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Chemical Studies on Tobacco Smoke

LI: Studies on Non-Volatile Nitrosamines in Tobacco*

by

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Recent studies have demonstrated the presence of N'-nitrosonornicotine (NNN; Fig. 1) in unburned smoke tobacco at levels between 0.3 and 9.0 ppm (1—5). Higher levels were found in certain types of snuff and chewing tobacco (1—3). Since this compound, like many nitrosamines, exhibits biological activity in certain experimental systems (6—8), reduction of its concentration in tobacco is an important goal. A study of some of the factors which may influence NNN concentration in tobacco or may reflect on its mode of formation has therefore been initiated. In addition, it is likely that NNN may serve as an indicator for other nitrosamines in tobacco. In this report, we describe the isolation of Z-*(syn)* and E-*(anti)* NNN from tobacco, the influence of curing and stalk position on NNN levels in tobacco, and the synthesis of two new nitrosamines which may be present in tobacco.

MATERIAL AND METHODS

Apparatus

Infrared spectra were measured on a Perkin-Elmer Model 267 spectrophotometer. uv spectra were recorded on a Cary Model 118 instrument. Nuclear magnetic resonance (nmr) spectra, obtained with a Hitachi-Perkin-Elmer Model R-24 60-MHz spectrometer, are reported as ppm (δ) values downfield from internal tetramethylsilane. Mass spectra were determined on a Hewlett-Packard Model 5982 dual source combined gas chromatograph-mass spectrometer system interfaced with a Hewlett-Packard Model 5933A computer. Elemental analyses were performed by Galbraith Laboratories, Knoxville, Tennessee. Liquid scintillation counting was done with a Nuclear Chicago Isocap 300 scintillation system.

Tobacco Samples

Commercial tobacco samples were obtained in New York from the open market. Burley tobacco plants *(Nicotiana tabacum* cv. Burley 21) varying in stages of growth and curing were grown under greenhouse conditions with low (approx. 50 lb./acre) and high (approx. 200 lb./acre) nitrogen fertilization. These Burley samples were sent from the United States Department of Agriculture to the American Health Foundation at dry ice temperature and analyzed immediately. For examination of the effects of leaf position on the stalk, Bright tobacco *(N. tabacum* cv. Coker 139) was produced by Dr. J. F. *Chaplin* at Kinston, N.C., with normal field management and curing practices.

High-Performance Liquid Chromatography (HPLC)

A Model ALC/GPC-202 high-performance liquid chromatograph (Waters Associates, Milford, Mass.) equipped with a Model 6000 solvent delivery system, and a Model LC-25 uv/visible detector was employed throughout this study. A 6 mm × 30 cm μ-Porasil column (Waters Assoc., Milford, Mass.) was used for HPLC determinations of NNN in tobacco. The eluting solvent was cyclohexane/chloroform/methanol (30%:68.6%:1.4%), with a flow rate of 1.0 ml/min, at a system pressure of 1,000 psi (68 bar). The sample to be injected was dissolved in cyclohexane/2-propanol (3:1), and introduced through a Model U6K septumless injector. The size of the injections ranged from 5 to 8 μl. The retention time for NNN was 13 minutes. For further details see reference (3).

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For the separation of the Z- and E-isomers of NNN, a μ-Bondapak/C_{18} column (Waters Associates) was employed with elution by 2.5% KH_2PO_4 and 5% CH_3CN in water, at a system pressure of 2000 psi and a column temperature of 0 °C, with a flow rate of 2 ml/min. The Z- and E-structures were assigned by nmr (9, 10).

Kinetics

A mixture of E- and Z-NNN (1.25 mg) was injected on the HPLC column; the peaks were baseline separated under the conditions described. Each peak was collected and the resulting solution was kept in a constant temperature bath at the appropriate temperature and pH. Kinetics were measured in the eluting solvent (2.5% KH_2PO_4 and 5% CH_3CN in H_2O). Solutions of the separated isomers were re-injected periodically and the ratio of E- and Z-isomers determined by measurement of peak areas. The rate constants were determined by standard methods for a first order reversible reaction (11).

