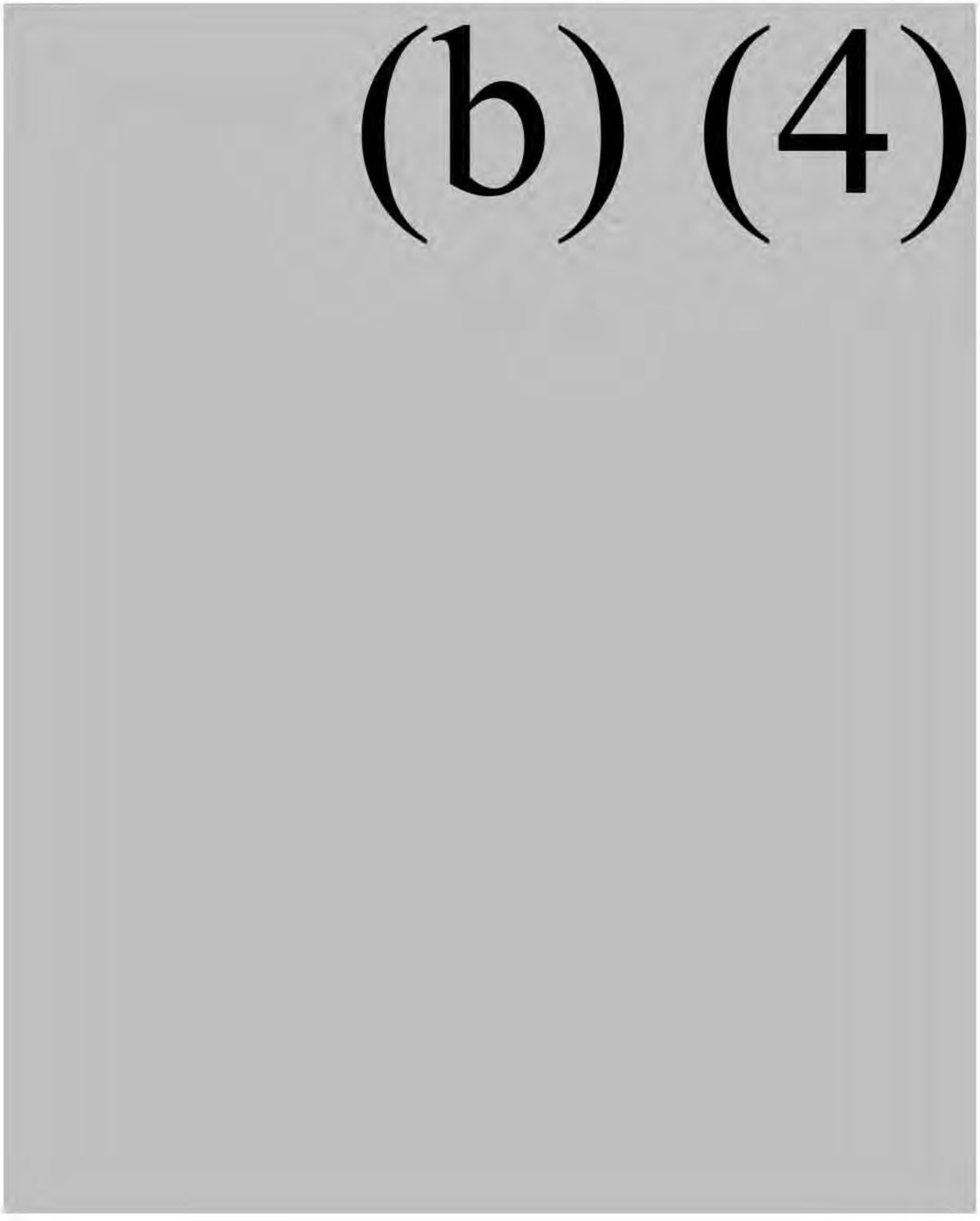
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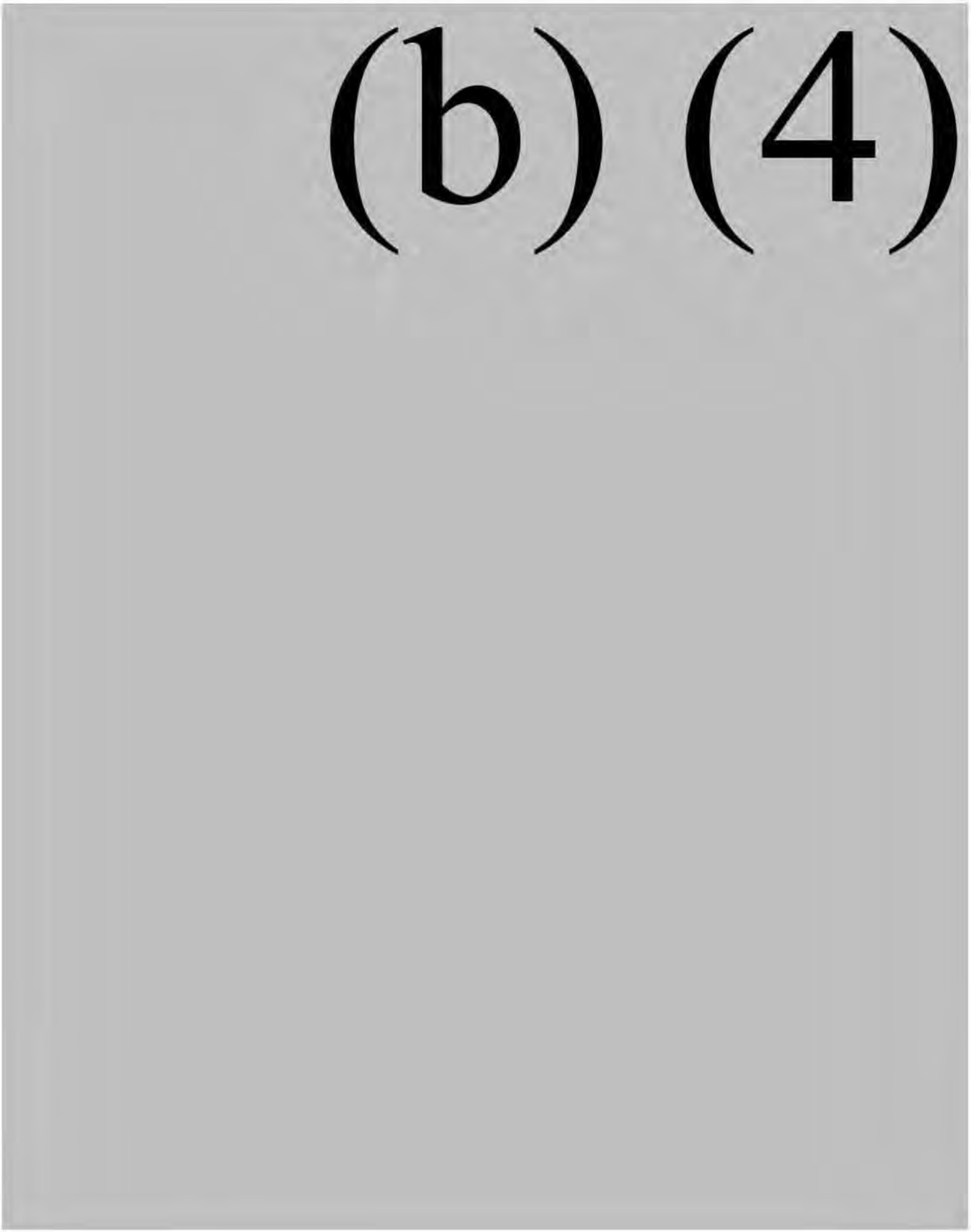
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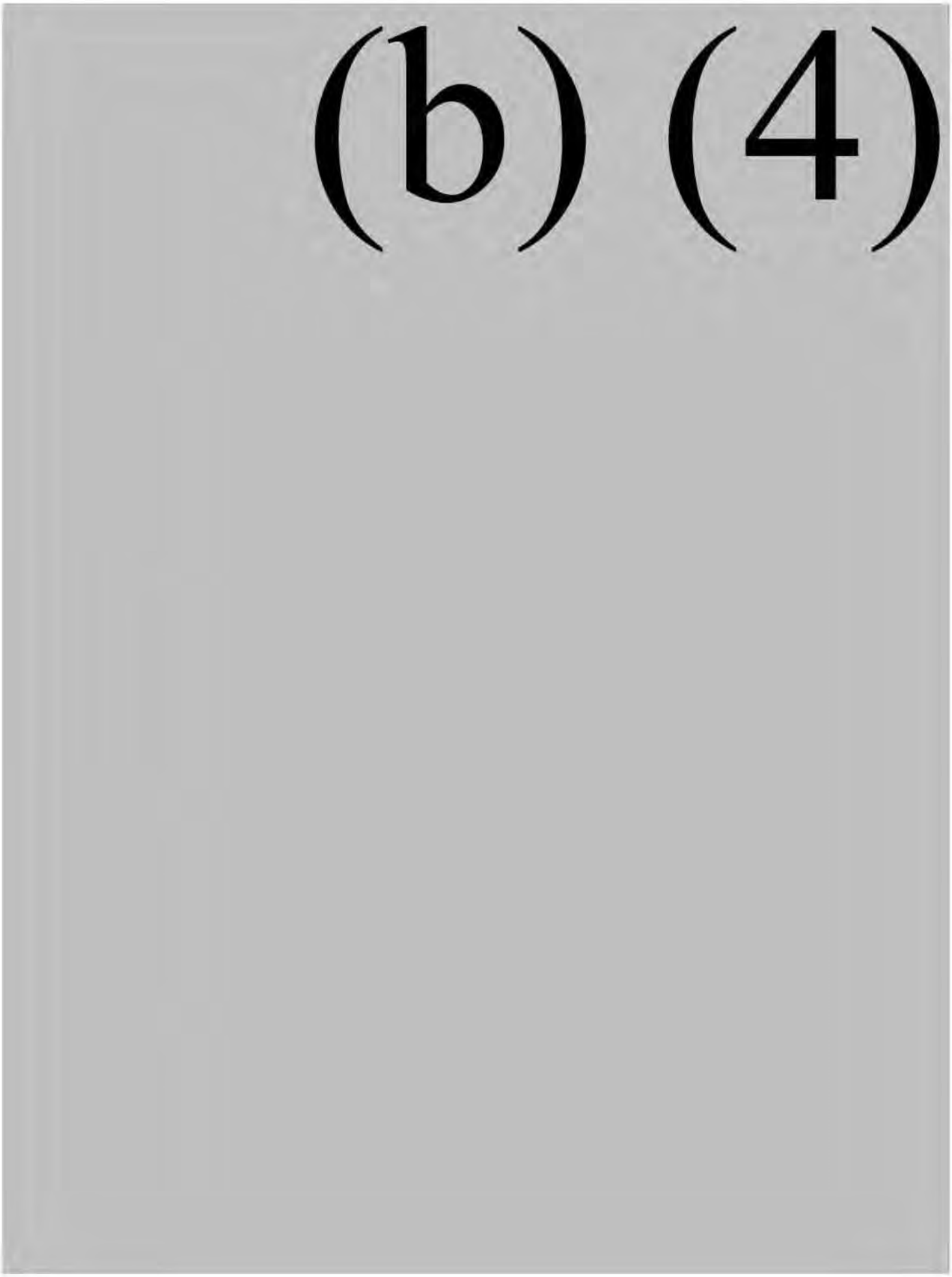

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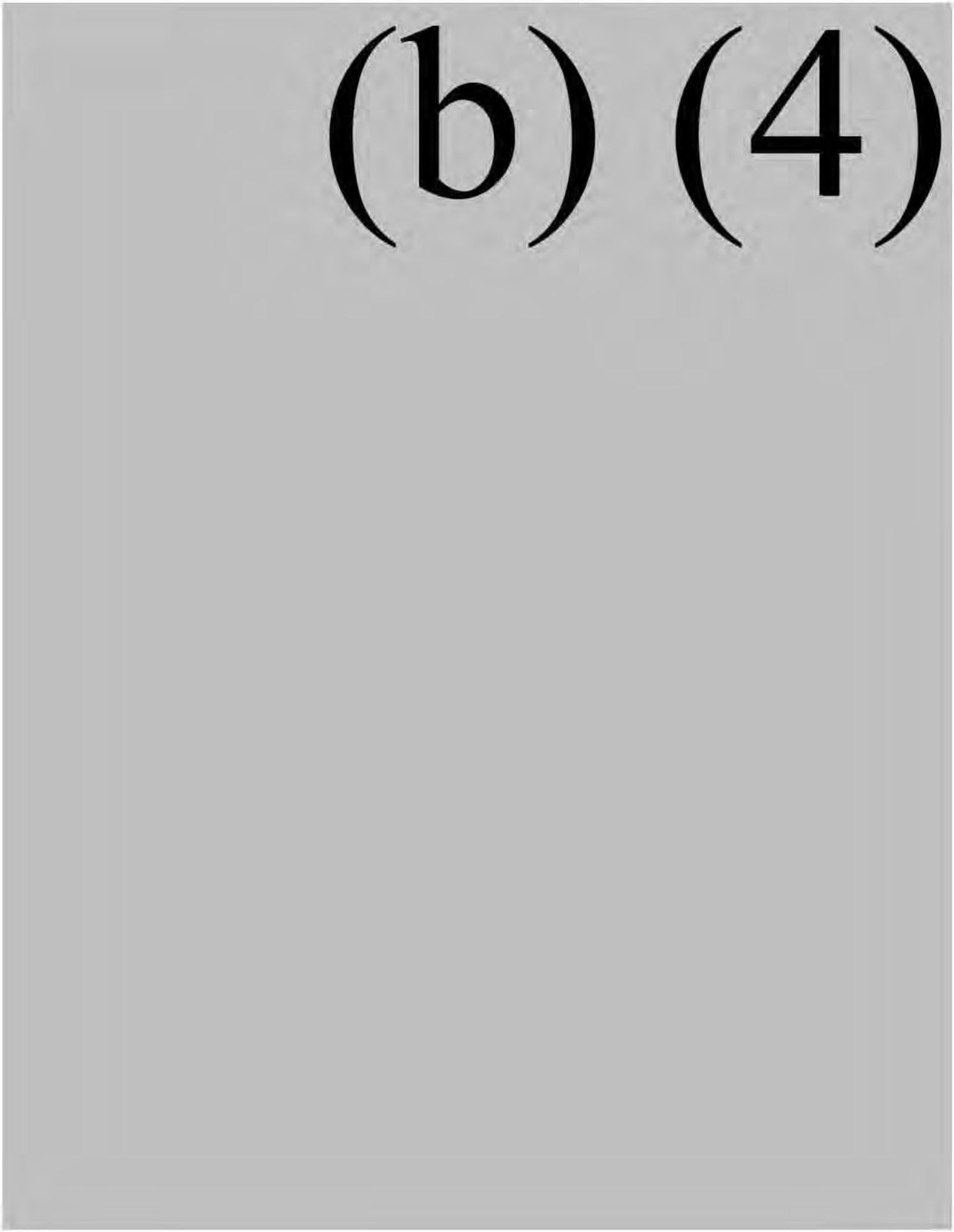
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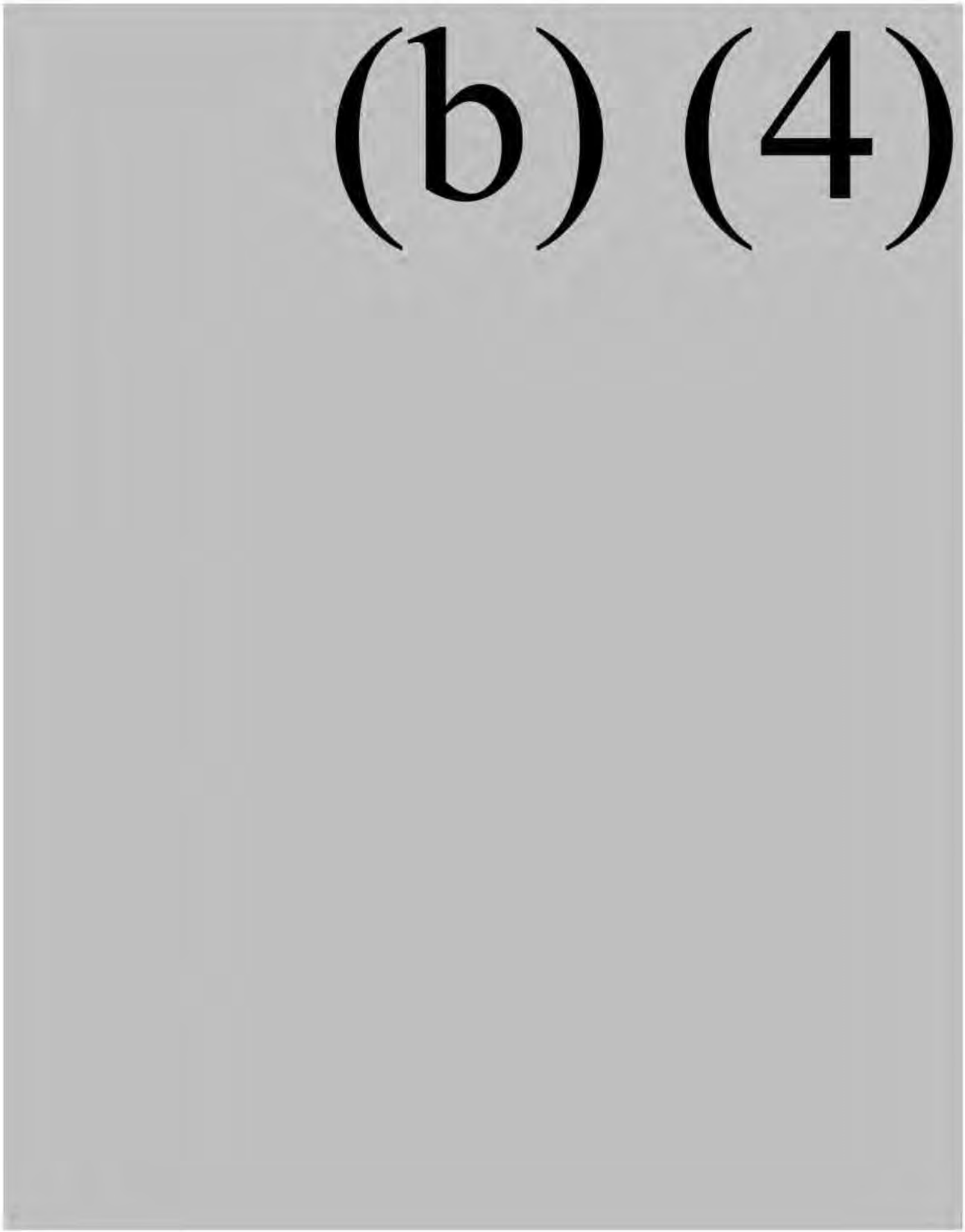
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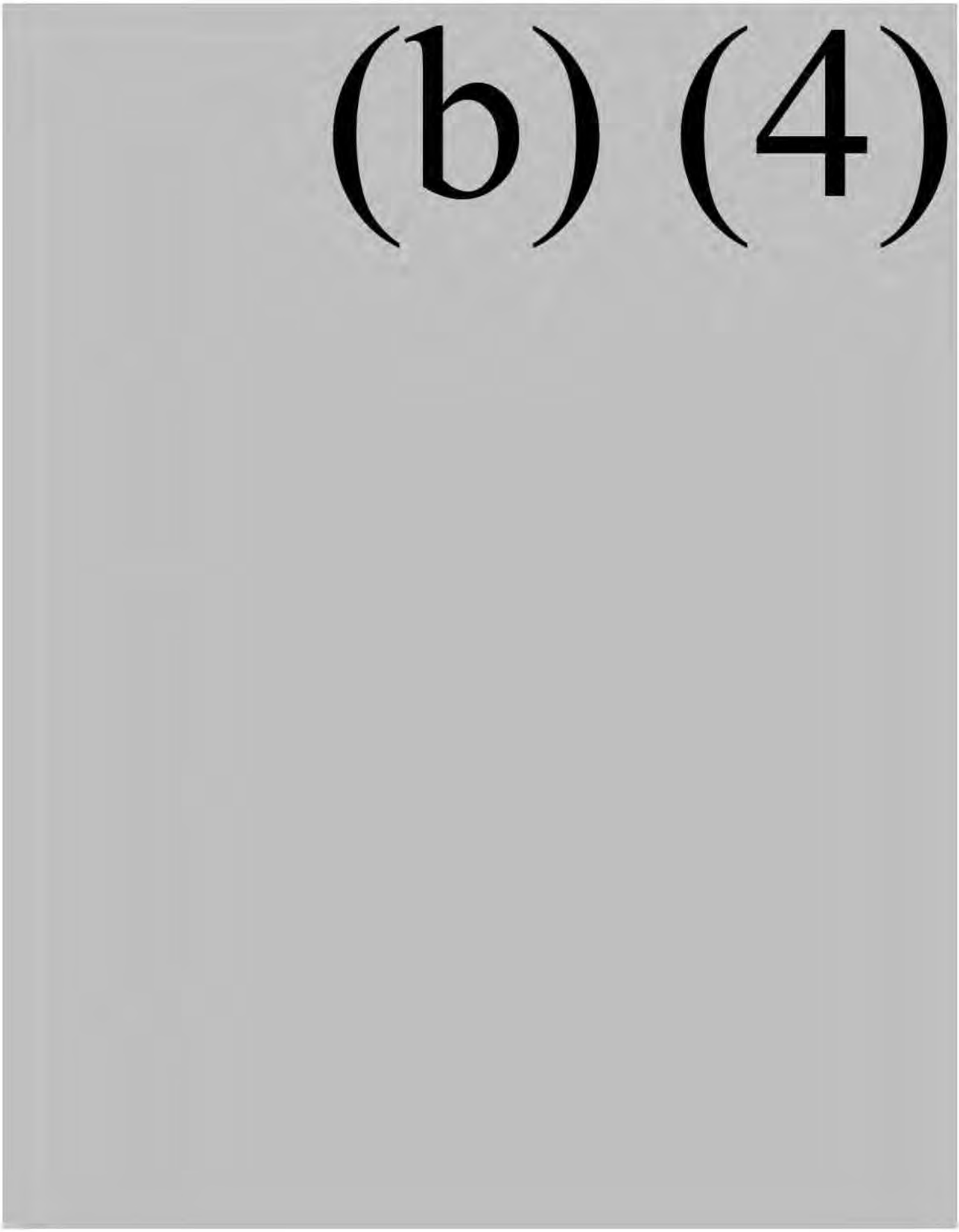
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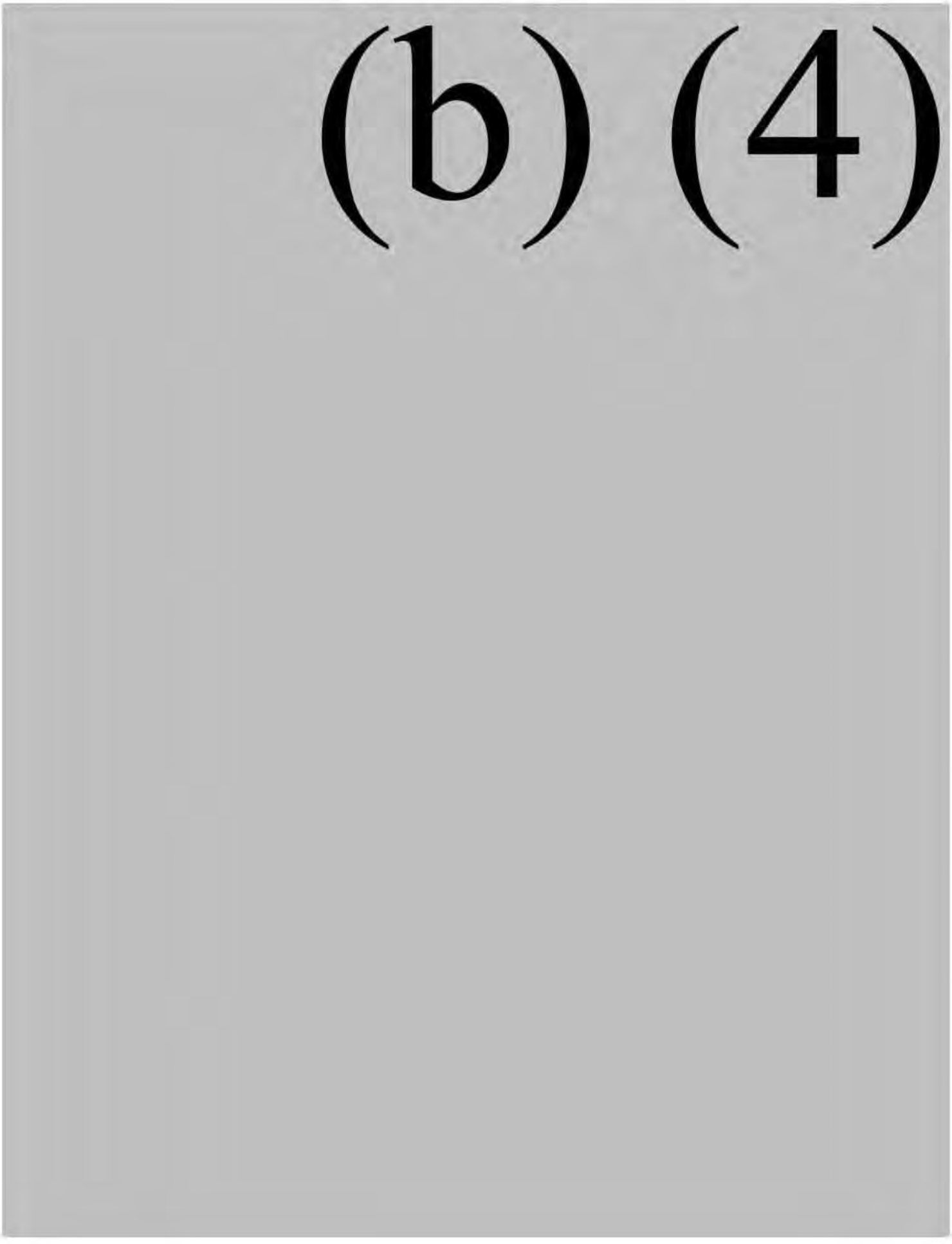
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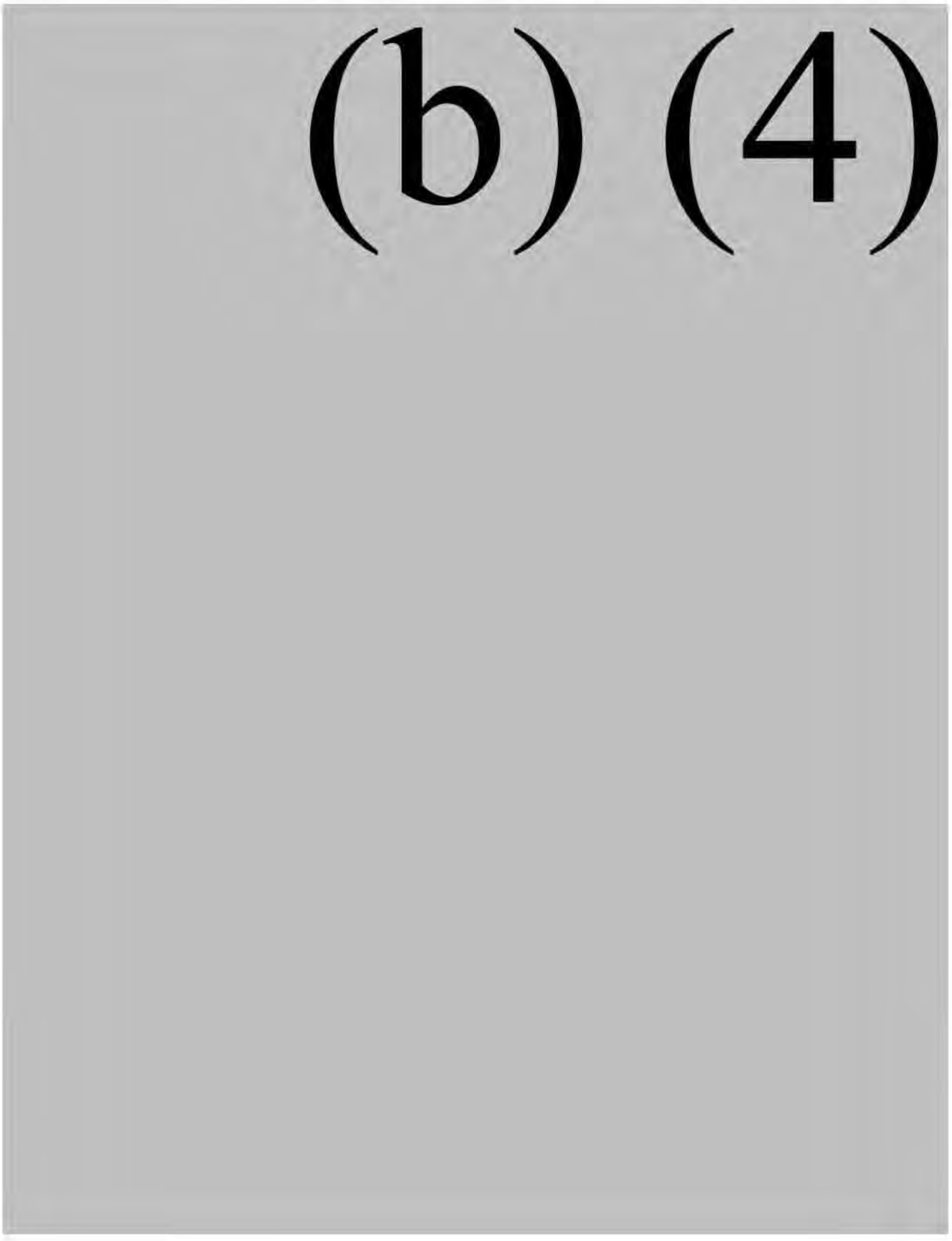
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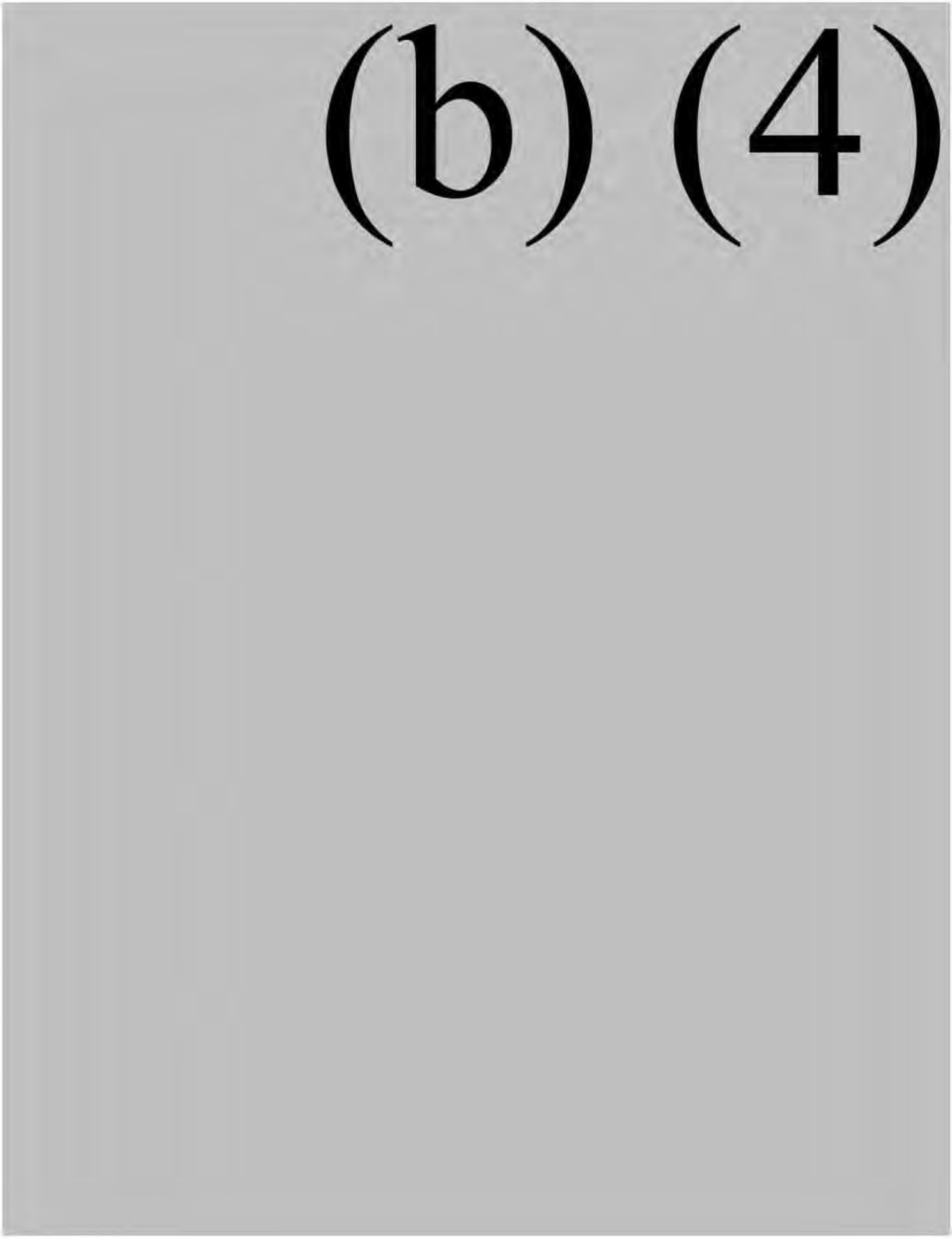
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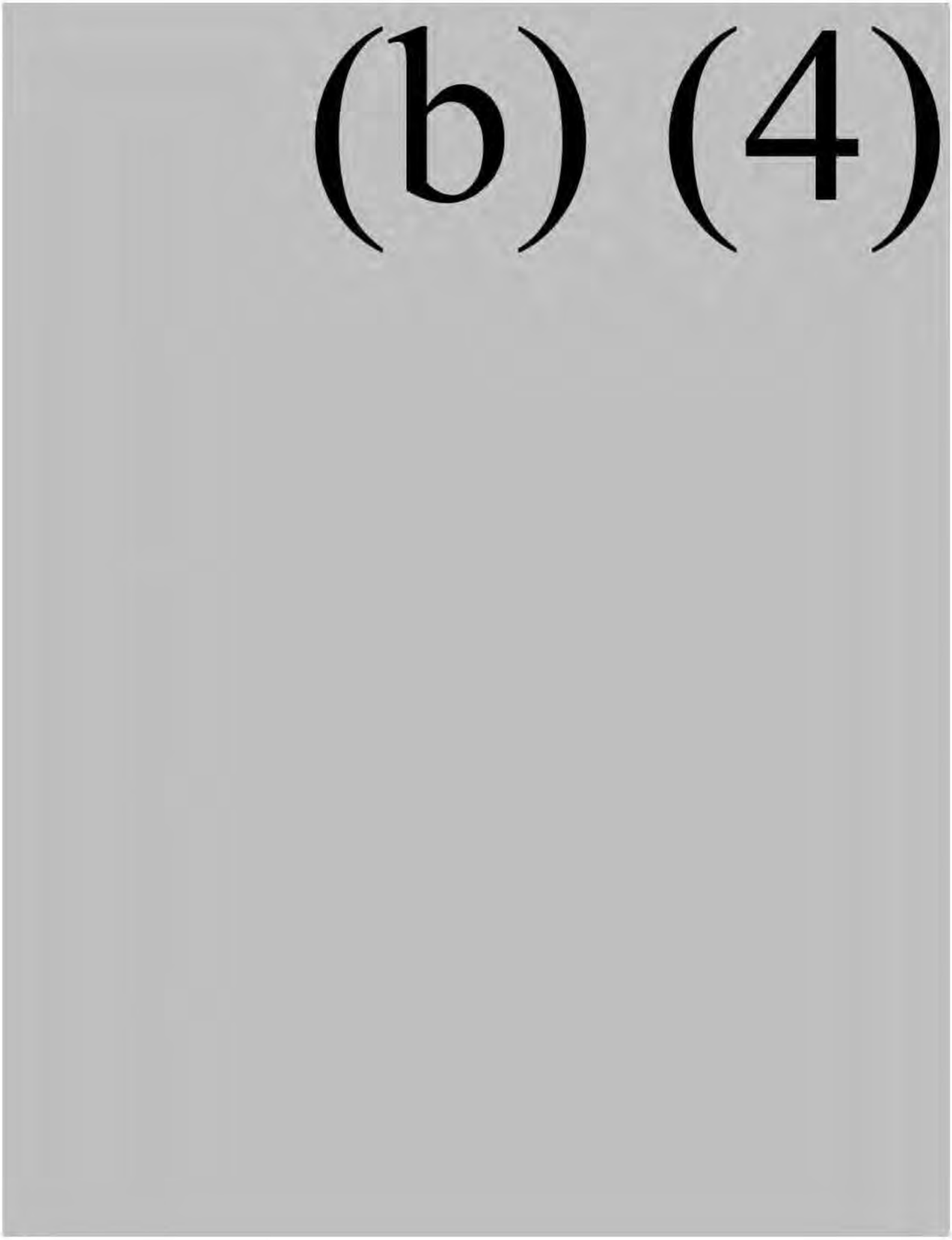
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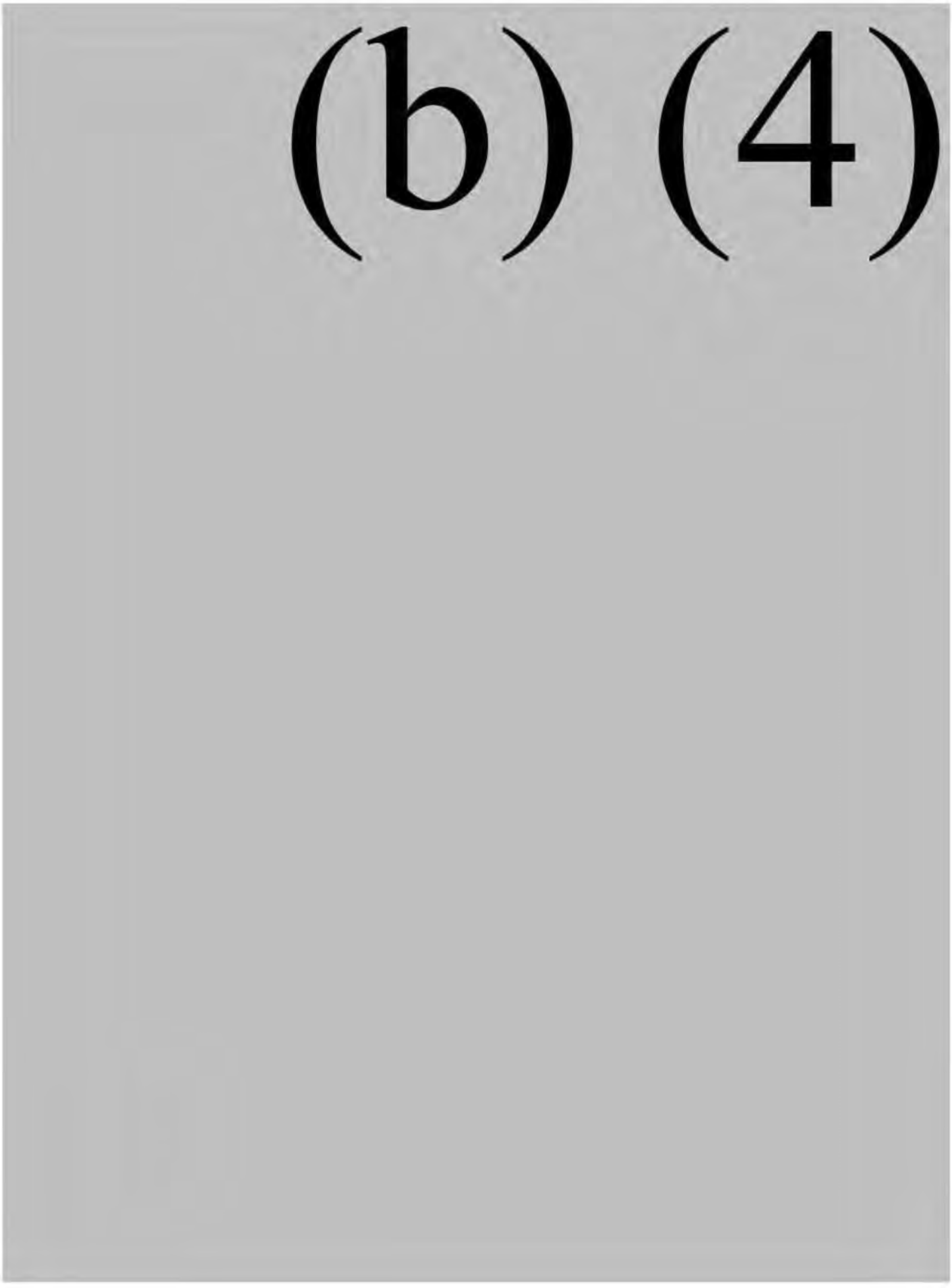
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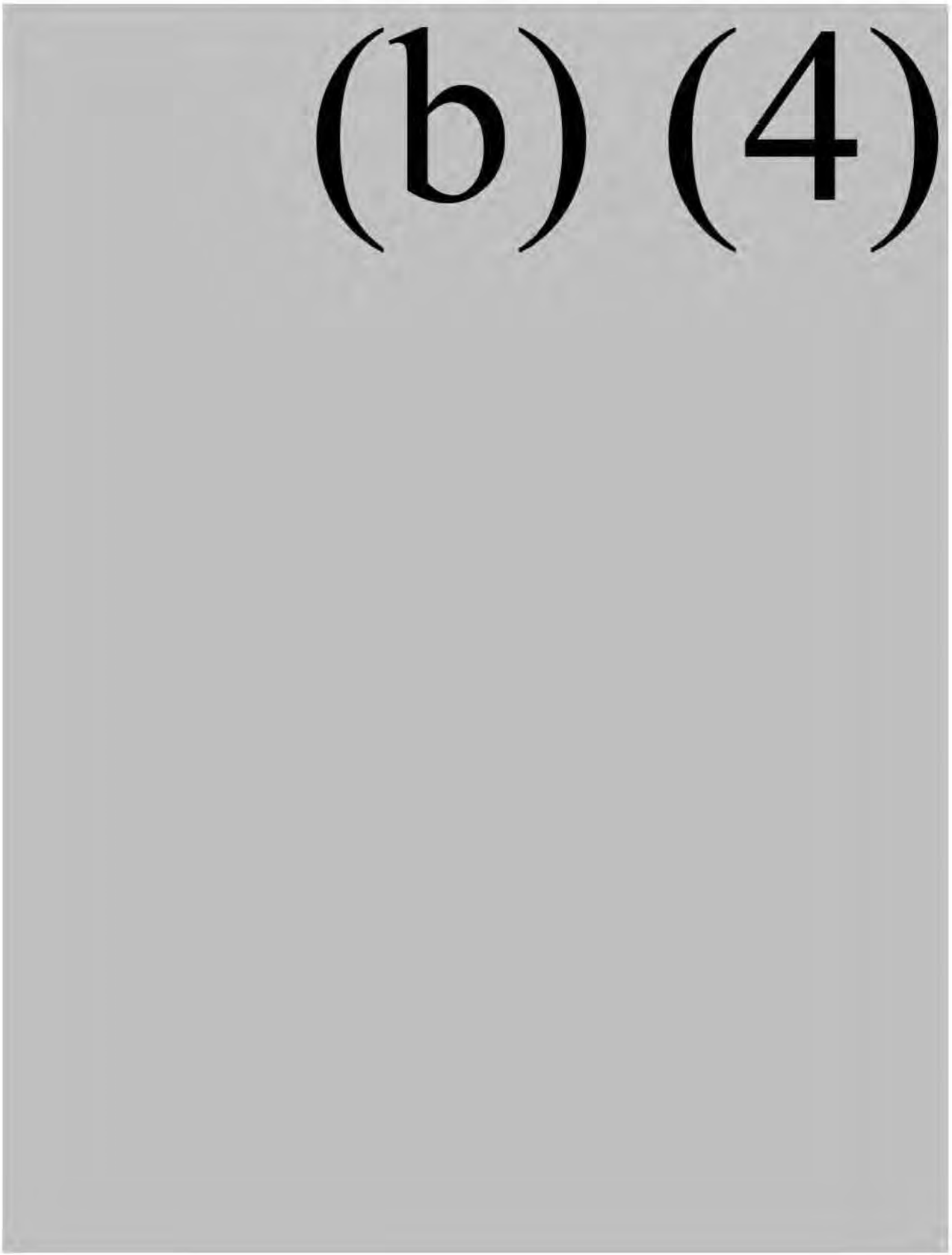
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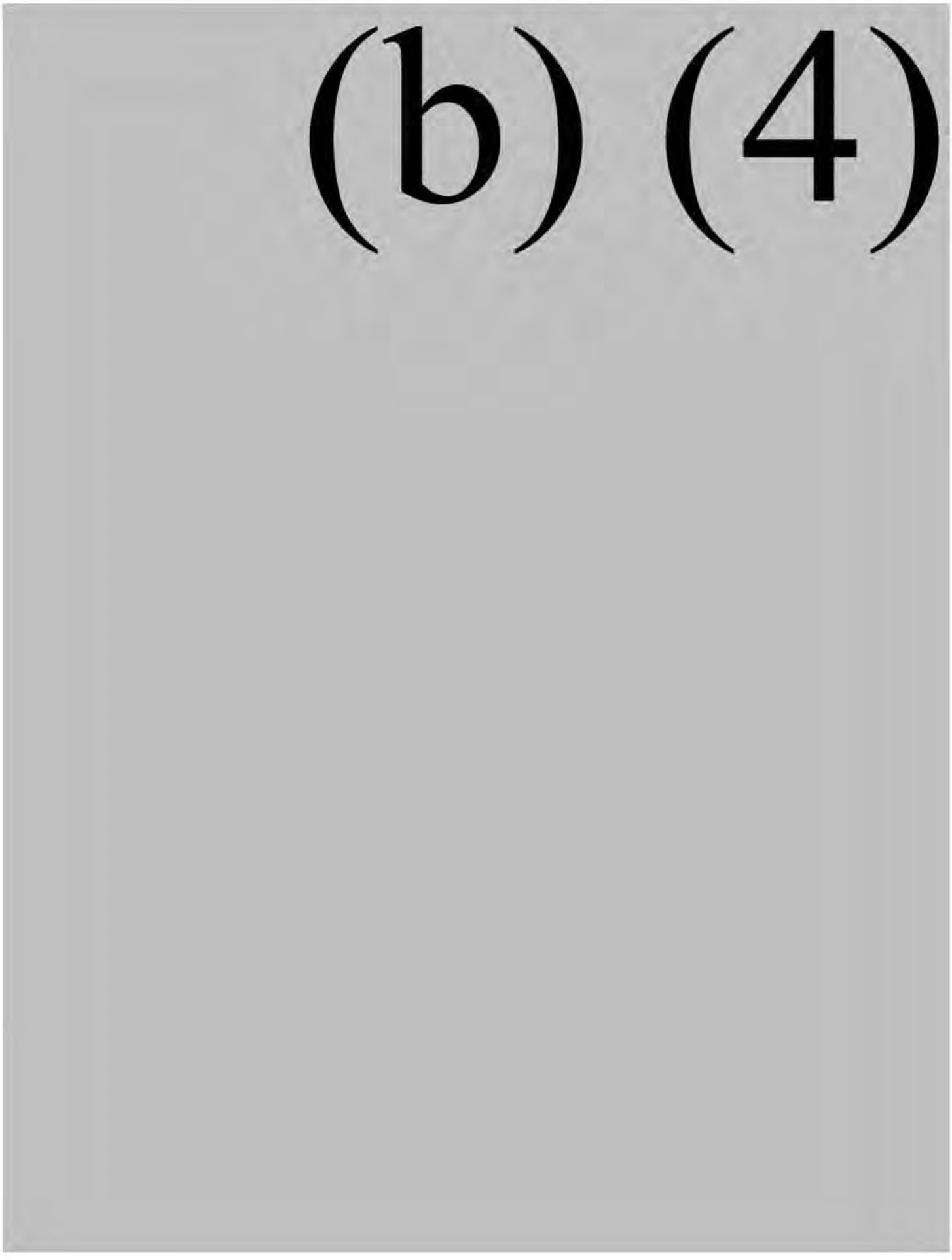
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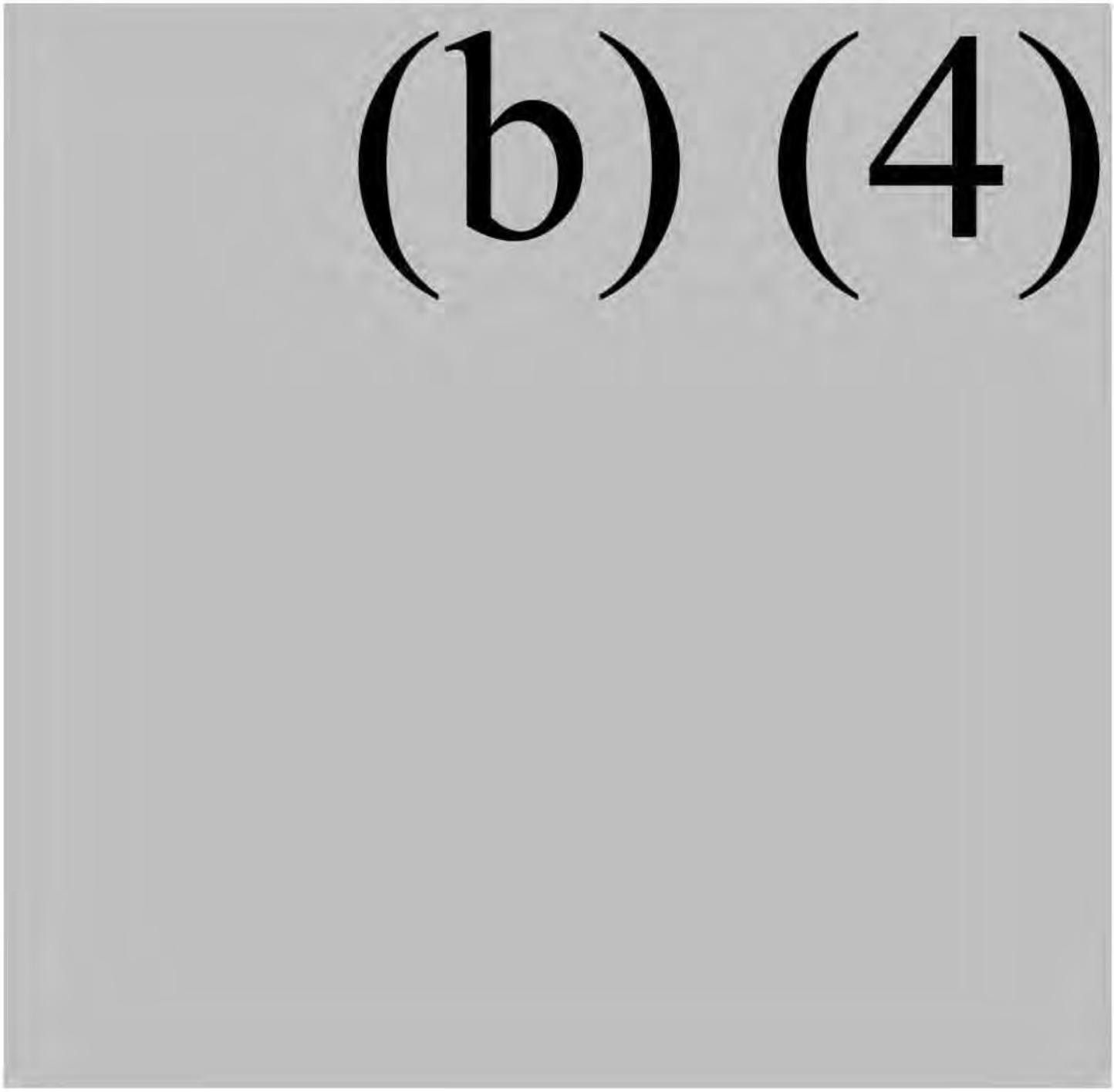

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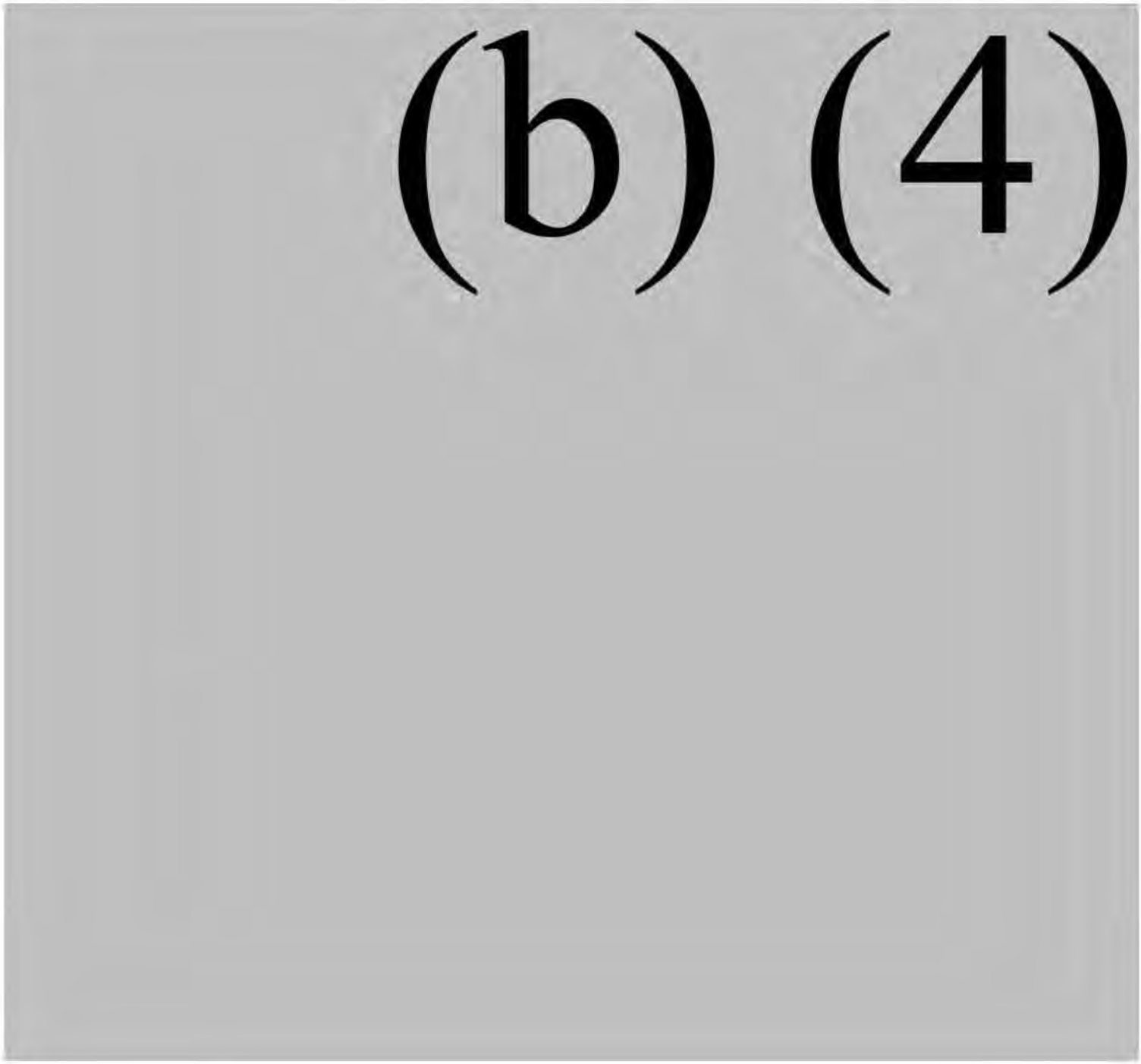
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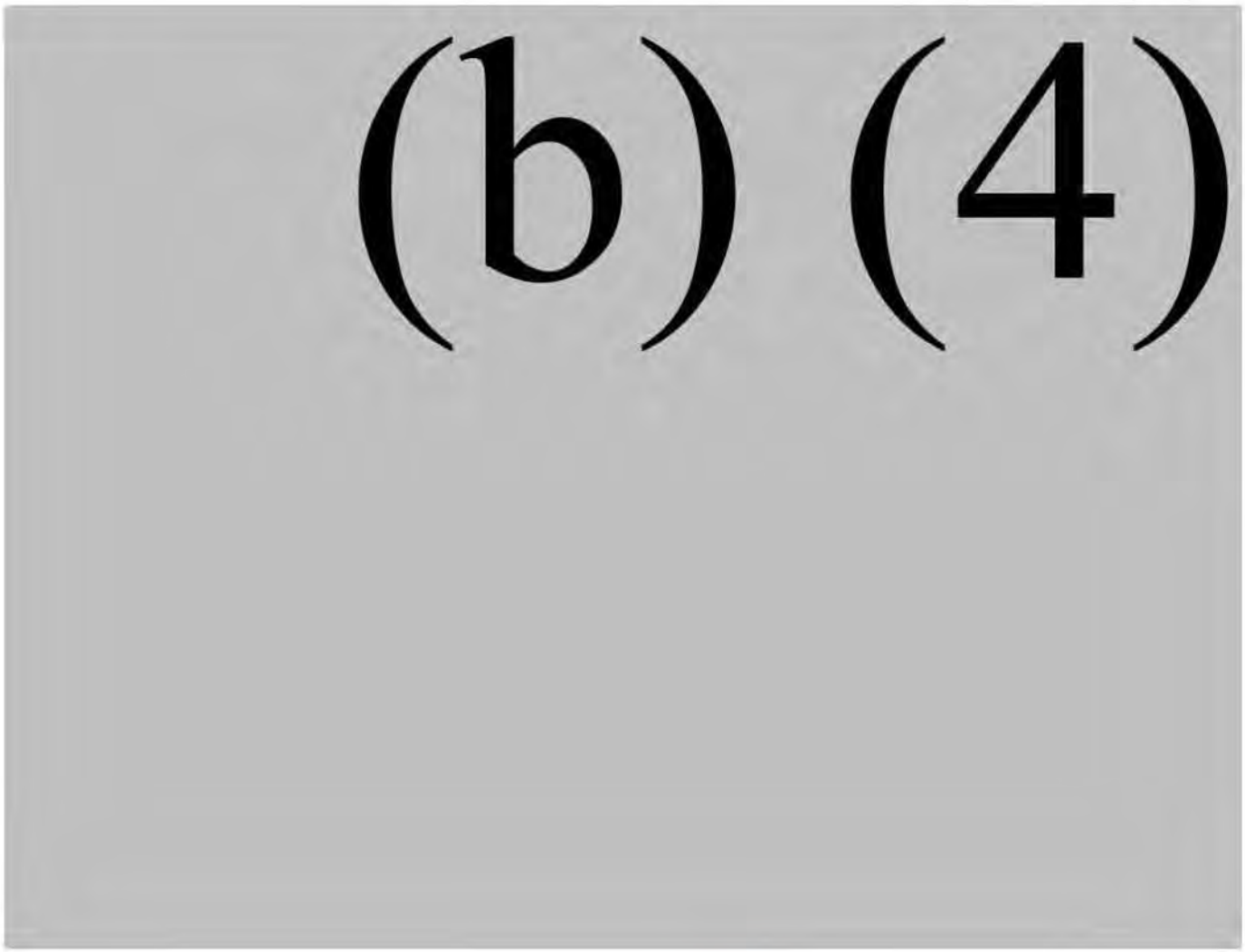

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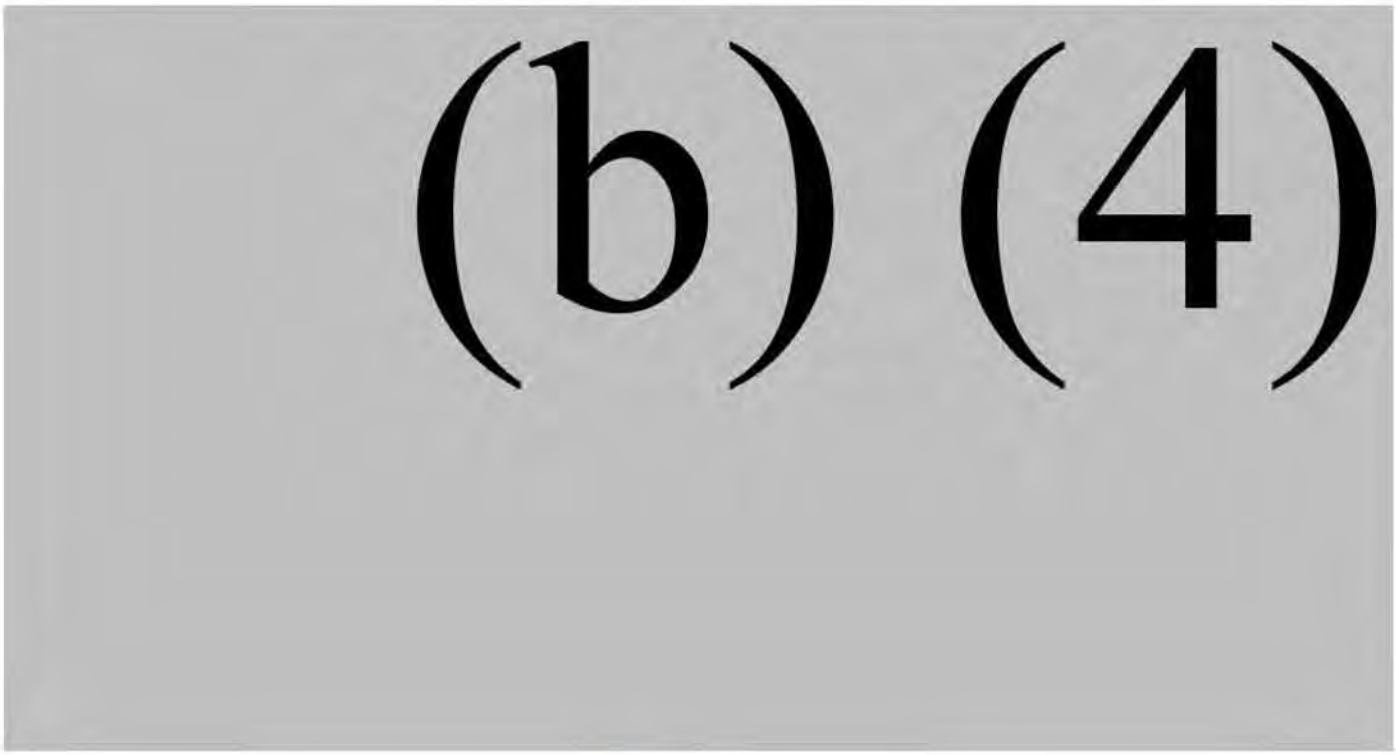

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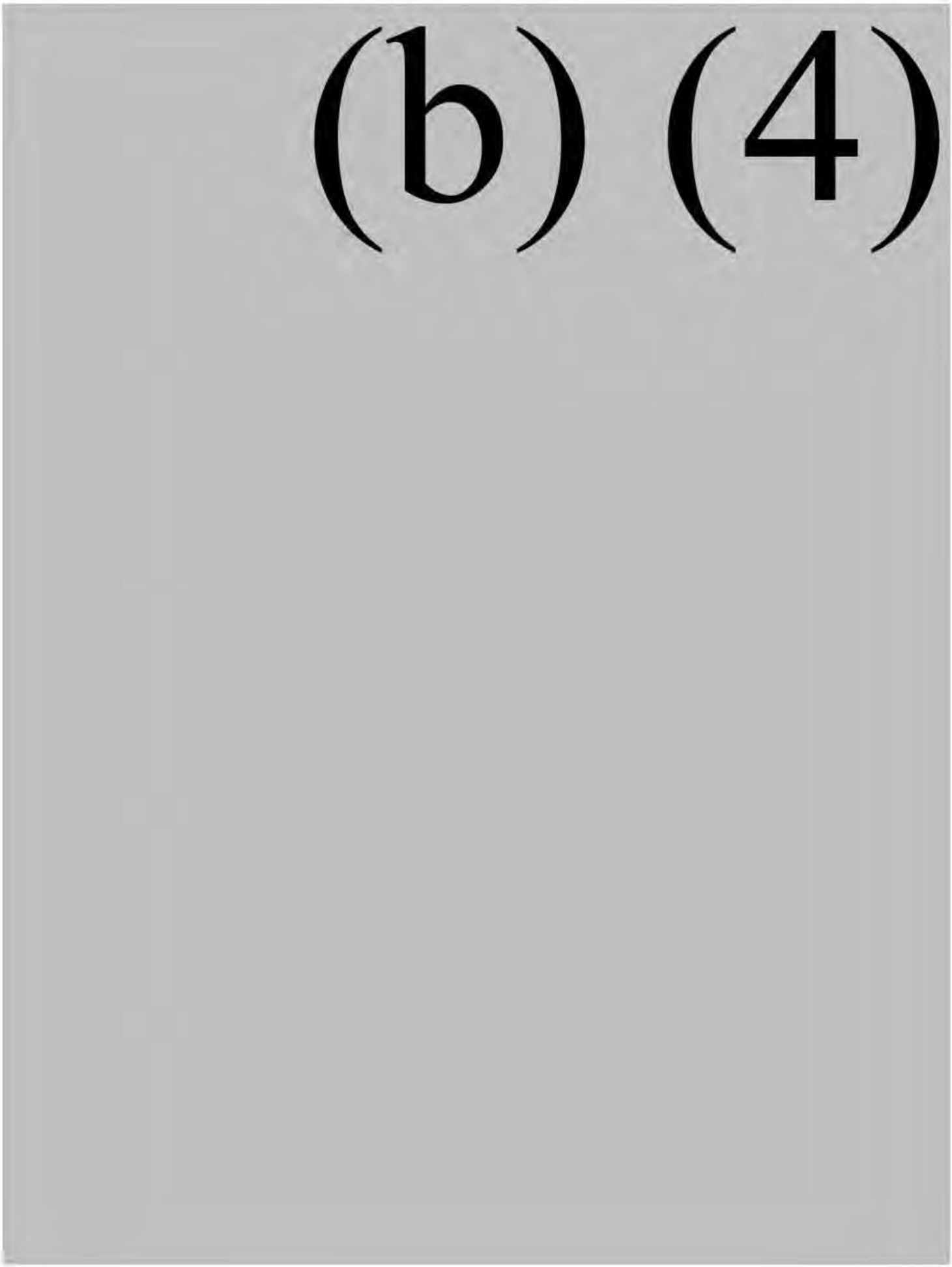
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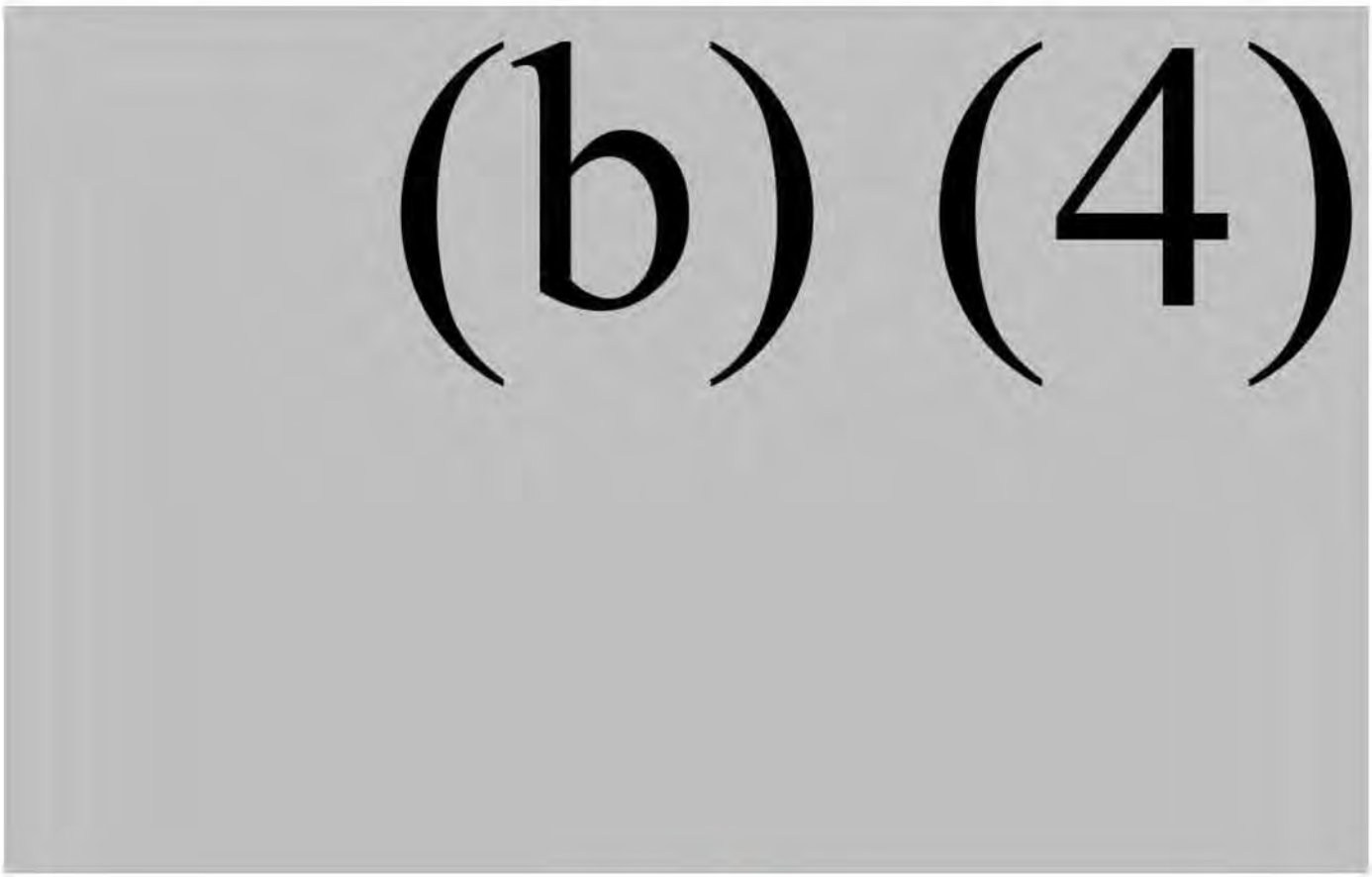

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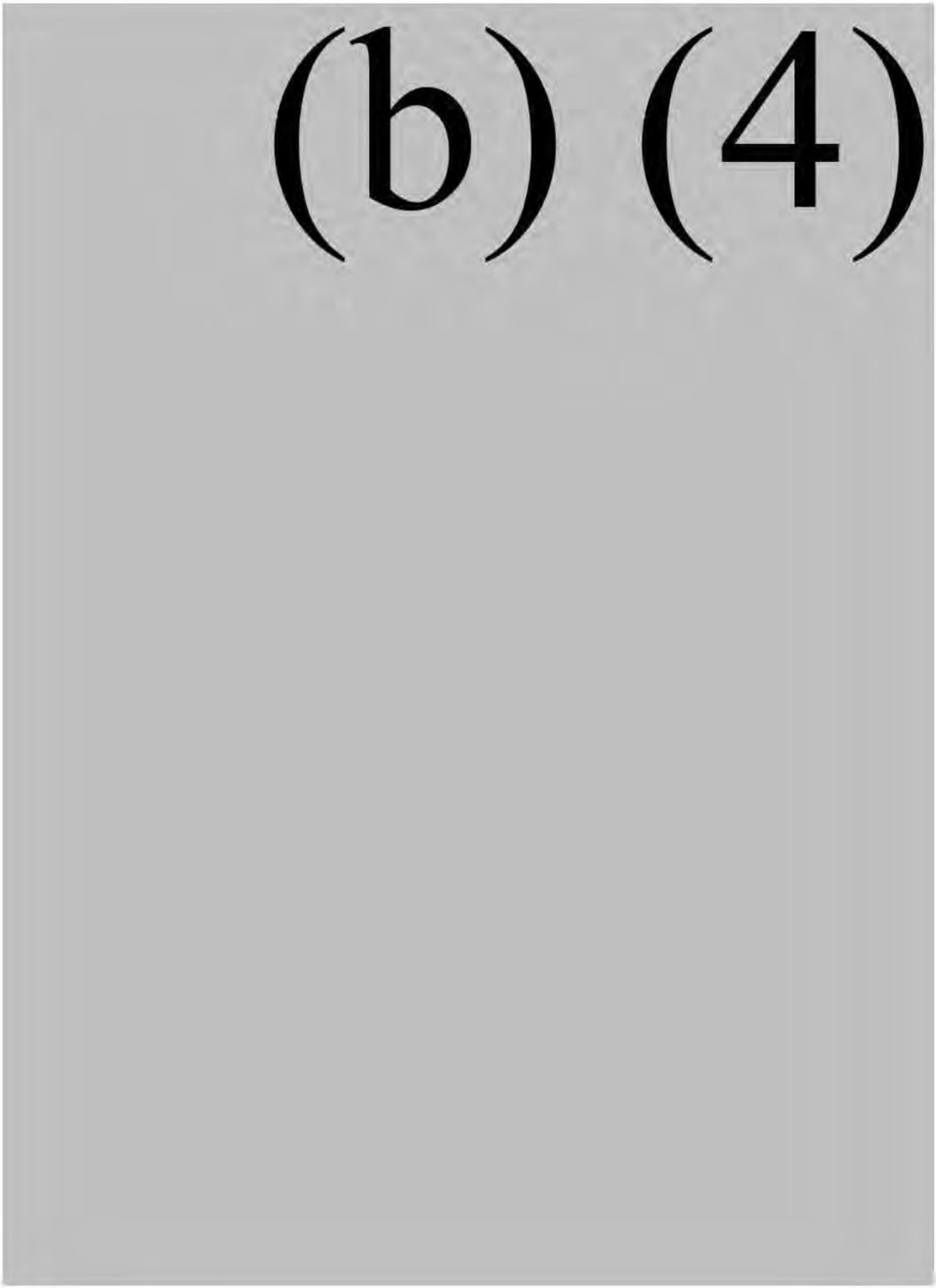
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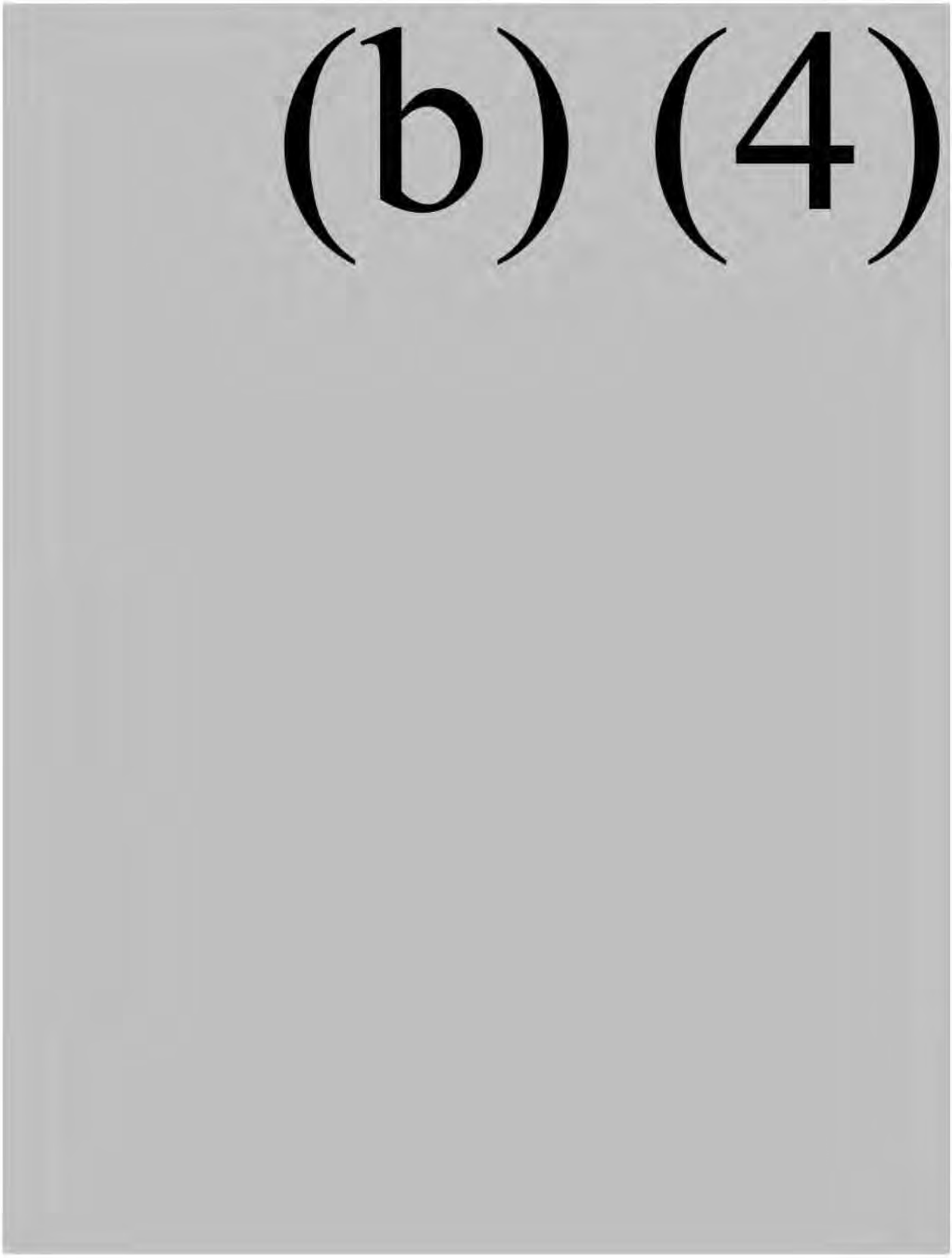
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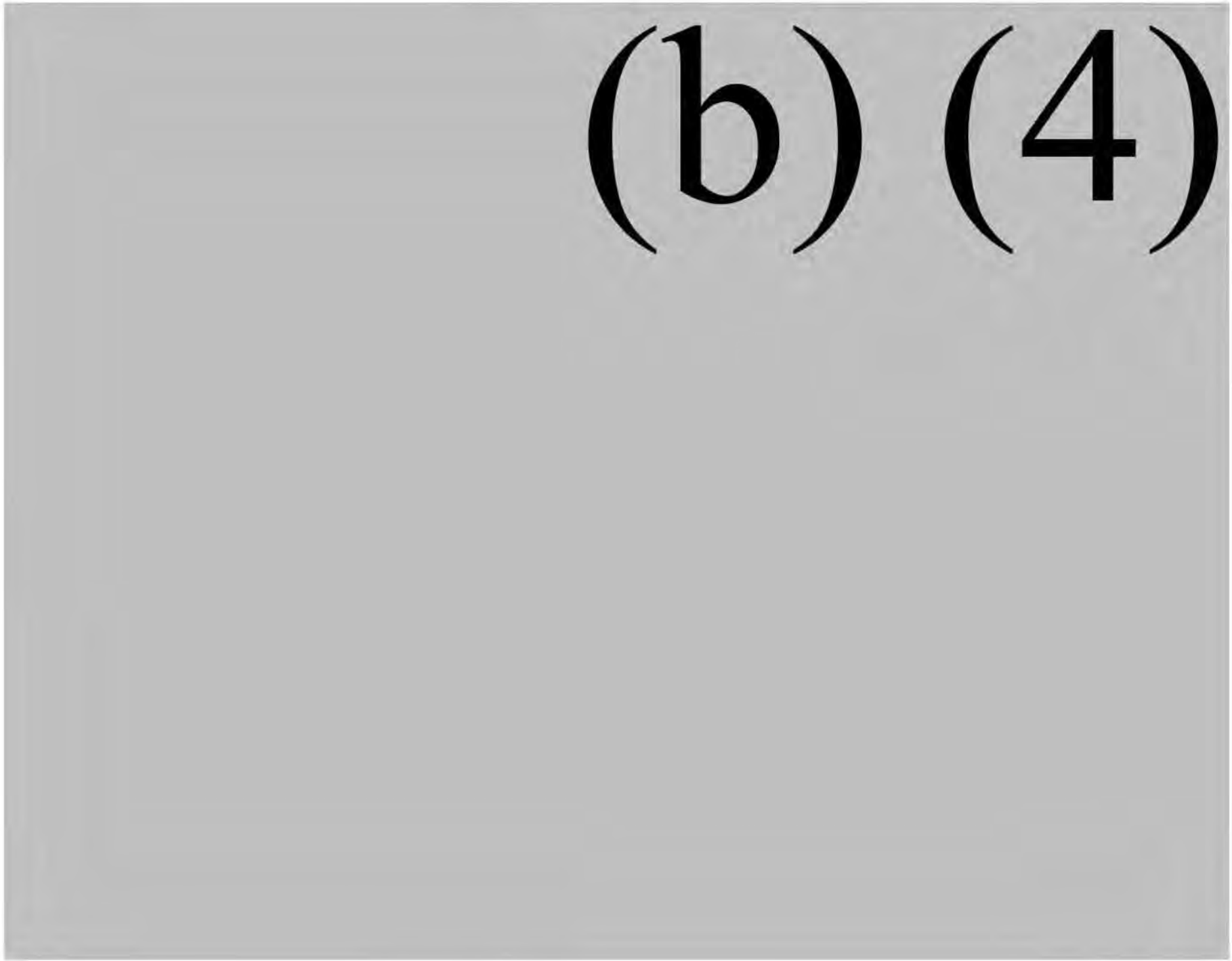
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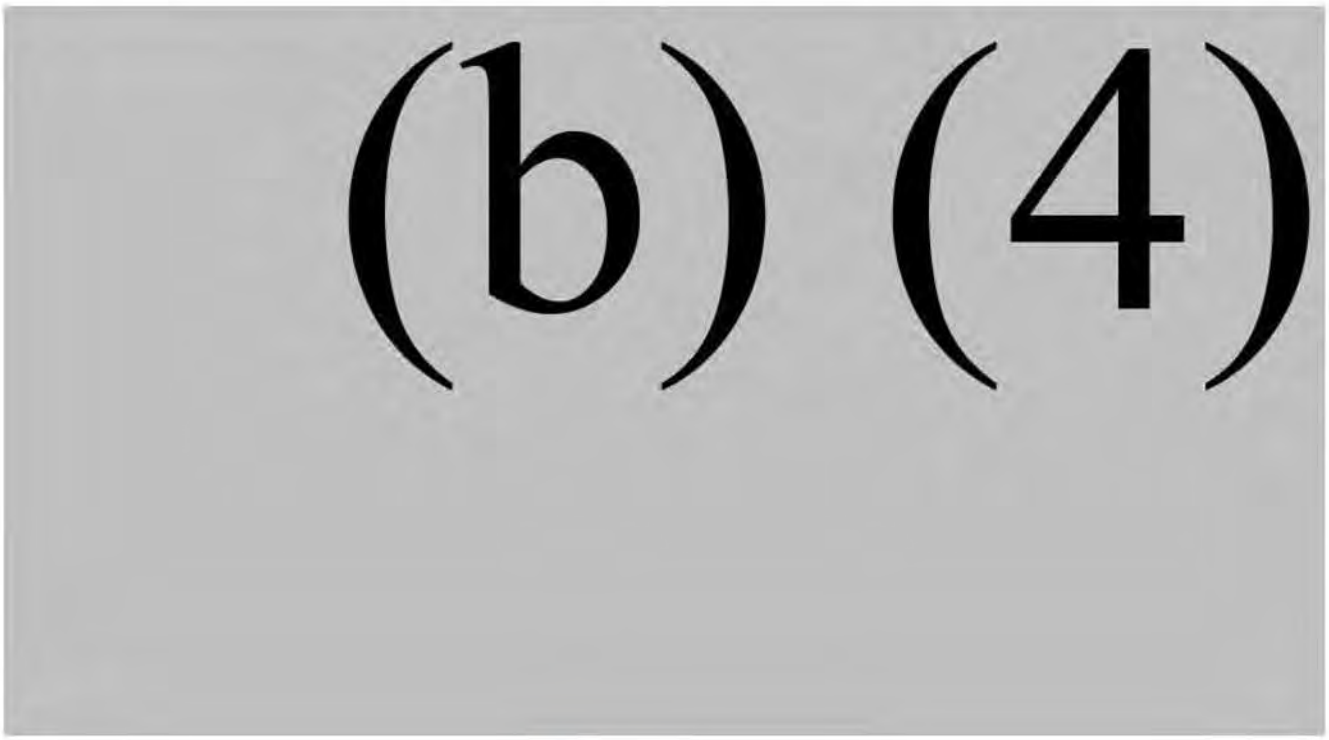

