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IDENTIFICATION AND OCCURRENCE OF TWO NEW *N*-NITROSAMINO ACIDS IN TOBACCO PRODUCTS: 3-(*N*-NITROSO-*N*-METHYLAMINO) PROPIONIC ACID AND 4-(*N*-NITROSO-*N*-METHYLAMINO) BUTYRIC ACID

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SUMMARY

Two new *N*-nitrosamino acids, 3-(*N*-nitroso-*N*-methylamino)propionic acid (CAS: 10478-42-9) and 4-(*N*-nitroso-*N*-methylamino)butyric acid (CAS: 61445-55-4) were isolated and identified for the first time in various types of tobacco, including snuff, chewing and pipe tobacco, cigars and cigarettes. Their levels ranged from 0.15 to 7.4 and 0 to 2.2 mg/kg of dry weight tobacco, respectively. For comparison, amounts of other *N*-nitrosamino acids like *N*-nitrosoproline (NPRO) and tobacco-specific-nitrosamines (TSNA) were determined in the same samples. The levels of *N*-nitrosamino acids were highly correlated with the levels of TSNA.

INTRODUCTION

Tobacco products have been reported to contain various *N*-nitroso compounds, including several volatile *N*-nitrosamines [3], *N*-nitrosodiethanolamine [1] and the TSNA such as *N'*-nitrosonornicotine (NNN), *N'*-nitrosoanatabine (NAT) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) [5]. All these nitrosamines are known carcinogens in a wide range of animal species [11]. Recently, the occurrence of non-carcinogenic NPRO was reported in several tobacco products [2], at levels that correlate well with the levels of TSNA. It was thus proposed to use NPRO levels as an indicator of *N*-nitrosation of amines in smokeless (uncombusted), processed tobacco [2].

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We recently analyzed saliva samples collected from tobacco chewers and chewers of betel quid containing tobacco for the presence of various *N*-nitrosamines, i.e. TSNA, betel-nut-specific nitrosamines and NPRO [8]. During the analysis by gas chromatography coupled with a thermal energy analyzer (GC-TEA), several unidentified substances were frequently detected, particularly in the fraction containing NPRO. These unknown compounds appeared to be *N*-nitrosamino acids like NPRO, because they were extractable in organic solvents only after the aqueous extract of the sample had been acidified, and then could be detected by GC-TEA only after esterification with diazomethane. Since various tobacco products were later found to contain the same unknown *N*-nitroso compounds, we have isolated and identified these substances.

The results presented below confirm the occurrence in various tobacco products of these two new *N*-nitrosamino acids, which have not been reported to occur in foodstuffs or other industrial products. NPRO and TSNA were determined in the same tobacco samples to allow comparison of the respective levels.

MATERIALS AND METHODS

Materials

All commercial tobacco products were obtained in local tobacco shops in Stockholm, Sweden, and in Lyon, France and in a grocery store in Henderson, NB, USA.

Reagents

3-(*N*-Nitroso-*N*-methylamino)propionic acid (NMPA) and 4-(*N*-nitroso-*N*-methylamino)butyric acid (NMBA) were synthesized by nitrosation of the respective amino acid precursor with aqueous sodium nitrite; 3-(*N*-methylamino)propionic acid and 4-(*N*-methylamino)butyric acid were prepared by condensing methylamine with 3-bromopropionic acid or 4-chlorobutyric acid (Fluka, Buchs, Switzerland), respectively. *N*-Nitroso-iso-nipecotic acid was synthesized by nitrosation of iso-nipecotic acid (Aldrich Chemical Co., Milwaukee, WI, U.S.A.). NPRO and *N*-nitroso-DL-pipecolic acid (NPIC) were synthesized according to the method of Lijinsky et al. [6]. The purity of the synthesized compounds was verified by thin-layer chromatography and by GC using either flame ionization, TEA or mass spectrometry (MS) for detection. TSNA (NNN, NAT, NNK) were kindly provided by Dr. D. Hoffmann and Dr. S.S. Hecht, Naylor Dana Institute for Disease Prevention, American Health Foundation, Valhalla, NY, U.S.A. All other chemicals were obtained commercially and were used without further purification.