Synthesis

4-(N-Methyl-N-nitrosamino)-1-(3-pyridyl)-1-butanone [3]: A solution of 4-(N-methyl)-1-(3-pyridyl)-1-butanone dihydrochloride (12) (2 g, 0.0080 mol) in 20 ml H_2O was adjusted to pH 4 by addition of aqueous 10 N NaOH and then $NaNO_2$ (1 g, 0.0140 mol) was added in one batch. After the mixture was stirred at room temperature overnight, it was made strongly alkaline with aqueous NaOH and extracted (6 × 200 ml) with methylene chloride. The combined methylene chloride extracts were dried (anhydrous K_2CO_3) and concentrated giving a residue of 1.2 g, which was purified on a silica-gel column with elution by benzene, chloroform, and chloroform:methanol (10:1). The product, a mixture of E- (72.7%) and Z- (27.3%) isomers (0.5 g, 0.002 mol, 30%), was obtained by crystallization from cyclohexane/benzene:

melting point 63–65 °C;

ir (film) 1690, 1586, 1420, 1336 cm^{-1};

nmr ($CDCl_3$), multiplet, 9.30–7.10 δ (4H, pyridyl-H); triplet, 4.20 δ, and triplet, 3.66 δ (2H, E- and Z-$C\underline{H}_2$-N); singlet, 3.74 δ, and singlet, 3.04 δ (3H, Z- and E-$C\underline{H}_3$-N); multiplet, 3.05 δ (2H, $C\underline{H}_2$-CO-pyr); multiplet, 2.20 δ (2H, -C-$C\underline{H}_2$-C-N);

mass spectrum, m/e (relative intensity), 177 (58), 106 (100), 78 (92);

uv (EtOH) λ_{max} (ε), 230 (14500), 341 (103);

analysis of [3]-dinitrophenylhydrazone: for $C_{15}H_{15}N_7O_5$
calculated C 49.61, H 4.42, N 25.31;
found C 49.57, H 4.48, N 25.24.

Dihydro-3-(3-pyridoyl)-2-(3H)-furanone [4]: A mixture of NaOMe (8 g, 0.1488 mol) in 40 ml dry benzene was distilled until 25 ml benzene was collected. To this suspension of NaOMe in benzene, a solution of ethyl nicotinate (16.4 g, 0.1086 mol) and butyrolactone (9.3 g, 0.1068 mol) was added. The mixture was stirred at room temperature for 5 hours and then allowed to stand overnight. Then dry benzene was added followed by 10 g glacial HAc and 40 ml H_2O.

The organic layer was separated and the aqueous layer was extracted with chloroform (4 × 200 ml). The benzene solution and chloroform extracts were combined, dried (Na_2SO_4) and concentrated. The residue (15 g) was purified by column chromatography on silica-gel with elution by benzene, benzene/chloroform (1/1) and chloroform giving [4] (11.5 g, 0.0600 mol, 60%):

ir (film) 1760, 1685, 1585, 1581, 1420, 1375 cm^{-1};

nmr ($CDCl_3$), multiplet, 9.28–7.40 δ (4H, pyridyl-H); multiplet, 5.10–4.15 δ (3H, -CO-C$\underline{H}$-CO- and O-$C\underline{H}_2$); multiplet, 2.76 δ (2H, -C-$C\underline{H}_2$-C-);

mass spectrum, m/e (relative intensity), 191 M^+ (12), 106 (100), 98 (99), 84 (84), 70 (78).

