Appendix B

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FLUORESCENCE MEASUREMENT OF THE EQUILIBRIUM DISSOCIATION CONSTANT FOR THE COMPLEXES BETWEEN ANTITHROMBIN III AND 21 NEW OLIGOSACCHARIDES

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INTRODUCTION

Antithrombin III (AT III), a protein that belongs to the serpin superfamily of the serine proteinase inhibitors, plays a critical role in the blood coagulation. AT III and the other inhibitory serpins share the property of forming irreversible complexes with their target enzymes through a unique suicide substrate-like inhibition mechanism that leads to a covalent acyl-enzyme complex [1]. AT III is the main inhibitor of thrombin and factor Xa, two enzymes involved in the coagulation cascade and that react at low rate with this serpin [2]. The highly negatively charged polysaccharide heparin is known to dramatically enhance the rate of inhibition of factor Xa and thrombin by AT III through its binding to the serpin *via* a specific pentasaccharide fragment present in about one third of the naturally occurring heparin chains [3, 4], a property widely used for the regulation of blood coagulation in therapeutic. The pentasaccharide-induced acceleration of factor Xa inhibition results from a conformational change of AT III that extrudes its reactive site loop from a partially buried position to a more accessible one [3, 5, 6] and from exosite interactions between the serpin and the clotting proteinase [7-9]. A bridging mechanism requiring longer heparin chains mainly accounts for the acceleration of thrombin inhibition [10].

The heparin/pentasaccharide-induced structure modification of AT III has been investigated by cristallography [5] and numerous spectroscopic techniques. It has been published that the AT III intrinsic fluorescence increases by 30-49 % when it binds to high affinity heparin or to the specific pentasaccharide. This fluorescence exaltation has been used by many investigators as a signal to quantitate the affinity of these compounds for AT III as well as to investigate by fast kinetic studies the mechanism by which they associate with the serpin [6, 11-14].

We report here a single blind investigation of the AT III-binding properties of 21 new oligosaccharides isolated from Enoxaparin, a low molecular weight heparin, performed by monitoring the alteration of the intrinsic fluorescence of the protein upon its reaction with these products.

MATERIAL

21 samples of the oligosaccharides were sent to us by Sanofi-Aventis (Drs. C. Viskov and A. Uzan, Centre de Recherche de Paris, Vitry-sur-Seine, France). Each sample (identified by a reference) was provided as a solution (about 500 µL) of known concentration prepared in 0.010 M Tris buffer pH 7.5 containing 0.150 M NaCl. For convenience we numbered the products from 1 to 21. The pentasaccharide (same origin) was provided for the purpose of a previous study. The concentration of its stock solution was calculated from the weight and using MW = 1818 Da. The pure human AT III preparation is a product from Hyphen BioMed (Neuville-sur-Oise, France). Its concentration in the stock solution was calculated from its absorbance at 280 nm using ε_{280nm} = 35560 $M^{-1}.cm^{-1}$. The serpin solution was found to contain at least 95 % of active protein as determined by an established procedure[15]. All other reagents are of analytical grade.

EXPERIMENTAL PROCEDURES

The equilibrium dissociation constants K_d for the interaction of the oligosaccharides with AT III has been assessed at 25° C in 0.050 M HEPES containing 0.100 M NaCl by equilibrium titration experiments in which the intrinsic fluorescence of the serpin was monitored upon reaction of a constant concentration of the protein with increasing concentrations of these compounds, a procedure already described by others [6, 12, 14, 16]. As a reference product we used the pentasaccharide.

The fluorescence intensities measurements (λ_{ex} = 280 nm, 3 or 5 nm band-pass, λ_{em} = 340 nm, 3, 5 or 10 nm band-pass) were performed using a RF5000 Shimadzu fluorospectrophotometer equipped with a thermostated cell holder. A typical fluorescence equilibrium titration experiment was done as follows: an aliquot of the buffered AT III stock solution was diluted (final concentration = 0.0172, 0.0345, 0.042, 0.069 or 0.52 µM) into the HEPES buffer contained in an 1 x 1 cm path quartz cuvette (final volume = 2.5 ml) and the fluorescence intensity was read at 340 nm prior consecutive additions of small volumes (0.5 – 2.0 µl) of the appropriate oligosaccharide solution. Before addition of AT III, the apparatus was set to zero by reading against the buffer alone. The reference of the oligosaccharides, the corresponding number, the concentration of the provided stock solutions and that of the solutions used for the purpose of the equilibrium titration experiments are given at Table I of the annex.

The best estimate of K_d has been determined by fitting the fluorescence data to the equilibrium binding equation:

$$\Delta f = \Delta f_{max} \frac{([ATIII]_0 + n[ols]_0 + K_d) - \sqrt{([ATIII]_0 + n[ols]_0 + K_d)^2 - 4n[ols]_0[ATIII]_0}}{2[ATIII]_0} \quad (1)$$

Where $[ATIII]_0$ and $[ols]_0$ stand for the AT III and oligosaccharide initial concentrations, respectively. Δf and Δf_{max} are the absolute change of fluorescence intensity for a given oligosaccharide concentration and that of the maximum fluorescence intensity change, respectively. n is the binding stoichiometry (ie the ratio of the concentration of the high affinity species present in the reaction mixture *vs* the total concentration of the studied product). Here, the value of this parameter was set to 1 since the analyzed solutions were supposed to contain a single chemically well defined product, each molecule of which was assumed to bind one molecule of AT III only. Non-linear regression analysis was done with the GraFit software (Erithacus Software).

RESULTS

The reaction of all analyzed products with AT III was accompanied by an 31.4 – 51.5 % increase of the intrinsic fluorescence of the protein at 340 nm. We used this property to follow the AT III : oligosaccharide complex formation by titrating serpin solutions by incremental amounts of oligosaccharide. The oligosaccharide concentration : fluorescence intensity data sets (given in the annex) were analyzed as described above to assess K_d. The figures (see annex) show that the theoretical curves generated using equation 1 and the best estimates of the equilibrium dissociation constant adequately fit the experimental data collected for the reaction of each compound with AT III. The value of K_d, and the maximal fluorescence increase for each AT III : oligosaccharide pair are summarized bellow at table I. The standard error on K_d, and the concentration of AT III used in each experiment are given with the corresponding figure (see annex).

TABLE I

Equilibrium dissociation constant (K_d) and maximal fluorescence enhancement for the various AT III : oligosaccharide interactions

product reference	product number	K_d (µM)	[a]AT III max fluorescence enhancement (%)
P33894-51-1	1	0.30350	41.4
P32650-55-1	2	0.11430	46.1
P32444-021-2	3	0.31190	33.2
P32650-067-1	4	0.20320	38.3
P32964-120	5	1.33820	39.4
P34279-47-04	6	0.00115	46.0
P32444-021-3	7	0.30650	38.7
P32650-061-1	8	0.20850	40.0
P32650-063-1	9	0.56760	46.3
P32077-090-2	10	0.14290	51.5
P33818-64-2	11	0.02110	35.2
P33818-62-3	12	0.05280	38.4
P33818-44-1	13	0.19620	36.5
P31404-012-1	14	0.06360	44.4
P33818-36-3	15	0.04240	31.4
P33818-64-1	16	0.08040	42.3
P33818-62-1	17	0.09210	44.0
P33818-62-4	18	0.18440	36.9
P33818-44-4	19	0.02630	38.6
P33818-22-4	20	0.46660	44.1
P34279-47-2	21	0.00149	49.0
pentasaccharide	--------	0.02080	33.4

[a]obtain from the fluorescence intensities measured in the presence of saturating oligosaccharide concentrations or from the fit of the experimental data by equation 1 (products 5 and 20)

CONCLUSION

The present single blind study was aimed at determining the equilibrium dissociation constant describing the affinity of 21 oligosaccharides, purified from Enoxaparin, for AT III. The measurements were done at 25° C in 0.050 M HEPES containing 0.100 M NaCl. A reference product, the pentasaccharide was submitted to the same analyses.

Together these data essentially provide to the chemists interesting informations regarding to the relationships between the chemical structure of the oligosaccharides and the stability of their complex with AT III. The set of equilibrium constants determined here may be considered as a photograph of the AT III-binding properties of the products taken under the used experimental conditions. For this reason they do not give informations on parameters such as the relative contributions of the ionic and non-ionic interactions involved in the formation of the complexes.

The interaction of AT III with the various products was fluorometrically followed by monitoring at 340 nm the intrinsic fluorescence of AT III with excitation at 280 nm. The reactions were accompanied by a fluorescence increase ranging from 31.4 % (product 15) to 51.5 % (product 10). Saturating concentrations of pentasaccharide induced a 33.4 % enhancement. Similar values have been reported for the interaction of AT III with high affinity heparin [14] and with small oligosaccharides [12].

All analyses were done assuming that each oligosaccharide solution contains one pure and well defined product only. On this basis we found that the K_d values for the binding of the 21 oligosaccharides to AT III are ranging from 1.33 µM (product 5) to 1.15 nM (product 6). Under the experimental conditions used the pentasaccharide binds to the protein with $K_d = 0.0208$ µM, a value that compares with published data [3, 12]. Similar values of K_d were found for products 11 and 19 (0.0211 and 0.0263 µM, respectively). 17 oligosaccharides (products 1, 2, 3, 4, 5, 7, 8, 9, 10, 12, 13, 14, 15, 16, 17, 18 and 20) form a significantly weaker complex with AT III than does the pentasaccharide. For 12 of them (products 1, 2, 3, 4, 5, 7, 8, 9, 10, 13, 18 and 20) fluorescence titrations yielded $K_d > 0.100$ µM. In contrast we found that products 6 and 21 form particularly tight complexes with the protein ($K_d = 1.15$ and 1.49 nM, respectively). Similar low K_d have been reported for complexes between AT III and synthetic pentadeca to nanodecasaccharides aimed at promoting thrombin inhibition [17, 18].

The tested oligosaccharides, present in Enoxaparin, have a large range of affinities for ATIII (the value of K_d is varying by three orders of magnitude). This is a characteristic

of Enoxaparin. It can be presumed that, in Enoxaparin, the mixture of these oligosaccharides contributes to the final affinity of this LMWH to AT III and finally to its effects on Haemostasis.