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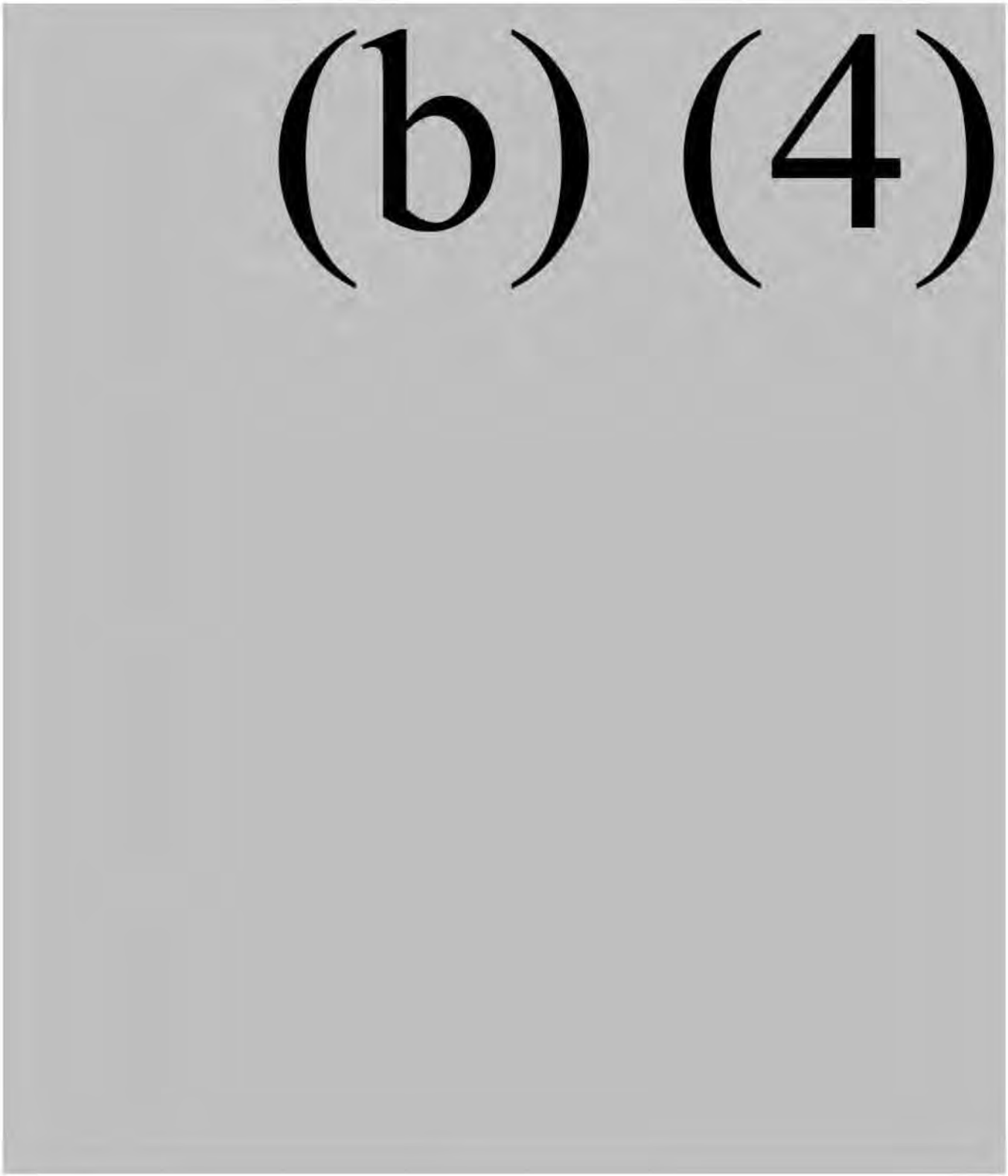
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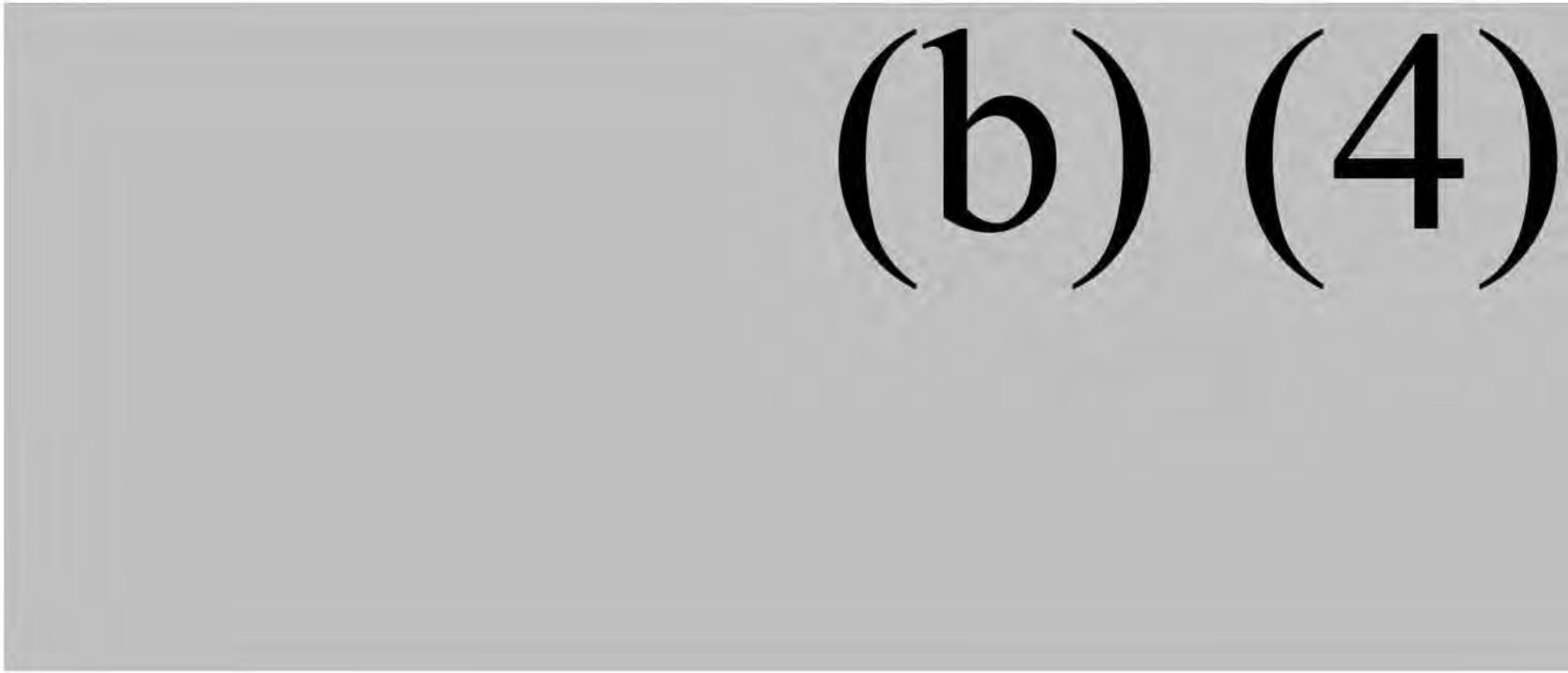
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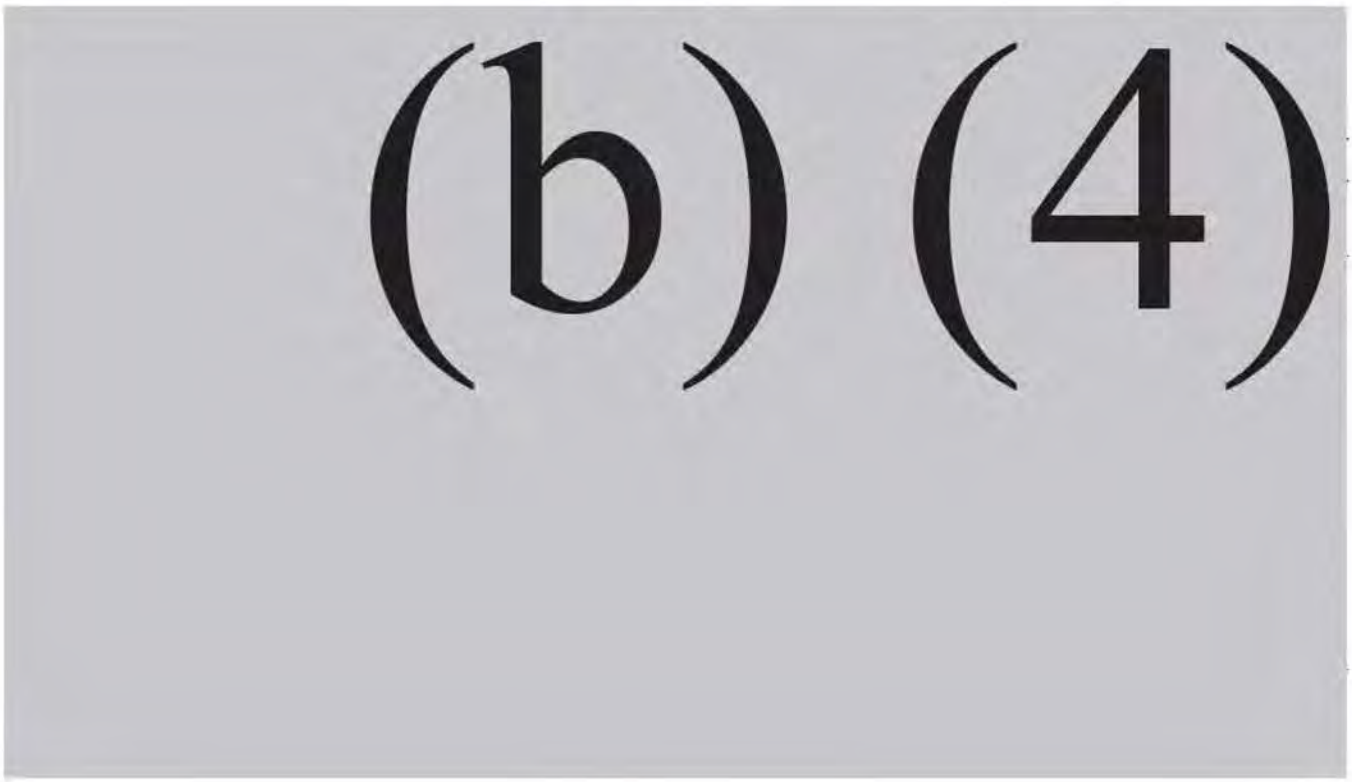

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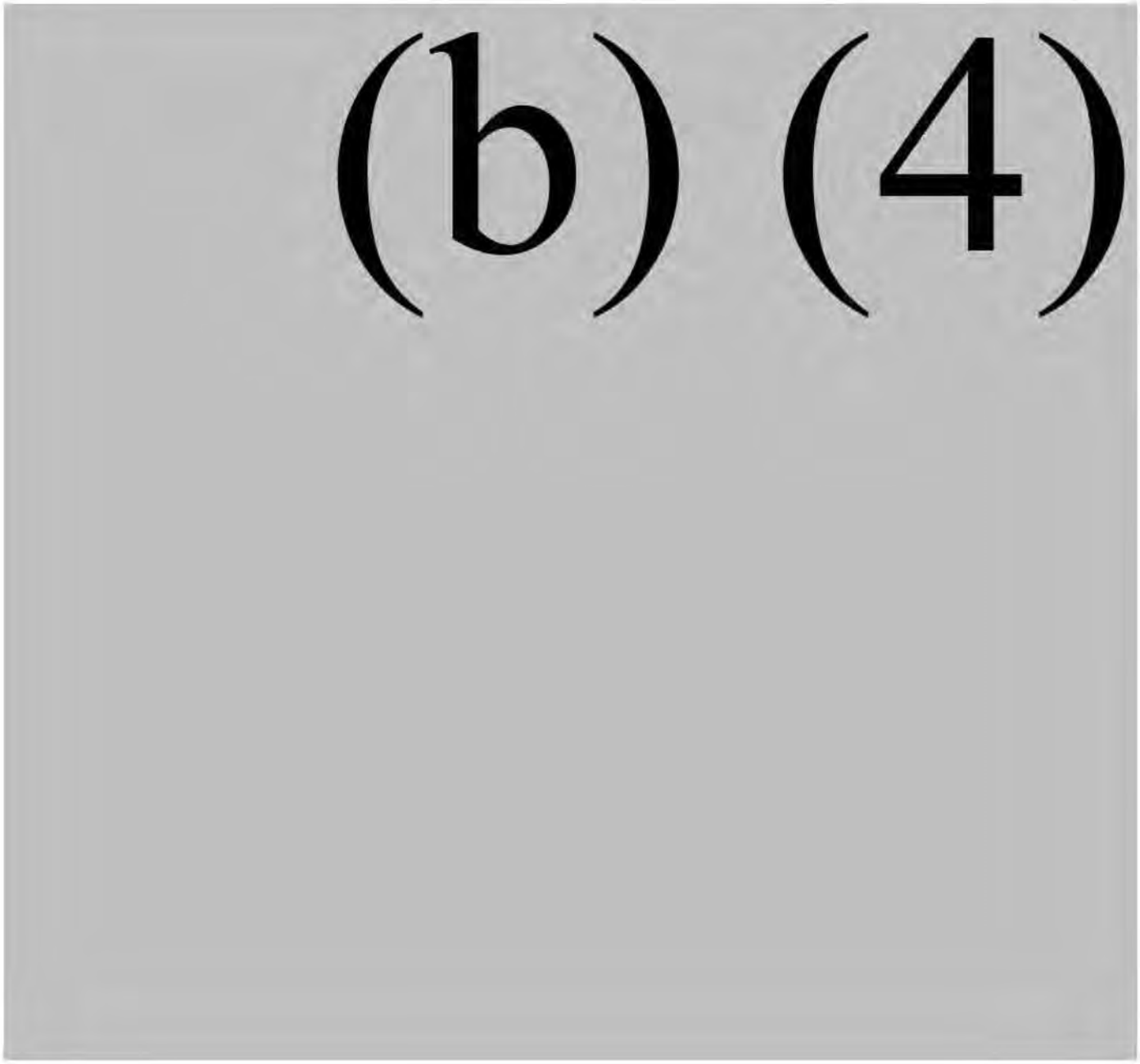
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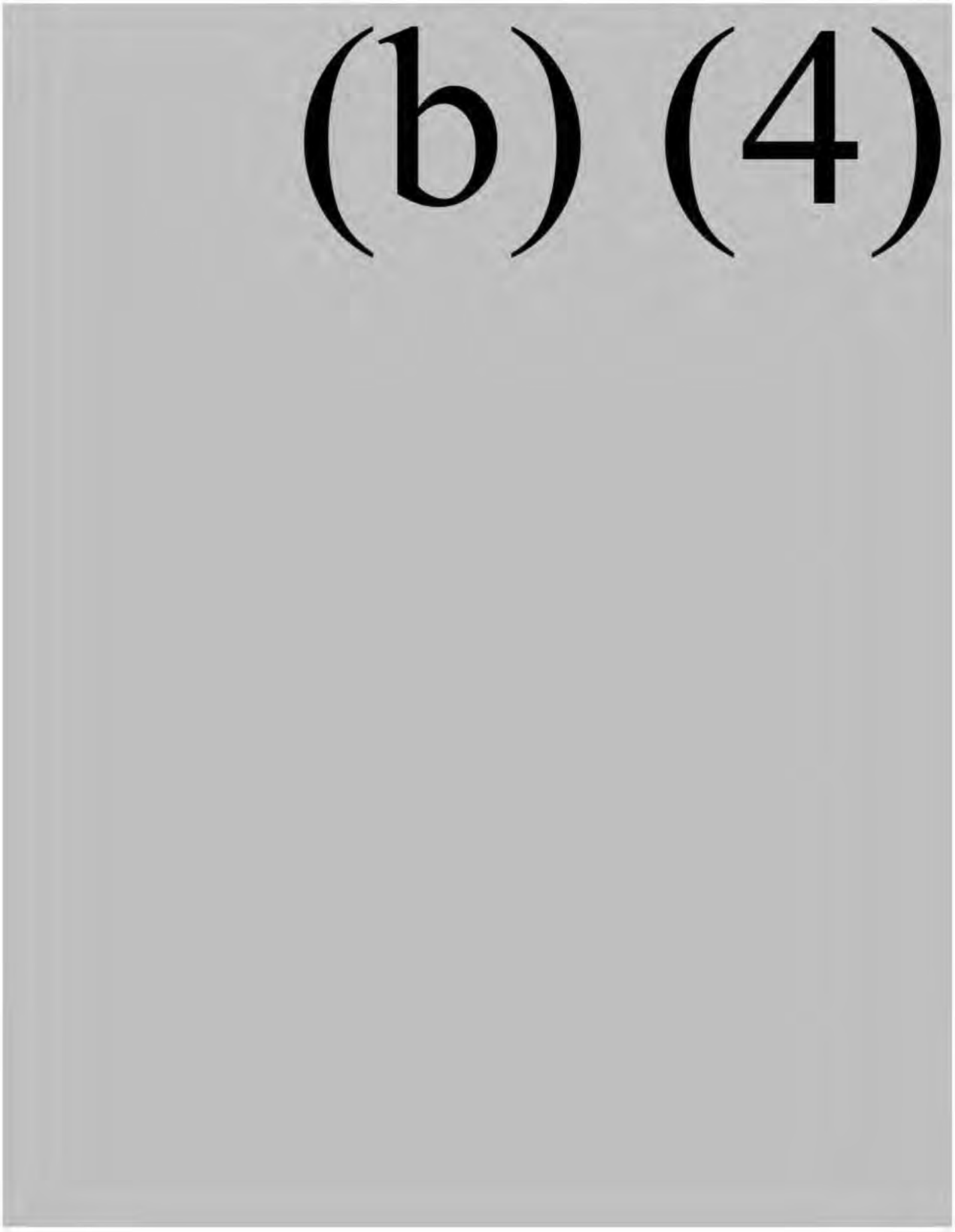
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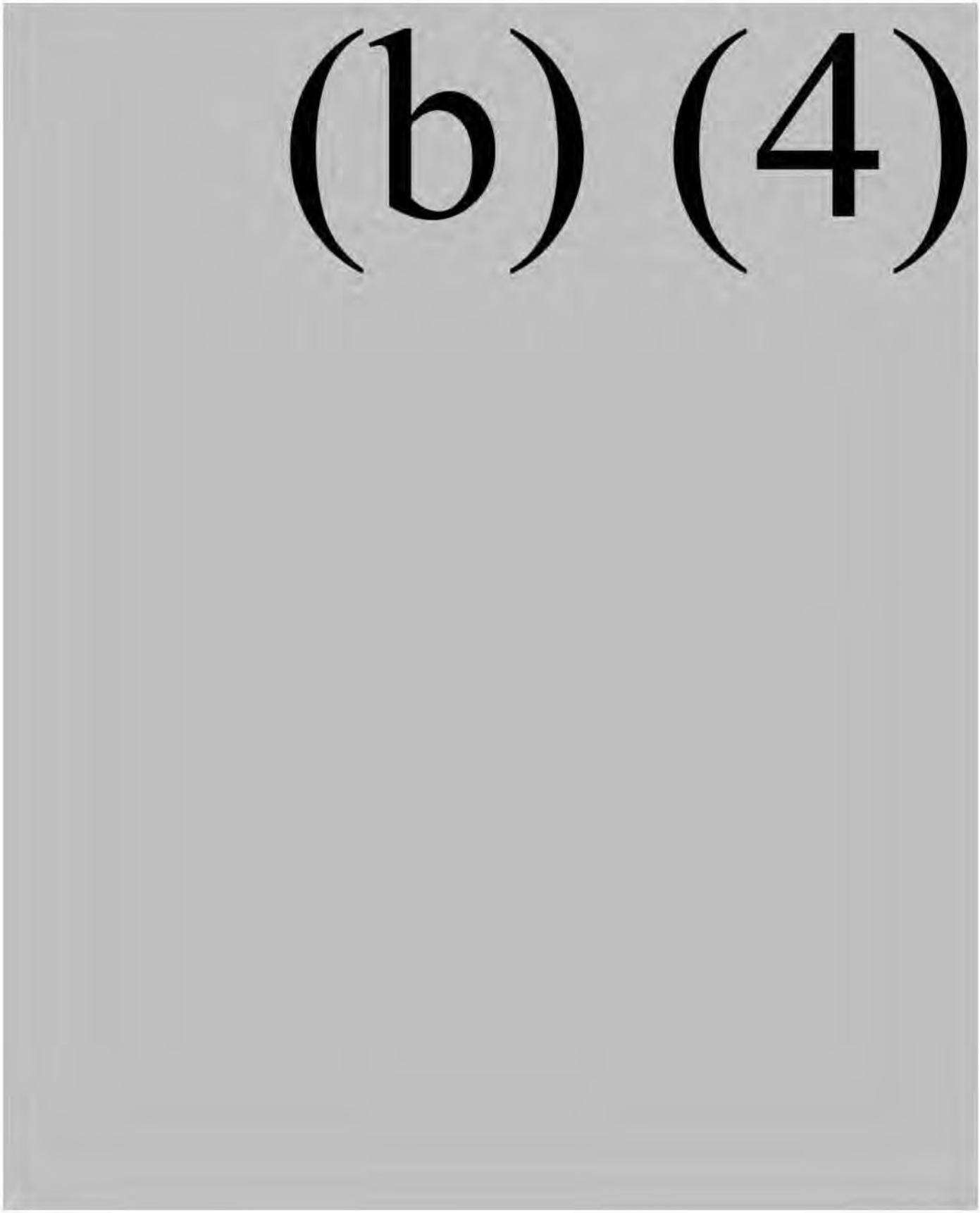
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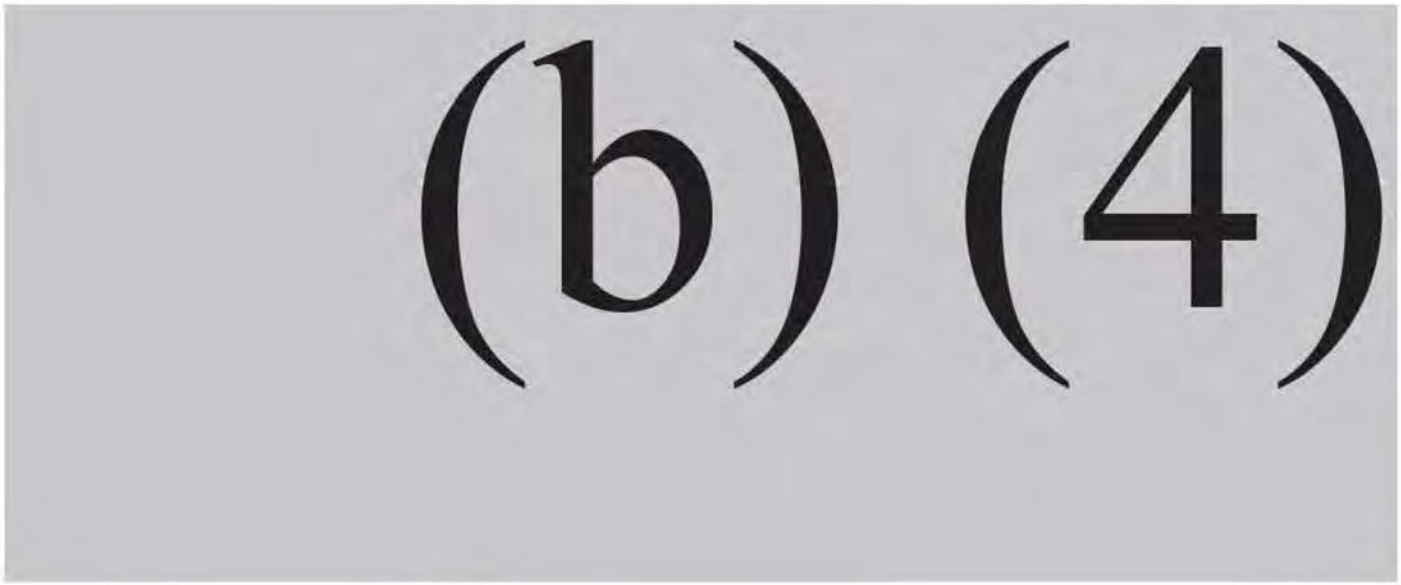