4-Hydroxy-1-(3-pyridyl)-1-butanone [5]: A solution of dihydro-3-(3-pyridoyl)-2-(3H)-furanone [4] (9.5 g, 0.050 mol) in 40 ml concentrated HCl was stirred at room temperature for 3 hours. The pH was adjusted to 12 by addition of aqueous NaOH (10 N) at 0 °C, and the mixture was extracted with chloroform (6 × 150 ml). The combined chloroform extracts were dried (K_2CO_3) and concentrated giving a residue (8.0 g) which was chromatographed on silica gel with elution by chloroform/methanol (99/1) to give [5] (6.8 g, 0.041 mol, 82%):

ir (film) 3336, 1690, 1585 cm^{-1};

nmr ($CDCl_3$), multiplet, 9.00–7.22 δ (4H, pyridyl-H); triplet, 3.67 δ (2H, $C\underline{H}_2$OH); triplet, 3.18 δ (2H, pyr-CO-$C\underline{H}_2$-); singlet, 2.60 δ (1H, -O$\underline{H}$); multiplet, 1.98 δ (2H, -C-$C\underline{H}_2$-C-);

mass spectrum, m/e (relative intensity), 166, M + 1 (37), 148 (30), 121 (62), 106 (100), 78 (64);

analysis of [5]-dinitrobenzoate (melting point 103–104 °C): for $C_{16}H_{13}N_3O_7$
calculated C 53.49, H 3.62, N 11.70;
found C 53.16, H 3.74, N 11.34.

4-N-Methylamino-4-(3-pyridyl)-butane-1-ol [6]: To a solution of anhydrous methylamine (11.4 g, 0.36 mol) in 150 ml absolute methanol was added 24 ml 5 N HCl in methanol, followed by 4-hydroxy-1-(3-pyridyl)-1-butanone [5] (9.6 g, 0.060 mol) and $NaBH_3CN$ (Aldrich Chemical Co., Inc., Milwaukee, Wi.), (2.3 g, 0.036 mol). The solution was stirred at room temperature for 3 days, and was then acidified (pH <2) with concentrated HCl. The methanol was removed *in vacuo* and the residue was dissolved in 60 ml H_2O and washed with ether (3 × 20 ml). The aqueous solution was made strongly alkaline with KOH pellets at 0 °C and then extracted with chloroform (6 × 150 ml). The combined chloro-

form extracts were dried (K_2CO_3) and concentrated, giving a residue (4.5 g) which was purified by column chromatography on silica-gel with elution by benzene, chloroform, and chloroform/methanol (90/10). Pure [6] (2.9 g, 0.014 mol, 28%) was obtained, with spectral properties as follows:

ir (film) 3300, 1590, 1578, 1475, 1425 cm^{-1};

nmr ($CDCl_3$), multiplet, 8.80–7.12 δ (4H, pyridyl-H); multiplet, 3.50 δ (4H, -HC-N, -CH_2-O and -N-H); singlet, 3.30 δ (1H, -O-H); singlet, 2.25 δ (3H, N-CH_3); multiplet, 1.20 δ (4H, -C-CH_2-CH_2-C-);

mass spectrum, m/e (relative intensity), 181, M + 1 (42), 121 (100);

analysis of [6]-dipicrate (melting point 165–166 °C): for $C_{22}H_{22}N_8O_{18}$
calculated C 41.38, H 3.45, N 17.56;
found C 41.44, H 3.41, N 17.60.

4-(N-Methyl-N-nitrosamino)-4-(3-pyridyl)-butane-1-ol [7]: A solution of 4-N-methylamino-4-(3-pyridyl)-butane-1-ol [6] (2.4 g, 0.013 mol) in 4 ml H_2O was adjusted to pH 4.0 with 2 N HCl. $NaNO_2$ (1.12 g, 0.016 mol) in 4 ml H_2O was added in one batch. The solution was stirred at room temperature overnight and worked up as in preparation of [3]. The amine [6] was converted to the nitrosamine [7] quantitatively. Spectral properties of [7]:

ir (film) 3360, 3025, 1590, 1578, 1430 cm^{-1};