REFERENCES

1 Gettins, P. G. (2002) Serpin structure, mechanism, and function. Chem Rev 102, 4751-4804

2 Craig, P., Olson, T. and Shore, J. (1989) Transient kinetics of heparin-catalyzed protease inactivation by antithrombin III. J. Biol. Chem. 264, 5452-5461

3 Olson, S., Björk, I., Sheffer, R., Craig, P., Shore, J. and Choay, J. (1992) Role of the antithrombin-binding pentasaccharide in heparin acceleration of antithrombin-proteinase reactions. Resolution of the antithrombin conformational change contribution to heparin rate enhancement. J. Biol. Chem. 267, 12528-12538

4 Choay, J., Petitou, M., Lormeau, J. C., Sinay, P., Casu, B. and Gatti, G. (1983) Structure-activity relationship in heparin: a synthetic pentasaccharide with high affinity for antithrombin III and eliciting high anti-factor Xa activity. Biochem Biophys Res Commun 116, 492-499

5 Jin, L., Abrahams, J. P., Skinner, R., Petitou, M., Pike, R. N. and Carrell, R. W. (1997) The anticoagulant activation of antithrombin by heparin. Proc. Natl. Acad. Sci. USA 94, 14683-14688

6 Huntington, J. A., Olson, S. T., Fan, B. and Gettins, P. G. W. (1996) Mechanism of Heparin Activation. Evidence for Reactive Center Loop Preinsertion with Expulsion upon Heparin Binding. Biochemistry 35, 8495-8503

7 Olson, S. T. and Chuang, Y. J. (2002) Heparin activates antithrombin anticoagulant function by generating new interaction sites (exosites) for blood clotting proteinases. Trends Cardiovasc Med 12, 331-338

8 Johnson, D. J., Li, W., Adams, T. E. and Huntington, J. A. (2006) Antithrombin-S195A factor Xa-heparin structure reveals the allosteric mechanism of antithrombin activation. Embo J 25, 2029-2037

9 Rezaie, A. R. (2006) Pentasaccharide enhances the inactivation of factor Xa by antithrombin by promoting the assembly of a Michaelis-type intermediate complex. Demonstration by rapid kinetic, surface plasmon resonance, and competitive binding studies. Biochemistry 45, 5324-5329

10 Olson, S. and Björk, I. (1991) Predominant contribution of surface approximation to the mechanism of heparin acceleration of the antithrombin-thrombin reaction. Elucidation from salt concentration effects. J. Biol. Chem. 266, 6353-6364

11 Schedin-Weiss, S., Arocas, V., Bock, S. C., Olson, S. T. and Bjork, I. (2002) Specificity of the basic side chains of Lys114, Lys125, and Arg129 of antithrombin in heparin binding. Biochemistry 41, 12369-12376

12 Desai, U. R., Petitou, M., Bjork, I. and Olson, S. T. (1998) Mechanism of heparin activation of antithrombin. Role of individual residues of the pentasaccharide activating sequence in the recognition of native and activated states of antithrombin. J. Biol. Chem. 273, 7478-7487

13 Olson, S., Srinivasan, K., Björk, I. and Shore, J. (1981) Binding of high affinity heparin to antithrombin III. Stopped flow kinetic studies of the binding interaction. J. Biol. Chem. 256, 11073-11079

14 Olson, S. and Shore, J. (1981) Binding of high affinity heparin to antithrombin III. characterization of the protein fluorescence enhancement. J. Biol. Chem. 256, 11065-11072

15 Boudier, C. and Bieth, J. G. (2001) The reaction of serpins with proteinases involves important enthalpy changes. Biochemistry 40, 9962-9967

16 Lin, P., Sinha, U. and Betz, A. (2001) Antithrombin binding of low molecular weight heparins and inhibition of factor Xa. Biochim Biophys Acta 1526, 105-113

17 Petitou, M., Duchaussoy, P., Driguez, P. A., Herault, J. P., Lormeau, J. C. and Herbert, J. M. (1999) New synthetic heparin mimetics able to inhibit thrombin and factor Xa. Bioorg Med Chem Lett 9, 1155-1160

18 Petitou, M., Herault, J. P., Bernat, A., Driguez, P. A., Duchaussoy, P., Lormeau, J. C. and Herbert, J. M. (1999) Synthesis of thrombin-inhibiting heparin mimetics without side effects. Nature 398, 417-422

Annex

to

FLUORESCENCE MEASUREMENT OF THE EQUILIBRIUM DISSOCIATION CONSTANT FOR THE COMPLEXES BETWEEN ANTITHROMBIN III AND 21 NEW OLIGOSACCHARIDES

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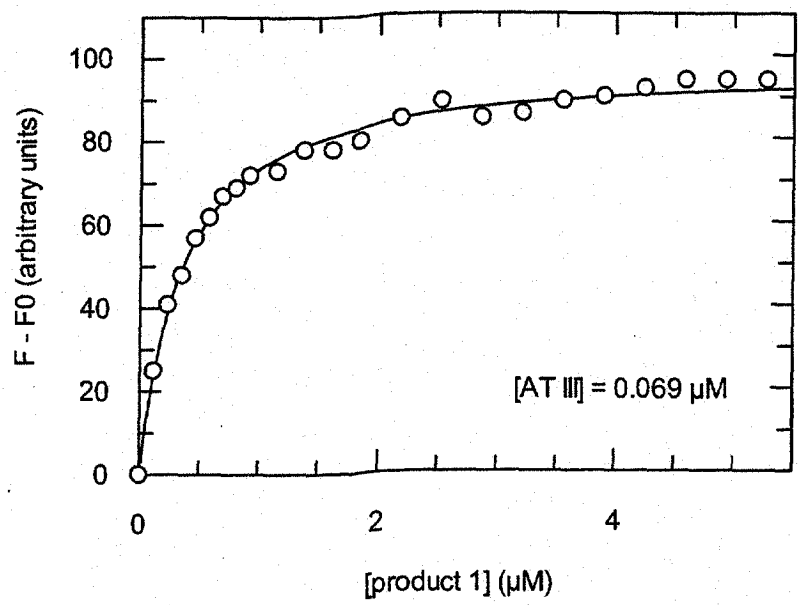
TABLE I

Products identification and concentration of the various oligosaccharide solutions used in this study.

products identification		[a]product concentration in the provided solutions (mM)	[b]product concentration in the working solutions (mM)
reference	number		
P33894-51-1	1	1.72	0.573
P32650-55-1	2	1.22	0.305
P32444-021-2	3	1.47	0.367
P32650-067-1	4	1.14	0.285
P32964-120	5	1.24	1.24
P34279-47-04	6	0.83	0.0166
P32444-021-3	7	1.27	1.27
P32650-061-1	8	1.12	0.56
P32650-063-1	9	1.31	0.655
P32077-090-2	10	1.05	0.525
P33818-64-2	11	1.07	0.107
P33818-62-3	12	1.03	0.206
P33818-44-1	13	0.44	0.44
P31404-012-1	14	1.39	0.278
P33818-36-3	15	0.88	0.088
P33818-64-1	16	1.01	0.202
P33818-62-1	17	0.97	0.194
P33818-62-4	18	1.11	1.11
P33818-44-4	19	0.37	0.0616
P33818-22-4	20	0.35	0.35
P34279-47-2	21	1.02	0.0102 and 0.0204
pentasaccharide	-------	-------	0.0916

[a]each product (except the heparin pentasaccharide) was provided diluted in 0.010 M tris buffer pH 7.5 containing 0.150 M NaCl.
[b]solutions used to perform the equilibrium titration experiments.



Parameter	Value	Std. Error
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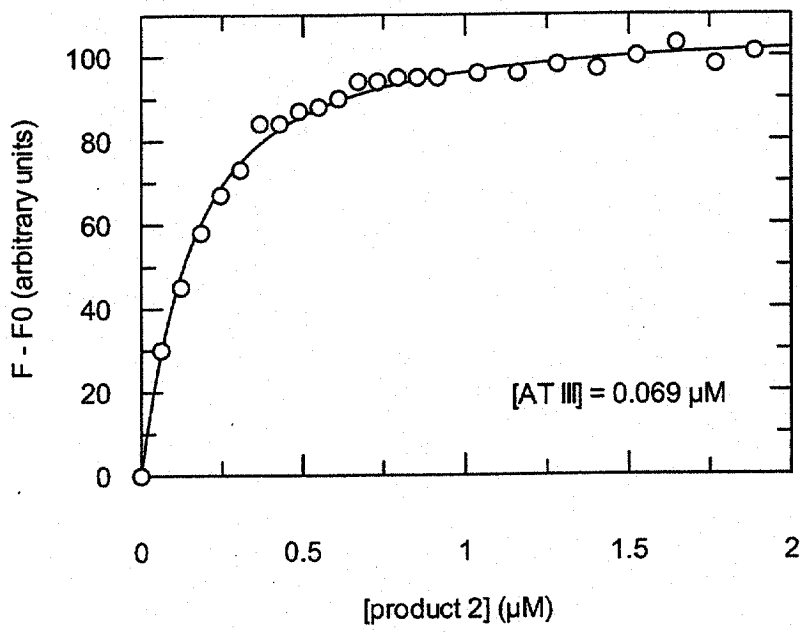

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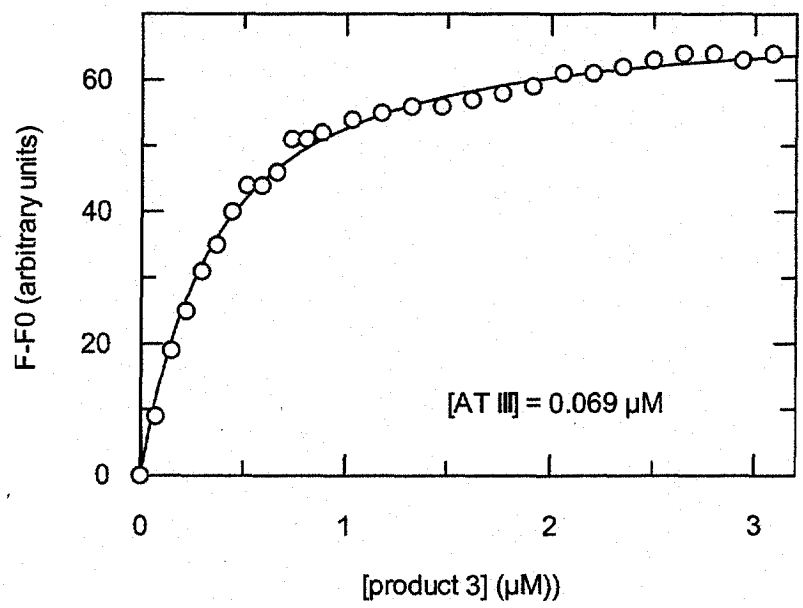

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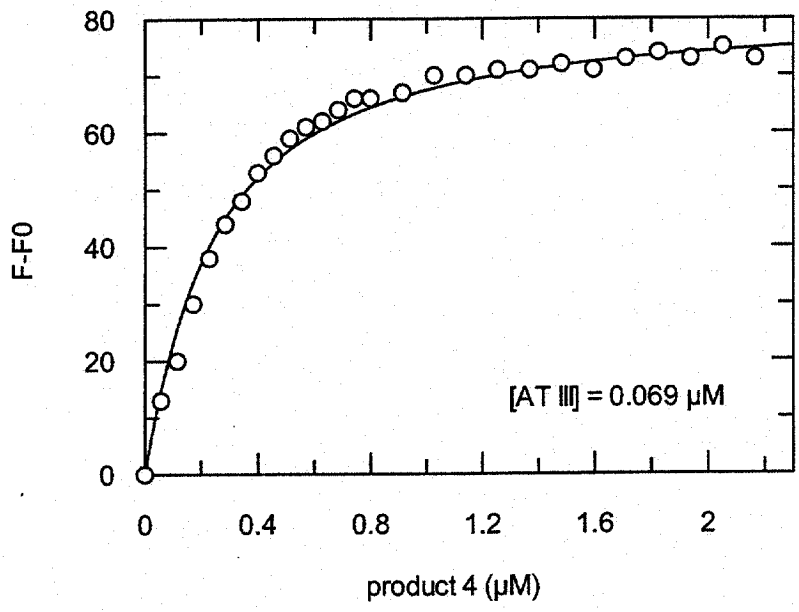

