Carcinogenesis vol.21 no.4 pp.839–843, 2000

SHORT COMMUNICATION

Enantiomeric composition of *N'*-nitrosonornicotine and *N'*-nitrosoanatabine in tobacco

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The tobacco-specific nitrosamines *N'*-nitrosonornicotine (NNN) and *N'*-nitrosoanatabine (NAT) are found in substantial quantities in unburned tobacco. Although this has been documented in many previous studies, no data are available on the enantiomeric composition of these nitrosamines, which both have a chiral center at their 2'-positions. We used chiral stationary phase gas chromatography with nitrosamine-selective detection to determine the enantiomeric composition of NNN and NAT in moist snuff, chewing tobacco, and cigarette tobacco. (*S*)-NNN comprised 75.0 ± 8.83% (SD) (*n* = 12) of total NNN while (*S*)-NAT comprised 82.6 ± 1.44% (*n* = 12) of total NAT. Levels of the (*S*)-enantiomers of NNN and NAT were generally similar to those of the corresponding secondary amines, nornicotine and anatabine, suggesting a precursor to product relationship. Nitrosation of (*S*)-nicotine at pH 7.0 produced >99% (*S*)-NNN. These results suggest that nornicotine is a significant precursor of NNN in tobacco. The results of this study provide new insights into the structures and precursors of tobacco-specific nitrosamines in tobacco products.

Since the first report documenting the presence of p.p.m. quantities of *N'*-nitrosonornicotine (NNN) in unburned tobacco (1), numerous studies have confirmed this observation and extended it to other tobacco-specific nitrosamines (reviewed in refs 2–5). Recent studies clearly demonstrate that levels of NNN in tobacco continue to be substantial (4–6). For example, NNN levels in the five leading brands of moist snuff sold in the USA range from 3.04 to 8.73 μg/g dry weight of tobacco (6). In cigarette tobacco, NNN levels in products from 12 different countries range from 45 to 12 454 ng/cigarette (4). NNN and the related tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) are the most abundant strong carcinogens in unburned tobacco (7). Tobacco products also contain considerable amounts of *N'*-nitrosoanatabine (NAT) and smaller quantities of *N'*-nitrosoanabasine (NAB) (2–5). Neither of these nitrosamines displays potent carcinogenicity in laboratory animals (2,3).

Abbreviations: GC-MS-SIM, gas chromatography–mass spectrometry with selected ion monitoring; GC-TEA, gas chromatography with detection by thermal energy analyzer; 5-MeNNN, 5-methyl-*N'*-nitrosonornicotine; NAB, *N'*-nitrosoanabasine; NAT, *N'*-nitrosoanatabine; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, *N'*-nitrosonornicotine.

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There has been a significant and alarming increase in the sales of moist snuff products in the USA (8). Sales of moist snuff increased from 7 700 000 kg in 1972 to 23 900 000 kg in 1994 (8). It has been estimated that each year in the USA ~824 000 young people experiment with smokeless tobacco and 304 000 become regular users (8). Among high school students, 15.8% of males and 1.5% of females were current smokeless tobacco users in 1997 (9). Snuff-dipping, as practiced in North America, is an accepted cause of oral cancer and the tobacco-specific nitrosamines NNN and NNK are believed to play an important role in oral cancer induction by smokeless tobacco products (2,3,7,10–13). In rats, a mixture of NNN and NNK applied to the oral cavity induces oral tumors (14).

NNN and NAT have chiral centers at their 2'-positions (Figure 1), but all studies carried out so far on the analysis of these nitrosamines in tobacco have reported data for racemic material. Enantiomers frequently differ in their biological effects. Therefore, in this study we have determined the enantiomeric composition of NNN and NAT in tobacco.

Nornicotine enantiomers were prepared as described (15). They were nitrosated as reported previously (16). The resulting NNN enantiomers were purified by silica gel (70–230 mesh) column chromatography with elution by $CHCl_3$/MeOH 98:2. (*S*)-NNN (195 mg, 1.1 mmol) and (*R*)-NNN (182 mg, 1.03 mmol) were isolated as yellowish oils which solidified to off-white solids upon standing at –20°C. The NMR and MS of (*S*)-NNN and (*R*)-NNN were identical to those of racemic NNN. Specific rotations (MeOH) were as follows: (*S*)-nornicotine $[\alpha]_D^{20} = -33.0$ (concentration, in g/100 ml, 0.98), literature value (15), –34.9; (*R*)-nornicotine $[\alpha]_D^{20} = +35.2$ (1.9), literature value (15), +34.9; (*S*)-NNN $[\alpha]_D^{20} = -154$ (0.85); (*R*)-NNN $[\alpha]_D^{20} = +159$ (1.5). NAT enantiomers were assigned by nitrosation of a methanol extract of smokeless tobacco which contains predominantly (*S*)-anatabine (17) and analysis for (*S*)-NAT by gas chromatography with detection by a thermal energy analyzer (GC-TEA) as described below.