nmr ($CDCl_3$), multiplet, 9.10–7.30 δ (4H, pyridyl-H); triplets, 6.42 δ and 5.80 δ (1H, Z- and E-, pyr-CH-N-NO); singlets, 5.40 and 3.98 δ (3H, Z- and E-CH_3-N-NO); multiplet, 3.75 δ (3H, -CH_2OH); multiplet, 2.50 δ (2H, CH_2-C-O); multiplet, 1.65 δ (2H, CH_2-C-N);

mass spectrum, m/e (relative intensity), 210, M + 1 (12), 150 (100), 132 (38), 117 (42), 85 (43), 83 (76);

analysis of [7]-dinitrobenzoate (m.p. 100–101 °C): for $C_{17}H_{17}N_5O_7$
calculated C 50.62, H 4.22, N 17.37;
found C 50.48, H 4.23, N 17.10.

4-(N-Methyl-N-nitrosamino)-4-(3-pyridyl)-butanal [2]: To a solution of 4-(N-methyl-N-nitrosamino)-4-(3-pyridyl)-butane-1-ol [7] (2 g, 0.0095 mol) in 35 ml dry benzene were added sequentially 35 ml dry dimethylsulfoxide, 0.8 ml dry pyridine, 0.3 ml freshly distilled trifluoroacetic acid and 6.2 g dicyclohexylcarbodiimide. The mixture was tightly stoppered and stirred at room temperature for 18 hours. After the addition of 30 ml benzene, the mixture was filtered. The aqueous solution was saturated with NaCl and extracted with chloroform (6 × 150 ml). The combined chloroform extracts were dried (Na_2SO_4) and concentrated. Most of the dimethyl sulfoxide was removed *in vacuo* at room temperature. The residue was purified by silica-gel chromatography with elution by benzene, chloroform, and chloroform-methanol (10/1) to give [2] (520 mg, 26%), a mixture of E-(81%) and Z-(19%) isomers:

ir (film) 2730, 1720, 1430 cm^{-1};

nmr ($CDCl_3$), singlet, 9.70 δ (1H, CHO); multiplet, 9.20–6.80 δ (4H, pyridyl-H); triplets, 6.20 δ and 5.59 δ (1H, Z- and E-pyr-CH-N-NO); singlets, 3.48 δ and 2.78 δ (3H, Z- and E-, CH_3-N-NO); multiplet, 2.65–2.40 δ (4H, C-(CH_2)$_2$-C=O);

mass spectrum, m/e (relative intensity), 207 (3), 177 (3), 148 (100), 120 (30), 119 (30), 92 (49);

uv (EtOH) λ_{max} (ε), 230 (6486), 258 (sh) (3726), 351 (69);

analysis of dimethone derivative (m.p. 155.0–155.5 °C): for $C_{26}H_{35}N_3O_5$
calculated C 66.52, H 7.46, N 8.96;
found C 66.70, H 7.64, N 8.75.

RESULTS AND DISCUSSION

Analysis of tobacco for NNN can now be accomplished by either gas chromatography (1, 2) or high-performance liquid chromatography (HPLC) (3). The latter method offers certain advantages including shorter analysis times and clean separation of Z- and E-NNN. This separation is achieved by reverse phase chromatography. A typical chromatogram of a mixture of Z- and E-NNN is shown in Figure 1. The structures of the Z- and E-isomers were assigned by determining the nmr spectrum of the same mixture; the protons α- to the nitrosamino functionality were discernable

Figure 1. Separation of E- and Z-isomers of NNN by high-speed liquid chromatography.