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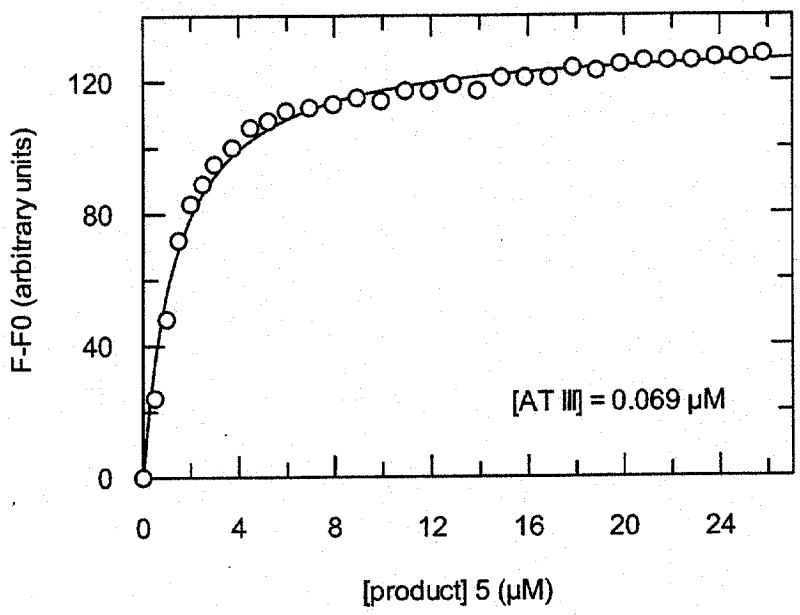

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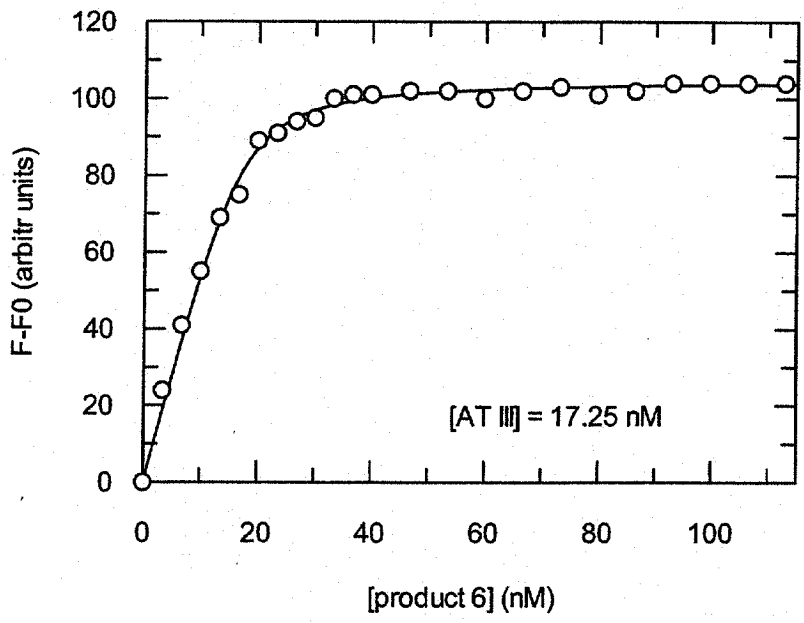

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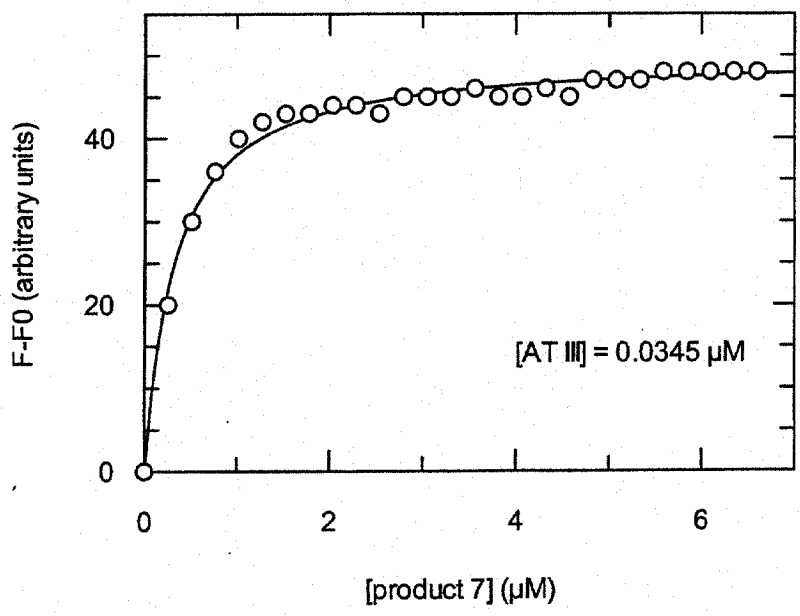

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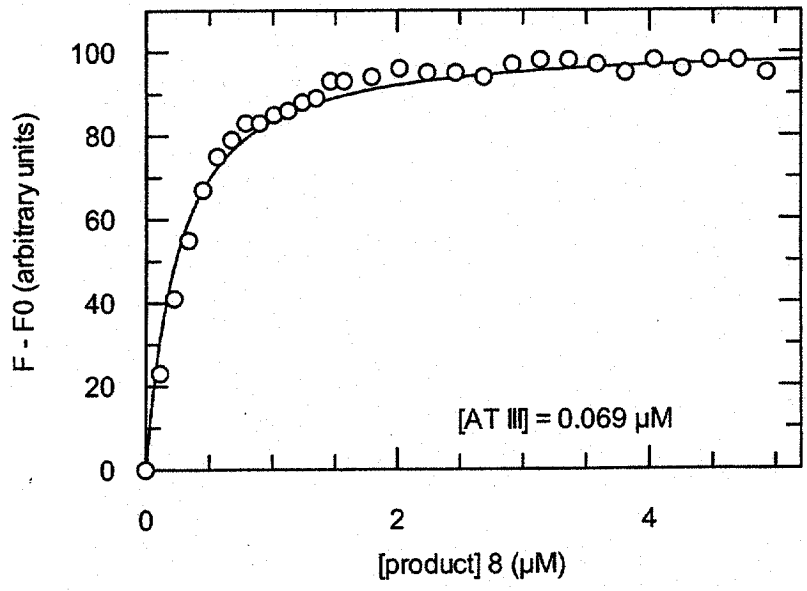

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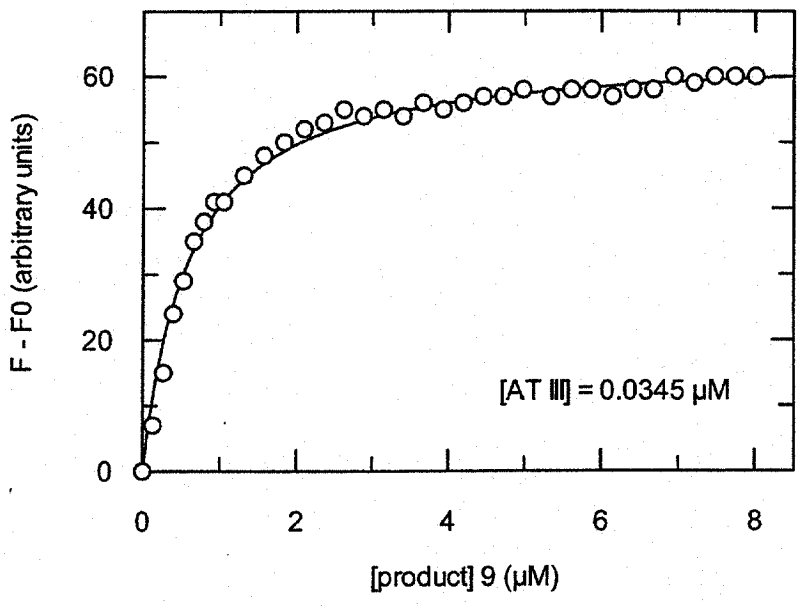

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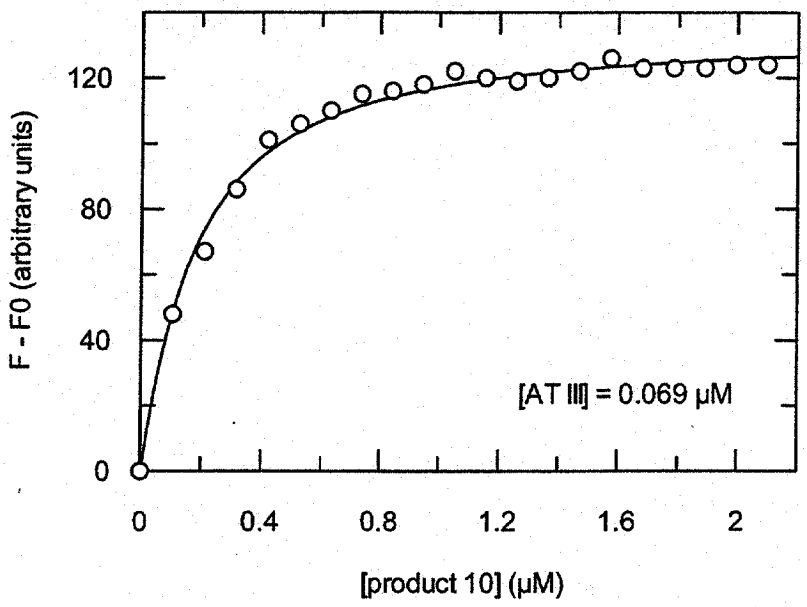

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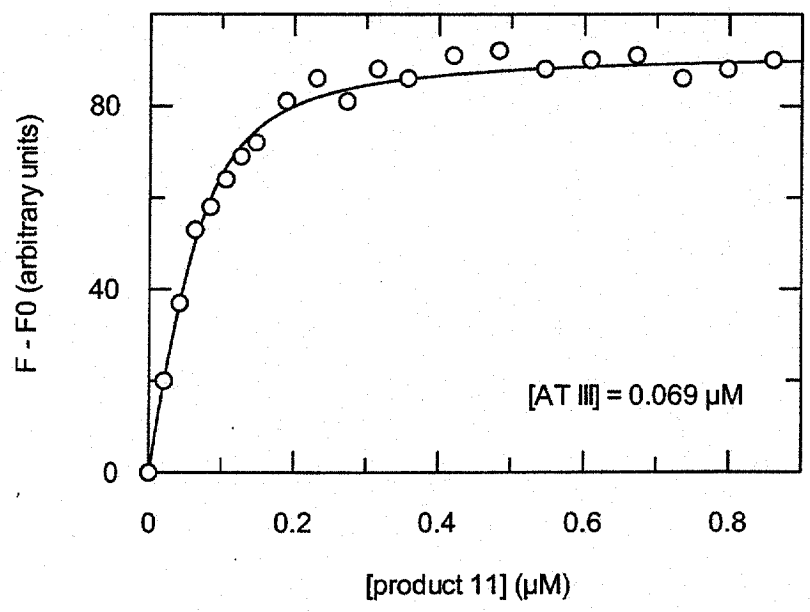