For Peer Review

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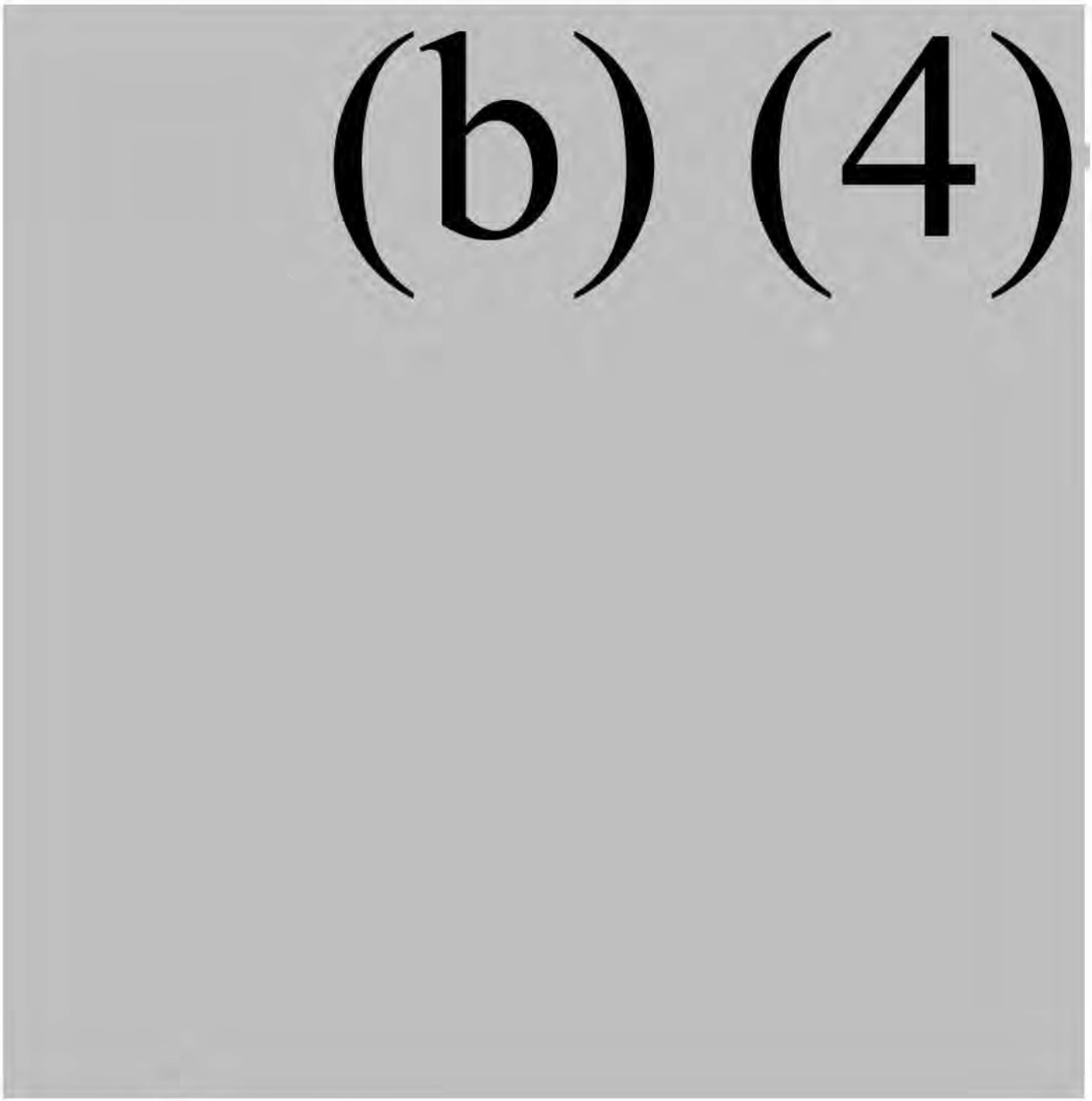
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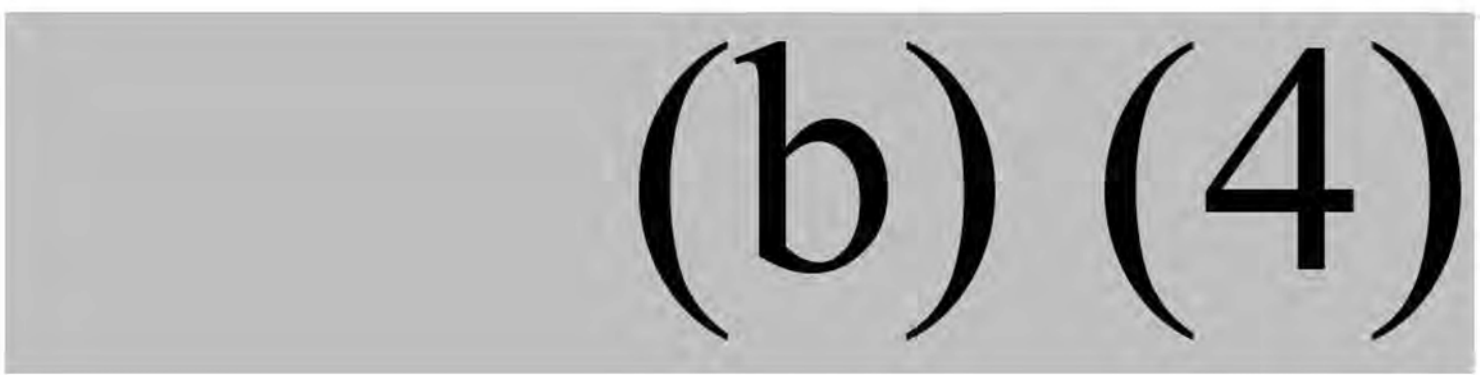
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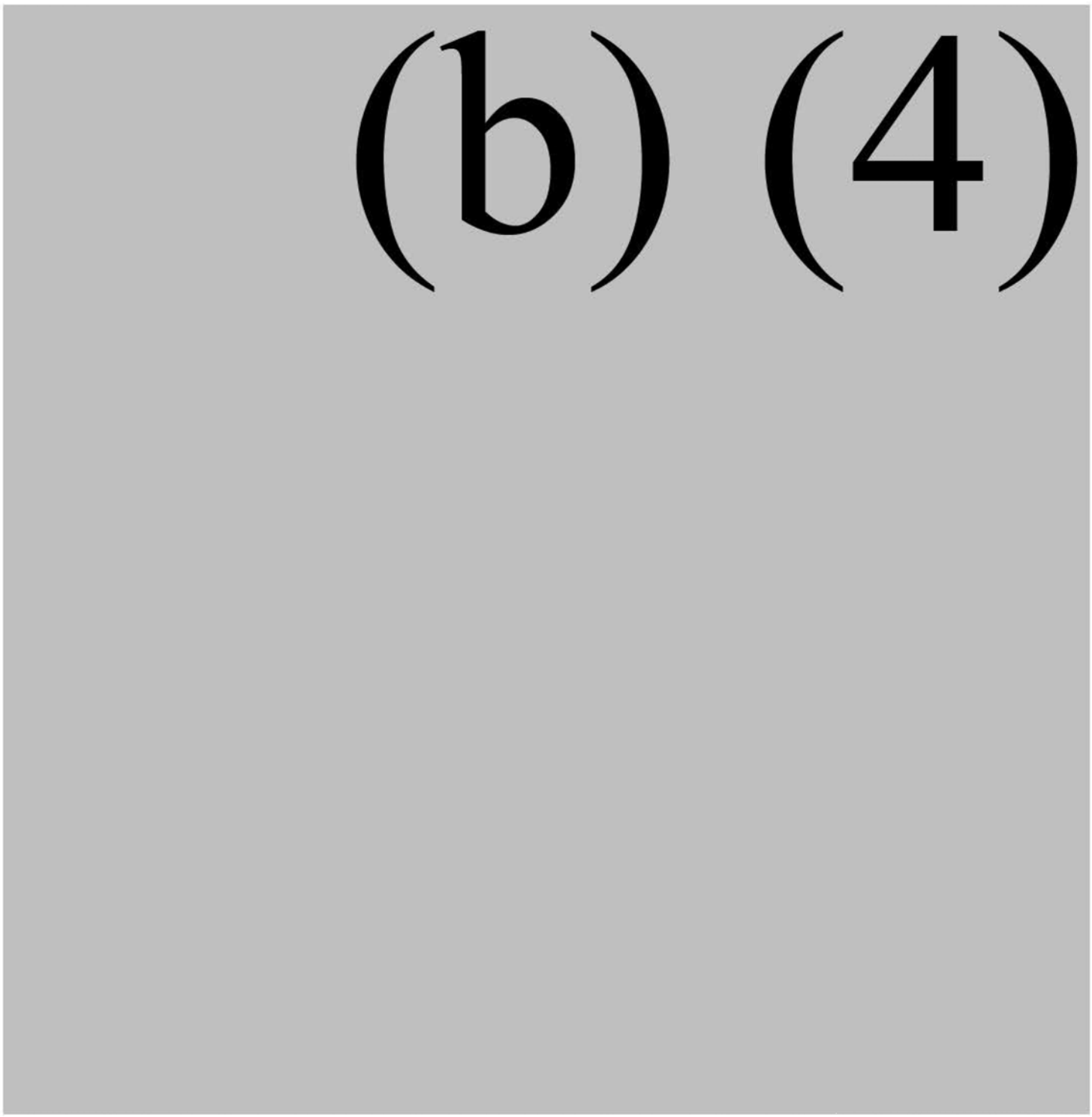
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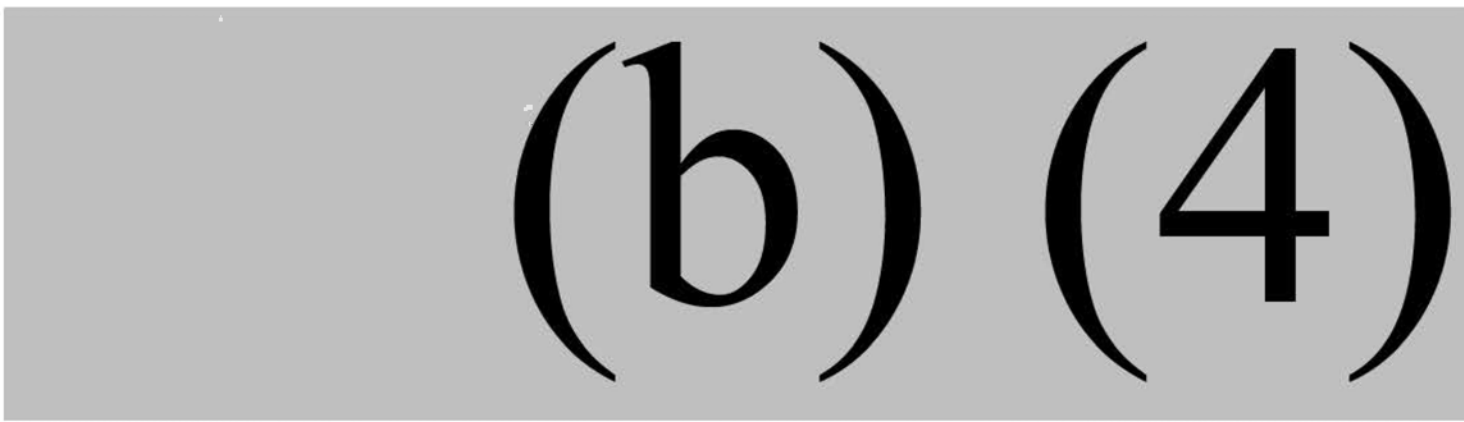
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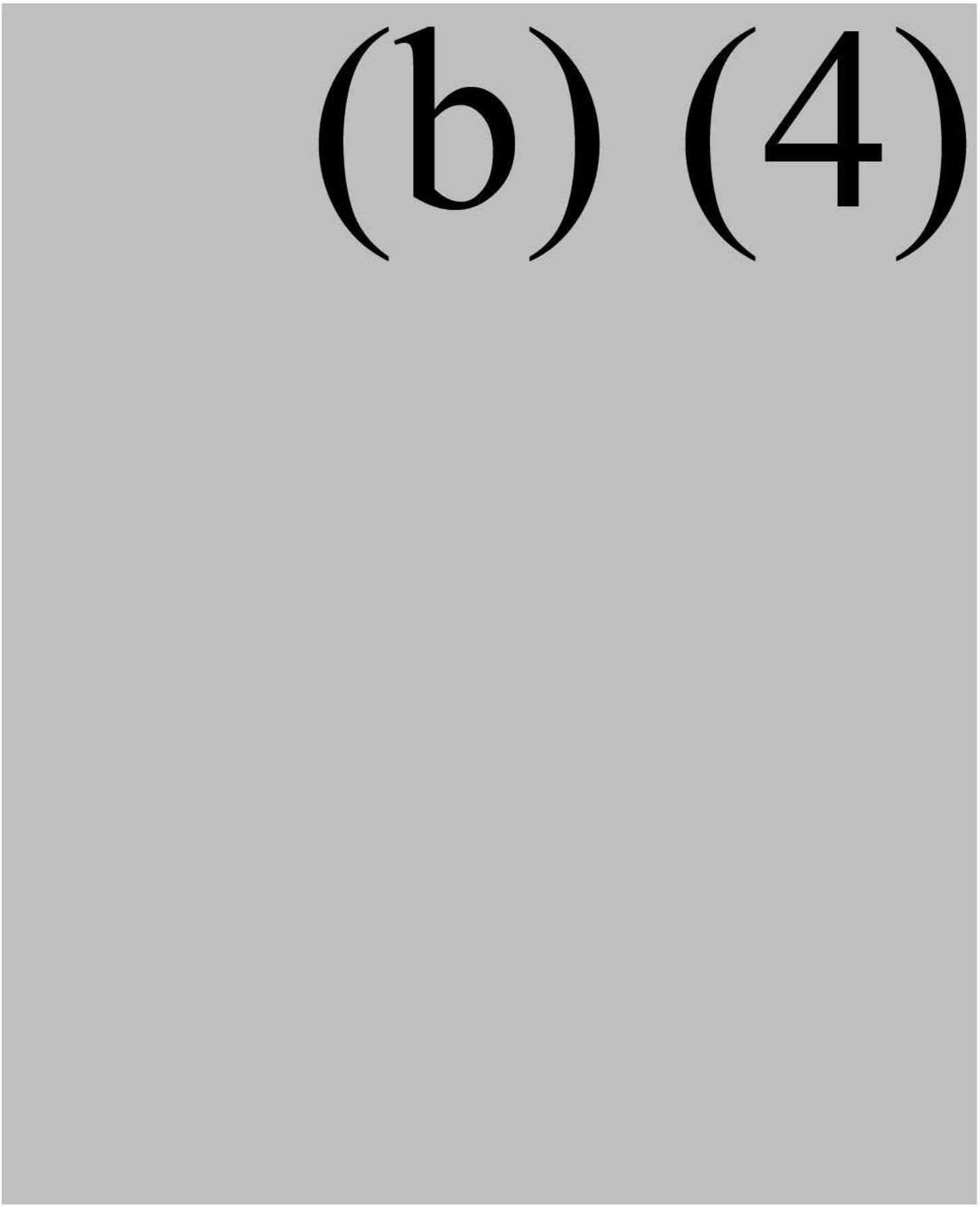
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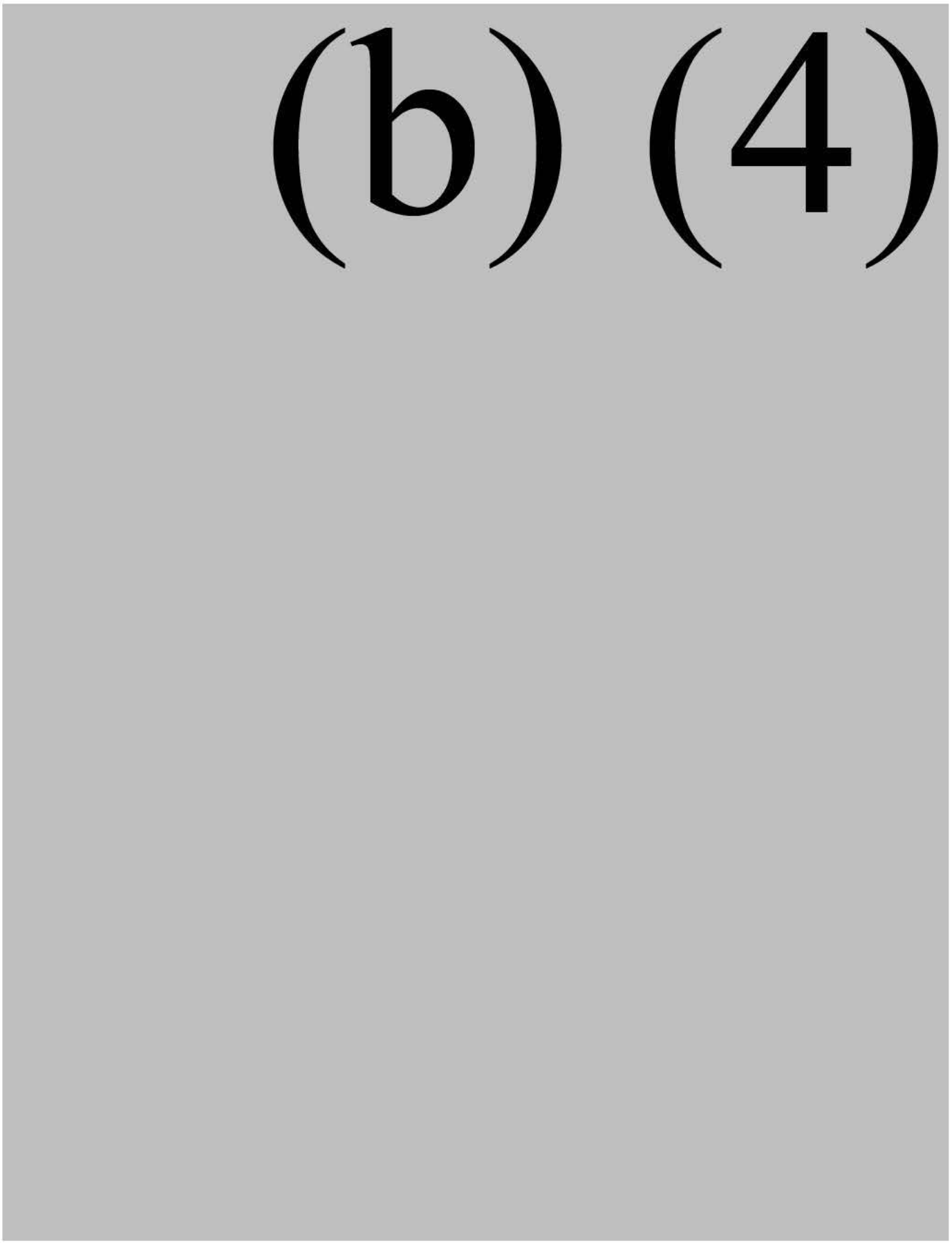
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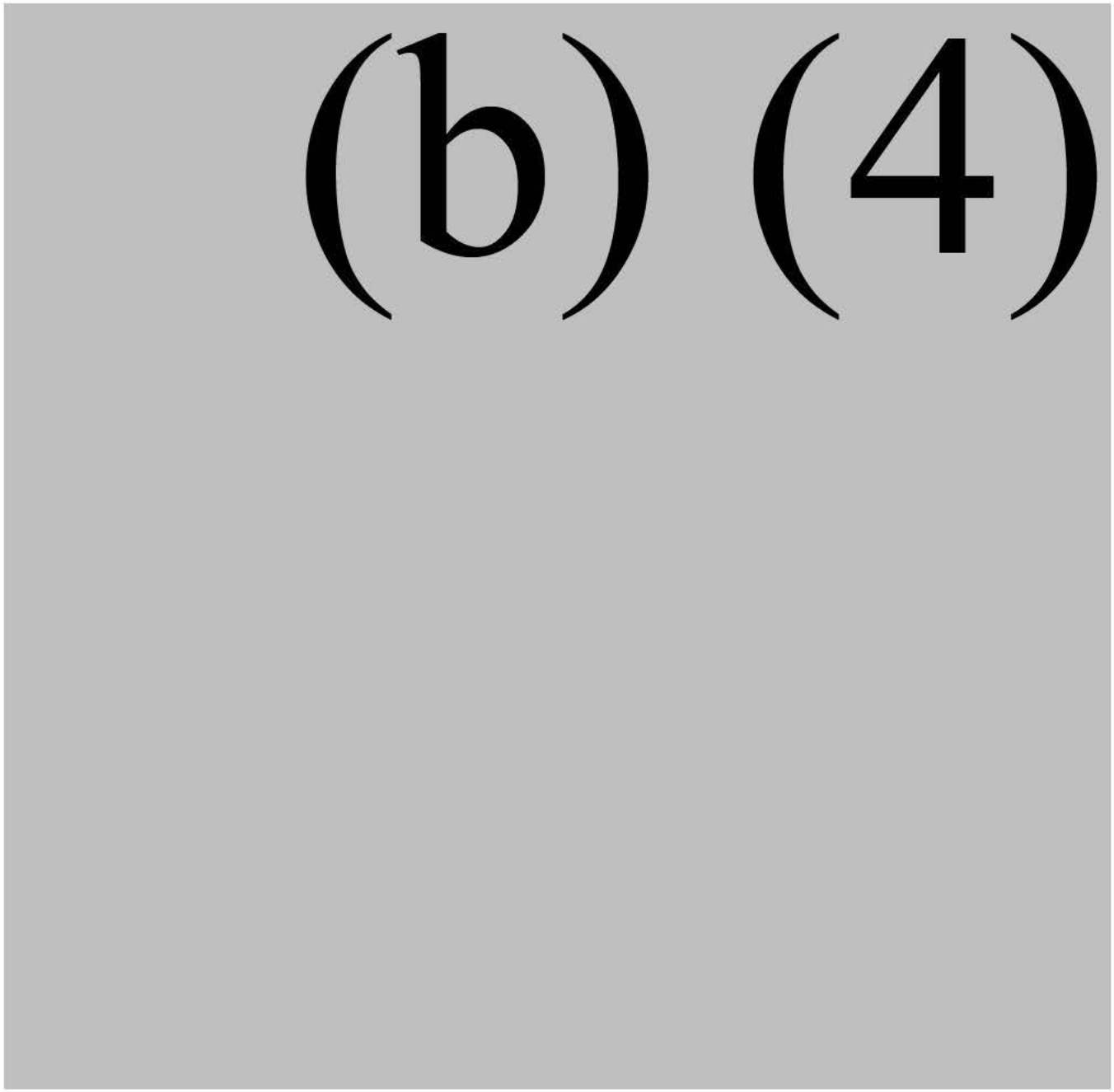
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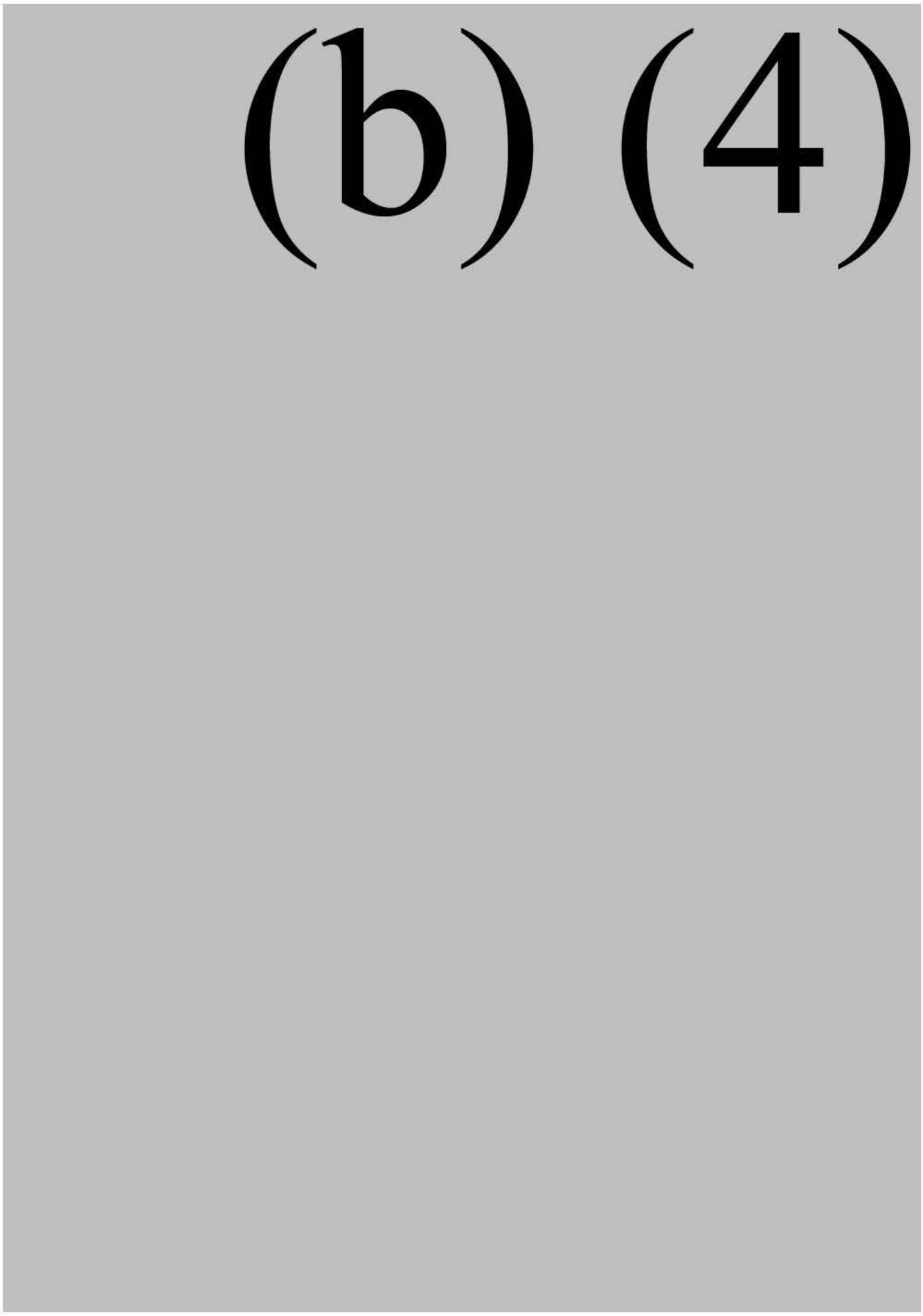
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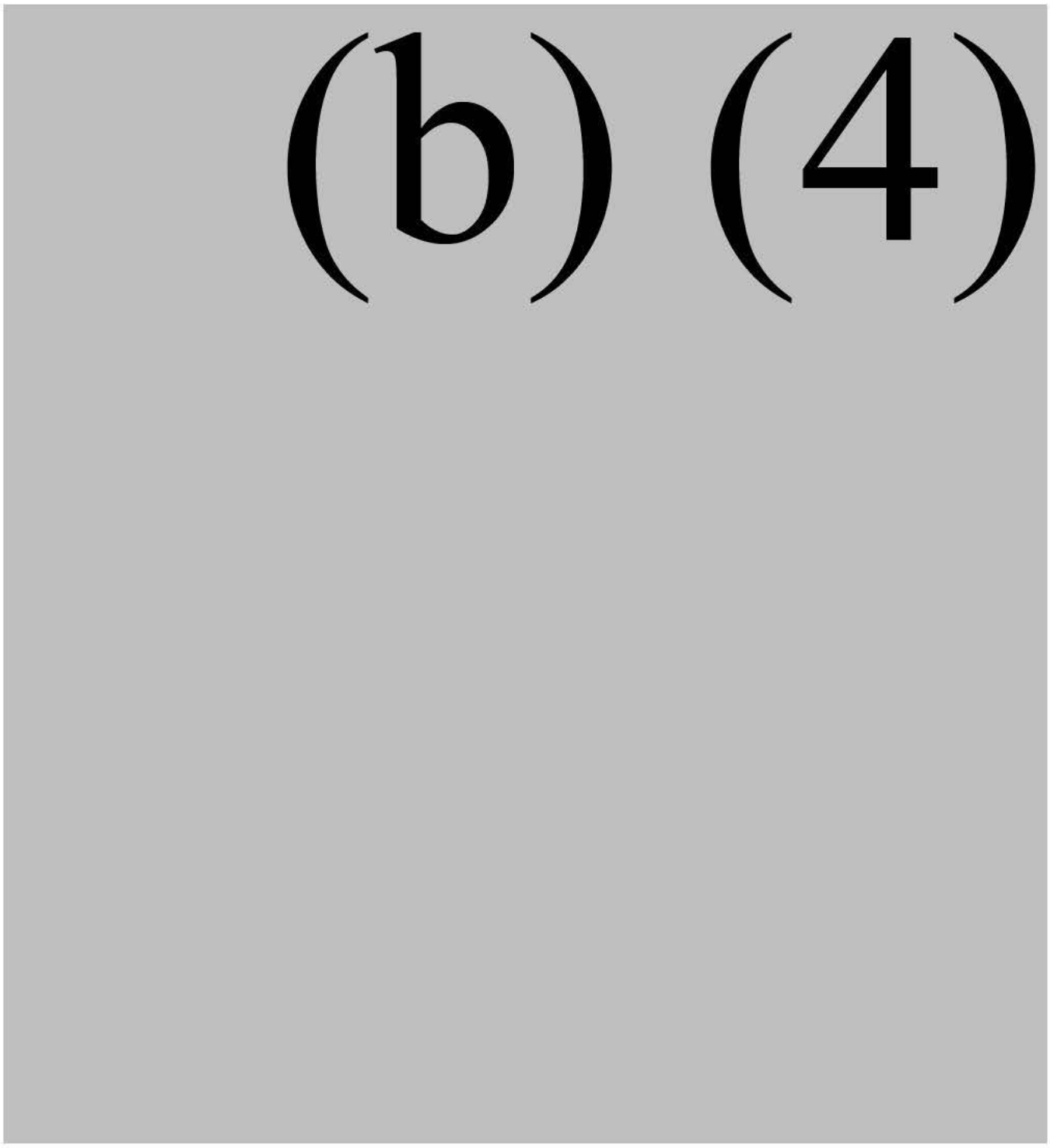
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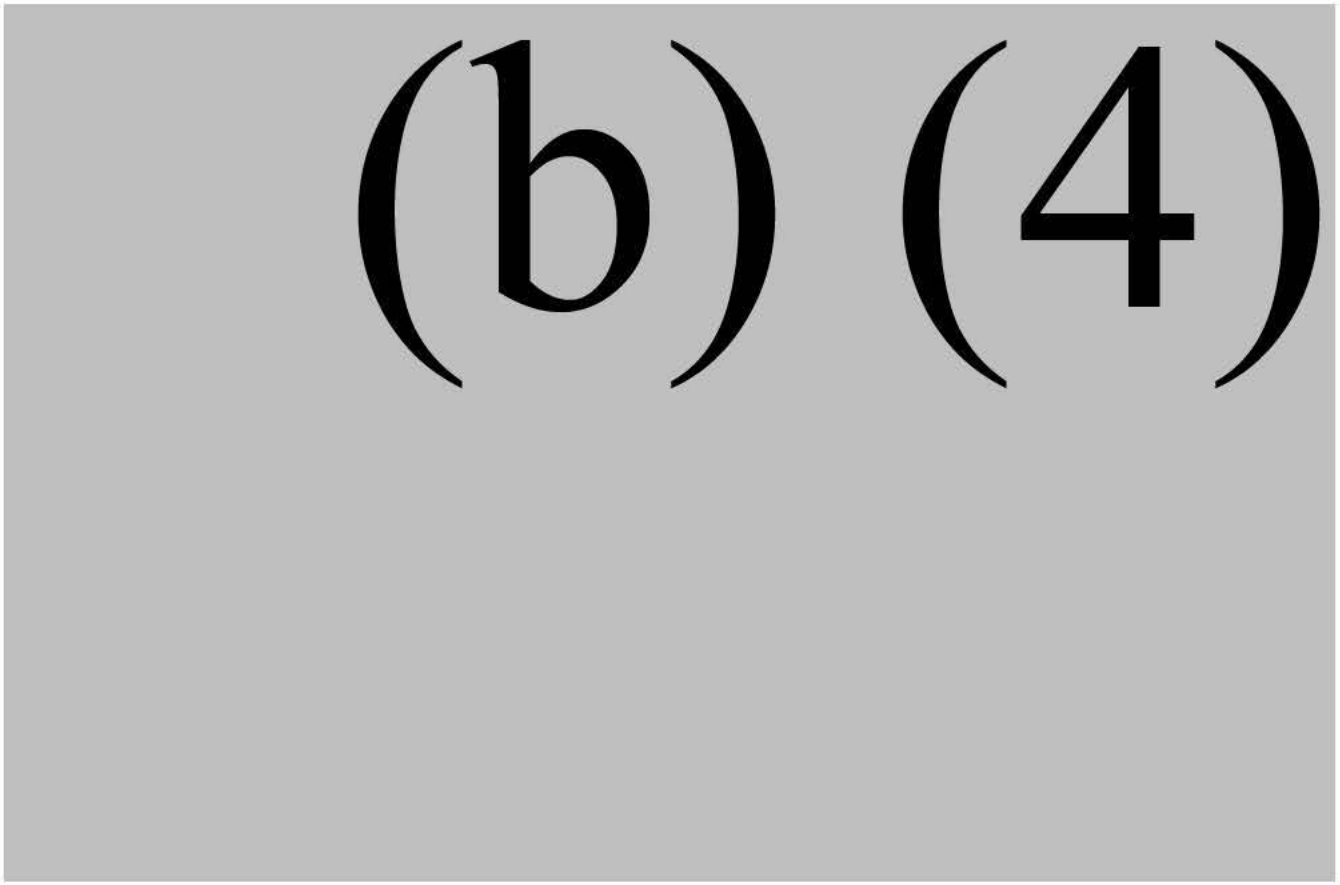
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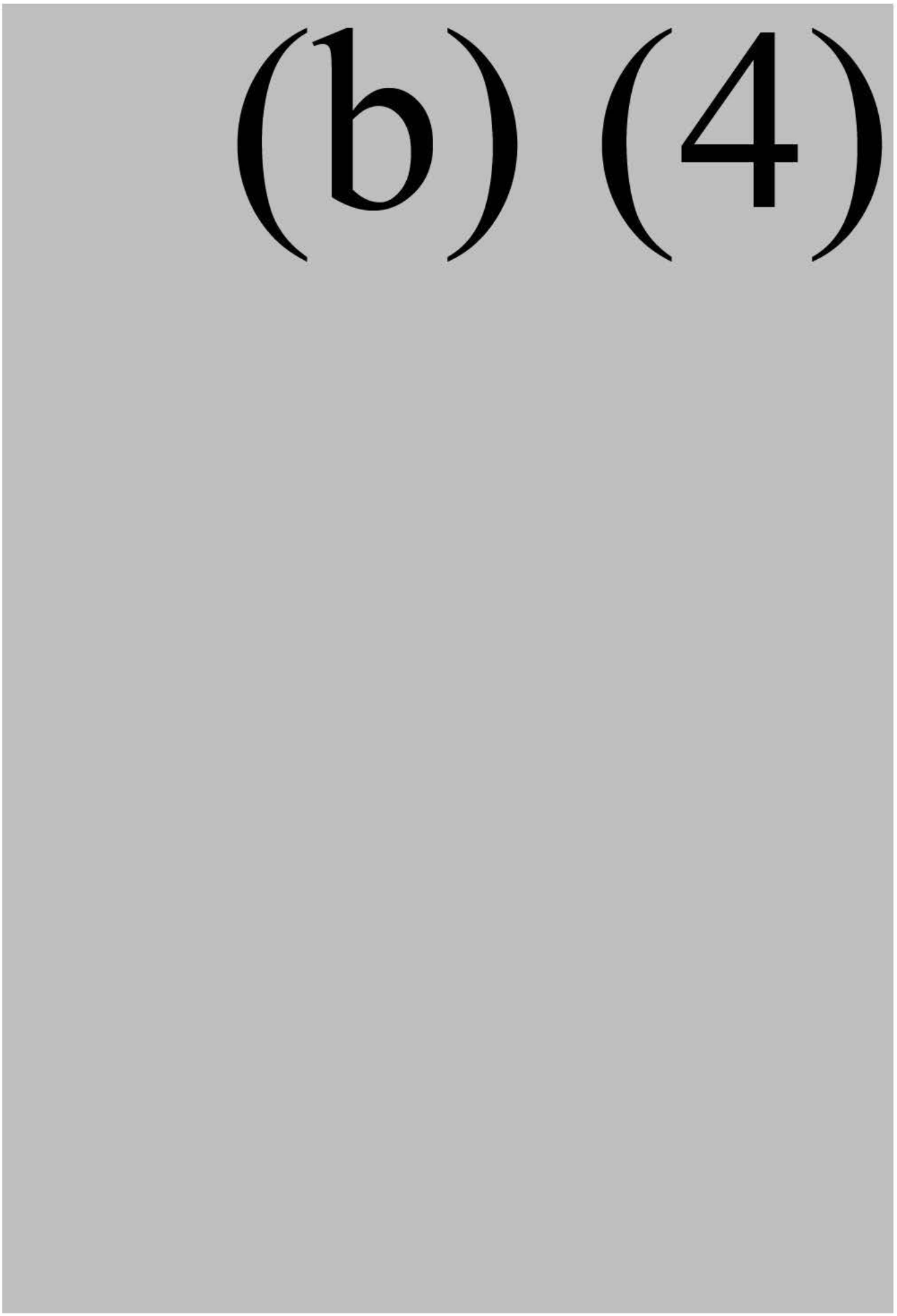
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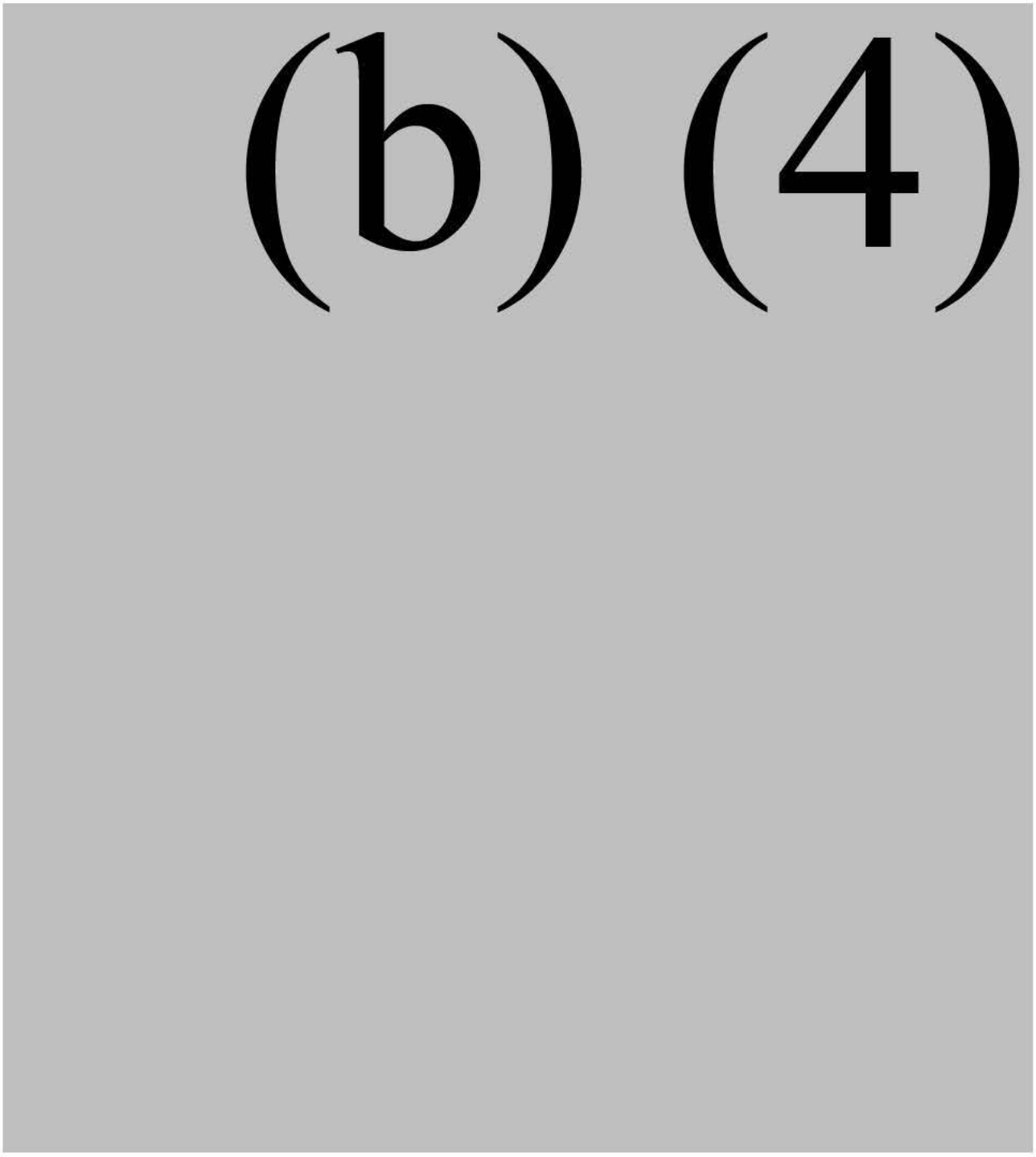
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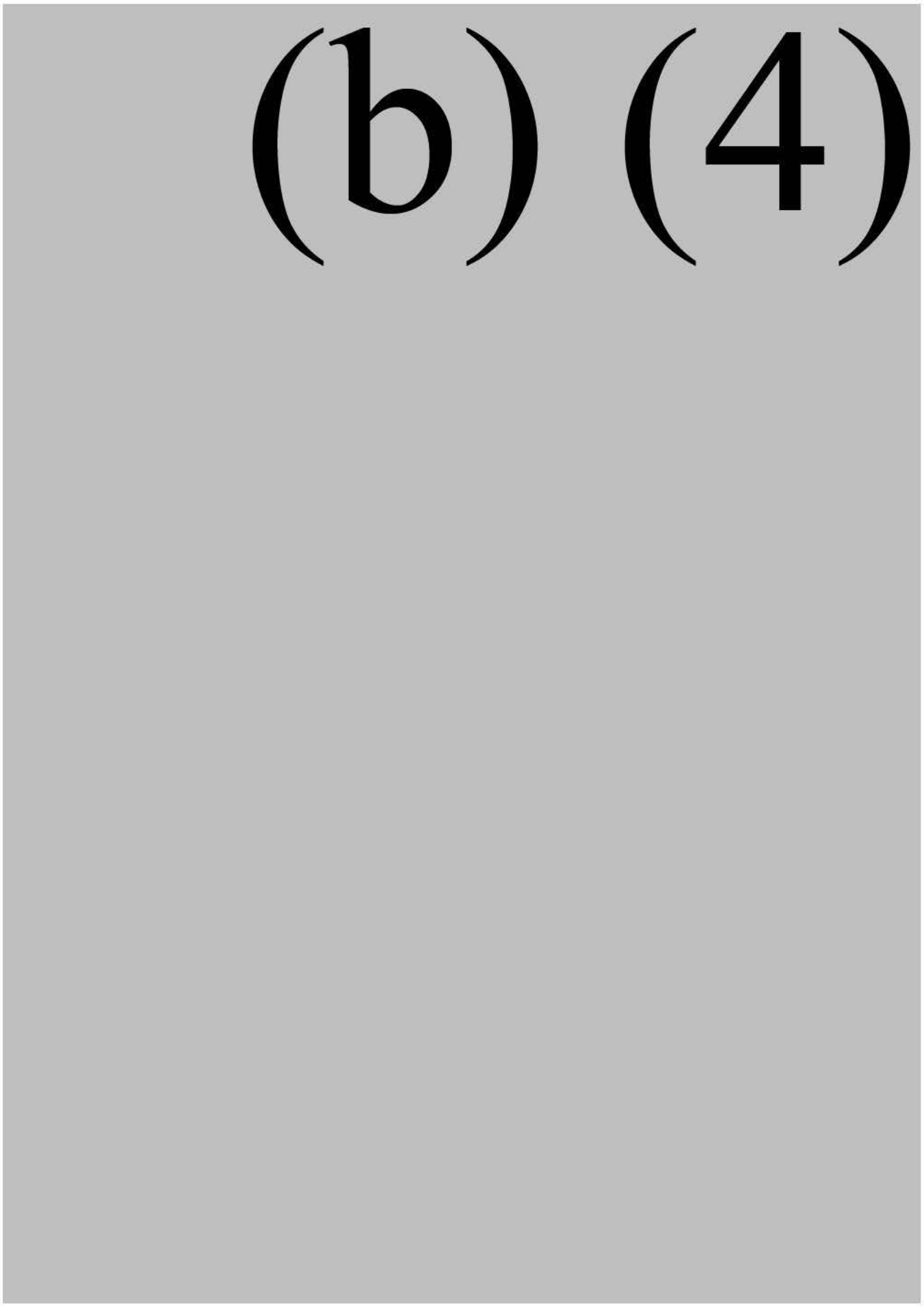
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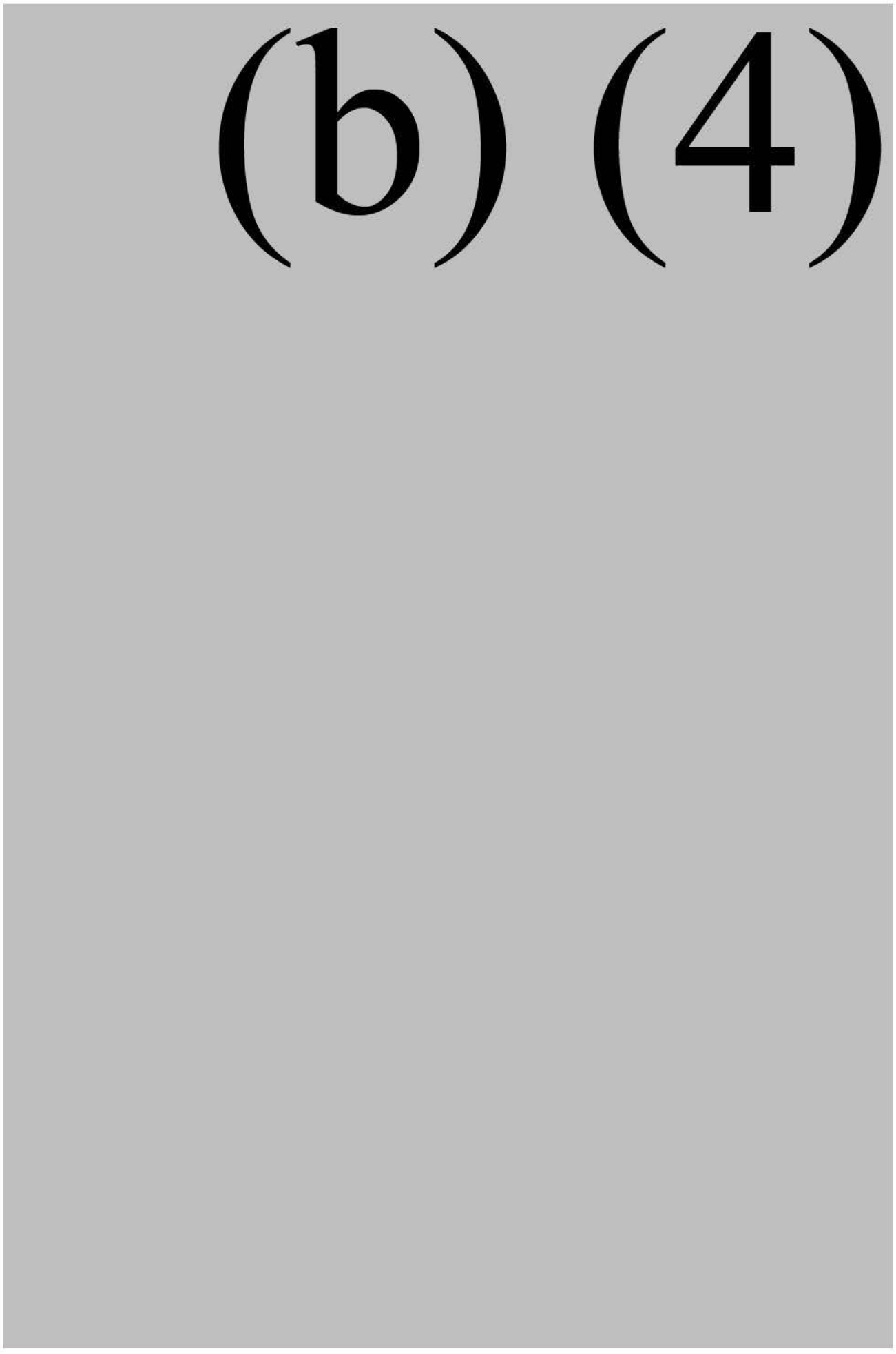
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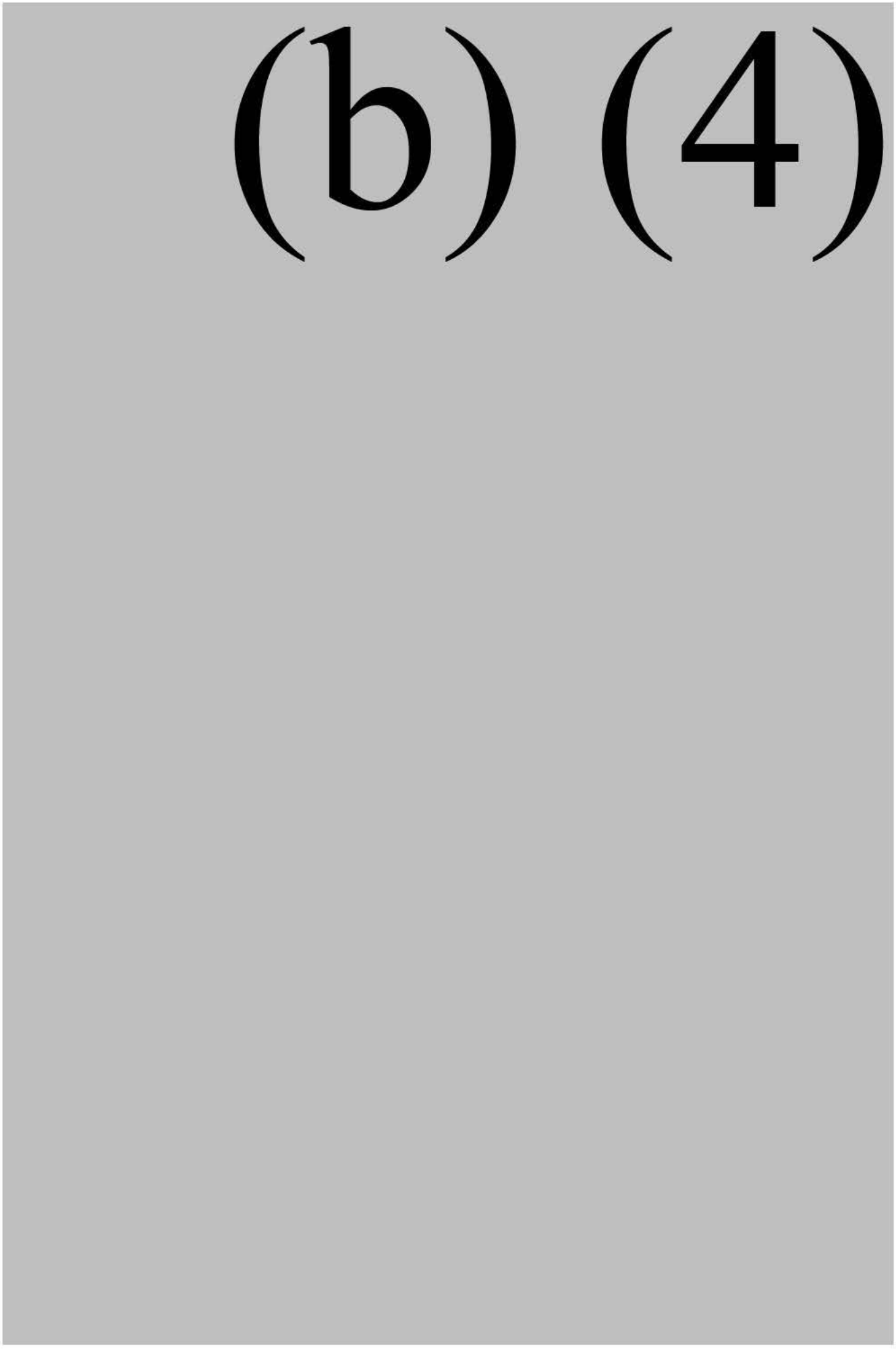
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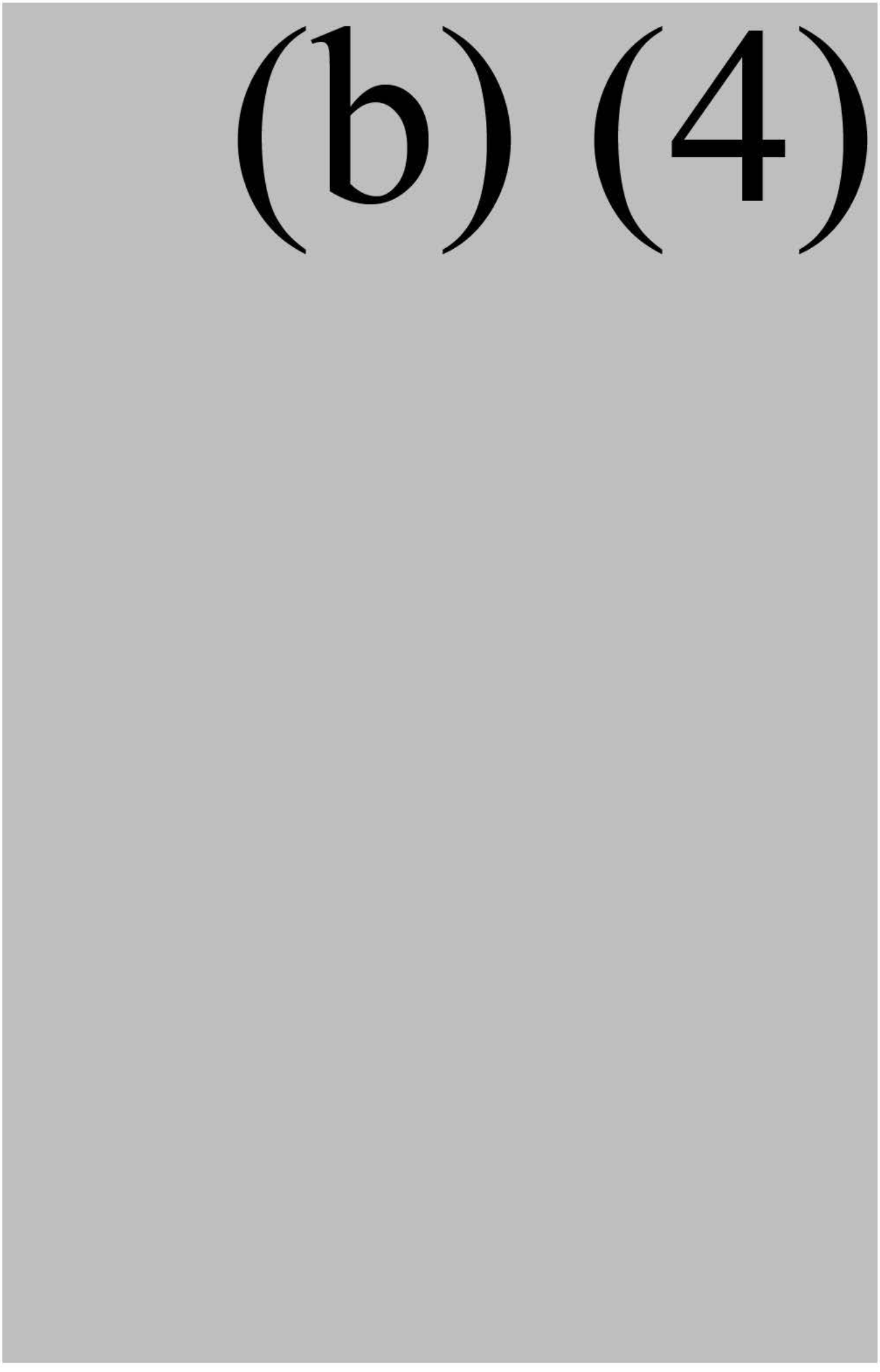
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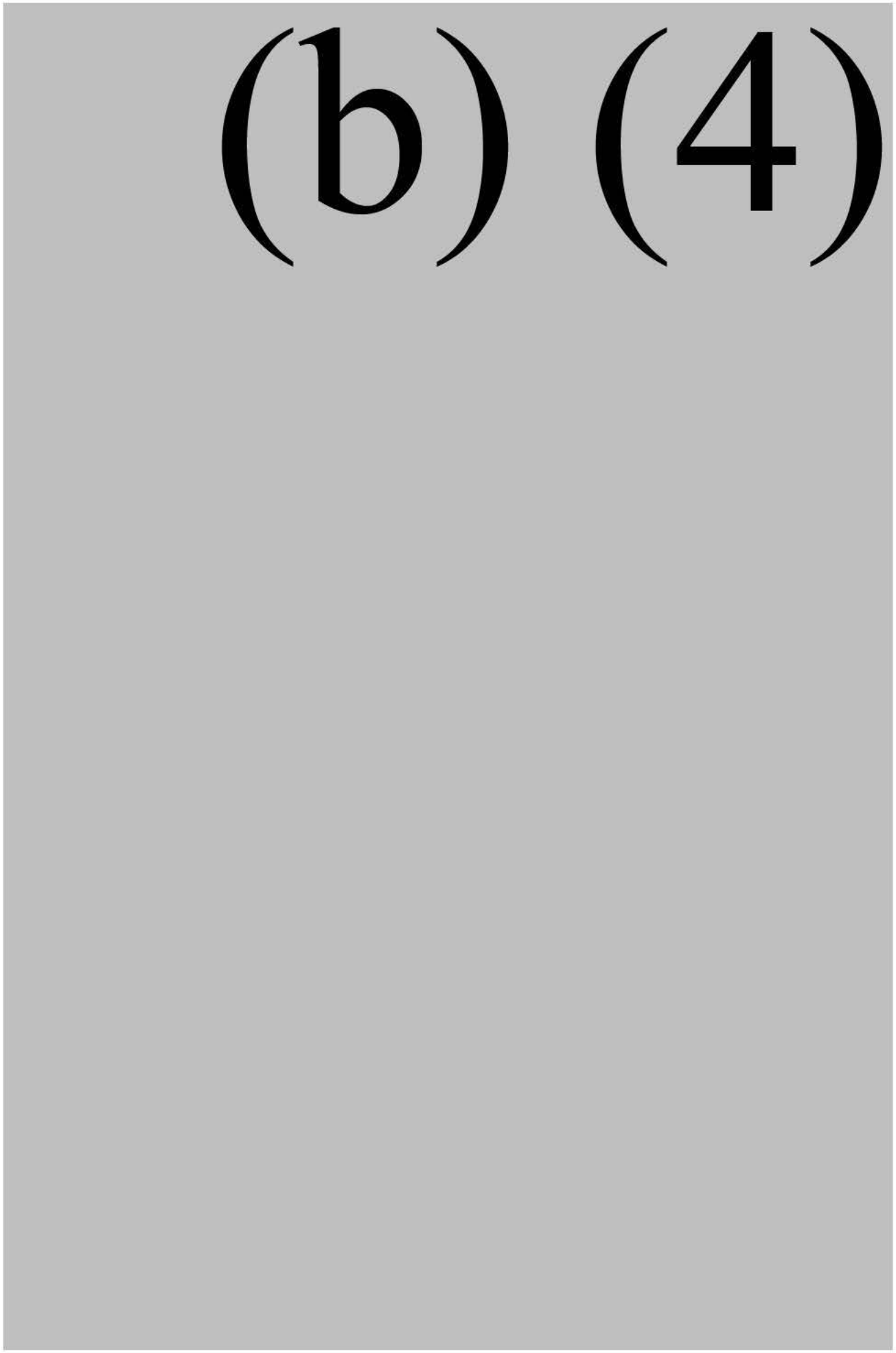
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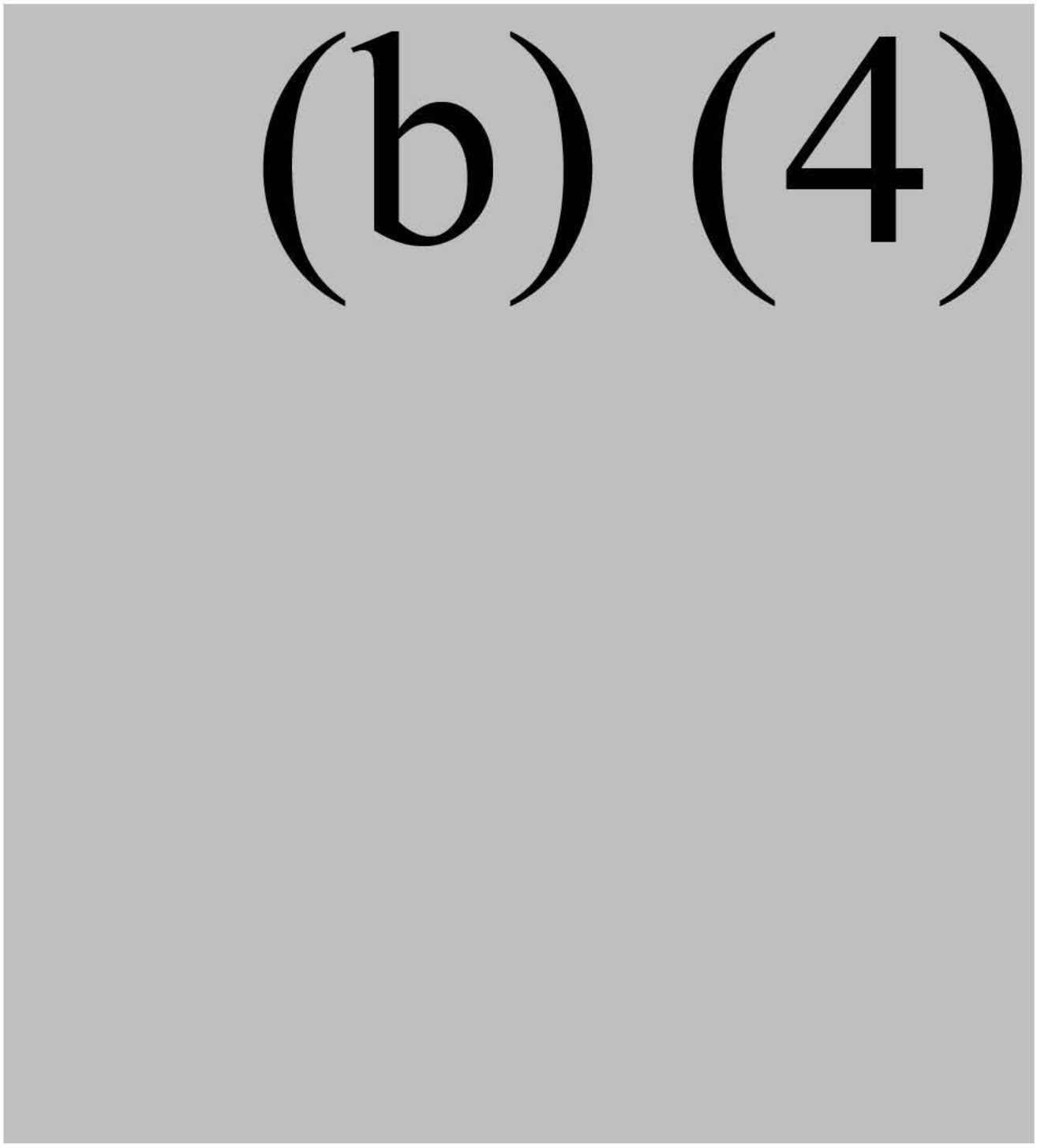
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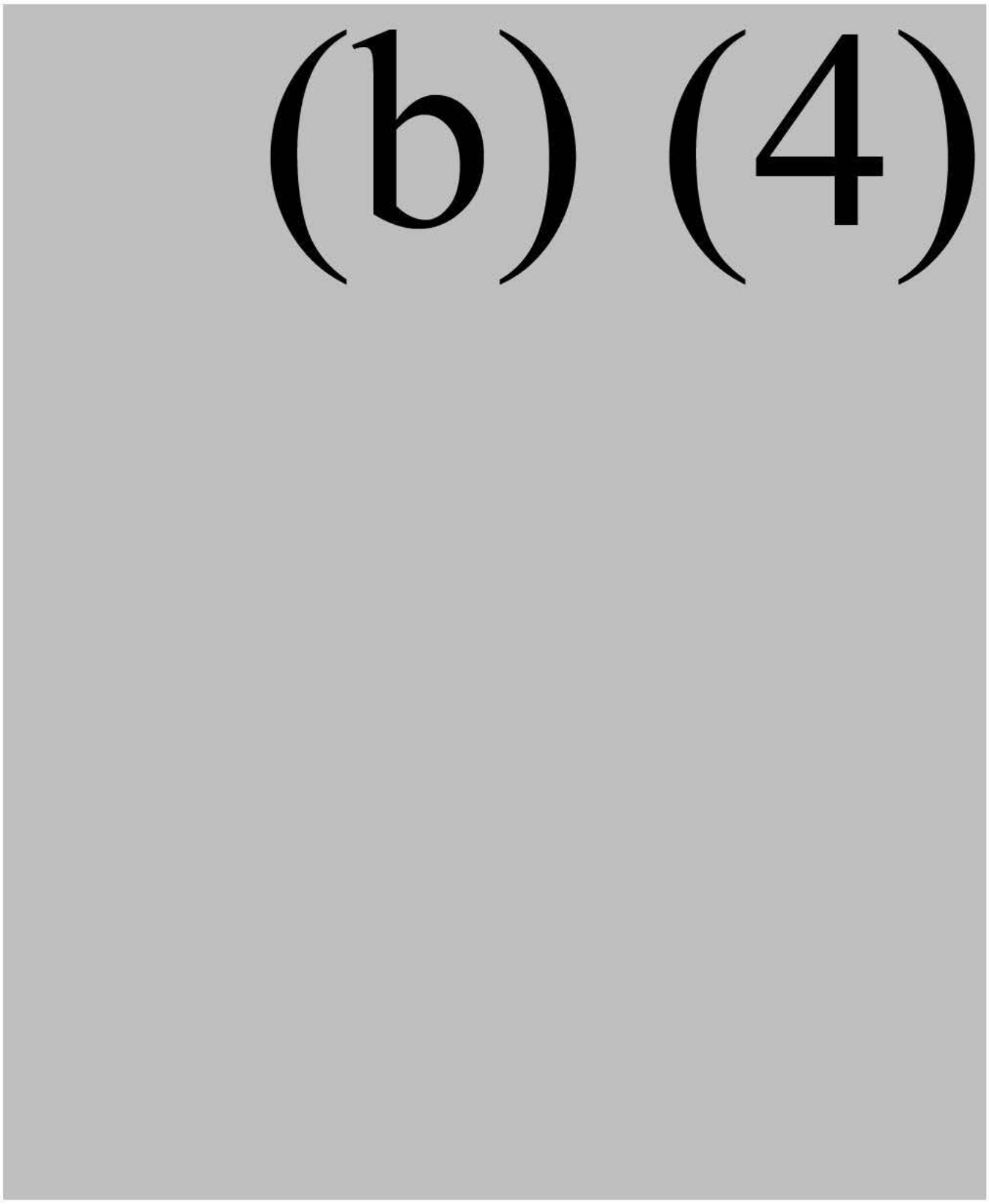
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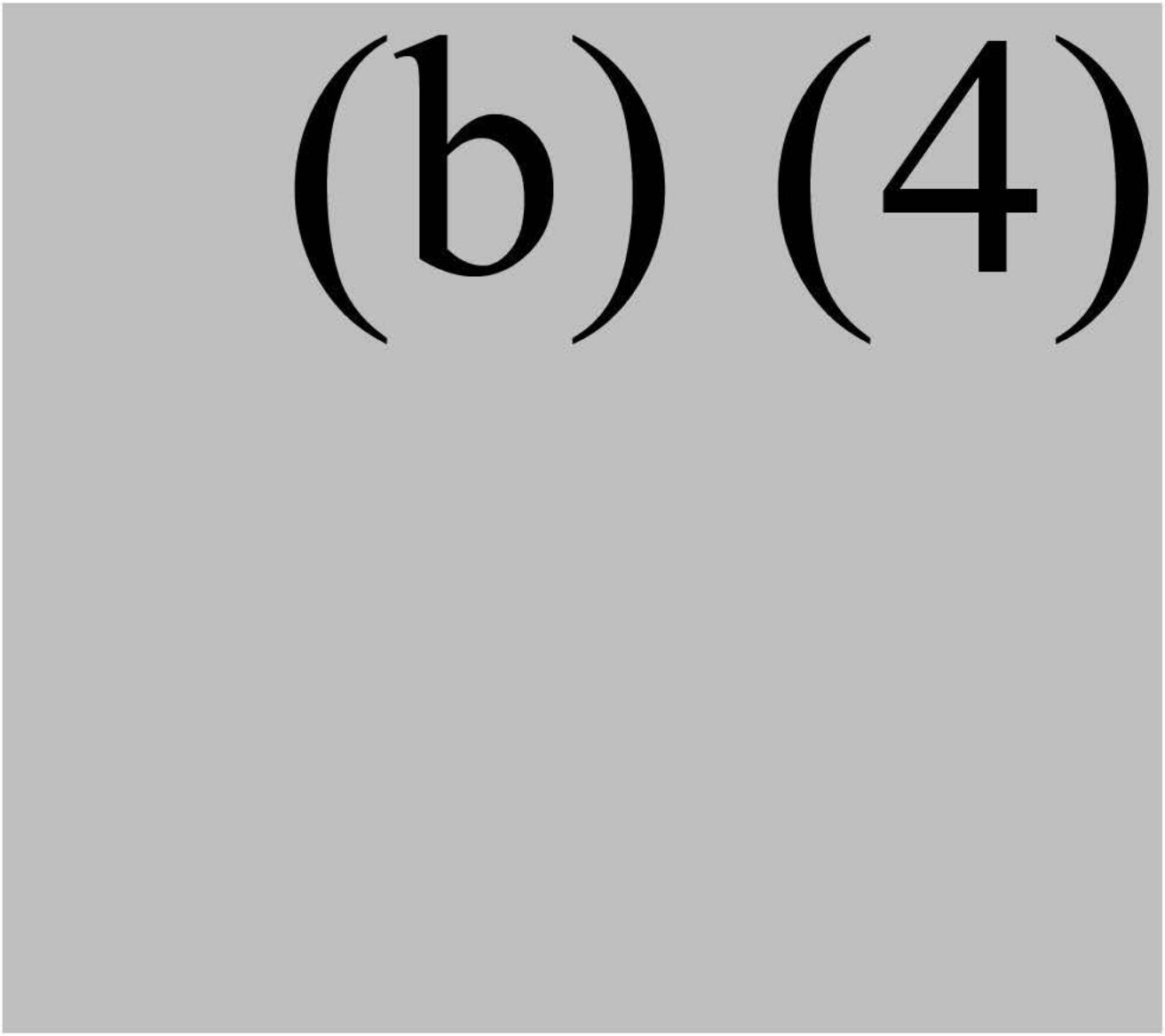
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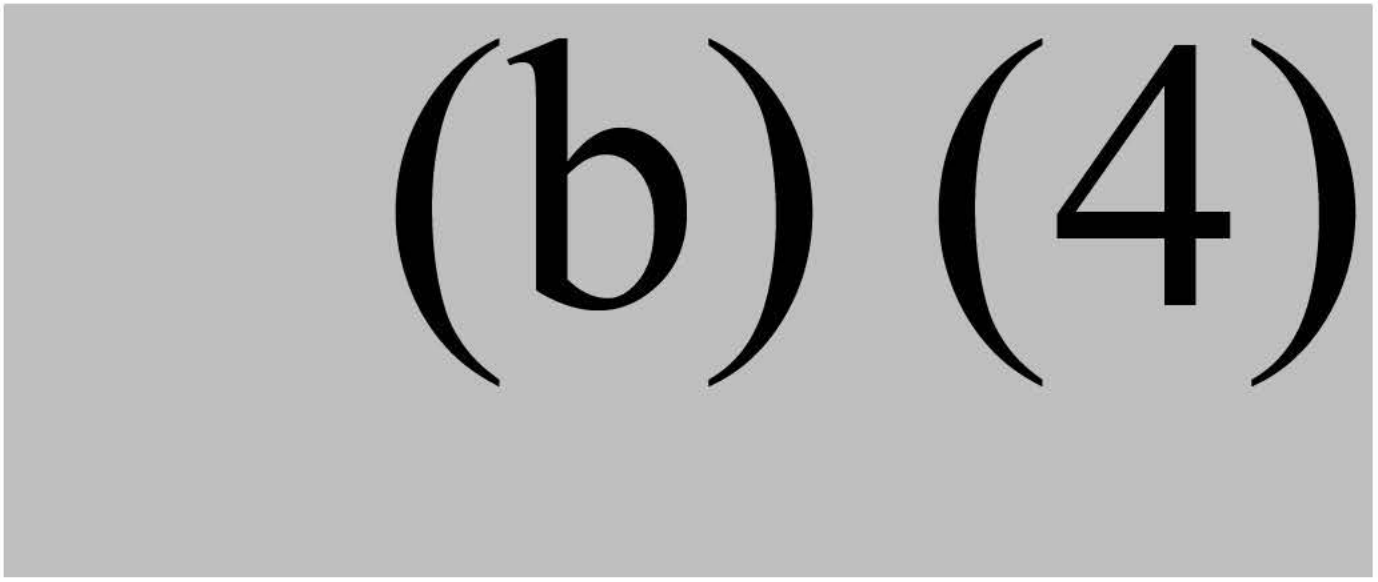
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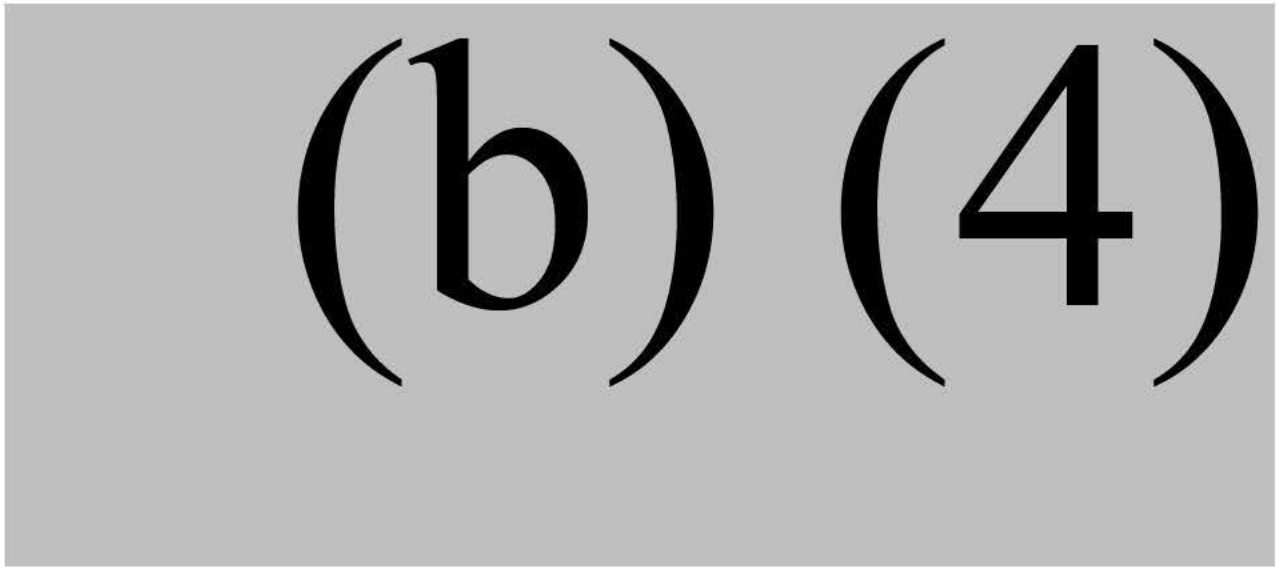
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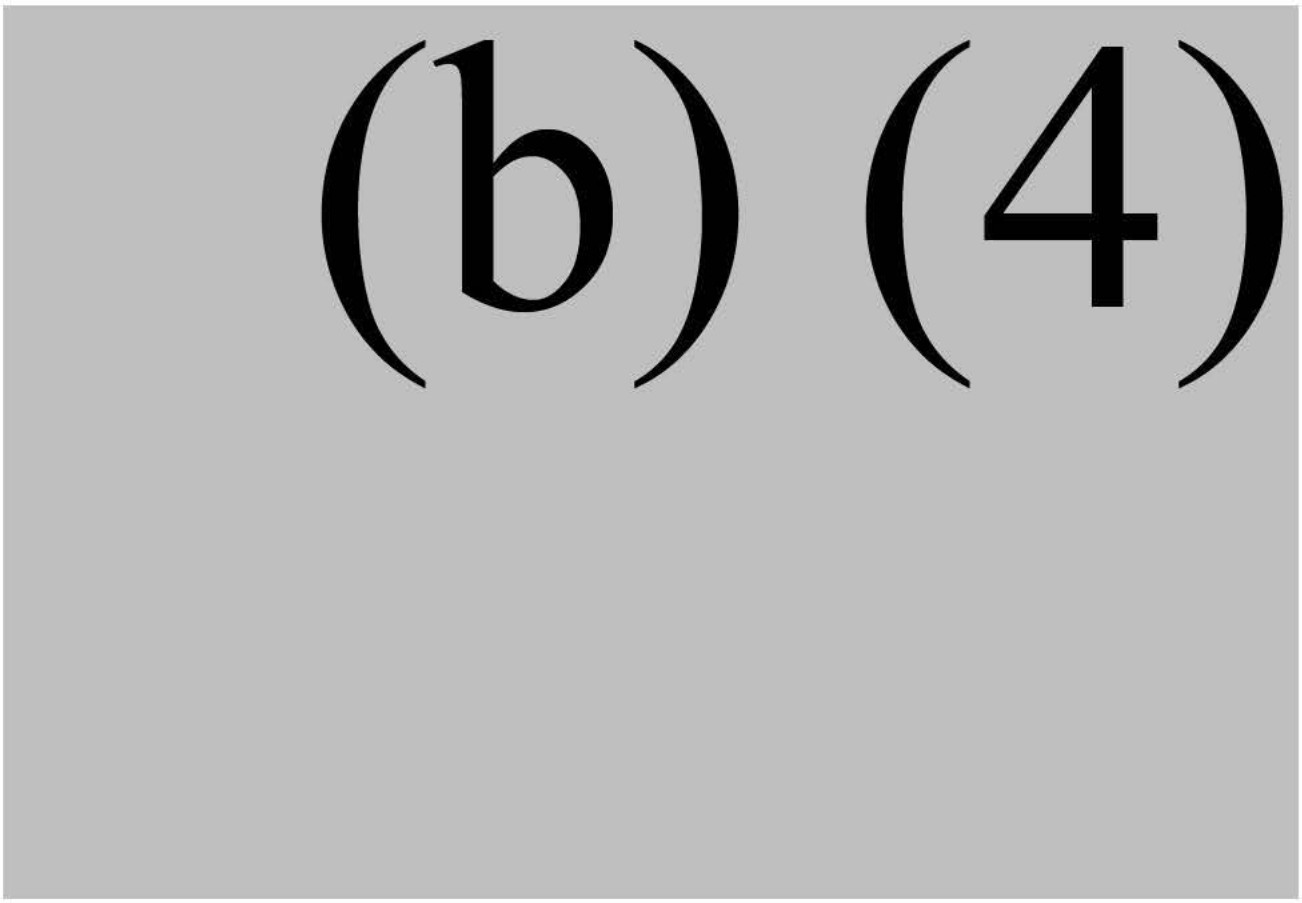
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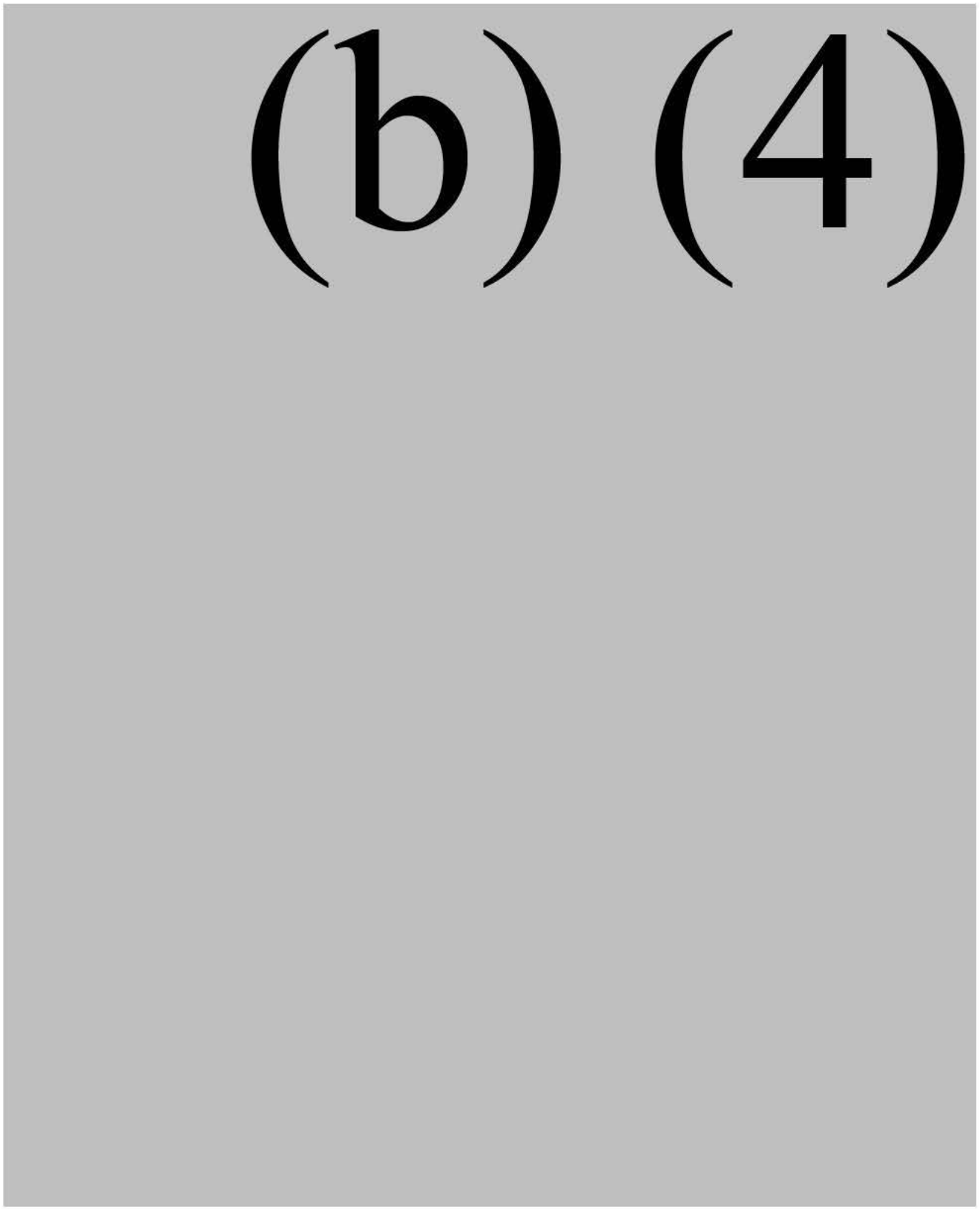
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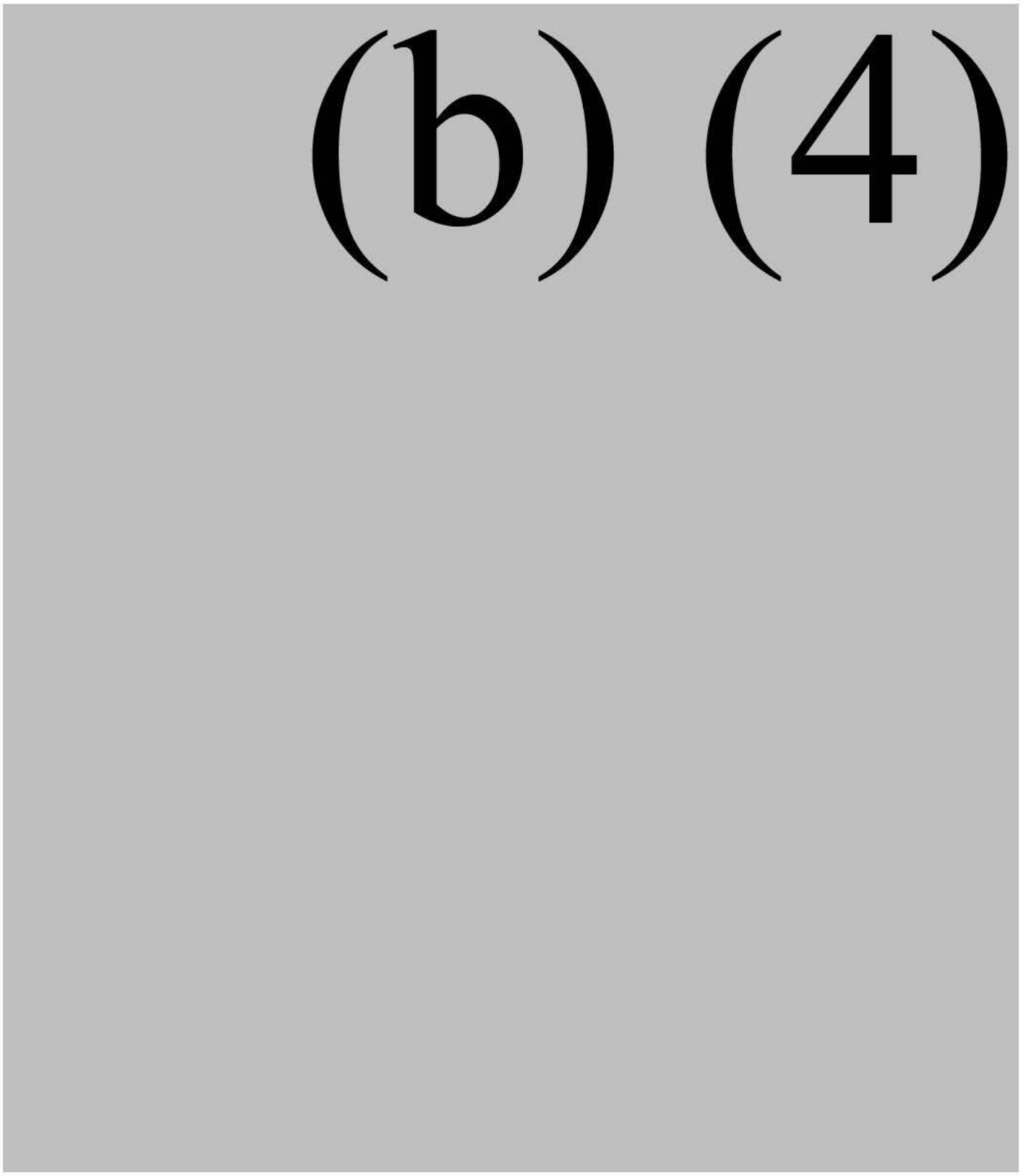
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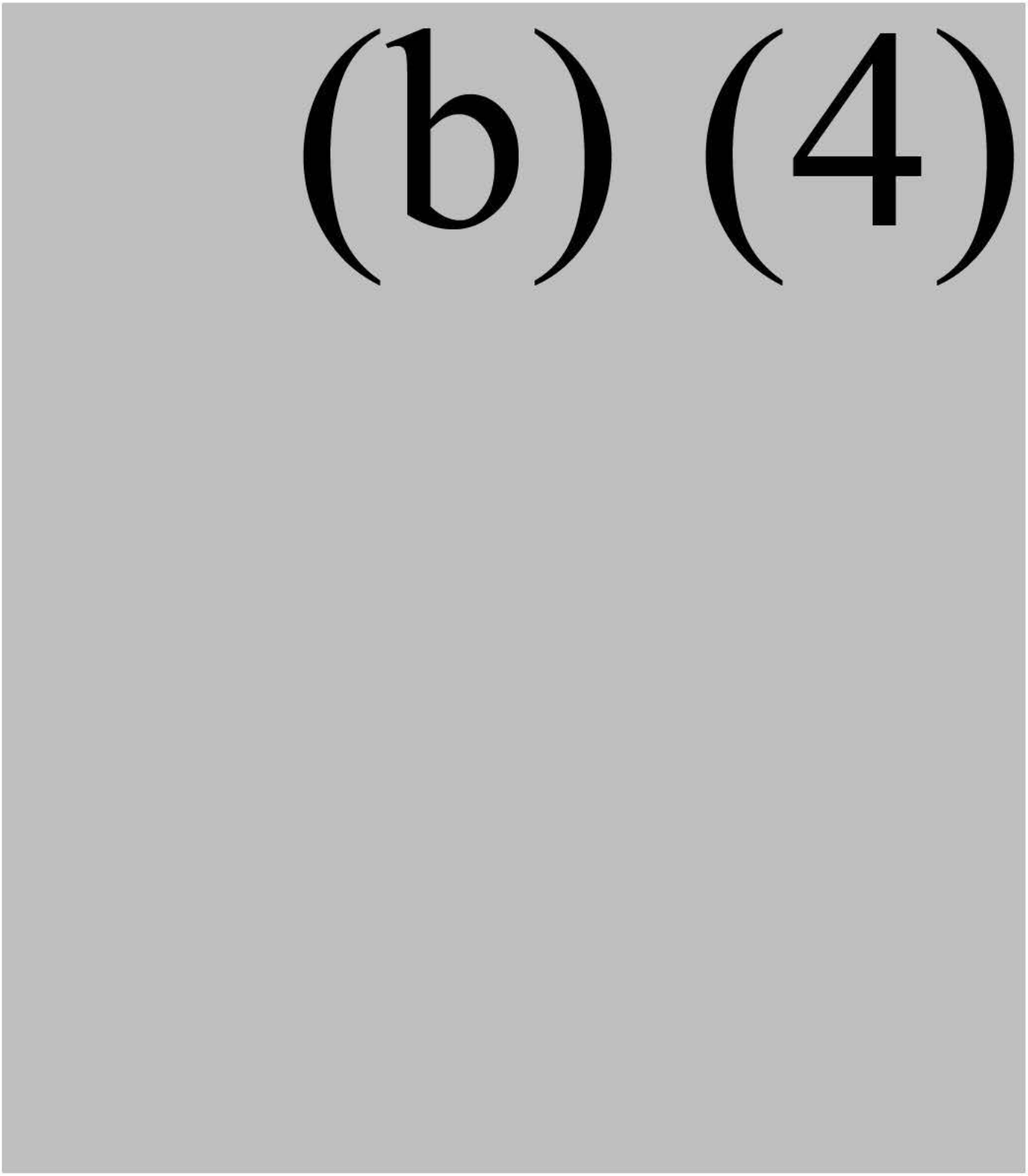
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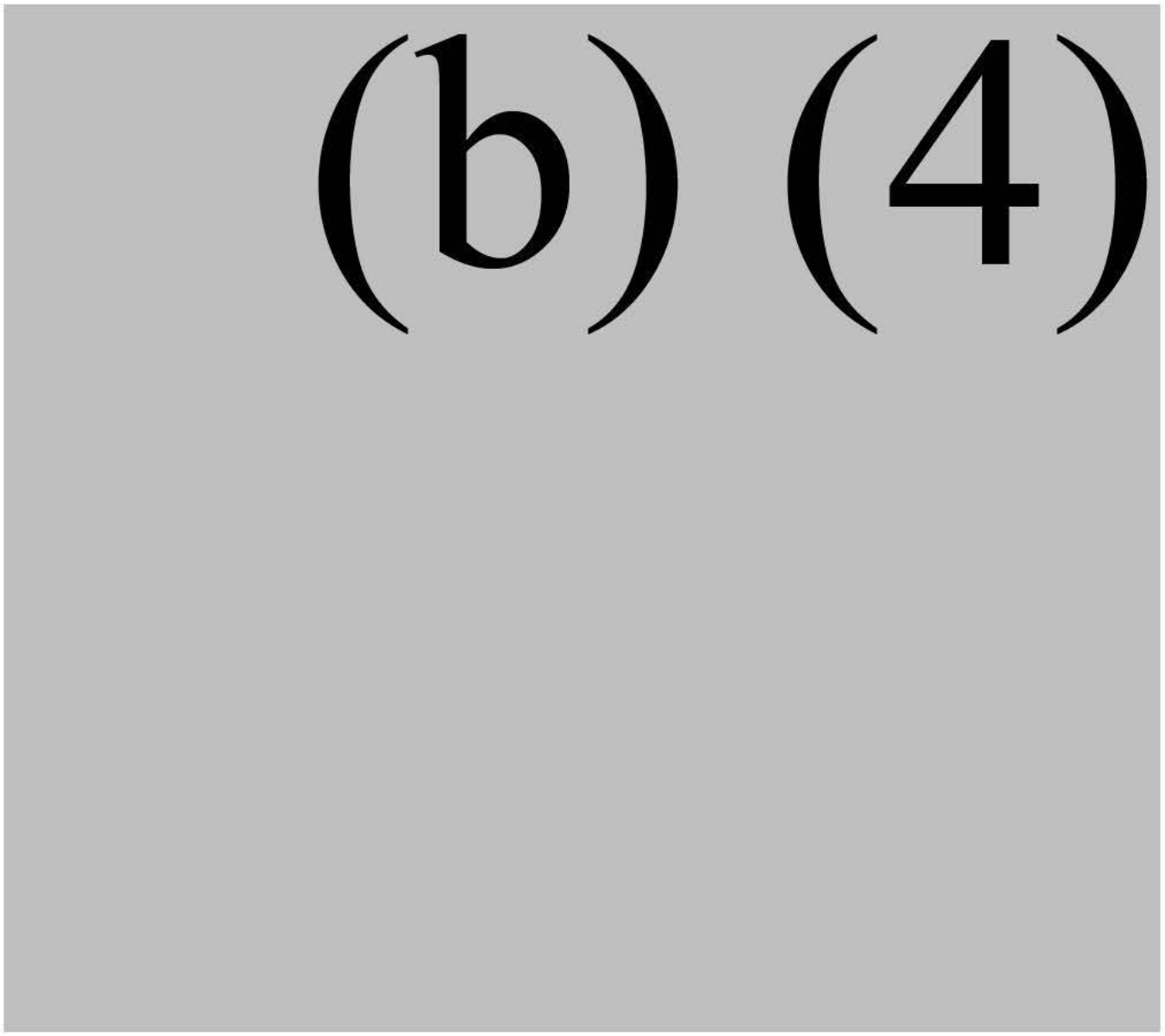