Isolation and identification of N-nitrosamino acids in tobacco

In order to compare chromatographic data on the unknown *N*-nitrosamines with those on authentic compounds, 250 g of pipe tobacco were

stirred for 1 h with 1 ℓ of boiling water, the aqueous extract was filtered through glass wool, and the filtrate was adjusted to pH 8—9 with aqueous sodium hydroxide and extracted 3 times with 1 ℓ of dichloromethane. The dichloromethane extract was discarded and the aqueous extract was then adjusted to pH 1 with concentrated sulfuric acid and extracted 3 times with 1 ℓ of 10% (v/v) methanol in dichloromethane. The combined methanol-dichloromethane extracts were dried over anhydrous sodium sulfate and evaporated to dryness in a rotary evaporator at 30°C. A volume of 10 ml diethyl ether was added to the residue, and the sample was derivatized with excess diazomethane. After evaporating the diazomethane at 50°C, the derivatized extract was applied to a 3.5 × 35 cm column of silica gel. The column was eluted successively with mixtures of dichloromethane in *n*-pentane (25%, 50%, 75% (by vol.)), followed by 100% dichloromethane and diethyl ether in dichloromethane (25%, 50%, 75% (by vol.)). The fraction containing the unknowns and other nitrosamino acids were concentrated individually in a Kuderna-Danish evaporator, and each sample was rechromatographed on a 1 × 20 cm column of silica gel by elution with the same solvent mixtures as above. Finally, 4 fractions (F1—F4) each containing one of the respective TEA-responsive compounds at a relatively high concentration, were obtained, and were analysed by GC-TEA on 3 different GC columns.

For structural confirmation by GC-MS, the TEA-responsive compounds were isolated from nitrosated tobacco, since incubation of aqueous tobacco extract with sodium nitrite at pH 3 was found to increase the concentrations of these compounds significantly. Thus, 25 g of pipe tobacco were extracted with 200 ml of boiling distilled water. Sodium (2 g) was added to the aqueous extract, and the pH was adjusted to 3.0 with concentrated hydrochloric acid. After incubation at 50°C for 2 h, the remaining nitrite was destroyed by addition of excess ammonium sulfamate. The sample was then extracted and purified as described above.

The MS analysis was performed with a Hewlett-Packard Model 5970A mass selective detector, coupled to a Perkin-Elmer Sigma 2B GC. The MS was scanned at unit resolution from 30 to 400 u at 200 u per second in the electron impact mode at 70 eV. A 25 m × 0.22 mm i.d. vitreous silica capillary column coated with SGE-BP 1 bonded stationary phase (film thickness = 0.25 μm) was employed. The column temperature was held at 100°C for 3 min before programming at 5°C/min to 200°C. Under these conditions, methyl esters of NMPA, NMBA, NPRO and NPIC had the retention times of 5.9, 8.7, 9.5 and 10.7 min, respectively.

Analysis of N-nitrosamino acids and TSNAs in tobacco

A 0.5-g sample of tobacco was extracted for 2 h with magnetic stirring with 30 ml distilled water containing 1 ml of 20% ammonium sulfamate in 3.6 N sulfuric acid and *N*-nitroso-iso-nipecotic acid and *N*-nitroso-*n*-butylbenzylamine as internal standards. The mixture was filtered through

glass wool. The filtrate was adjusted to pH 8—9 with 3 N sodium hydroxide and extracted 3 times with an equal volume of dichloromethane in the presence of 10 g sodium chloride. The dichloromethane extracts were dried over anhydrous sodium sulfate and concentrated in a Kuderna-Danish evaporator at 47°C. The concentrated dichloromethane extract was used for the analysis of TSNA. The aqueous layer was then adjusted to pH 1 with 3.6 N sulfuric acid and extracted 3 times with an equal volume of ethyl acetate. The combined ethyl acetate extracts were dried over anhydrous sodium sulfate and evaporated to dryness in a rotary evaporator at 50°C. The residue was dissolved in 2 ml diethylether and *N*-nitrosamino acids were derivatized with excess diazomethane.

A Varian model 1445-10 GC was interfaced with a TEA model 543 (Thermo Electron Corpn. Waltham, MA, U.S.A.). A 2 m × 3 mm (i.d.) glass column packed with 10% UCW-982 on Chromosorb W-HP (80—100 mesh) was employed to quantitate *N*-nitrosamines. The column temperatures for the analysis of TSNA and methyl esters of *N*-nitrosamino acids were 200°C and 150°C, respectively. The flow rate of carrier gas (argon) was 40 ml/min. The injection port temperature was 250°C.

Under these conditions, the recovery of NMPA and NMBA, with which tobacco samples were spiked at a concentration of 1 mg/kg, was 89% and 98%, respectively, and that of TSNA and other *N*-nitrosamino acids was more than 70%. The detection limit for each compound was 10—50 μg/kg of tobacco. The moisture content of the tobacco was determined by drying each sample in air at 110°C.