5-Methyl-*N'*-nitrosonornicotine (5-MeNNN) was synthesized from methyl 5-methylnicotinate (Lancaster Synthesis Inc., Windham, NH) as described for NNN (16). The overall yield was 24.2%, giving 283 mg (1.48 mmol) of a yellowish solid, mp 58–59°C. Its spectral properties were as follows: ^{1}H NMR ($CDCl_3$); δ 8.41 (0.63 H, *s*, pyr-6H, *E* isomer), 8.37 (0.63 H, *s*, pyr-2H, *E* isomer), 8.32 (0.37 H, *s*, pyr-6H, *Z* isomer), 8.20 (0.37 H, *s*, pyr-2H, *Z* isomer), 7.32 (0.66 H, *s*, pyr-4H, *E* isomer), 7.14 (0.34 H, *s*, pyr-4H, *Z* isomer), 5.67 (0.63 H, *t*, 2'H, *E* isomer), 5.22 (0.37 H, *t*, 2'H, *Z* isomer), 4.71–4.40 (0.74 H, *m*, 5'H, *Z* isomer), 3.94–3.67 (1.26 H, *m*, 5'H, *E* isomer), 2.53 (1.26 H, *m*, 3'H, *E* isomer), 2.35 (2 H, *s*, 5-CH_3, *E* isomer), 2.31 (1H, *s*, 5-CH_3, *Z* isomer), 2.24–1.96 (2.75 H, *m*, 4'H, *E* + *Z* isomer; 3'H, *Z* isomer); ESI MS *m/z* $[M+H]^+$ 192.

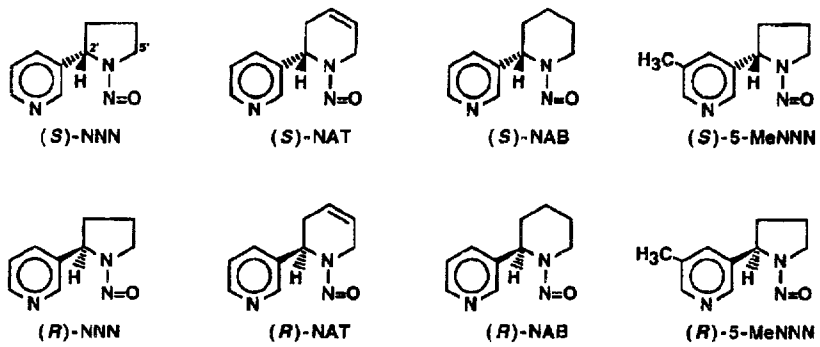

Fig. 1. Structures of the enantiomers of NNN, NAT, NAB and 5-MeNNN.

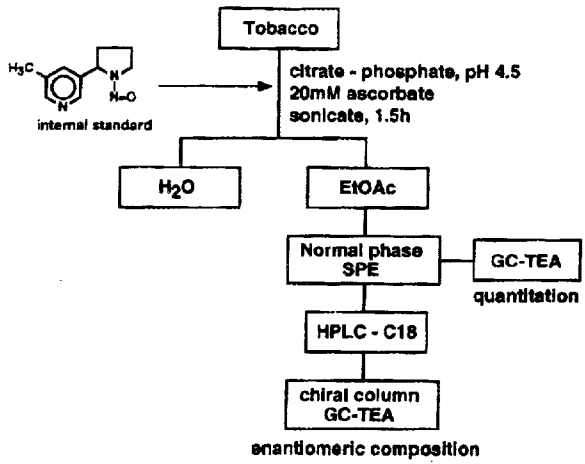

Fig. 2. Scheme for analysis of tobacco-specific nitrosamines in tobacco. SPE, solid phase extraction.

Table I. GC retention times of nitrosamine enantiomers[a]

Compound	Retention time (min)
(*S*)-NNN	33.8
(*R*)-NNN	36.1
(*S*)-NAT	35.4
(*R*)-NAT	37.8
(*S*)-NAB	36.5
(*R*)-NAB	37.8
(*S*)-5-MeNNN	43.7
(*R*)-5-MeNNN	45.6

[a]On a Cyclosil-B column with conditions as described in Materials and methods.