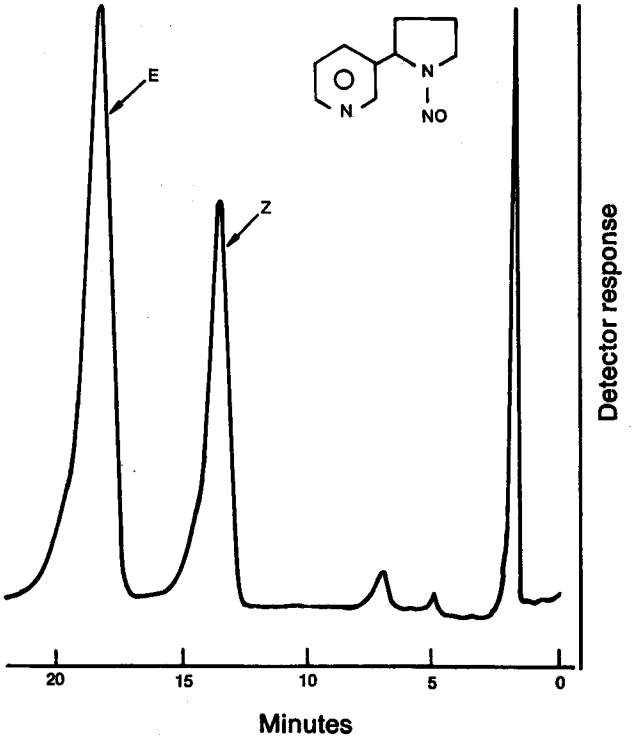


Table 1. Rate constants and equilibrium constants for the interconversion of Z- and E-N'-nitrosonornicotine.

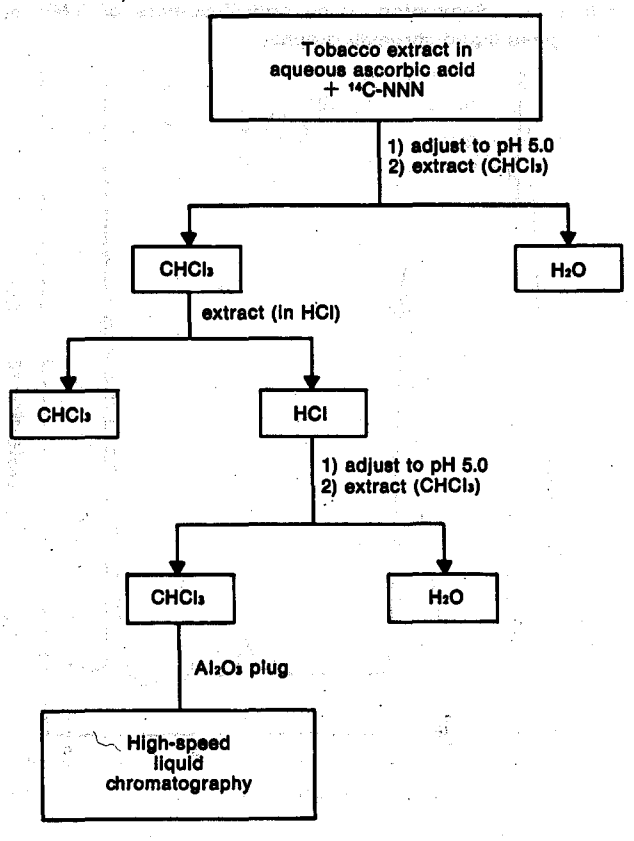
Temperature (°C)	pH	$t_{1/2}$ (min)	Rate constants (min^{-1}) k_f	k_r	Equilibrium constant K	Equilibrium % of E-
0.0	4.5	730	5.7×10^{-4}	3.8×10^{-4}	1.50	60
25.0	4.5	40	1.1×10^{-2}	6.5×10^{-3}	1.70	63
37.0	5.0	10	4.4×10^{-2}	2.5×10^{-2}	1.77	64
25.0	2.0	21	1.8×10^{-2}	1.4×10^{-2}	1.33	57
25.0	7.0	51	9.2×10^{-3}	4.3×10^{-3}	2.16	68

$$Z \underset{k_r}{\overset{k_f}{\rightleftharpoons}} E$$

for each isomer, as previously described (10). The separation achieved by HPLC allowed measurement of the rates of interconversion of the two isomers, and these data are presented in Table 1. The reaction was first order and reversible with an energy of activation of 19 kcal/mol, in good agreement with previously reported data for other nitrosamines (13, 14). The rate of interconversion increased at lower pH.