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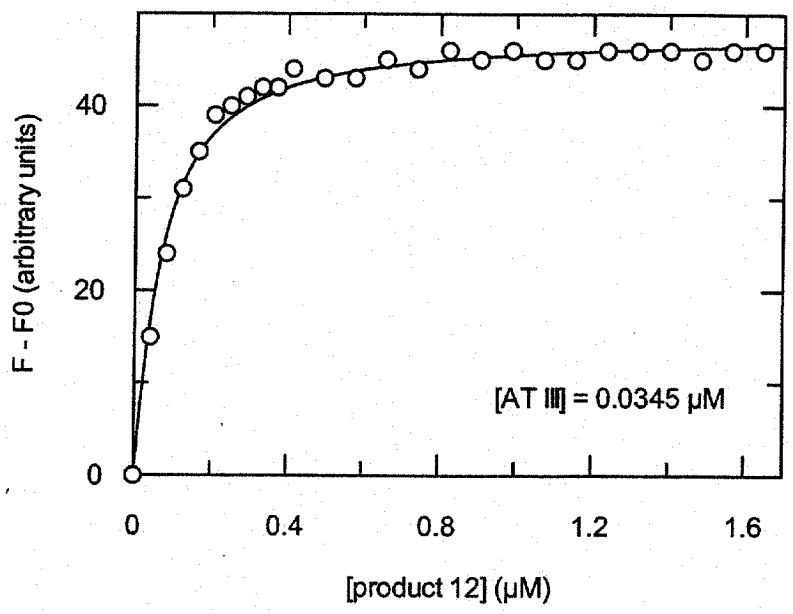

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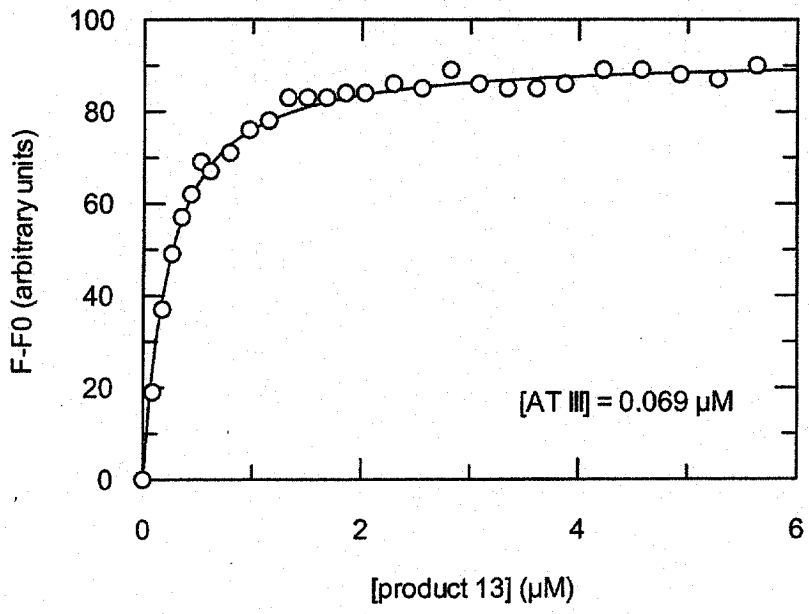

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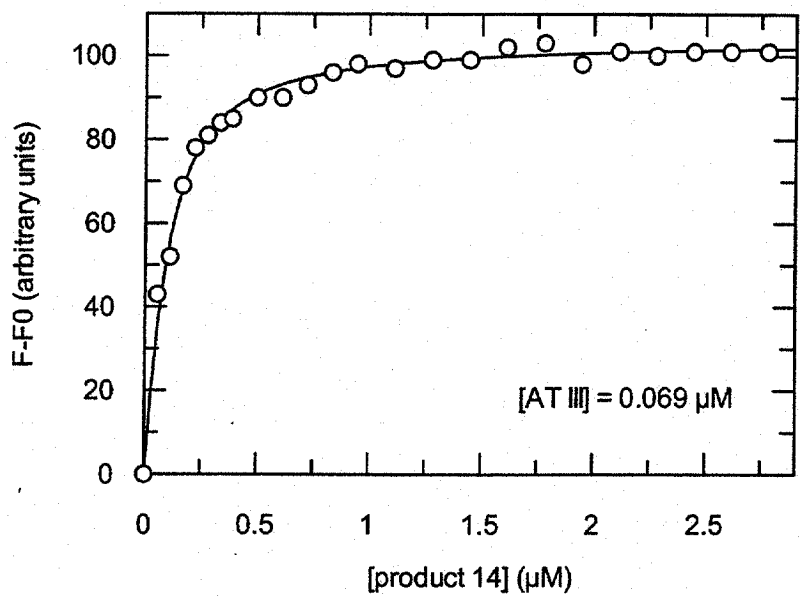

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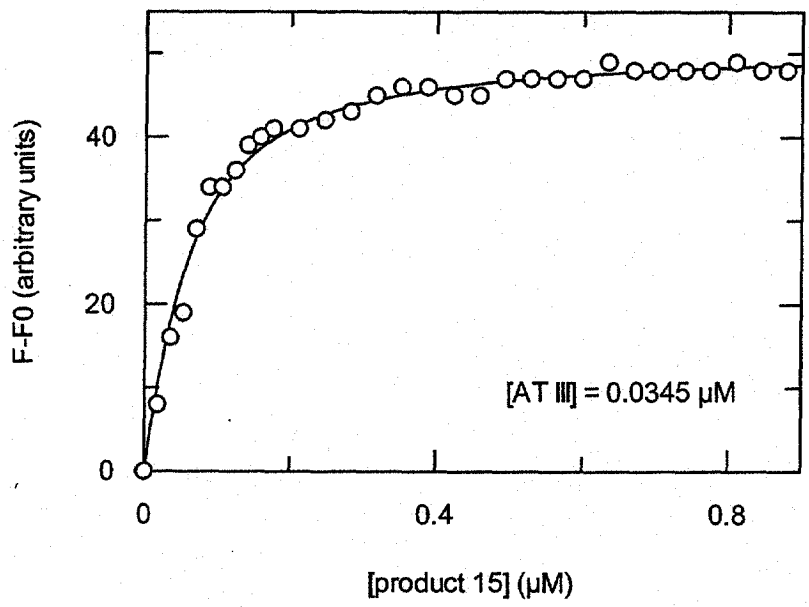

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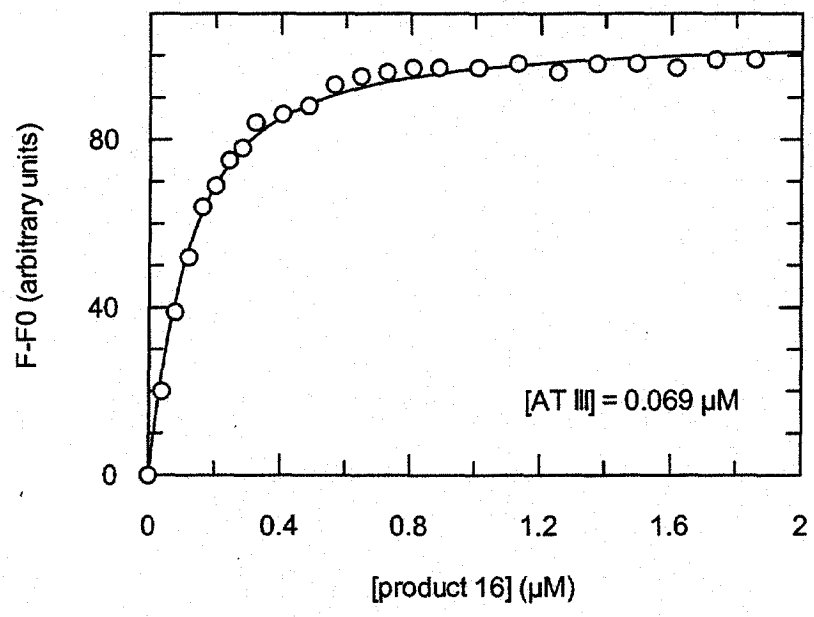

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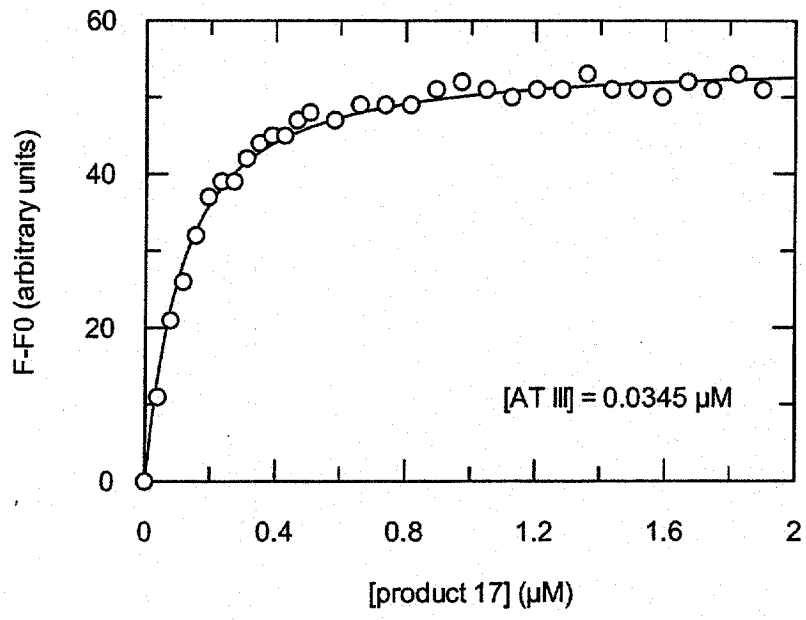