Commercial cigarettes and smokeless tobacco products were purchased on the open market, in April 1999. Research cigarettes and research smokeless tobacco products were chosen for comparison to the enantiomeric composition of nornicotine and anatabine (17). These were obtained from the Tobacco and Health Research Institute, University of Kentucky.

The analytical method is summarized in Figure 2 and is similar to that published previously (18). Extraction buffer, pH 4.5, was prepared from 2 l of 0.1 M citric acid, 1.65 l of 0.2 M Na_2HPO_4 and ascorbic acid to a final concentration of 20 mM. Tobacco was chopped in a blender into pieces ~1 mm^2. A 2.0 g portion of tobacco and 500 ng 5-MeNNN internal standard were added to 40 ml of extraction buffer and the mixture was sonicated for 1.5 h. It was filtered and the tobacco residue was rinsed once with buffer. The filtrates were combined, the pH was adjusted to 5.0 and the solution was extracted three times with 20 ml of ethyl acetate. The extracts were concentrated to dryness, redissolved in 0.5 ml of ethyl acetate and applied to a SEP-PAK Plus silica solid phase extraction cartridge containing 690 mg silica (Waters Corp., Milford, MA) and pre-equilibrated with methylene chloride. The cartridge was eluted with methylene chloride (5 ml) and methylene chloride/ethyl acetate 50:50 (5 ml), then with ethyl acetate (10 ml). The ethyl acetate eluant contained the analytes. An aliquot was set aside for quantitation of total NNN, NAB and NAT as described below. The remainder was brought to dryness, redissolved in 1 ml of H_2O and further purified by reverse phase HPLC using a 250×4.6 mm Bondclone C-18 column (Phenomenex, Torrance, CA) eluted at 1 ml/min as follows: 15% MeOH in H_2O for 5 min, then a 20 min gradient to 45% MeOH in H_2O, then a 5 min gradient back to 15% MeOH, and a hold for 15 min. 3-Acetylpyridine (50 μg) and 3-pyridylcarbinol acetate (50 μg) were used as retention time markers. Fraction 1 containing NNN was collected from 16–22 min, e.g. between the apices of the two marker peaks. Fraction 2, which contained NAB, NAT and 5-MeNNN, was collected between 22 and 36 min. These fractions were concentrated to dryness and transferred to a 100 μl vial. The sample was redissolved in CH_3CN for analysis by GC-TEA (Thermedics Detection Inc., Chelmsford, MA) using a 30 m×0.25 mm i.d., 0.25 μm film thickness Cyclosil-B column (J & W Scientific, Folsom, CA). This column has a 30% heptakis-(2,3-di-*O*-methyl-6-*O*-t-butyldimethylsilyl)-β-cyclodextrin chiral selector physically dispersed in the DB-1701 stationary phase [14% (cyanopropylphenyl)methylpolysiloxane]. The column was operated with a 2 m×0.53 mm i.d. deactivated fused silica precolumn. The GC was operated in the splitless mode. The initial temperature of the oven was 60°C for 2 min, then it was programmed at 12°C/min to 170°C, then held for 35 min, then increased at 12°C/min to 210°C, and held for 5 min. The carrier gas was He at 1 ml/min. Retention times of the NNN, NAB, NAT and 5-MeNNN enantiomers are summarized in Table I. For quantitation of total NNN, NAT and NAB we used a 30 m×0.32 mm i.d, 0.25 μm film thickness DB-1301 column [6% (cyanopropylphenyl) methylpolysiloxane; J. & W. Scientific] and a 2 m×0.53 mm i.d. deactivated fused silica precolumn. The flow rate was 2.6 ml/min He and splitless injection at 225°C was used. The oven temperature program was 80°C for 2 min, then 12°C/