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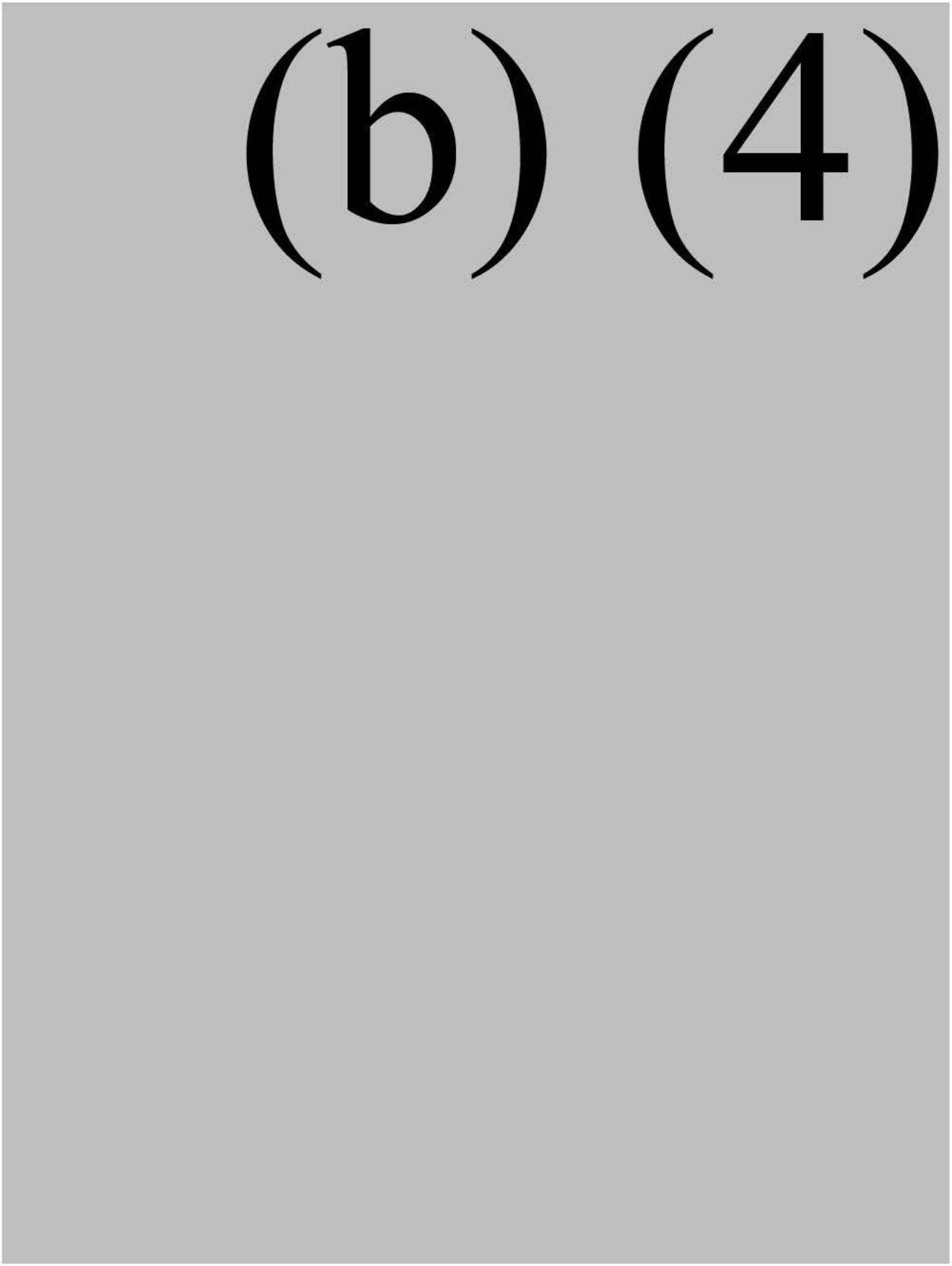
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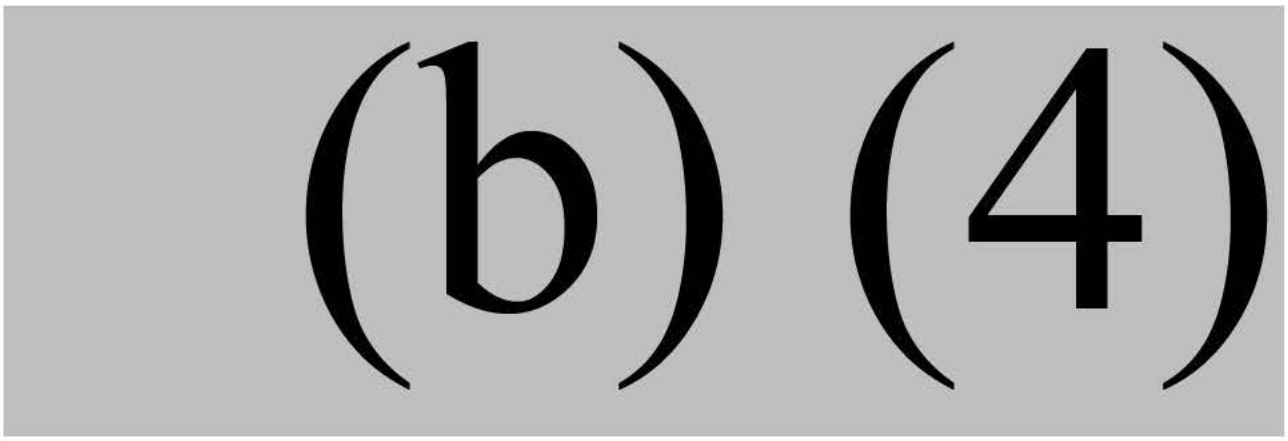
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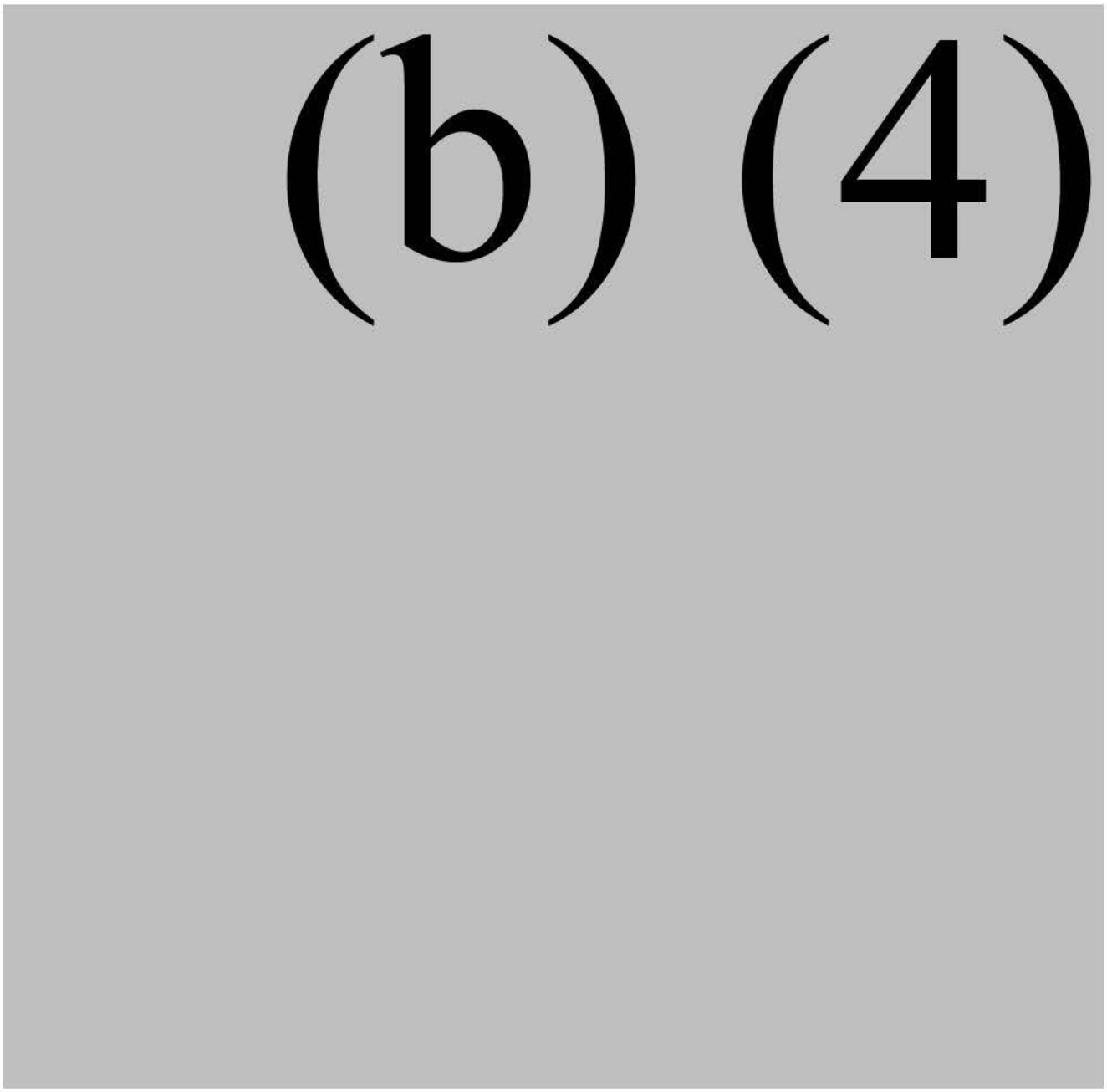
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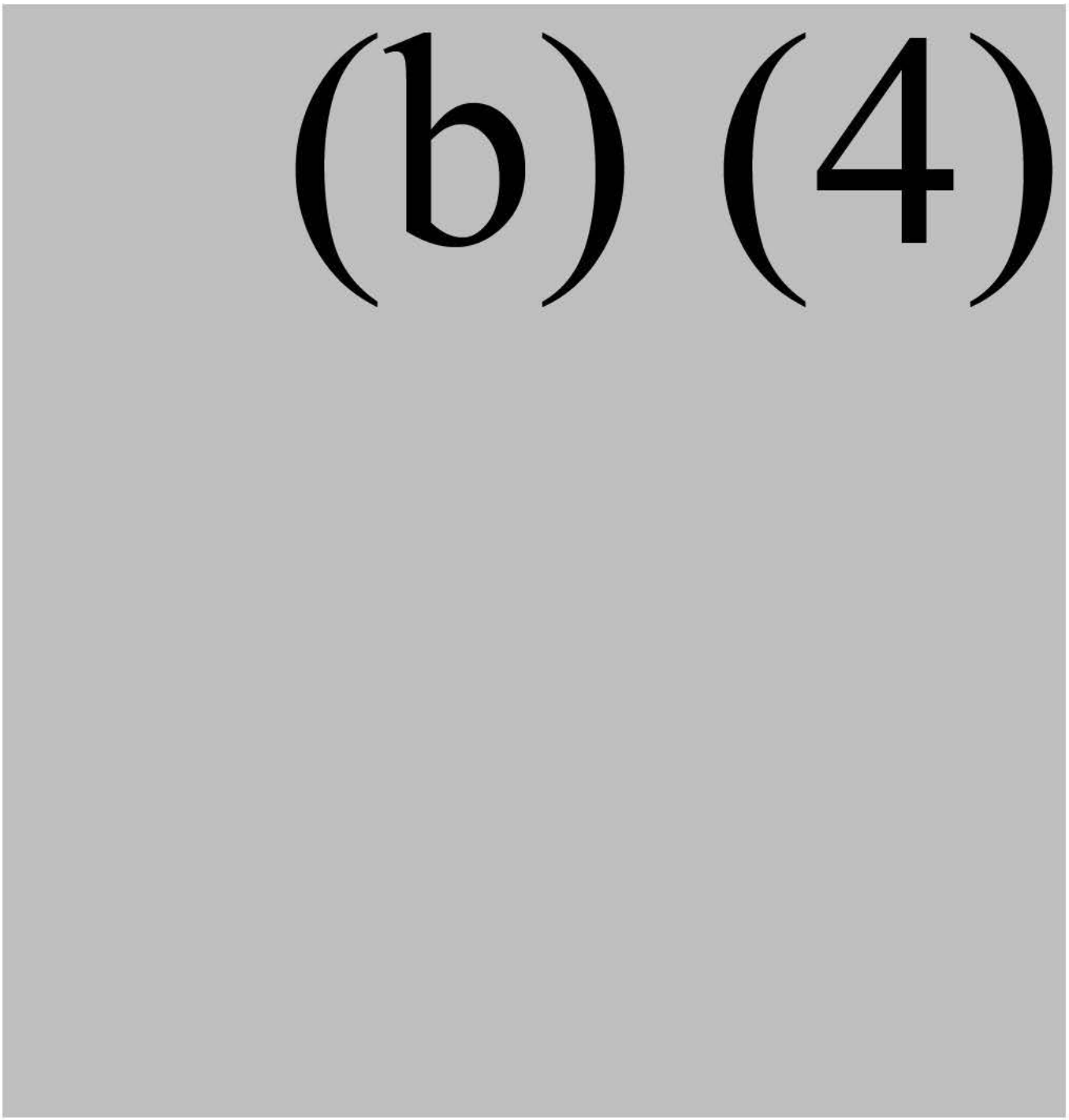
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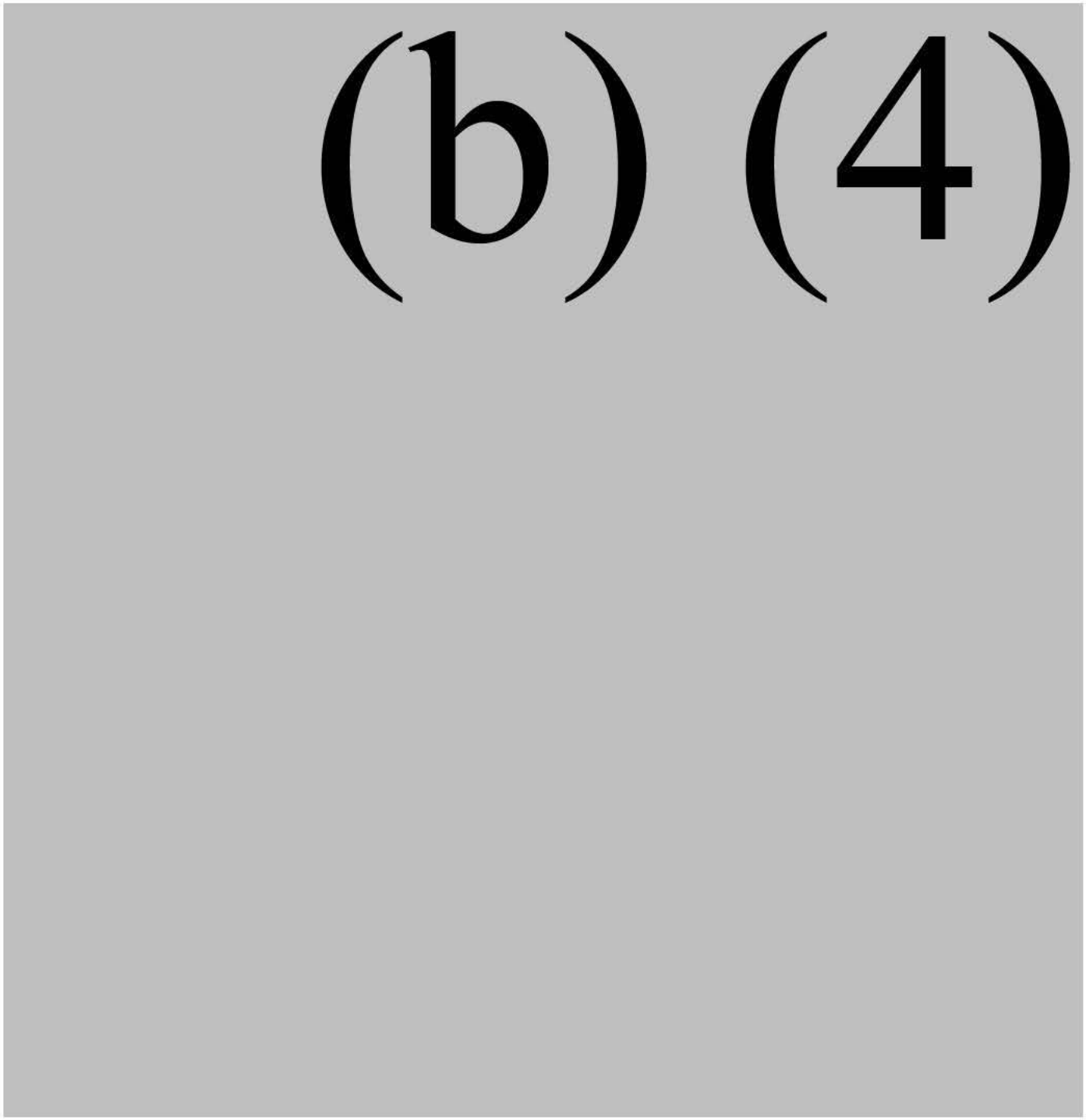
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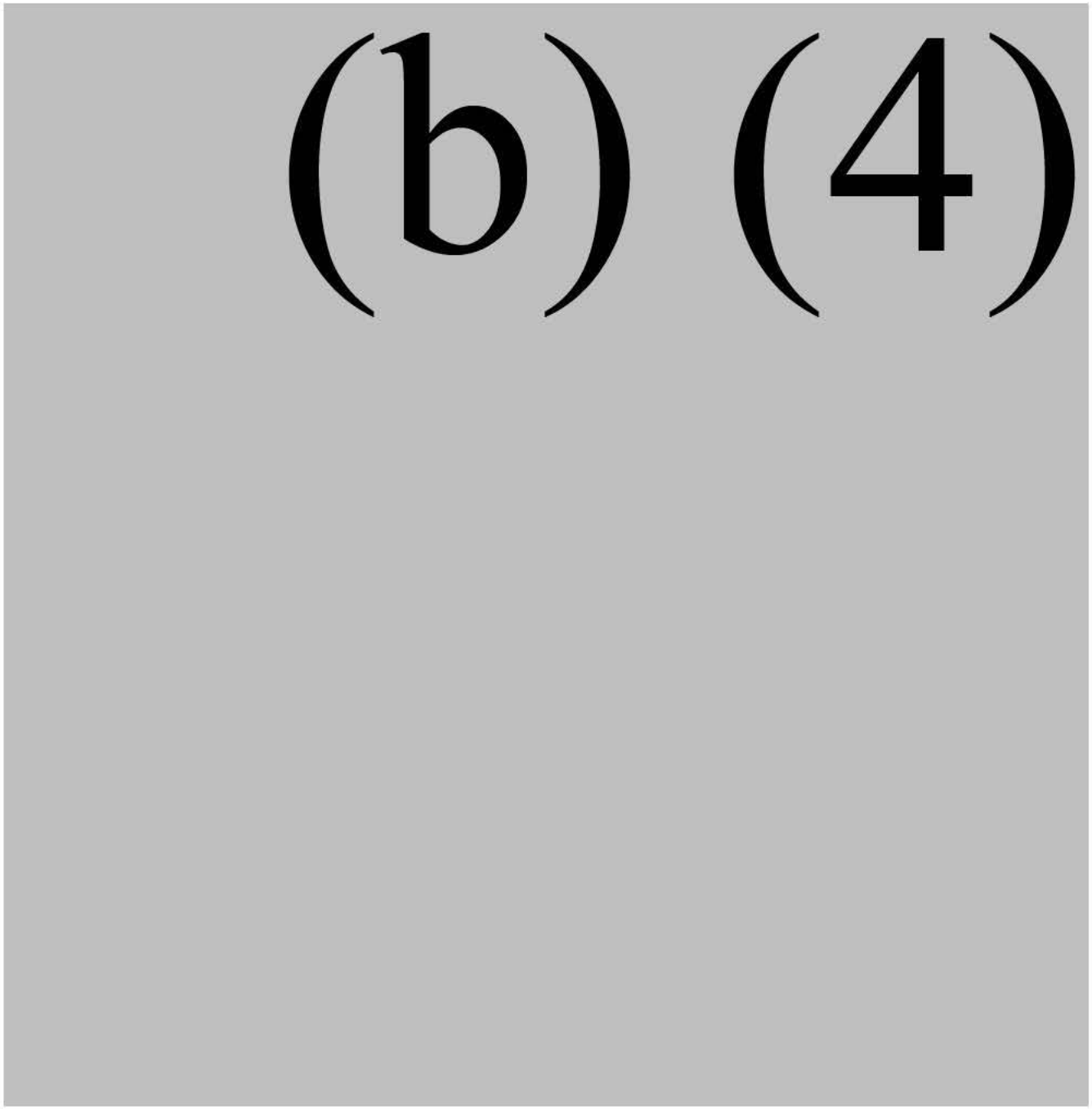
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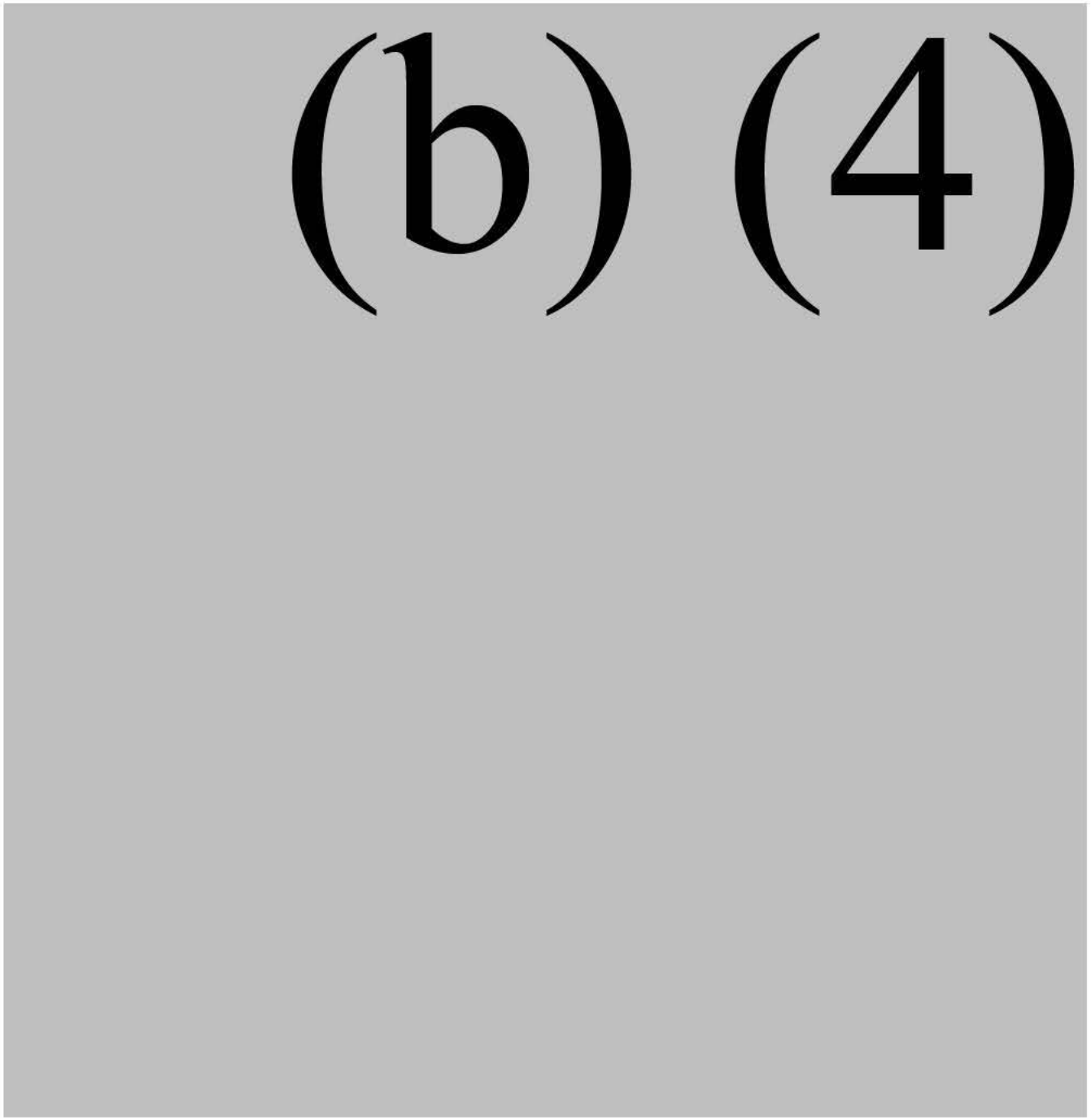
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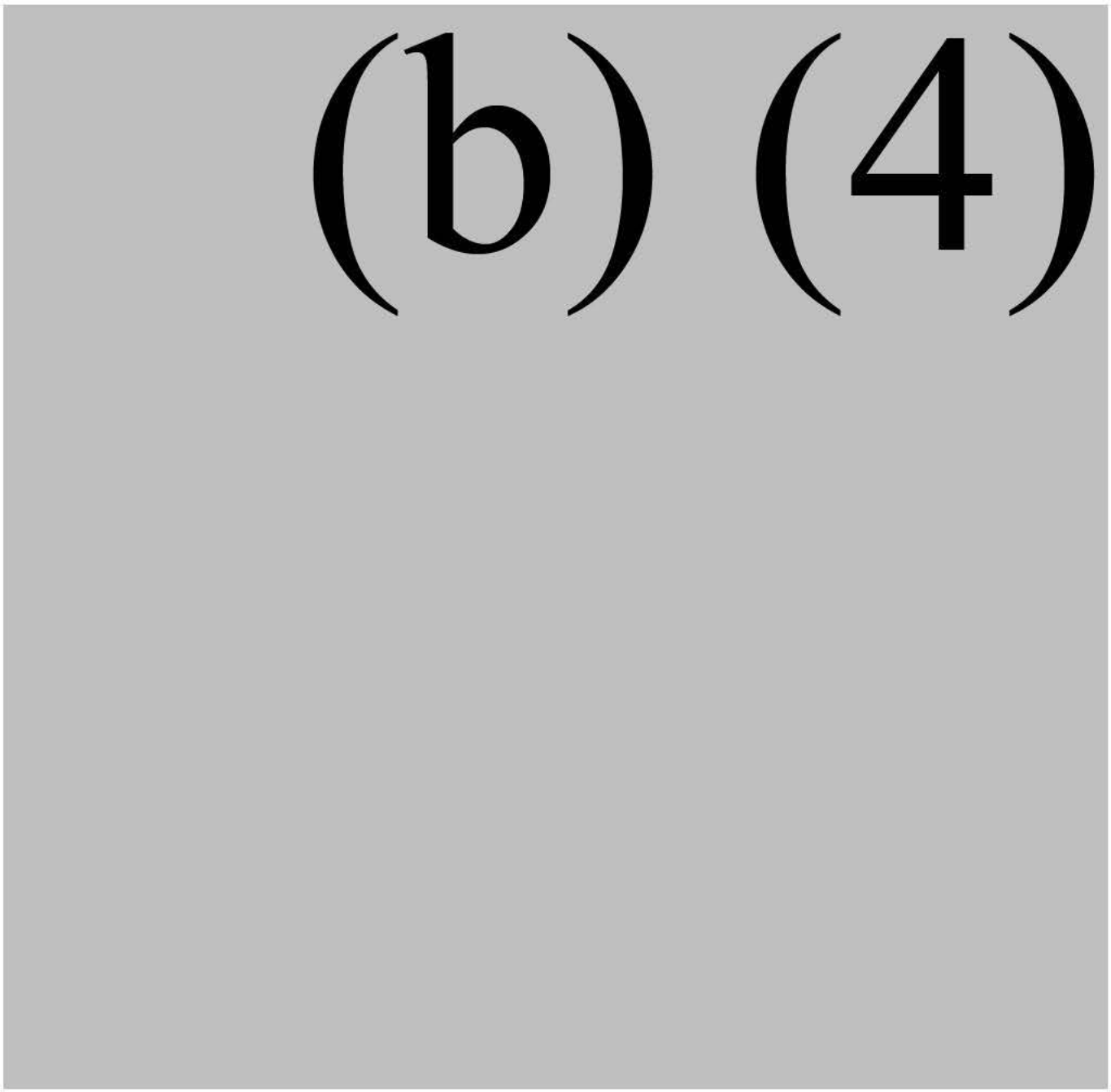
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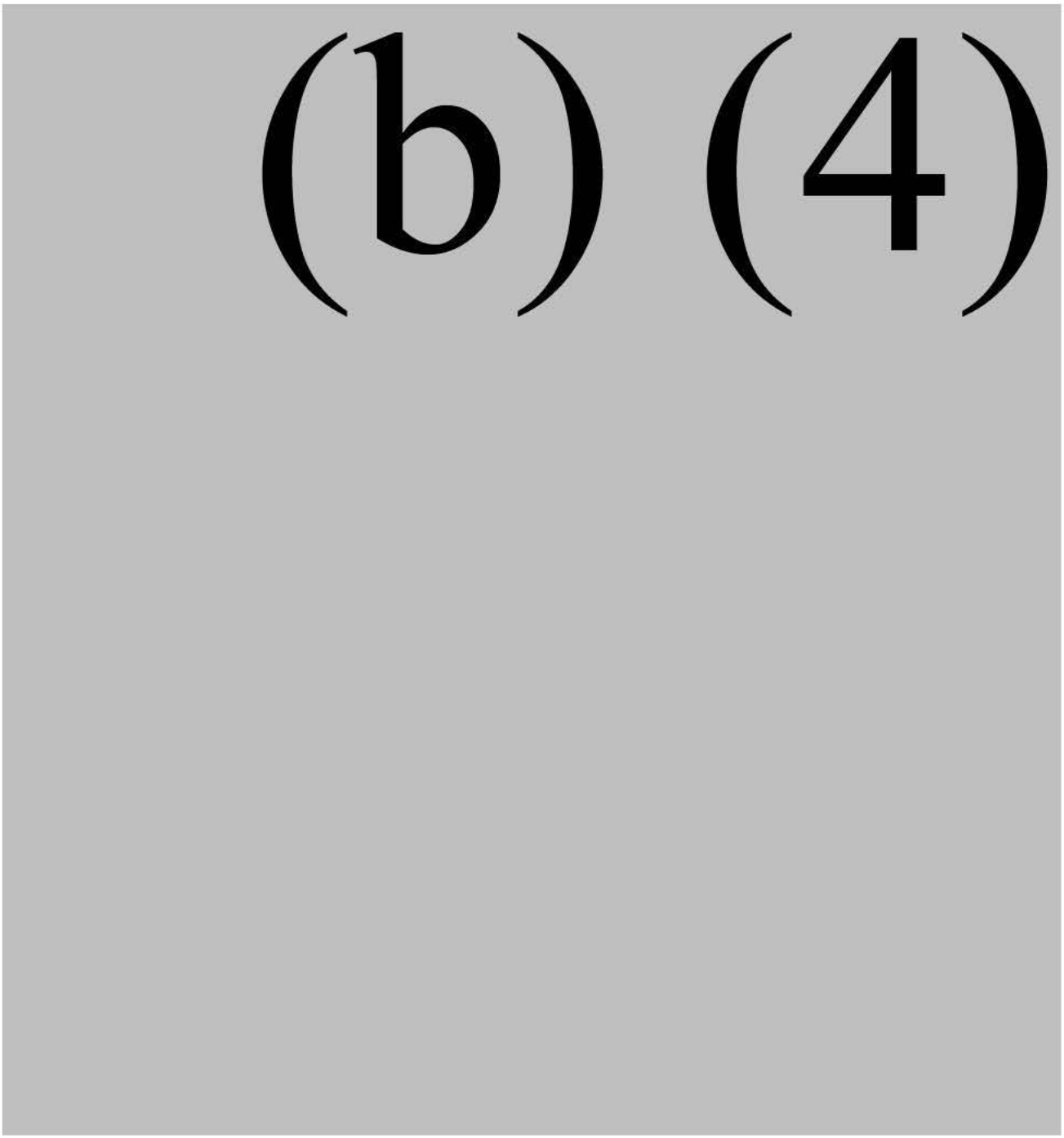
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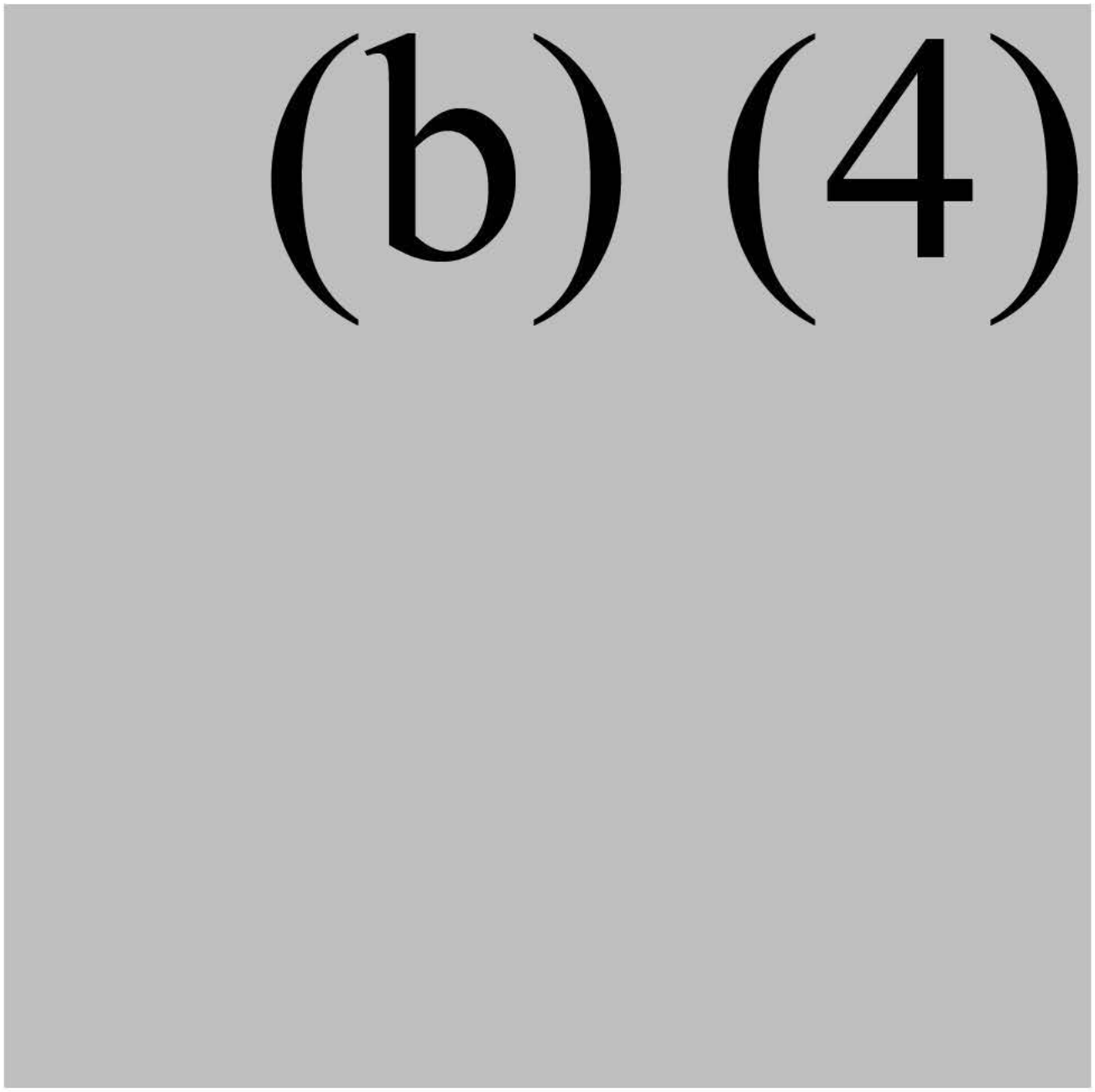
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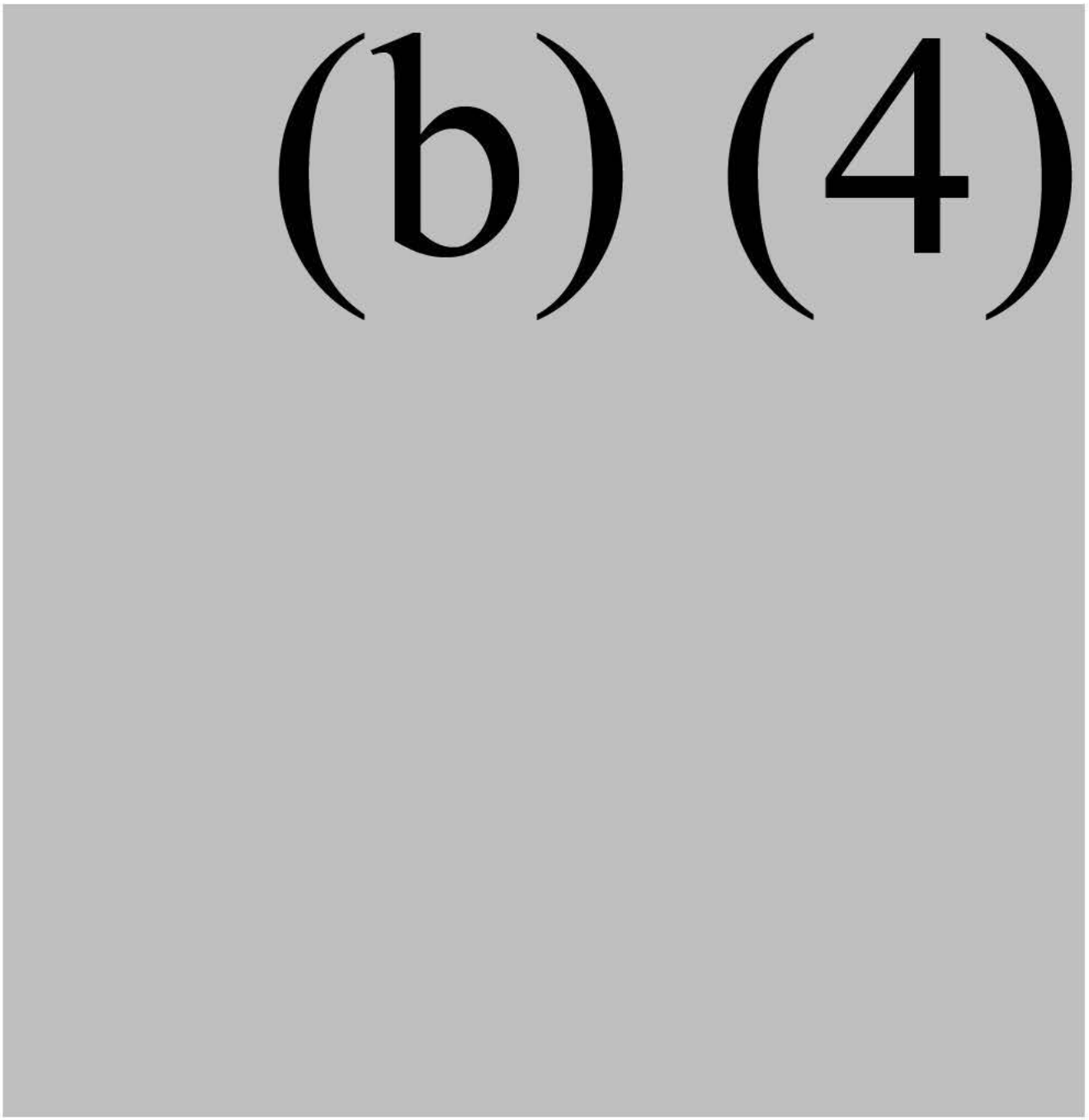
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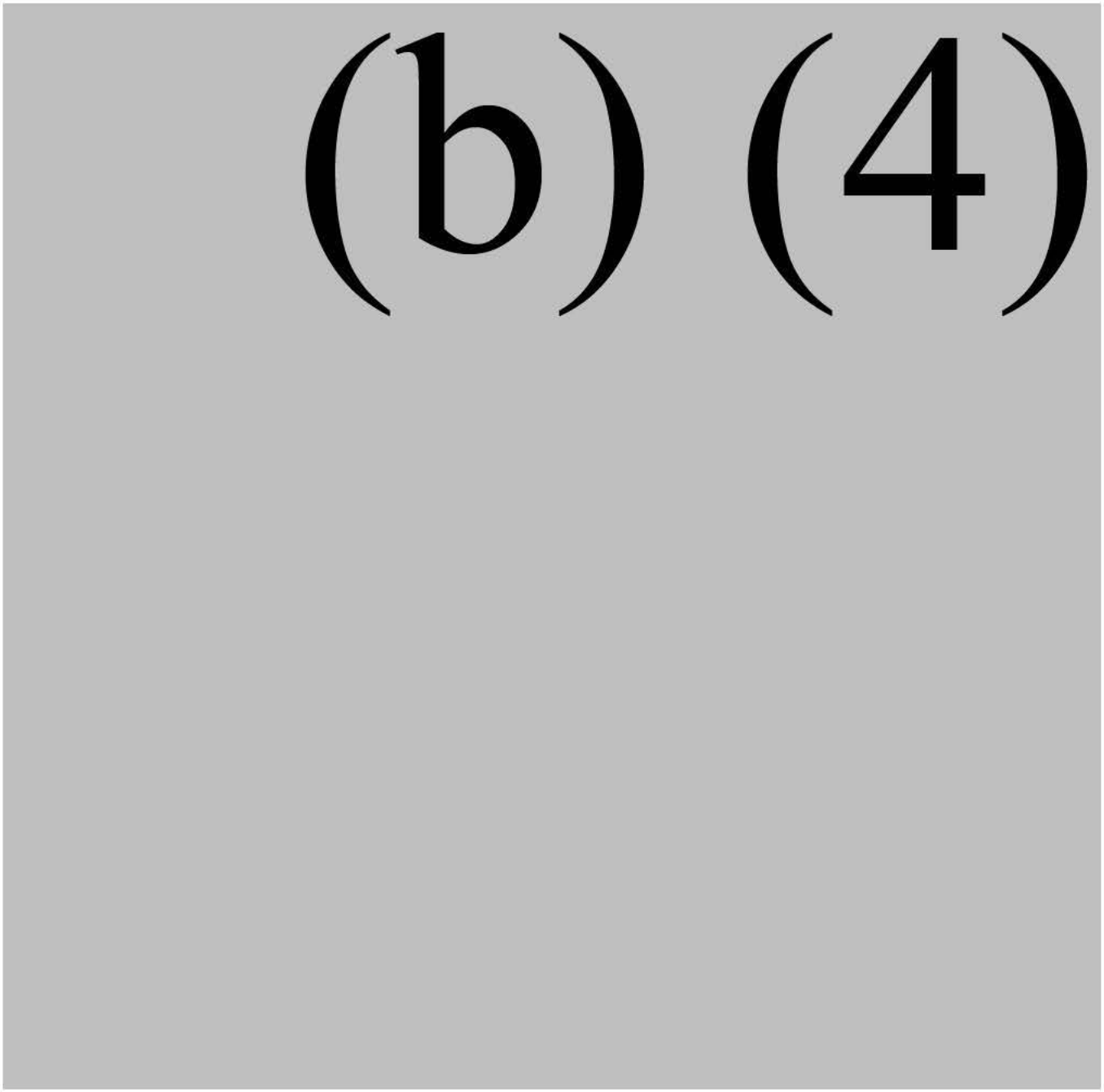
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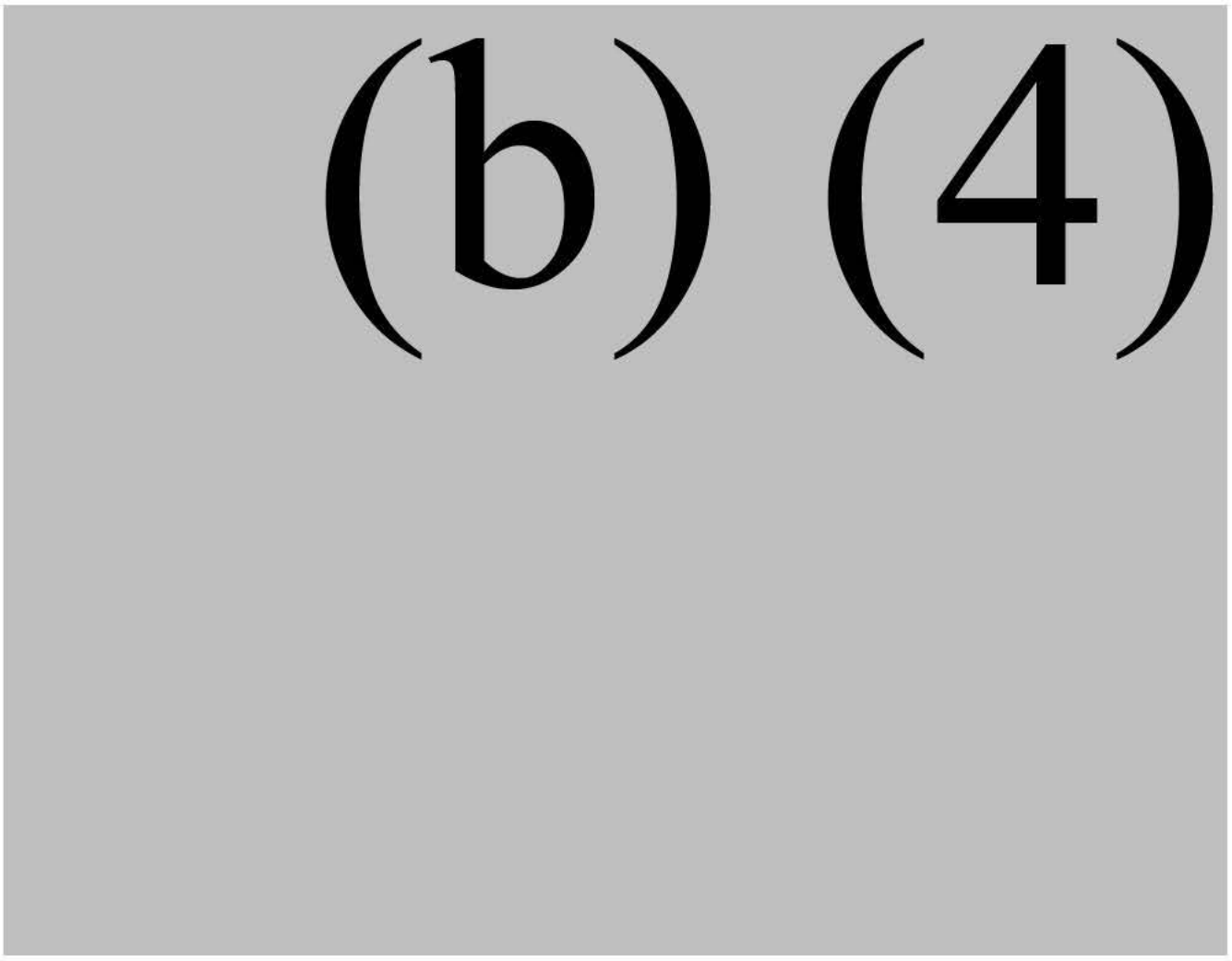
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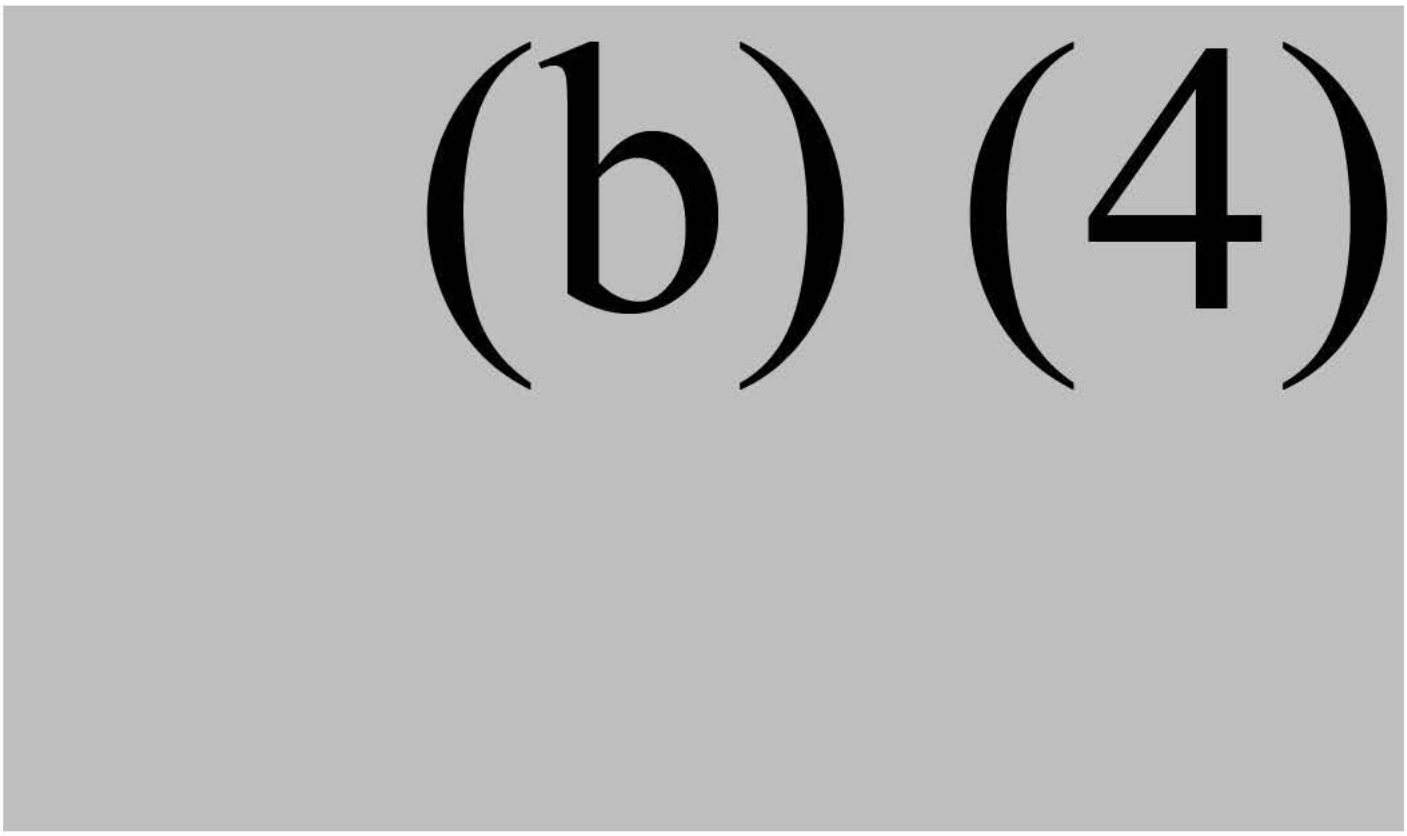
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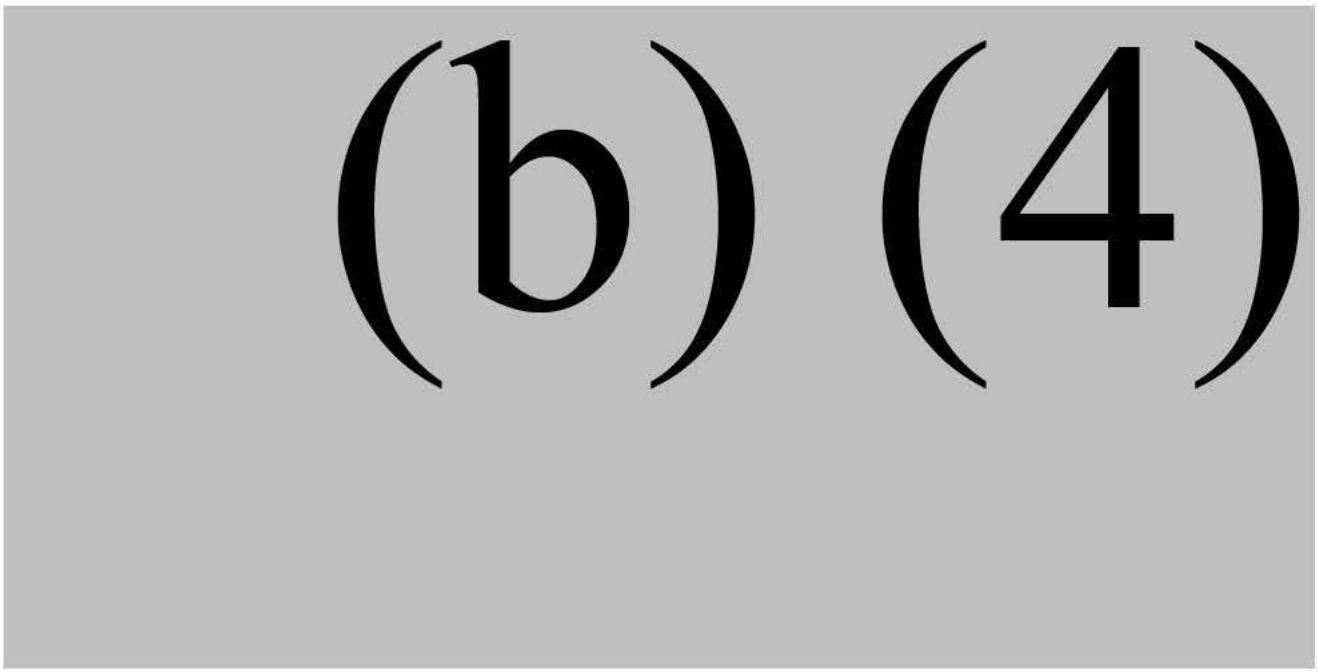
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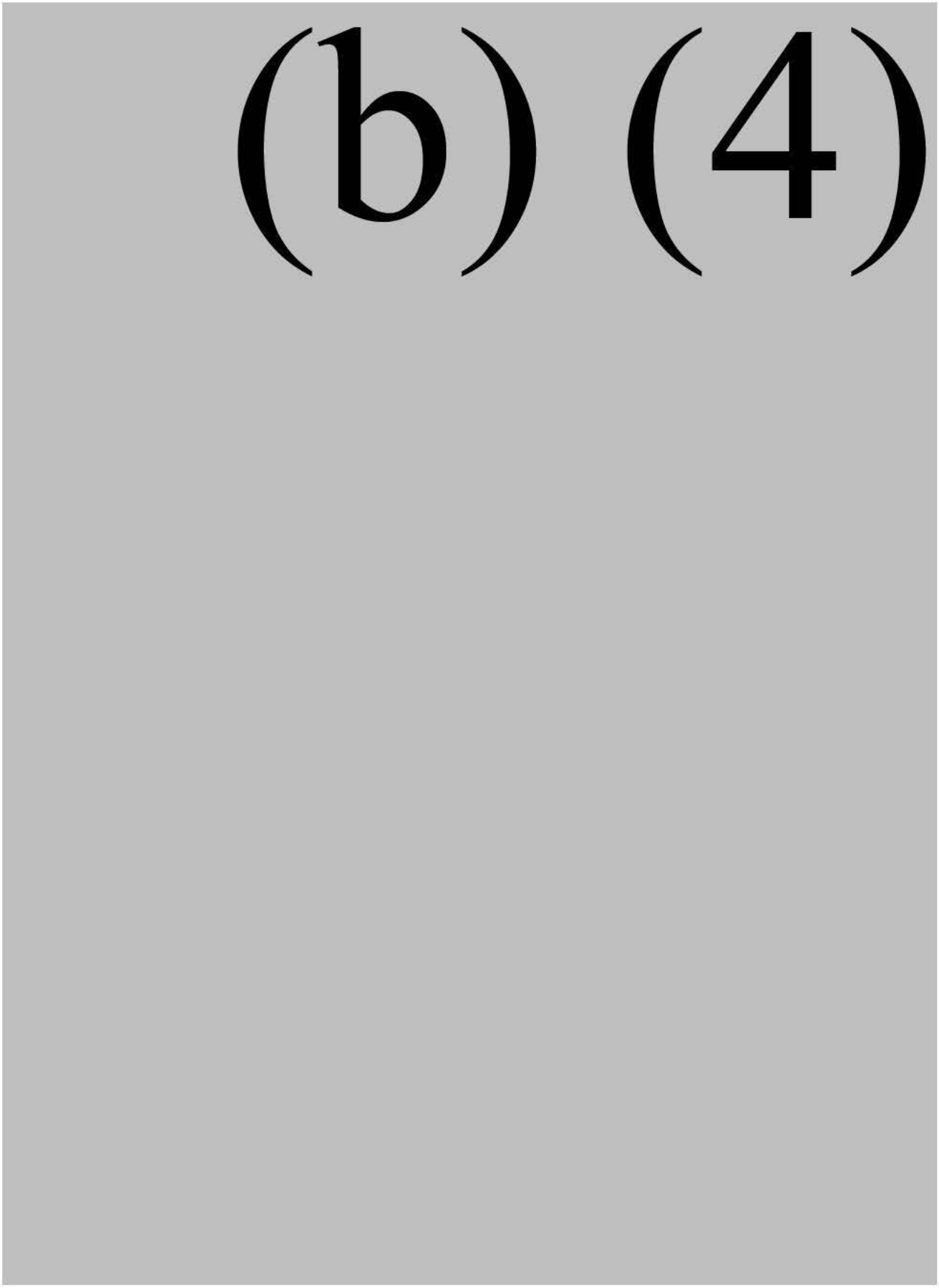
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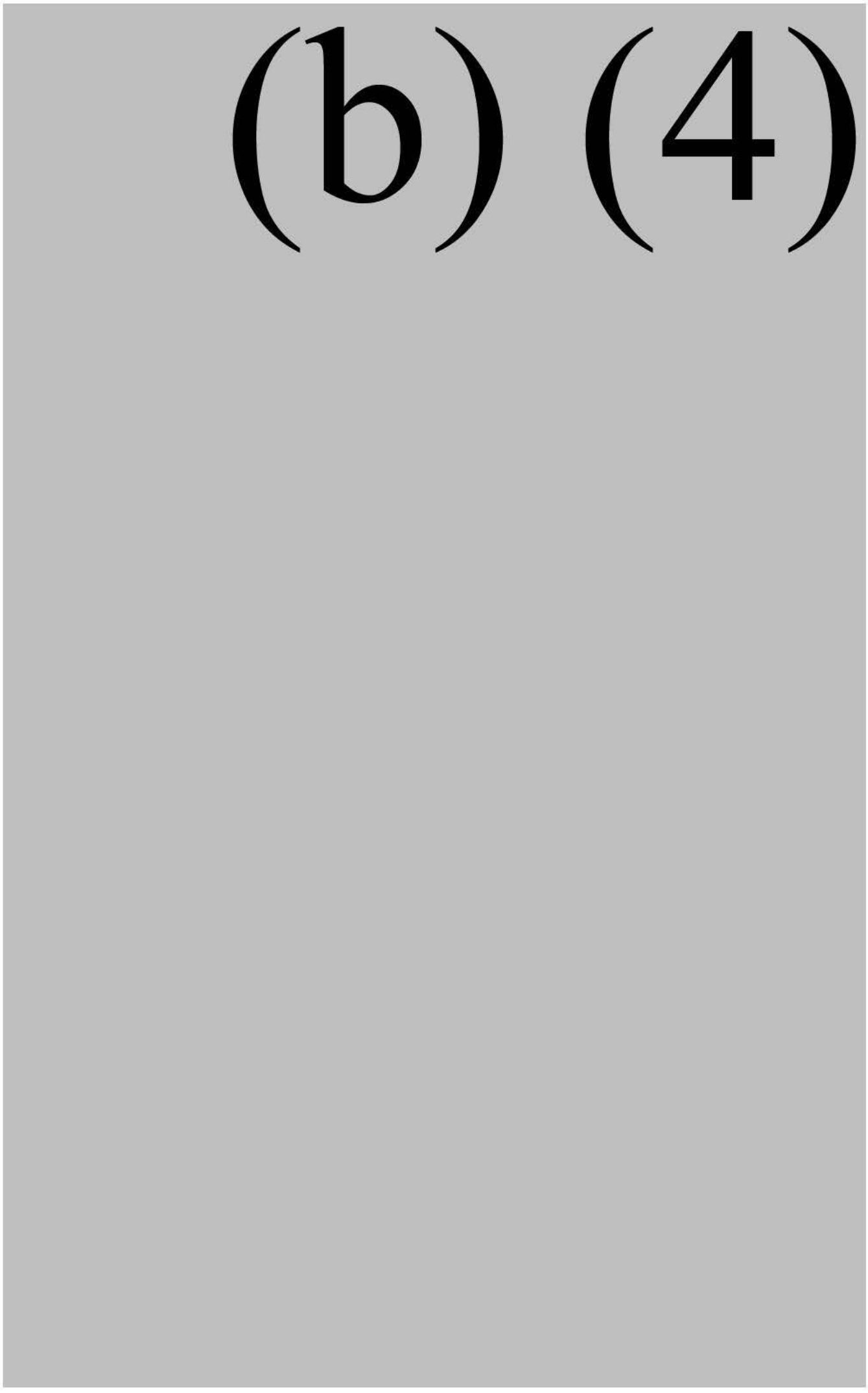
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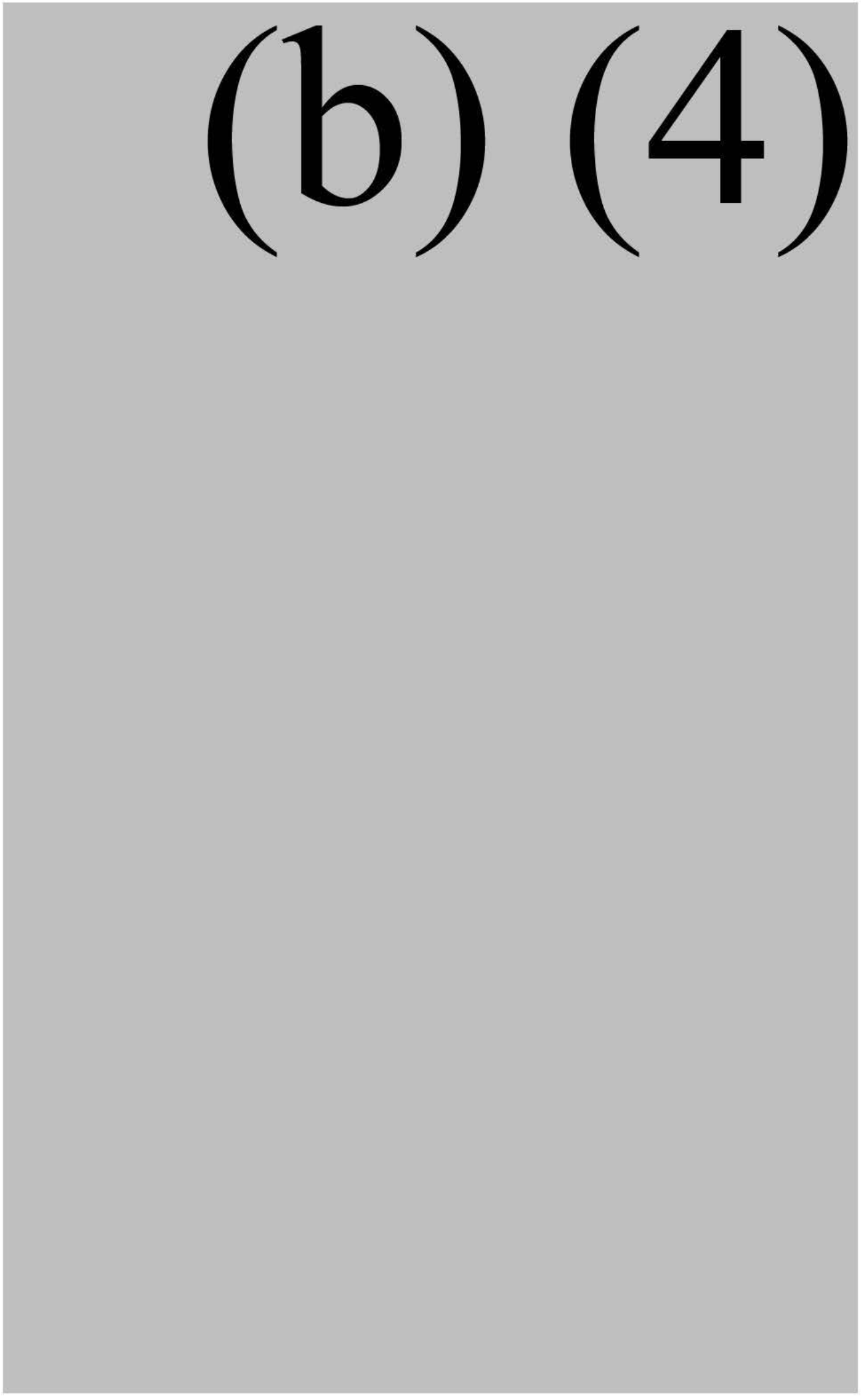
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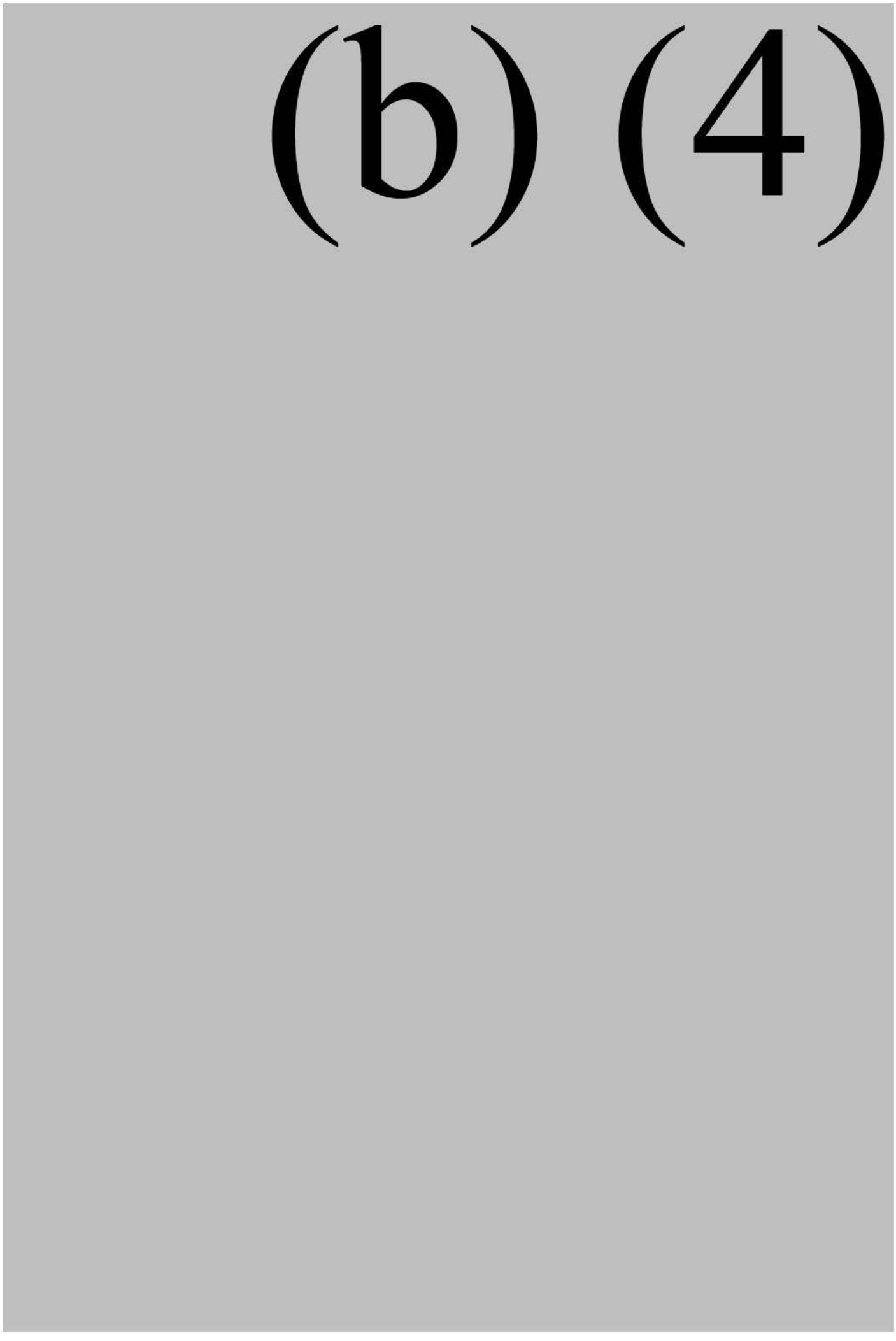
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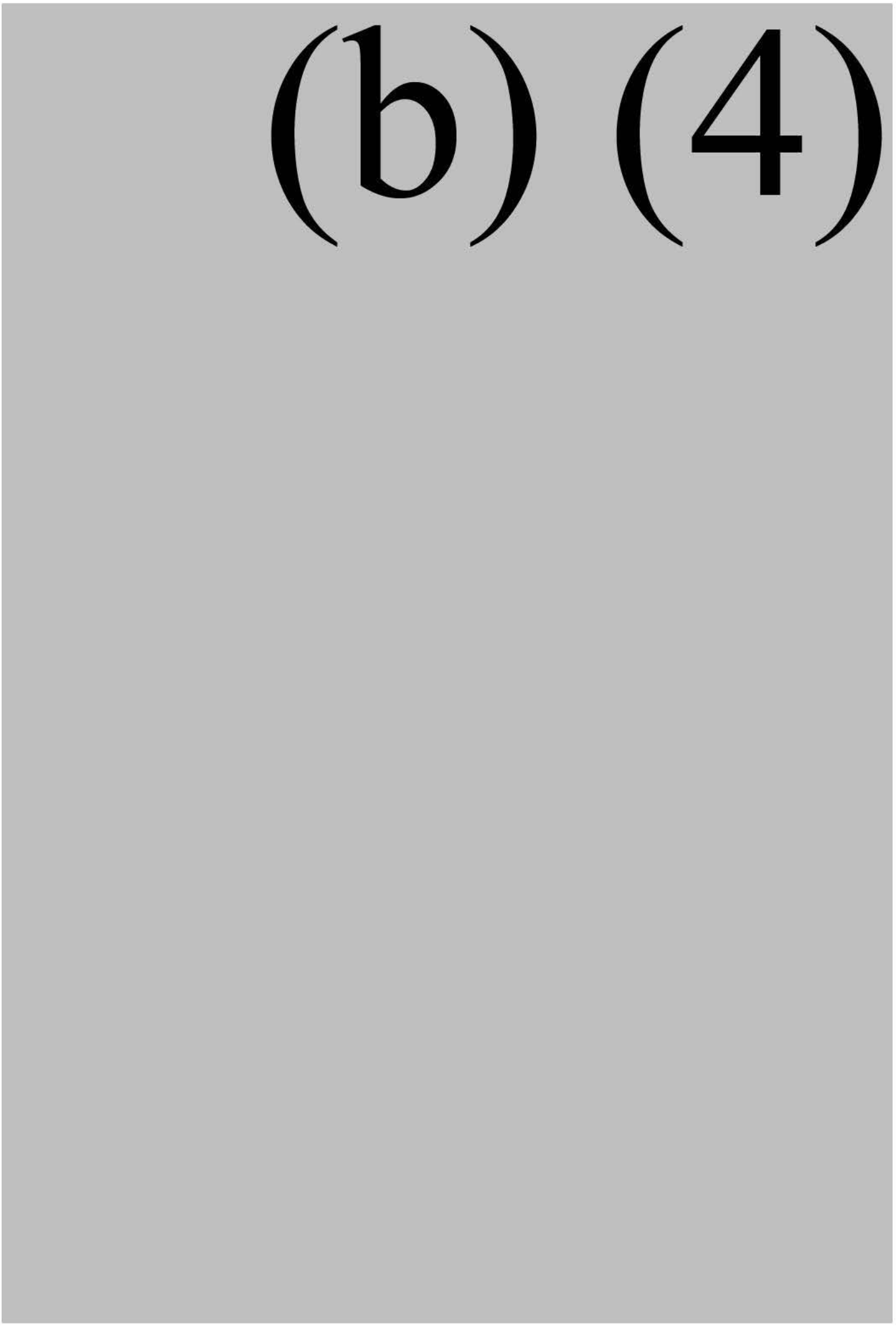
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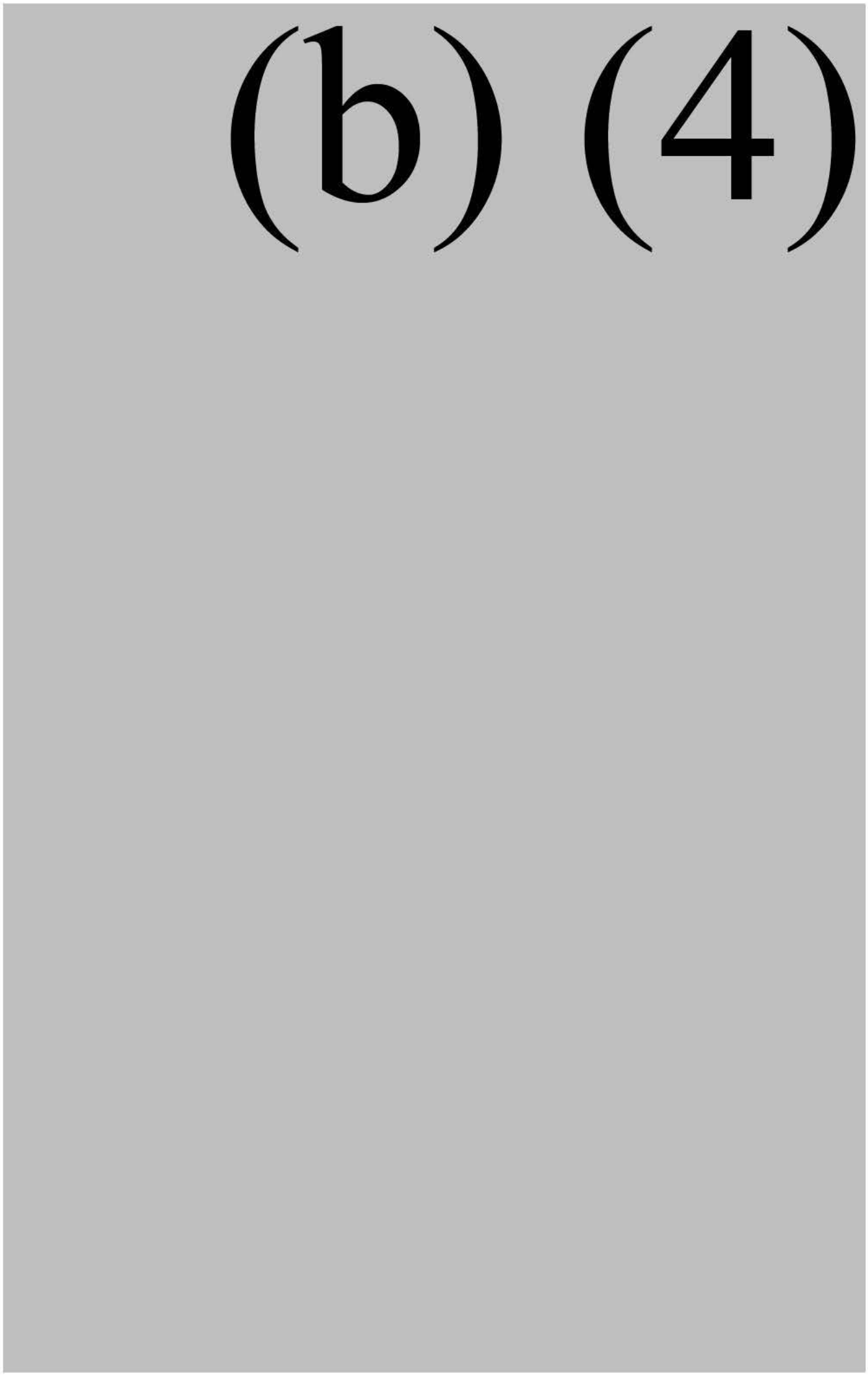
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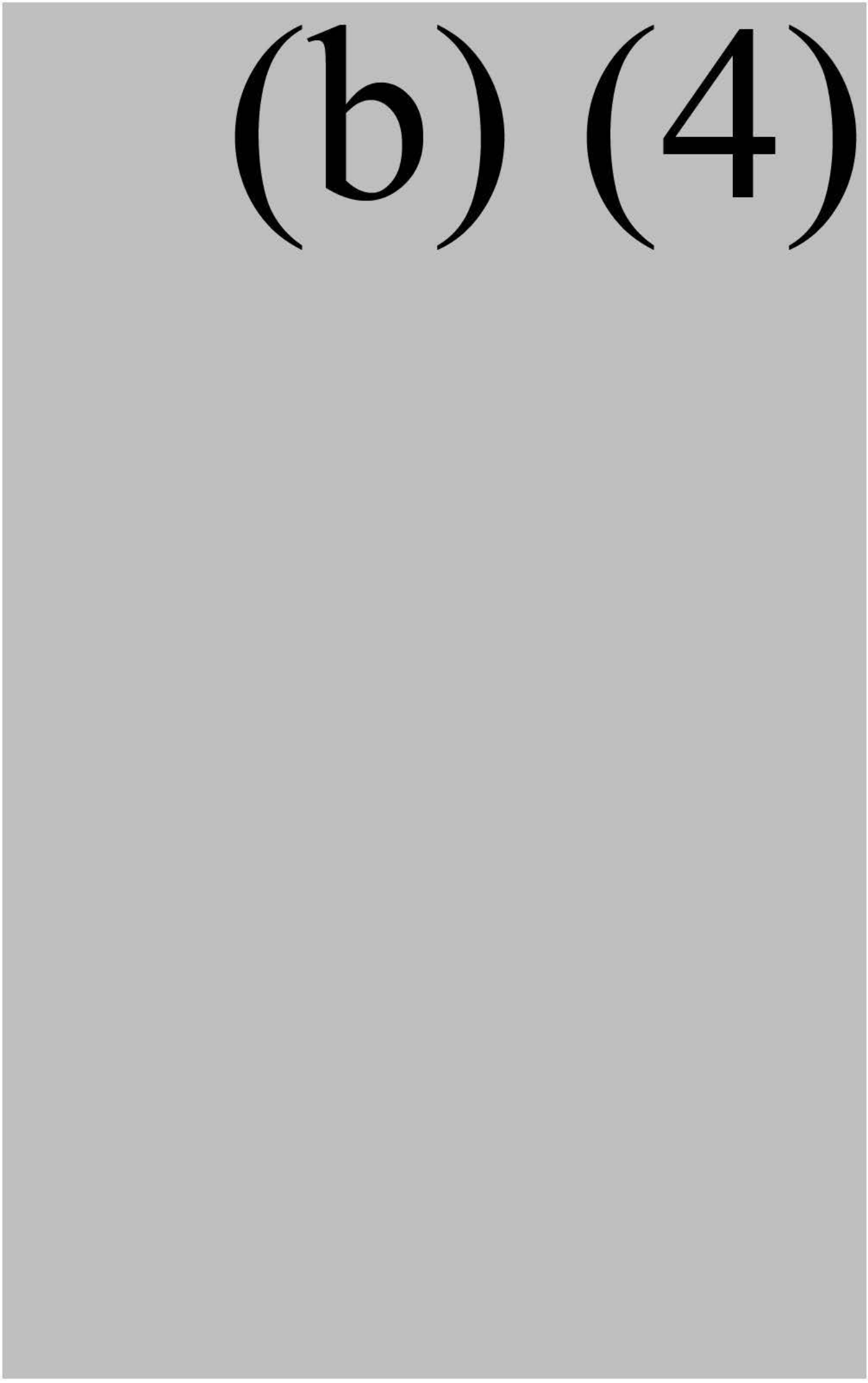
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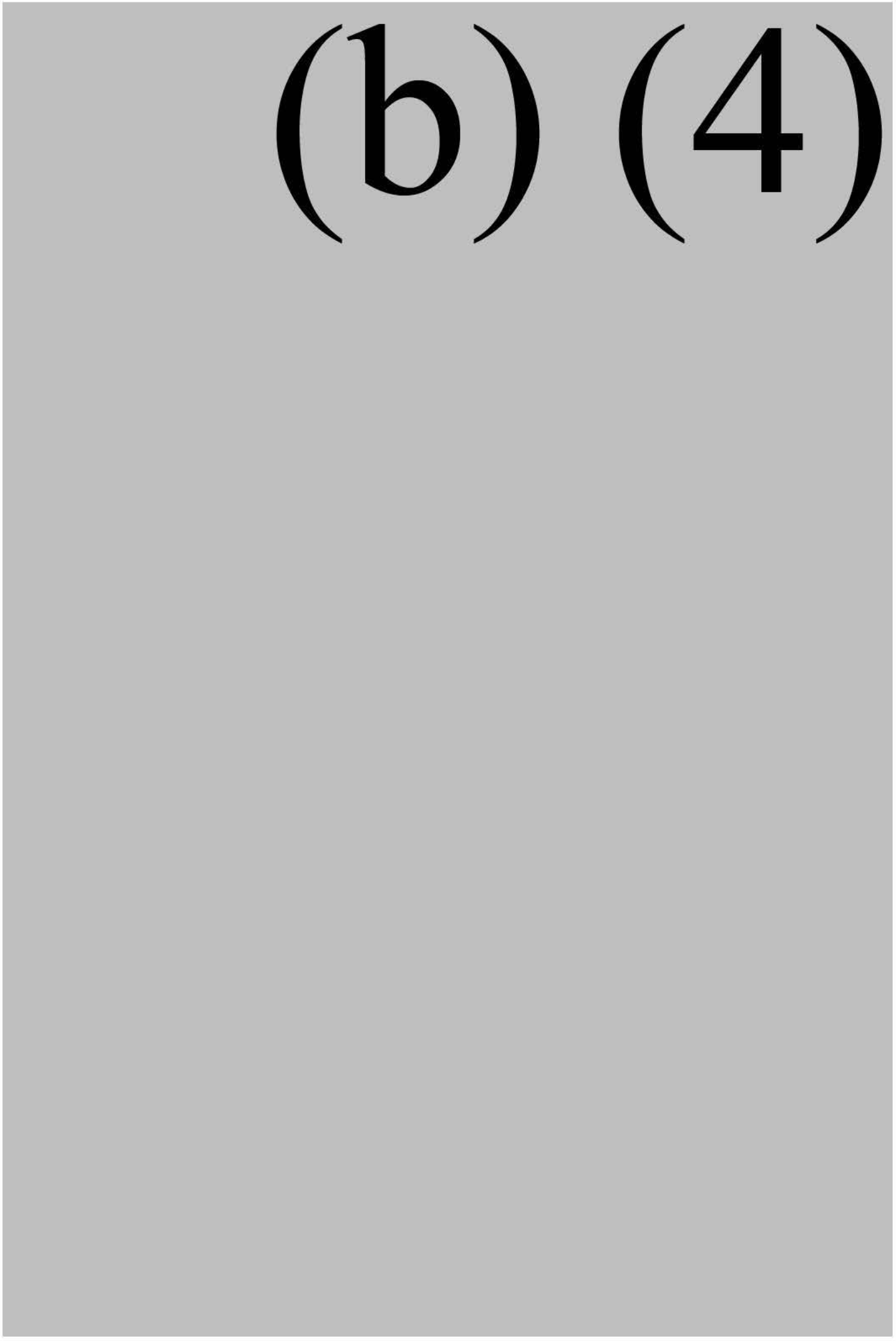
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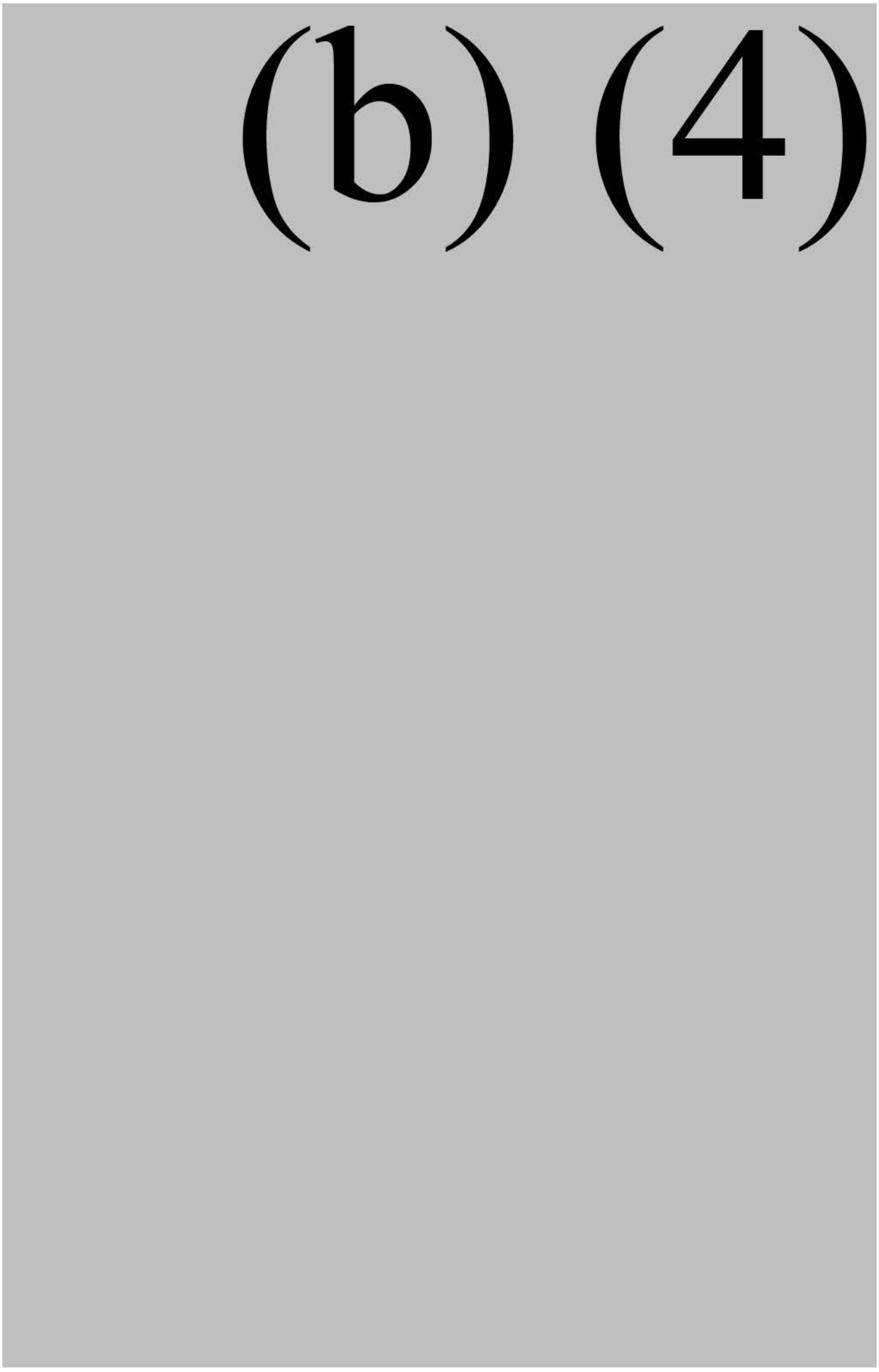
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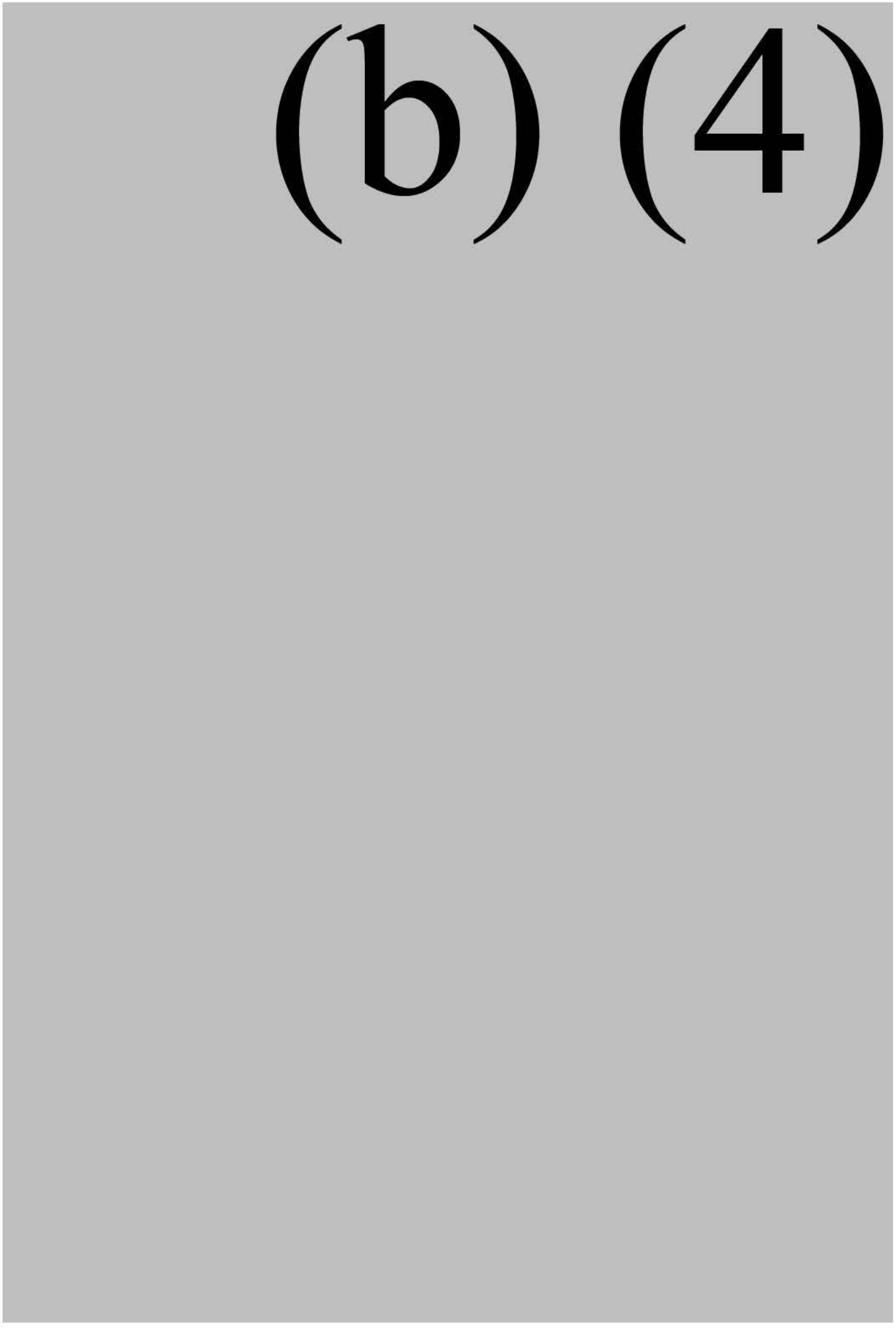
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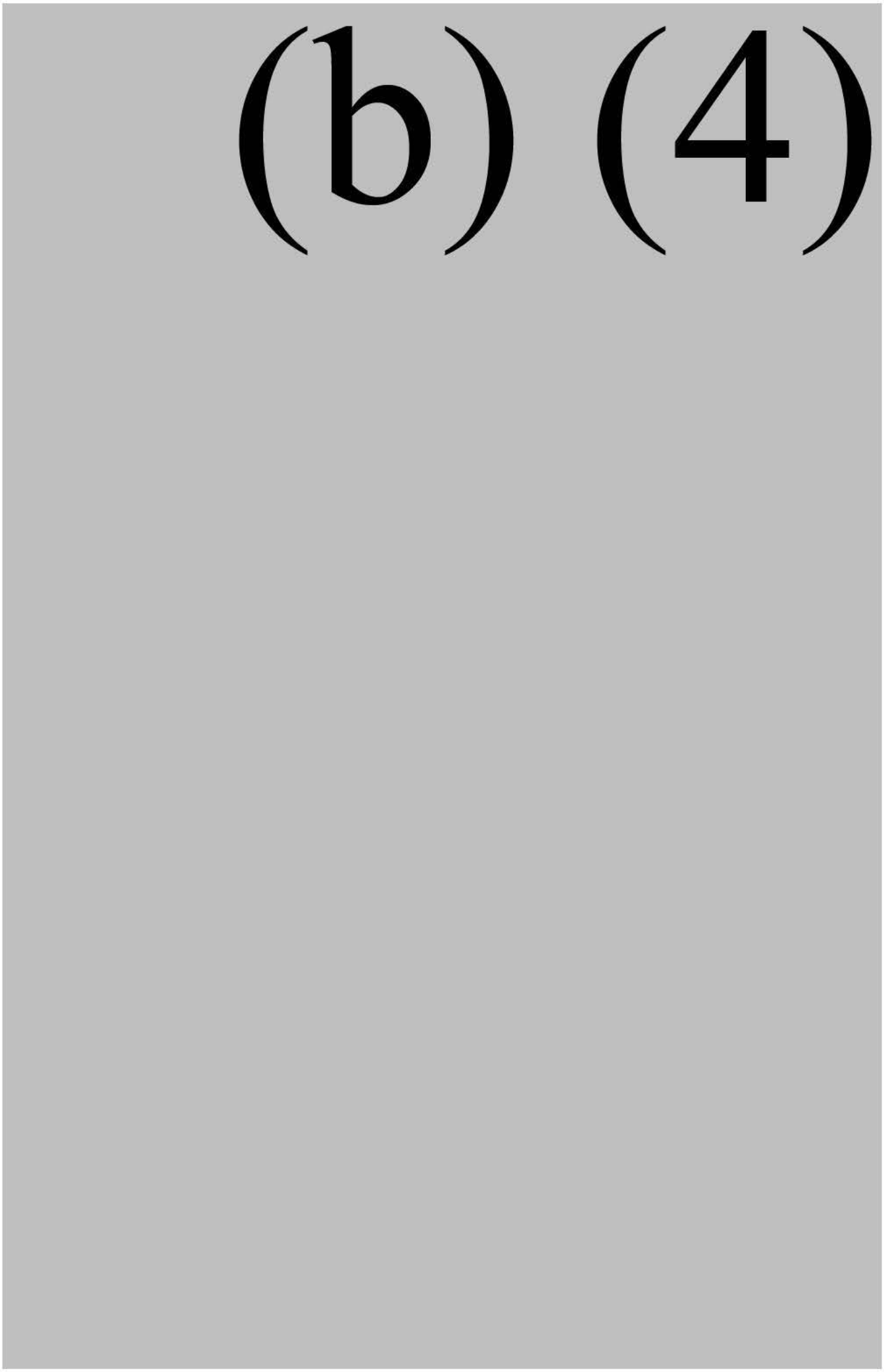
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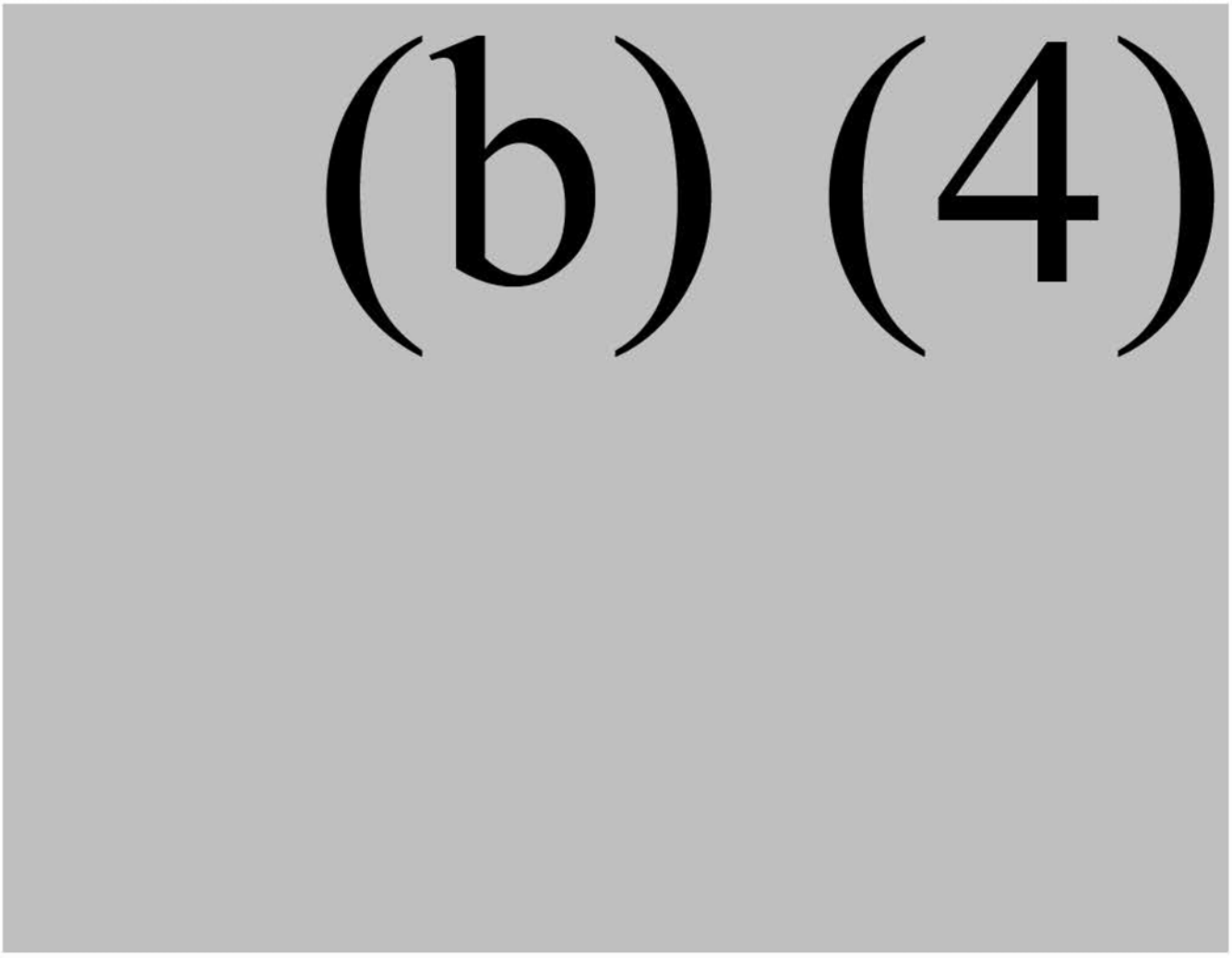