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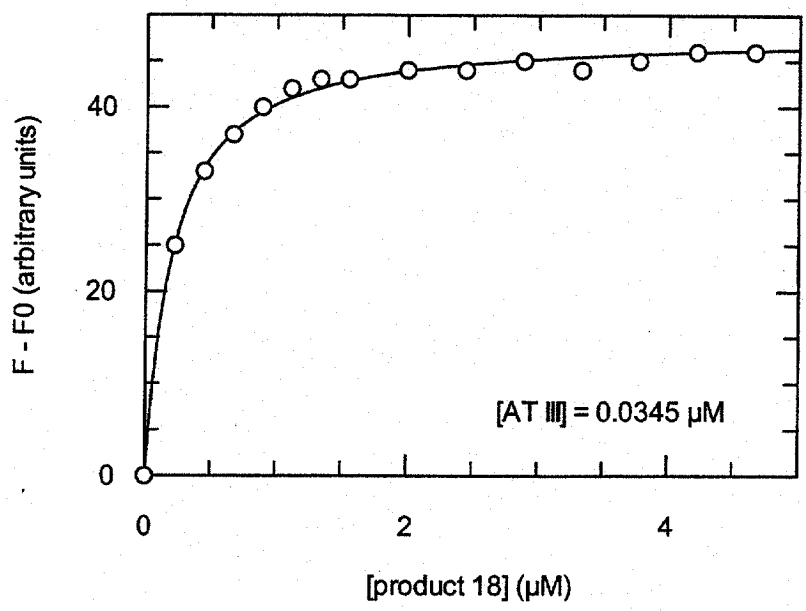

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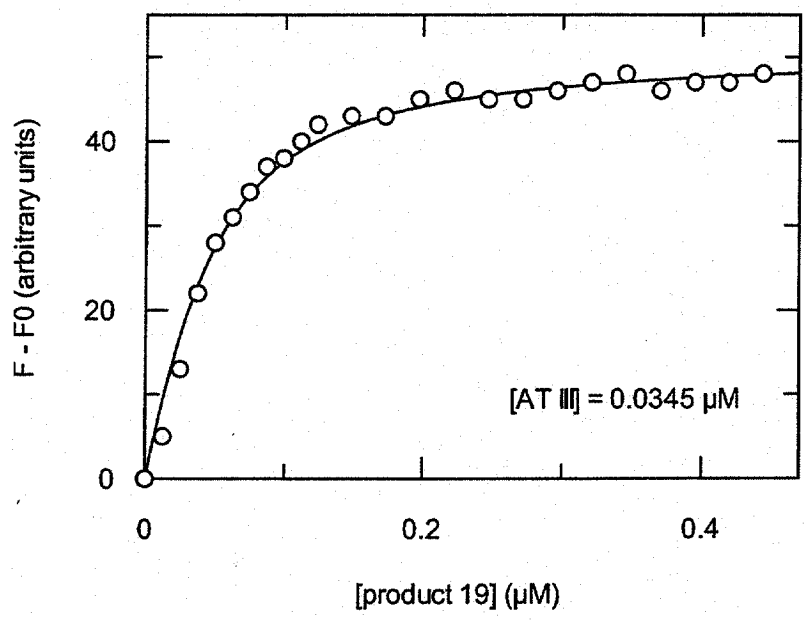

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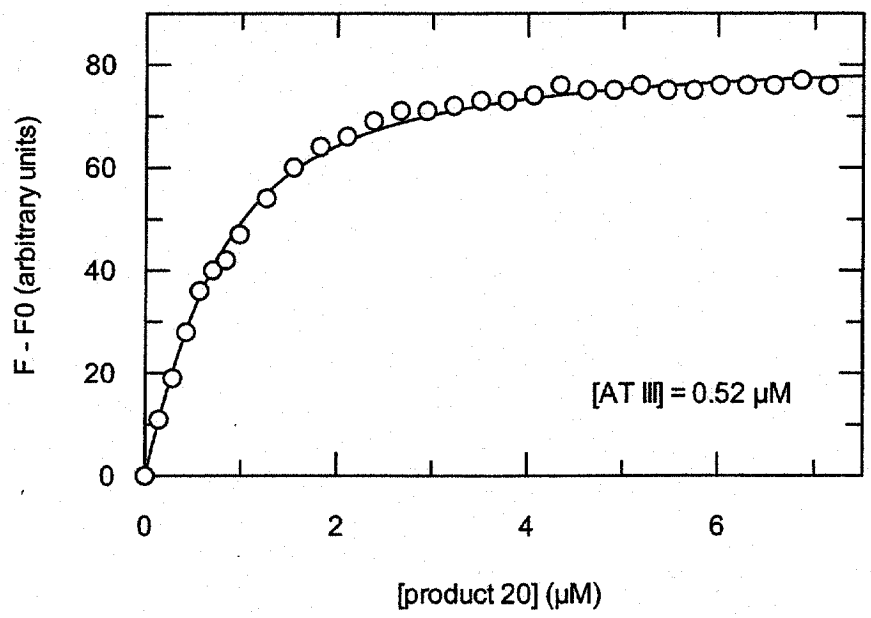

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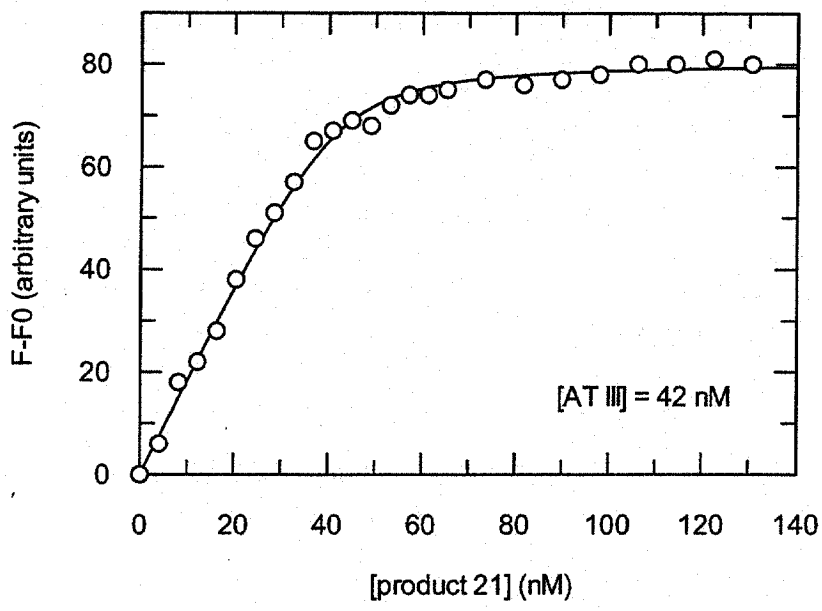

Parameter	Value	Std. Error
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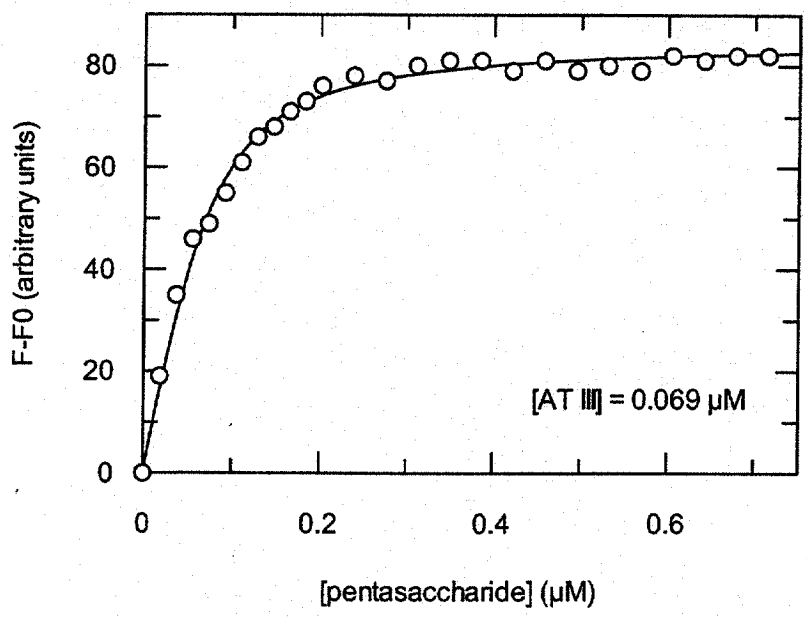
pentasaccharide



Parameter	Value	Std. Error
Kd (μM)	0.0208	0.0010

	1	2	3	4
Type				
Column Name	product 1 (µL)	product 1 (µM)	F (arbitr units)	F-F0 (arbitr units)
1	0.0000	0.0000	234.0000	0.0000
2	0.5000	0.1147	259.0000	25.0000
3	1.0000	0.2293	275.0000	41.0000
4	1.5000	0.3440	282.0000	48.0000
5	2.0000	0.4587	291.0000	57.0000
6	2.5000	0.5733	296.0000	62.0000
7	3.0000	0.6880	301.0000	67.0000
8	3.5000	0.8027	303.0000	69.0000
9	4.0000	0.9173	306.0000	72.0000
10	5.0000	1.1467	307.0000	73.0000
11	6.0000	1.3760	312.0000	78.0000
12	7.0000	1.6053	312.0000	78.0000
13	8.0000	1.8347	314.0000	80.0000
14	9.5000	2.1787	319.0000	85.0000
15	11.0000	2.5227	323.0000	89.0000
16	12.5000	2.8667	319.0000	85.0000
17	14.0000	3.2107	320.0000	86.0000
18	15.5000	3.5547	323.0000	89.0000
19	17.0000	3.8987	324.0000	90.0000
20	18.5000	4.2427	326.0000	92.0000
21	20.0000	4.5867	328.0000	94.0000
22	21.5000	4.9307	328.0000	94.0000
23	23.0000	5.2739	328.0000	94.0000

	1	2	3	4
Type				
Column Name	product2 (µL)	product2 (µM)	F (arbitr units)	F-F0 (arbitr units)
1	0.0000	0.0000	234.0000	0.0000
2	0.5000	0.0610	264.0000	30.0000
3	1.0000	0.1220	279.0000	45.0000
4	1.5000	0.1830	292.0000	58.0000
5	2.0000	0.2440	301.0000	67.0000
6	2.5000	0.3050	307.0000	73.0000
7	3.0000	0.3660	318.0000	84.0000
8	3.5000	0.4270	318.0000	84.0000
9	4.0000	0.4880	321.0000	87.0000
10	4.5000	0.5490	322.0000	88.0000
11	5.0000	0.6100	324.0000	90.0000
12	5.5000	0.6710	328.0000	94.0000
13	6.0000	0.7320	328.0000	94.0000
14	6.5000	0.7930	329.0000	95.0000
15	7.0000	0.8540	329.0000	95.0000
16	7.5000	0.9150	329.0000	95.0000
17	8.5000	1.0370	330.0000	96.0000
18	9.5000	1.1590	330.0000	96.0000
19	10.5000	1.2810	332.0000	98.0000
20	11.5000	1.4030	331.0000	97.0000
21	12.5000	1.5250	334.0000	100.0000
22	13.5000	1.6470	337.0000	103.0000
23	14.5000	1.7690	332.0000	98.0000
24	15.5000	1.8900	335.0000	101.0000