RESULTS AND DISCUSSION

Figure 1A and B show typical GC-TEA chromatograms obtained from the analysis of the *N*-nitrosamino acid fraction of the tobacco extract (after esterification with diazomethane) on 2 different GC columns, UCW-982 and FFAP. All of the tobacco products analysed so far contain TEA-responsive substances corresponding to peaks 1 and 3, at relatively high concentrations. Minor amounts of compounds corresponding to peaks 2 and 4 were detected occasionally.

In order to establish their structures, the TEA-responsive compounds were extracted from 250 g of pipe tobacco and purified by silica gel column chromatography, as described in 'Materials and Methods'. Subsequently, 4 fractions, F1—F4, containing peaks 1, 2, 3 and 4, respectively as the major TEA-responsive substance, were obtained. The retention times on 3 different GC columns (10% UCV-982, 5% FFAP, 3% OV-17) of each unknown compound were compared with those of about 40 synthetic *N*-nitroso derivatives of amino acids, including *N*-alkylamino and cyclic amino acids. Peaks 1 and 2 were subsequently found to have identical GC retention times as the methyl esters of NMPA and NMBA, respectively. Similarly, the retention

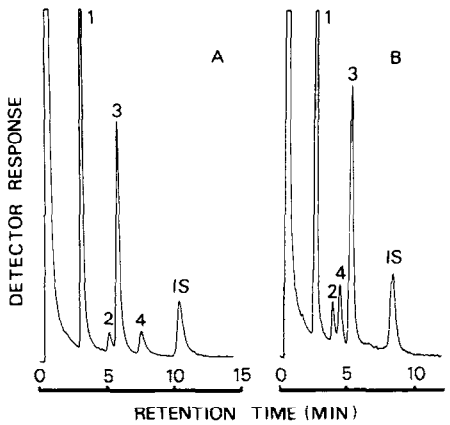

Fig. 1. Typical GC-TEA chromatograms of tobacco extract after esterification with diazomethane (A) A 2 m × 3 mm i.d. glass column packed with 10% UCW-982 on Chromosorb W-HP (80—100 mesh) was employed at a column oven temperature of 150°C. (B) A 2 m × 3 mm i.d. glass column packed with 5% FFAP on Chromosorb W-HP (80—100 mesh) was employed at a column oven temperature of 190°C. Peaks 1, 2, 3 and 4 were identified as NMPA, NMBA, NPRO and NPIC, respectively. IS is internal standard.

times of peak 3 and 4 were shown to be identical with those of the methyl esters of NPRO and NPIC, respectively.

In order to obtain direct structural confirmation, a comparison was made of the mass spectra of authentic *N*-nitrosamino acids (as methyl esters) and of the GC peaks, eluting at the same retention time as the compounds in the chromatogram of the purified tobacco extracts. For this experiment, we isolated and identified the TEA-responsive compounds from nitrosated tobacco, since nitrosation of the aqueous tobacco extract resulted in a notable increase in the amounts of these compounds. The mass spectra of the compounds isolated from the nitrosated tobacco and those of the methyl esters of NMPA, NMBA, NPRO and NPIC are shown in Fig. 2. Although the mass spectra of the isolated compounds contained additional interfering peaks, all major fragment ion peaks, including M^+ and $(M-30)^+$ (loss of NO), were present at intensities similar to those seen in the mass spectra of the authentic synthetic compounds.

The good agreement between the mass spectra and retention times of the TEA-responsive compounds and the methyl esters of standard *N*-nitrosamino acids indicates that they are identical. Thus, tobacco products contain at least 4 *N*-nitrosamino acids, namely NMPA, NMBA, NPIC and NPRO; the first 3 compounds were identified for the first time in tobacco products.

Table 1 summarizes the amounts of the 4 *N*-nitrosamino acids present in various types of tobacco products, including snuff, chewing tobacco,