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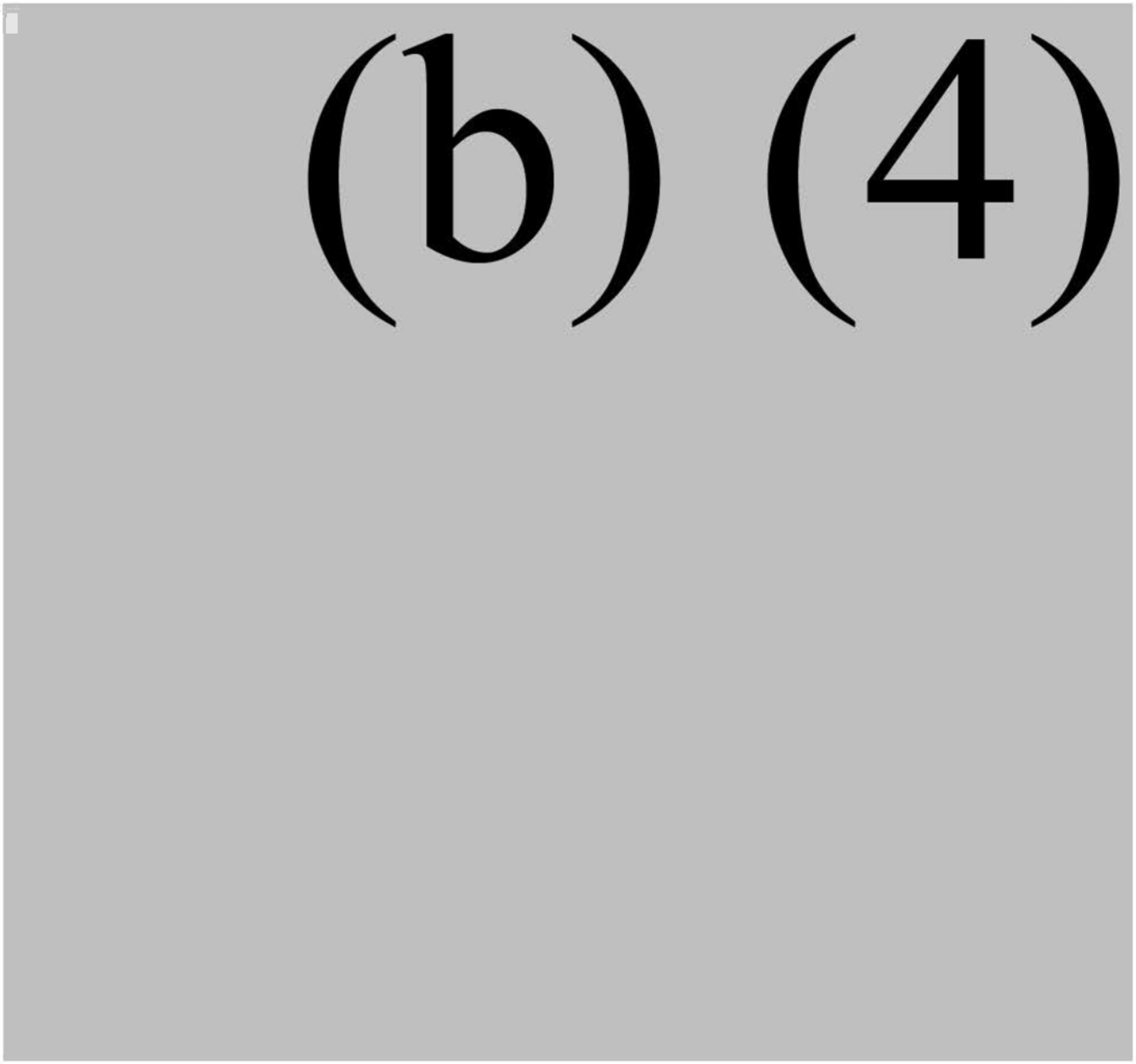