Since the half-life for isomerization was relatively long (730 min) at 0 °C, the ratio of Z- and E-NNN in tobacco could be determined. Fine-cut chewing tobacco which had previously been shown to contain 90.6 μg/g NNN (3) was analyzed by HPLC (see Figure 2) with the entire analysis performed at 0 °C. The NNN peak was collected from the μ-Porasil column and was re-injected on the C_{18}-μ-Bondapak column. The amounts of E- and Z-NNN were 72% and 28%, respectively. Since the analysis consumed 6 hours, the equilibrium

Figure 2. Analysis of tobacco for NNN by high-speed liquid chromatography.



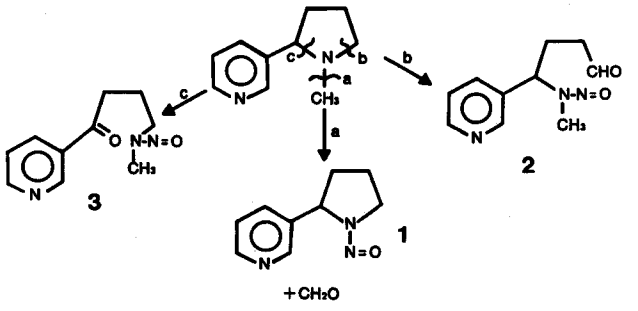
position in fine-cut chewing tobacco was estimated to be 76% E- and 24% Z-NNN (68.8 μg/g E-NNN and 21.7 μg/g Z-NNN). Since the pH of this tobacco was 7.6, the E/Z ratio was approximately that which would be expected in solution at 25 °C. Thus, there appears to be no special feature of this particular tobacco product which would significantly alter the E/Z ratio of NNN, even if only one isomer were initially formed.

Since our previous results indicated that NNN may have formed during curing of tobacco (2), we determined NNN concentrations in tobacco at various growth and curing stages. The stages examined were [1] at flowering, [2] after topping, [3] at harvest, [4] half-cured, and [5] after curing. The results are summarized in Table 2. We did not detect NNN (detection limit = 40 ng/g dry tobacco) in uncured tobacco. In tobacco grown on high N-fertilization, the presence of NNN was confirmed beginning at half-cured stage. The concentration of NNN was higher in the high N than in the low N fertilized samples, in which NNN was detected only after complete curing.

Since stalk position influences the concentration of many tobacco components (15, 16), including nicotine, cigarettes made from the eight stalk positions of a Bright tobacco variety (Coker 139) were analyzed for NNN. The results are presented in Table 3. Slightly higher values were observed for positions 1 and 3 (bottom leaf being number 1), but there appears to be no definite trend in relation to stalk positions. This is in marked contrast to observations for nicotine which generally showed an increase from the lower to the higher positions (15, 16). Nitrate concentrations were slightly higher in positions 1–3 than in positions 4–8.

Either nicotine or nornicotine could be a precursor for NNN in tobacco. Previously, we presented data which indicated that NNN probably derived principally from nicotine (2). This hypothesis was based on the observation that N'-nitrosoanabasine (NAB) was not present in tobacco within our detection limits. Since nornicotine and anabasine were nitrosated at similar rates (17), one would have expected significant concentrations of NAB in tobacco, if the secondary amines were the precursors. If nicotine gave rise to NNN in tobacco by a presumed oxidative cleavage of bond a (Figure 3) and nitrosation (path a), then two other nitrosamines, 4-(N-methyl-N-nitrosamino)-4-(3-pyridyl)-butanal [2]

Figure 3. Formation of nitrosamines from nicotine.



and 4-(N-methyl-N-nitrosamino)-1-(3-pyridyl)-1-butanone [3], might be expected to be formed by cleavage of bonds b and c, respectively. The formation of these compounds from nicotine was first suggested by *Klus* and *Kuhn* (5). The synthesis of these nitrosamines was, therefore, undertaken.