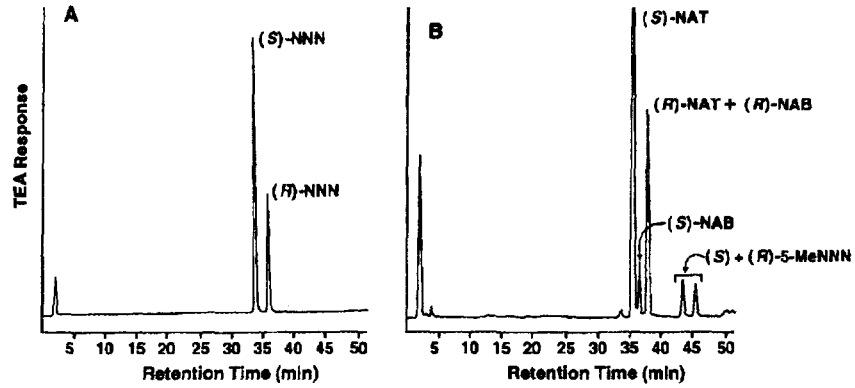

Fig. 3. Gas chromatography of fractions 1 and 2 containing (A) NNN and (B) NAT and NAB. 5-MeNNN is the internal standard. See Materials and methods for details of the preparation of fractions 1 and 2.

Table II. Enantiomeric composition of NNN, NAT, nornicotine and anatabine in tobacco[a]

Product	Total amounts (μg/g dry weight)			(*S*)-NNN (%)	(*S*)-Nornicotine[b] (%)	(*S*)-NAT (%)	(*S*)-Anatabine[b] (%)
	NNN	NAT	NAB				
Commercial cigarettes							
Non-filter	3.6	1.5	0.10	73	NA[c]	82	NA
Filter	5.3	2.0	0.12	74	NA	83	NA
Research cigarettes[d]							
1A3	0.32	0.39	0.050	63	72.3	81	82.6
1R3	2.3	1.5	0.088	78	70.6	83	85.9
2R1F	1.3	1.1	0.058	70	81.6	83	81
4A1	1.1	0.19	0.012	95	96.0	85	80
Commercial smokeless tobacco							
Moist snuff 1	4.4	2.9	0.23	67	NA	82	NA
Moist snuff 2	8.2	7.5	0.53	67	NA	82	NA
Chewing tobacco	0.72	0.44	0.028	78	NA	85	NA
Research smokeless tobacco							
1S1, chewing tobacco	1.8	0.79	0.046	83	87.4	83	83.6
1S2, dry snuff	12	7.7	1.6	82	88.0	80	89.2
1S3, moist snuff	6.2	3.5	0.51	70	75.6	82	86.7

[a]Determined by chiral column gas chromatography as described in Materials and methods.
[b]Data from Armstrong *et al.* (17).
[c]NA, not available.
[d]1A3, a blend of low nicotine Burley and high nicotine flue cured tobaccos; 1R3, Tobacco and Health Research Institute equivalent to the experimental blend used by the National Cancer Institute as their standard for experimental work; 2R1F, filter version of the 2R1 standard reference cigarette; 4A1, a blend of low nicotine Burley and low nicotine flue cured tobaccos.

min to 150°C, then a hold for 15 min, then an increase at 12°C/min to 200°C, then a hold for 10 min. NNN eluted at 19.4 min, NAT at 20.4 min, NAB at 21.8 min and 5-MeNNN at 25.7 min.

Chiral column gas chromatograms of HPLC fraction 1, containing NNN, and fraction 2, containing NAT, NAB and 5-MeNNN, are illustrated in Figure 3A and B. The identities of the nitrosamines were confirmed by gas chromatography–mass spectrometry with selected ion monitoring (GC-MS-SIM) analysis of 1R3 cigarette tobacco as well as 1S2 and 1S3 snuff. A Finnigan TSQ-7000 instrument was operated in the positive ion chemical ionization mode. GC conditions were the same as those used for GC-TEA analysis. Selected ion monitoring of *m/z* 178 (M+H) and 148 (M+H-NO) was employed for analysis of NNN, *m/z* 190 and 160 for NAT and *m/z* 192 and 162 for NAB. In the GC-MS-SIM chromatograms of fraction A, peaks were observed only at the correct retention times for (*S*)- and (*R*)-NNN. The ratios of (*S*)- to (*R*)-NNN were similar to those observed by GC-TEA. Analysis of fraction B similarly produced peaks for (*S*)- and (*R*)-NAT and (*S*)- and (*R*)-NAB.

GC-TEA was used for quantitation of the enantiomers. The GC-TEA chromatograms of fraction 1 were exceptionally clean (Figure 3A) and the NNN enantiomers were readily quantified. We did not attempt to quantify the enantiomers of NAB (fraction 2, Figure 3B) because the peaks were small and (*R*)-NAB co-eluted with (*R*)-NAT. The results are summarized in Table II.