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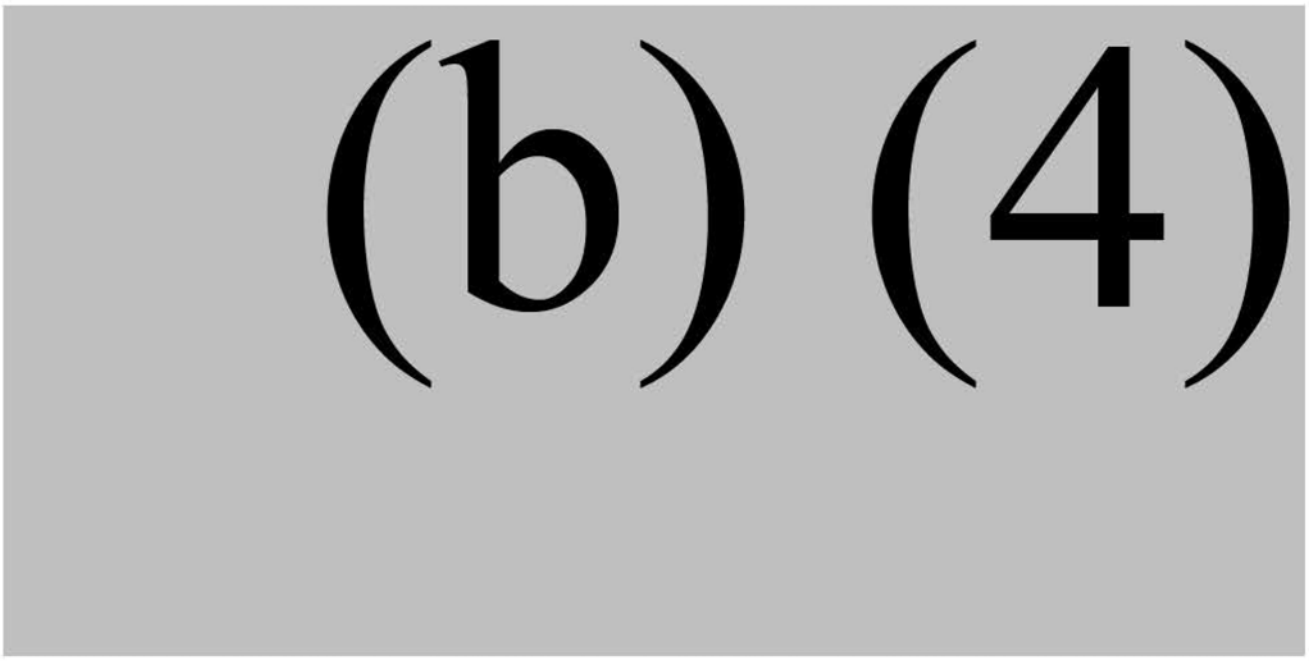
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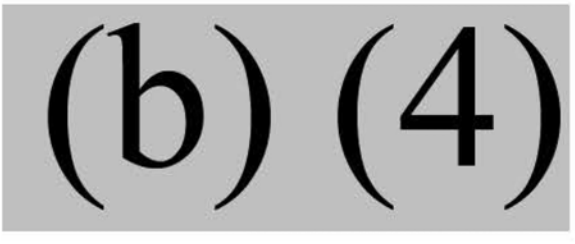
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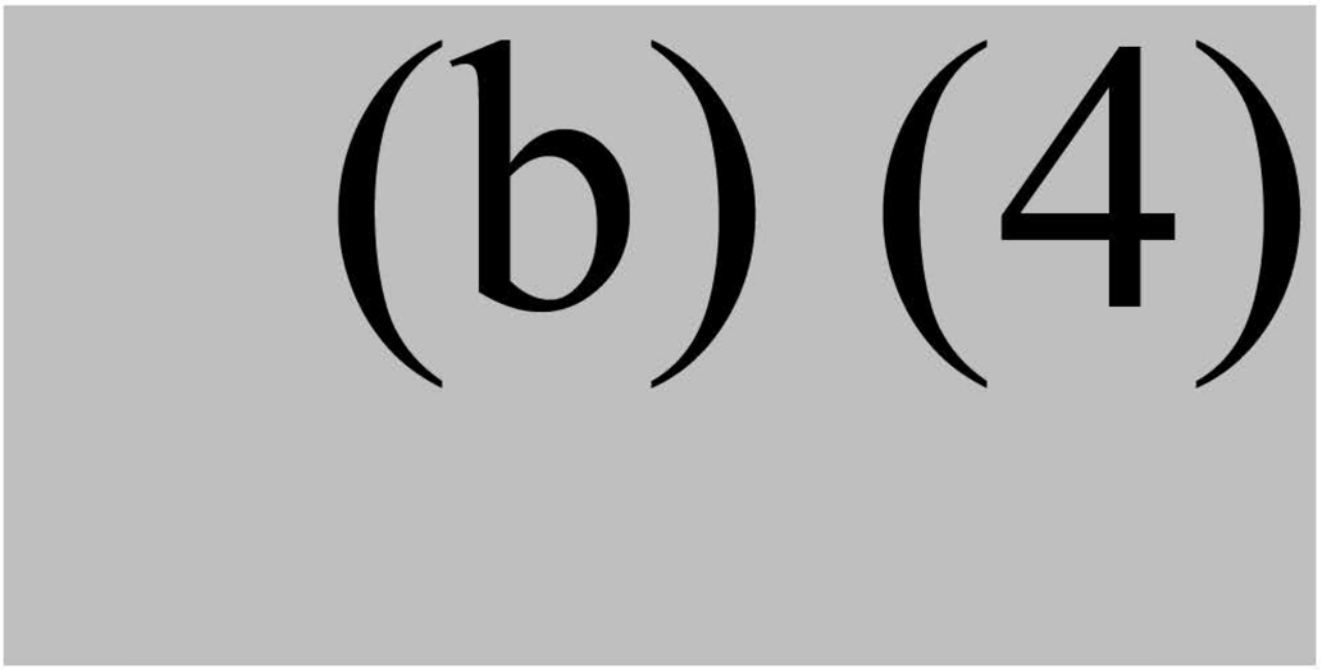
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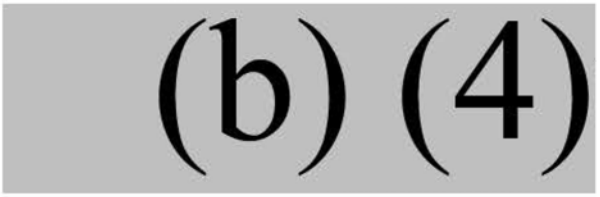
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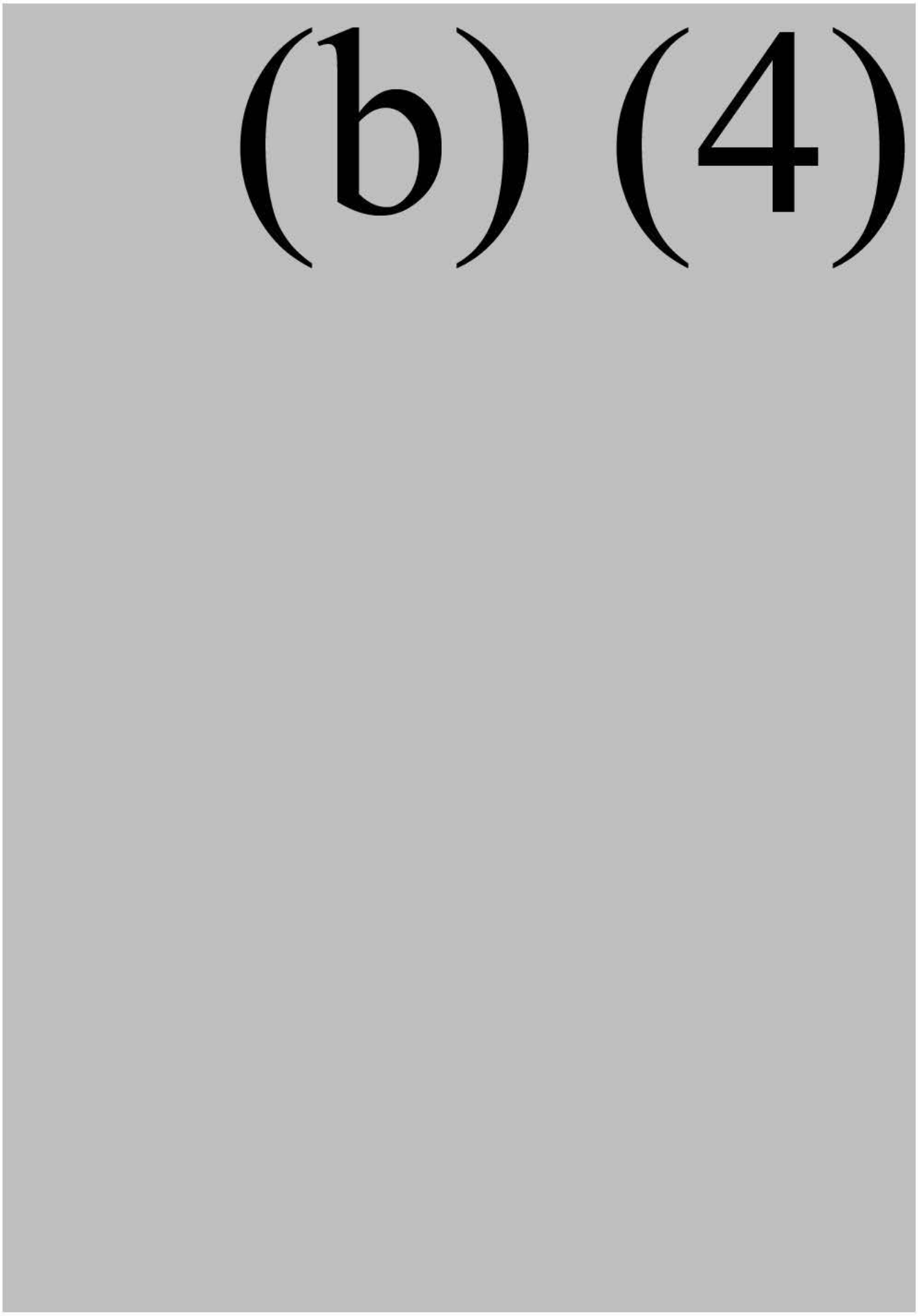
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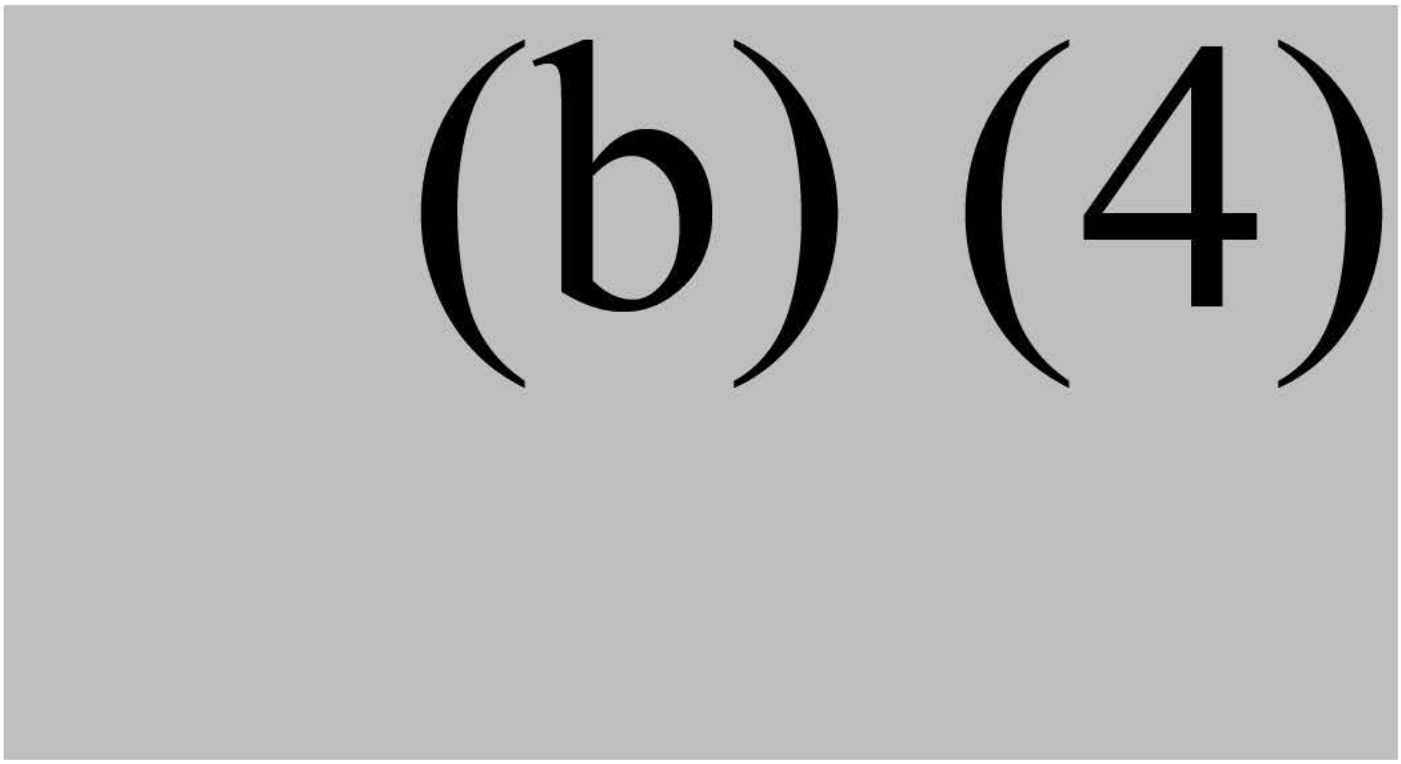
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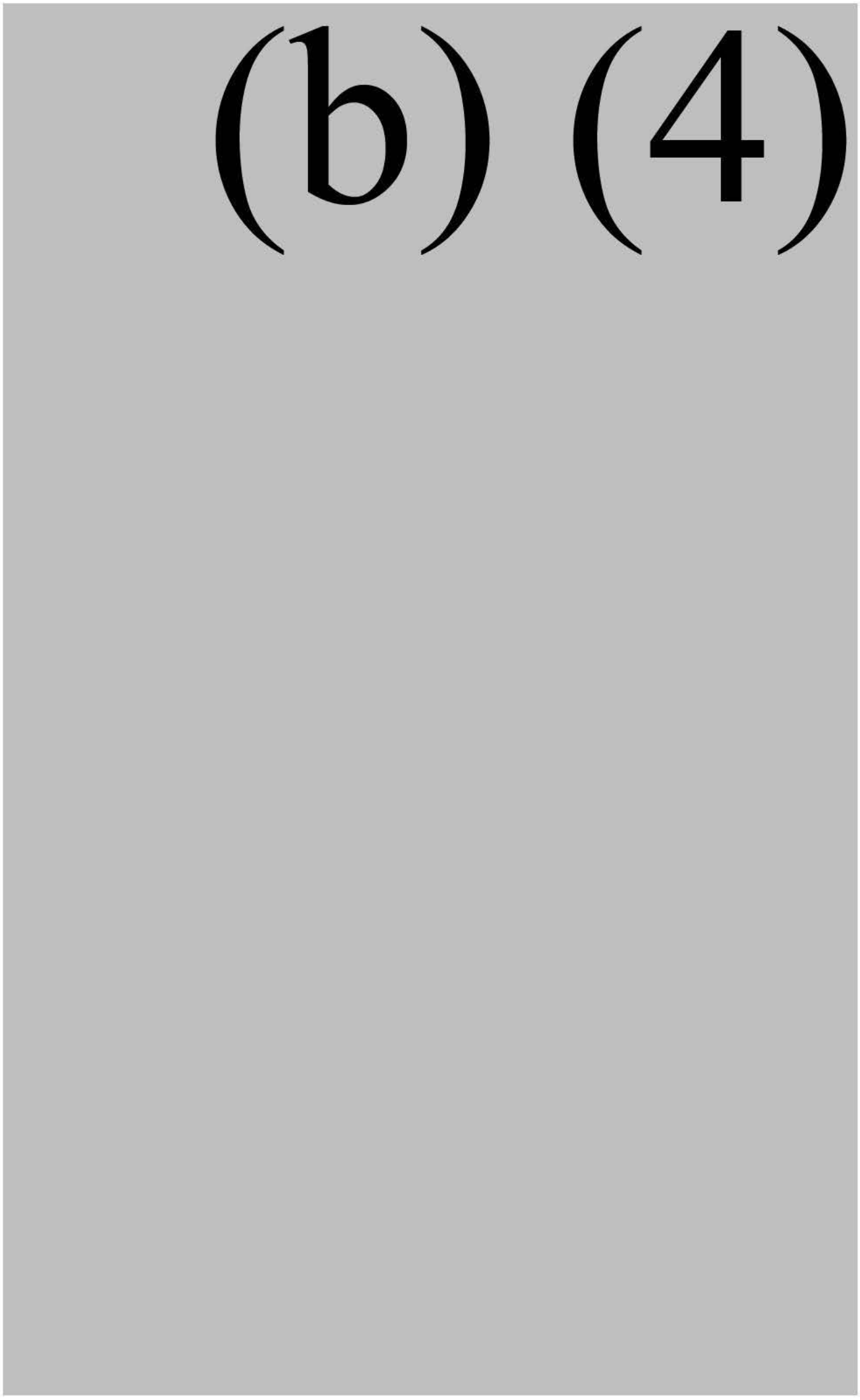
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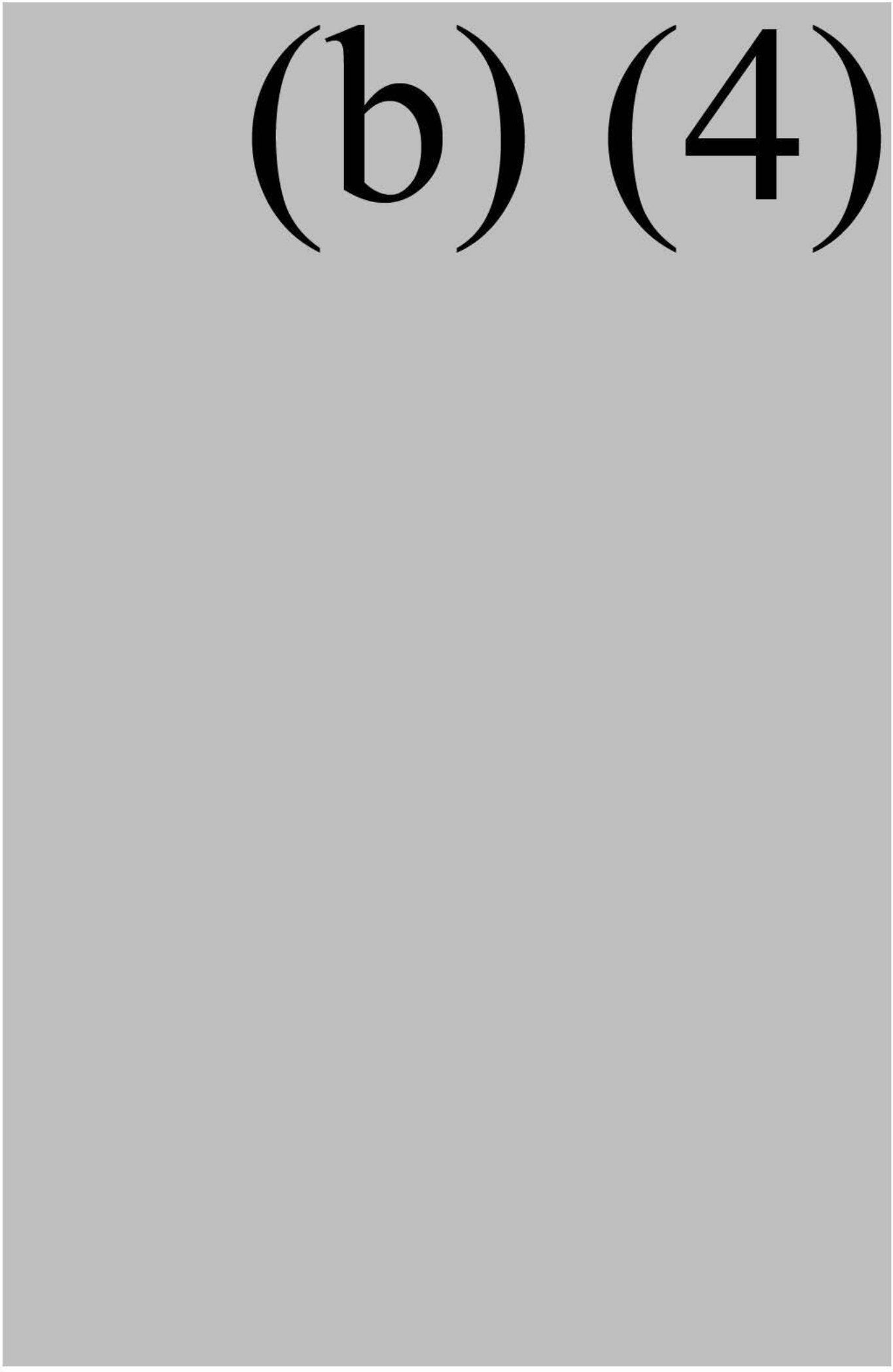
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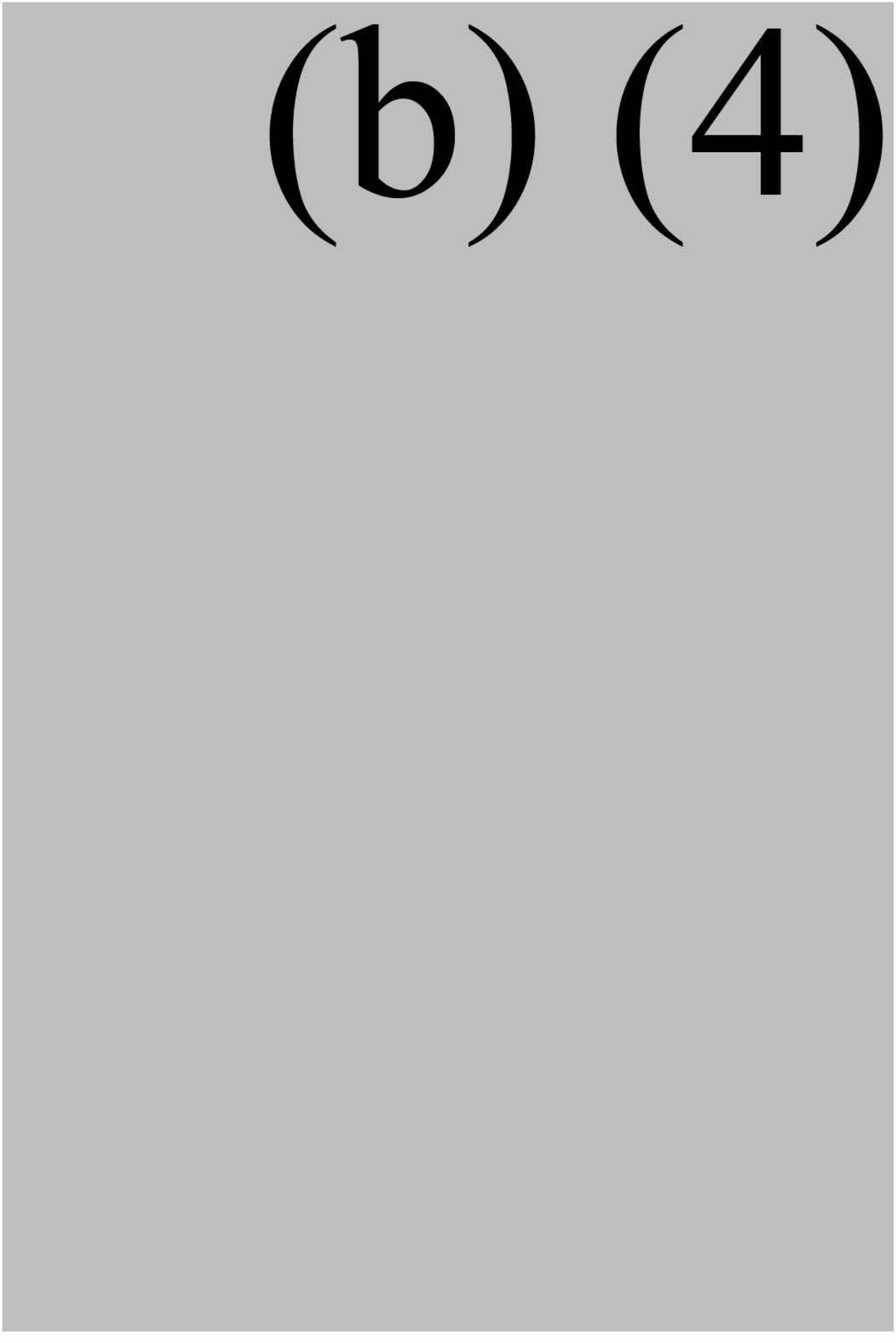
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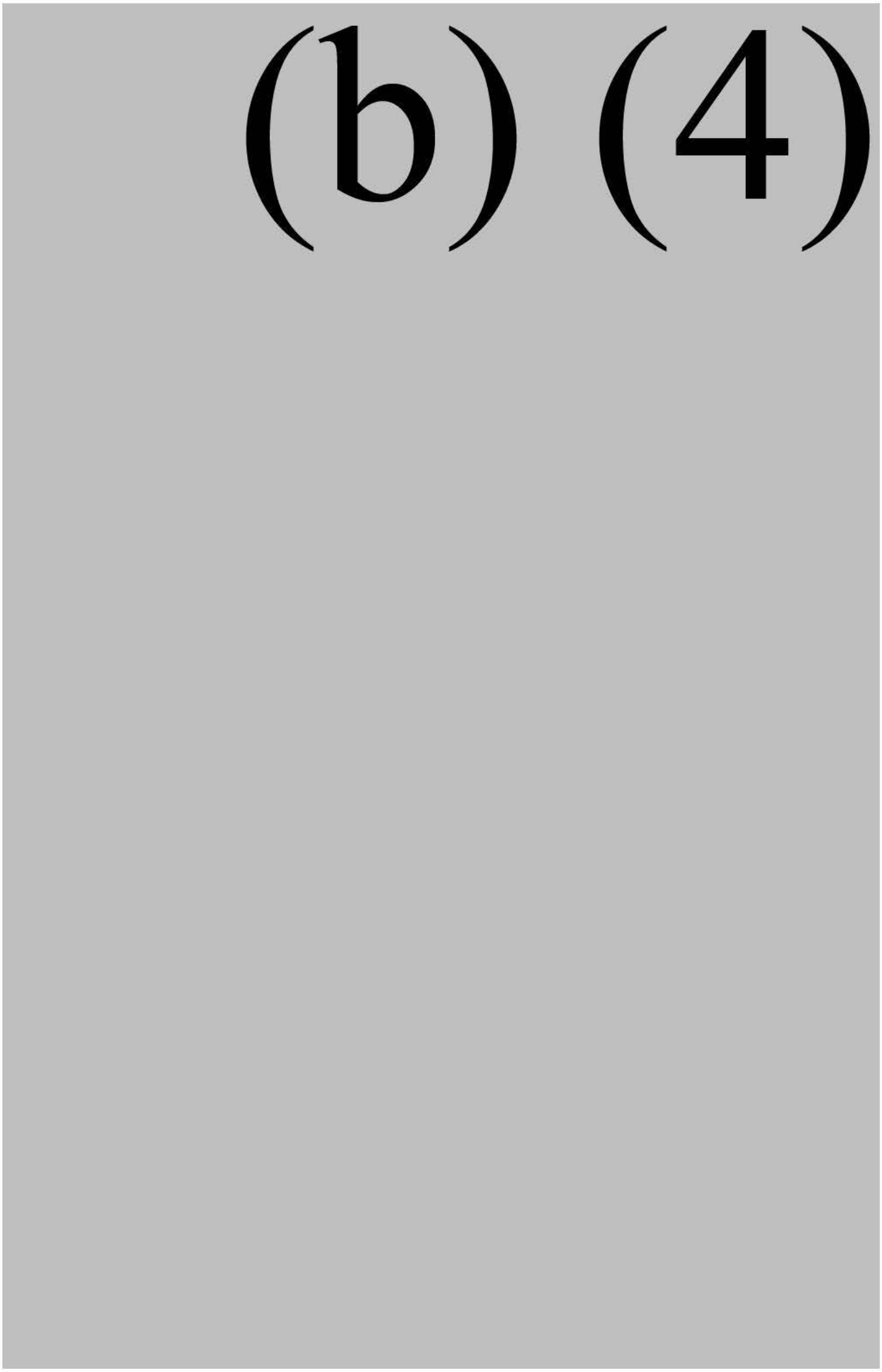
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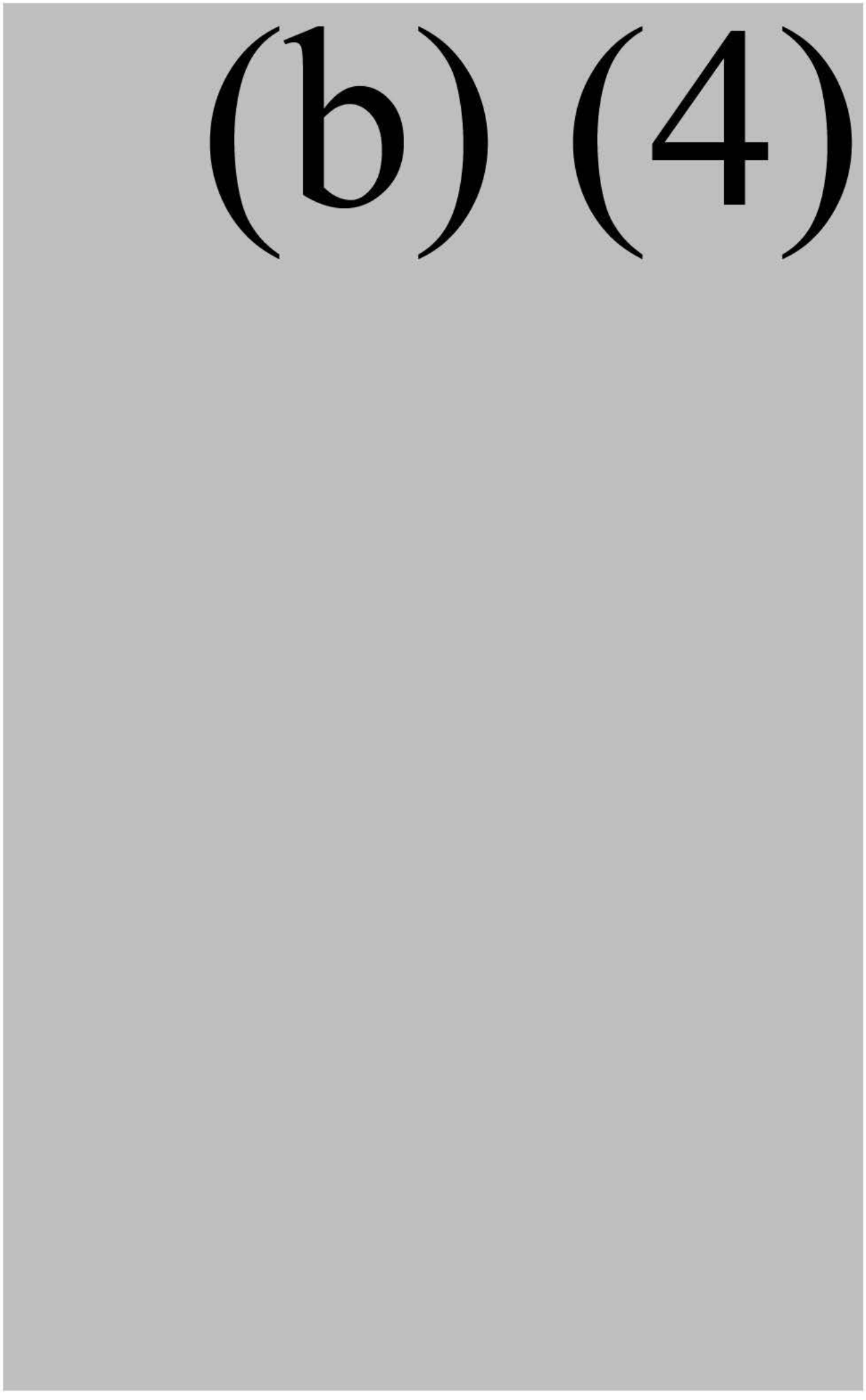
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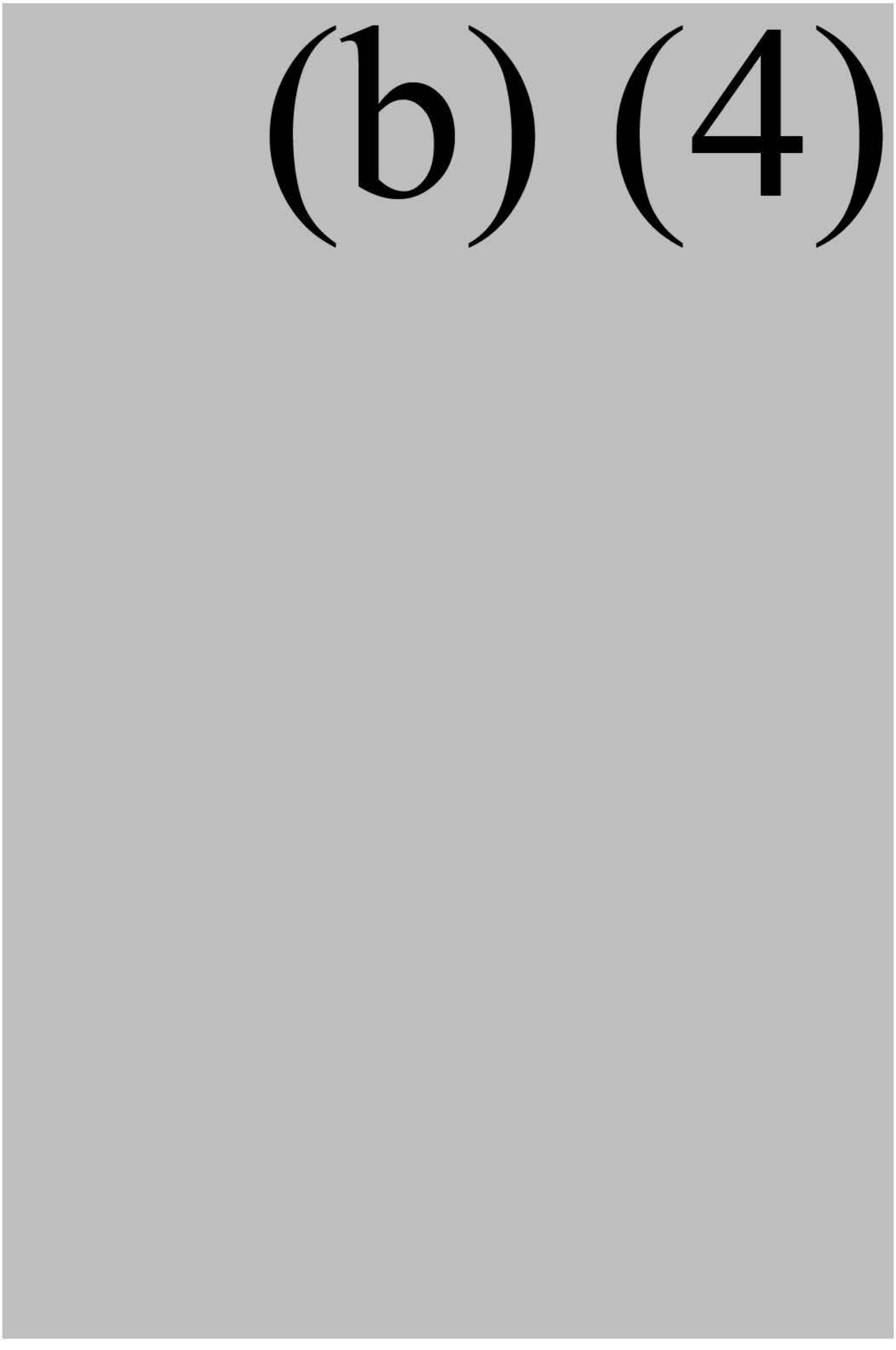
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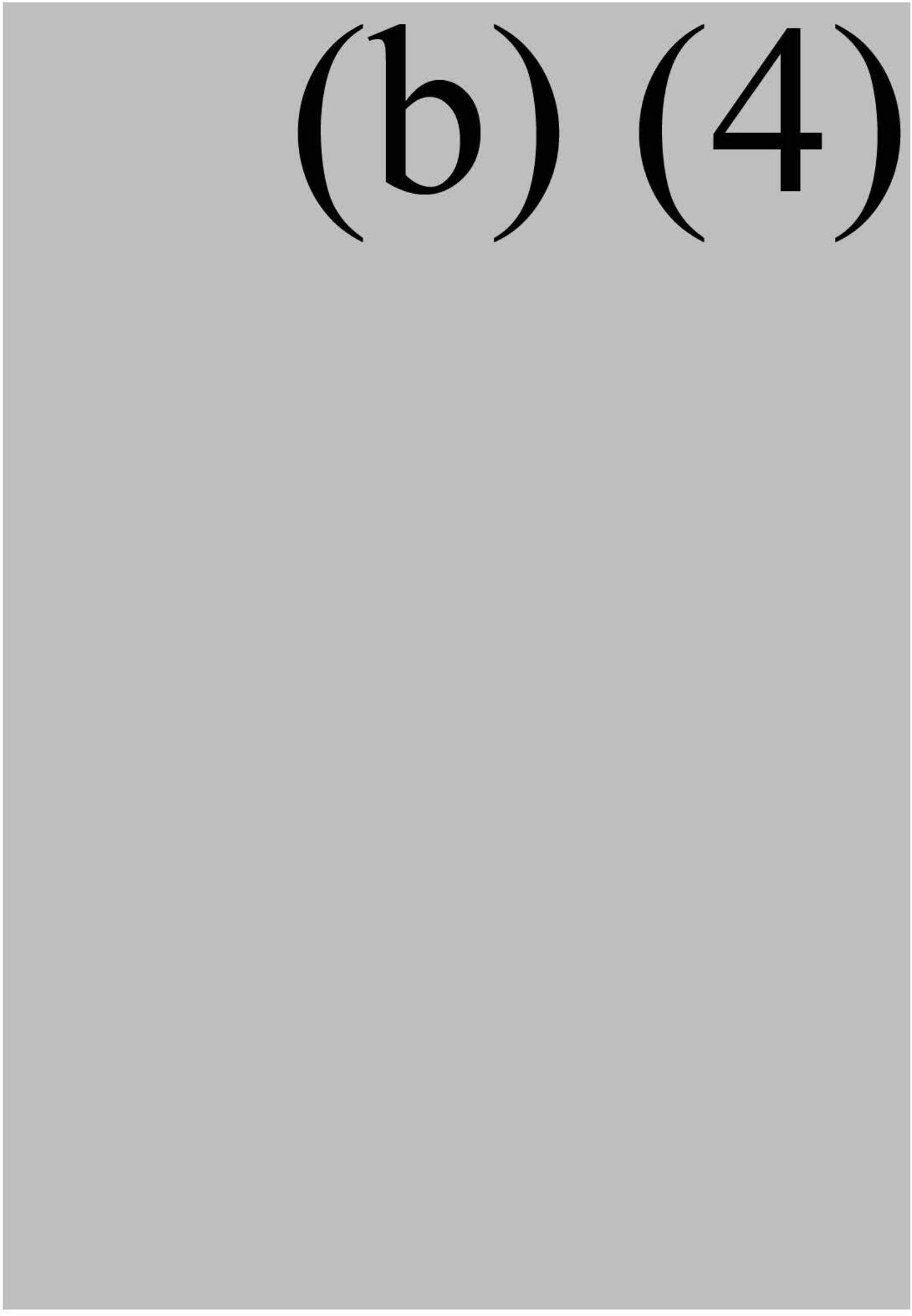
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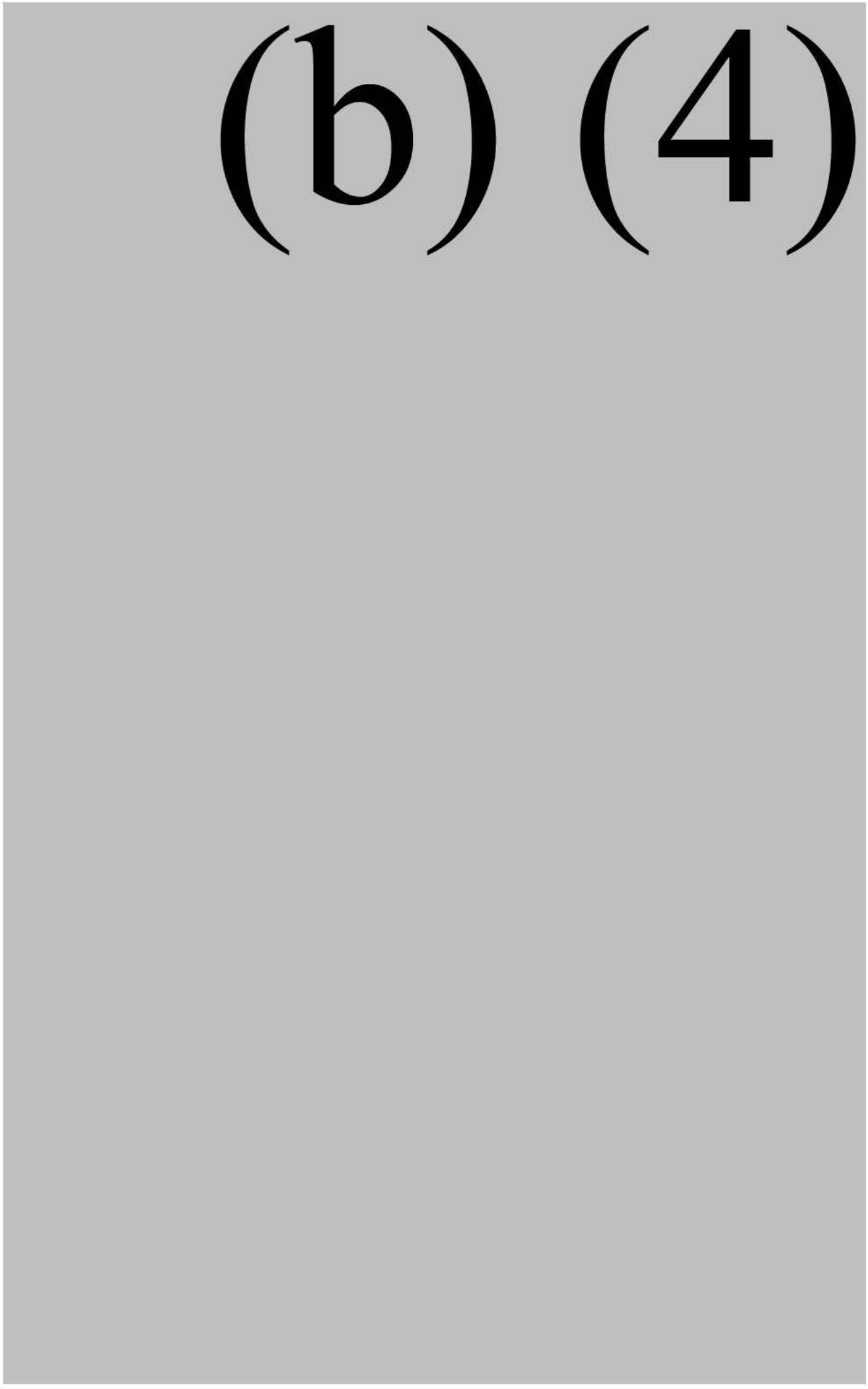
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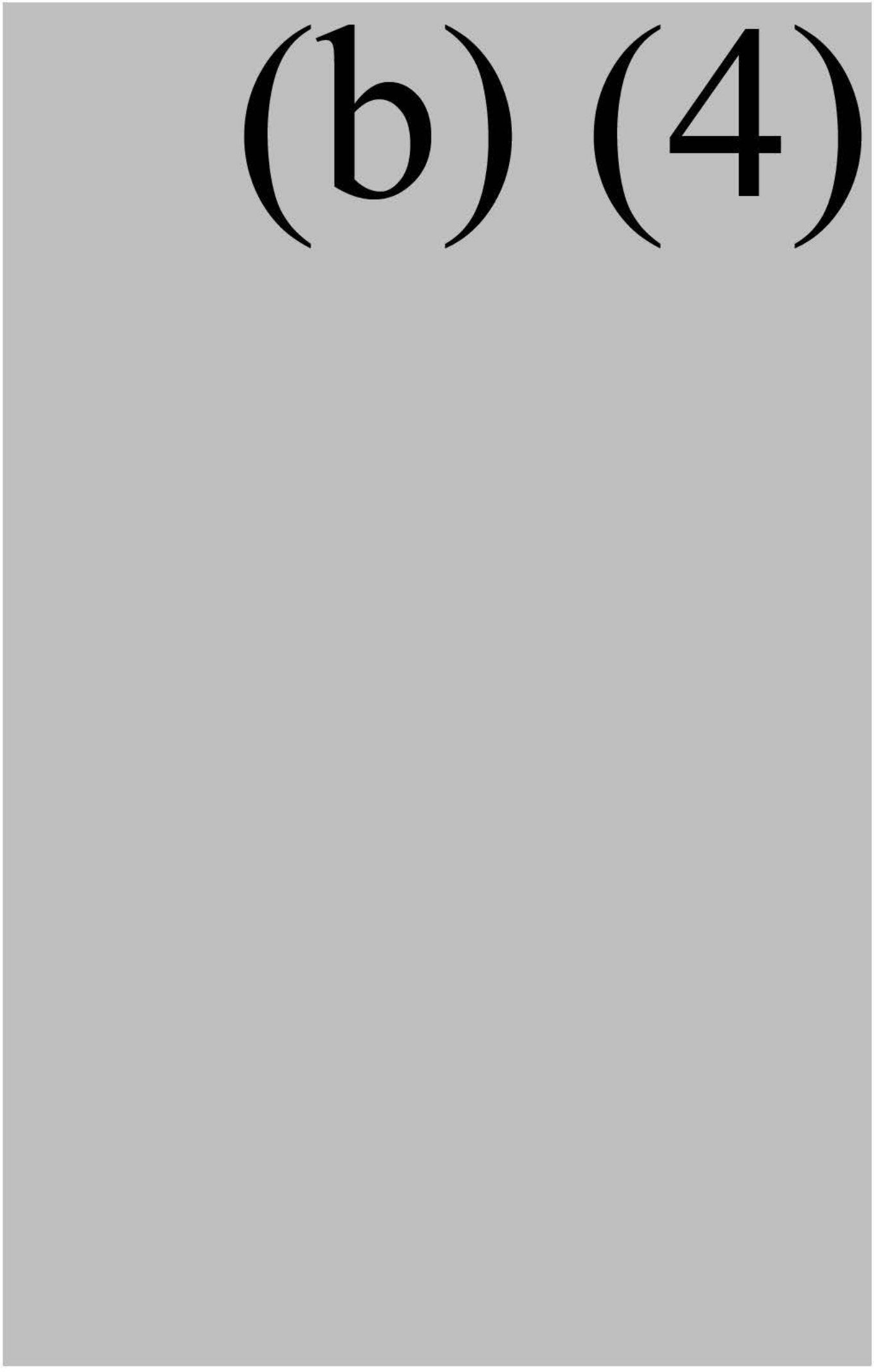
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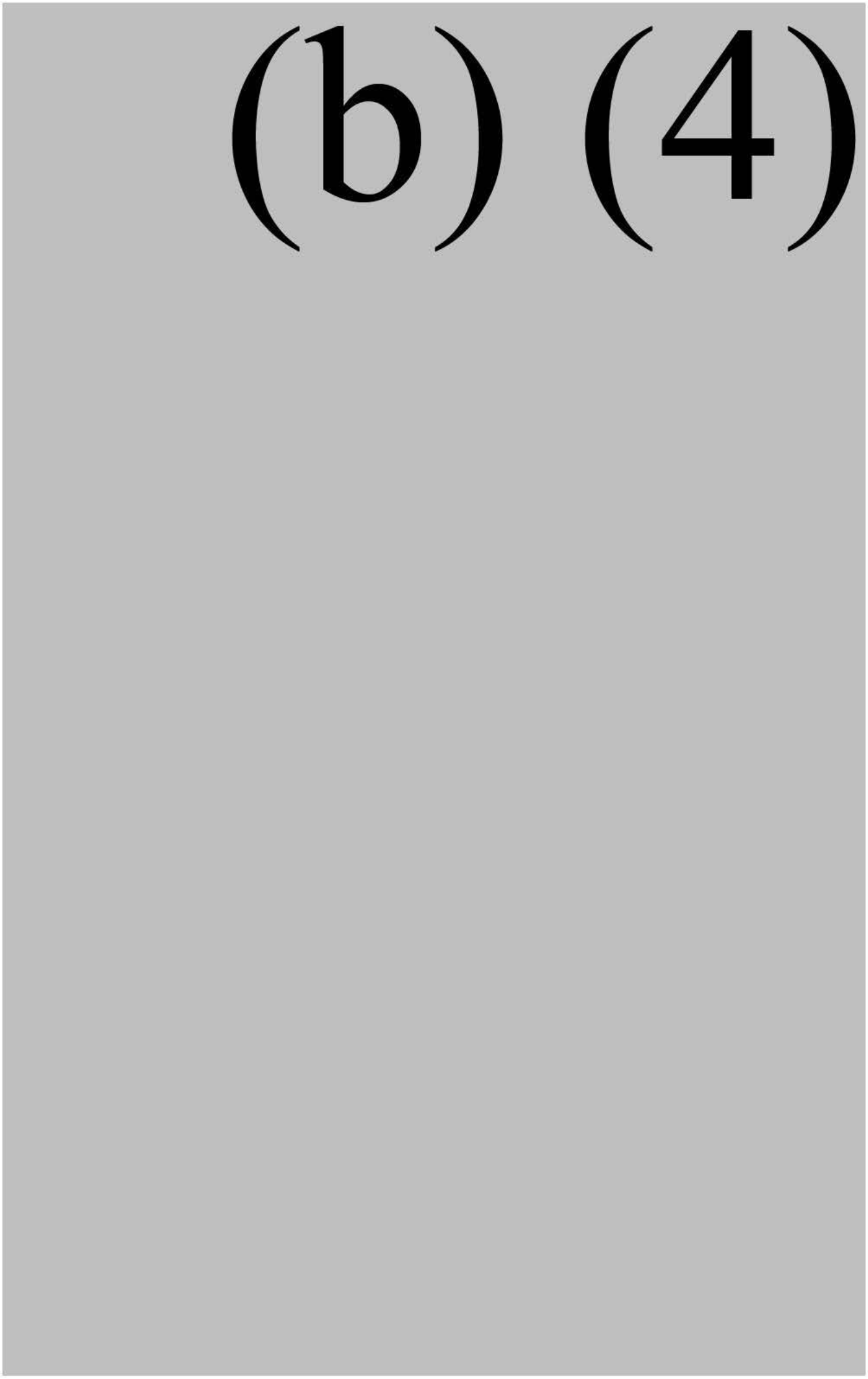
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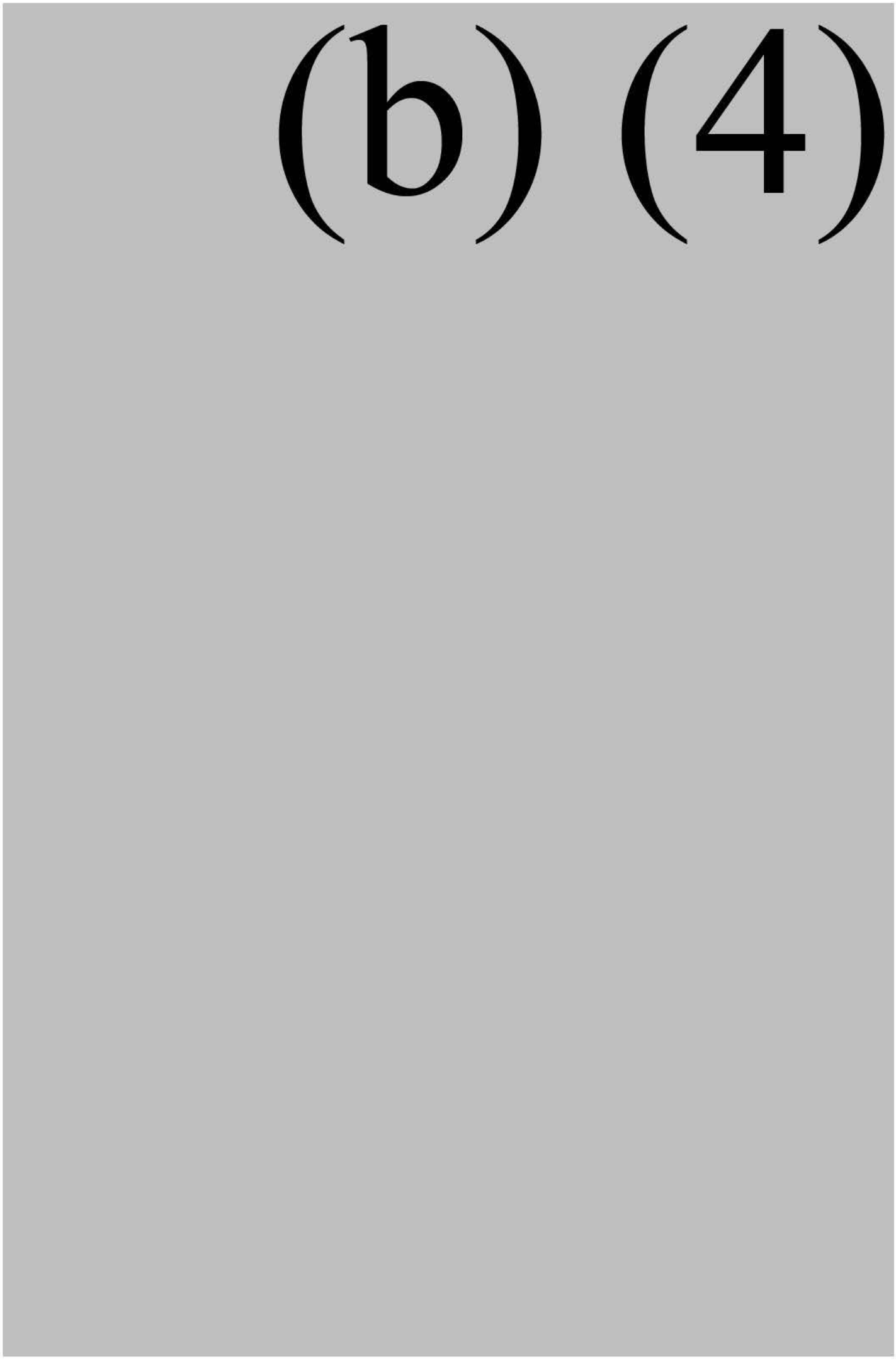
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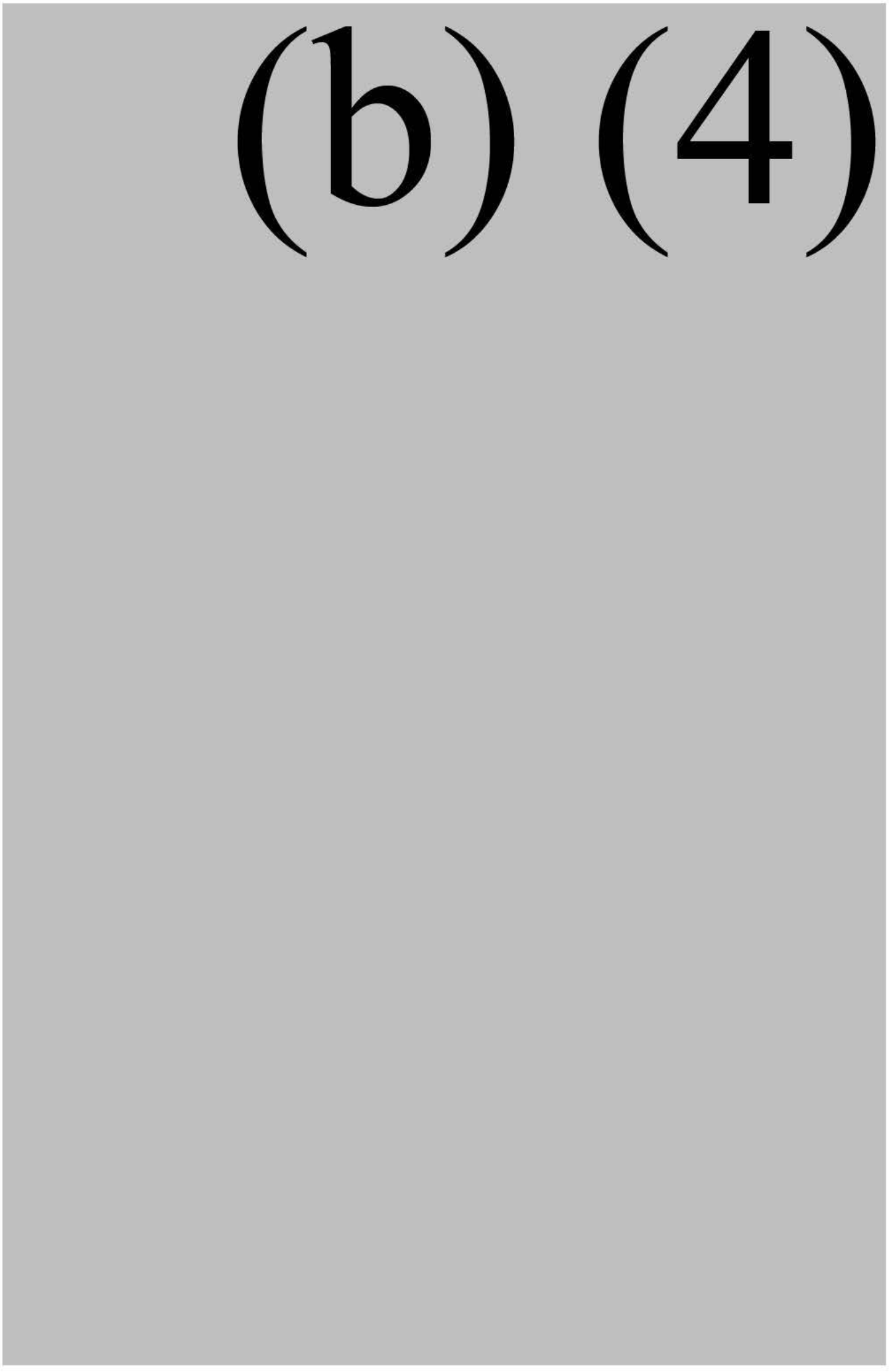
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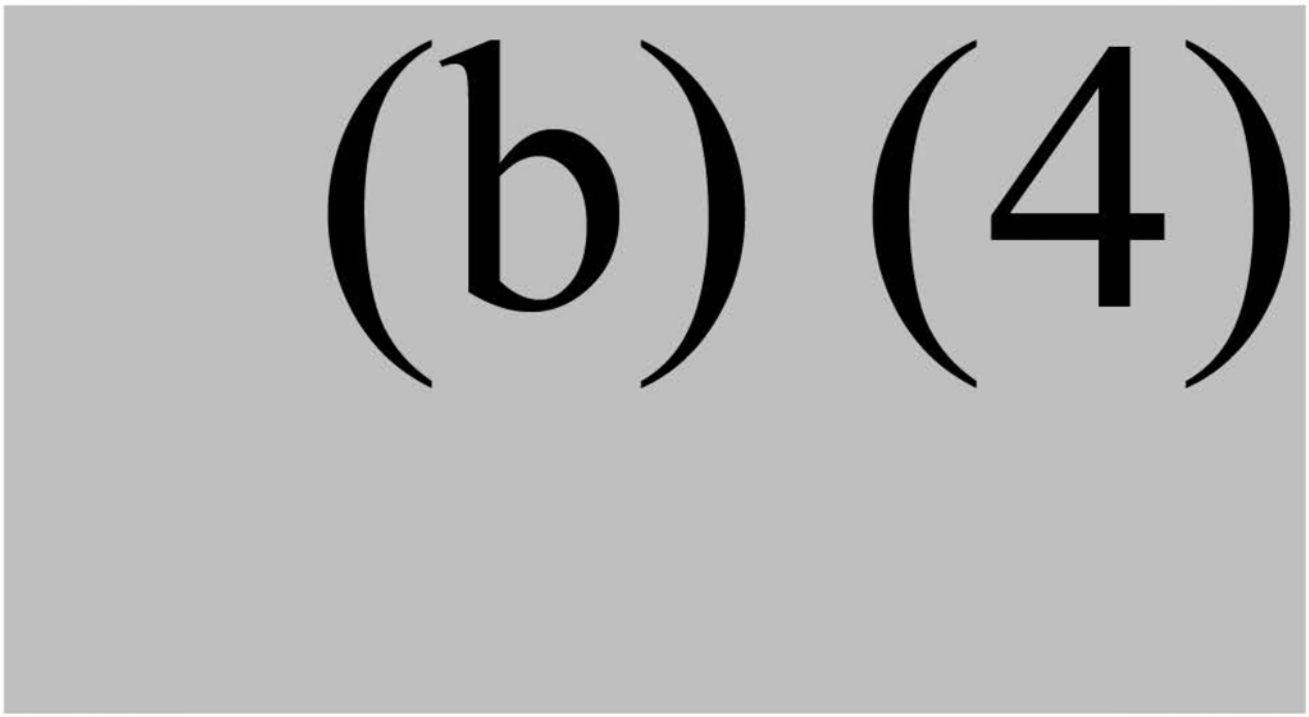
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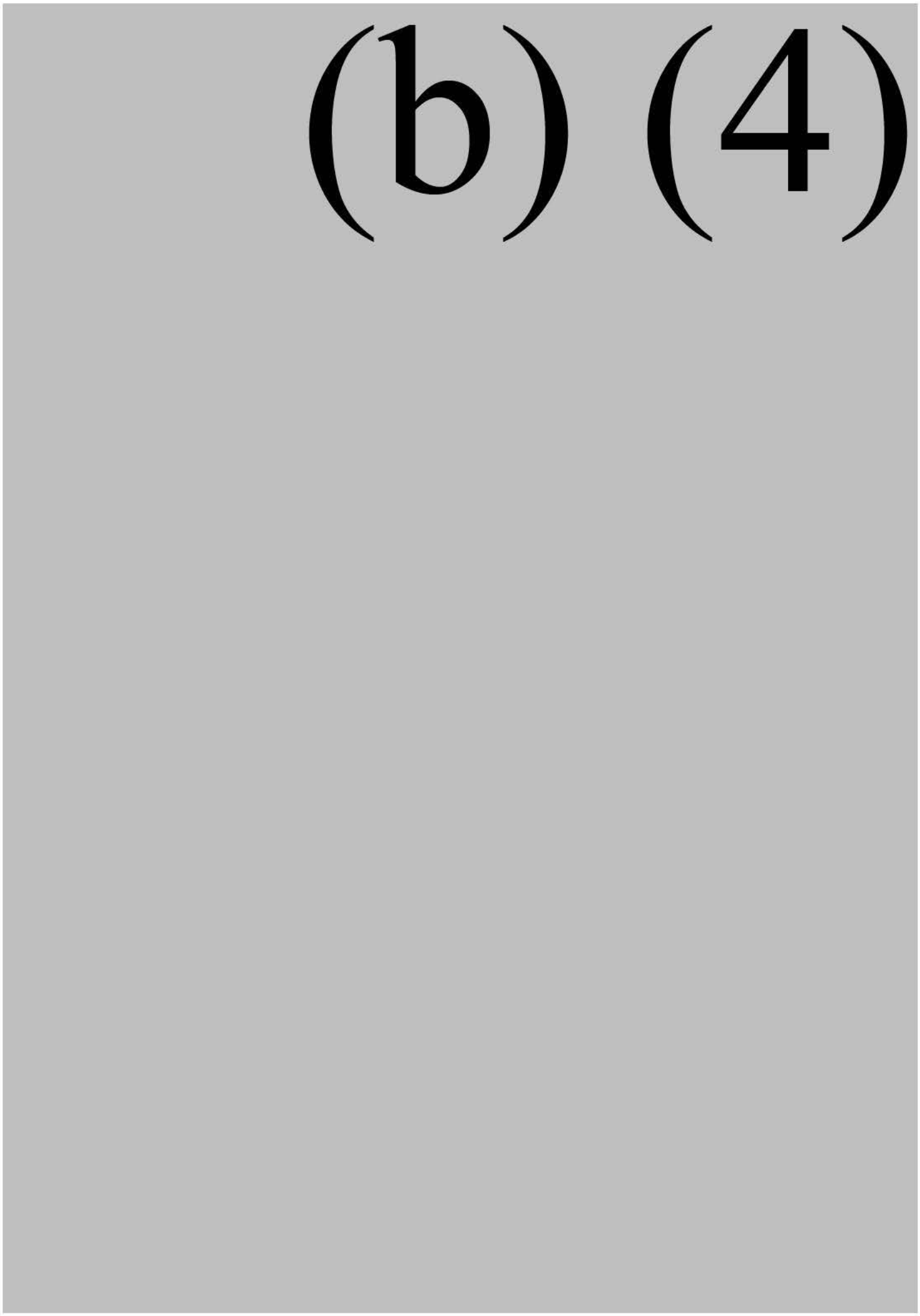
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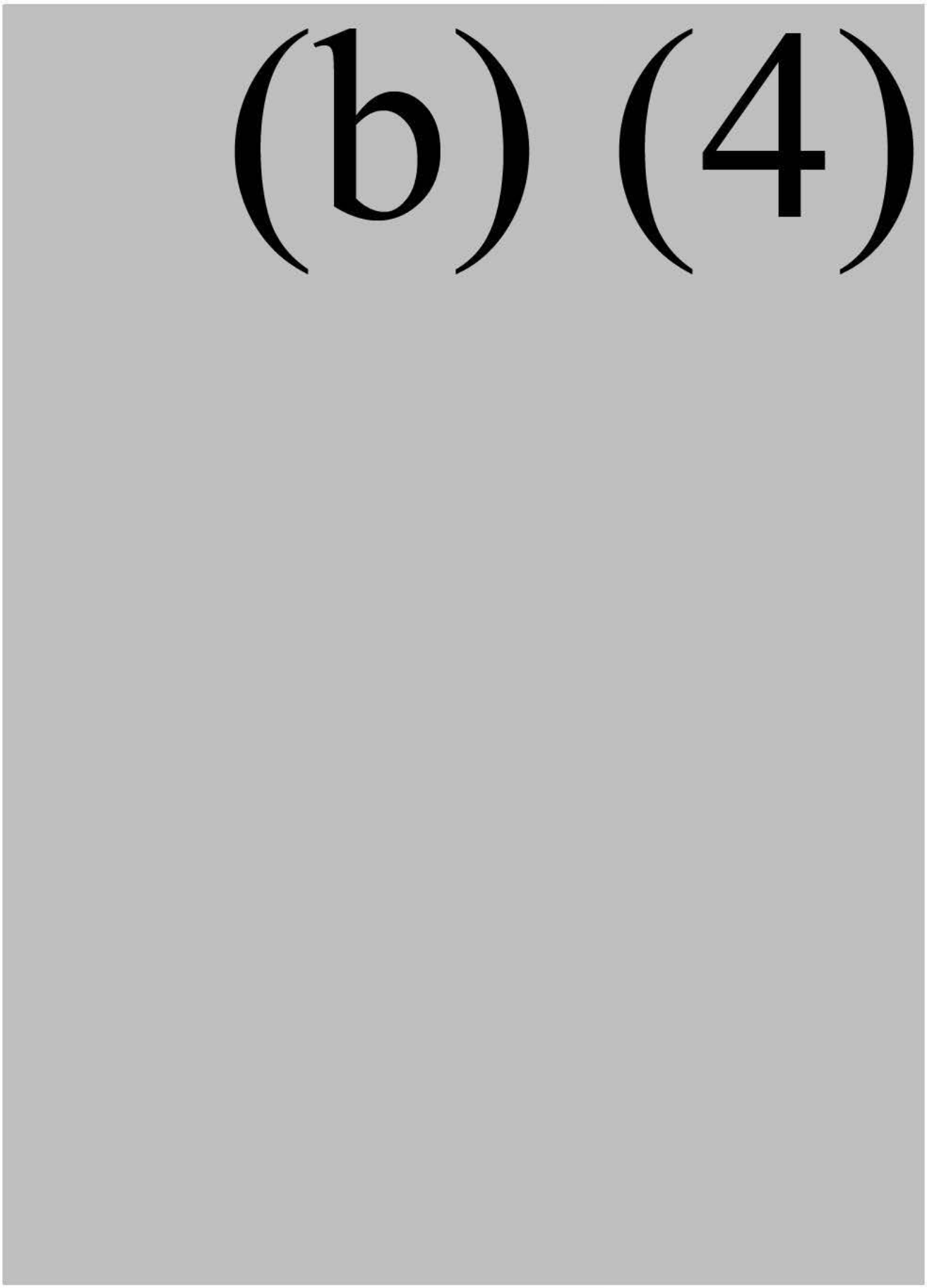
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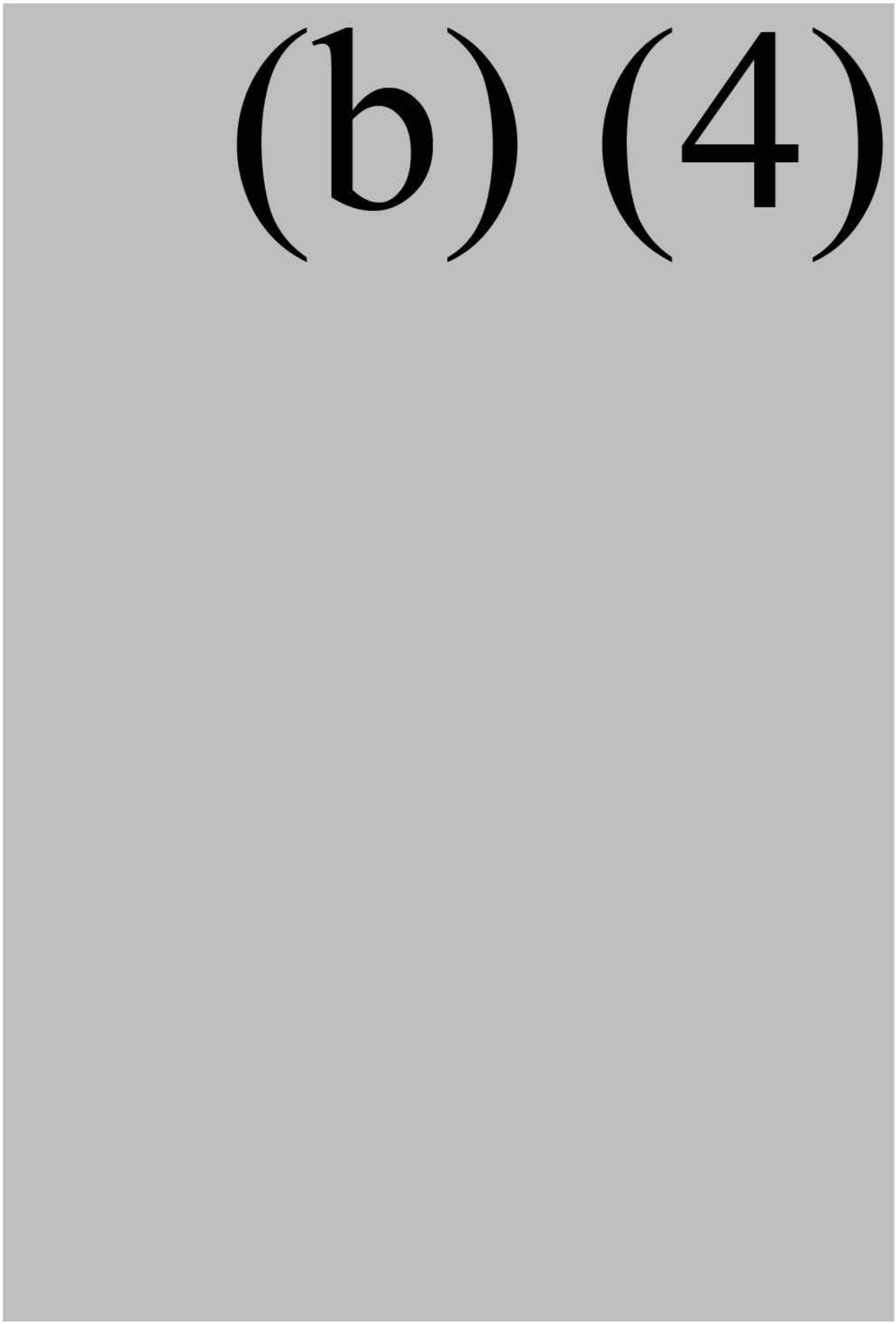
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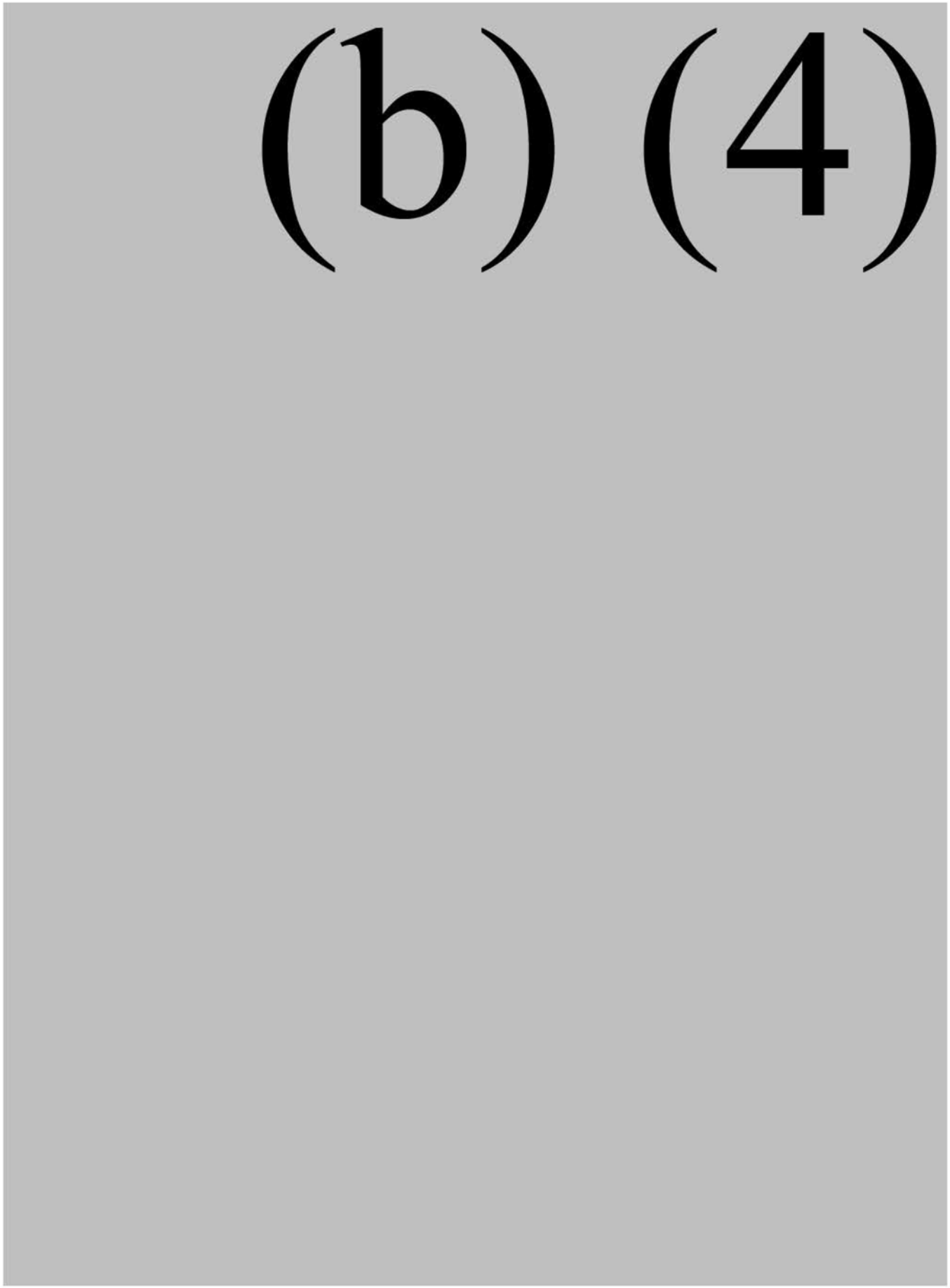
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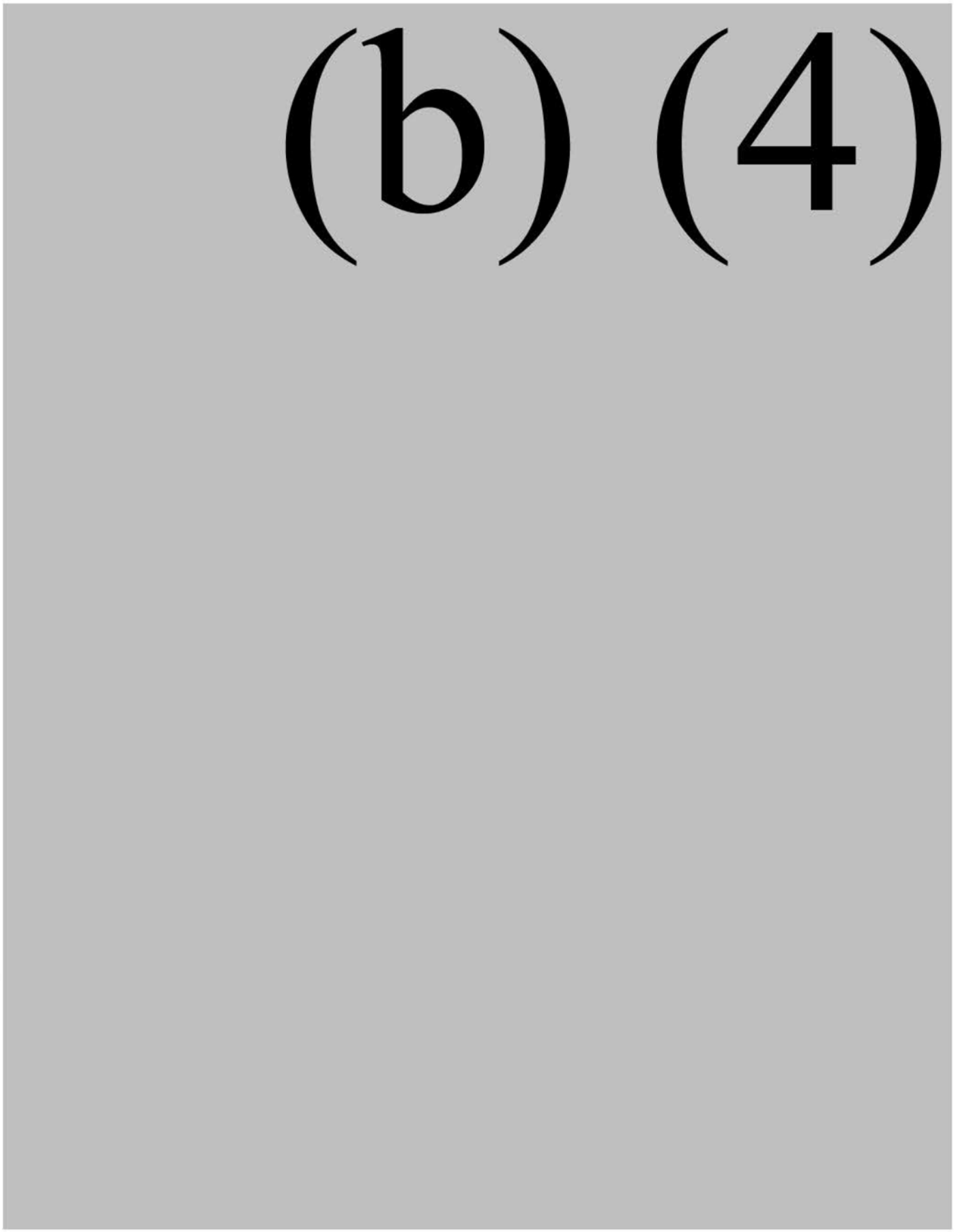
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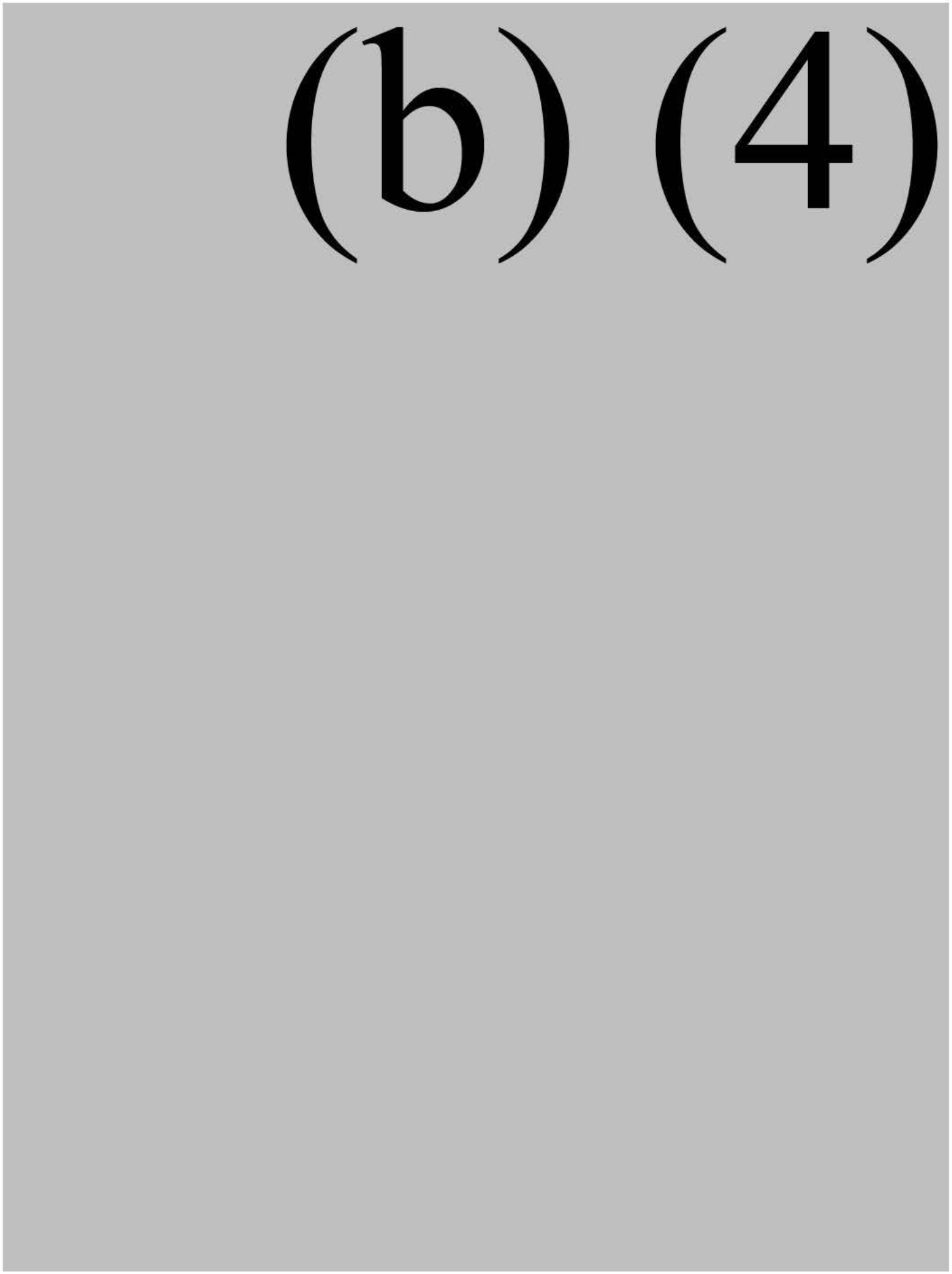
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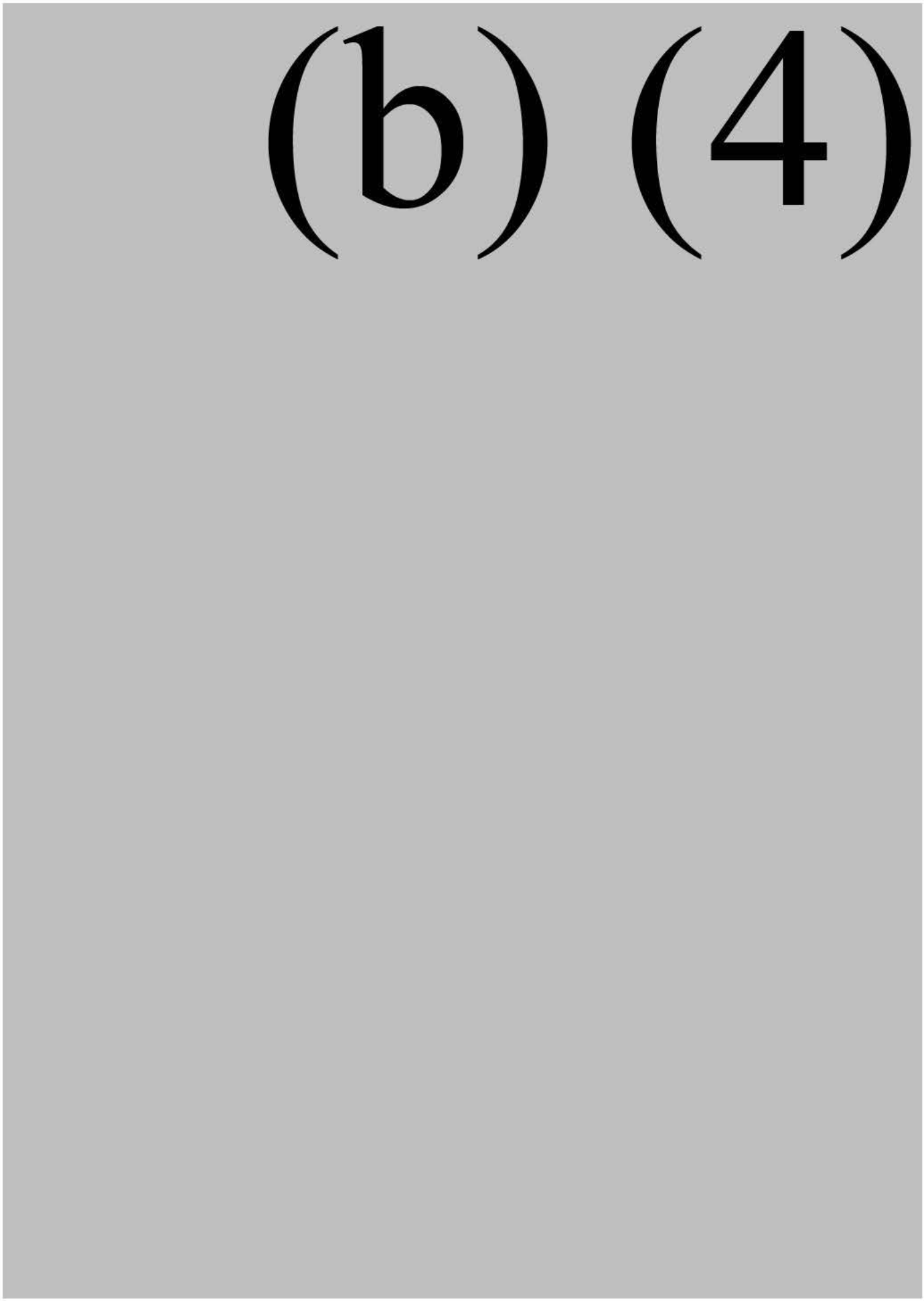
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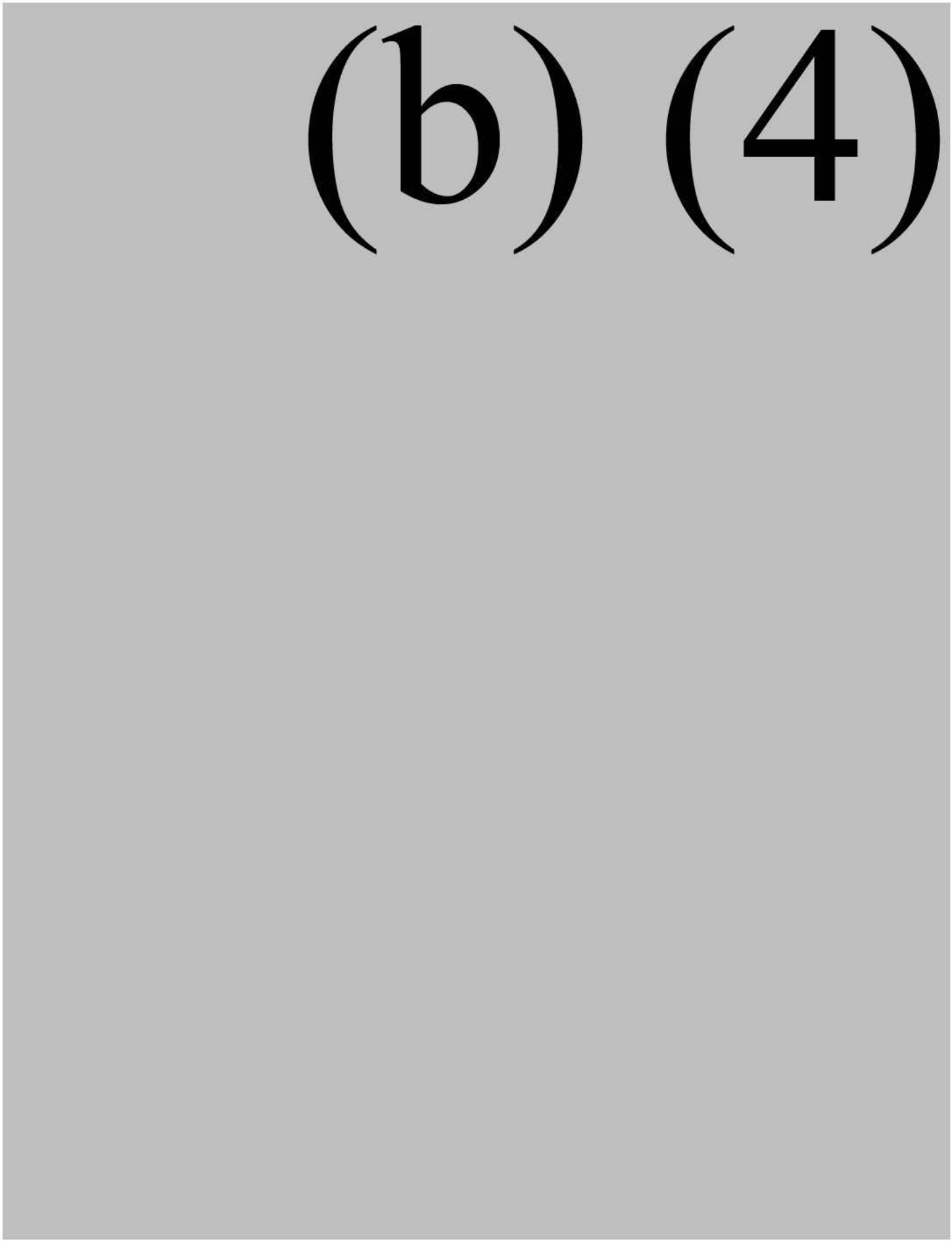
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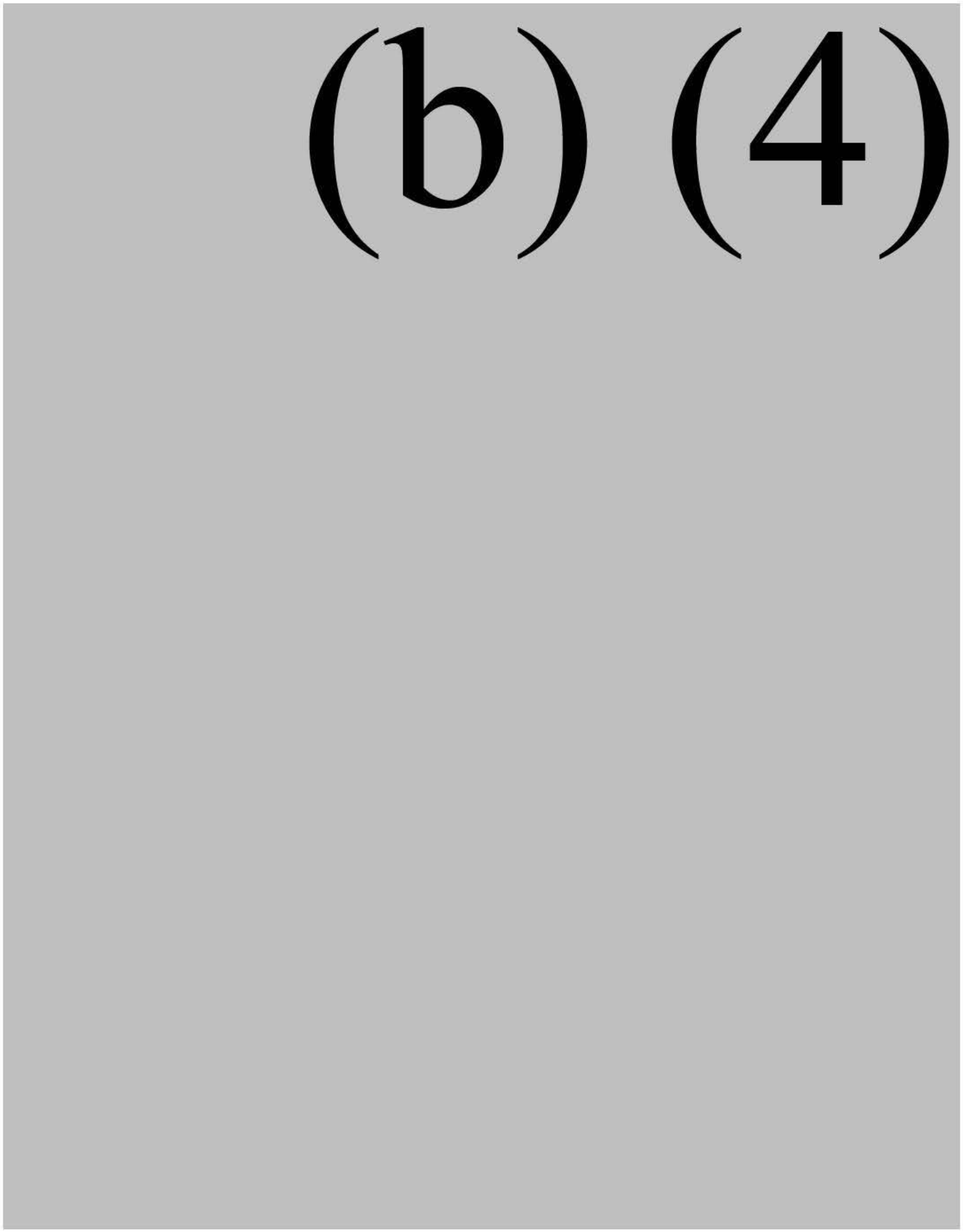
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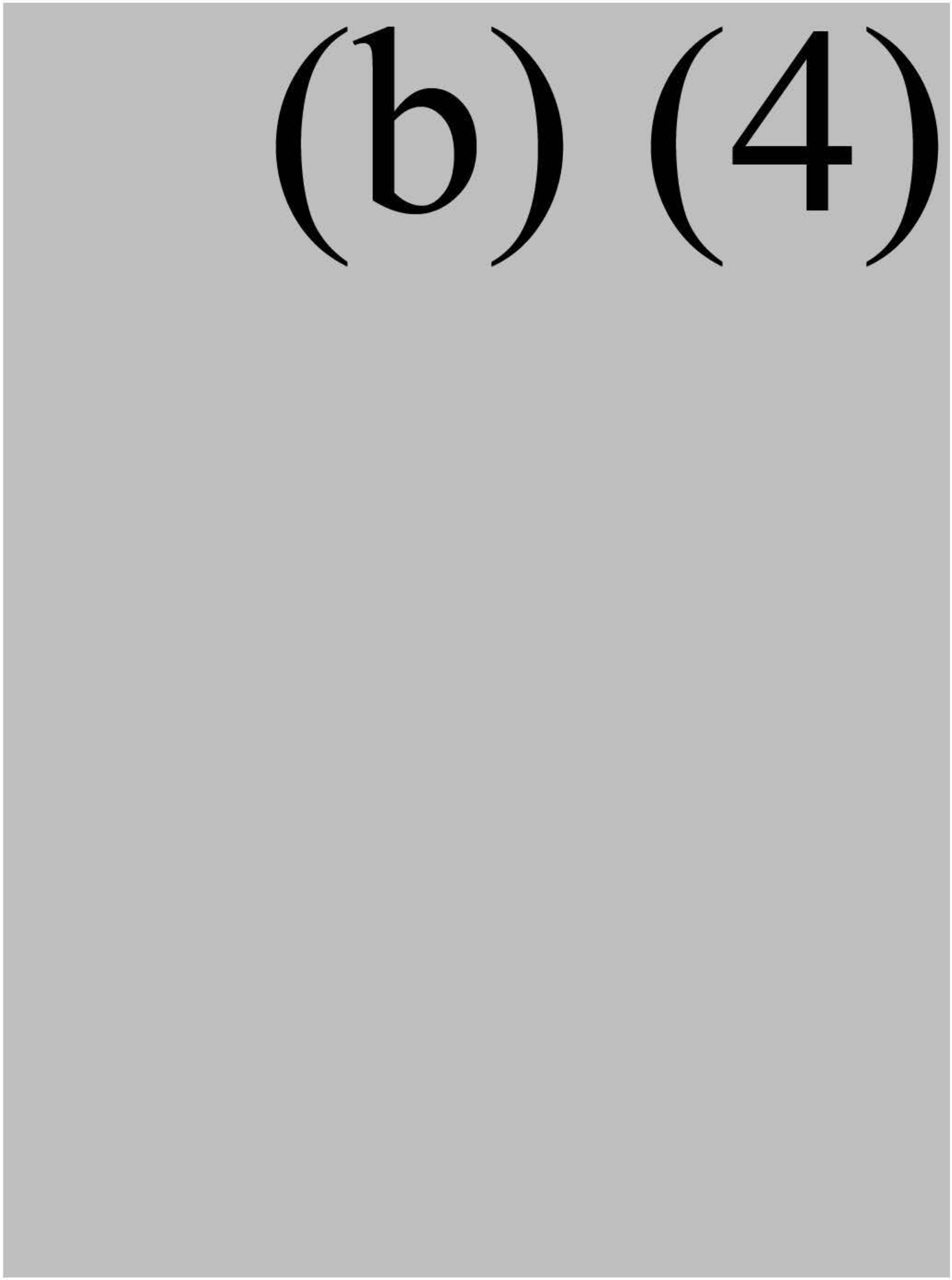
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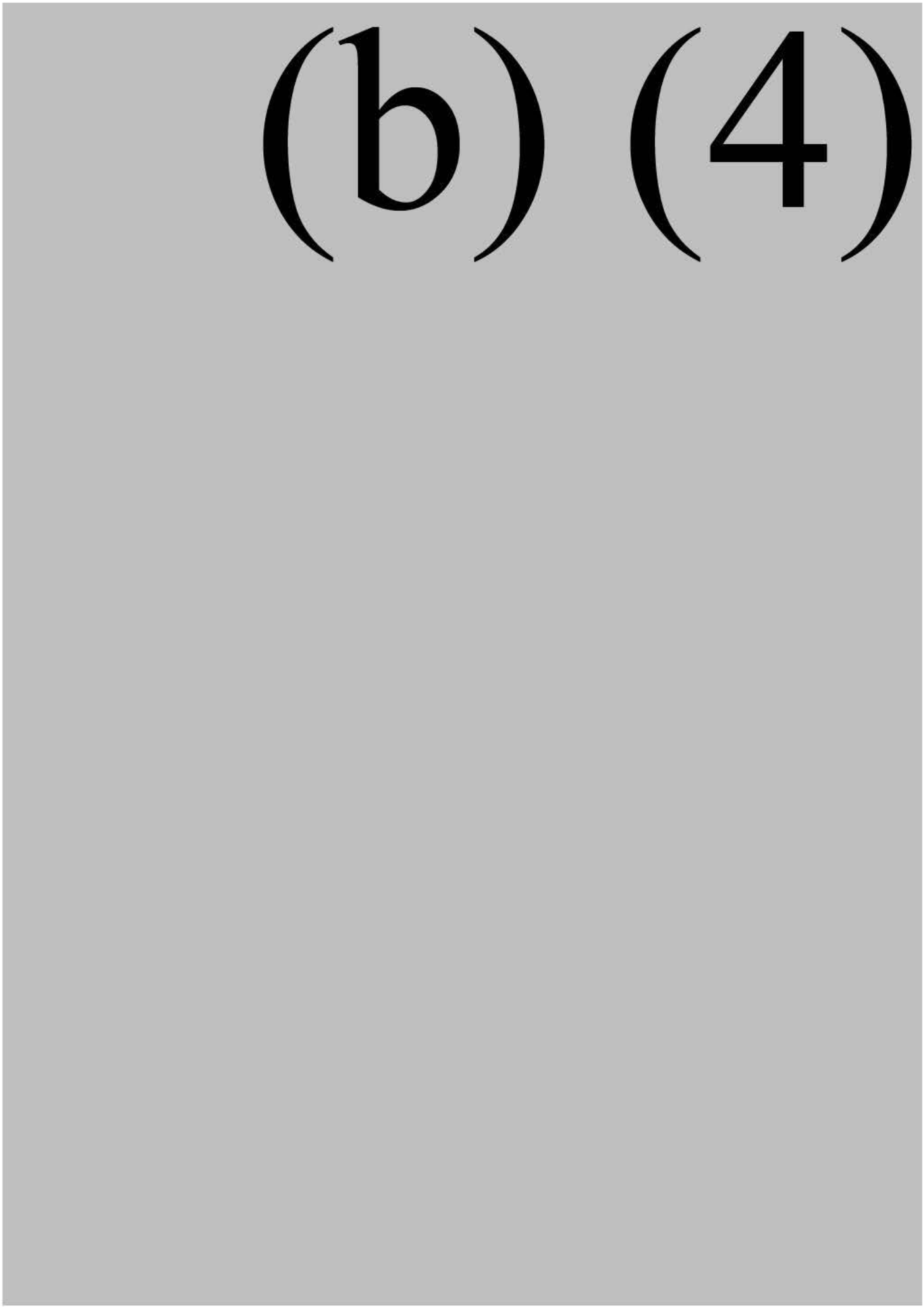
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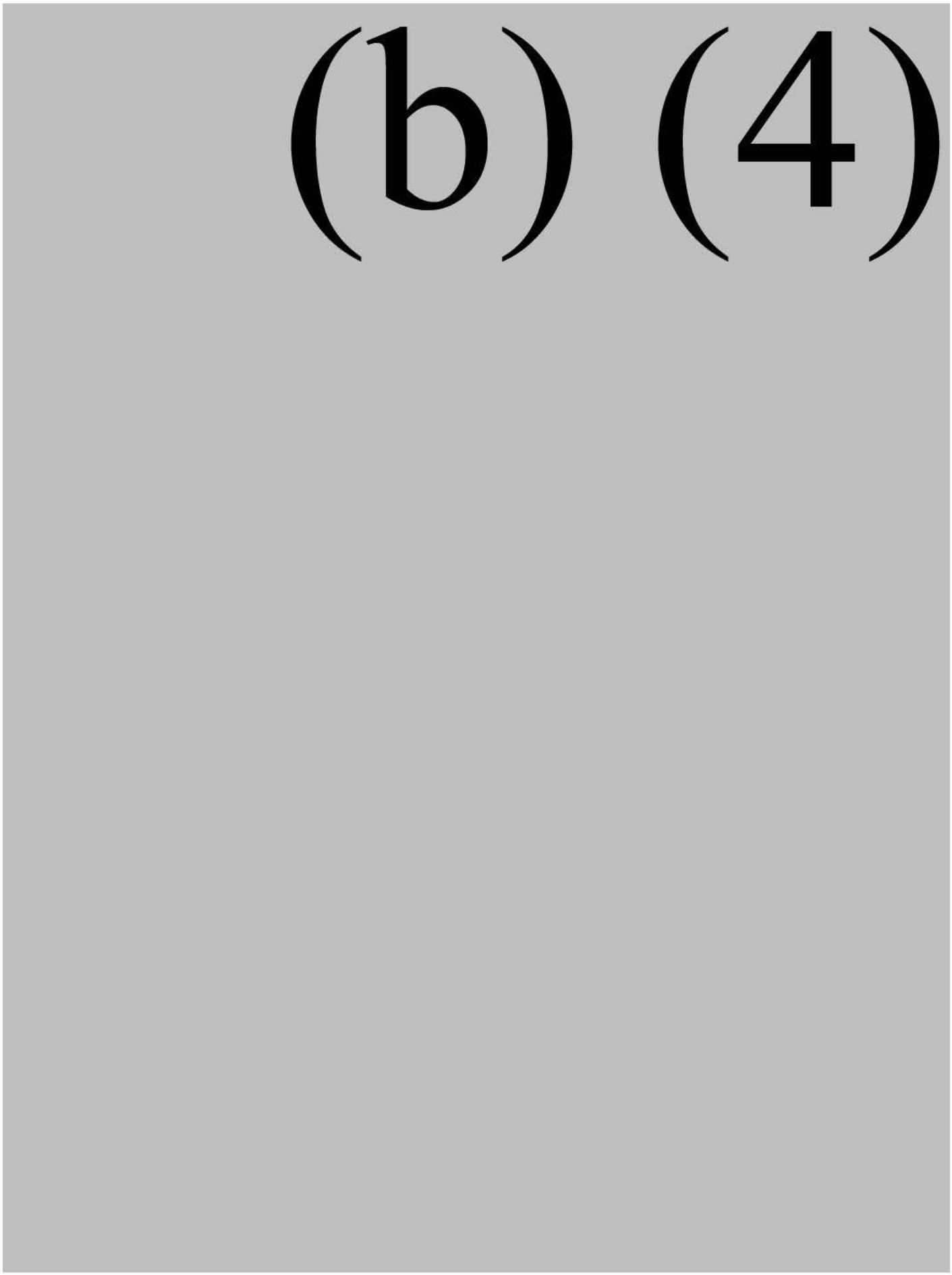
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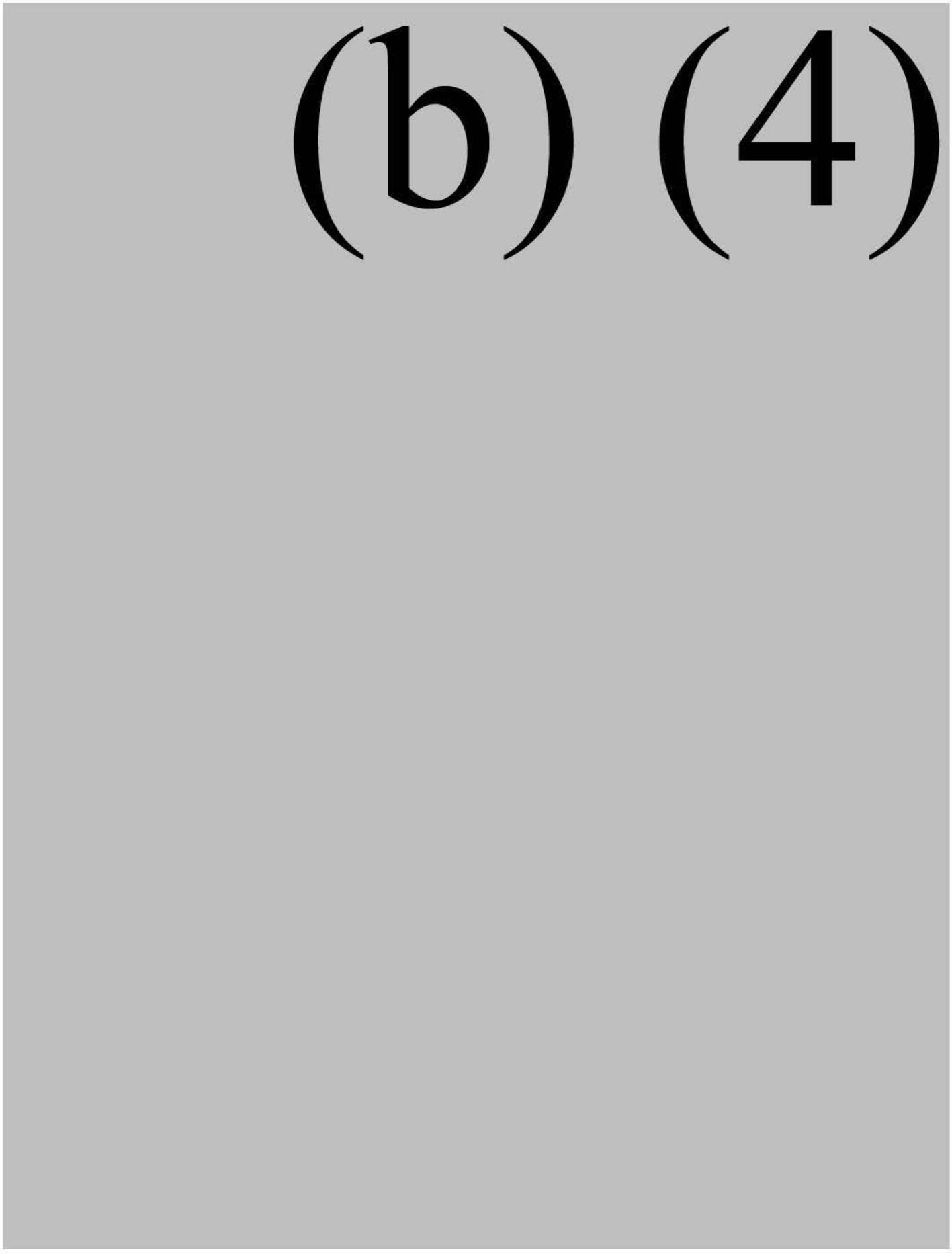
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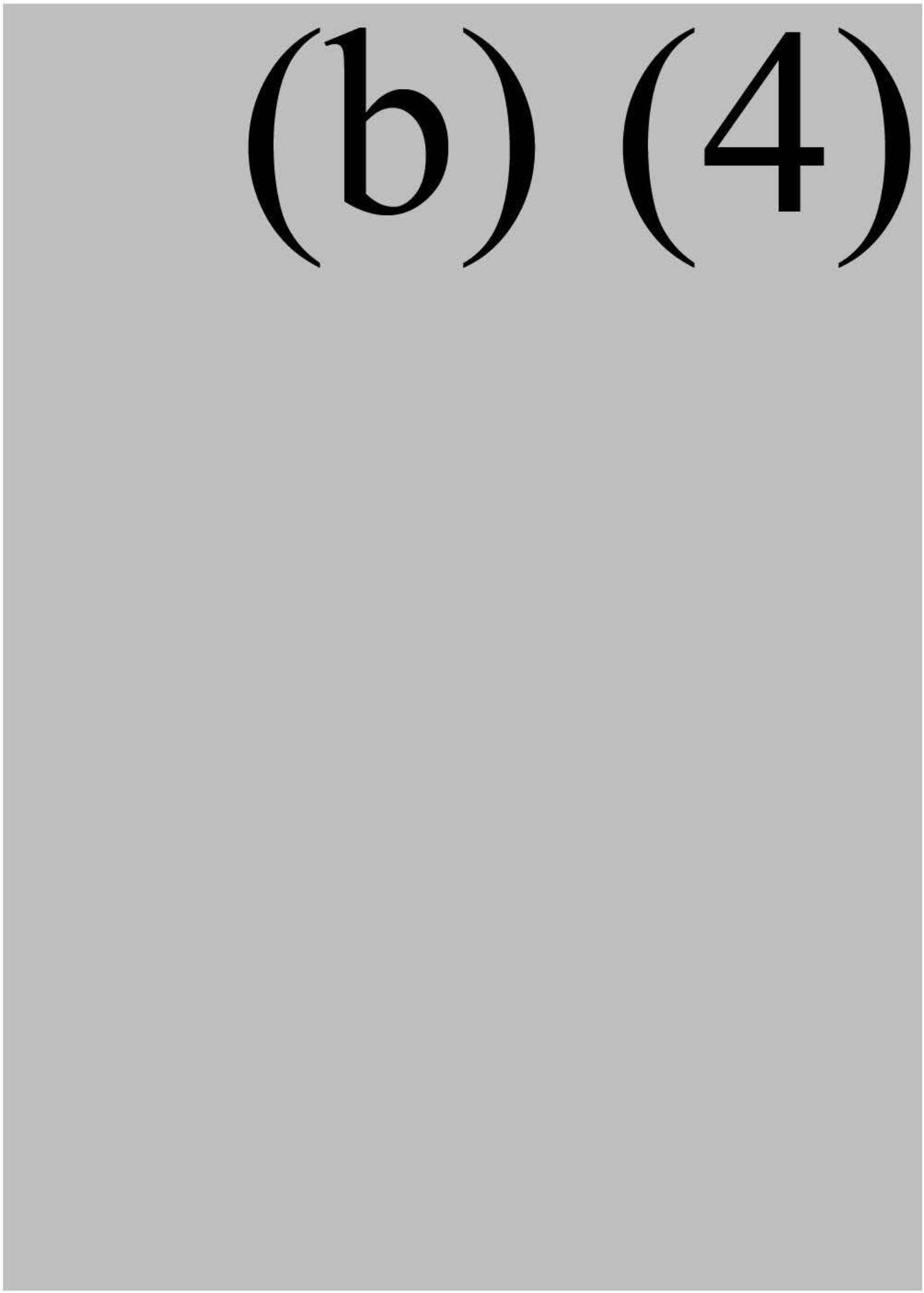
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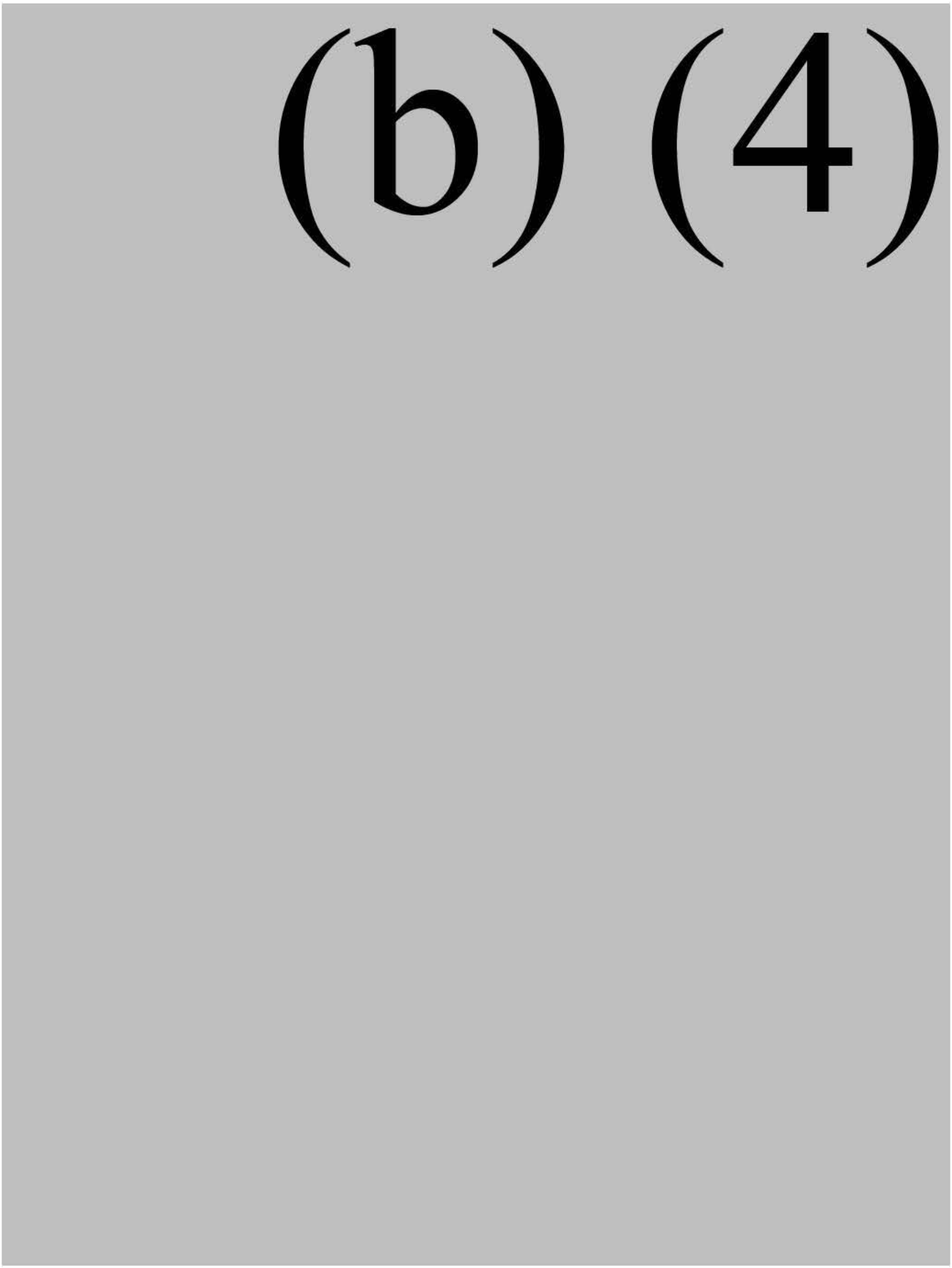
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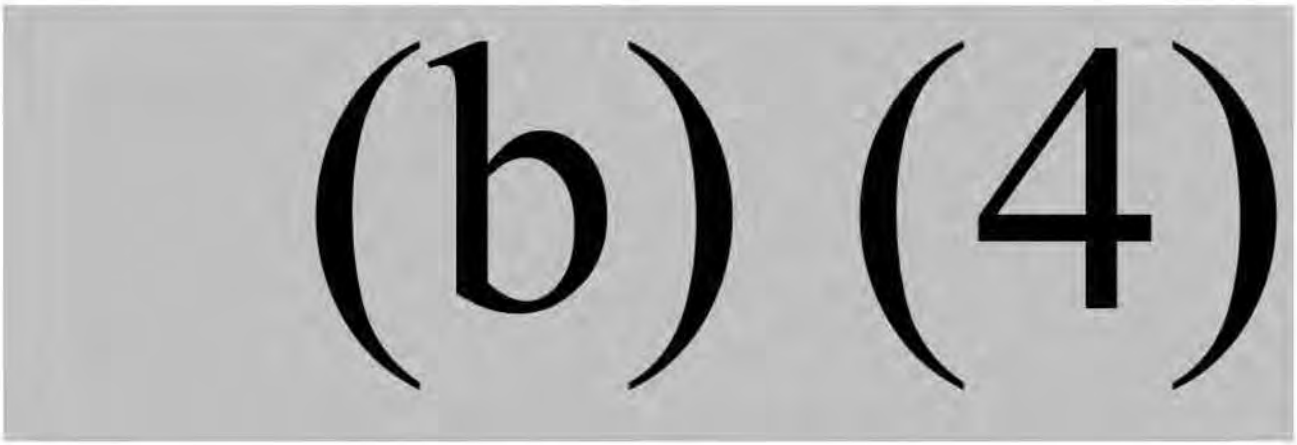

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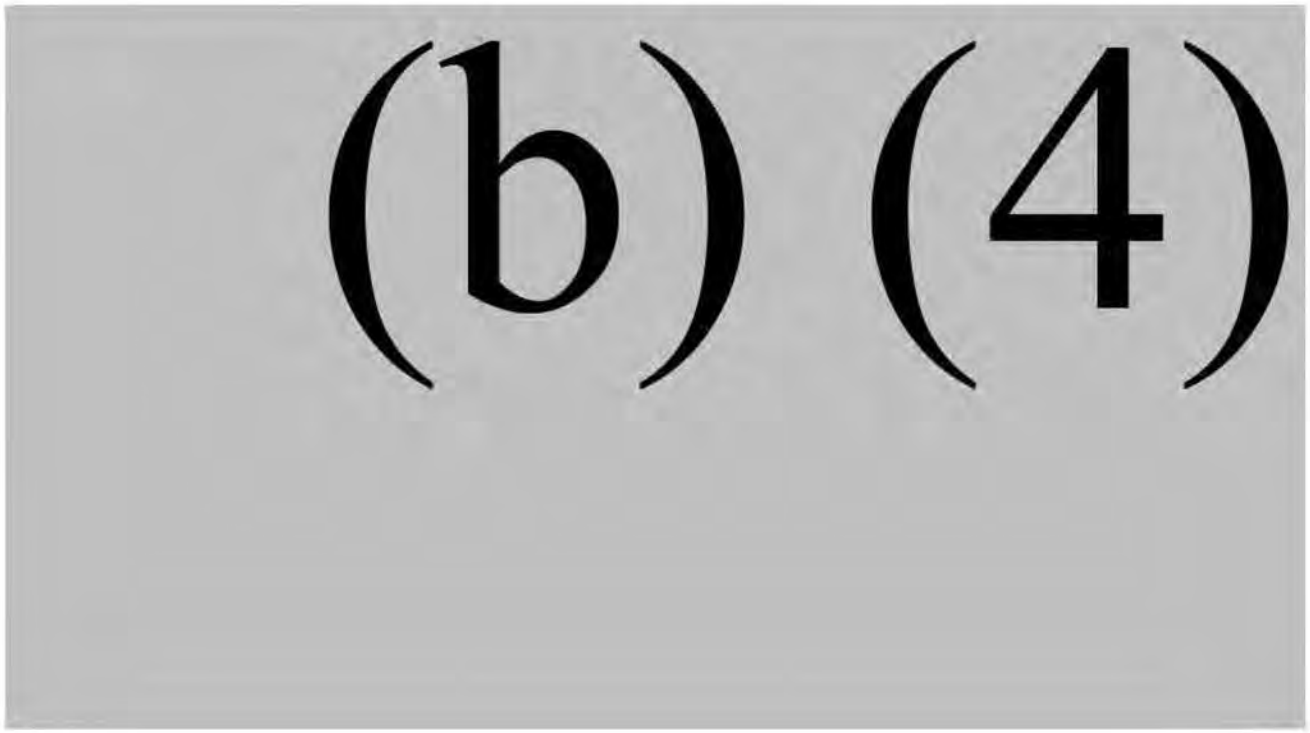

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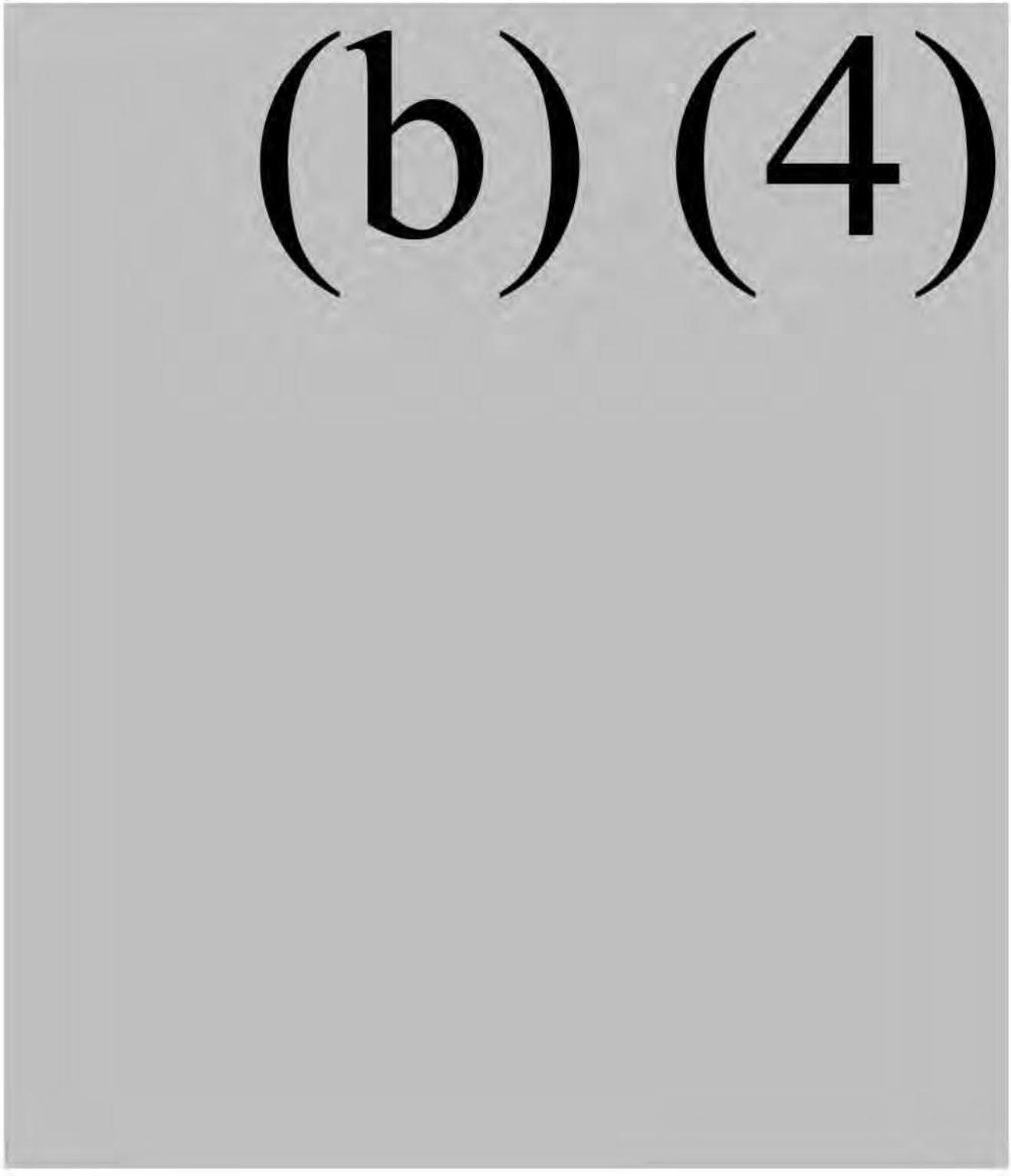

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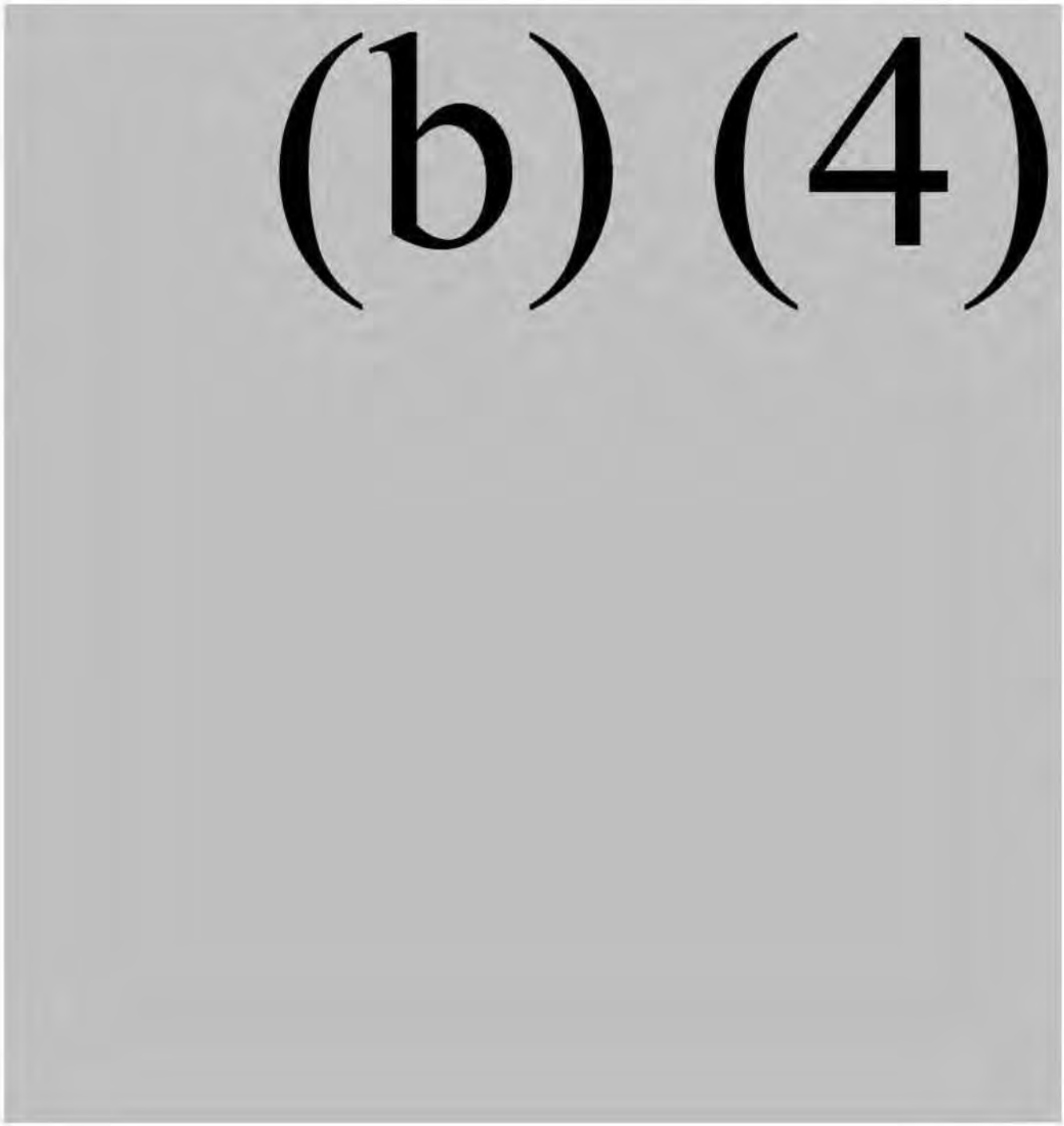

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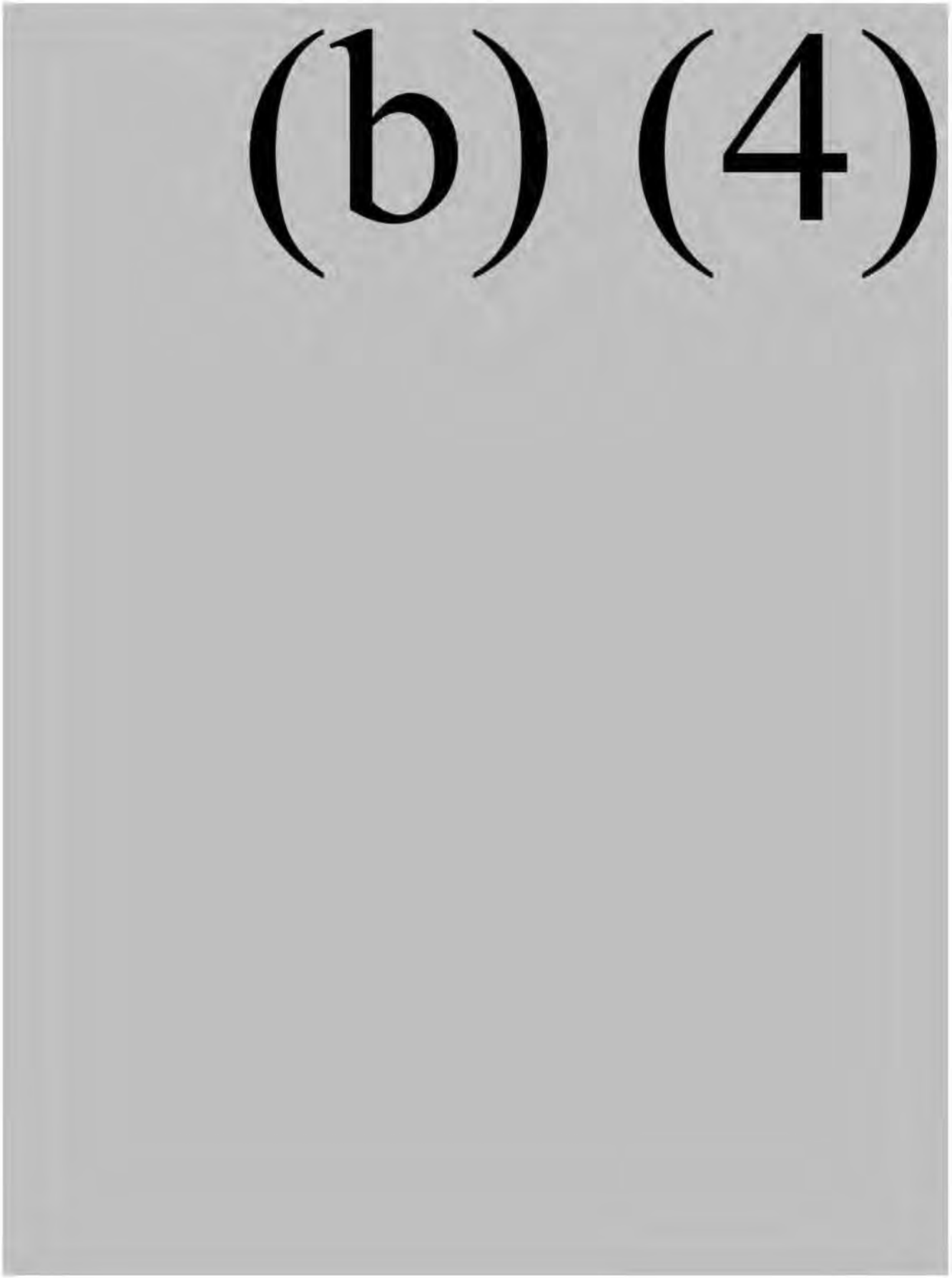