	1	2	3	4
Type				
Column Name	product 3 (µL)	product 3 (µM)	F (arbitr units)	F-F0 (arbitr units)
1	0.0000	0.0000	186.0000	0.0000
2	0.5000	0.0735	195.0000	9.0000
3	1.0000	0.1470	205.0000	19.0000
4	1.5000	0.2205	211.0000	25.0000
5	2.0000	0.2940	217.0000	31.0000
6	2.5000	0.3675	221.0000	35.0000
7	3.0000	0.4410	226.0000	40.0000
8	3.5000	0.5145	230.0000	44.0000
9	4.0000	0.5880	230.0000	44.0000
10	4.5000	0.6615	232.0000	46.0000
11	5.0000	0.7350	237.0000	51.0000
12	5.5000	0.8085	237.0000	51.0000
13	6.0000	0.8820	238.0000	52.0000
14	7.0000	1.0290	240.0000	54.0000
15	8.0000	1.1760	241.0000	55.0000
16	9.0000	1.3230	242.0000	56.0000
17	10.0000	1.4700	242.0000	56.0000
18	11.0000	1.6170	243.0000	57.0000
19	12.0000	1.7640	244.0000	58.0000
20	13.0000	1.9110	245.0000	59.0000
21	14.0000	2.0580	247.0000	61.0000
22	15.0000	2.2050	247.0000	61.0000
23	16.0000	2.3500	248.0000	62.0000
24	17.0000	2.4990	249.0000	63.0000
25	18.0000	2.6460	251.0000	64.0000
26	19.0000	2.7930	250.0000	64.0000
27	20.0000	2.9400	249.0000	63.0000
28	21.0000	3.0870	250.0000	64.0000

	1	2	3	4
Type				
Column Name	product 4 (µL)	product 4 (µM)	F (arbitr units)	F-F0 (arbitr units)
1	0.0000	0.0000	214.0000	0.0000
2	0.5000	0.0570	227.0000	13.0000
3	1.0000	0.1140	234.0000	20.0000
4	1.5000	0.1710	244.0000	30.0000
5	2.0000	0.2280	252.0000	38.0000
6	2.5000	0.2850	258.0000	44.0000
7	3.0000	0.3420	262.0000	48.0000
8	3.5000	0.3990	267.0000	53.0000
9	4.0000	0.4560	270.0000	56.0000
10	4.5000	0.5130	273.0000	59.0000
11	5.0000	0.5700	275.0000	61.0000
12	5.5000	0.6270	276.0000	62.0000
13	6.0000	0.6840	278.0000	64.0000
14	6.5000	0.7410	280.0000	66.0000
15	7.0000	0.7980	280.0000	66.0000
16	8.0000	0.9120	281.0000	67.0000
17	9.0000	1.0260	284.0000	70.0000
18	10.0000	1.1400	284.0000	70.0000
19	11.0000	1.2540	285.0000	71.0000
20	12.0000	1.3680	285.0000	71.0000
21	13.0000	1.4820	286.0000	72.0000
22	14.0000	1.5960	285.0000	71.0000
23	15.0000	1.7100	287.0000	73.0000
24	16.0000	1.8240	288.0000	74.0000
25	17.0000	1.9380	287.0000	73.0000
26	18.0000	2.0520	289.0000	75.0000
27	19.0000	2.1660	287.0000	73.0000

	1	2	3	4
Type				
Column Name	product 5 (µL)	product 5 (µM)	F (arbitr units)	F-F0 (arbitr units)
1	0.0000	0.0000	337.0000	0.0000
2	1.0000	0.4960	361.0000	24.0000
3	2.0000	0.9920	385.0000	48.0000
4	3.0000	1.4880	409.0000	72.0000
5	4.0000	1.9840	420.0000	83.0000
6	5.0000	2.4800	426.0000	89.0000
7	6.0000	2.9760	432.0000	95.0000
8	7.5000	3.7200	437.0000	100.0000
9	9.0000	4.4640	443.0000	106.0000
10	10.5000	5.2080	445.0000	108.0000
11	12.0000	5.9520	448.0000	111.0000
12	14.0000	6.9440	449.0000	112.0000
13	16.0000	7.9360	450.0000	113.0000
14	18.0000	8.9280	452.0000	115.0000
15	20.0000	9.9200	451.0000	114.0000
16	22.0000	10.9120	454.0000	117.0000
17	24.0000	11.9040	454.0000	117.0000
18	26.0000	12.8960	456.0000	119.0000
19	28.0000	13.8880	454.0000	117.0000
20	30.0000	14.8800	458.0000	121.0000
21	32.0000	15.8720	458.0000	121.0000
22	34.0000	16.8640	458.0000	121.0000
23	36.0000	17.8560	461.0000	124.0000
24	38.0000	18.8480	460.0000	123.0000
25	40.0000	19.8400	462.0000	125.0000
26	42.0000	20.8320	463.0000	126.0000
27	44.0000	21.8240	463.0000	126.0000
28	46.0000	22.8160	463.0000	126.0000
29	48.0000	23.8080	464.0000	127.0000
30	50.0000	24.8000	464.0000	127.0000
31	52.0000	25.7920	465.0000	128.0000

	1	2	3	4
Type				
Column Name	product 6 (µL)	product 6 (nM)	F (arbitr units)	F-F0 (arbitr units)
1	0.0000	0.0000	228.0000	0.0000
2	0.5000	3.3200	252.0000	24.0000
3	1.0000	6.6400	269.0000	41.0000
4	1.5000	9.9600	283.0000	55.0000
5	2.0000	13.2800	297.0000	69.0000
6	2.5000	16.6000	303.0000	75.0000
7	3.0000	19.9200	317.0000	89.0000
8	3.5000	23.2400	319.0000	91.0000
9	4.0000	26.5600	322.0000	94.0000
10	4.5000	29.8800	323.0000	95.0000
11	5.0000	33.2000	328.0000	100.0000
12	5.5000	36.5200	329.0000	101.0000
13	6.0000	39.8400	329.0000	101.0000
14	7.0000	46.4800	330.0000	102.0000
15	8.0000	53.1200	330.0000	102.0000
16	9.0000	59.7600	328.0000	100.0000
17	10.0000	66.4000	330.0000	102.0000
18	11.0000	73.0400	331.0000	103.0000
19	12.0000	79.6800	329.0000	101.0000
20	13.0000	86.3200	330.0000	102.0000
21	14.0000	92.9600	332.0000	104.0000
22	15.0000	99.6000	332.0000	104.0000
23	16.0000	106.2400	332.0000	104.0000
24	17.0000	112.8800	332.0000	104.0000

	1	2	3	4
Type				
Column Name	product 7 (µL)	product 7 (µM)	F (arbitr units)	F-F0 (arbitr units)
1	0.0000	0.0000	129.0000	0.0000
2	0.5000	0.2540	149.0000	20.0000
3	1.0000	0.5080	159.0000	30.0000
4	1.5000	0.7620	165.0000	36.0000
5	2.0000	1.0160	169.0000	40.0000
6	2.5000	1.2700	171.0000	42.0000
7	3.0000	1.5240	172.0000	43.0000
8	3.5000	1.7780	172.0000	43.0000
9	4.0000	2.0320	173.0000	44.0000
10	4.5000	2.2860	173.0000	44.0000
11	5.0000	2.5400	172.0000	43.0000
12	5.5000	2.7940	174.0000	45.0000
13	6.0000	3.0480	174.0000	45.0000
14	6.5000	3.3020	174.0000	45.0000
15	7.0000	3.5560	175.0000	46.0000
16	7.5000	3.8100	174.0000	45.0000
17	8.0000	4.0640	174.0000	45.0000
18	8.5000	4.3180	175.0000	46.0000
19	9.0000	4.5720	174.0000	45.0000
20	9.5000	4.8260	176.0000	47.0000
21	10.0000	5.0800	176.0000	47.0000
22	10.5000	5.3340	176.0000	47.0000
23	11.0000	5.5880	177.0000	48.0000
24	11.5000	5.8420	177.0000	48.0000
25	12.0000	6.0960	177.0000	48.0000
26	12.5000	6.3500	177.0000	48.0000
27	13.0000	6.6040	177.0000	48.0000

	1	2	3	4
Type				
Column Name	product 8 (µL)	product 8 (µM)	F (arbitr units)	F - F0 (arbitr units)
1	0.0000	0.0000	255.0000	0.0000
2	0.5000	0.1120	278.0000	23.0000
3	1.0000	0.2240	296.0000	41.0000
4	1.5000	0.3360	310.0000	55.0000
5	2.0000	0.4480	322.0000	67.0000
6	2.5000	0.5600	330.0000	75.0000
7	3.0000	0.6720	334.0000	79.0000
8	3.5000	0.7840	338.0000	83.0000
9	4.0000	0.8960	338.0000	83.0000
10	4.5000	1.0080	340.0000	85.0000
11	5.0000	1.1200	341.0000	86.0000
12	5.5000	1.2320	343.0000	88.0000
13	6.0000	1.3440	344.0000	89.0000
14	6.5000	1.4560	348.0000	93.0000
15	7.0000	1.5680	348.0000	93.0000
16	8.0000	1.7920	349.0000	94.0000
17	9.0000	2.0160	351.0000	96.0000
18	10.0000	2.2400	350.0000	95.0000
19	11.0000	2.4640	350.0000	95.0000
20	12.0000	2.6880	349.0000	94.0000
21	13.0000	2.9120	352.0000	97.0000
22	14.0000	3.1360	353.0000	98.0000
23	15.0000	3.3600	353.0000	98.0000
24	16.0000	3.5840	352.0000	97.0000
25	17.0000	3.8080	350.0000	95.0000
26	18.0000	4.0320	353.0000	98.0000
27	19.0000	4.2560	351.0000	96.0000
28	20.0000	4.4800	353.0000	98.0000
29	21.0000	4.7040	353.0000	98.0000
30	22.0000	4.9280	350.0000	95.0000

	1	2	3	4
Type				
Column Name	product 9 (µL)	product 9 (µM)	F (arbitr units)	F - F0 (arbitr units)
1	0.0000	0.0000	138.0000	0.0000
2	0.5000	0.1310	145.0000	7.0000
3	1.0000	0.2620	153.0000	15.0000
4	1.5000	0.3930	162.0000	24.0000
5	2.0000	0.5240	167.0000	29.0000
6	2.5000	0.6550	173.0000	35.0000
7	3.0000	0.7860	176.0000	38.0000
8	3.5000	0.9170	179.0000	41.0000
9	4.0000	1.0480	179.0000	41.0000
10	5.0000	1.3100	183.0000	45.0000
11	6.0000	1.5720	186.0000	48.0000
12	7.0000	1.8340	188.0000	50.0000
13	8.0000	2.0960	190.0000	52.0000
14	9.0000	2.3580	191.0000	53.0000
15	10.0000	2.6200	193.0000	55.0000
16	11.0000	2.8820	192.0000	54.0000
17	12.0000	3.1440	193.0000	55.0000
18	13.0000	3.4060	192.0000	54.0000
19	14.0000	3.6680	194.0000	56.0000
20	15.0000	3.9300	193.0000	55.0000
21	16.0000	4.1920	194.0000	56.0000
22	17.0000	4.4540	195.0000	57.0000
23	18.0000	4.7160	195.0000	57.0000
24	19.0000	4.9780	196.0000	58.0000
25	20.0000	5.3400	195.0000	57.0000
26	21.0000	5.6070	196.0000	58.0000
27	22.0000	5.8740	196.0000	58.0000
28	23.0000	6.1410	195.0000	57.0000
29	24.0000	6.4080	196.0000	58.0000
30	25.0000	6.6750	196.0000	58.0000
31	26.0000	6.9420	198.0000	60.0000
32	27.0000	7.2090	197.0000	59.0000
33	28.0000	7.4760	198.0000	60.0000
34	29.0000	7.7430	198.0000	60.0000
35	30.0000	8.0100	198.0000	60.0000