TABLE 1

N-NITROSAMINO ACIDS AND TOBACCO-SPECIFIC-*N*-NITROSAMINES IN VARIOUS TOBACCO PRODUCTS

Sample	Country	Moisture (%)	*N*-Nitrosamino acid (mg/kg dry wt of tobacco)				TSNA (mg/kg dry wt of tobacco)		
			NMPA	NMBA	NPRO	NPIC	NNN	NAT	NNK
Snuff									
1	Sweden	49	4.01	0.21	12.4	0.39	15.8	18.1	2.51
2	Sweden	52	3.24	0.23	10.5	0.32	20.9	10.4	2.59
3	Sweden	52	3.23	0.15	7.10	0.50	14.6	11.1	2.15
4	Sweden	55	3.09	0.24	9.31	0.26	15.7	7.15	2.23
5	Sweden	51	2.92	0.20	6.21	0.22	154	7.51	1.89
6	Sweden	52	4.21	0.13	11.5	0.30	15.2	8.89	1.70
7	Sweden	21	0.38	0.03	2.48	0.06	10.7	14.7	0.41
8	Sweden	33	0.93	0.05	1.10	n.d.[a]	14.4	7.18	0.98
9	Sweden	53	4.40	n.d.	29.5	5.56	11.7	8.23	2.89
10	Sweden	40	0.51	n.d.	6.42	0.14	2.26	4.44	0.19
11	Sweden	22	1.84	0.08	0.89	n.d.	9.91	6.23	0.99
12	Sweden	36	2.66	0.26	5.83	0.25	18.5	21.4	2.95
13	USA	23	7.42	2.24	9.02	0.39	9.10	3.74	1.26
14	USA	21	1.25	0.07	0.47	n.d.	4.17	2.42	0.68
15	USA	27	1.50	0.12	2.14	0.08	4.41	6.57	0.88
16	USA	45	4.64	0.99	36.5	3.06	125	228	10.8
17	USA	48	5.40	1.77	50.9	6.08	135	339	13.6

Chewing tobacco									
18	Belgium	52	1.63	0.09	3.30	0.14	7.38	0.97	0.13
Cigarettes									
19	France	7	7.01	0.34	5.61	0.24	18.6	6.40	1.53
20	France	11	4.13	0.28	3.63	0.17	15.3	9.97	1.99
21	France	11	0.16	n.d.	1.83	n.d.	4.78	2.89	0.80
22	France	11	0.36	0.03	1.98	n.d.	4.77	3.85	1.04
23	USA	8	0.15	n.d.	1.29	n.d.	4.03	2.97	0.73
24	USA	12	0.31	0.06	1.50	n.d.	7.90	5.81	1.25
Cigars									
25	Netherlands	5	2.23	0.18	0.47	0.07	6.75	4.56	2.85
26	Netherlands	4	2.89	0.27	1.90	0.34	53.1	20.4	4.25
Pipe tobacco									
27	UK	18	0.43	0.03	1.58	0.12	3.00	2.51	0.64
28	France	12	1.92	0.08	1.20	n.d.	6.88	4.85	1.13
29	Netherlands	15	1.60	0.10	0.49	0.04	3.81	1.99	n.d.

[a]n.d., not detected.