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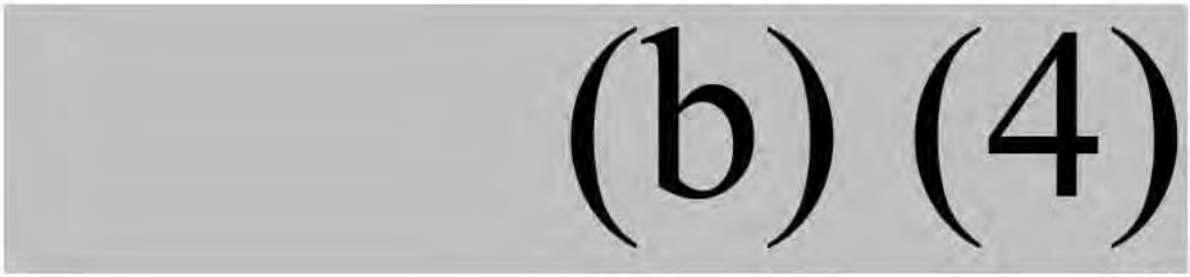
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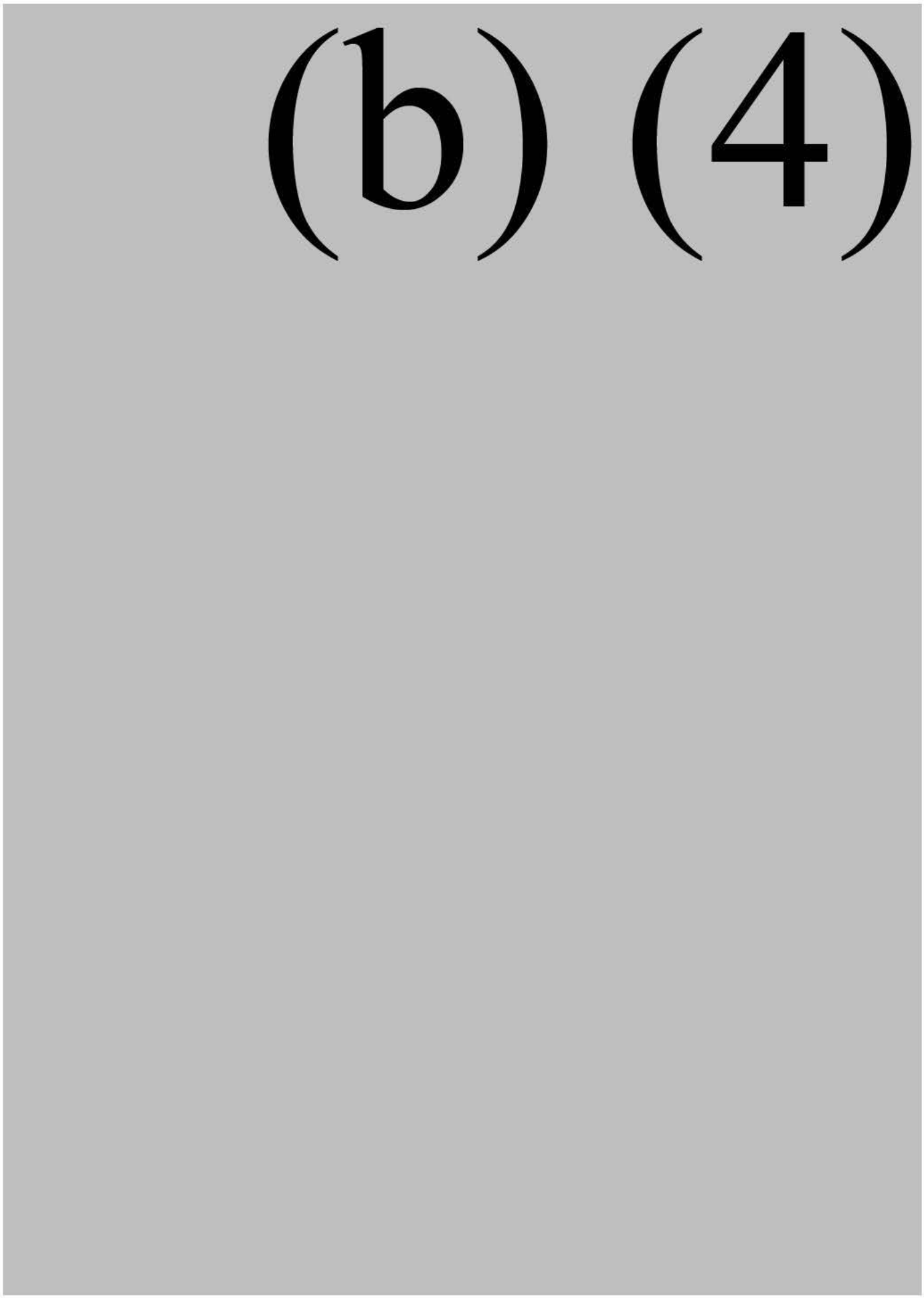
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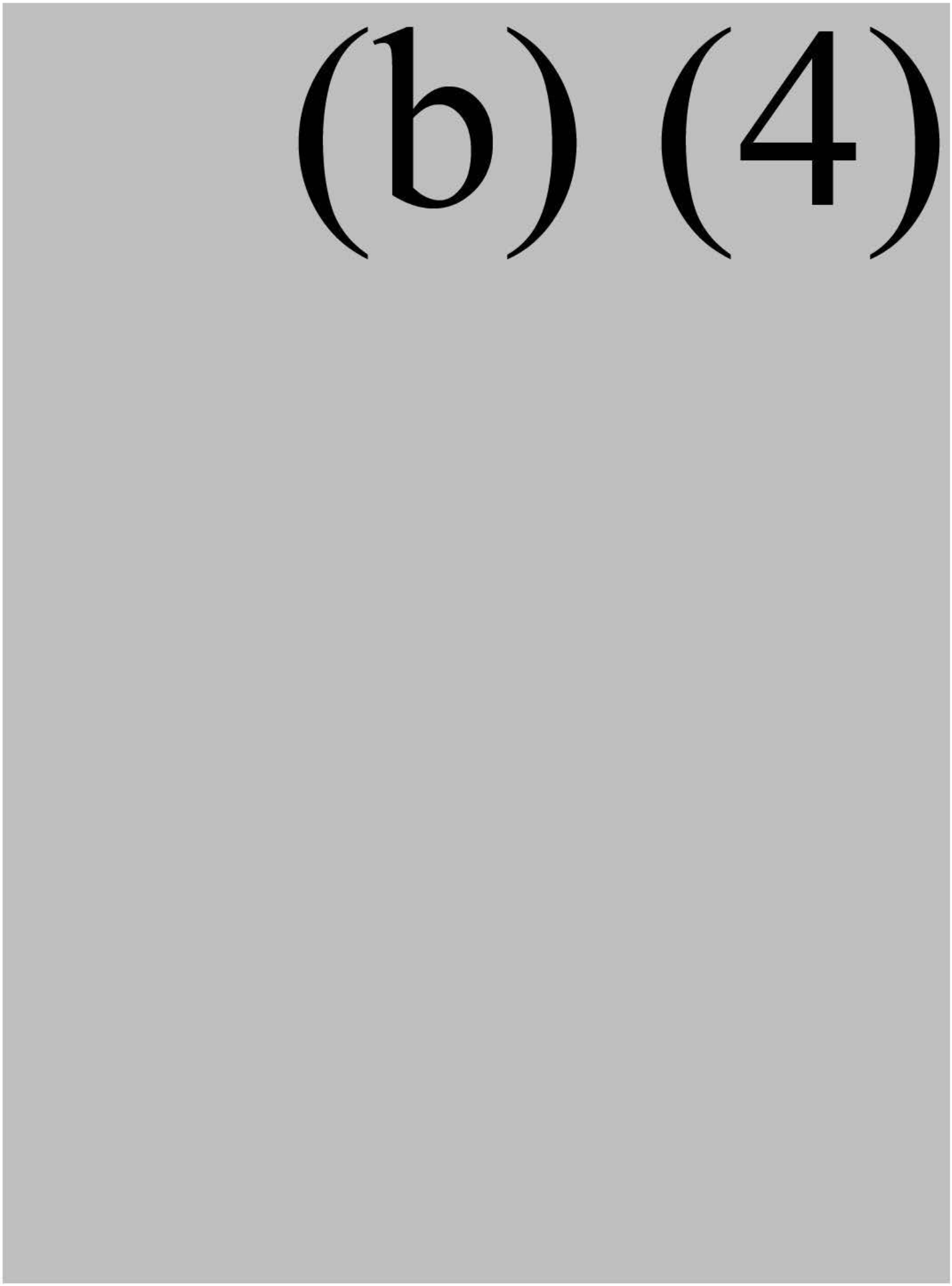
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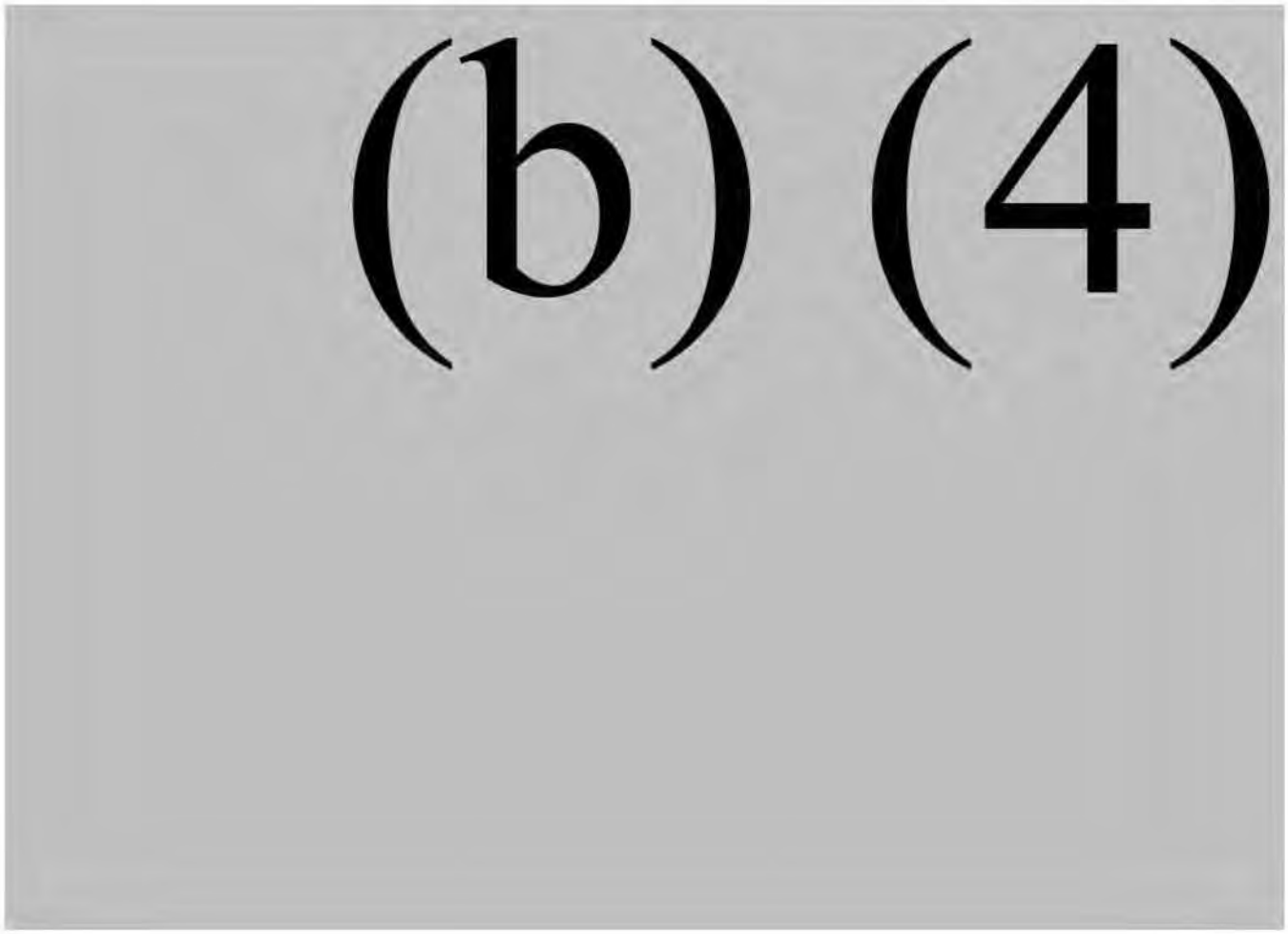

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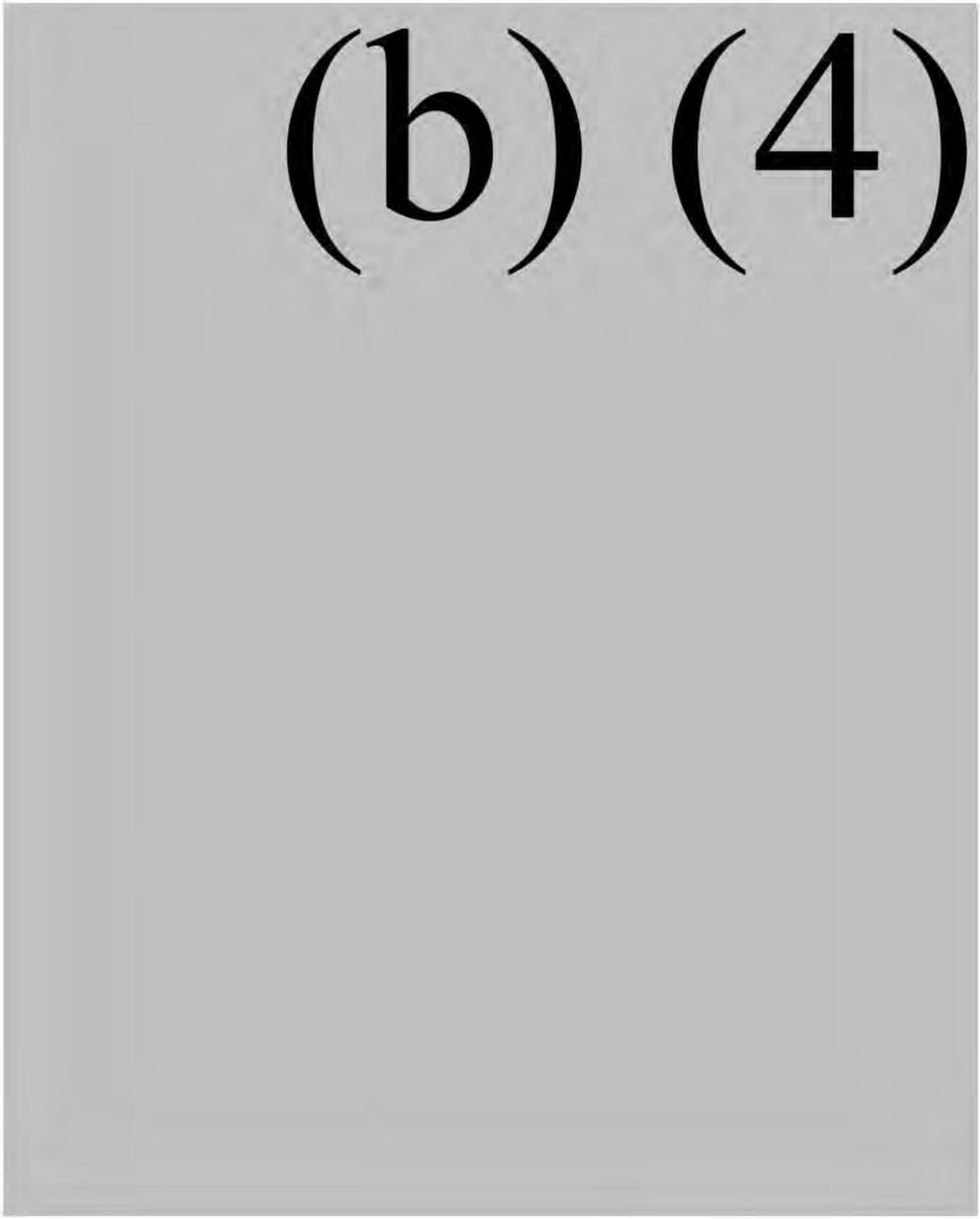

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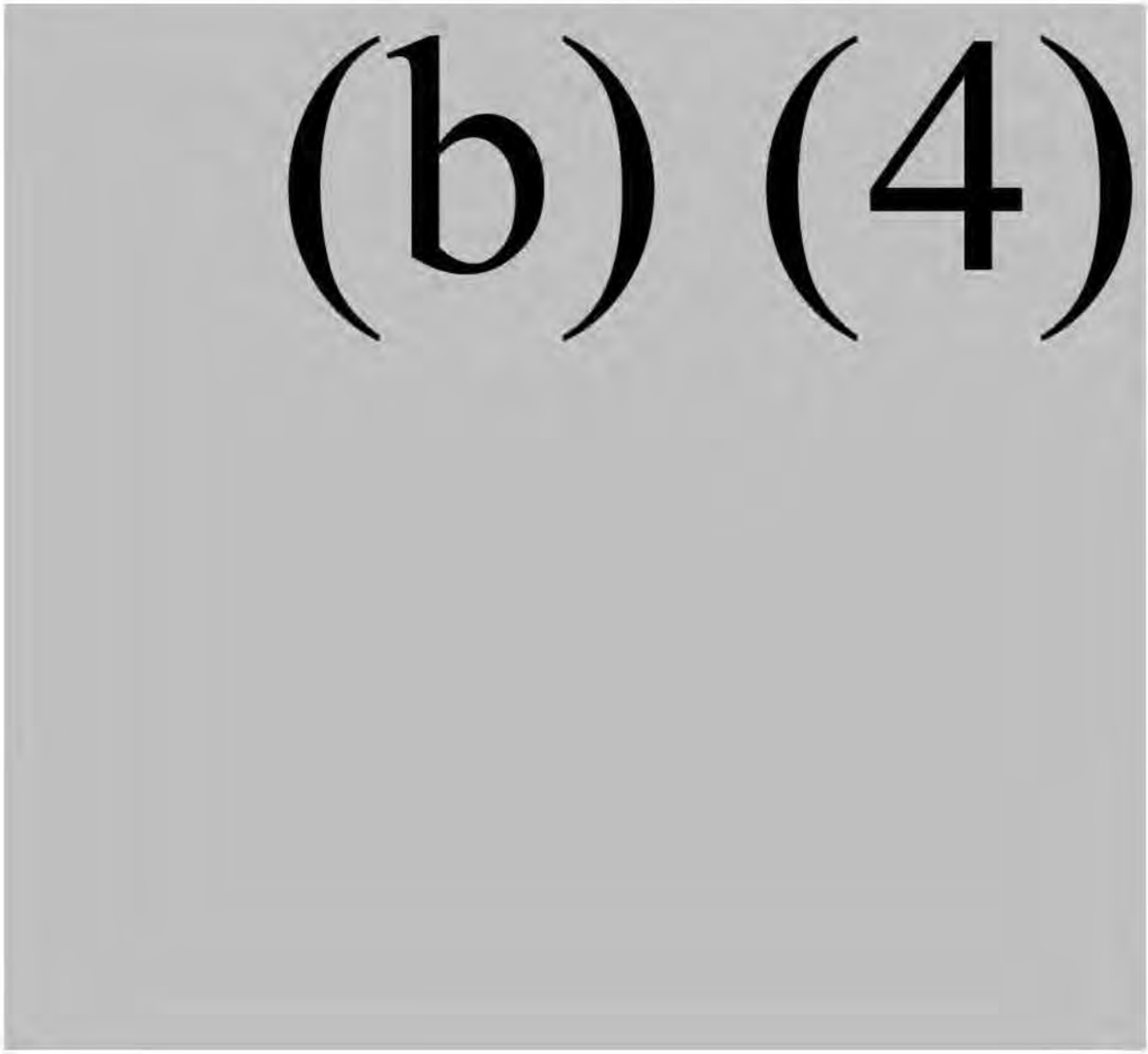

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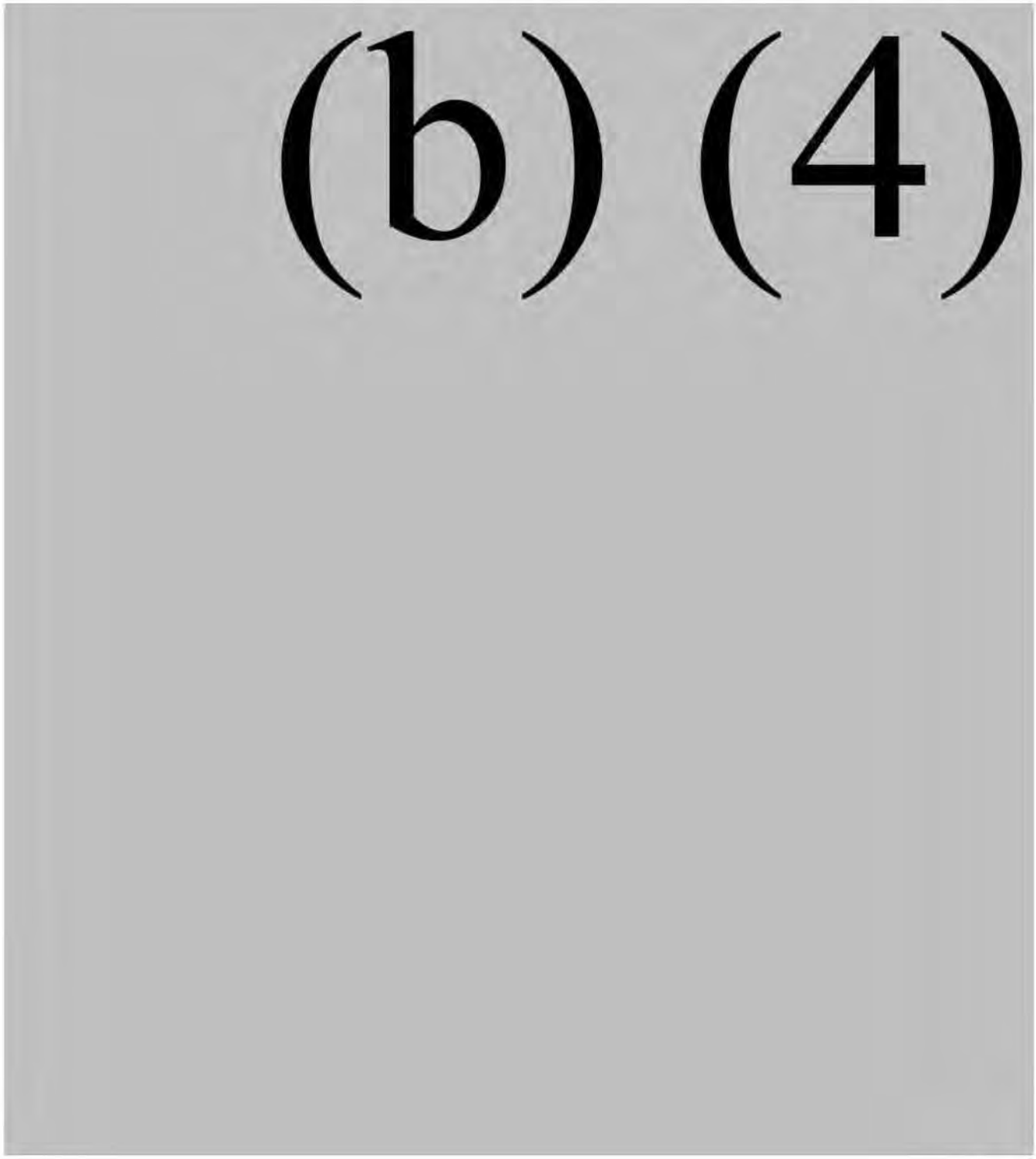

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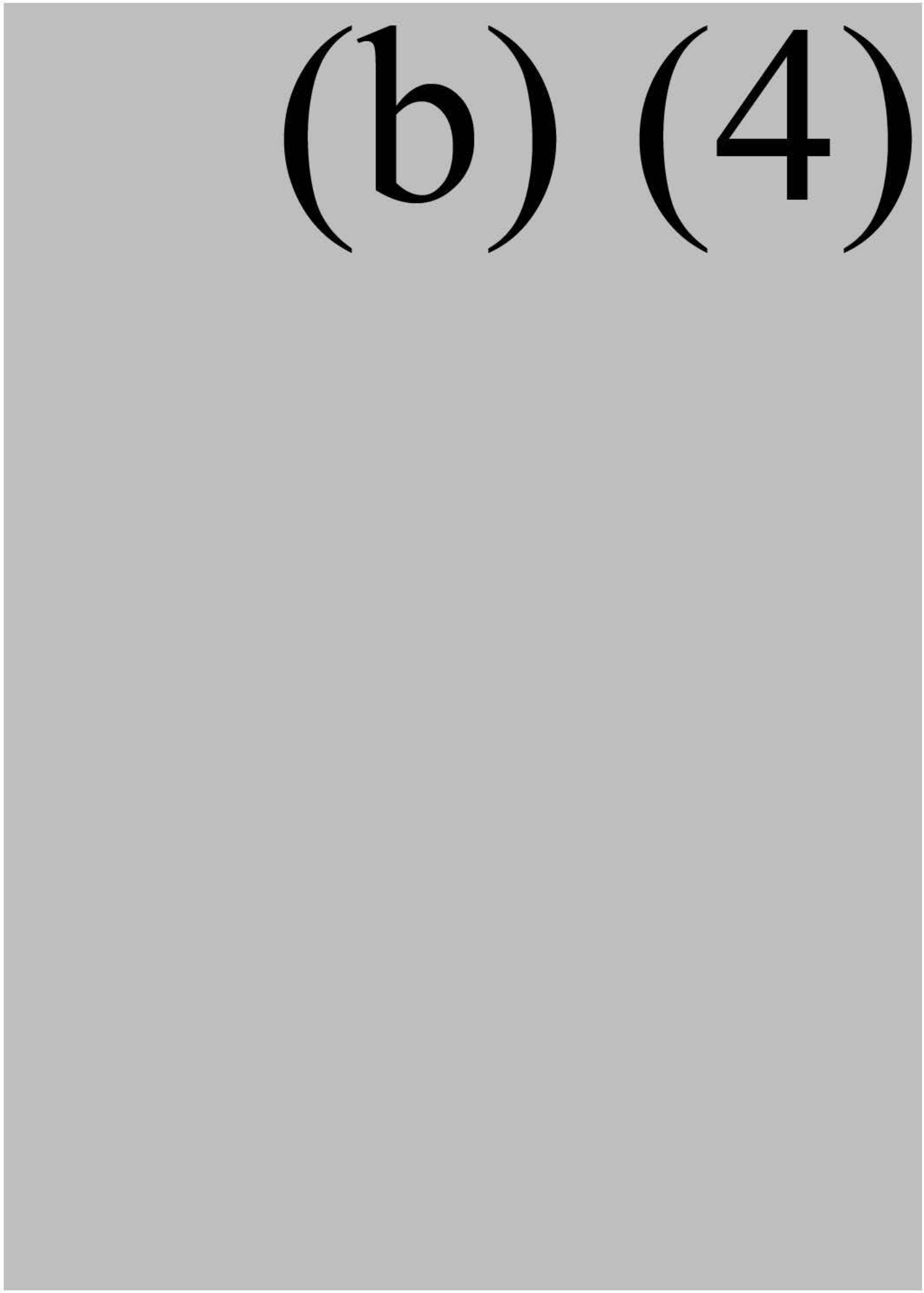
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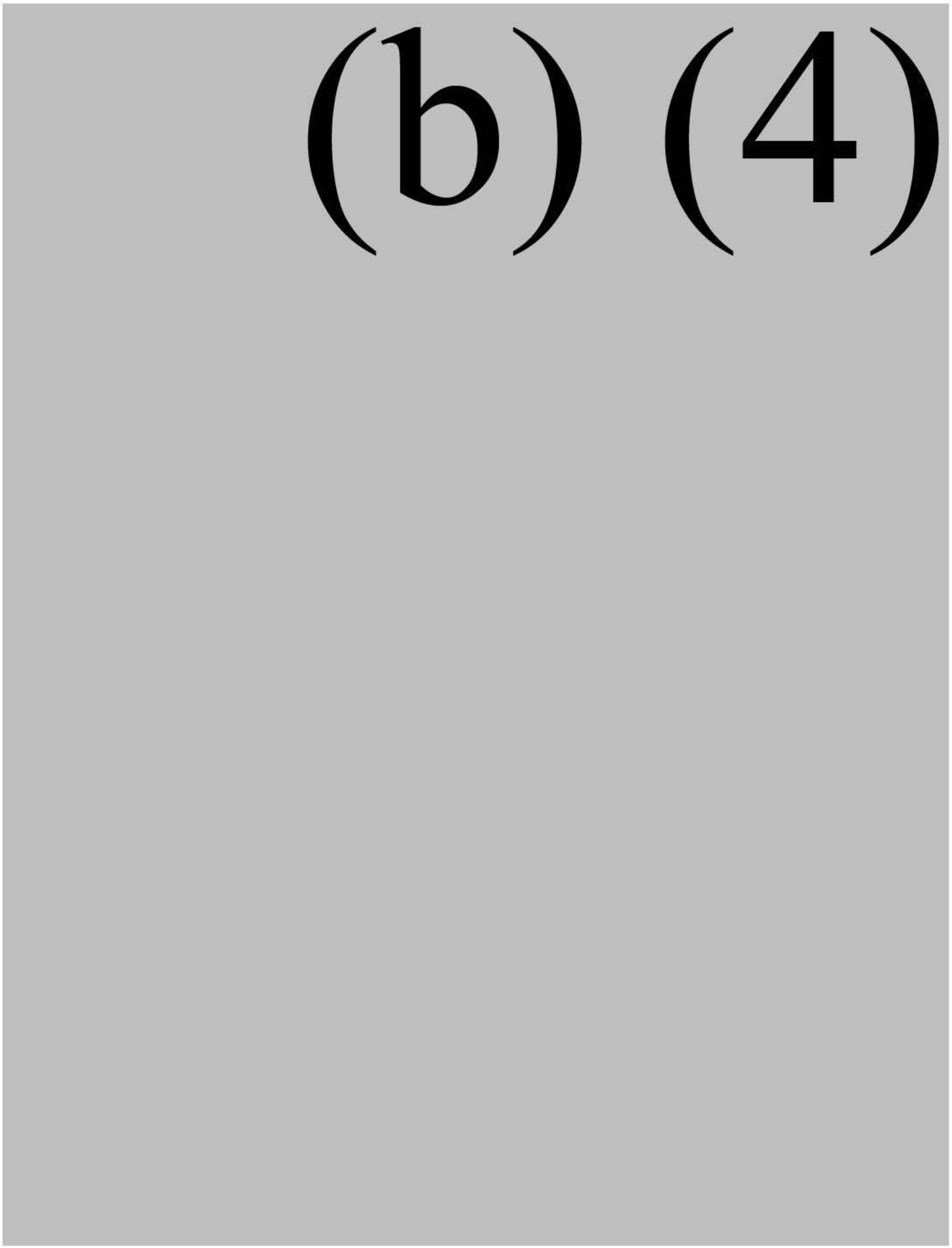
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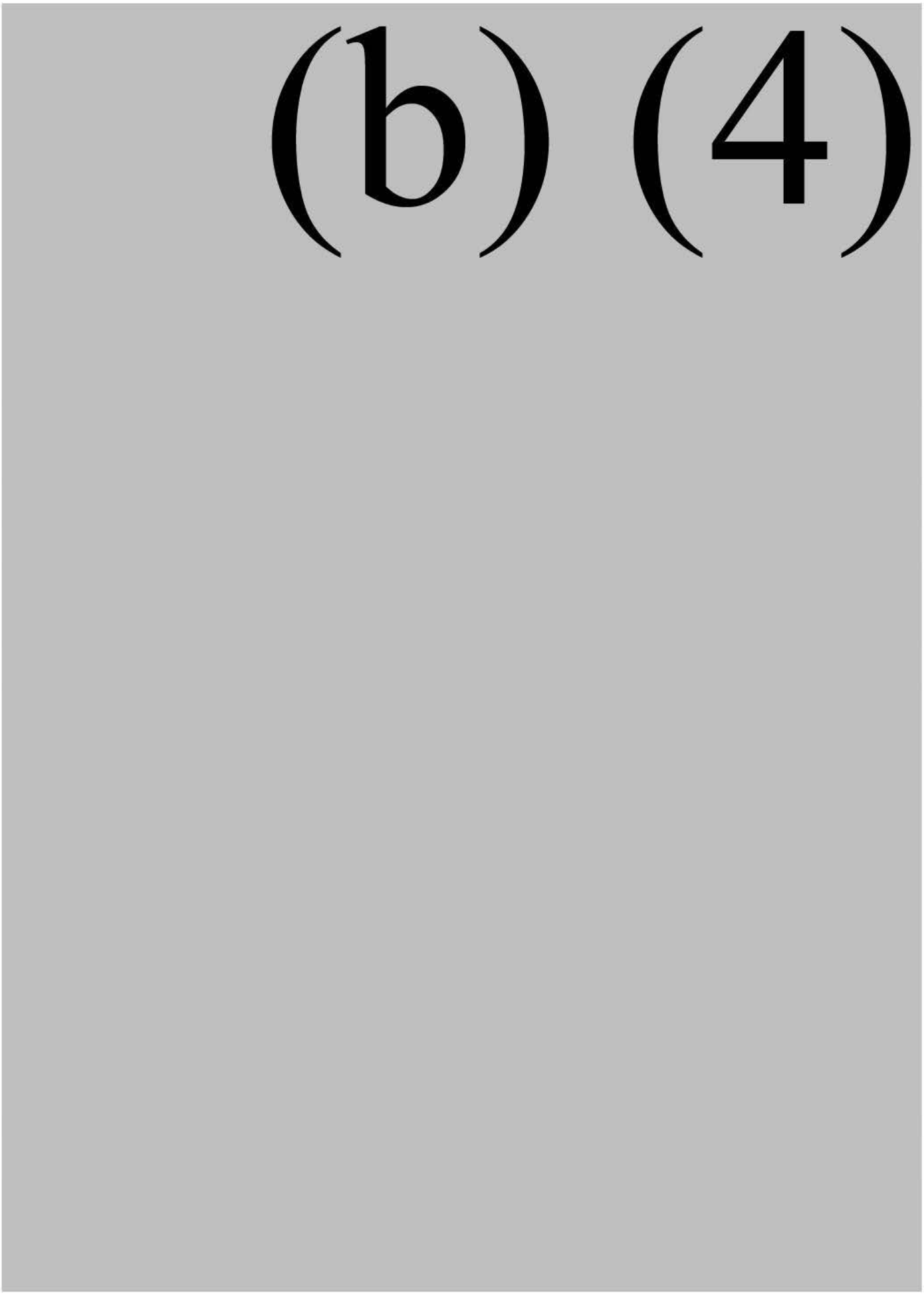
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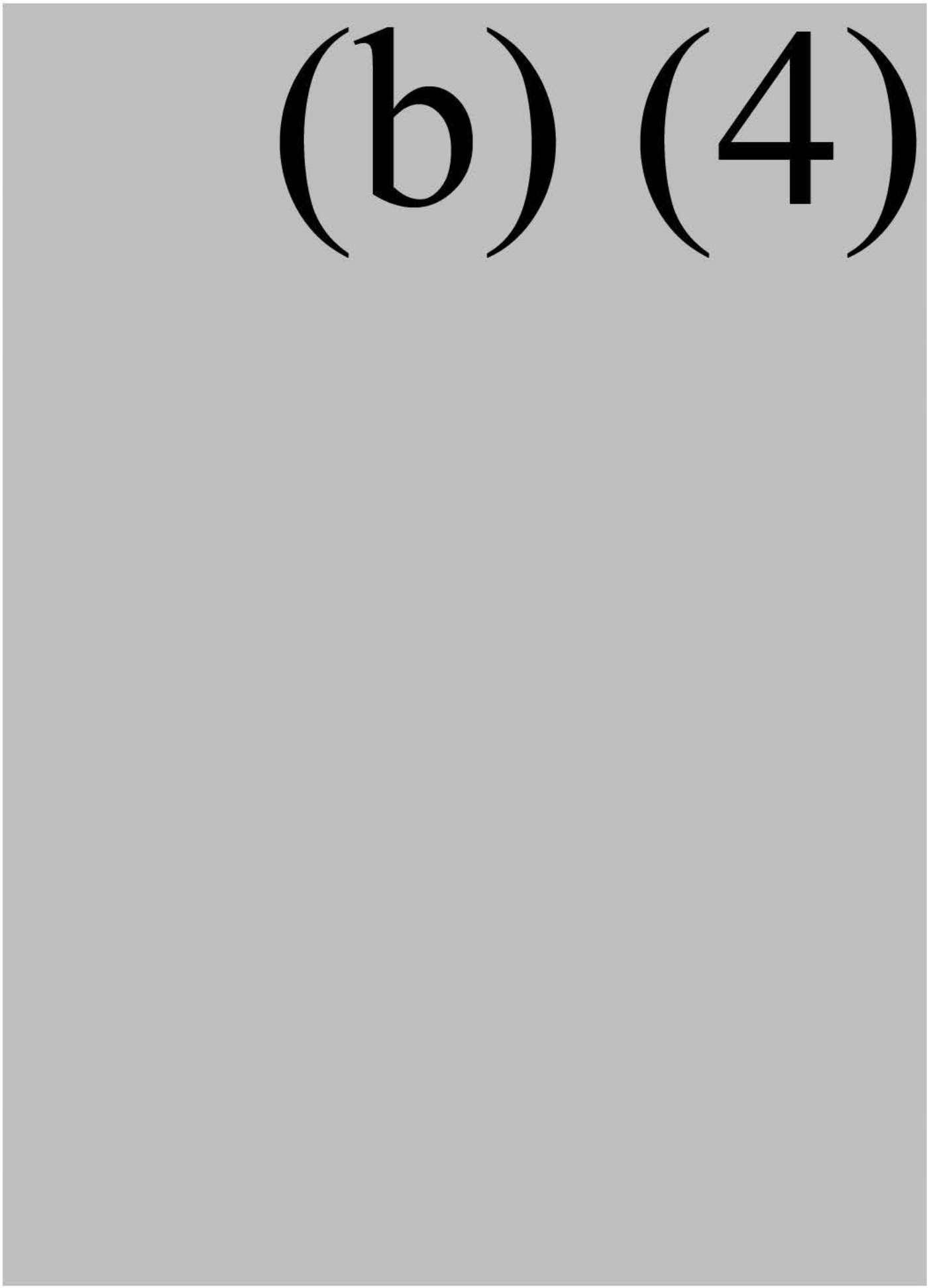
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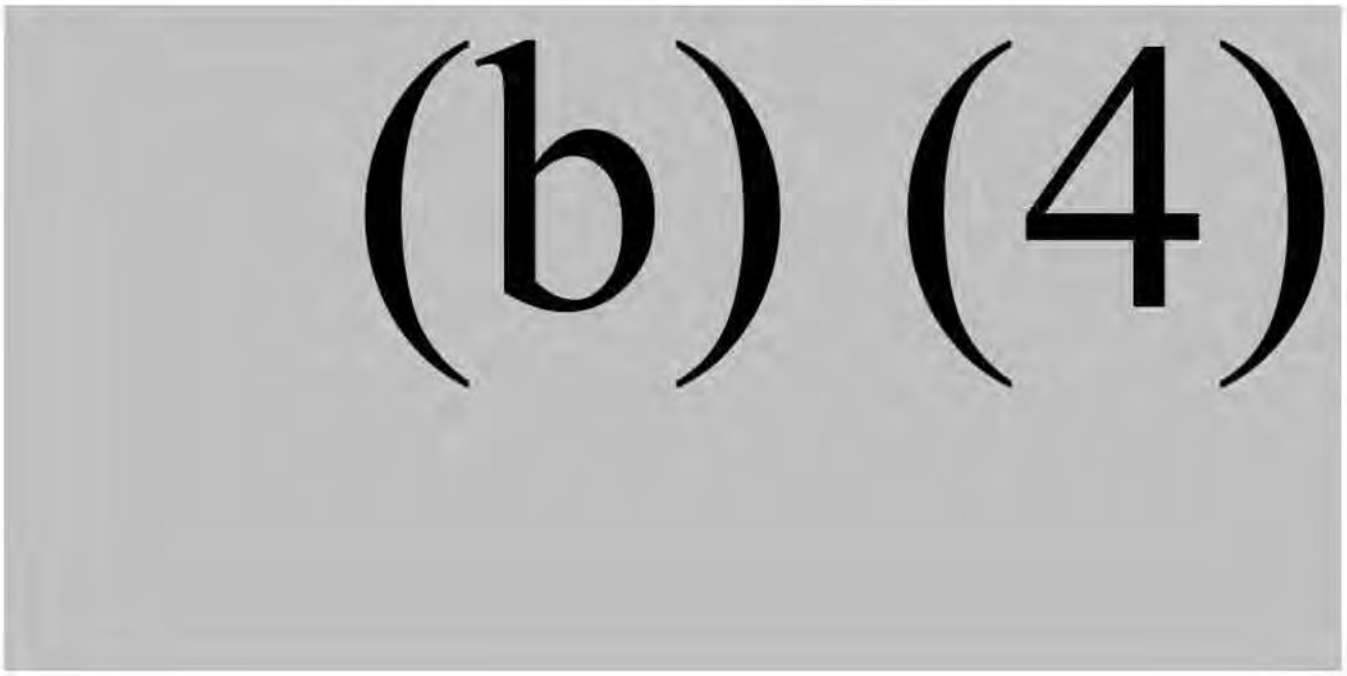

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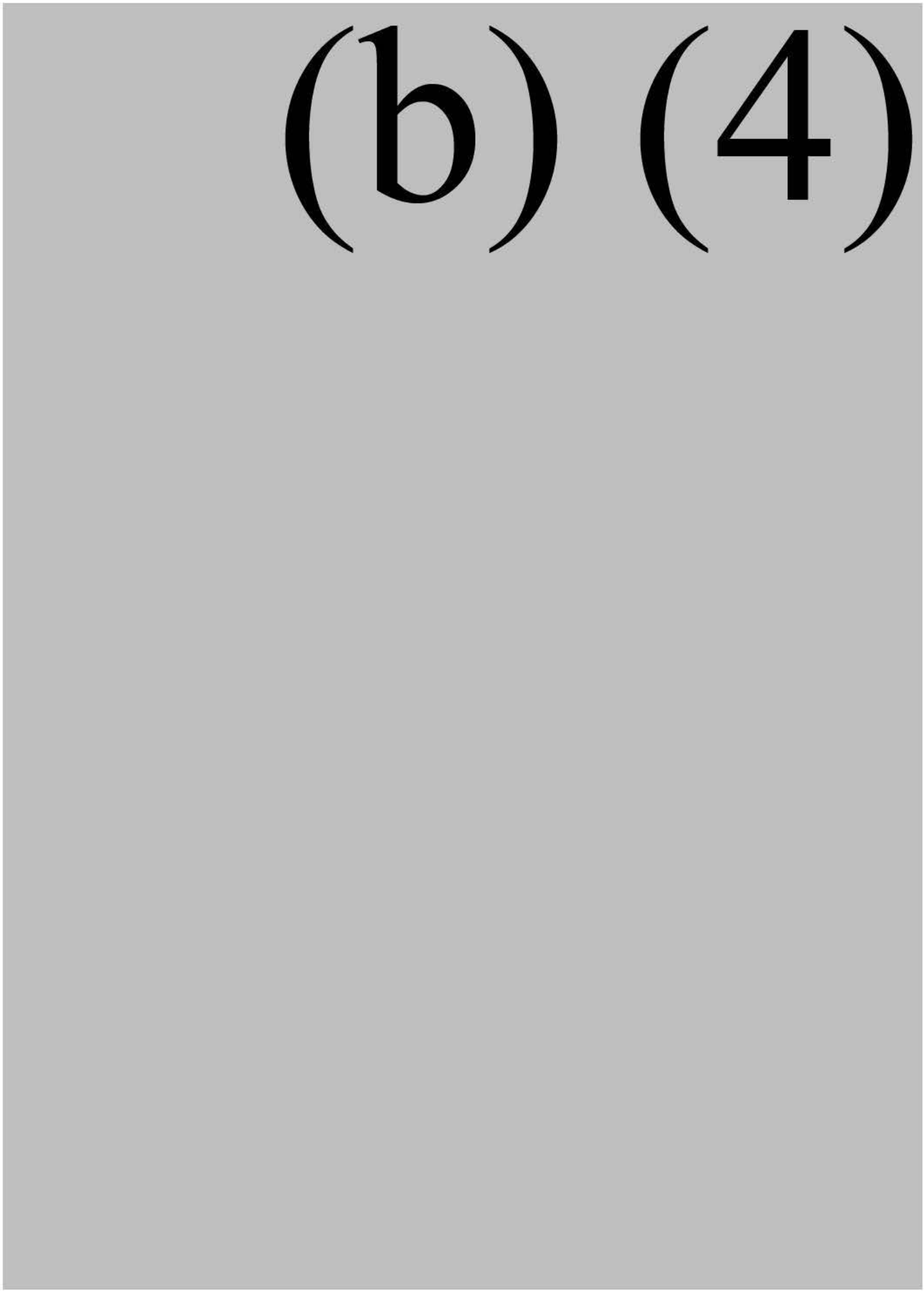
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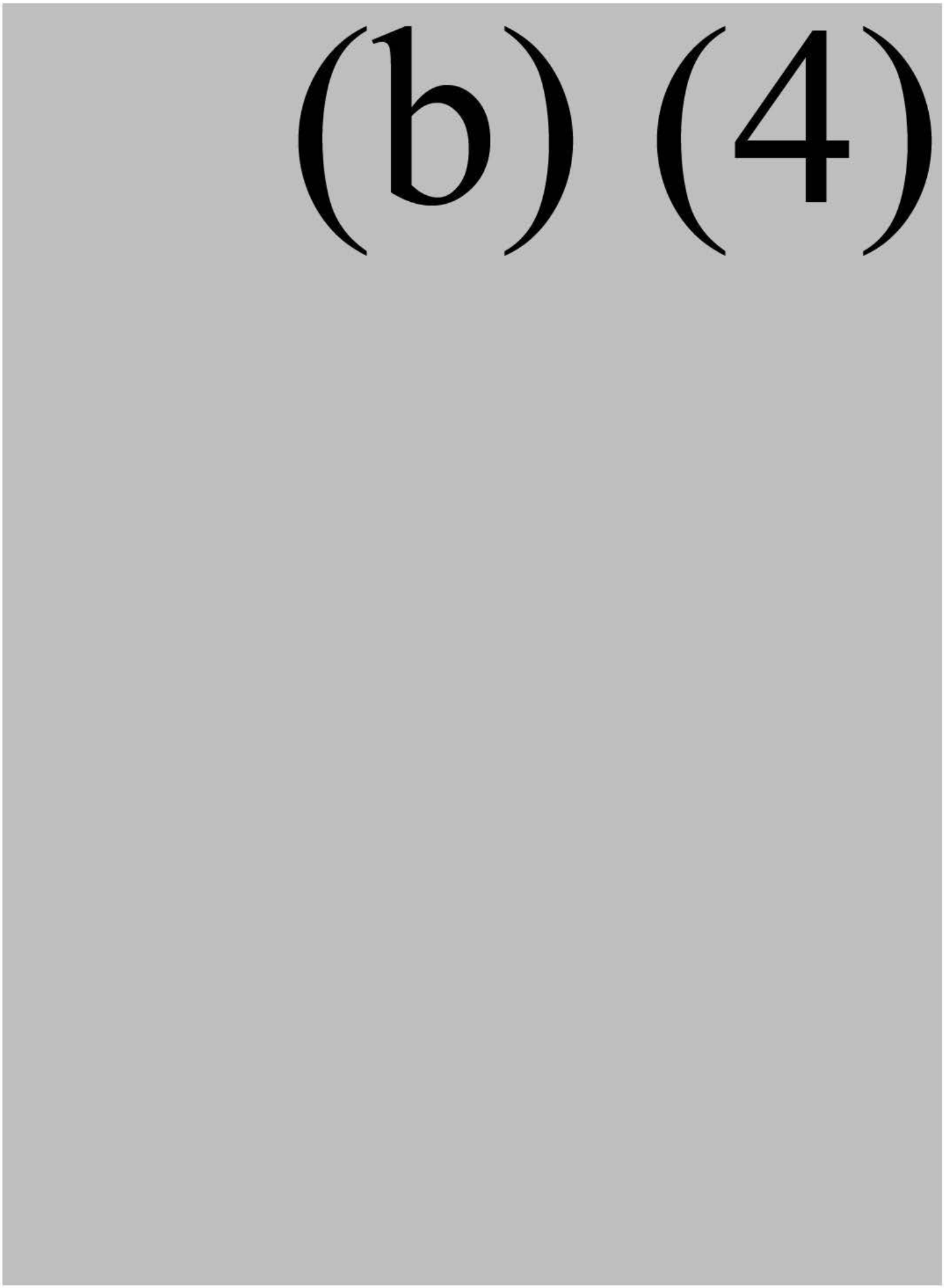
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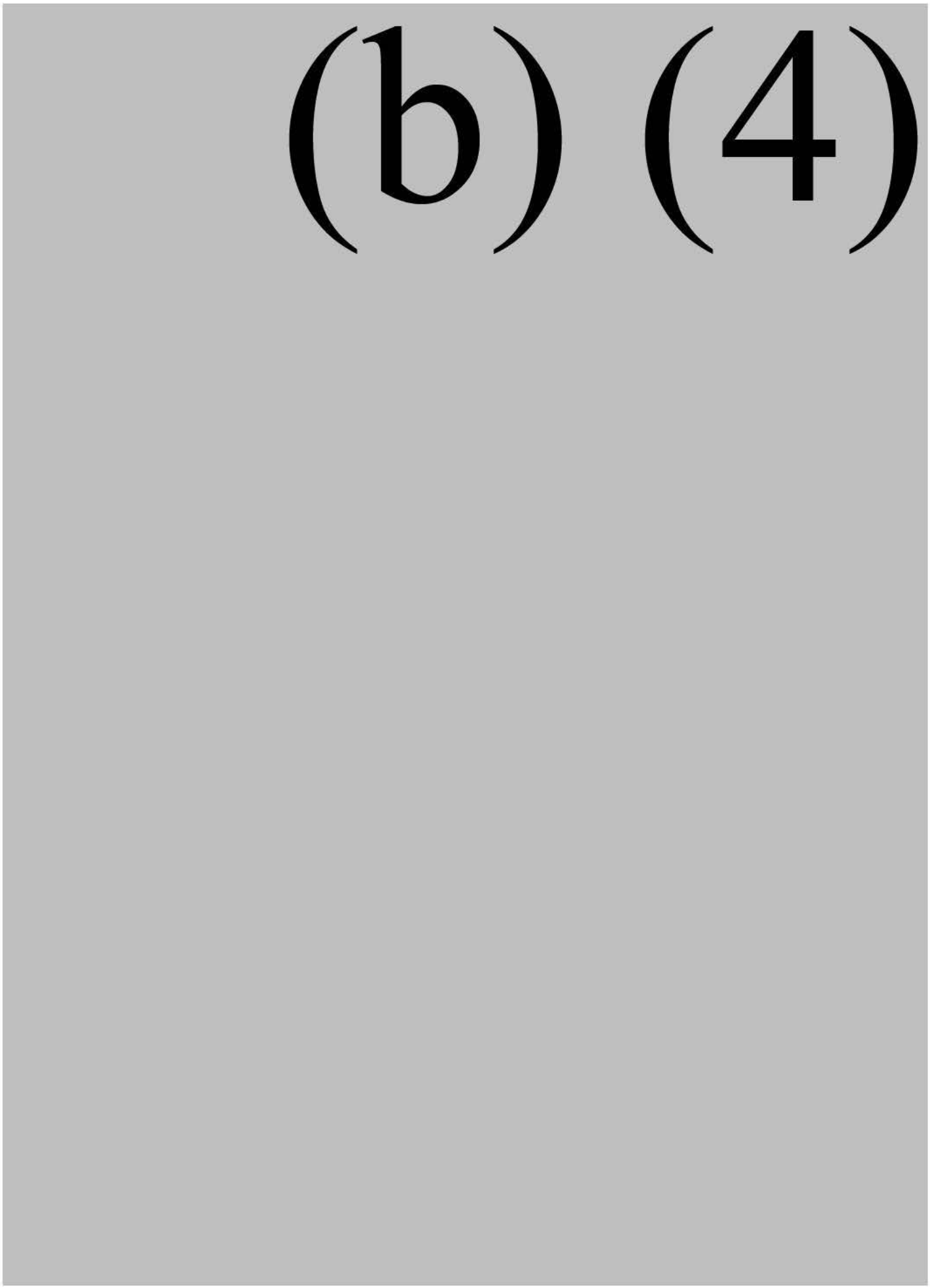
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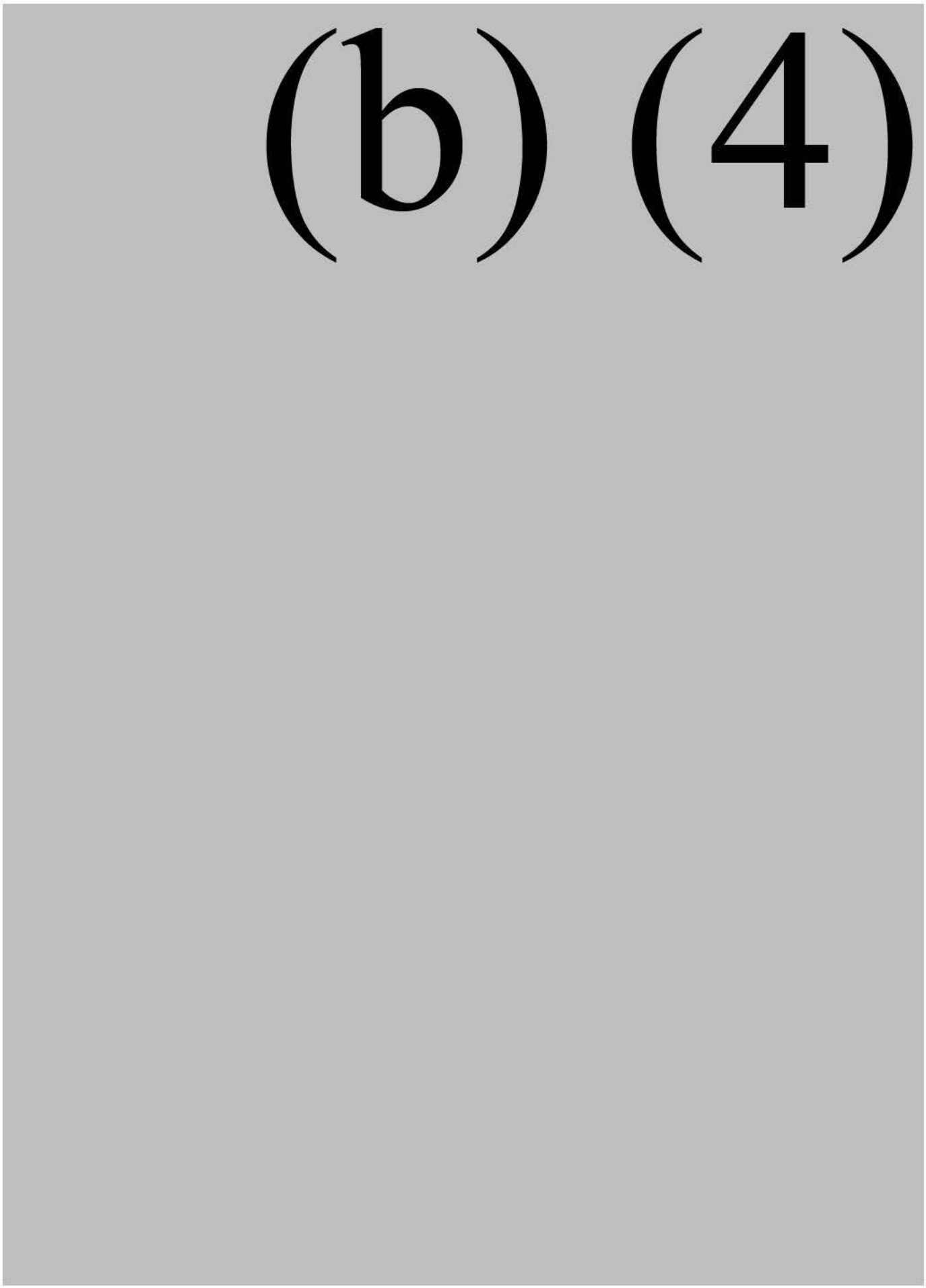
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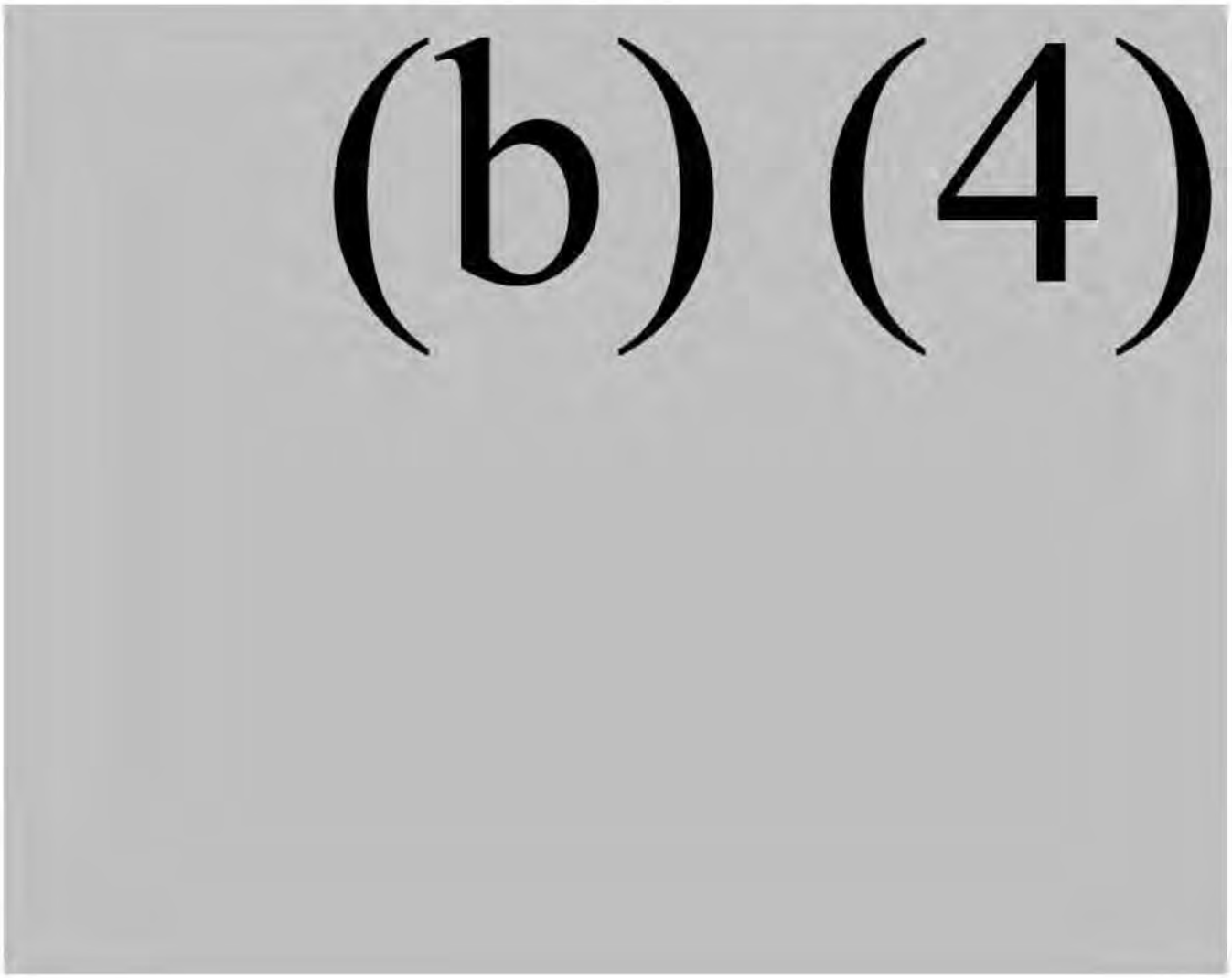

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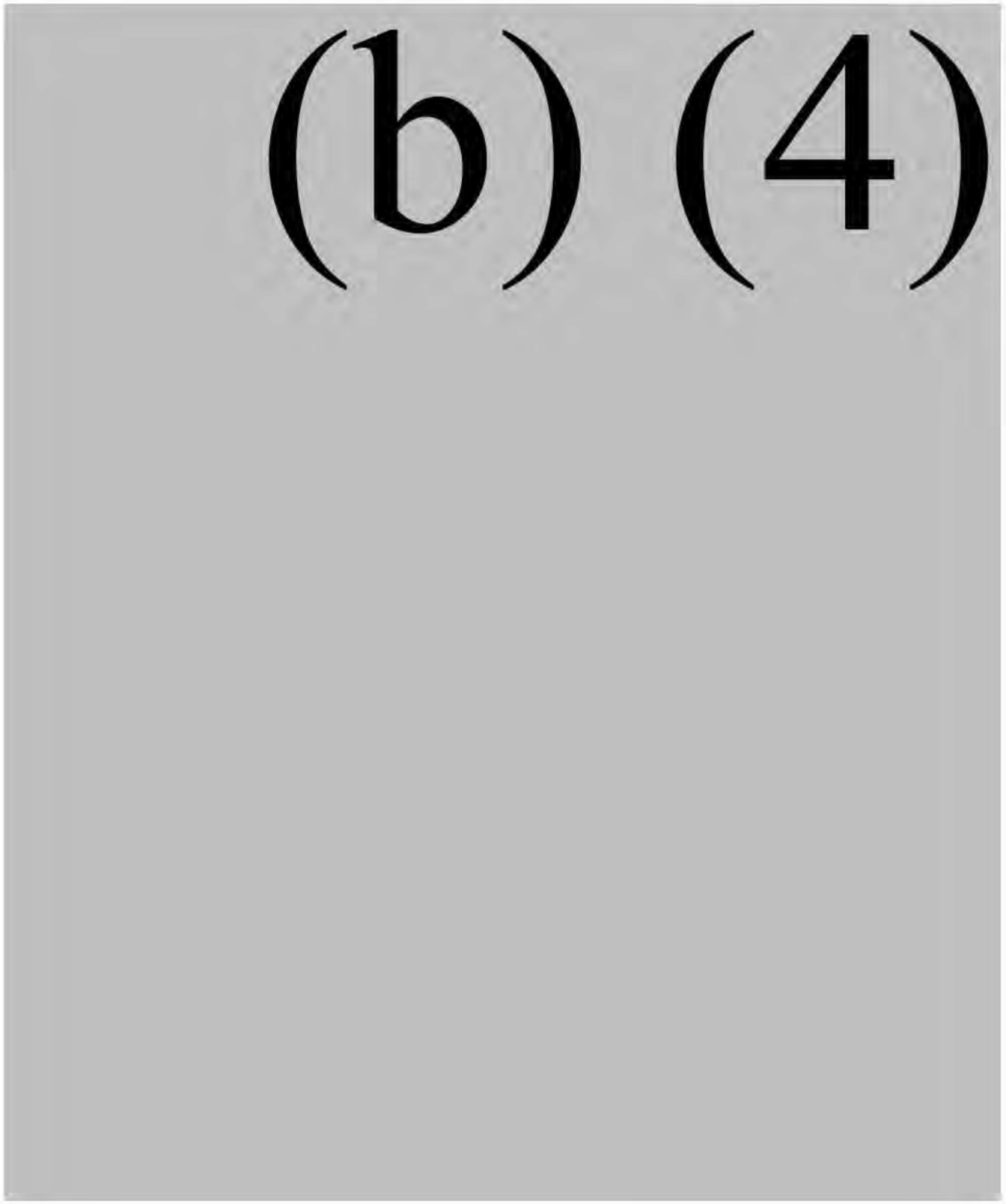

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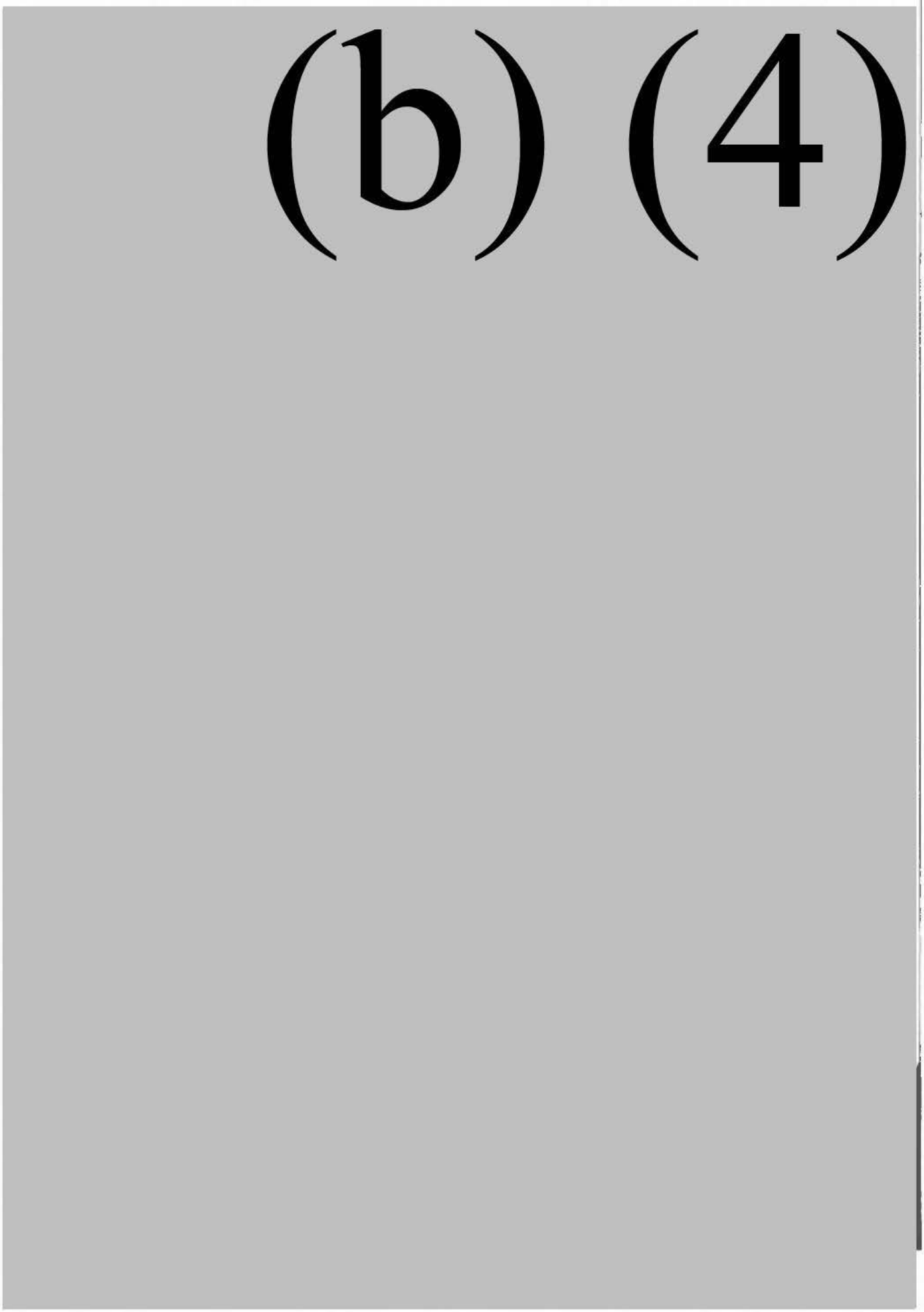
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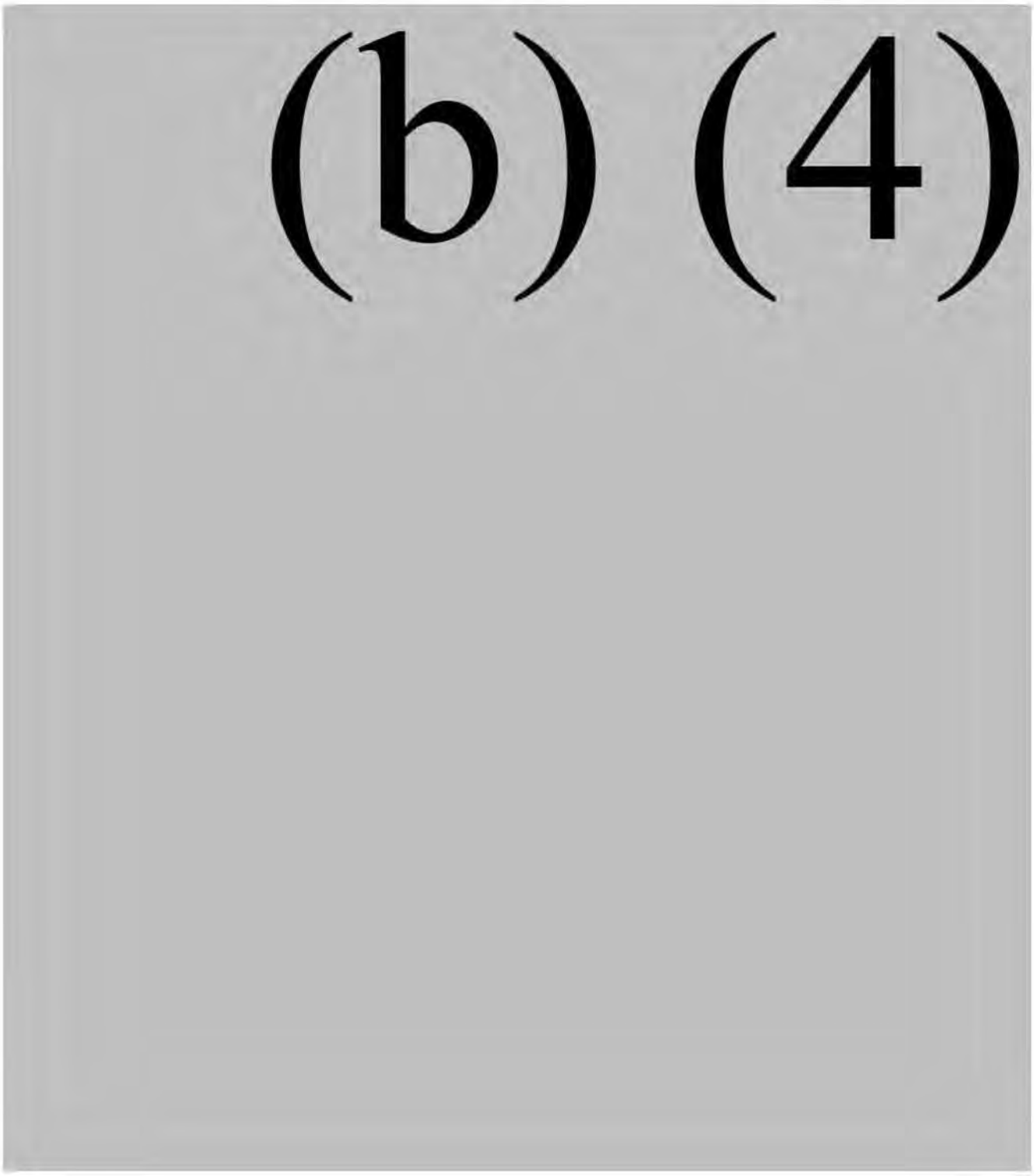

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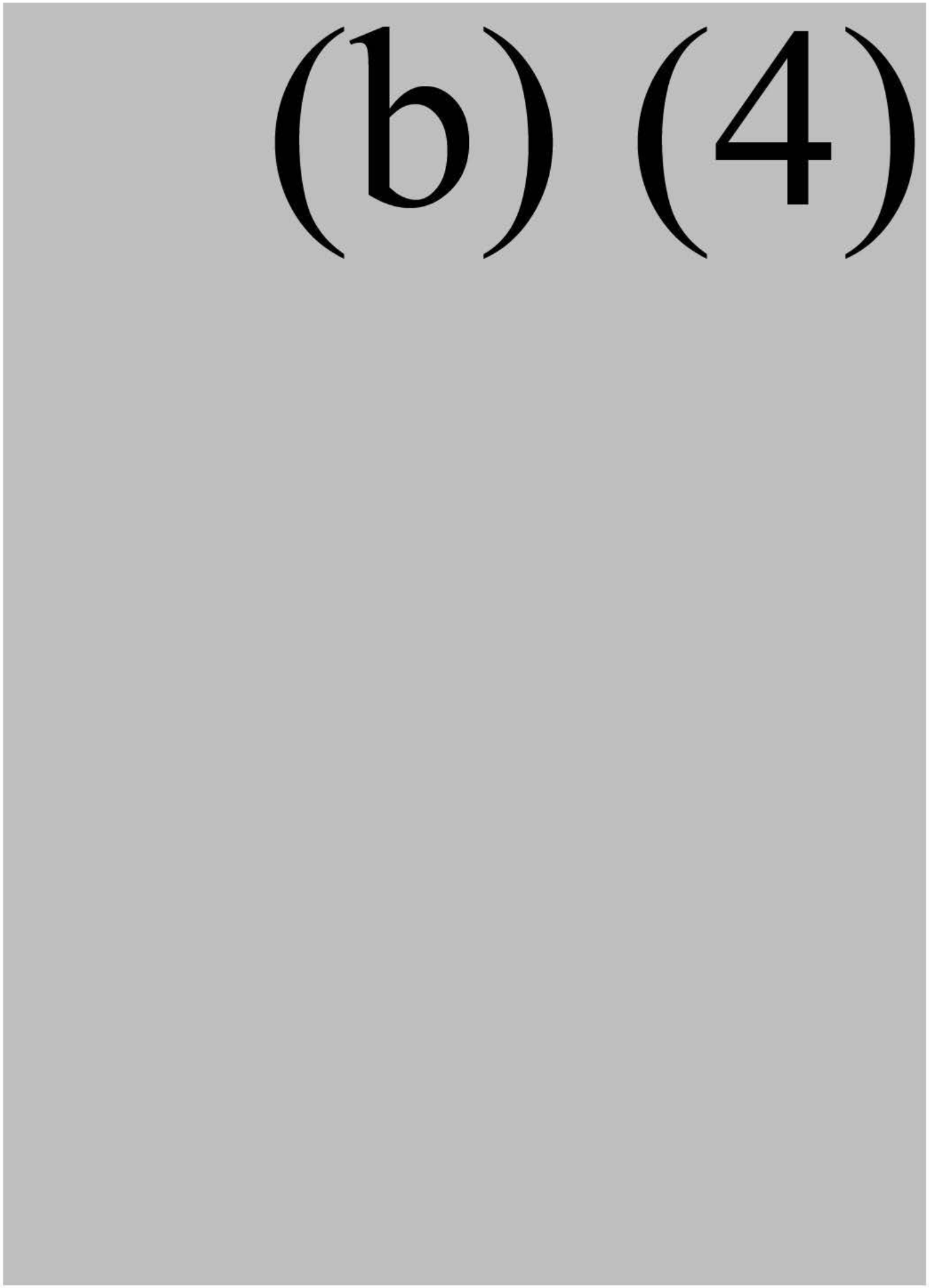
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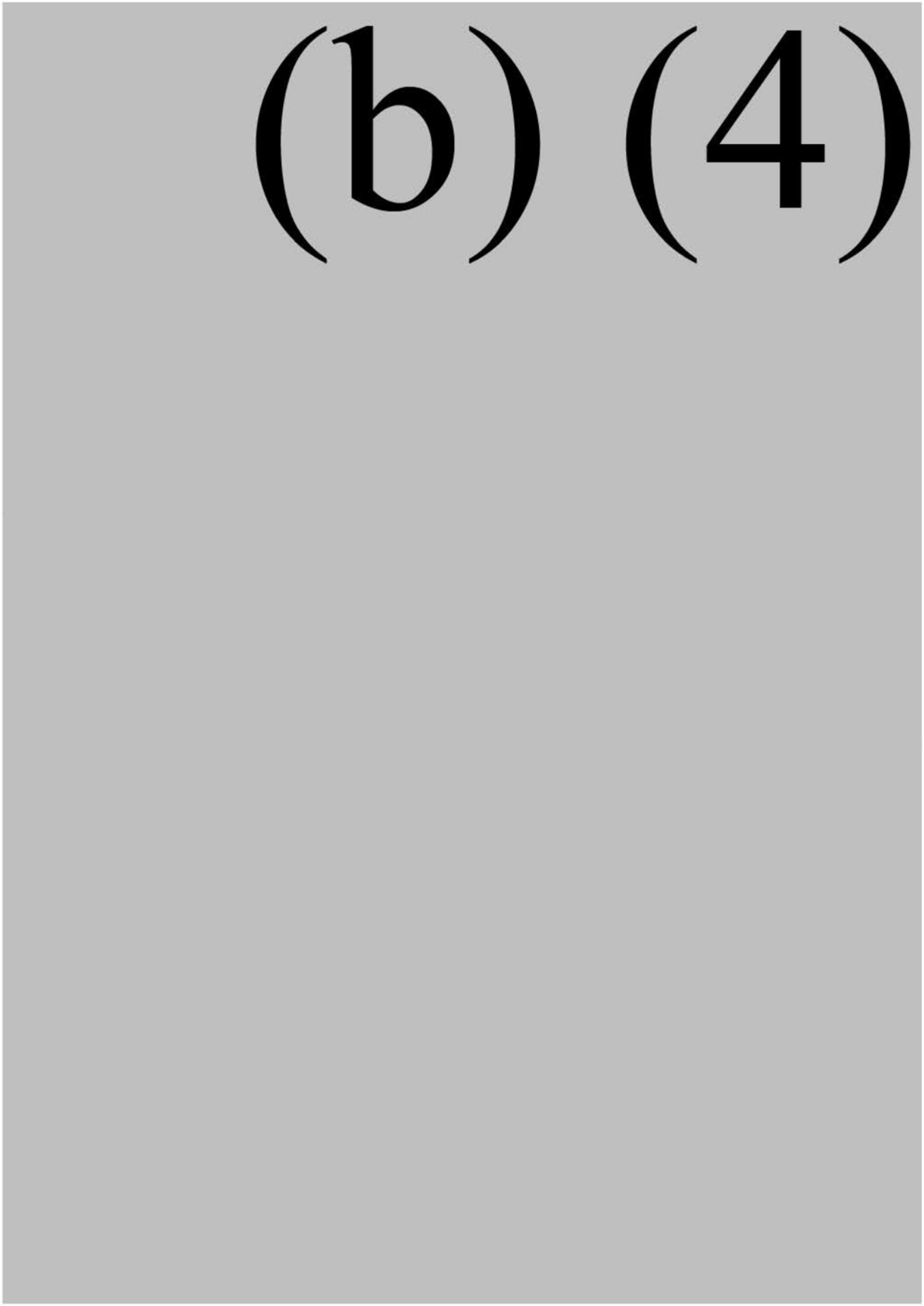
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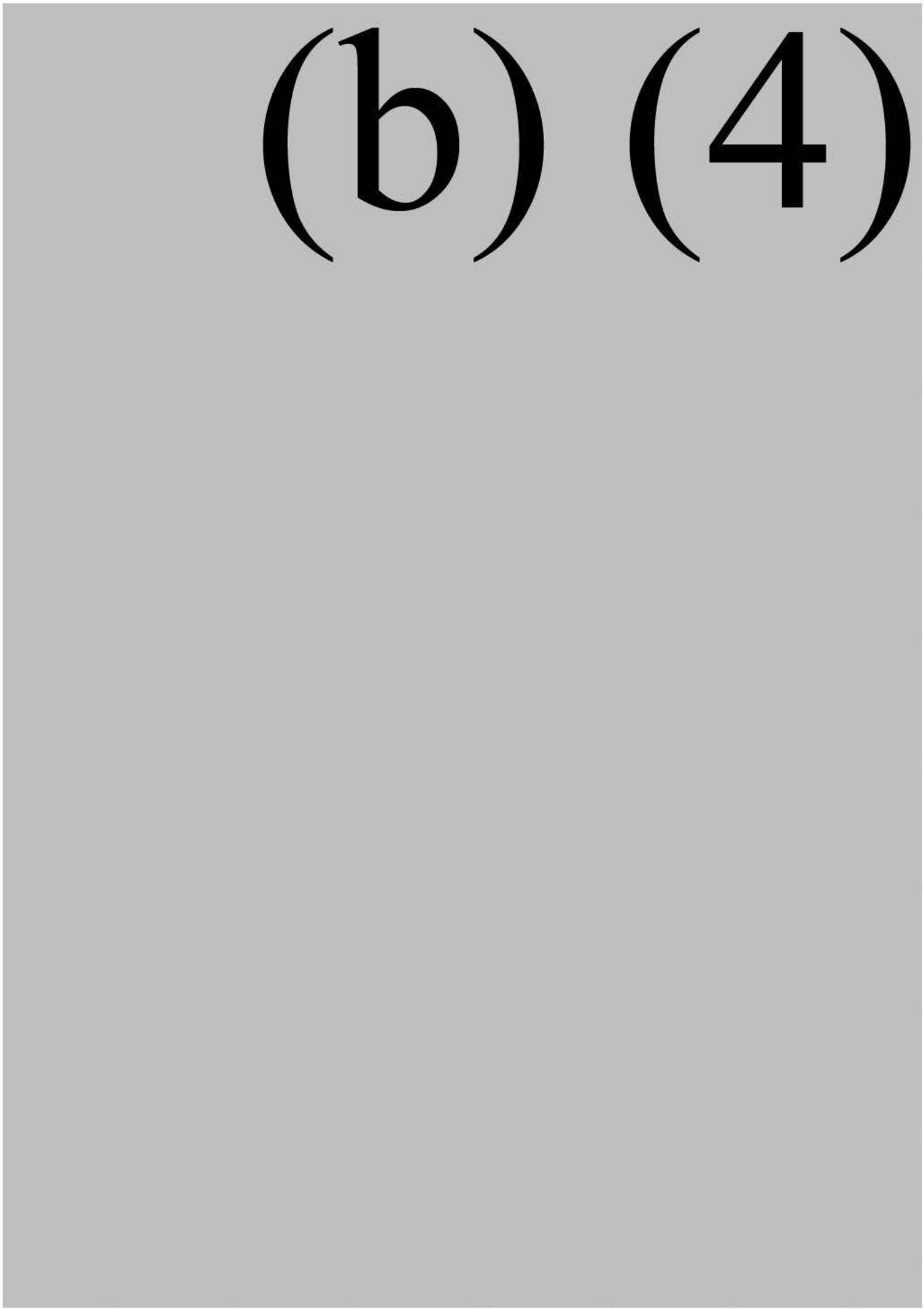
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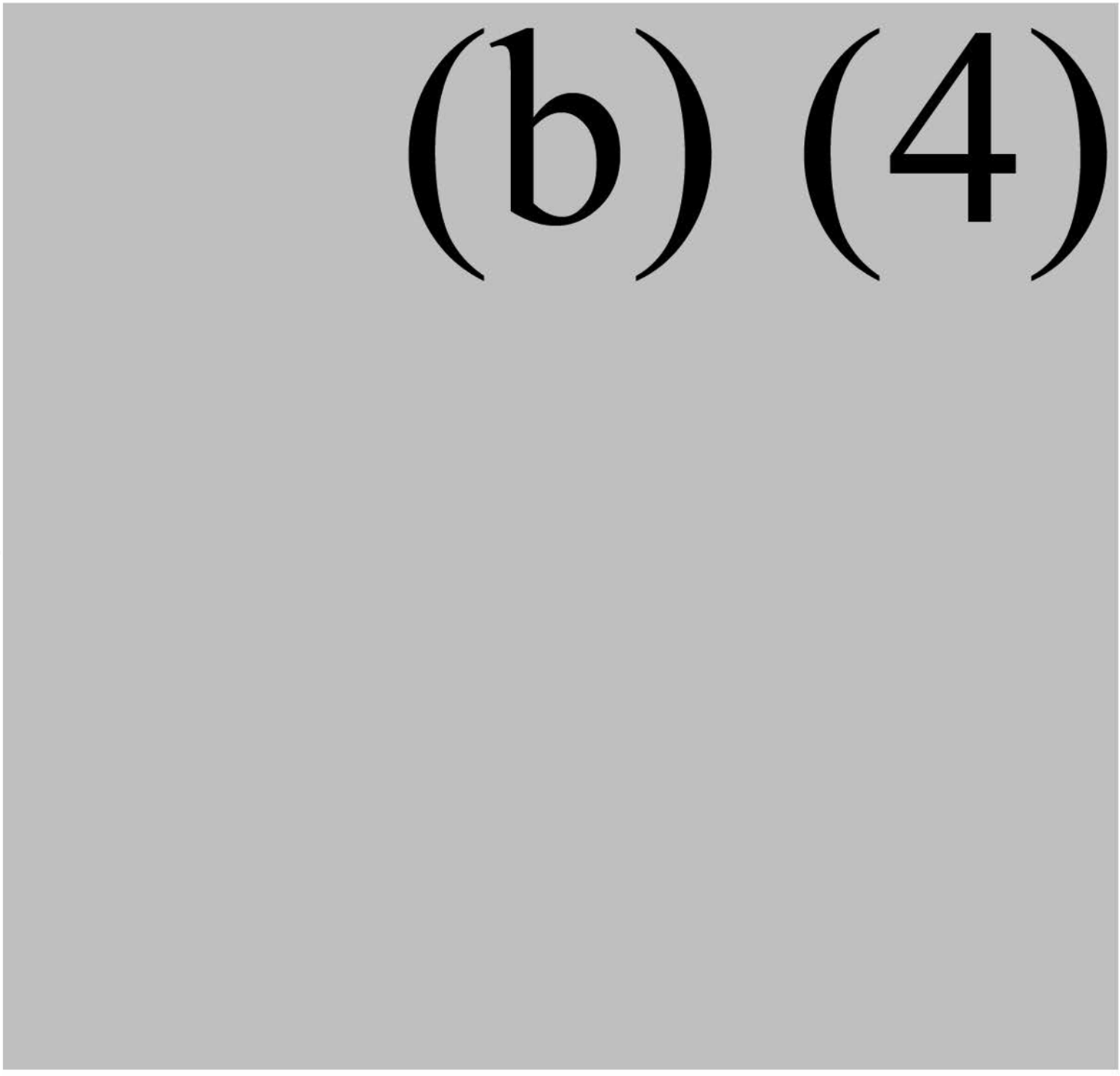

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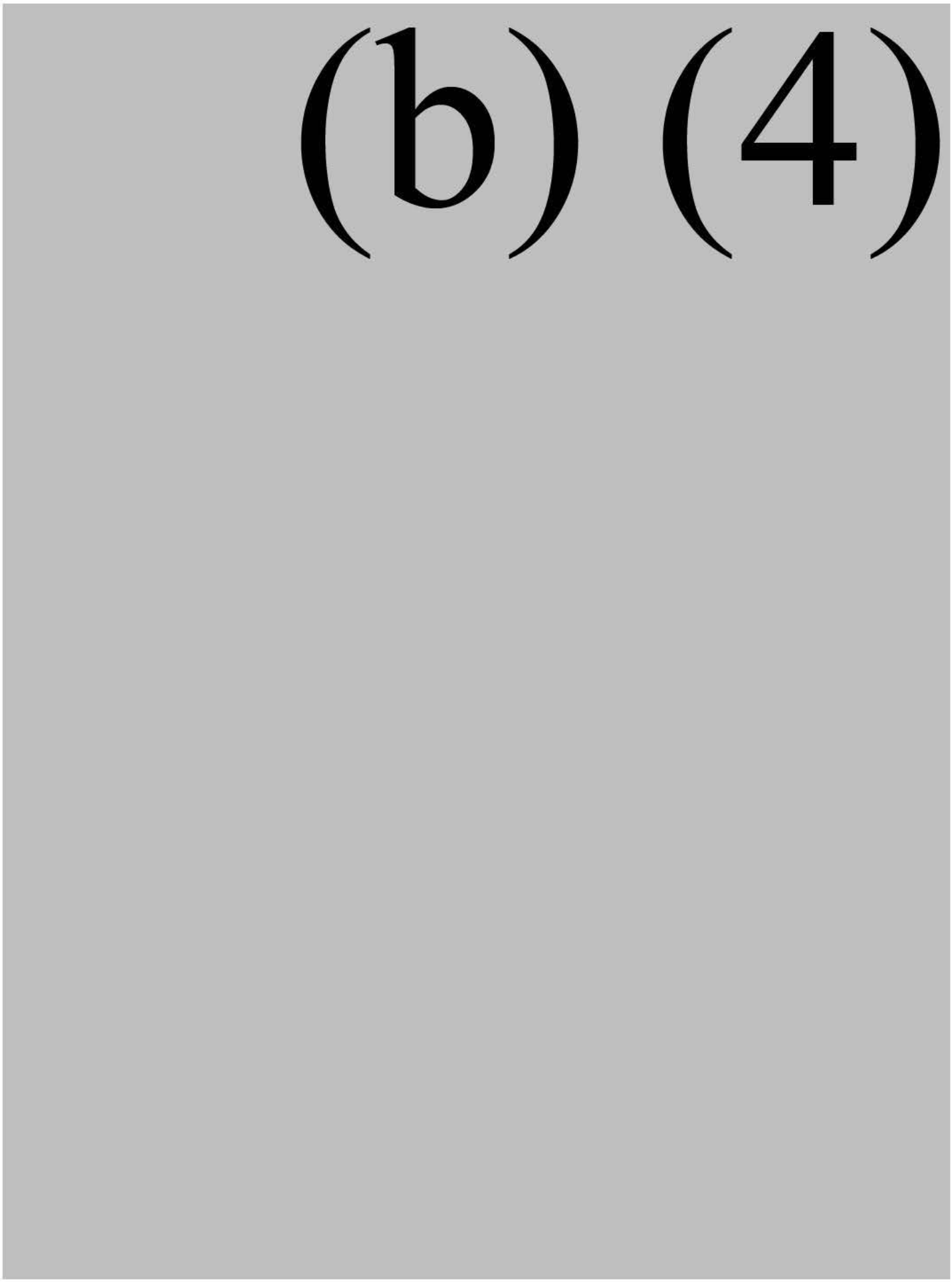
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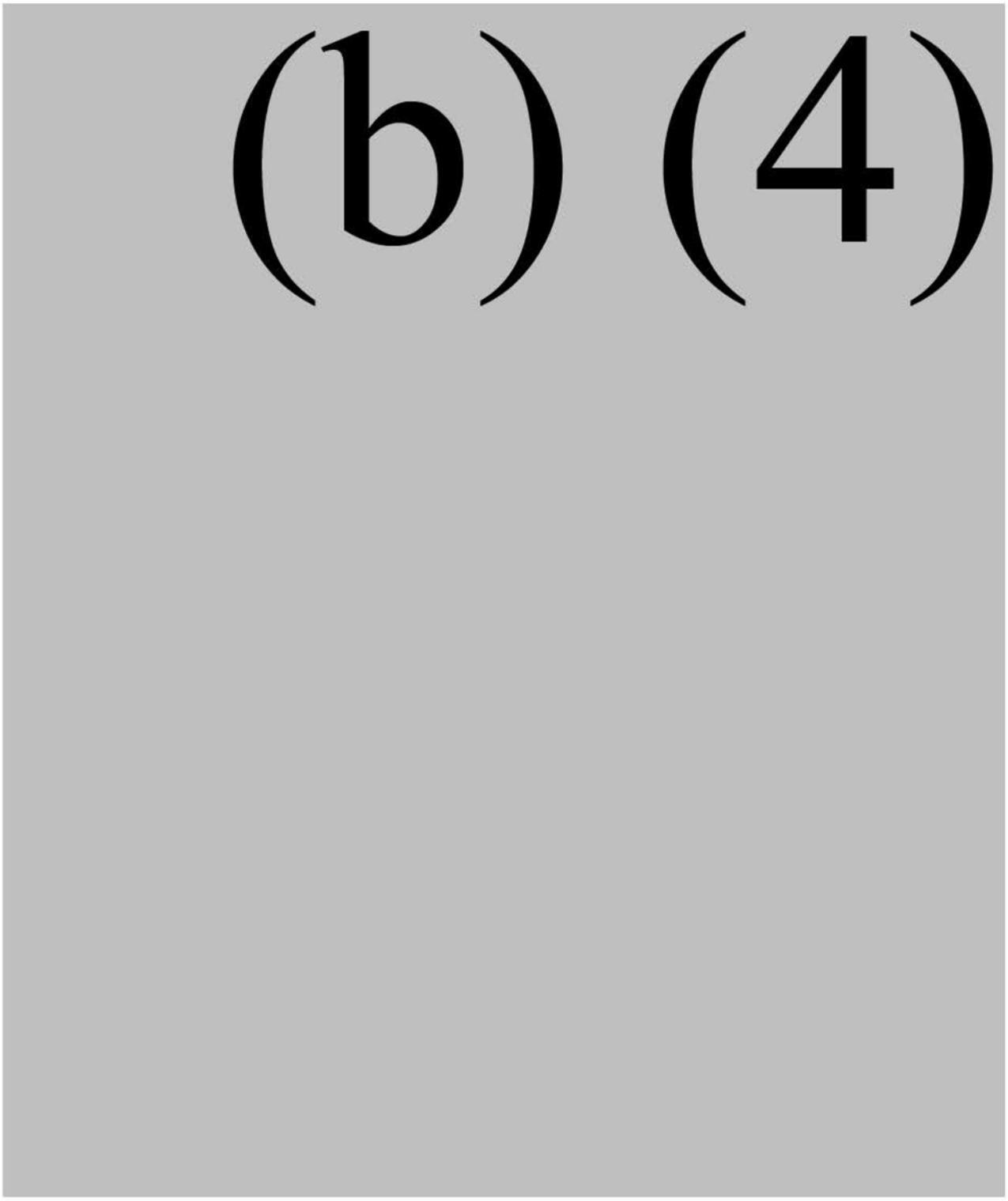