	1	2	3	4
Type				
Column Name	product 10 (µL)	product 10 (µM)	F (arbitr units)	F - F0 (arbitr units)
1	0.0000	0.0000	262.0000	0.0000
2	0.5000	0.1050	310.0000	48.0000
3	1.0000	0.2100	329.0000	67.0000
4	1.5000	0.3150	348.0000	86.0000
5	2.0000	0.4200	363.0000	101.0000
6	2.5000	0.5250	368.0000	106.0000
7	3.0000	0.6300	372.0000	110.0000
8	3.5000	0.7350	377.0000	115.0000
9	4.0000	0.8400	378.0000	116.0000
10	4.5000	0.9450	380.0000	118.0000
11	5.0000	1.0500	384.0000	122.0000
12	5.5000	1.1550	382.0000	120.0000
13	6.0000	1.2600	381.0000	119.0000
14	6.5000	1.3650	382.0000	120.0000
15	7.0000	1.4700	384.0000	122.0000
16	7.5000	1.5750	388.0000	126.0000
17	8.0000	1.6800	385.0000	123.0000
18	8.5000	1.7850	385.0000	123.0000
19	9.0000	1.8900	385.0000	123.0000
20	9.5000	1.9950	386.0000	124.0000
21	10.0000	2.1000	386.0000	124.0000

	1	2	3	4
Type				
Column Name	product 11 (µL)	product 11 (µM)	F (arbitr units)	F - F0 (arbitr units)
1	0.0000	0.0000	261.0000	0.0000
2	0.5000	0.0210	281.0000	20.0000
3	1.0000	0.0420	298.0000	37.0000
4	1.5000	0.0630	314.0000	53.0000
5	2.0000	0.0840	319.0000	58.0000
6	2.5000	0.1050	325.0000	64.0000
7	3.0000	0.1260	330.0000	69.0000
8	3.5000	0.1470	333.0000	72.0000
9	4.5000	0.1890	342.0000	81.0000
10	5.5000	0.2310	347.0000	86.0000
11	6.5000	0.2730	342.0000	81.0000
12	7.5000	0.3150	349.0000	88.0000
13	8.5000	0.3570	347.0000	86.0000
14	10.0000	0.4200	352.0000	91.0000
15	11.5000	0.4830	353.0000	92.0000
16	13.0000	0.5460	349.0000	88.0000
17	14.5000	0.6090	351.0000	90.0000
18	16.0000	0.6720	352.0000	91.0000
19	17.5000	0.7350	347.0000	86.0000
20	19.0000	0.7980	349.0000	88.0000
21	20.5000	0.8610	351.0000	90.0000

	1	2	3	4
Type				
Column Name	product 12 (µL)	product 12 (µM)	F (arbitr units)	F - F0 (arbitr units)
1	0.0000	0.0000	125.0000	0.0000
2	0.5000	0.0412	140.0000	15.0000
3	1.0000	0.0824	149.0000	24.0000
4	1.5000	0.1236	156.0000	31.0000
5	2.0000	0.1648	160.0000	35.0000
6	2.5000	0.2060	164.0000	39.0000
7	3.0000	0.2472	165.0000	40.0000
8	3.5000	0.2884	166.0000	41.0000
9	4.0000	0.3296	167.0000	42.0000
10	4.5000	0.3708	167.0000	42.0000
11	5.0000	0.4120	169.0000	44.0000
12	6.0000	0.4944	168.0000	43.0000
13	7.0000	0.5768	168.0000	43.0000
14	8.0000	0.6592	170.0000	45.0000
15	9.0000	0.7416	169.0000	44.0000
16	10.0000	0.8240	171.0000	46.0000
17	11.0000	0.9064	170.0000	45.0000
18	12.0000	0.9888	171.0000	46.0000
19	13.0000	1.0712	170.0000	45.0000
20	14.0000	1.1536	170.0000	45.0000
21	15.0000	1.2360	171.0000	46.0000
22	16.0000	1.3184	171.0000	46.0000
23	17.0000	1.4008	171.0000	46.0000
24	18.0000	1.4832	170.0000	45.0000
25	19.0000	1.5656	171.0000	46.0000
26	20.0000	1.6480	171.0000	46.0000

	1	2	3	4
Type				
Column Name	product 13 (µL)	product 13 (µM)	F (arbitr units)	F - F0 (arbitr units)
1	0.0000	0.0000	252.0000	0.0000
2	0.5000	0.0880	271.0000	19.0000
3	1.0000	0.1760	289.0000	37.0000
4	1.5000	0.2640	301.0000	49.0000
5	2.0000	0.3520	309.0000	57.0000
6	2.5000	0.4400	314.0000	62.0000
7	3.0000	0.5280	321.0000	69.0000
8	3.5000	0.6160	319.0000	67.0000
9	4.5000	0.7920	323.0000	71.0000
10	5.5000	0.9680	328.0000	76.0000
11	6.5000	1.1440	330.0000	78.0000
12	7.5000	1.3200	335.0000	83.0000
13	8.5000	1.4960	335.0000	83.0000
14	9.5000	1.6720	335.0000	83.0000
15	10.5000	1.8480	336.0000	84.0000
16	11.5000	2.0240	336.0000	84.0000
17	13.0000	2.2880	338.0000	86.0000
18	14.5000	2.5520	337.0000	85.0000
19	16.0000	2.8160	341.0000	89.0000
20	17.5000	3.0800	338.0000	86.0000
21	19.0000	3.3440	337.0000	85.0000
22	20.5000	3.6080	337.0000	85.0000
23	22.0000	3.8720	338.0000	86.0000
24	24.0000	4.2240	341.0000	89.0000
25	26.0000	4.5760	341.0000	89.0000
26	28.0000	4.9280	340.0000	88.0000
27	30.0000	5.2800	339.0000	87.0000
28	32.0000	5.6320	342.0000	90.0000

	1	2	3	4
Type				
Column Name	product 14 (µL)	product 14 (µM)	F (arbitr units)	F-F0 (arbitr units)
1	0.0000	0.0000	234.0000	0.0000
2	0.5000	0.0556	277.0000	43.0000
3	1.0000	0.1120	286.0000	52.0000
4	1.5000	0.1668	303.0000	69.0000
5	2.0000	0.2224	312.0000	78.0000
6	2.5000	0.2780	315.0000	81.0000
7	3.0000	0.3336	318.0000	84.0000
8	3.5000	0.3892	319.0000	85.0000
9	4.5000	0.5004	324.0000	90.0000
10	5.5000	0.6116	324.0000	90.0000
11	6.5000	0.7228	327.0000	93.0000
12	7.5000	0.8340	330.0000	96.0000
13	8.5000	0.9452	332.0000	98.0000
14	10.0000	1.1120	331.0000	97.0000
15	11.5000	1.2788	333.0000	99.0000
16	13.0000	1.4456	333.0000	99.0000
17	14.5000	1.6124	336.0000	102.0000
18	16.0000	1.7792	337.0000	103.0000
19	17.5000	1.9460	332.0000	98.0000
20	19.0000	2.1128	335.0000	101.0000
21	20.5000	2.2796	334.0000	100.0000
22	22.0000	2.4464	335.0000	101.0000
23	23.5000	2.6132	335.0000	101.0000
24	25.0000	2.7800	335.0000	101.0000

	1	2	3	4
Type				
Column Name	product 15 (µL)	product 15 (µM)	F (arbitr units)	F-F0 (arbitr units)
1	0.0000	0.0000	118.0000	0.0000
2	0.5000	0.0176	126.0000	8.0000
3	1.0000	0.0352	134.0000	16.0000
4	1.5000	0.0528	137.0000	19.0000
5	2.0000	0.0704	147.0000	29.0000
6	2.5000	0.0880	152.0000	34.0000
7	3.0000	0.1056	152.0000	34.0000
8	3.5000	0.1232	154.0000	36.0000
9	4.0000	0.1408	157.0000	39.0000
10	4.5000	0.1584	158.0000	40.0000
11	5.0000	0.1760	159.0000	41.0000
12	6.0000	0.2112	159.0000	41.0000
13	7.0000	0.2464	160.0000	42.0000
14	8.0000	0.2816	161.0000	43.0000
15	9.0000	0.3168	163.0000	45.0000
16	10.0000	0.3520	164.0000	46.0000
17	11.0000	0.3872	164.0000	46.0000
18	12.0000	0.4224	163.0000	45.0000
19	13.0000	0.4576	163.0000	45.0000
20	14.0000	0.4928	165.0000	47.0000
21	15.0000	0.5280	165.0000	47.0000
22	16.0000	0.5632	165.0000	47.0000
23	17.0000	0.5984	165.0000	47.0000
24	18.0000	0.6336	167.0000	49.0000
25	19.0000	0.6688	166.0000	48.0000
26	20.0000	0.7040	166.0000	48.0000
27	21.0000	0.7392	166.0000	48.0000
28	22.0000	0.7744	166.0000	48.0000
29	23.0000	0.8096	167.0000	49.0000
30	24.0000	0.8448	166.0000	48.0000
31	25.0000	0.8800	166.0000	48.0000

	1	2	3	4
Type				
Column Name	product 16 (µL)	product 16 (µM)	F (arbitr units)	F-F0 (arbitr units)
1	0.0000	0.0000	248.0000	0.0000
2	0.5000	0.0404	268.0000	20.0000
3	1.0000	0.0808	287.0000	39.0000
4	1.5000	0.1212	300.0000	52.0000
5	2.0000	0.1616	312.0000	64.0000
6	2.5000	0.2020	317.0000	69.0000
7	3.0000	0.2424	323.0000	75.0000
8	3.5000	0.2828	326.0000	78.0000
9	4.0000	0.3232	332.0000	84.0000
10	5.0000	0.4040	334.0000	86.0000
11	6.0000	0.4848	336.0000	88.0000
12	7.0000	0.5656	341.0000	93.0000
13	8.0000	0.6464	343.0000	95.0000
14	9.0000	0.7272	344.0000	96.0000
15	10.0000	0.8080	345.0000	97.0000
16	11.0000	0.8888	345.0000	97.0000
17	12.5000	1.0100	345.0000	97.0000
18	14.0000	1.1312	346.0000	98.0000
19	15.5000	1.2524	344.0000	96.0000
20	17.0000	1.3736	346.0000	98.0000
21	18.5000	1.4948	346.0000	98.0000
22	20.0000	1.6160	345.0000	97.0000
23	21.5000	1.7372	347.0000	99.0000
24	23.0000	1.8584	347.0000	99.0000