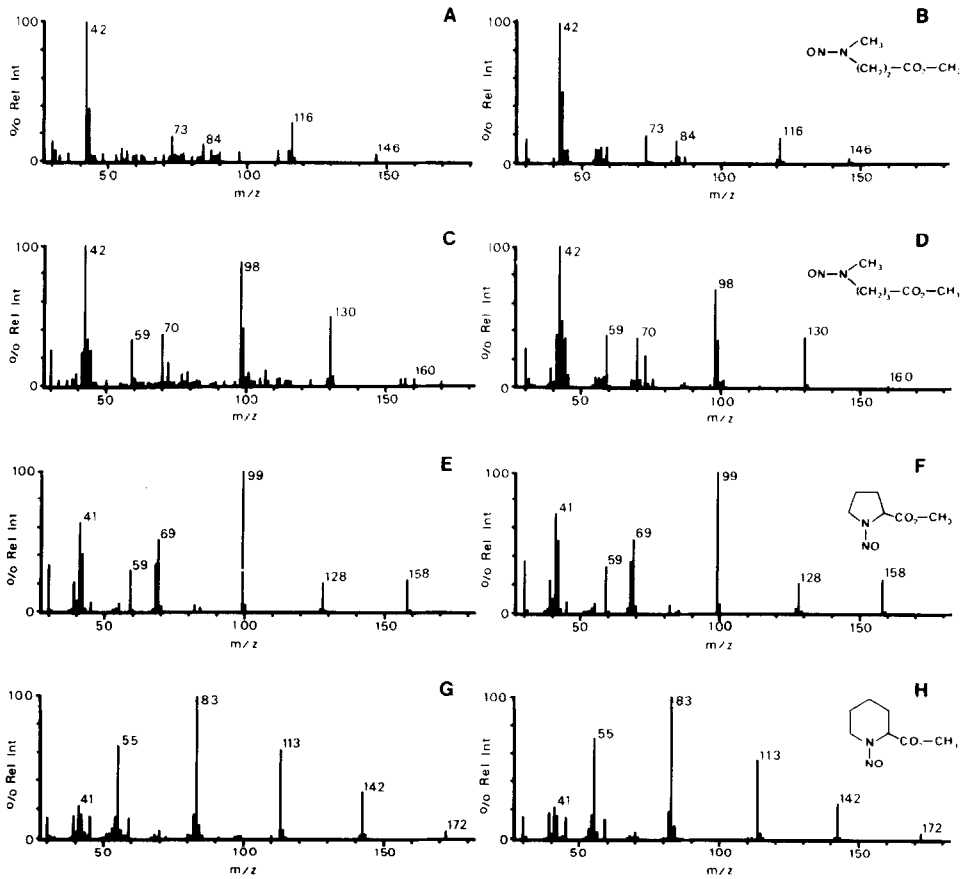

Fig. 2. Mass spectra of methyl esters of *N*-nitrosamino acids isolated from nitrosated tobacco (A, C, E, G) and those of reference compounds NMPA (B), NMBA (D), NPRO (F), NPIC (H).

cigarettes, cigars and pipe tobacco. For comparison, the levels of TSNA were determined in the same samples. The concentration of NMPA was in the range of 0.15 to 7.4, and that of NMBA in the range of 0 to 2.2 mg/kg dry wt of tobacco, respectively. NPRO occurred at relatively high concentrations, ranging from 0.5 to 50.9 mg/kg. NPIC was present as a minor product, at levels ranging from 0 to 6.1 mg/kg. In agreement with earlier findings [2,5], comparatively larger amounts of NNN and other TSNA were found especially in snuff.

The Spearman rank correlation coefficients (ρ) between the levels of the individual *N*-nitrosamines detected in 29 commercial tobacco products (Table 1) were determined (data not shown). In addition to the NPRO-level that has been shown to correlate well with the levels of TSNA [2], the levels of either NMPA, NMBA or NPIC were found to be significantly correlated

with each level of NNN, NAT, and NNK. Thus, the sum of the *N*-nitrosamino acids (NMPA, NMBA, NPRO and NPIC) are highly correlated with the sum of TSNA (NNN, NAT and NNK), ($\rho = 0.717$; $P < 0.01$); this relationship can be further used as an indicator for the formation of TSNA in various processed tobaccos.

To our knowledge, no published report has hitherto appeared on the occurrence of NMPA and NMBA in food and industrial products, and the origin of these two *N*-nitrosamino acids in tobacco is unknown. However, NMPA and NMBA have been isolated and identified as urinary metabolites of *N*-nitrosamines, such as *N*-nitroso-*N*-methyl-*n*-propylamine and *N*-nitroso-*n*-butyl-4-hydroxybutylamine, following oral administration to rats [9,12]. We recently reported the occurrence of these compounds in human saliva collected from tobacco chewers and chewers of betel quid with tobacco [8]. In addition, during the analysis of human urine samples collected from several countries, we occasionally detected peaks corresponding to NMPA and NMBA. At present, however, no data are available to confirm whether the presence of these compounds in human urine is due to use of tobacco.

Toxic and other adverse biological effects of NMPA and NMBA have not been extensively studied. Since it has been reported that a related compound, N-nitrososarcosine, produces liver tumours in mice [13] and esophageal carcinomas in rats [4] and that NMBA is weakly mutagenic in *Salmonella typhimurium* [7], further studies on the biological significance of these compounds are desirable.

Although we have investigated the occurrence of the four *N*-nitrosamino acids in various tobacco products, no data are yet available, on whether tobacco smoke contains these compounds. Brunnemann et al. [2] studied the effect of smoking on the fate of NPRO present in cigarettes. After spiking cigarettes with 5 mg NPRO each, about 0.14% was transferred into main-stream smoke, whereas a large portion served as the precursor (via decarboxylation) for *N*-nitrosopyrrolidine formation in the smoke. Whether NMPA and NMBA are also decarboxylated during cigarette smoking to yield *N*-nitrosomethylethylamine and *N*-nitrosomethyl-*n*-propylamine, respectively, both potent carcinogens in experimental animals [4,10], should now be investigated, particularly in view of the finding that cigarettes and mainstream and sidestream cigarette smoke contain these volatile *N*-nitrosamines [3]. In order to clarify their origin, investigations on their possible decarboxylation during the processing of tobacco and the smoking of cigarettes are warranted.

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