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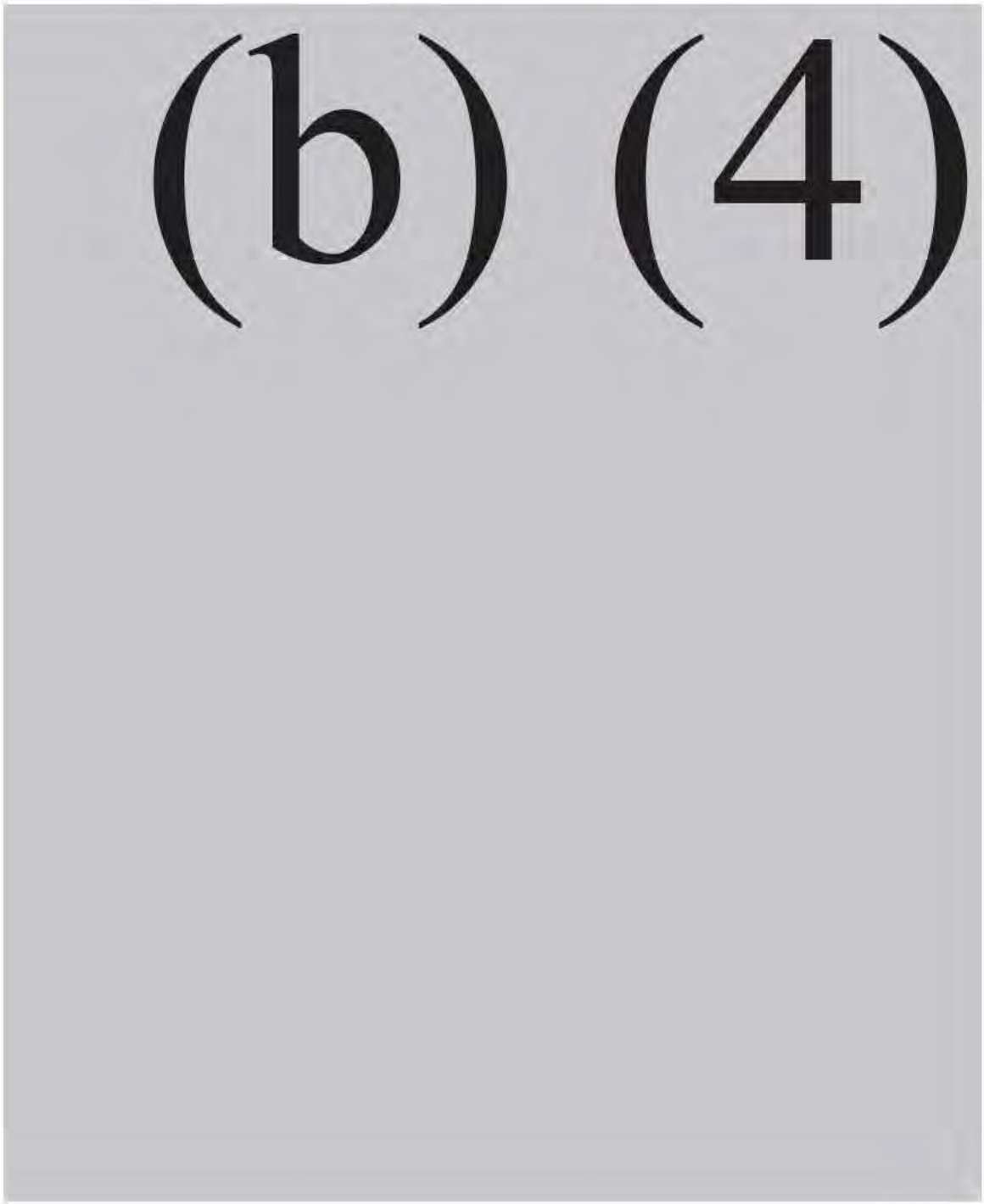
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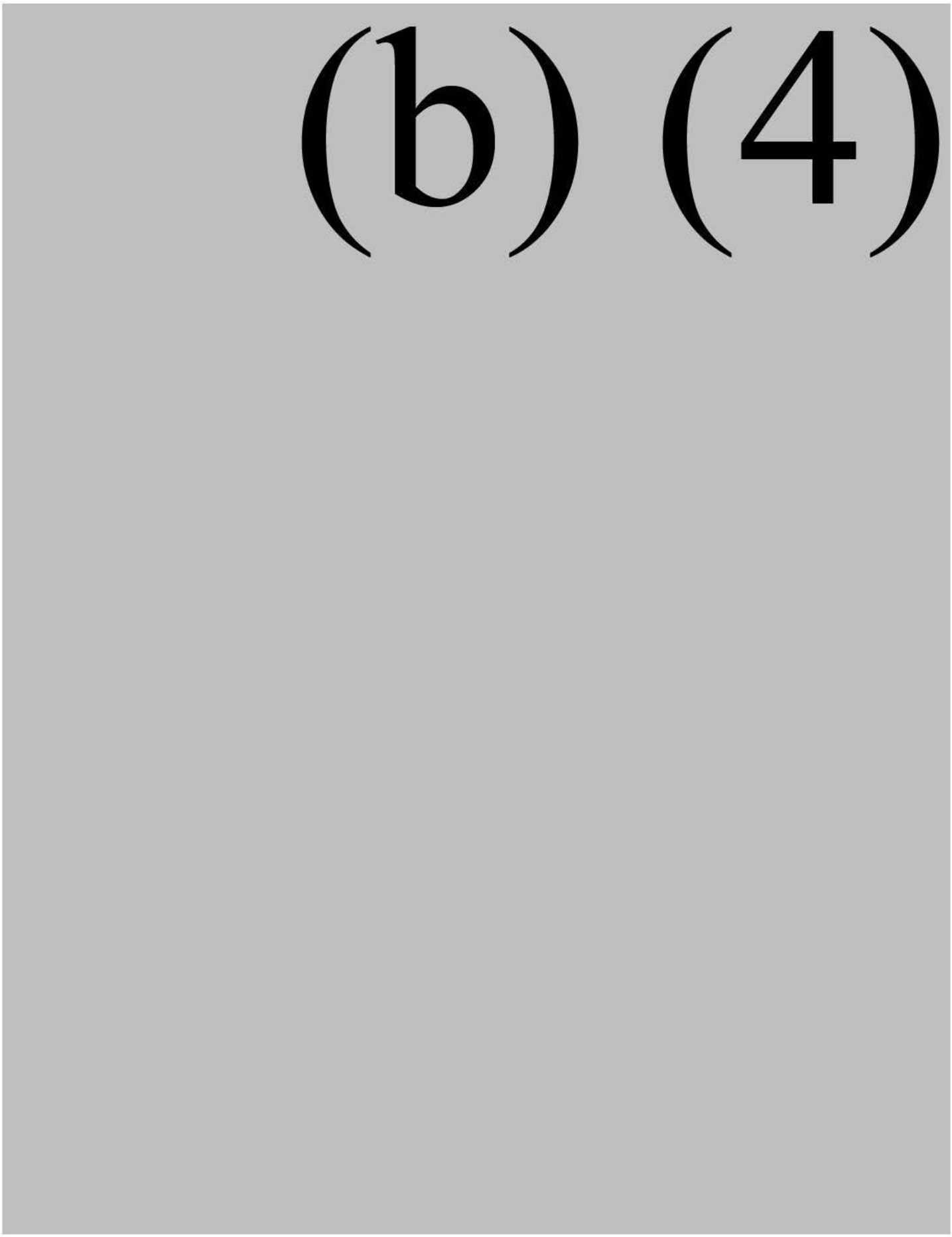
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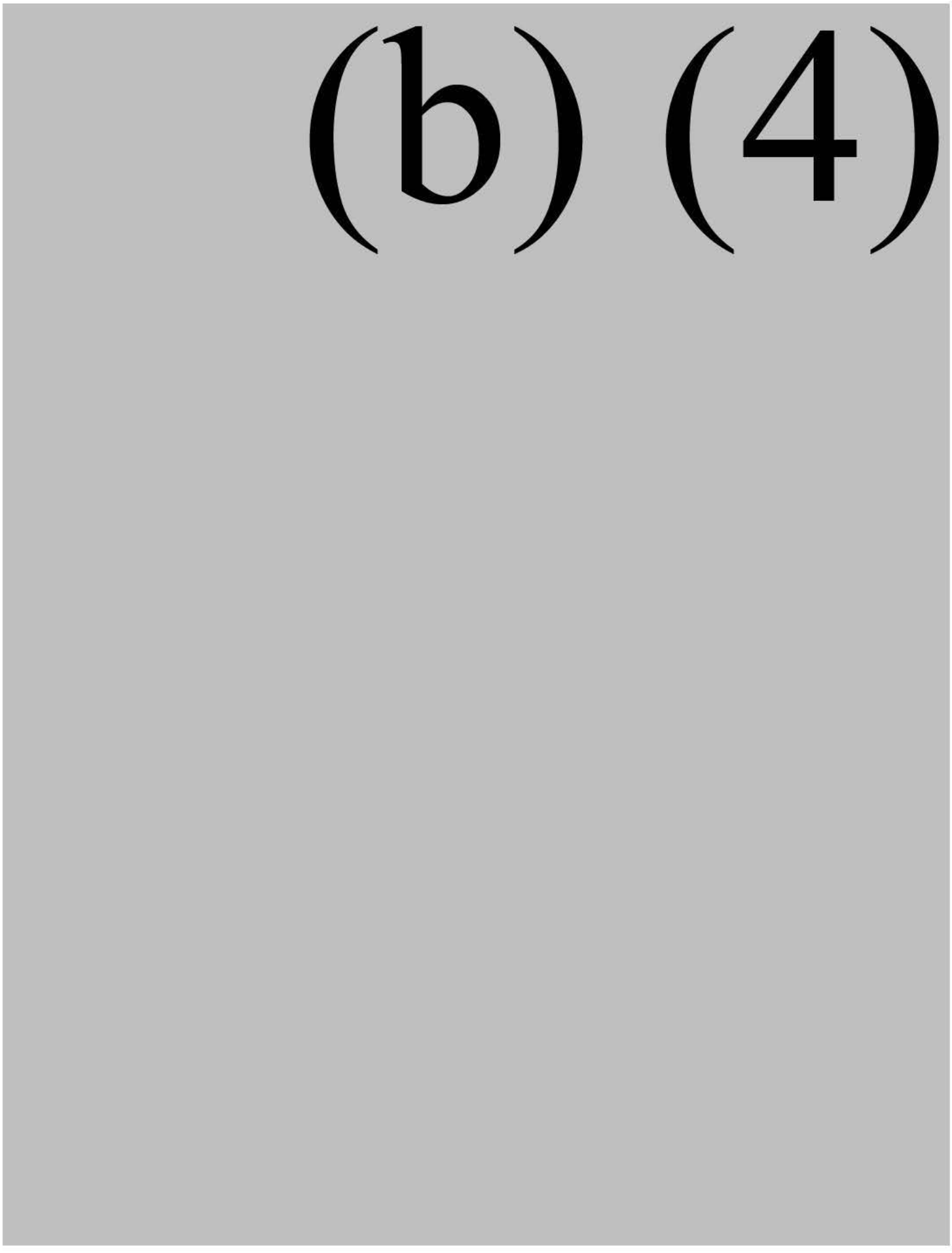
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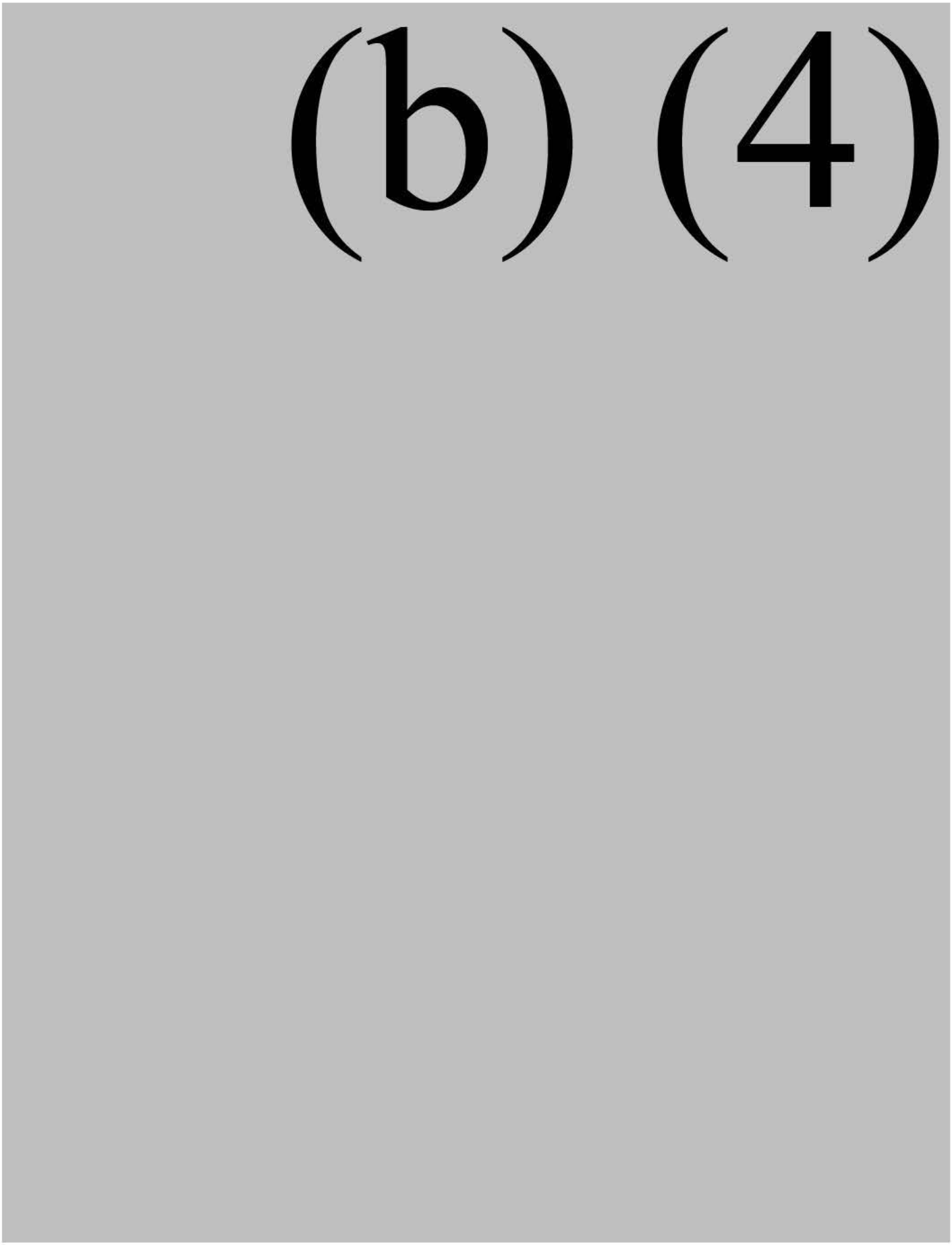
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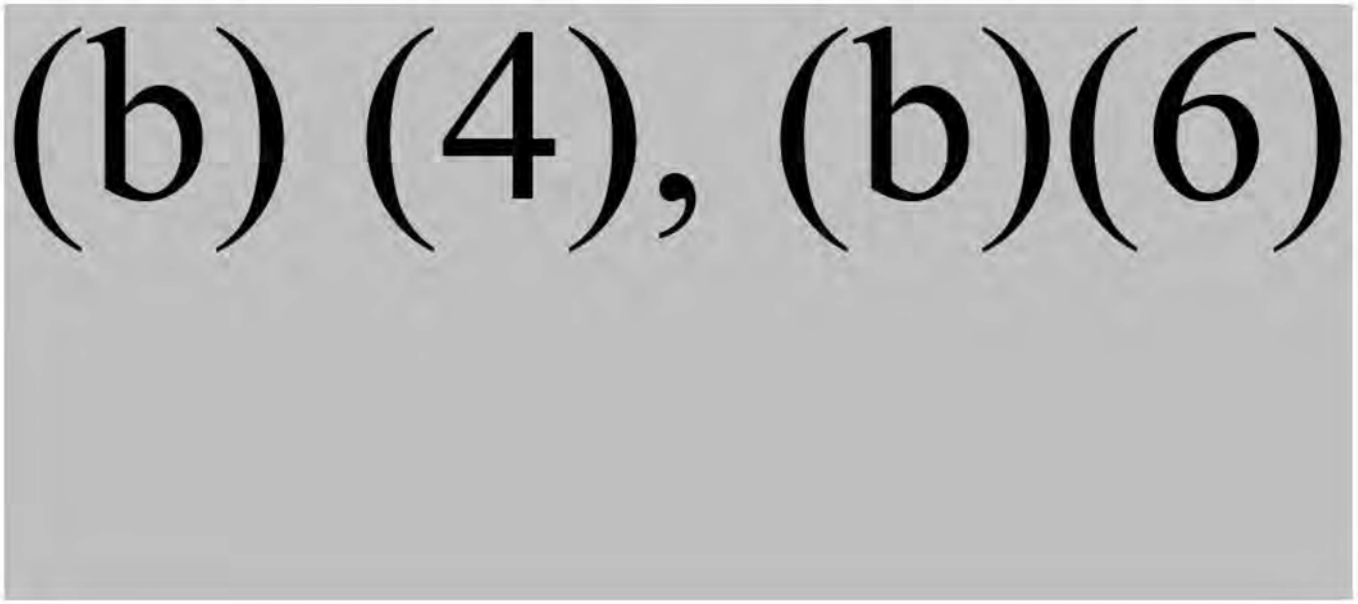
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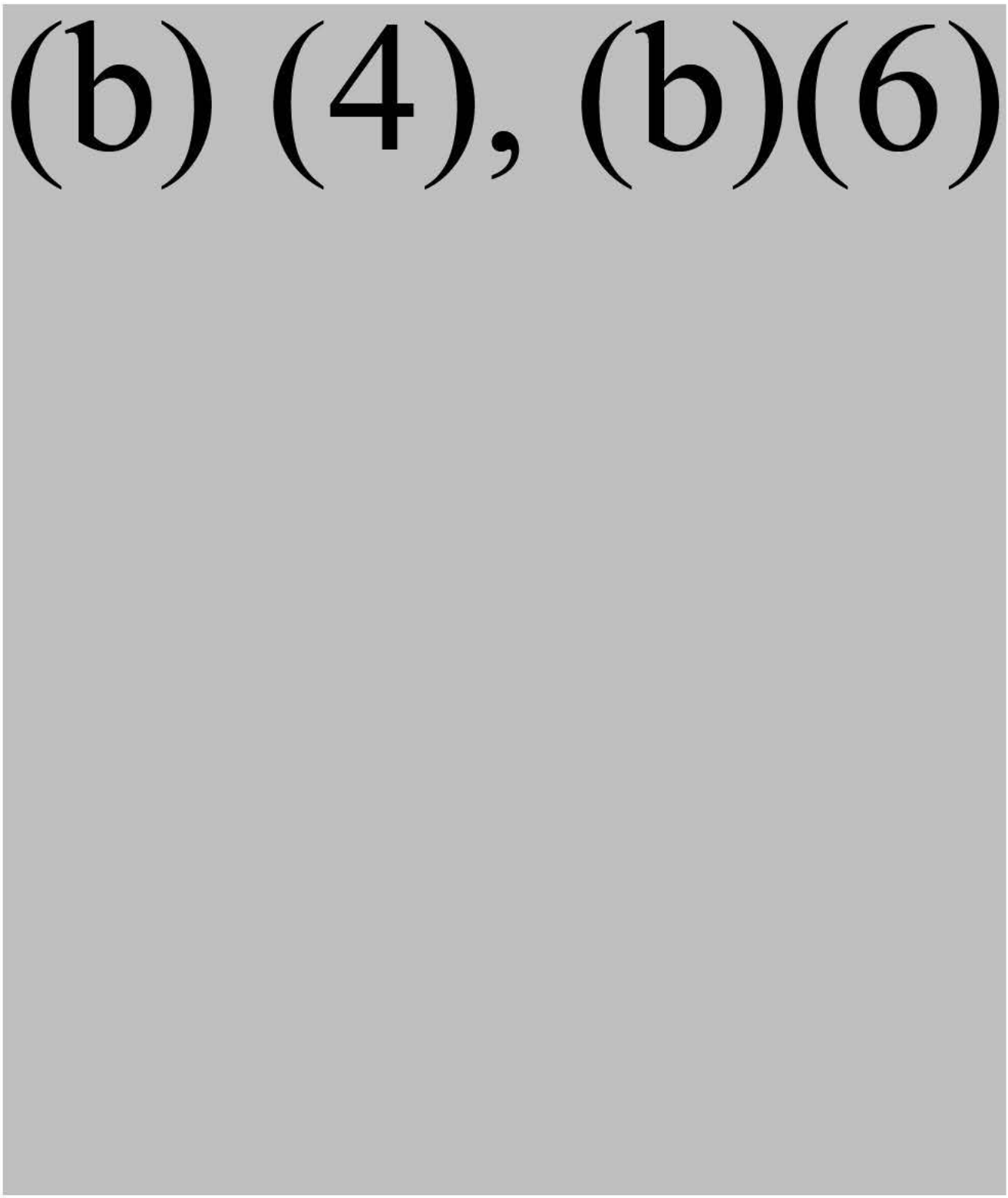
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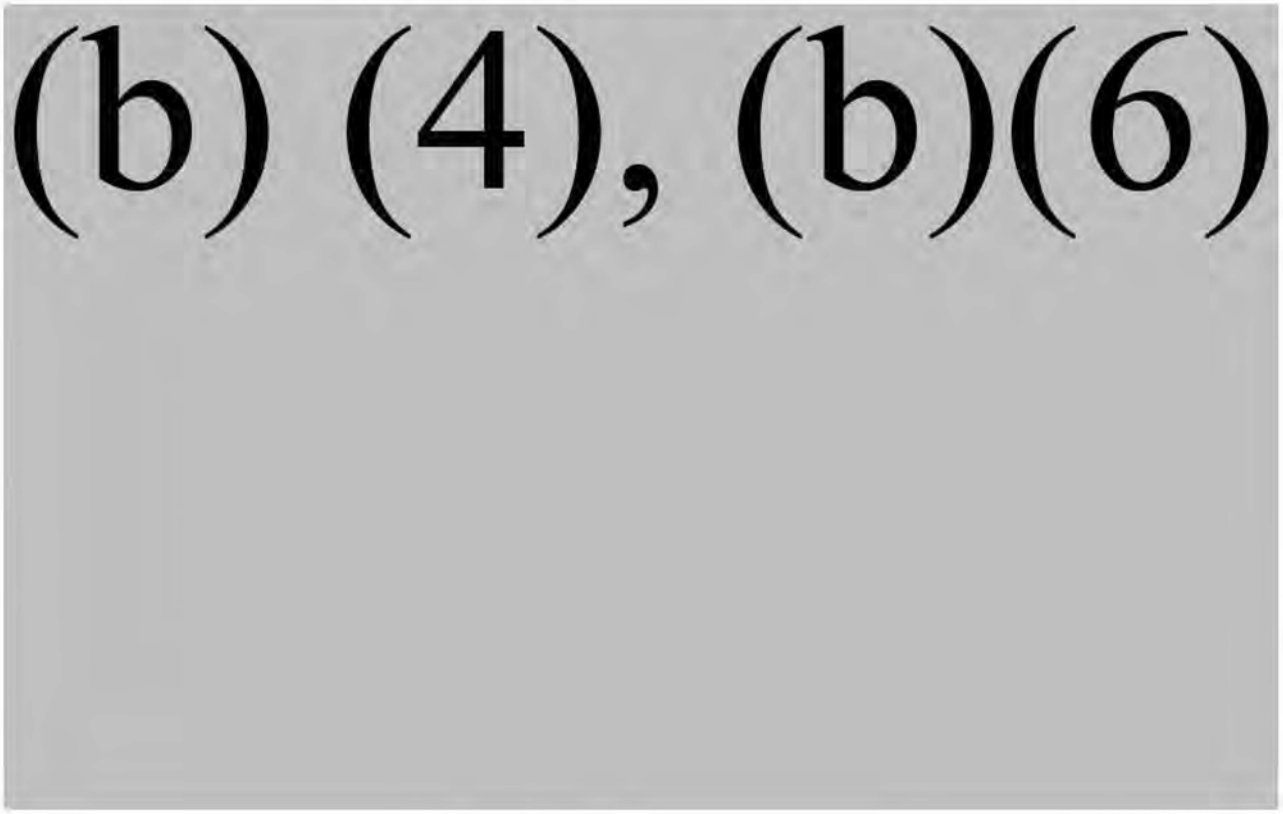
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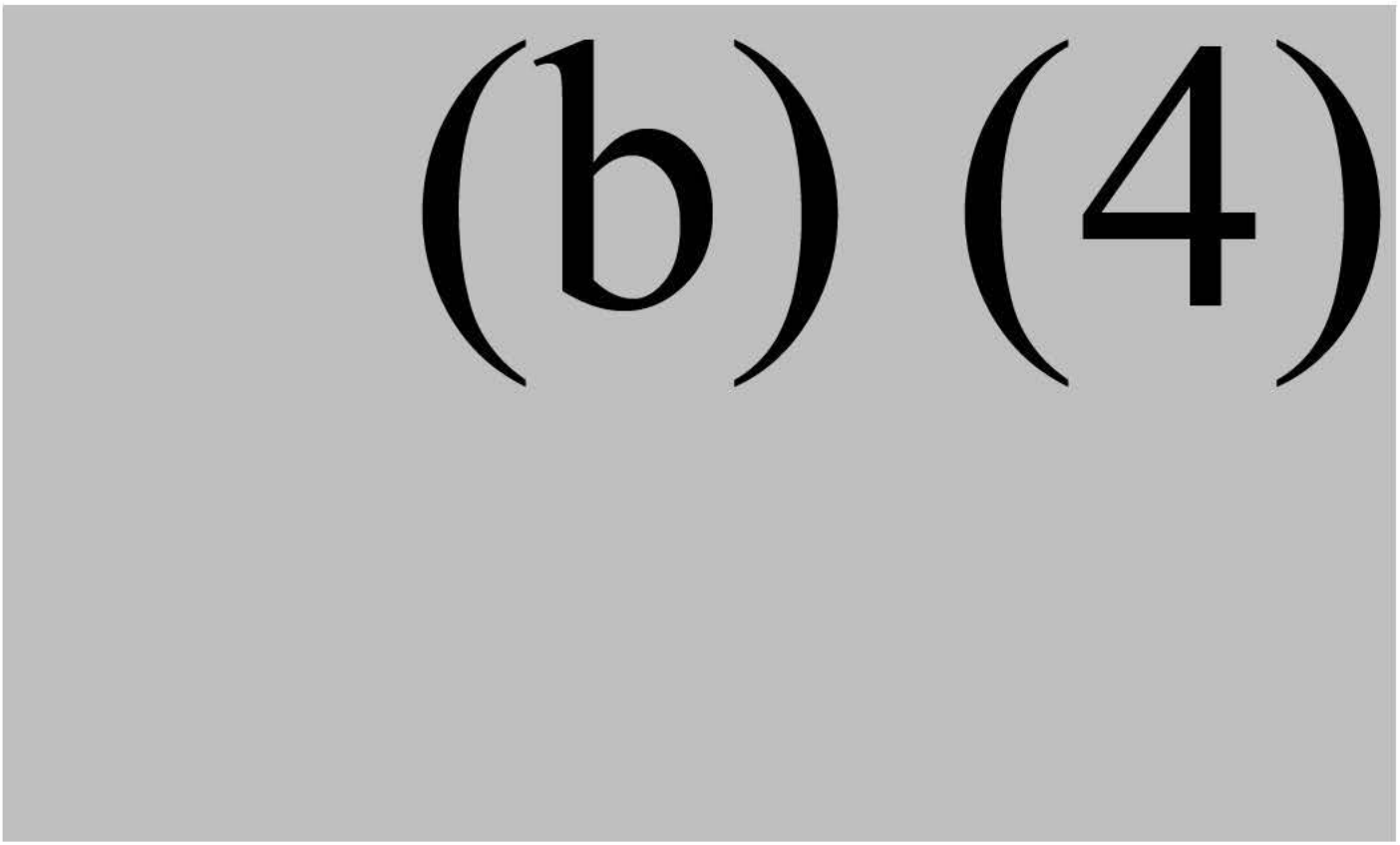
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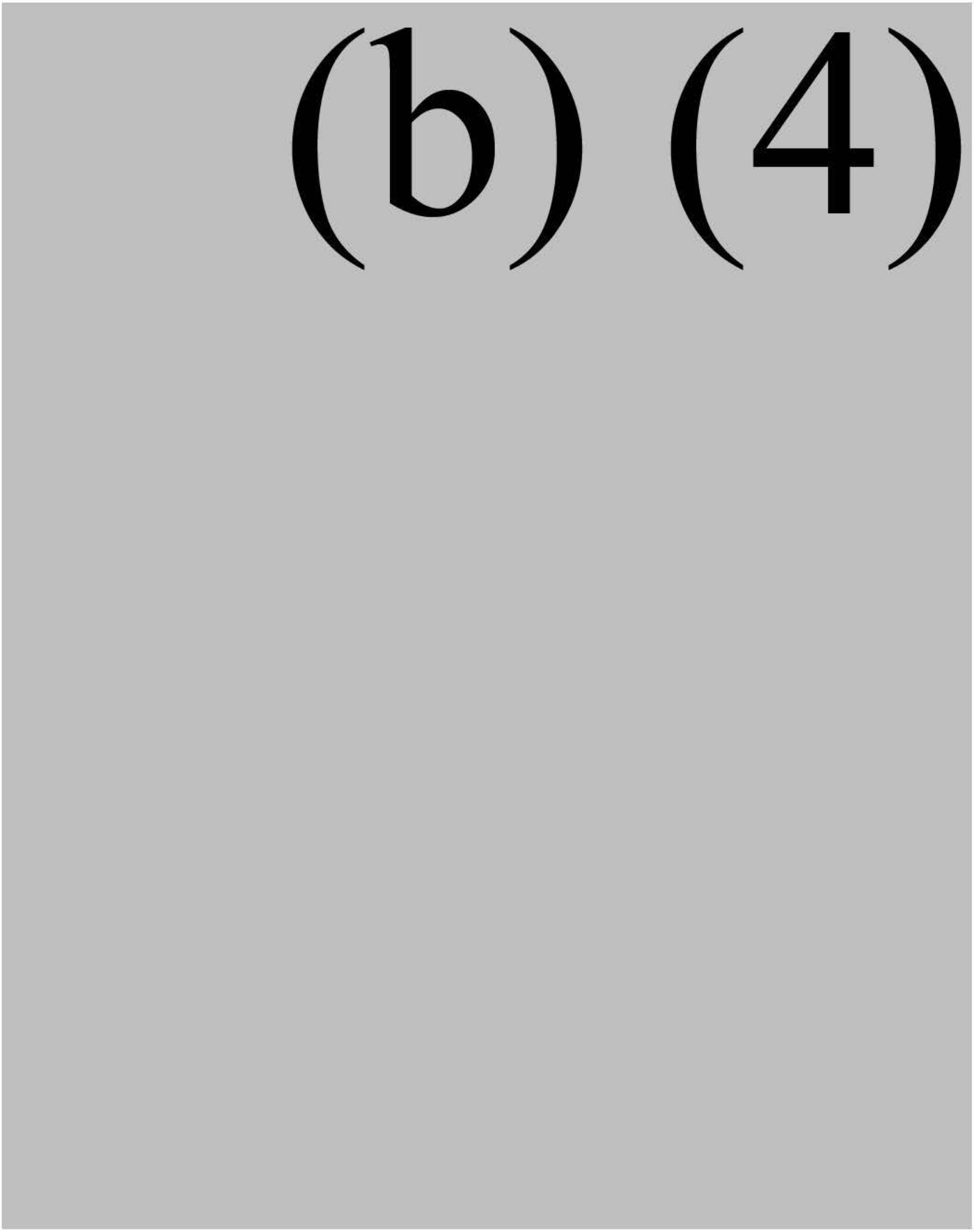
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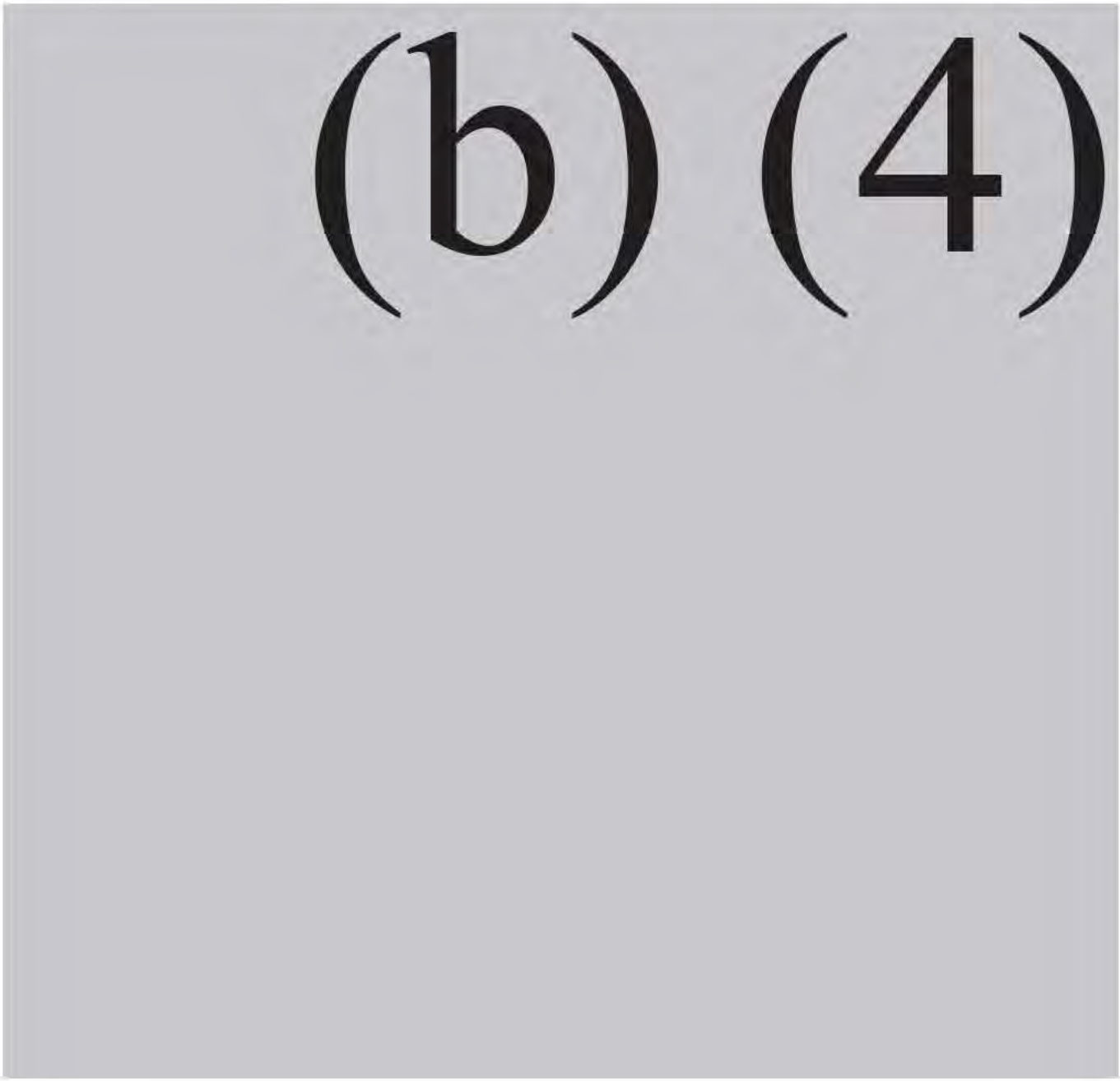
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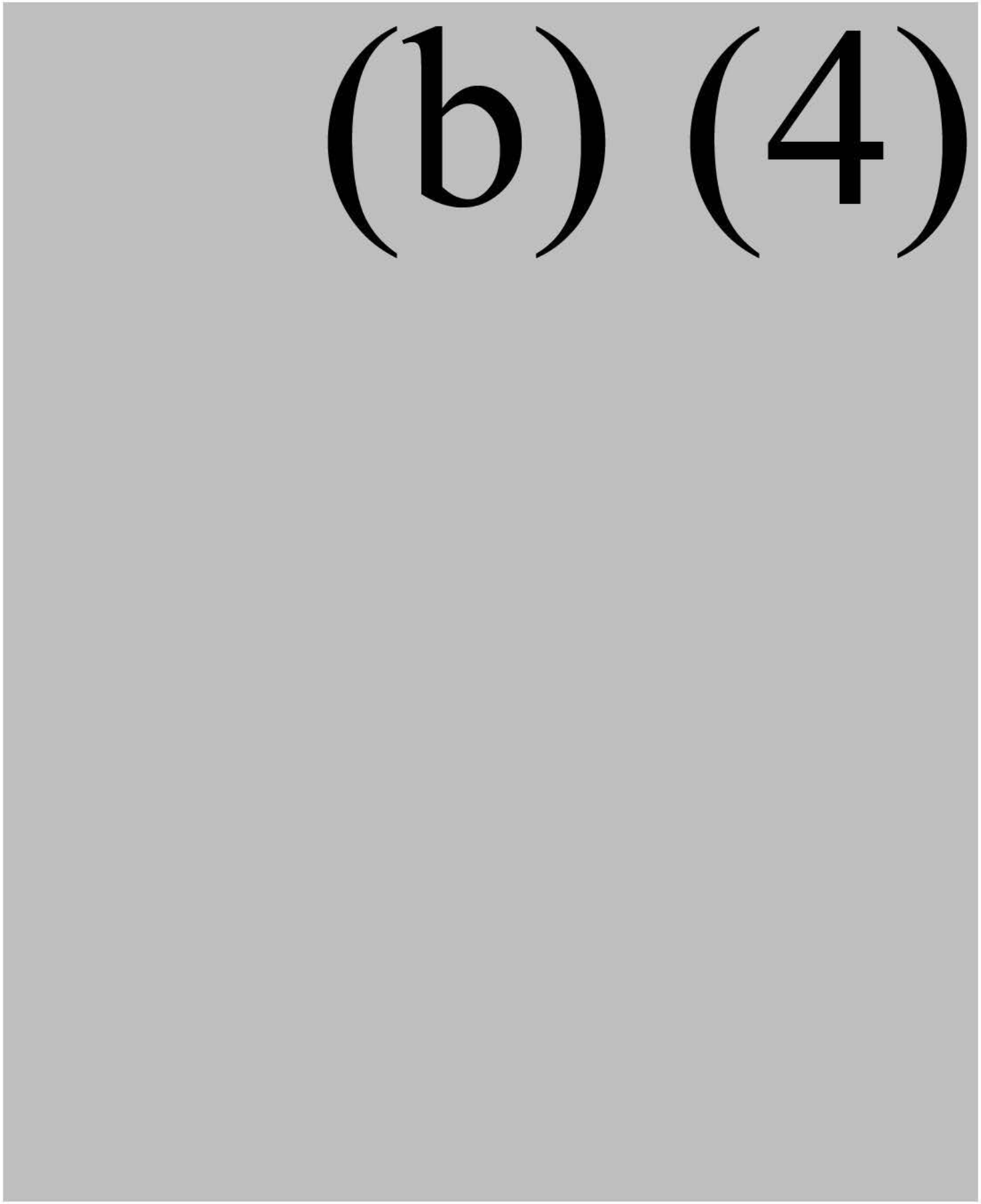
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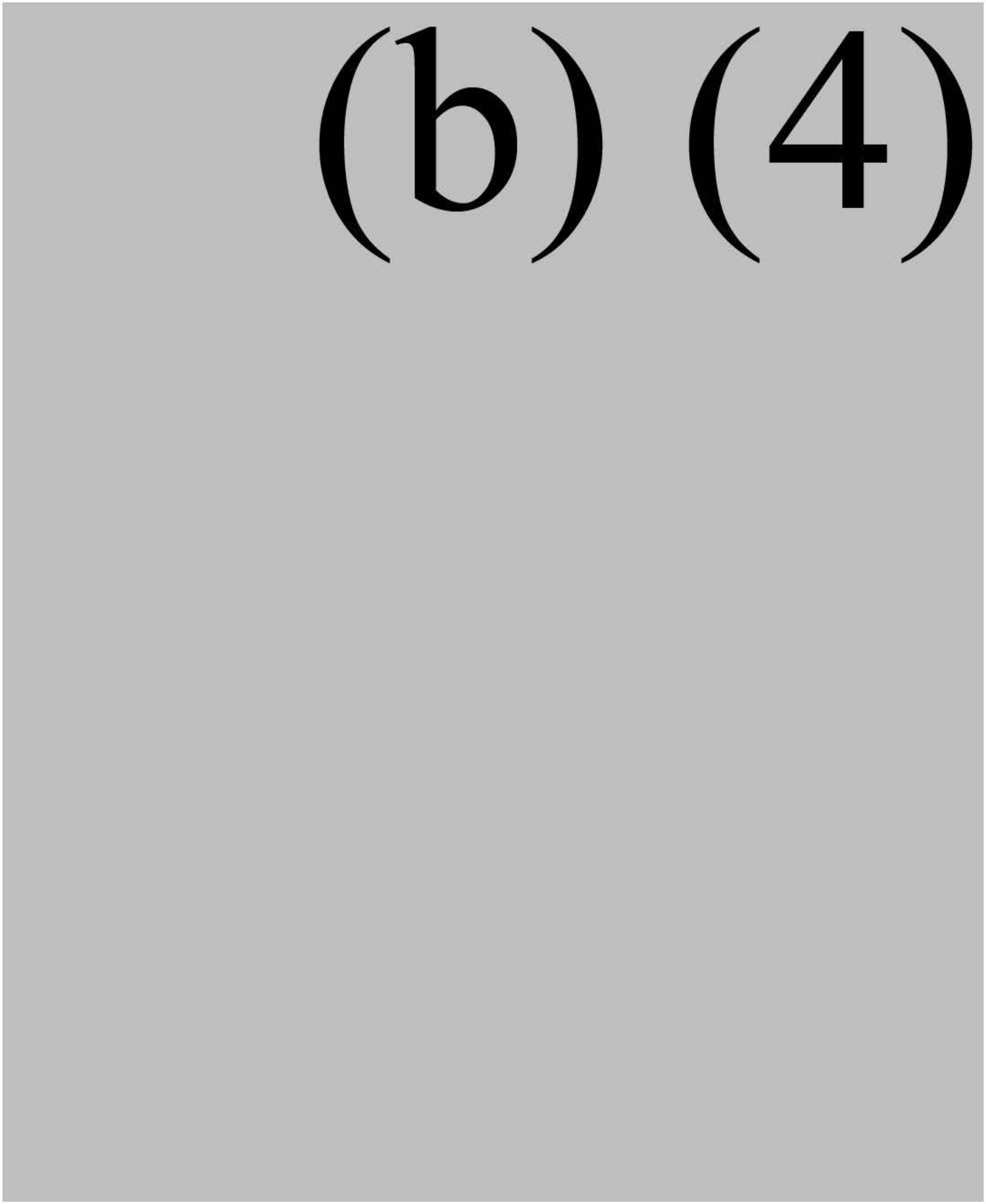
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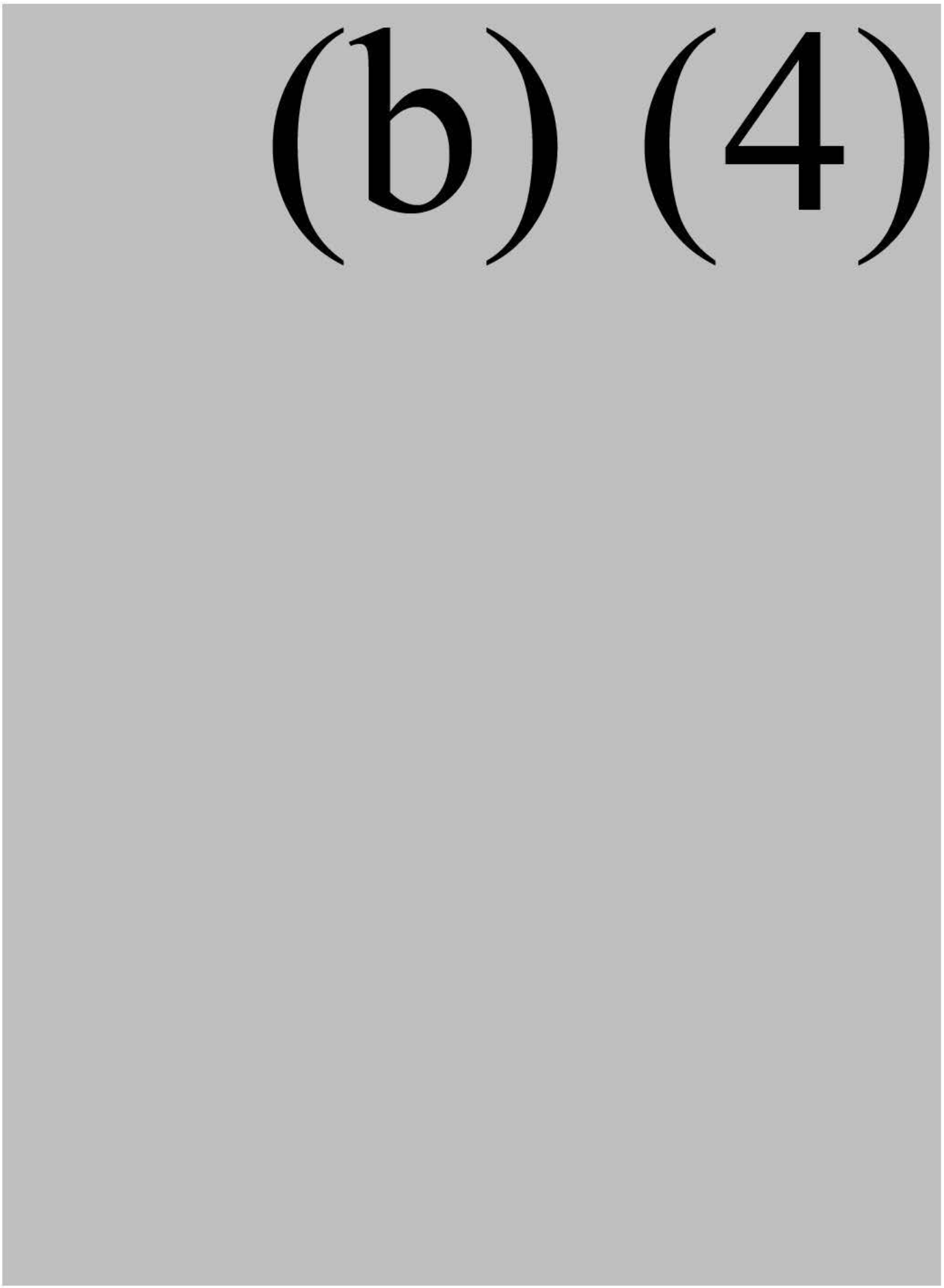
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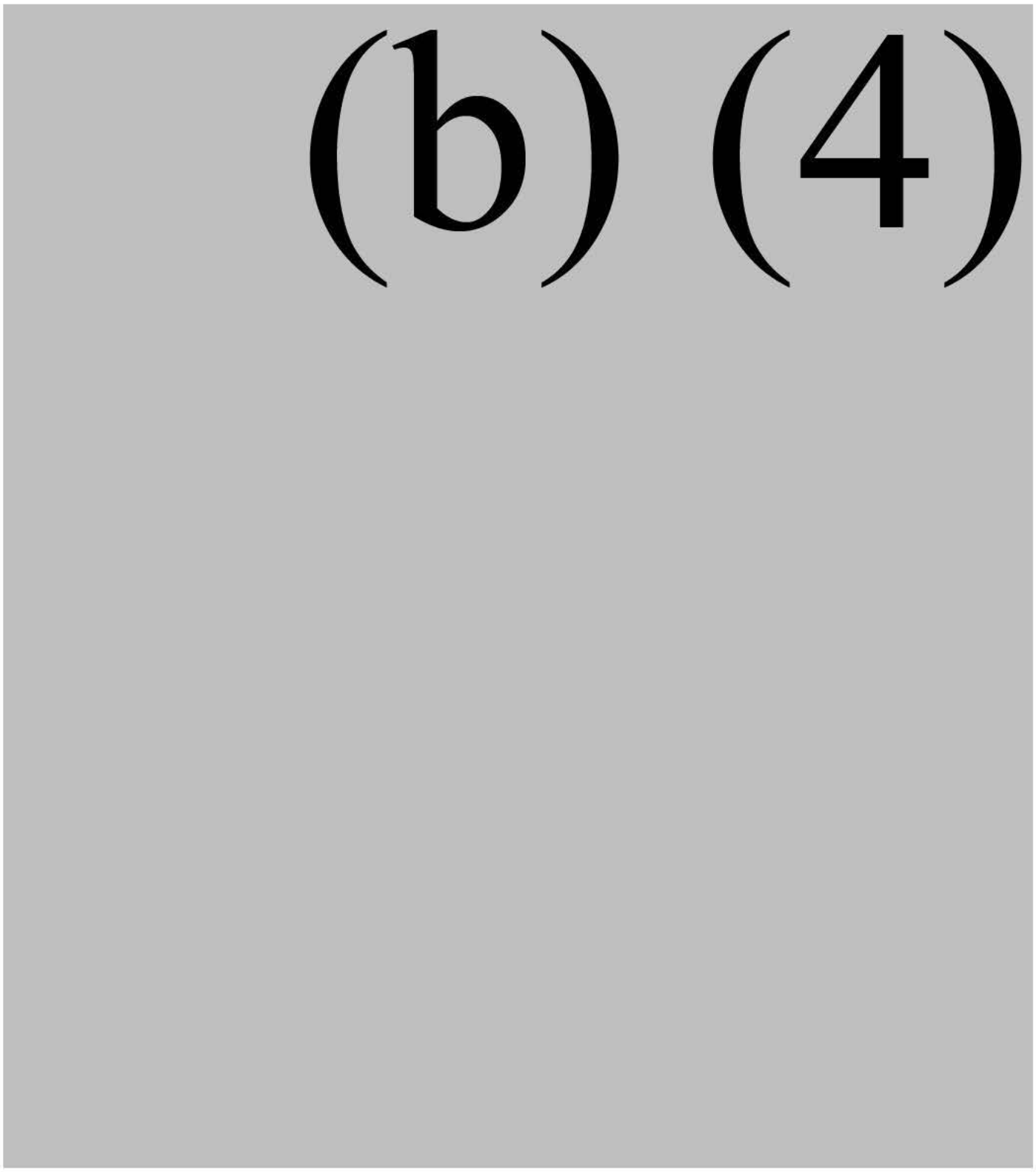
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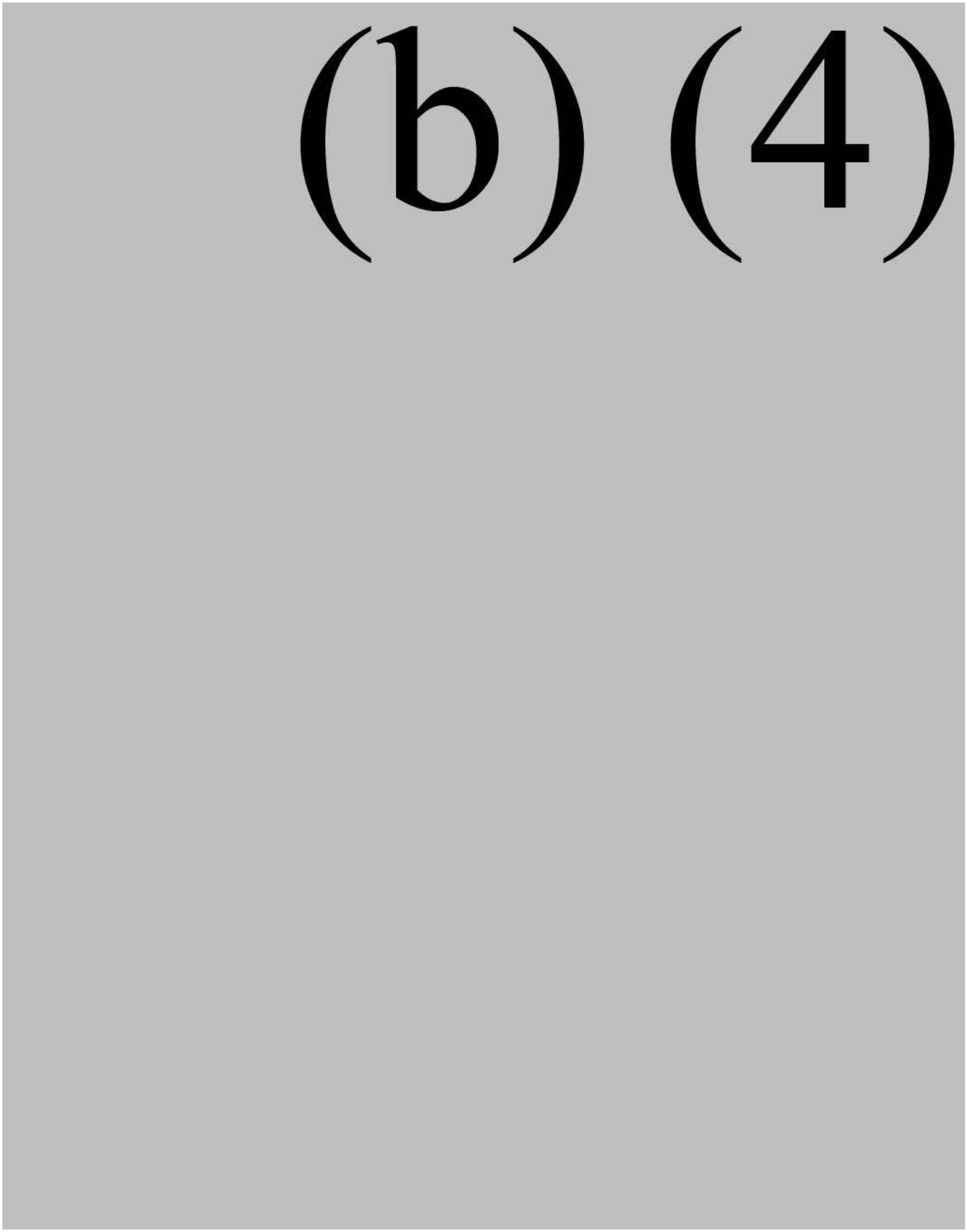
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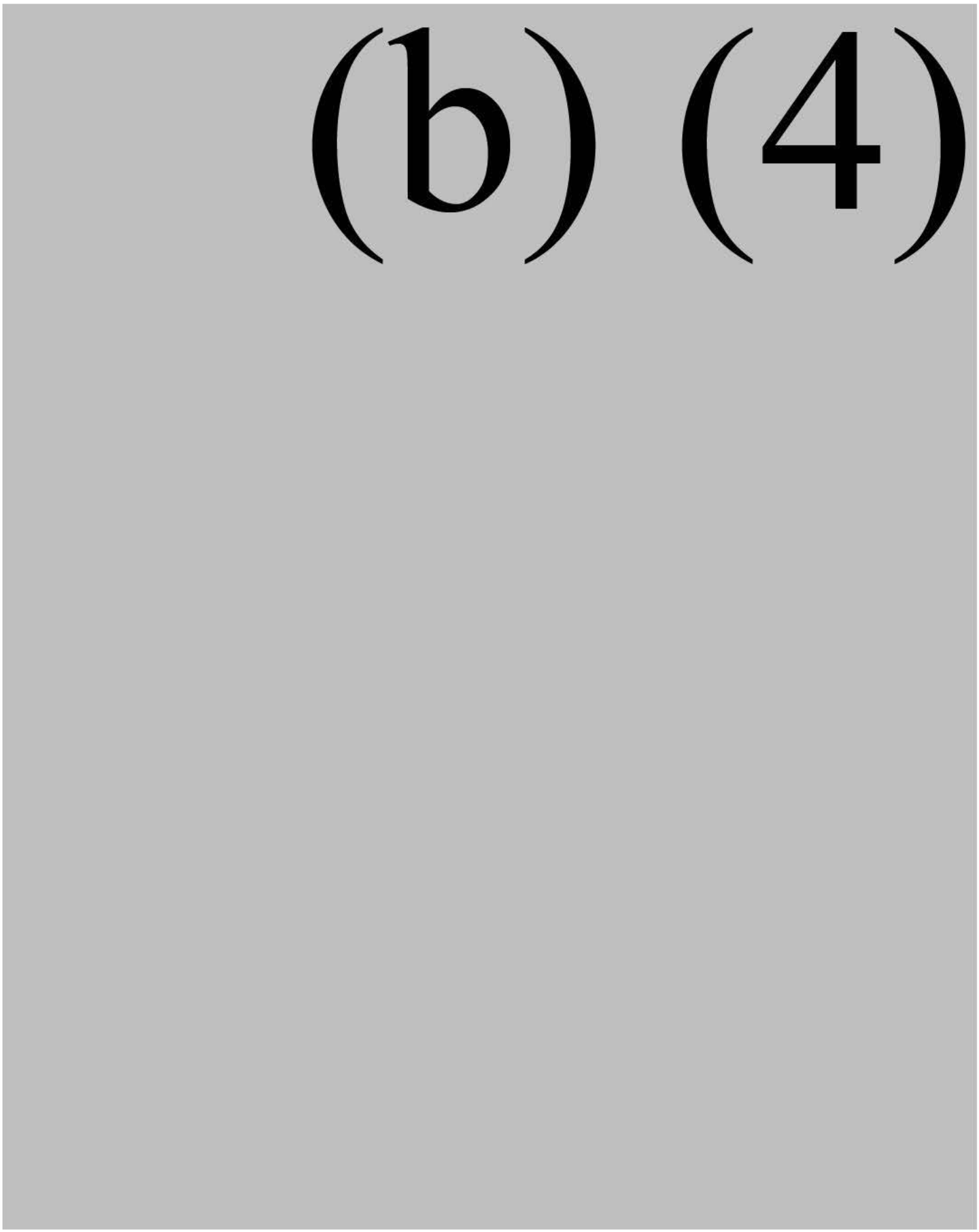
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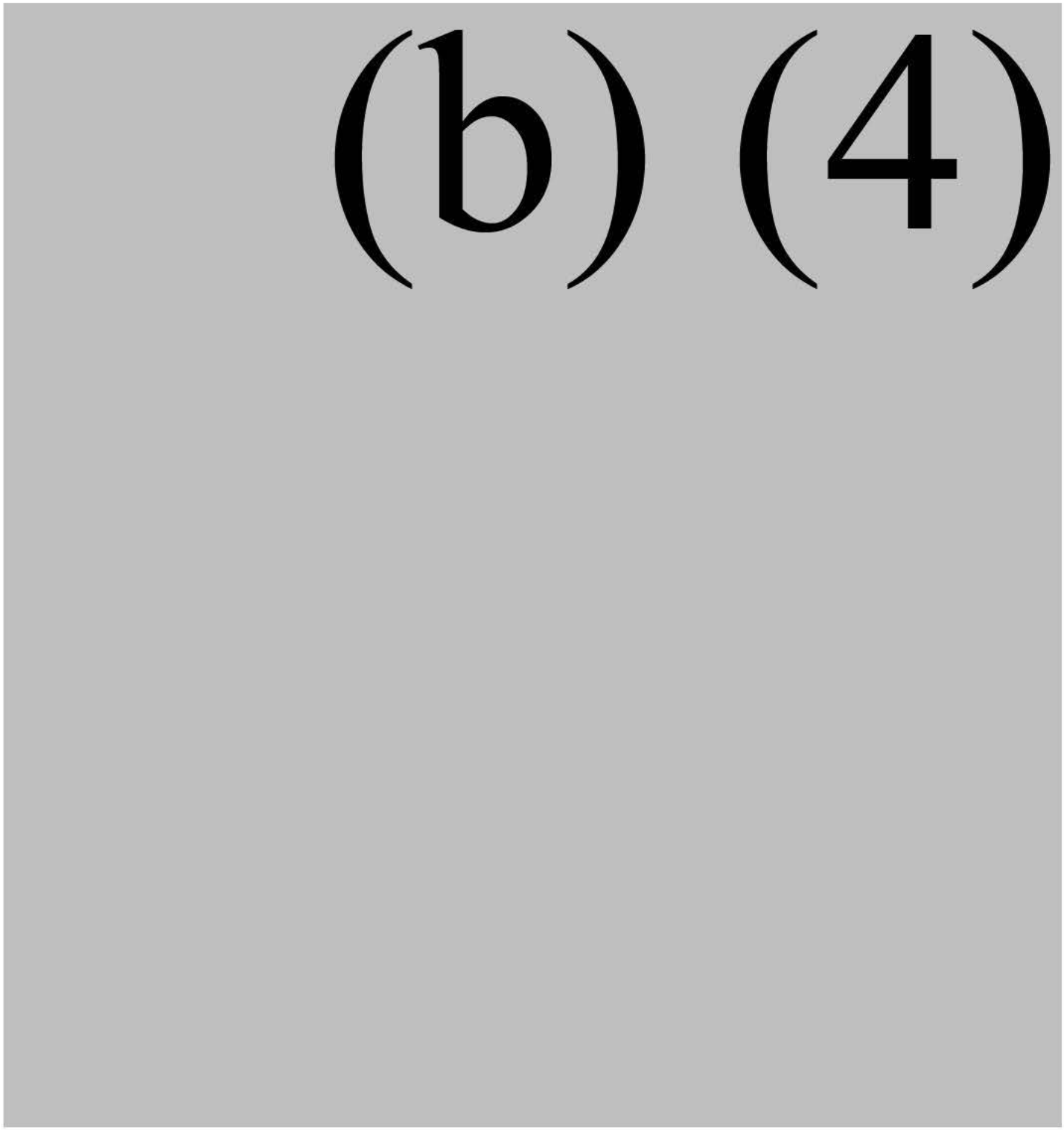
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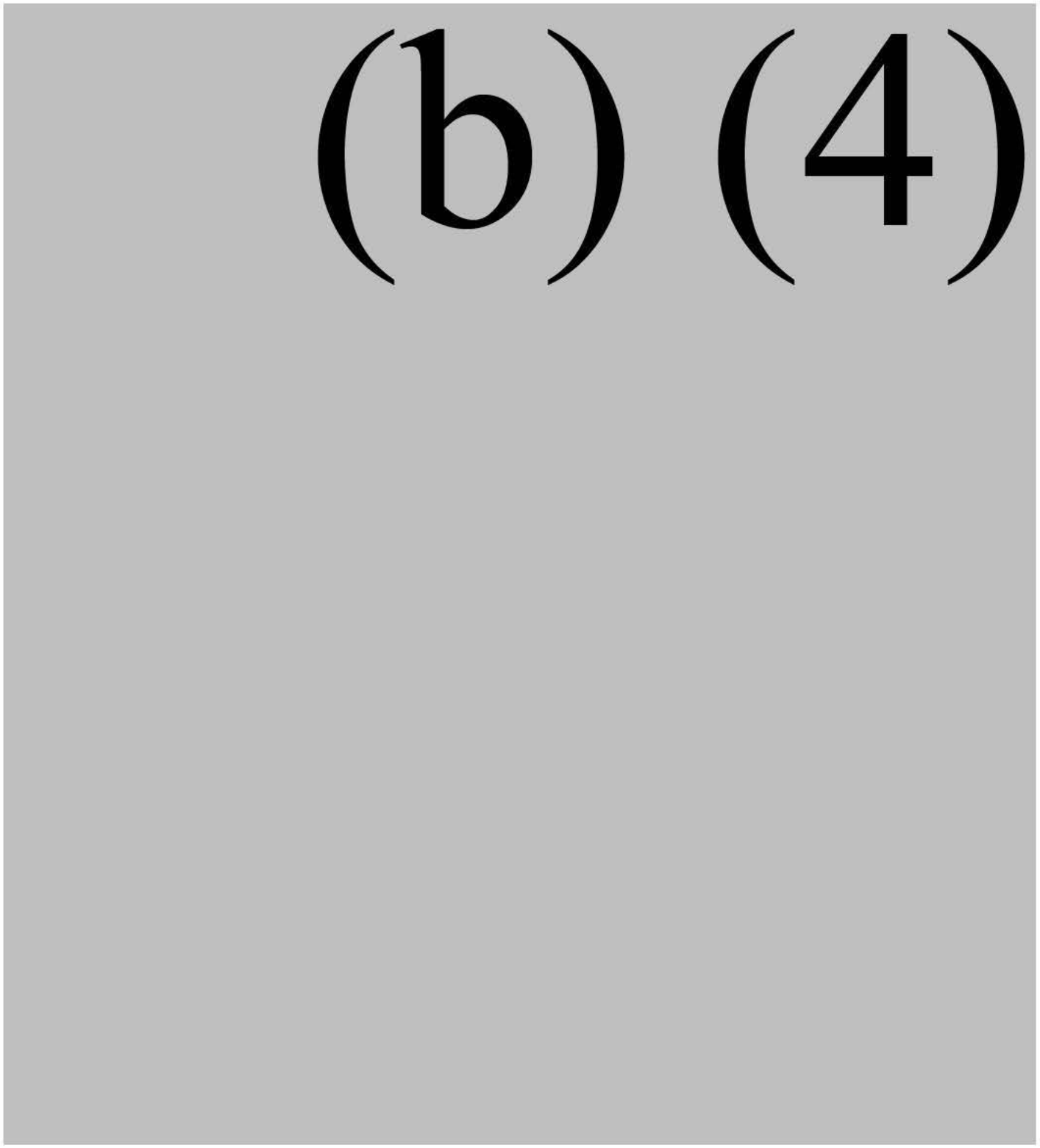
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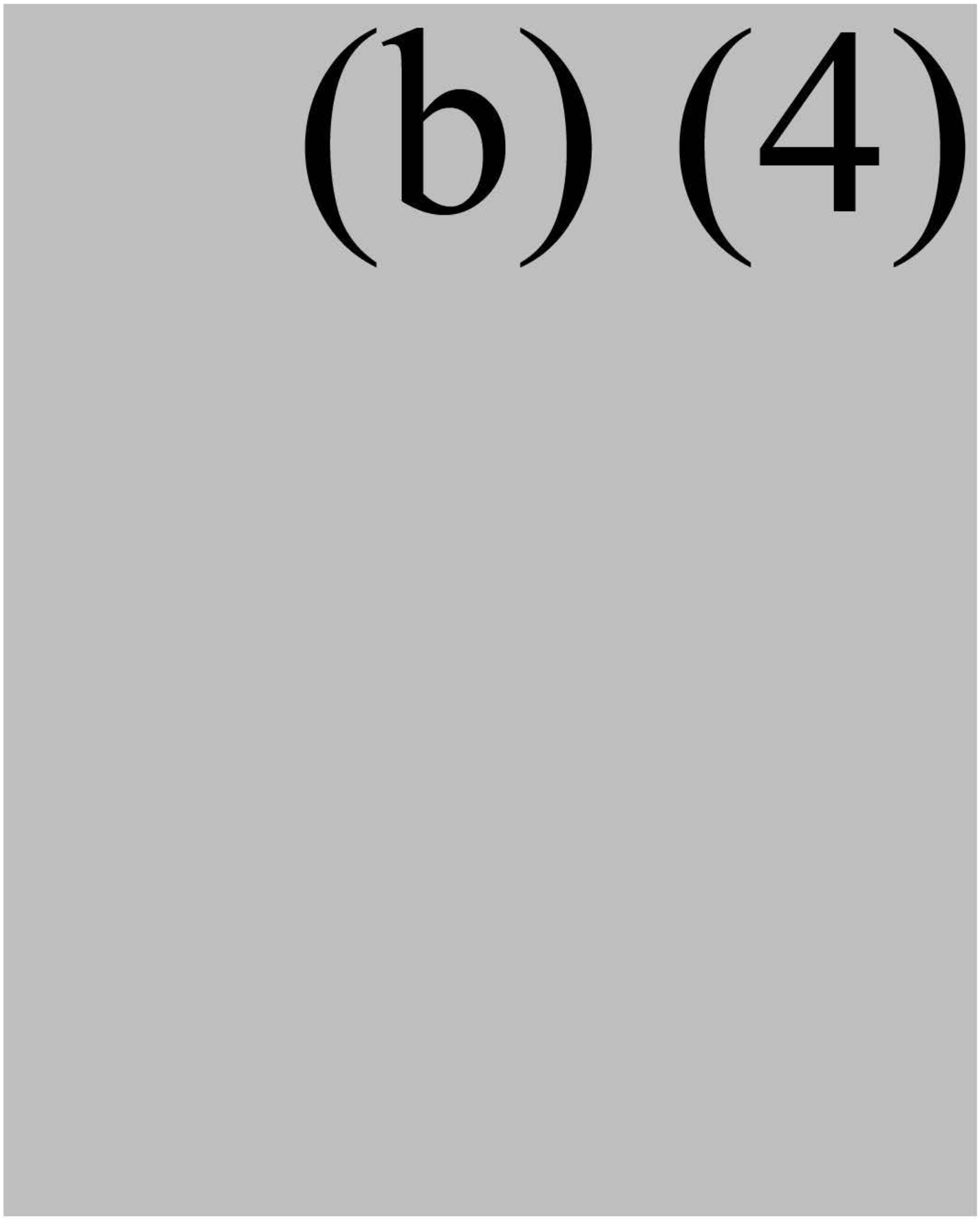
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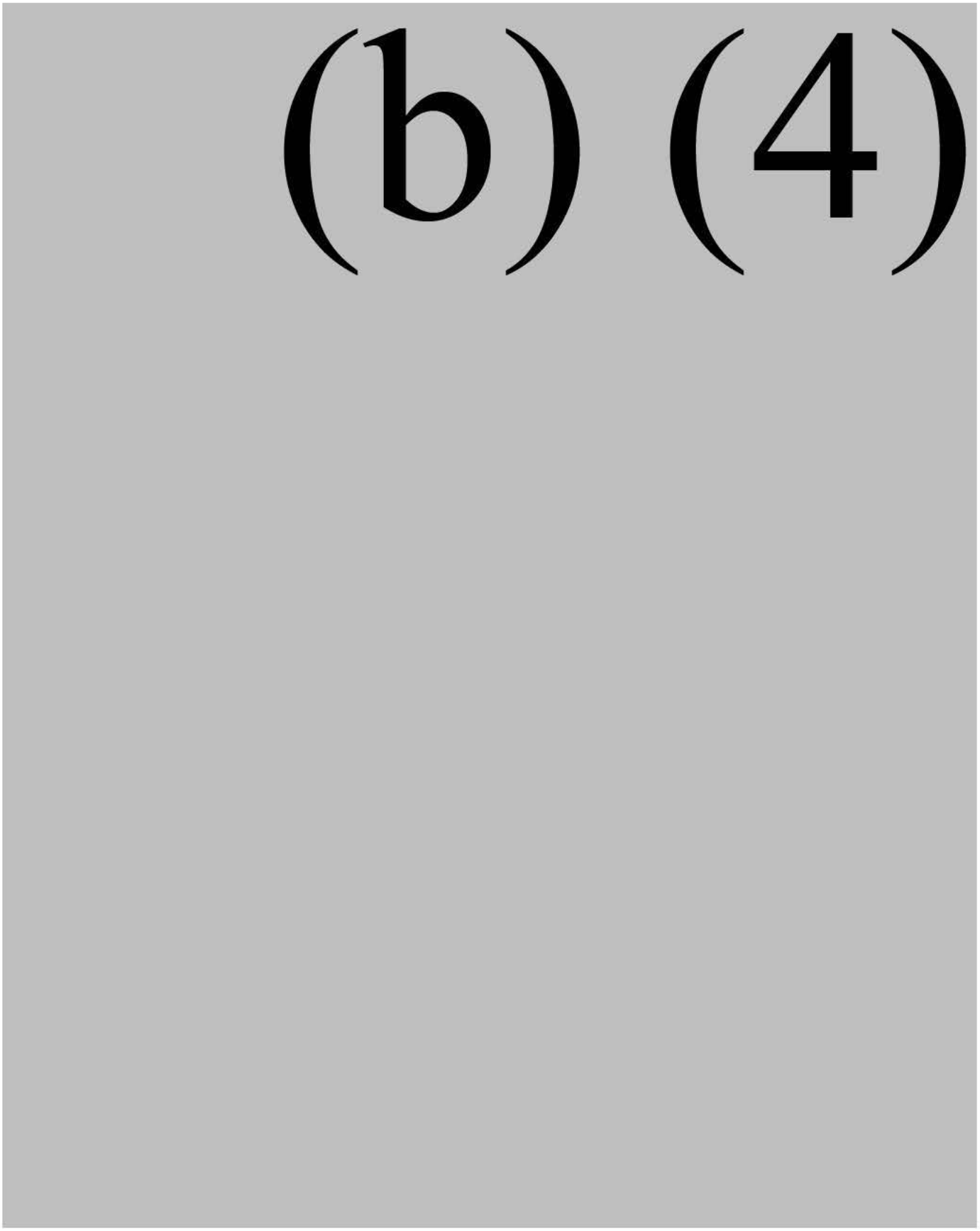
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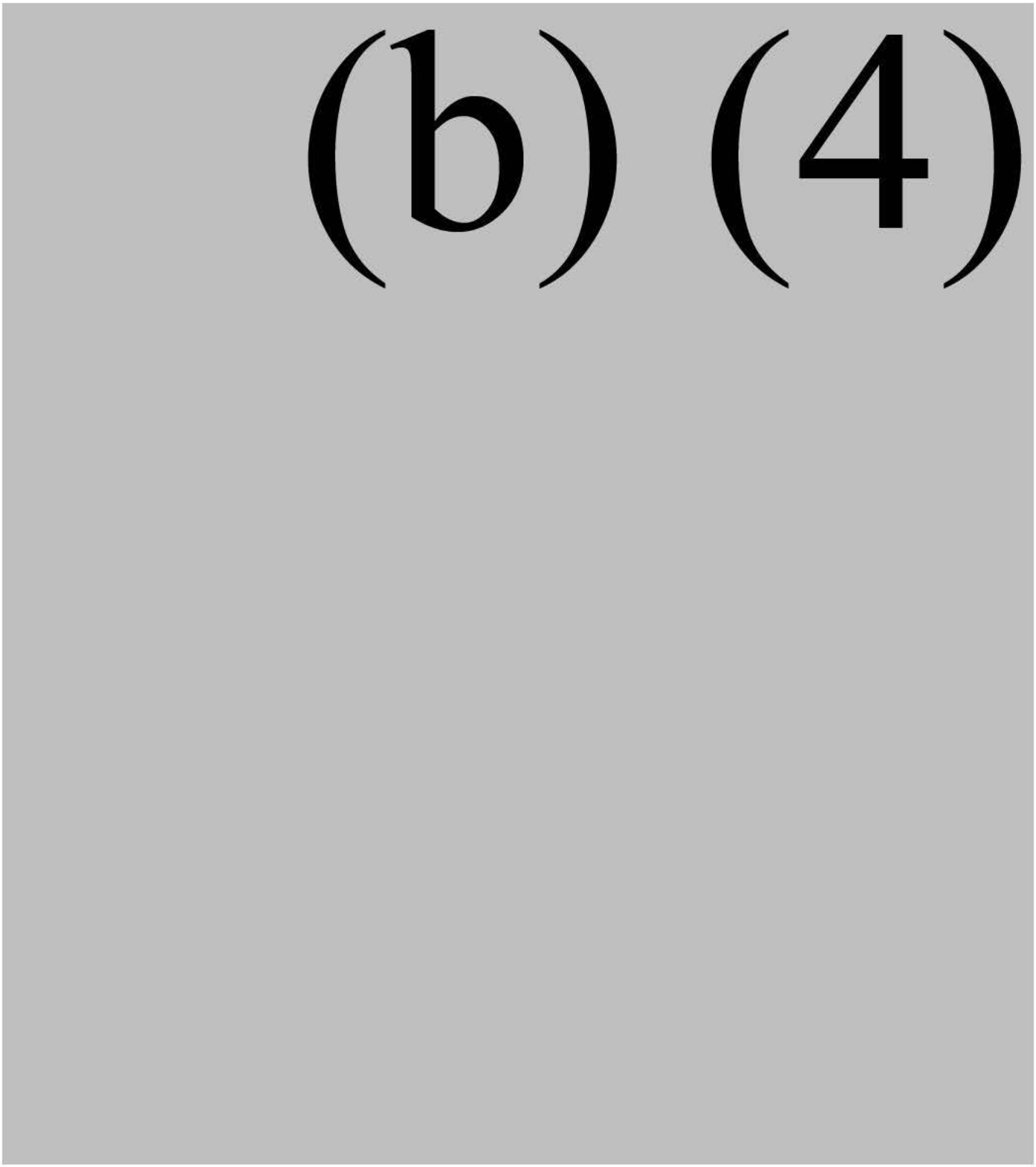
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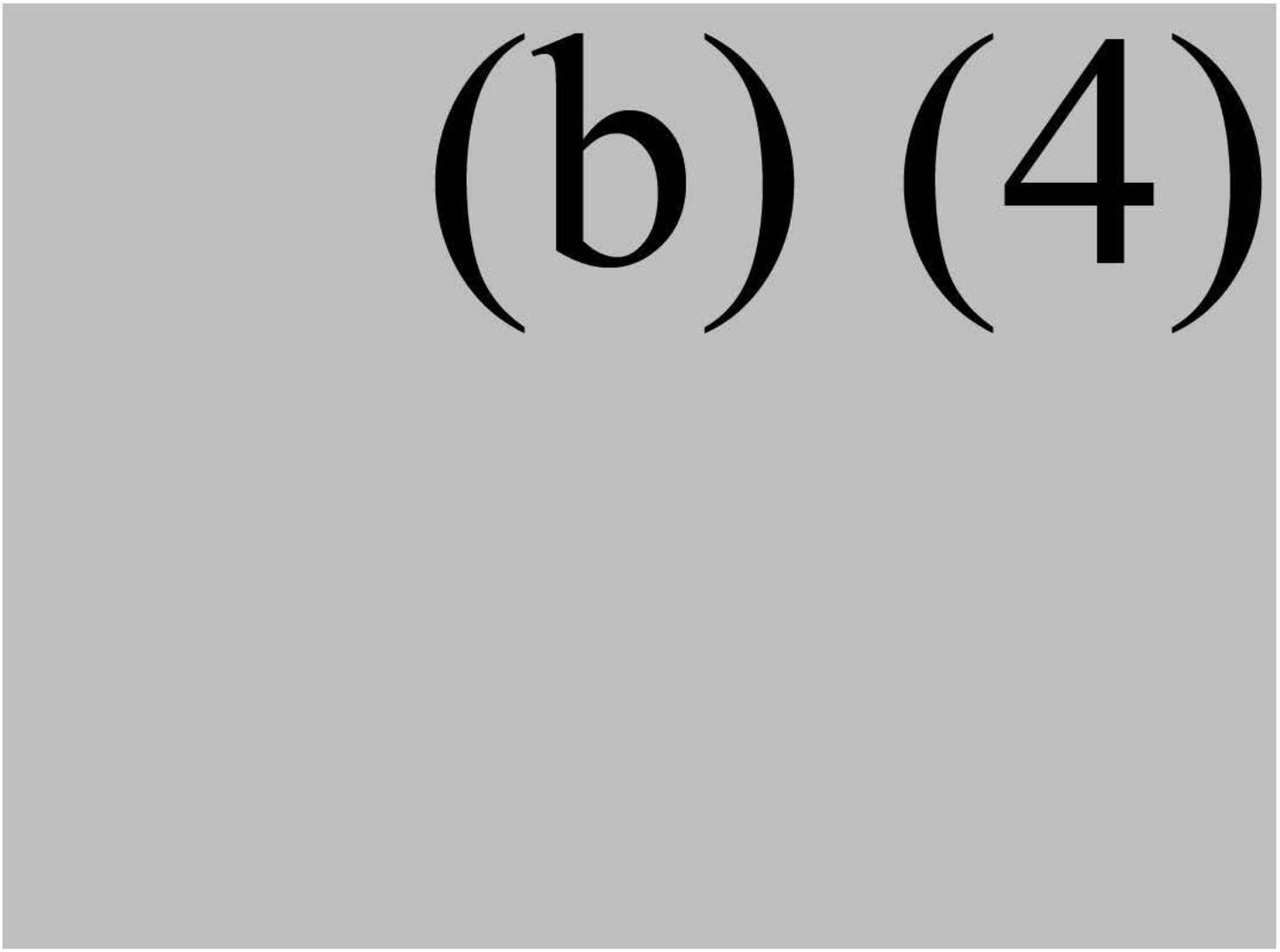
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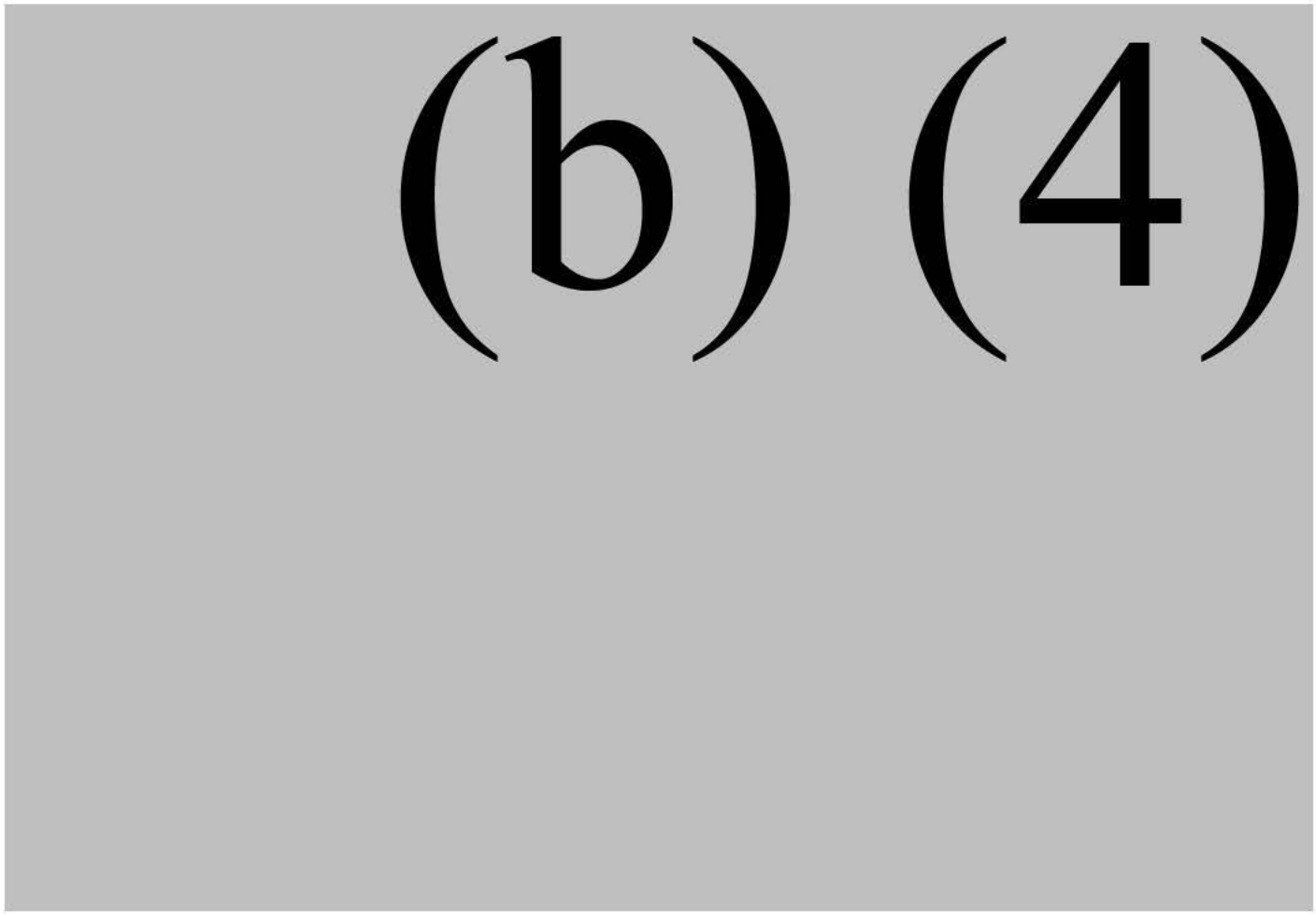
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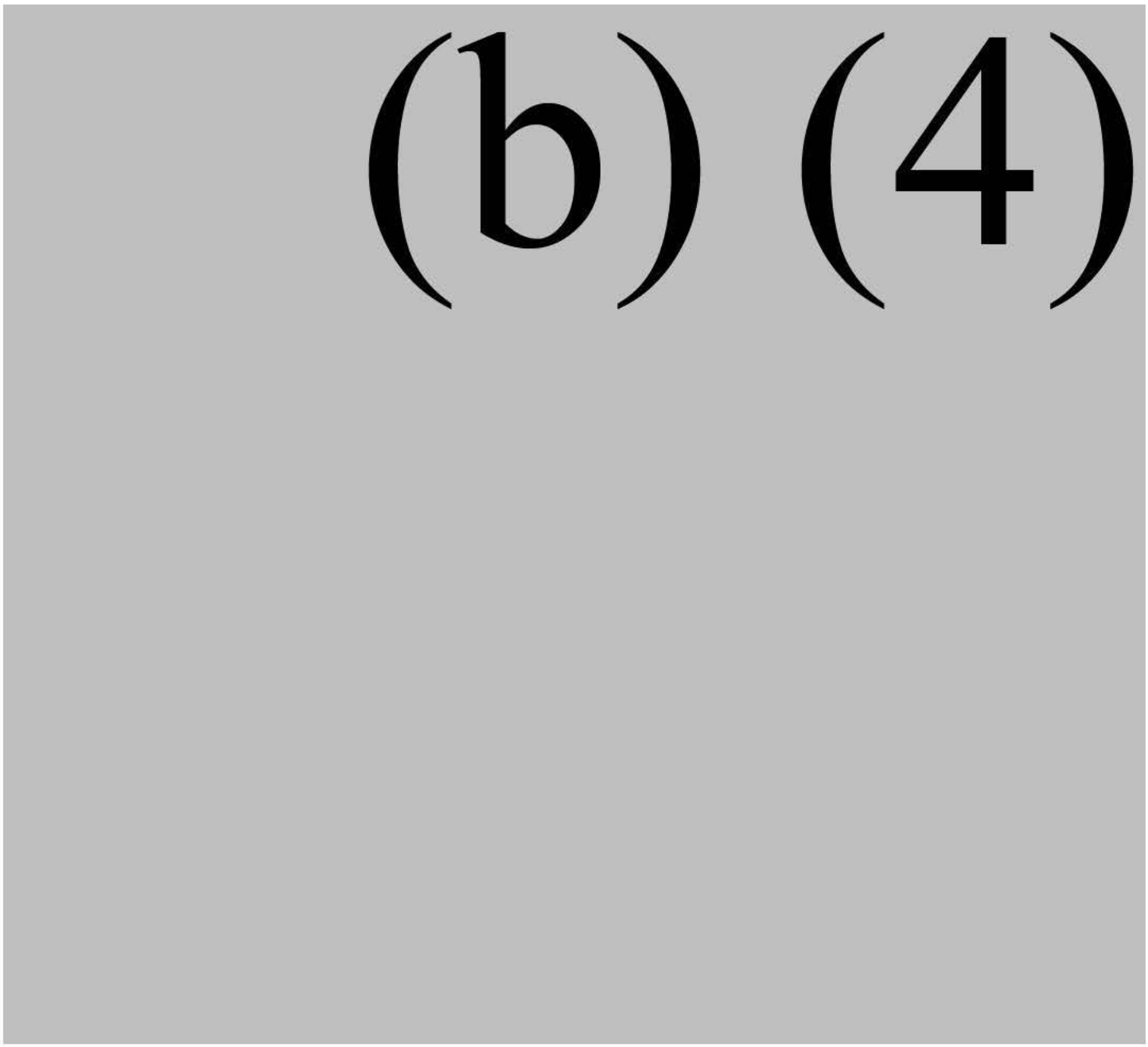
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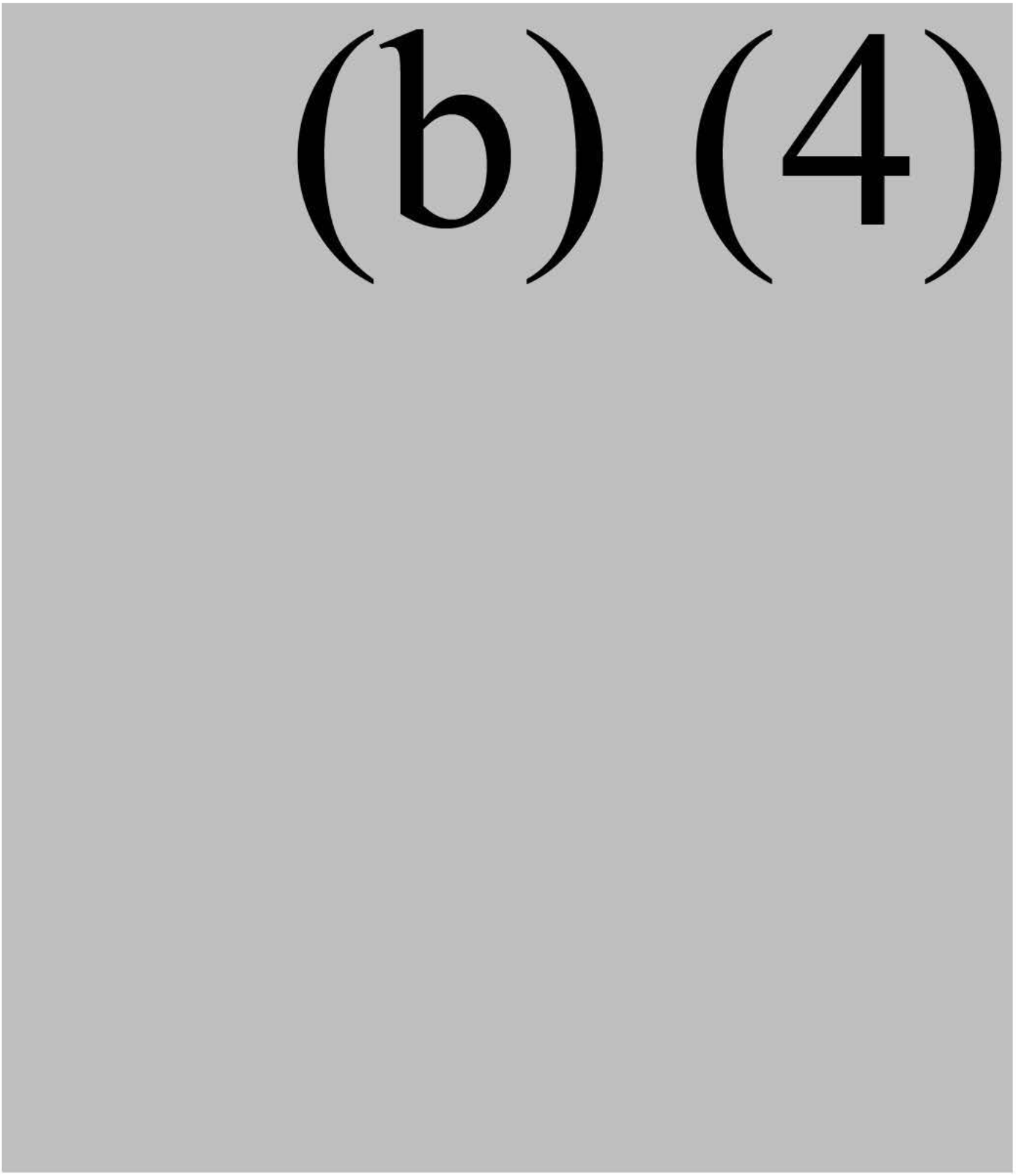
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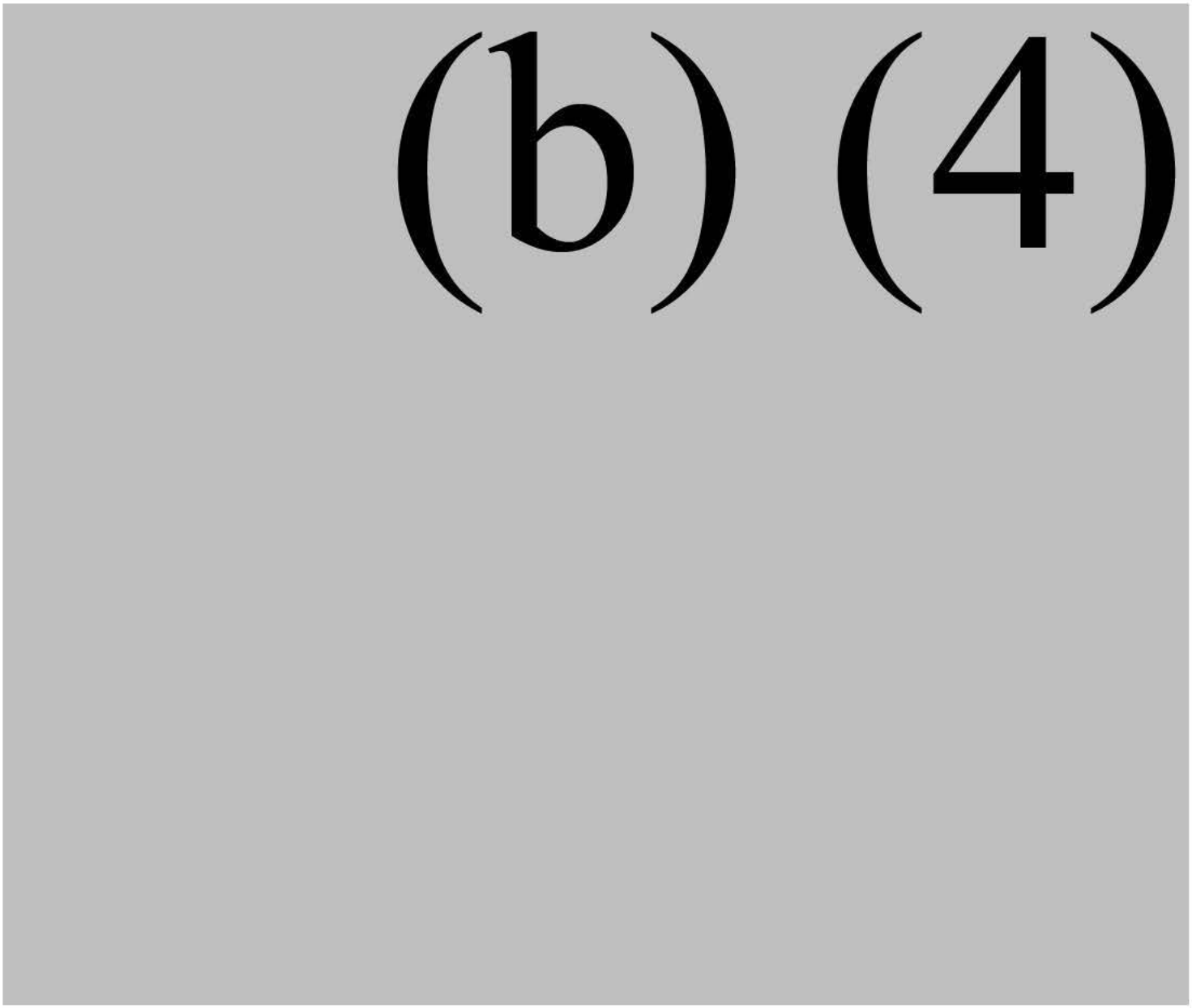
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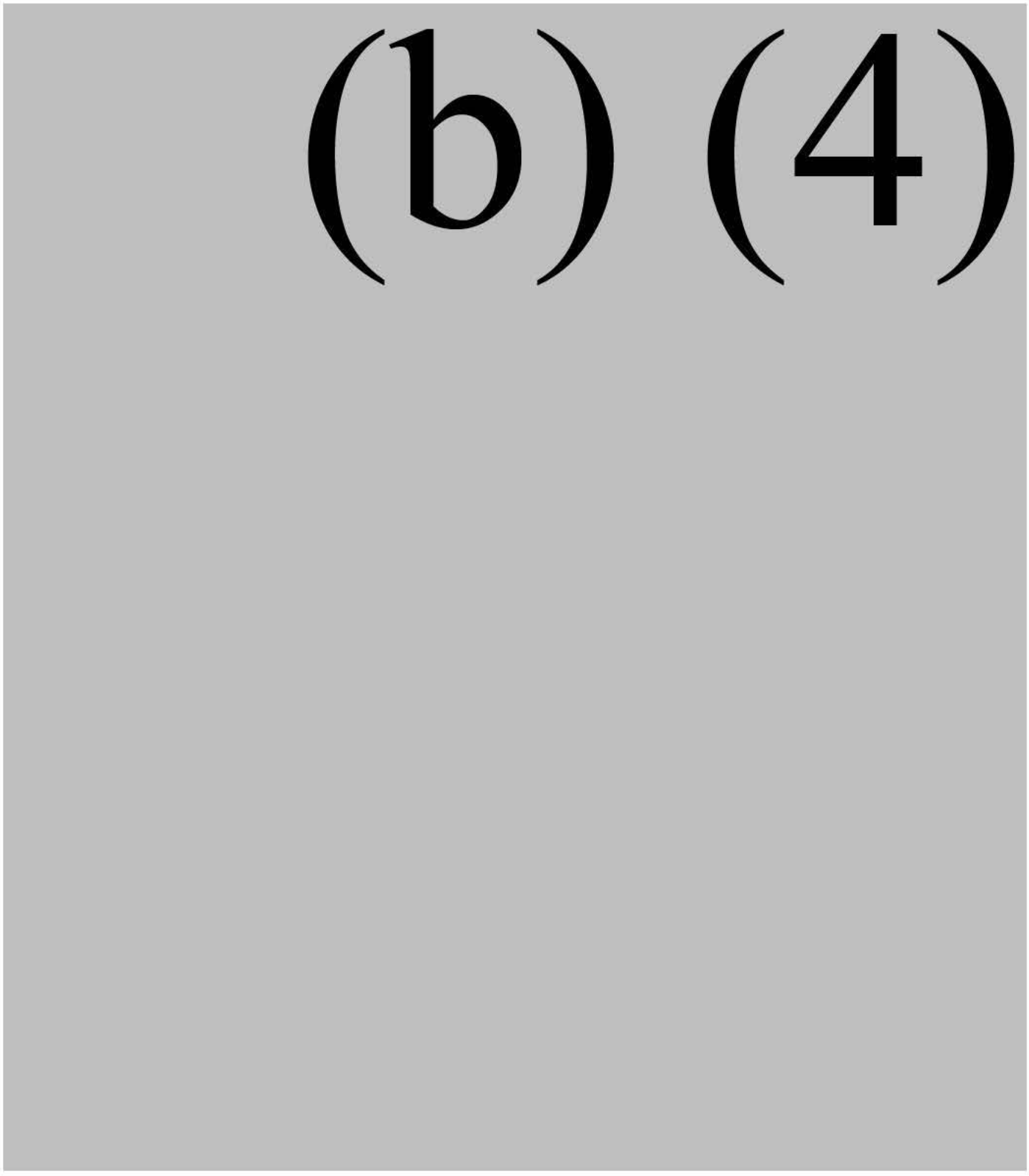
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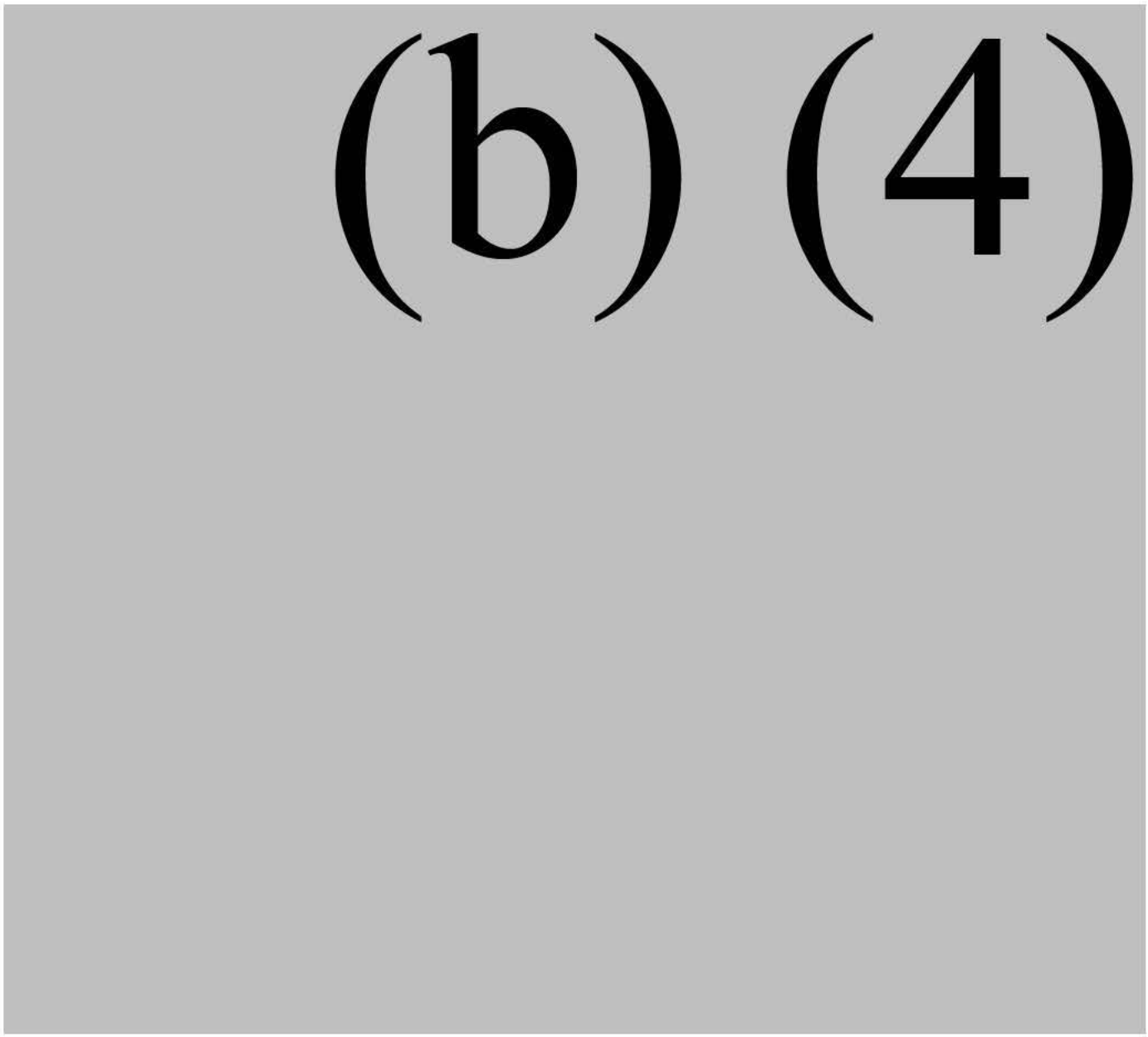
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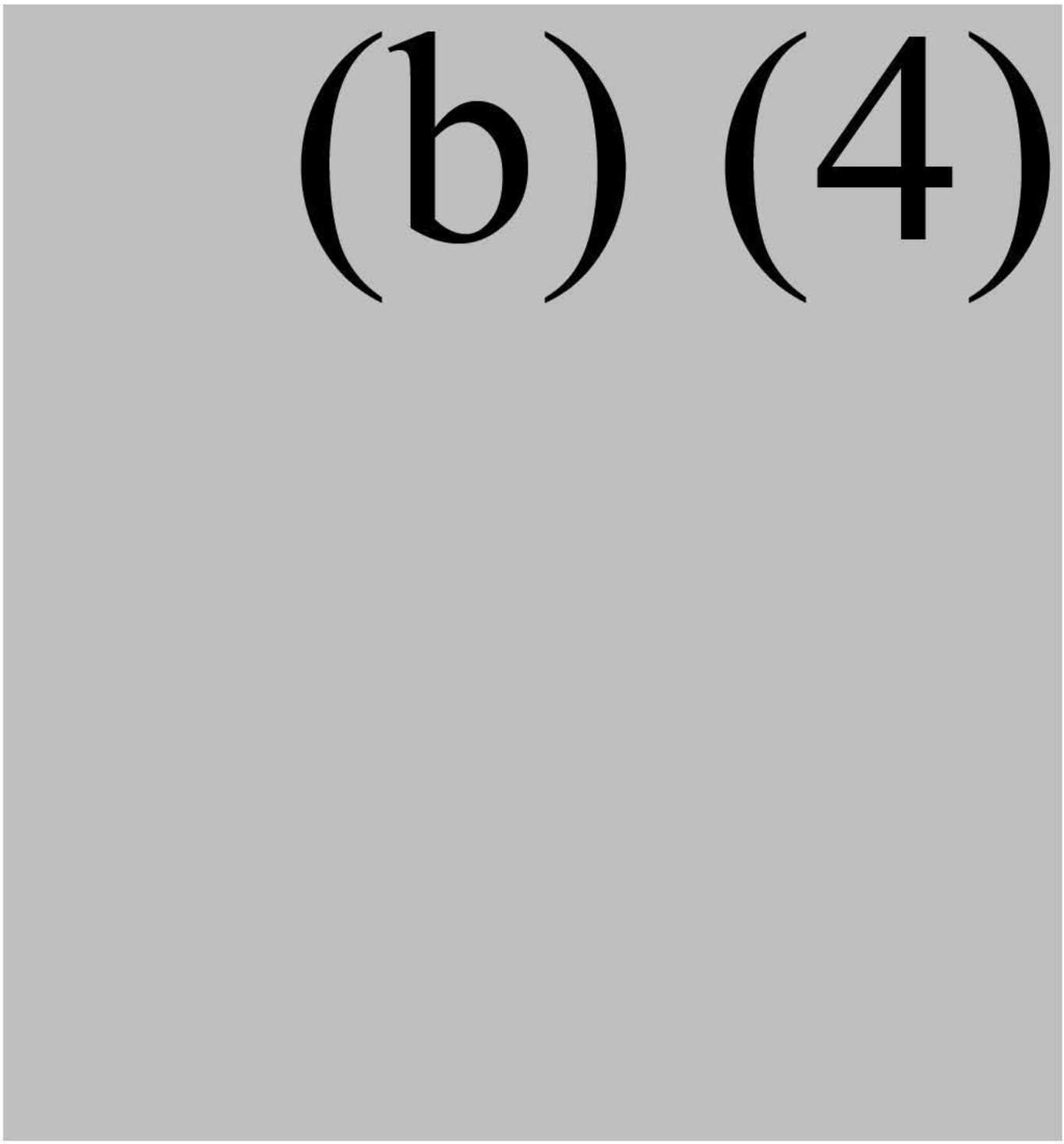
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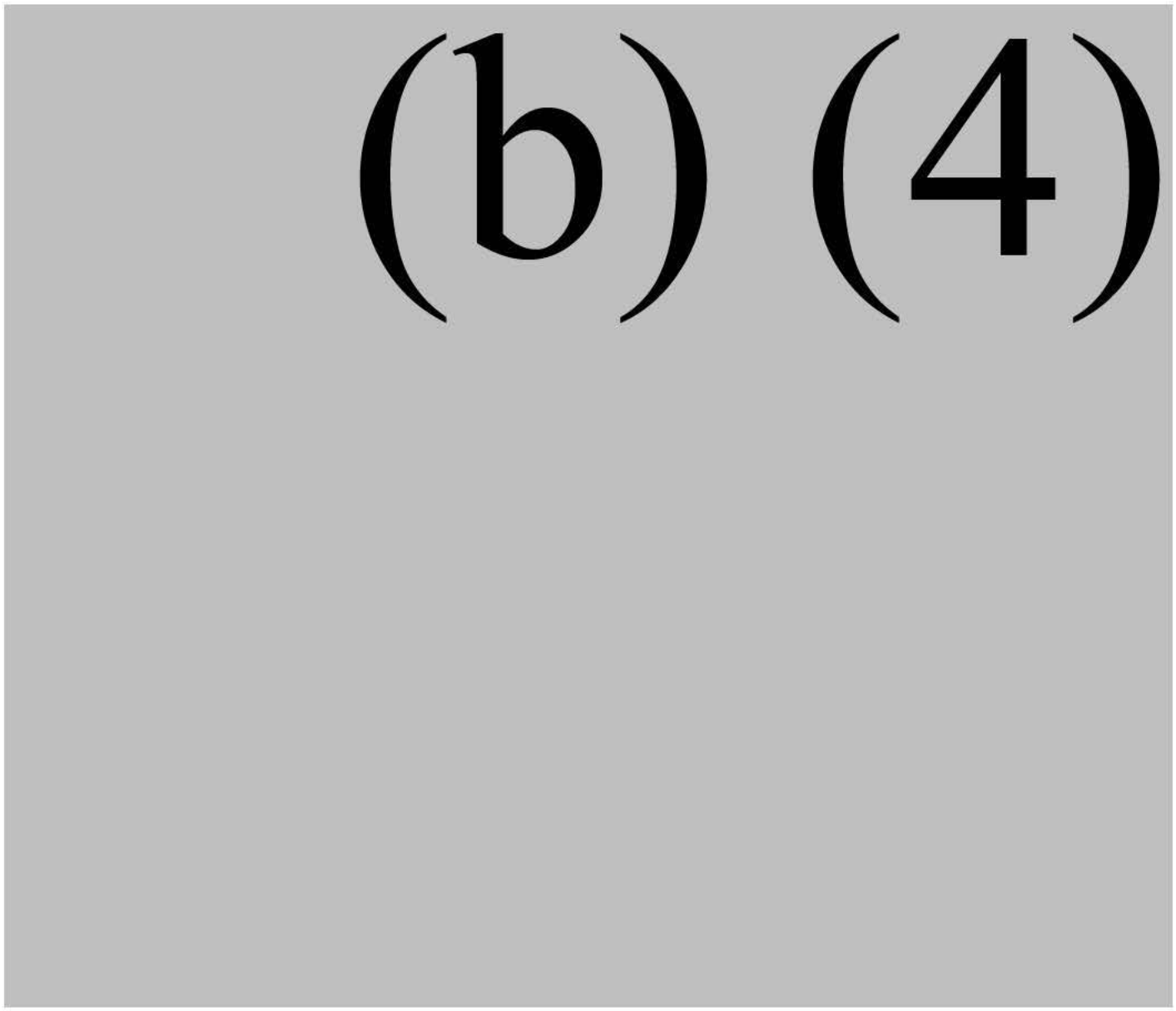
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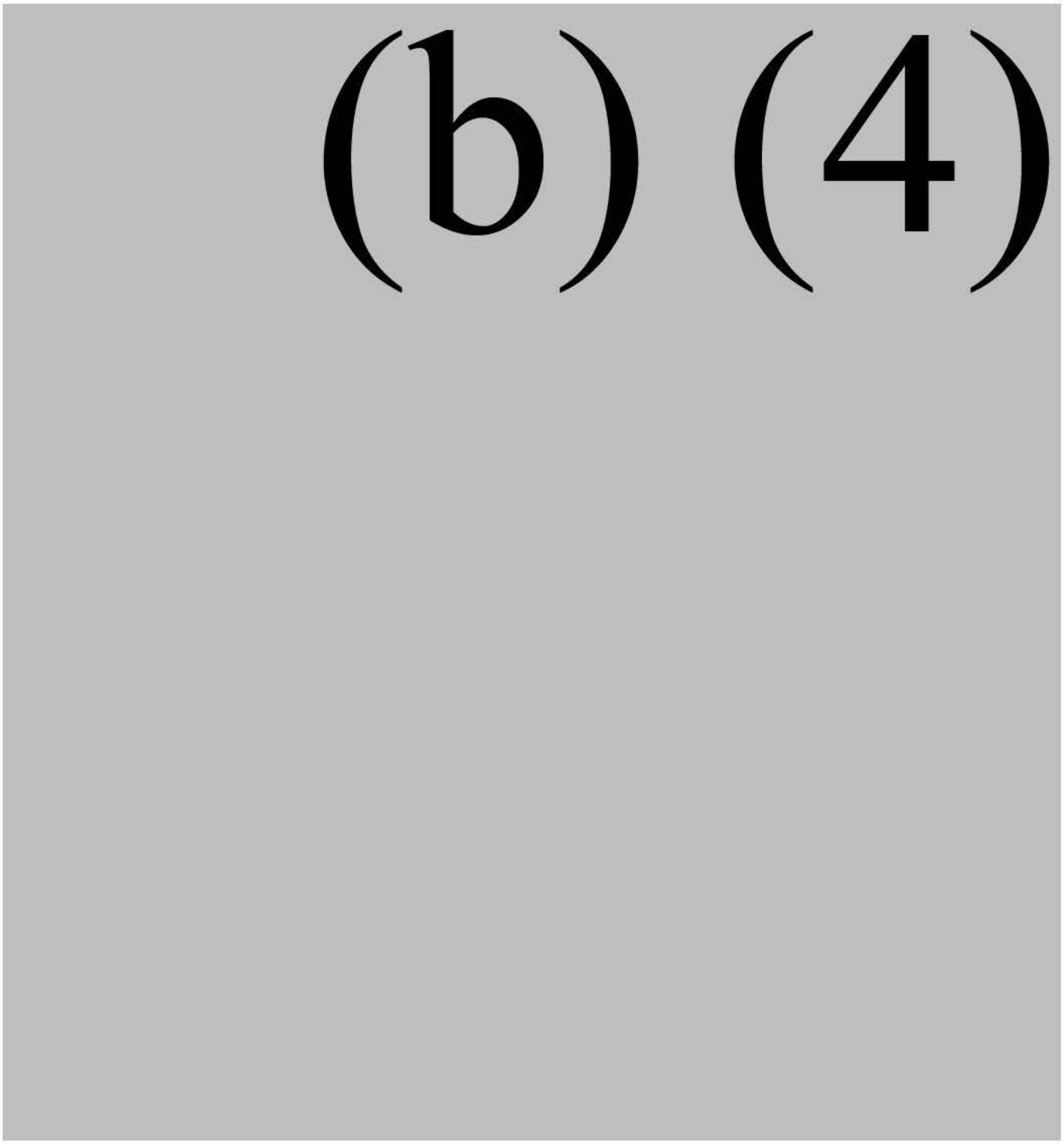
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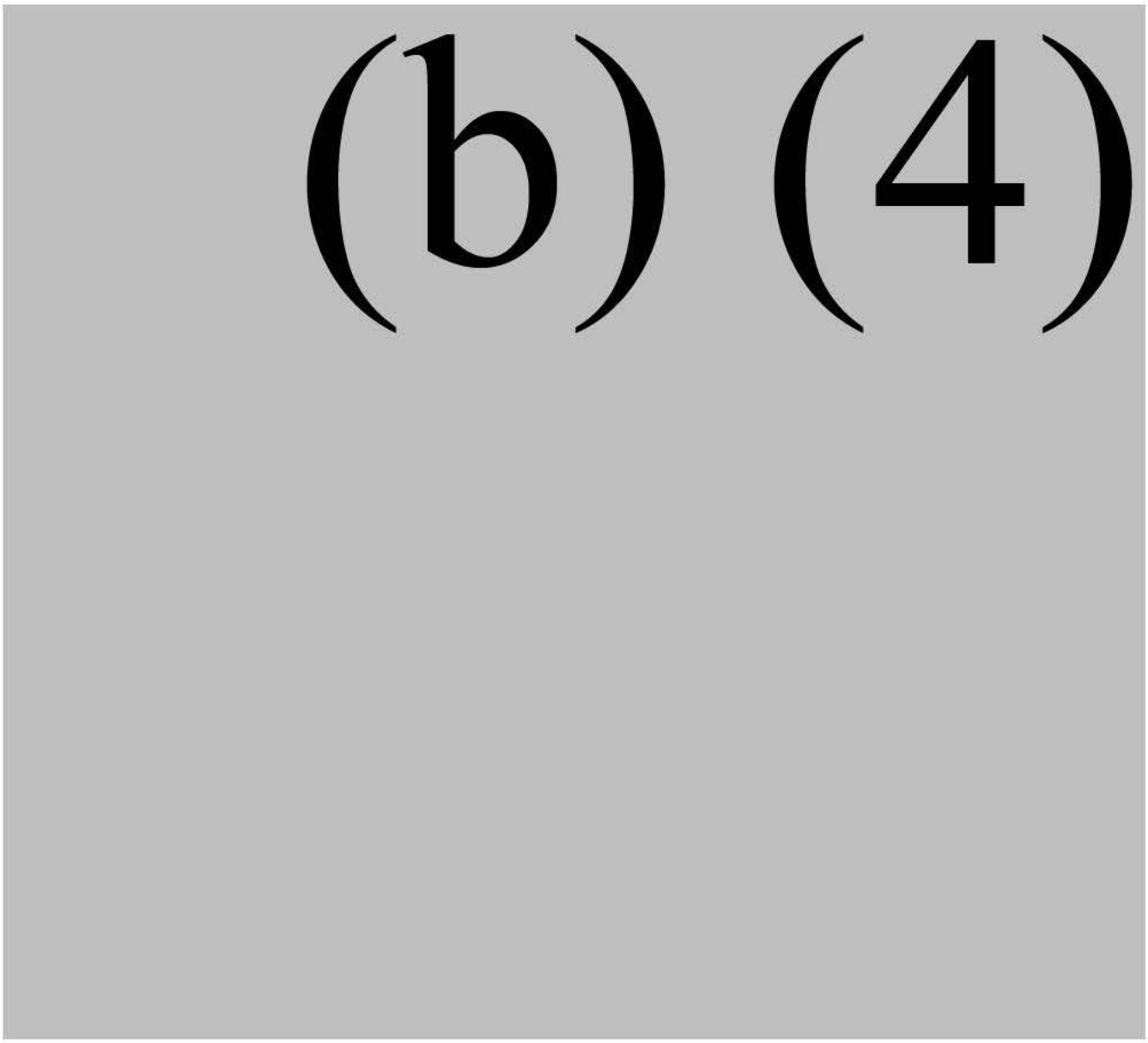
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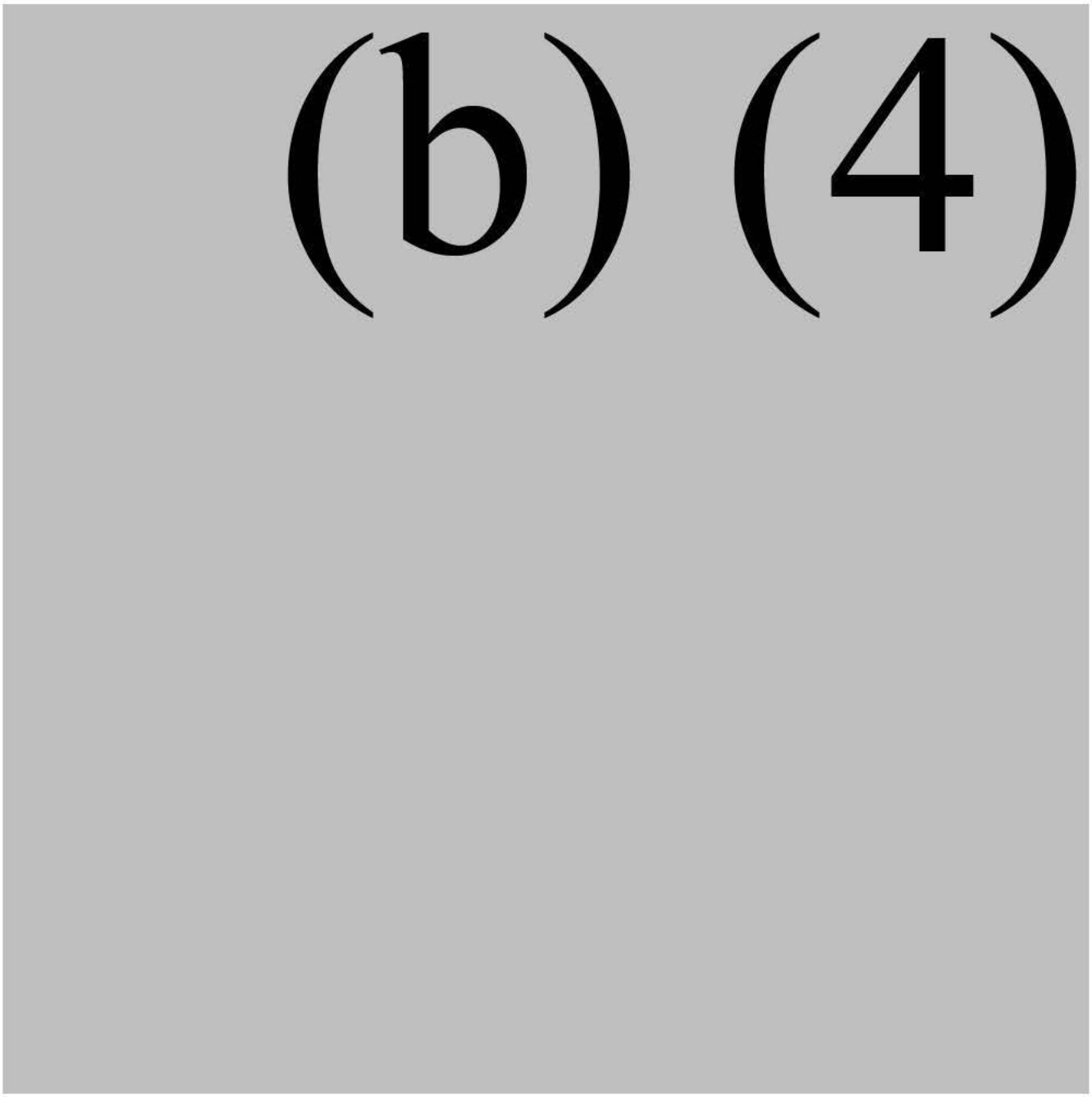
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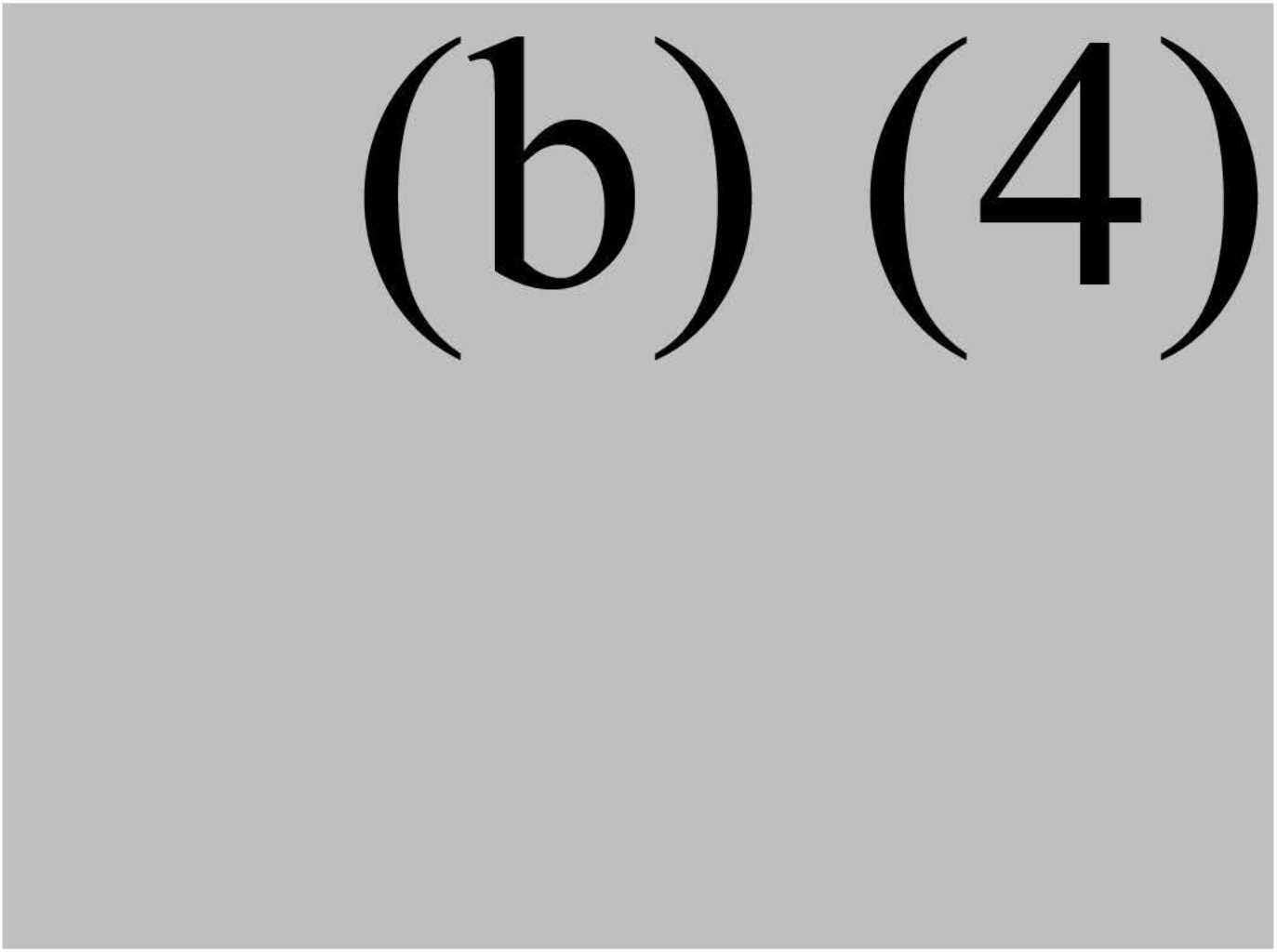
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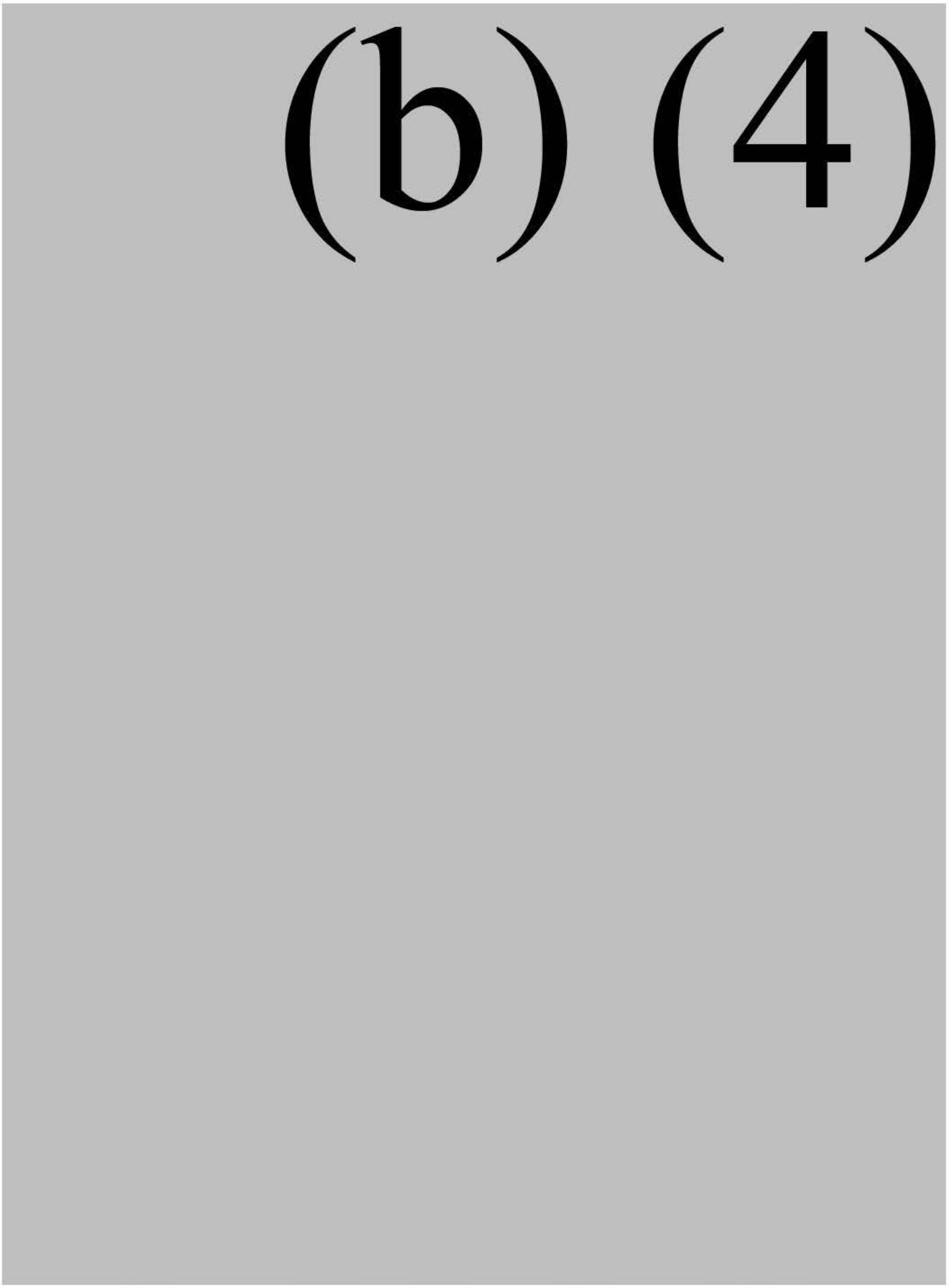
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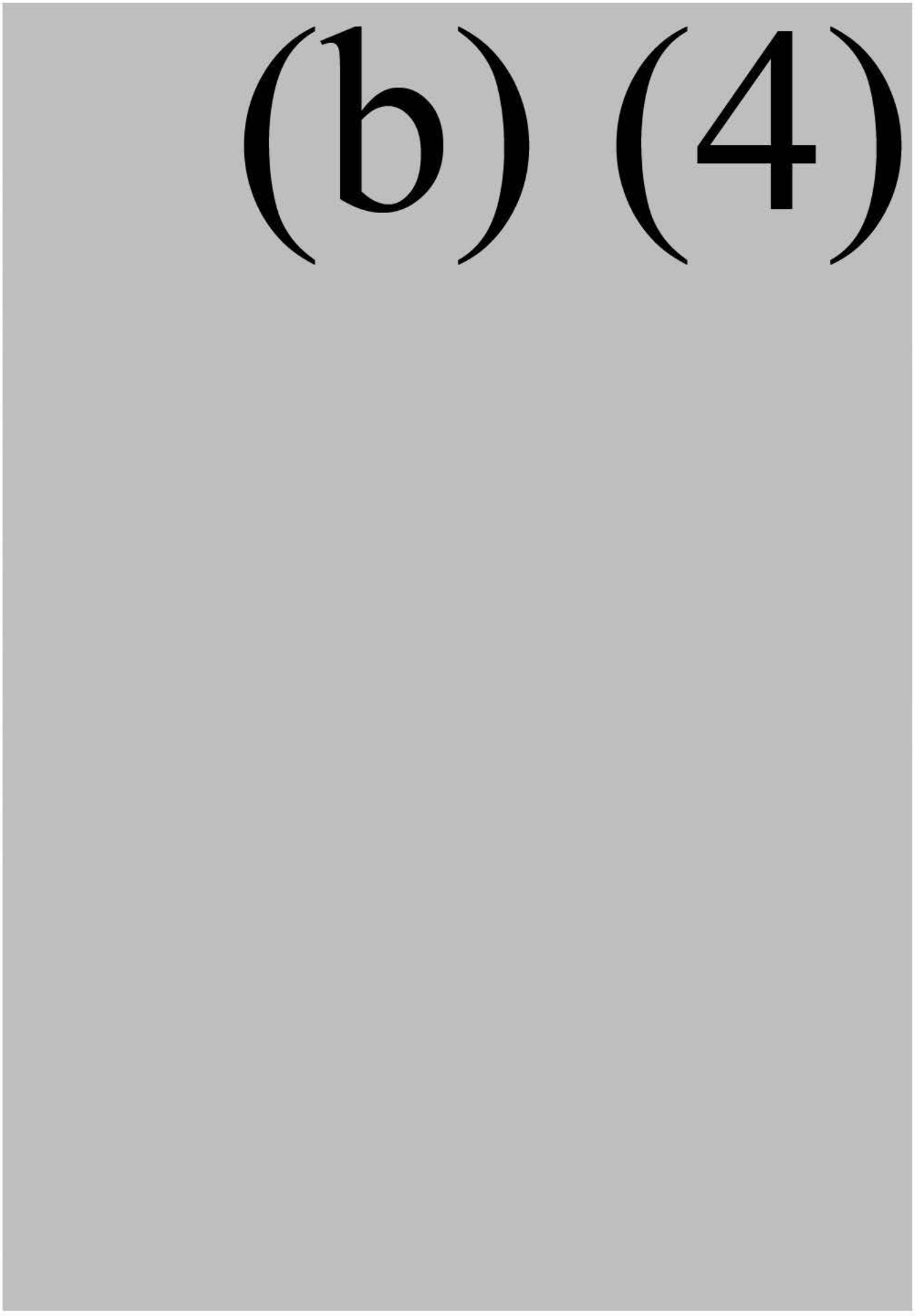
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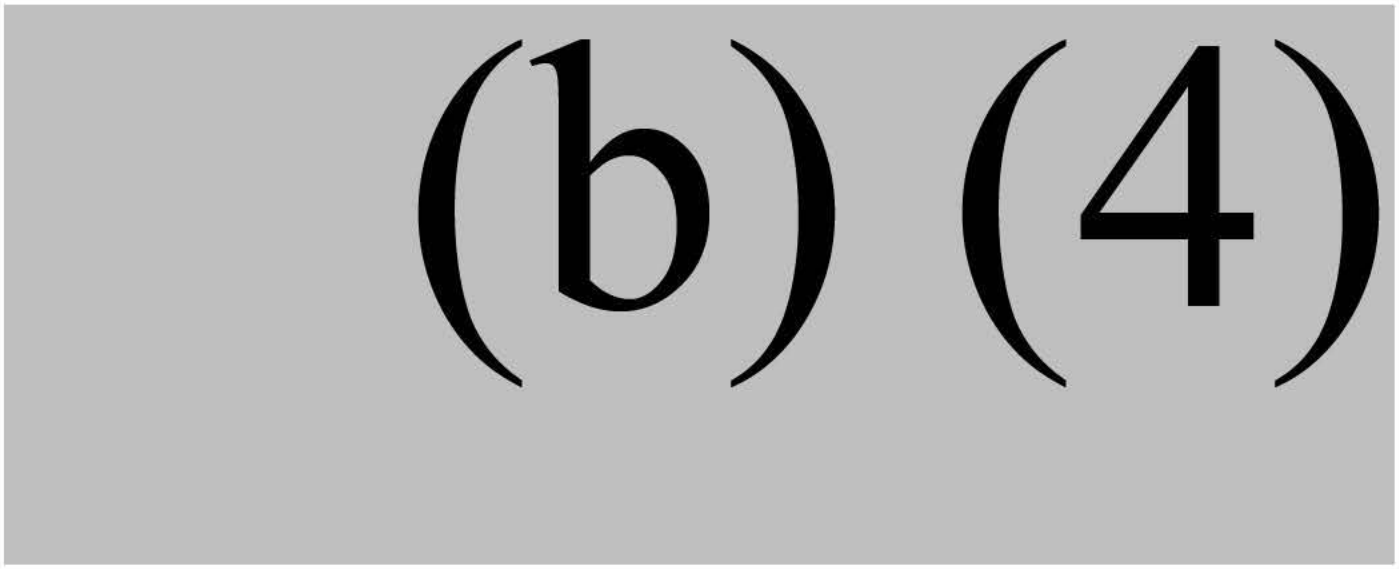
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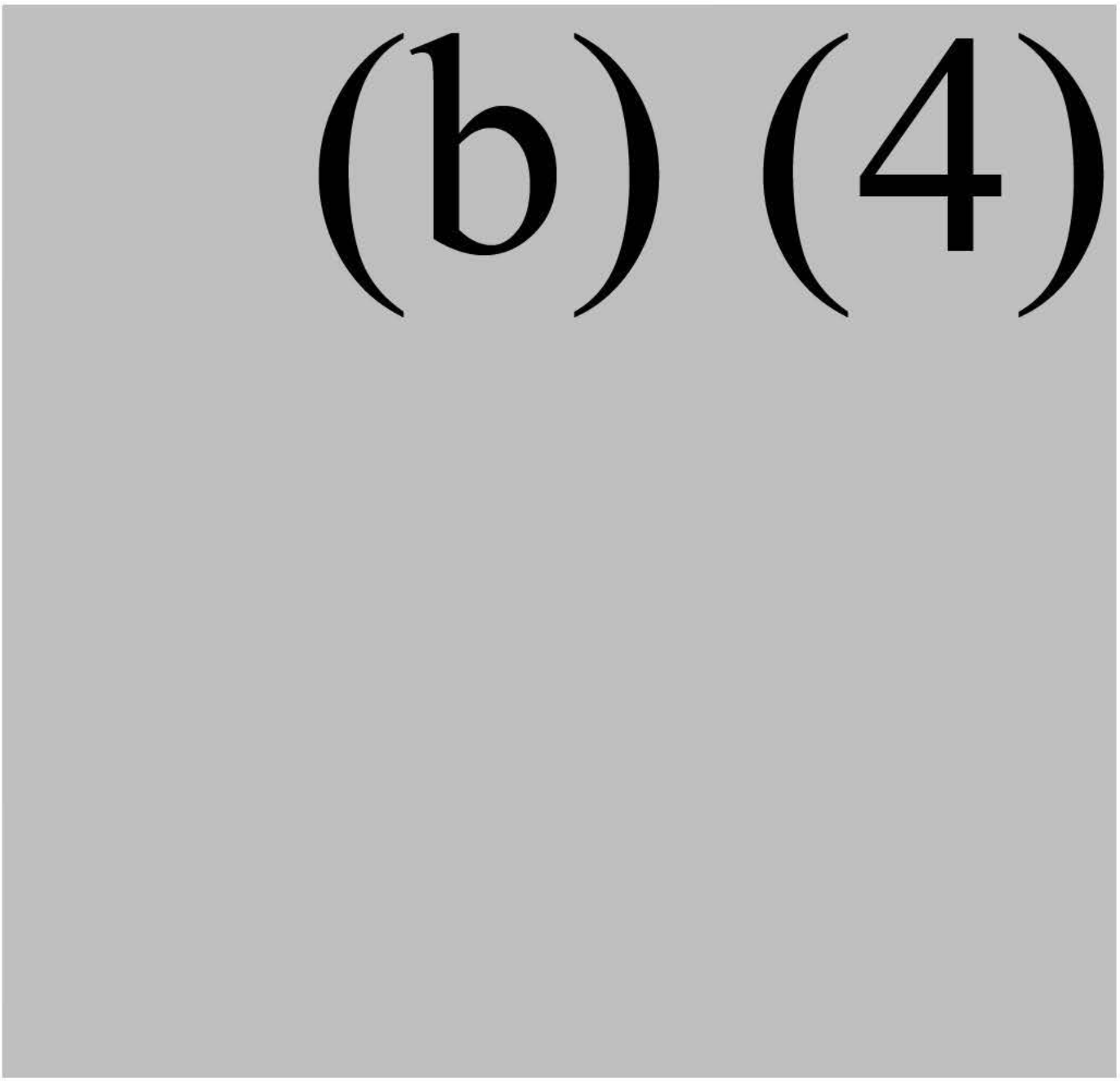
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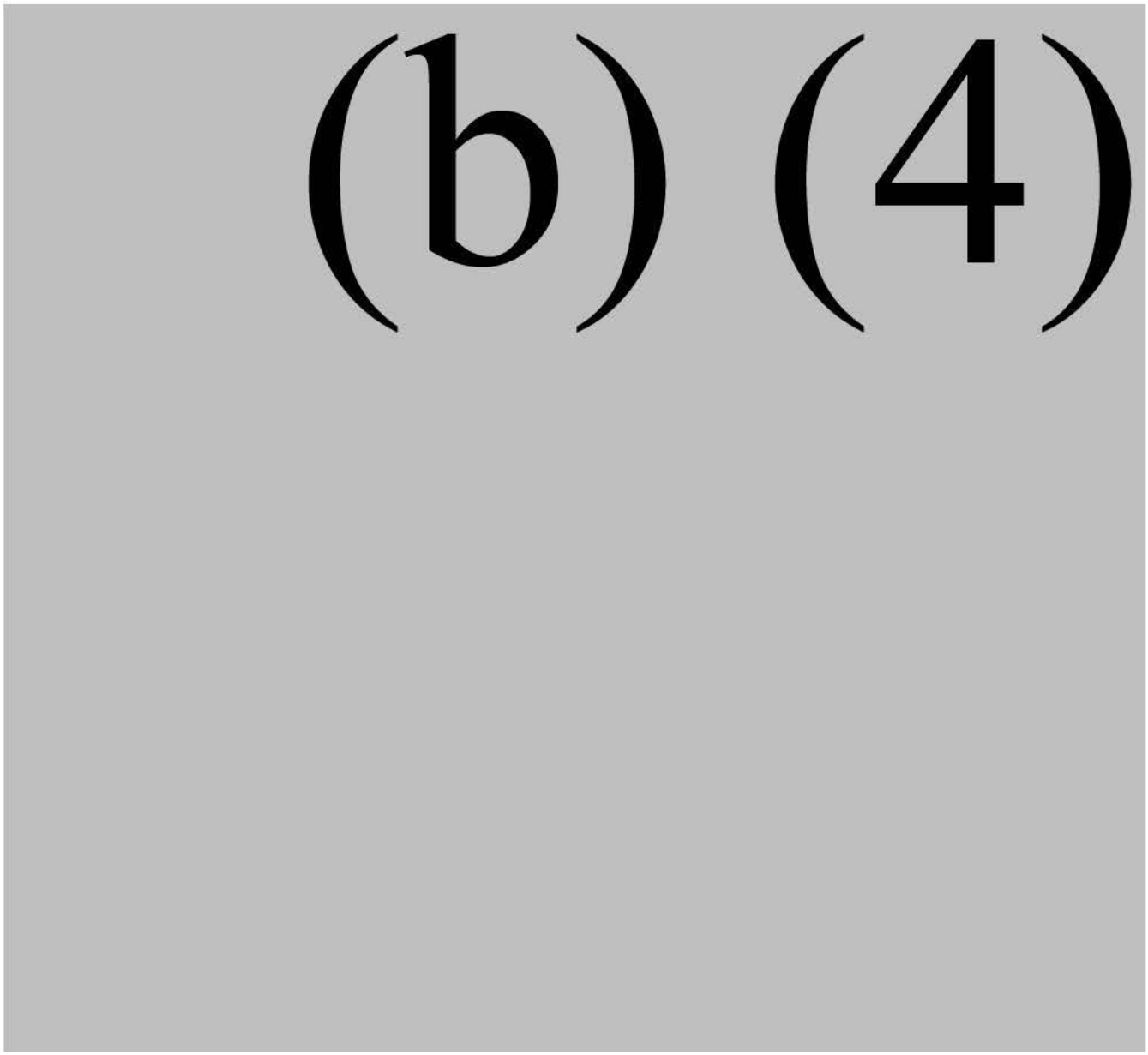
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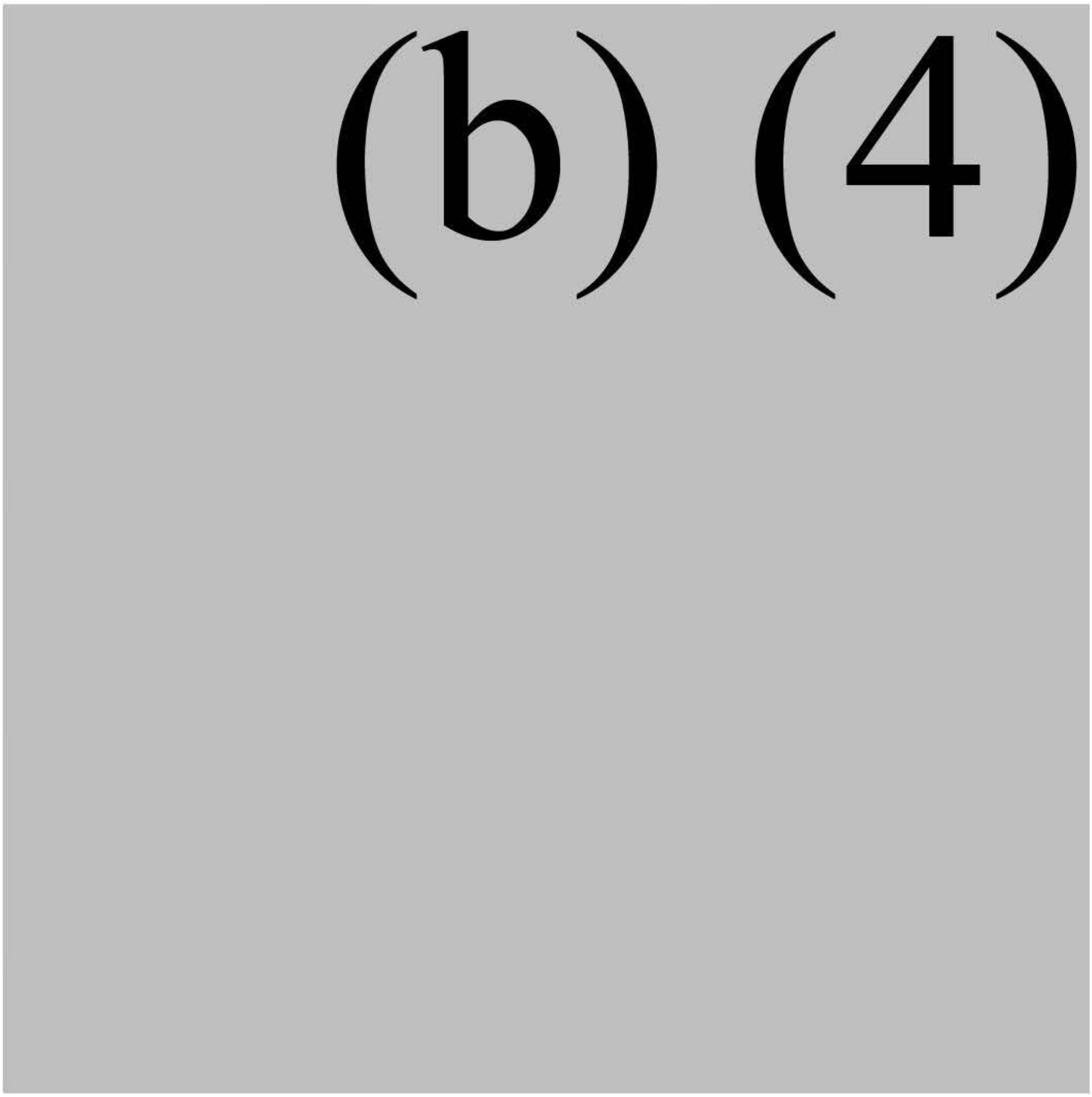
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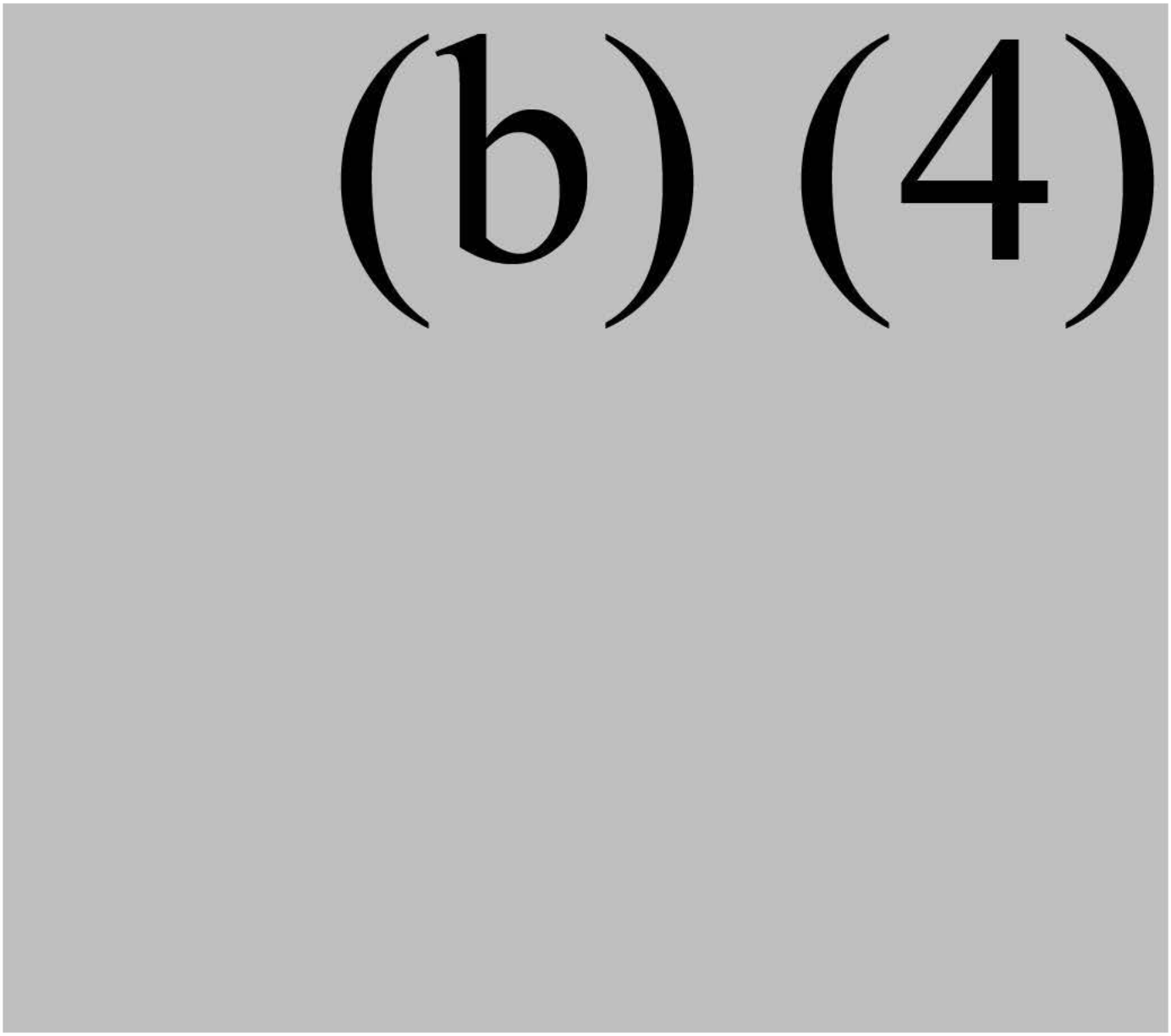
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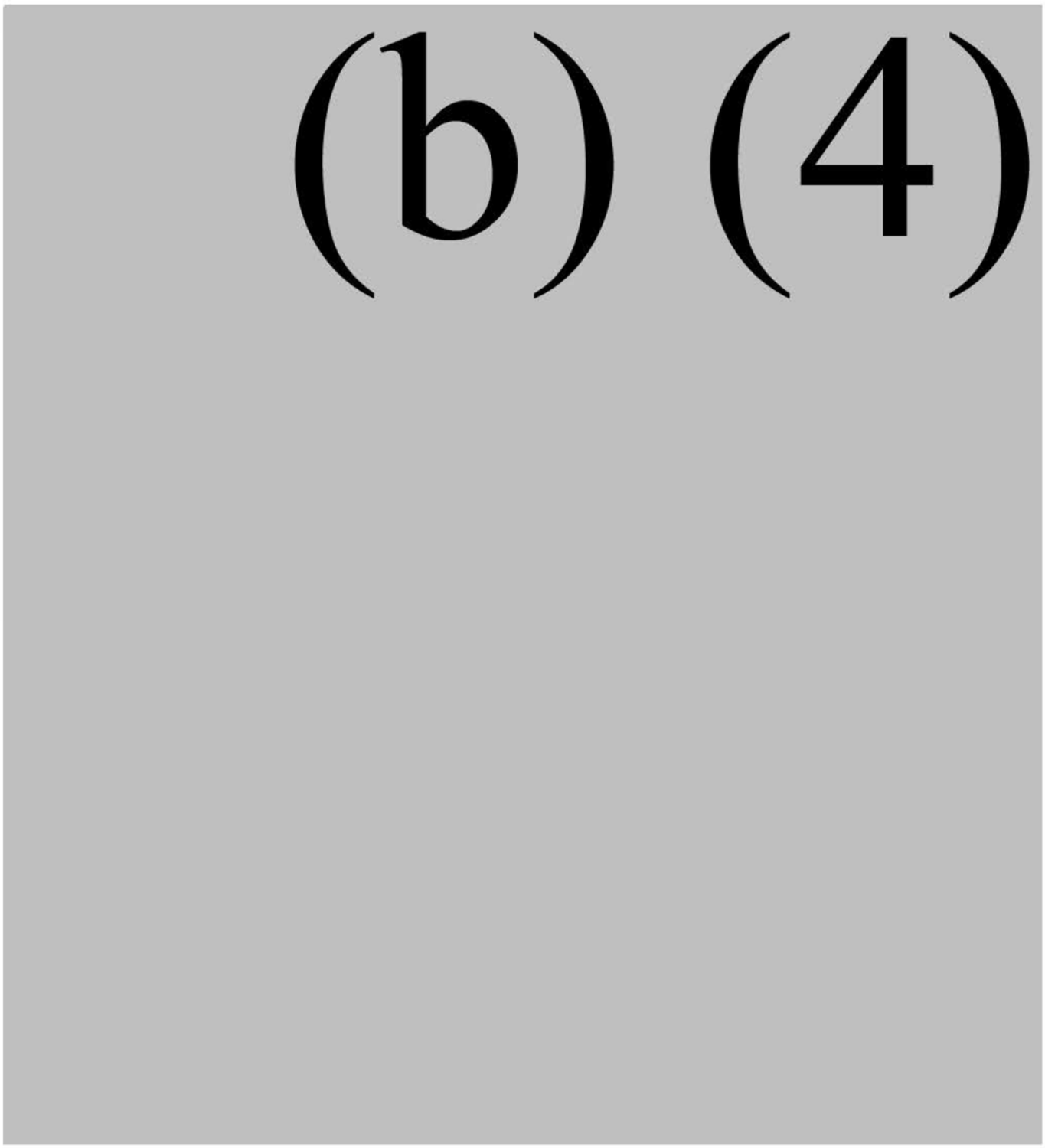
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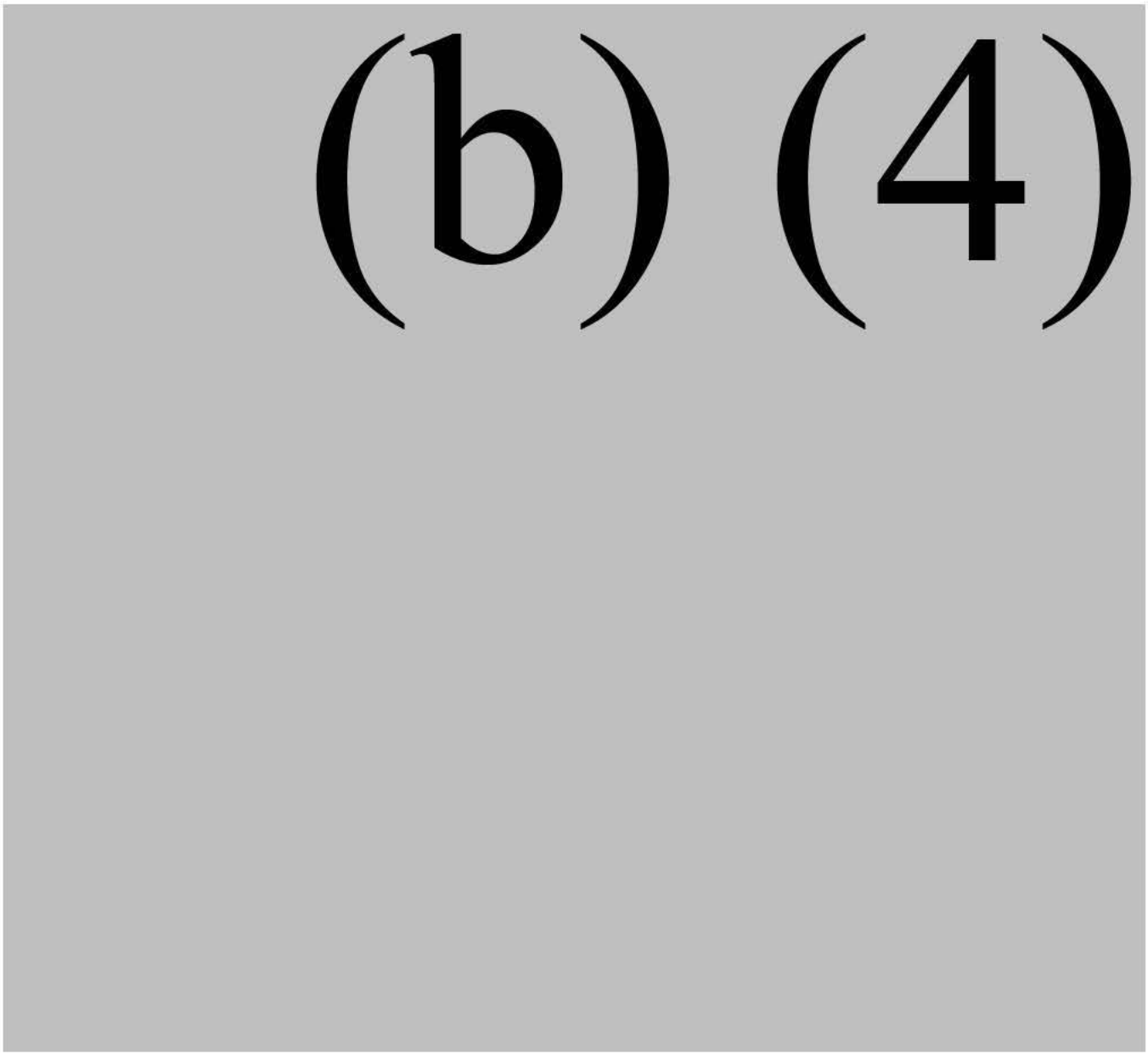
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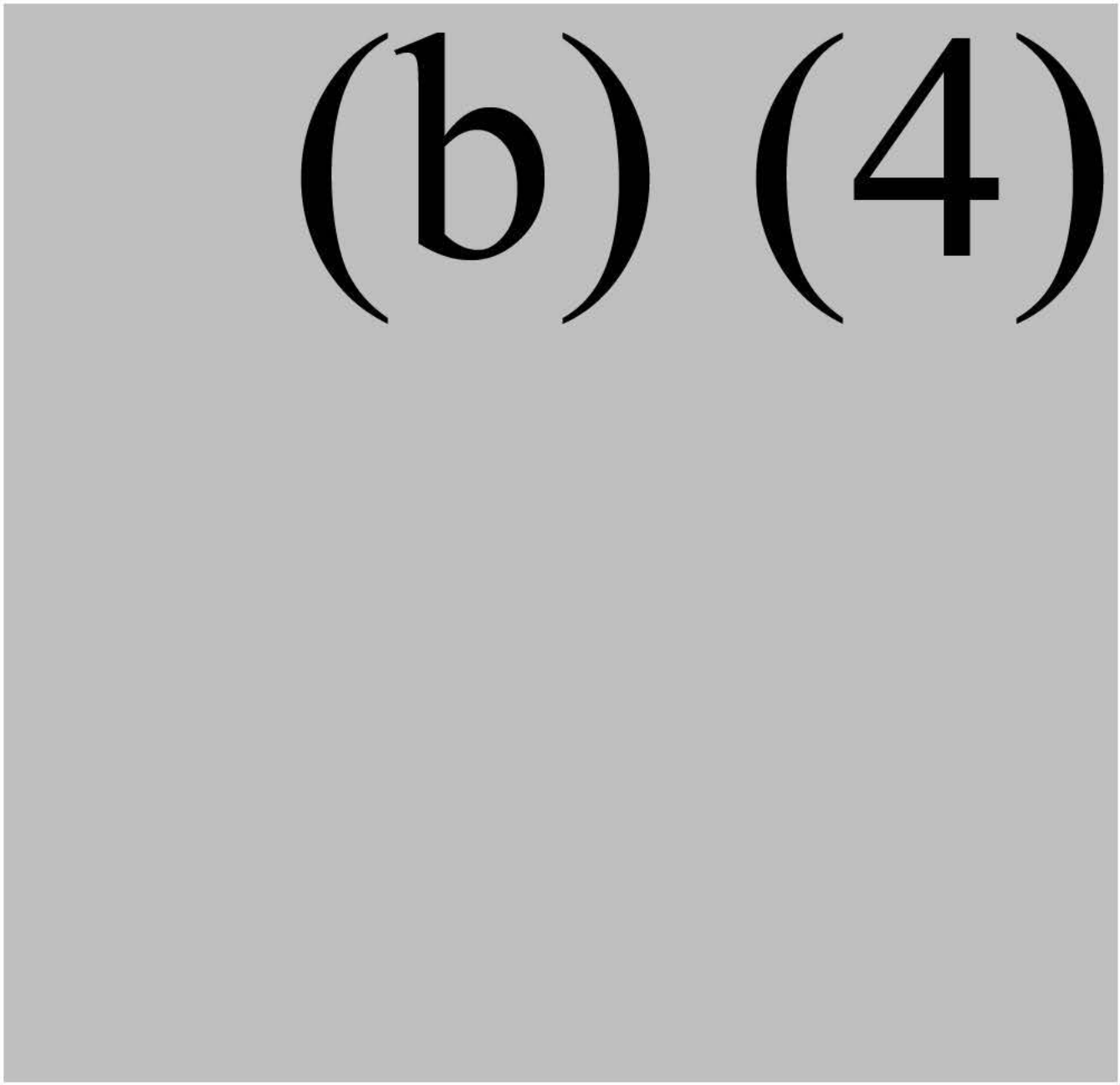
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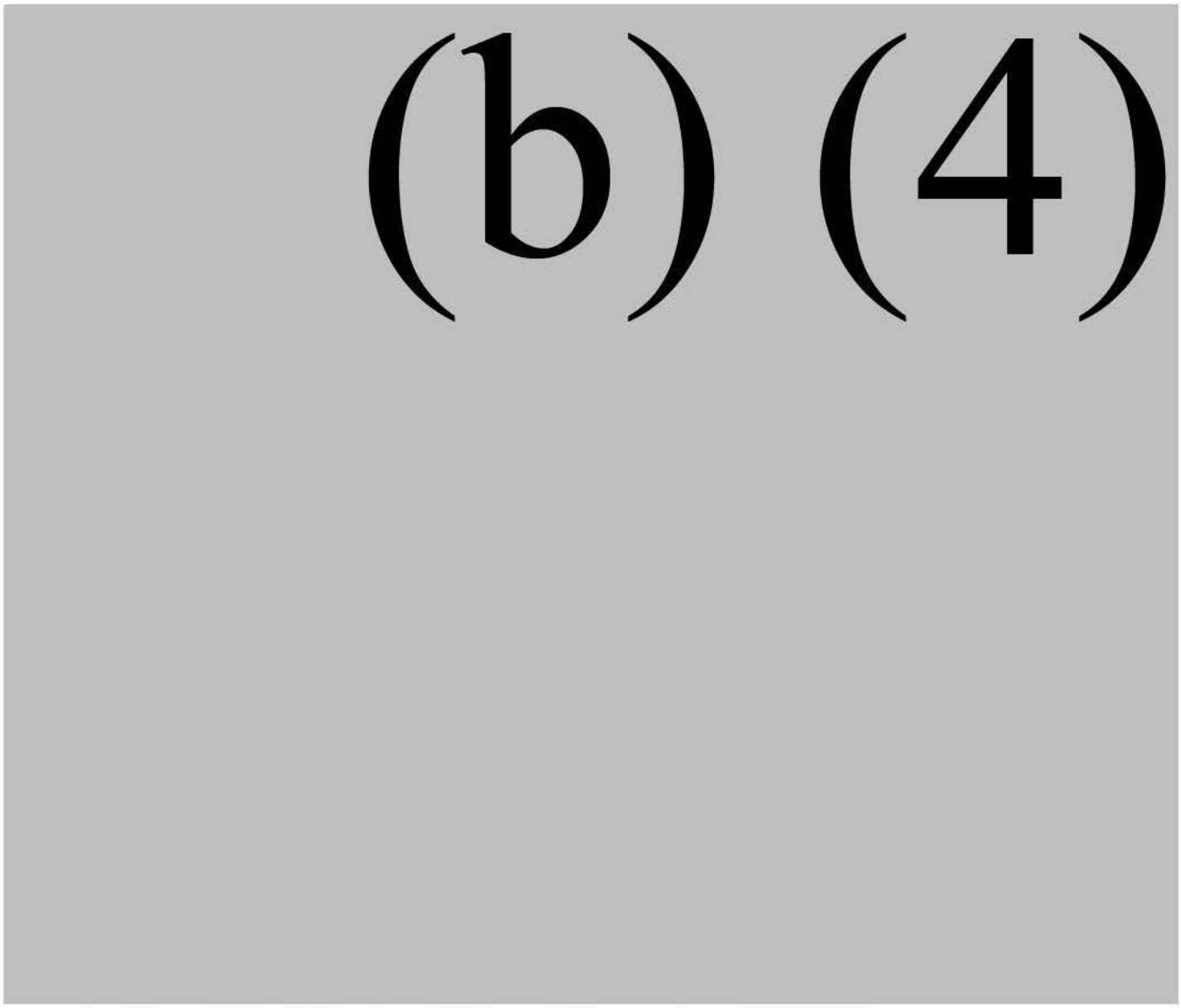
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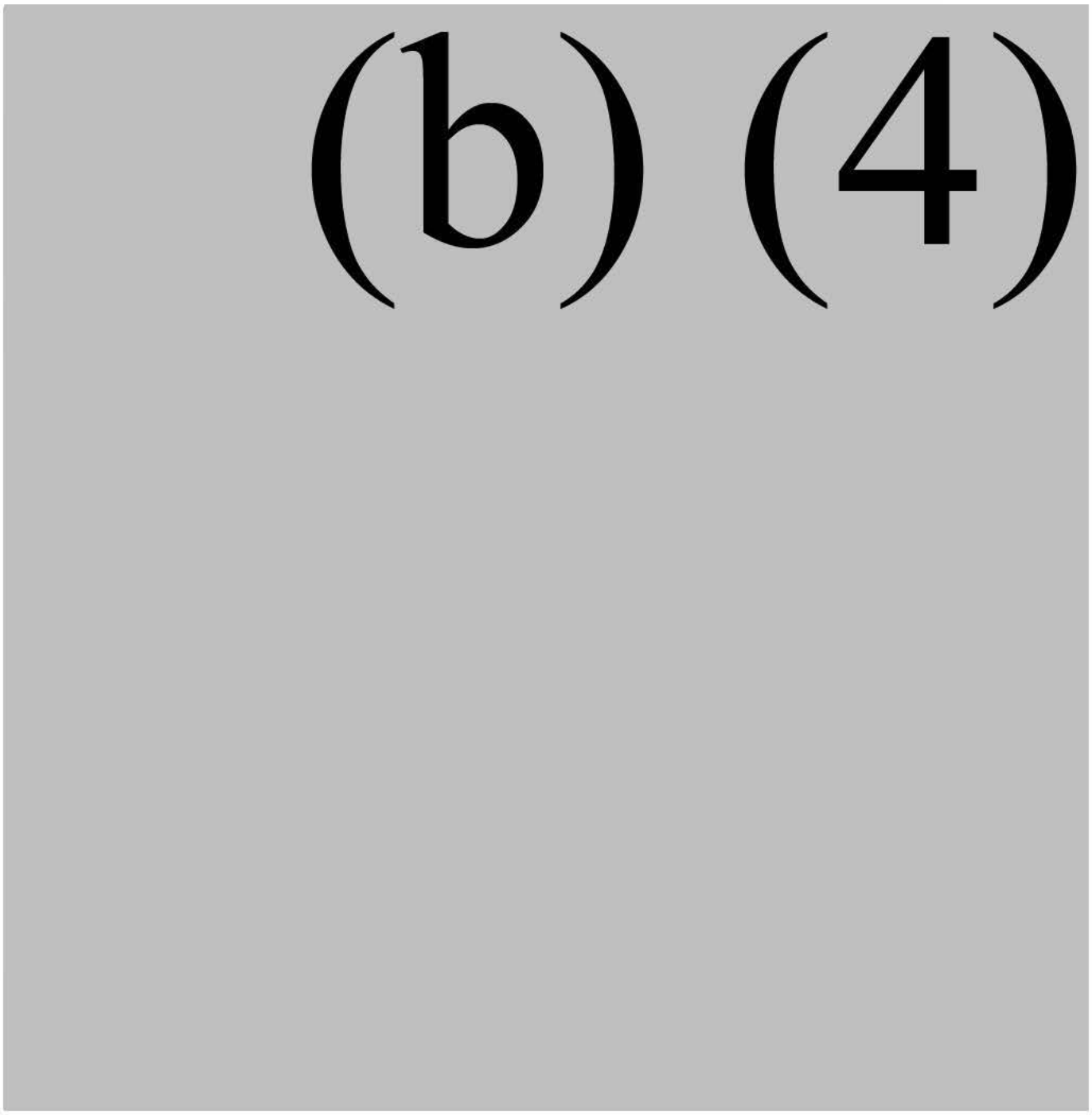
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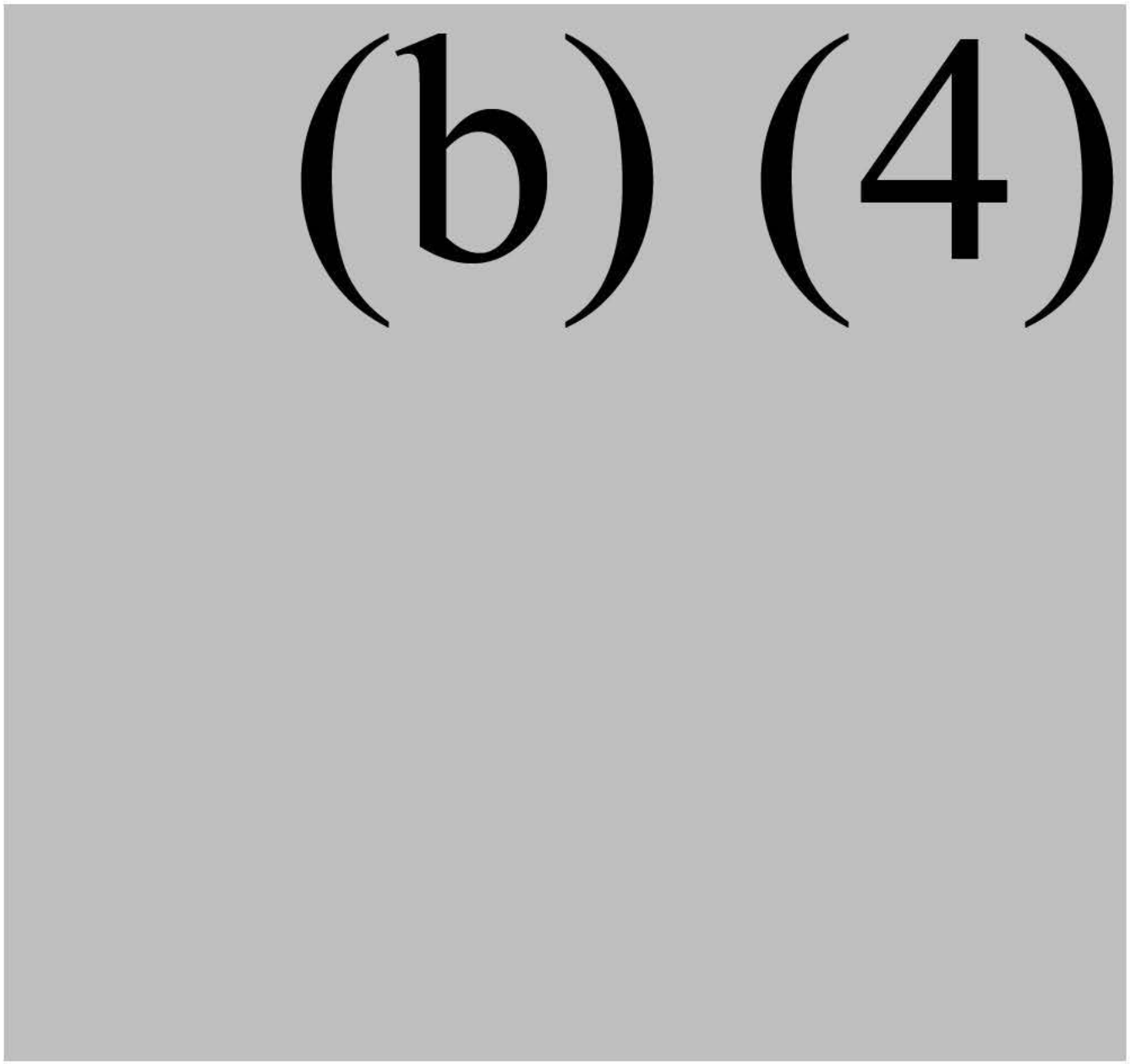
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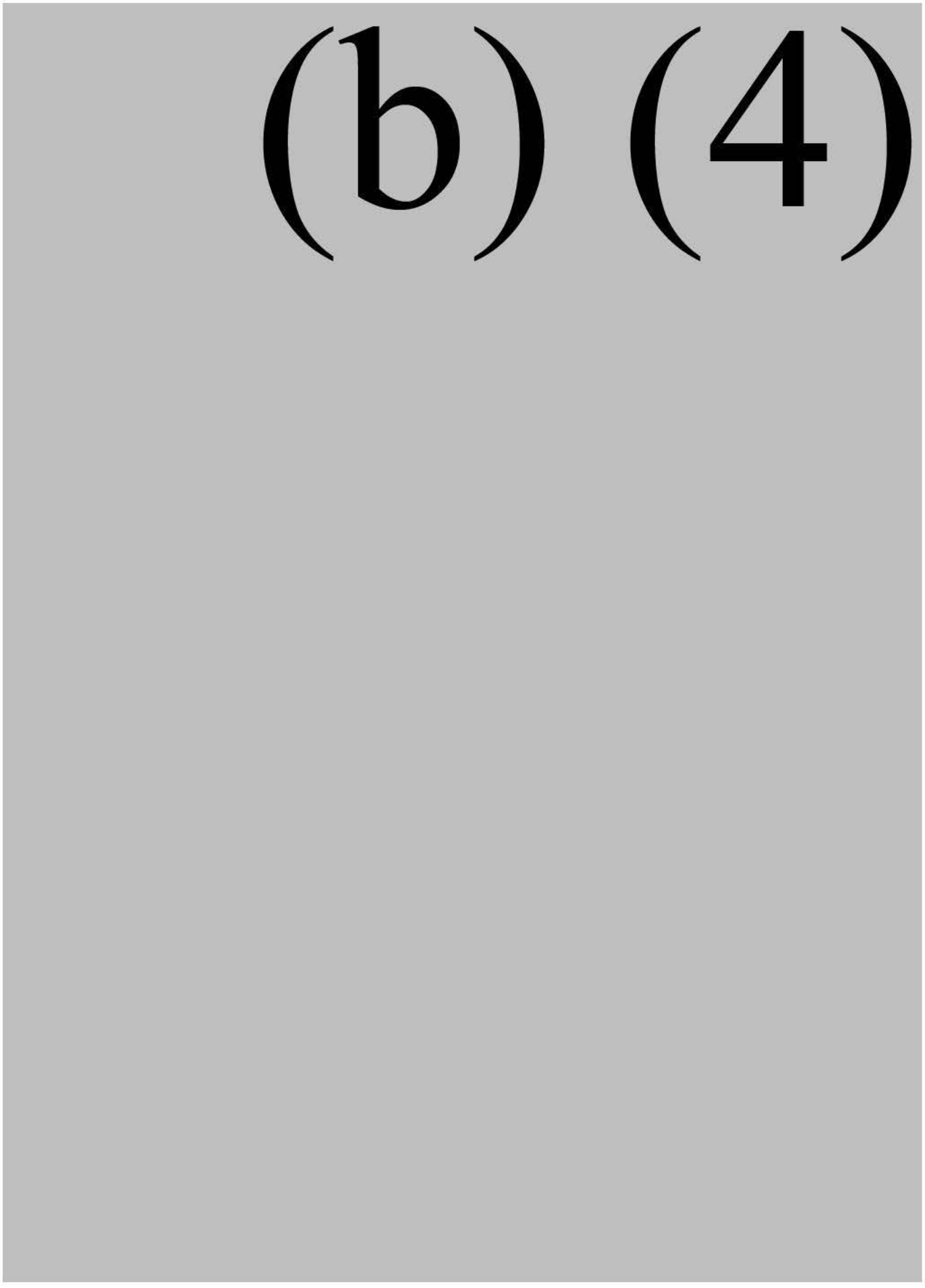
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