Total amounts of the nitrosamines were consistent with published data (1–6). With respect to chirality, the (*S*)-enantiomers of both NNN and NAT predominated. (*S*)-NNN comprised 75.0 ± 8.83% (SD) (n = 12) of total NNN while (*S*)-NAT comprised 82.6 ± 1.44% (n = 12) of total NAT. The percent (*S*)-enantiomer was significantly lower for NNN than for NAT (P = 0.0075, t-test). The variation in levels of (*S*)-NNN, ranging from 63 to 95% of the total, was greater

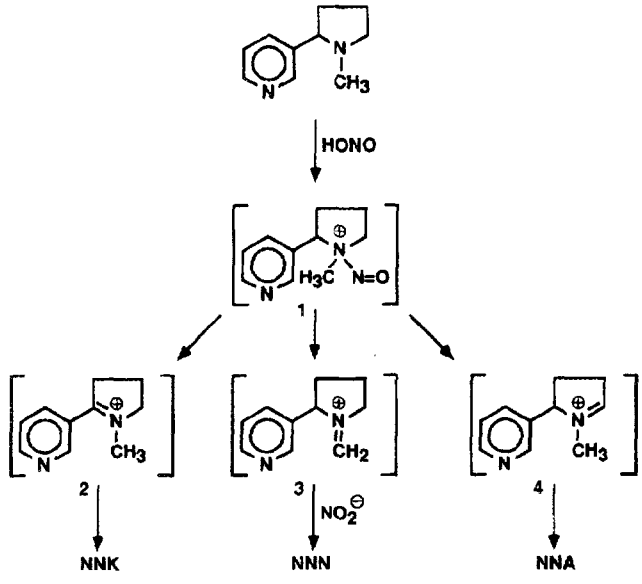

Fig. 4. Proposed mechanism for the nitrosation of nicotine via iminium ions (see ref. 22).

than that of (*S*)-NAT (80–85%). There were generally similar levels of the (*S*)-enantiomer in the nitrosamines and the corresponding secondary amines, based on seven samples for which data for both were available.

The similarity between the enantiomeric composition of the secondary amines and the corresponding nitrosamines suggests that nornicotine is the precursor to NNN and anatabine is the precursor to NAT. However, our previous studies indicated that nicotine, not nornicotine, is the major precursor to NNN in tobacco (19–21). The formation of NNN from nicotine is believed to proceed via iminium ion **3** (Figure 4; 22). This iminium ion is also hypothesized to be an intermediate in the biosynthesis of nornicotine from nicotine in the tobacco plant (23). If both NNN and nornicotine were formed from nicotine via a common intermediate such as **3**, their enantiomeric compositions would be similar. Therefore, although nicotine in tobacco is >99% (*S*)-nicotine (24), conversion to nornicotine or NNN in tobacco could result in loss of optical purity if iminium ion **3** were in equilibrium with **2**, leading to values more similar to those in Table II [e.g. 63–95% (*S*)-NNN and 70.6–96.0% (*S*)-nornicotine]. To test this possibility, we allowed (*S*)-nicotine (0.1 mmol, >99% (*S*) based on ref. 24; Aldrich) to react with $NaNO_2$ (0.5 mmol) in 15 ml of 0.1 M KH_2PO_4 buffer, pH 7.0, at room temperature for 17 h. These conditions have been previously shown to produce NNN, NNK and 4-(methylnitrosamino)-4-(3-pyridyl)butanal in yields of 0.1–0.2% (22). The enantiomeric composition of the NNN produced in this reaction was >99% (*S*)-NNN. These results demonstrate that, if **3** is an intermediate in the conversion of nicotine to NNN, racemization of **3** does not occur under these conditions. Thus, the enantiomeric composition of NNN in tobacco is closer to that of nornicotine than nicotine. These results suggest that nornicotine is a more important precursor of NNN in tobacco than nicotine. This is not consistent with our previous data (19–21) and at present we have no explanation for the discordant results. It is possible, however, that the conditions of our *in vitro* experiment are quite different from those prevailing in tobacco.

The results of this study provide new information relevant to the potential role of NNN as a causative agent for cancers associated with the use of moist snuff and other tobacco products. Our results demonstrate that (*S*)-NNN predominates in moist snuff, chewing tobacco and cigarette tobacco. Virtually all previous metabolic and carcinogenicity studies of NNN have been carried out with racemic material. In our ongoing studies, we hope to define these properties for the individual enantiomers.

Acknowledgement

This study was supported by grant CA-81301 from the National Cancer Institute.

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Received October 6, 1999; revised November 18, 1999; accepted November 29, 1999