	1	2	3	4
Type				
Column Name	product 17 (µL)	product 17 (µM)	F (arbitr units)	F-F0 (arbitr units)
1	0.0000	0.0000	125.0000	0.0000
2	0.5000	0.0388	136.0000	11.0000
3	1.0000	0.0776	146.0000	21.0000
4	1.5000	0.1164	151.0000	26.0000
5	2.0000	0.1552	157.0000	32.0000
6	2.5000	0.1940	162.0000	37.0000
7	3.0000	0.2328	164.0000	39.0000
8	3.5000	0.2716	164.0000	39.0000
9	4.0000	0.3104	167.0000	42.0000
10	4.5000	0.3492	169.0000	44.0000
11	5.0000	0.3880	170.0000	45.0000
12	5.5000	0.4268	170.0000	45.0000
13	6.0000	0.4656	172.0000	47.0000
14	6.5000	0.5044	173.0000	48.0000
15	7.5000	0.5820	172.0000	47.0000
16	8.5000	0.6596	174.0000	49.0000
17	9.5000	0.7372	174.0000	49.0000
18	10.5000	0.8148	174.0000	49.0000
19	11.5000	0.8924	176.0000	51.0000
20	12.5000	0.9700	177.0000	52.0000
21	13.5000	1.0476	176.0000	51.0000
22	14.5000	1.1252	175.0000	50.0000
23	15.5000	1.2028	176.0000	51.0000
24	16.5000	1.2804	176.0000	51.0000
25	17.5000	1.3580	178.0000	53.0000
26	18.5000	1.4356	176.0000	51.0000
27	19.5000	1.5132	176.0000	51.0000
28	20.5000	1.5908	175.0000	50.0000
29	21.5000	1.6684	177.0000	52.0000
30	22.5000	1.7460	176.0000	51.0000
31	23.5000	1.8236	178.0000	53.0000
32	24.5000	1.9012	176.0000	51.0000

	1	2	3	4
Type				
Column Name	product 18 (µL)	product 18 (µM)	F (arbitr units)	F - F0 (arbitr units)
1	0.0000	0.0000	130.0000	0.0000
2	0.5000	0.2220	155.0000	25.0000
3	1.0000	0.4440	163.0000	33.0000
4	1.5000	0.6660	167.0000	37.0000
5	2.0000	0.8880	170.0000	40.0000
6	2.5000	1.1100	172.0000	42.0000
7	3.0000	1.3320	173.0000	43.0000
8	3.5000	1.5540	173.0000	43.0000
9	4.5000	1.9980	174.0000	44.0000
10	5.5000	2.4420	174.0000	44.0000
11	6.5000	2.8860	175.0000	45.0000
12	7.5000	3.3300	174.0000	44.0000
13	8.5000	3.7740	175.0000	45.0000
14	9.5000	4.2180	176.0000	46.0000
15	10.5000	4.6620	176.0000	46.0000

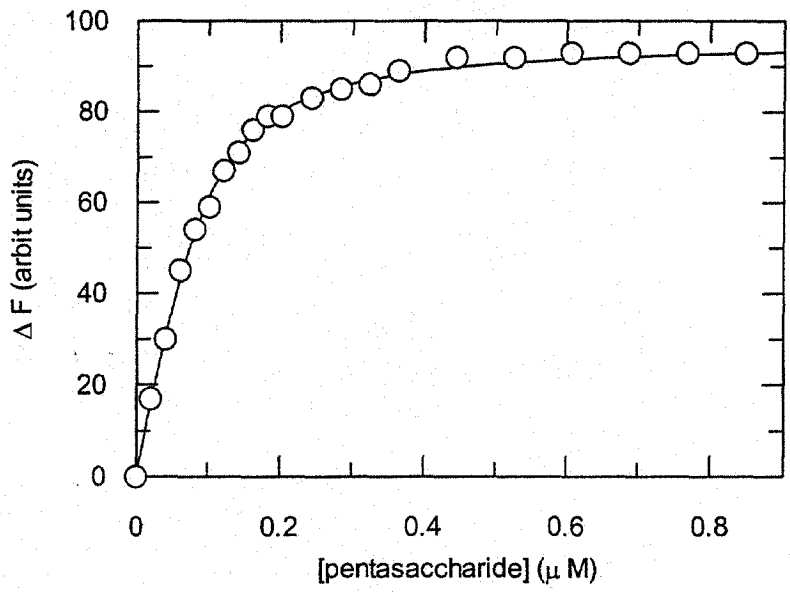
	1	2	3	4
Type				
Column Name	product 19 (µL)	product 19 (µM)	F (arbitr units)	F - F0 (arbitr units)
1	0.0000	0.0000	132.0000	0.0000
2	0.5000	0.0123	137.0000	5.0000
3	1.0000	0.0247	145.0000	13.0000
4	1.5000	0.0370	154.0000	22.0000
5	2.0000	0.0493	160.0000	28.0000
6	2.5000	0.0617	163.0000	31.0000
7	3.0000	0.0740	166.0000	34.0000
8	3.5000	0.0863	169.0000	37.0000
9	4.0000	0.0987	170.0000	38.0000
10	4.5000	0.1110	172.0000	40.0000
11	5.0000	0.1233	174.0000	42.0000
12	6.0000	0.1480	175.0000	43.0000
13	7.0000	0.1727	175.0000	43.0000
14	8.0000	0.1973	177.0000	45.0000
15	9.0000	0.2220	178.0000	46.0000
16	10.0000	0.2467	177.0000	45.0000
17	11.0000	0.2713	177.0000	45.0000
18	12.0000	0.2960	178.0000	46.0000
19	13.0000	0.3207	179.0000	47.0000
20	14.0000	0.3453	180.0000	48.0000
21	15.0000	0.3700	178.0000	46.0000
22	16.0000	0.3947	179.0000	47.0000
23	17.0000	0.4193	179.0000	47.0000
24	18.0000	0.4440	180.0000	48.0000

	1	2	3	4
Type				
Column Name	product 20 (µL)	product 20 (µM)	F (arbitr units)	F - F0 (arbitr units)
1	0.0000	0.0000	188.0000	0.0000
2	1.0000	0.1400	199.0000	11.0000
3	2.0000	0.2800	207.0000	19.0000
4	3.0000	0.4200	216.0000	28.0000
5	4.0000	0.5600	224.0000	36.0000
6	5.0000	0.7000	228.0000	40.0000
7	6.0000	0.8400	230.0000	42.0000
8	7.0000	0.9800	235.0000	47.0000
9	9.0000	1.2600	242.0000	54.0000
10	11.0000	1.5400	248.0000	60.0000
11	13.0000	1.8200	252.0000	64.0000
12	15.0000	2.1000	254.0000	66.0000
13	17.0000	2.3800	257.0000	69.0000
14	19.0000	2.6600	259.0000	71.0000
15	21.0000	2.9400	259.0000	71.0000
16	23.0000	3.2200	260.0000	72.0000
17	25.0000	3.5000	261.0000	73.0000
18	27.0000	3.7800	261.0000	73.0000
19	29.0000	4.0600	262.0000	74.0000
20	31.0000	4.3400	264.0000	76.0000
21	33.0000	4.6200	263.0000	75.0000
22	35.0000	4.9000	263.0000	75.0000
23	37.0000	5.1800	264.0000	76.0000
24	39.0000	5.4600	263.0000	75.0000
25	41.0000	5.7400	263.0000	75.0000
26	43.0000	6.0200	264.0000	76.0000
27	45.0000	6.3000	264.0000	76.0000
28	47.0000	6.5800	264.0000	76.0000
29	49.0000	6.8600	265.0000	77.0000
30	51.0000	7.1400	264.0000	76.0000

	1	2	3	4
Type				
Column Name	product 21 (µL)	product 21 (nM)	F (arbitr units)	F-F0 (arbitr units)
1	incr. adds of 1/100 stock solution			
2	0.0000	0.0000	163.0000	0.0000
3	1.0000	4.0800	169.0000	6.0000
4	2.0000	8.1600	181.0000	18.0000
5	3.0000	12.2400	185.0000	22.0000
6	4.0000	16.3200	191.0000	28.0000
7	5.0000	20.4000	201.0000	38.0000
8	6.0000	24.4800	209.0000	46.0000
9	7.0000	28.5600	214.0000	51.0000
10	8.0000	32.6400	220.0000	57.0000
11	9.0000	36.7200	228.0000	65.0000
12	10.0000	40.8000	230.0000	67.0000
13	11.0000	44.8800	232.0000	69.0000
14	12.0000	48.9600	231.0000	68.0000
15	13.0000	53.0400	235.0000	72.0000
16	14.0000	57.1200	237.0000	74.0000
17	15.0000	61.2000	237.0000	74.0000
18	16.0000	65.2800	238.0000	75.0000
19	incr. adds of 1/50 stock solution			
20	17.0000	73.4400	240.0000	77.0000
21	18.0000	81.6000	239.0000	76.0000
22	19.0000	89.7600	240.0000	77.0000
23	20.0000	97.9200	241.0000	78.0000
24	21.0000	106.0800	243.0000	80.0000
25	22.0000	114.2400	243.0000	80.0000
26	23.0000	122.4000	244.0000	81.0000
27	24.0000	130.5600	243.0000	80.0000

	1	2	3	4
Type				
Column Name	penta (µL)	penta (µM)	F (arbitr units)	F-F0 (arbitr units)
1	0.0000	0.0000	254.0000	0.0000
2	0.5000	0.0183	273.0000	19.0000
3	1.0000	0.0367	289.0000	35.0000
4	1.5000	0.0550	300.0000	46.0000
5	2.0000	0.0733	303.0000	49.0000
6	2.5000	0.0917	309.0000	55.0000
7	3.0000	0.1100	315.0000	61.0000
8	3.5000	0.1283	320.0000	66.0000
9	4.0000	0.1467	322.0000	68.0000
10	4.5000	0.1650	325.0000	71.0000
11	5.0000	0.1833	327.0000	73.0000
12	5.5000	0.2017	330.0000	76.0000
13	6.5000	0.2383	332.0000	78.0000
14	7.5000	0.2750	331.0000	77.0000
15	8.5000	0.3110	334.0000	80.0000
16	9.5000	0.3480	335.0000	81.0000
17	10.5000	0.3850	335.0000	81.0000
18	11.5000	0.4217	333.0000	79.0000
19	12.5000	0.4583	335.0000	81.0000
20	13.5000	0.4950	333.0000	79.0000
21	14.5000	0.5310	334.0000	80.0000
22	15.5000	0.5683	333.0000	79.0000
23	16.5000	0.6050	336.0000	82.0000
24	17.5000	0.6417	335.0000	81.0000
25	18.5000	0.6783	336.0000	82.0000
26	19.5000	0.7150	336.0000	82.0000

PENTASACCHARIDE



Parameter	Value	Std. Error
equilibrium constant (micro M)	0.0254	0.0024
stoichio	1.0205	0.0460

[ATIII] = 0.091 μM