For the preparation of nitrosamino ketone [3] the parent hydrochloride (pseudooxynicotine dihydrochloride) was prepared as previously described (12) and nitrosated under standard conditions. The product was characterized by ir, nmr, uv, mass spectrometry and elemental analysis. For the nitrosamino aldehyde [2], a more circuitous approach was necessary since the parent amino aldehyde cyclized readily (18). The synthetic scheme is outlined in Figure 4. Condensation of ethyl nicotinate with butyrolactone gave dihydro-3-(3-pyridoyl)-2-(3H)-furanone [4] which was hydrolyzed with aqueous HCl to give 4-hydroxy-1-(3-pyridyl)-1-butanone [5]. The latter was allowed to react with sodium cyanoborohydride and methylamine (19) to yield 4-N-methylamino-4-(3-pyridyl)-butane-1-ol [6]. Nitrosation of the amino alcohol [6] gave the corresponding nitrosamino alcohol [7] which was oxidized with DMSO and dicyclohexyl carbodiimide (20)

Table 2. N'-nitrosonornicotine in tobacco leaves.

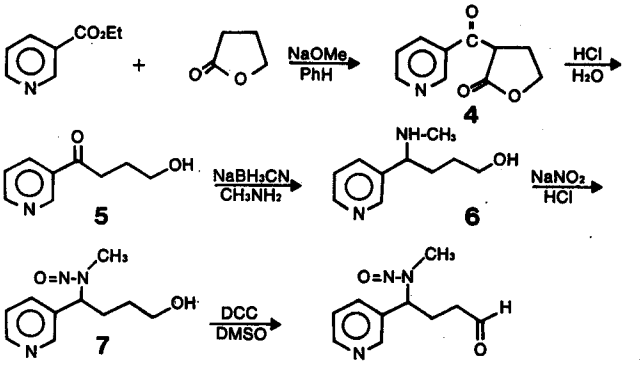
Burley tobacco	Stage of leaf analysis				
	Flowering	Topping	Harvest	Half-way cured	Full-cured
High N	ND*	ND	ND	0.9 µg/g	1.1 µg/g
Low N	ND	ND	ND	ND	0.5 µg/g

* ND: not detected.

Table 3. NNN in stalk positions of flue-cured tobacco (Coker 139).

Stalk position	NNN µg/g	Nicotine mg/g	NO_3^- mg/g
1	2.4	4.2	1.8
2	0.9	5.5	1.5
3	1.8	8.7	1.7
4	0.7	13.4	1.0
5	1.3	15.8	1.0
6	1.3	18.9	0.8
7	1.3	22.2	1.4
8	0.8	22.4	0.8

Figure 4. Synthesis of 4-(N-methyl-N-nitrosamino)-4-(3-pyridyl)-butanal.



to the nitrosamino aldehyde [2]. All spectral data were in accord with the structural features of this nitrosamino aldehyde. This oxidation proved to be a general method for preparation of nitrosamino aldehydes (21).

Currently, tobacco is being analyzed for the nitrosamino aldehyde [2] and the nitrosamino ketone [3] derived from nicotine. While the results of these analyses are not yet complete, preliminary experiments indicate the probable presence of these two compounds in certain types of unburned tobacco, although at levels considerably lower than NNN.

SUMMARY

Various factors which might contribute to the presence of or reflect on the formation of N'-nitrosonornicotine (NNN) or related nitrosamines in tobacco were studied. Using high-performance liquid chromatography (HPLC), the E- and Z-isomers of NNN were clearly separated and the rates of interconversion were determined. The E/Z ratio in tobacco approximated that observed in solution at similar pH and temperature. The influence of curing and stalk position on NNN levels in tobacco was determined. NNN was not detected in green tobacco but was detected in air-cured leaves from the same crop. No significant correlation was observed among stalk position and NNN levels in one variety of Bright tobacco. Since NNN may derive from nicotine, two new nitrosamines, a nitrosamino ketone and a nitrosamino aldehyde, which could theoretically arise from nicotine, were synthesized. Analysis of tobacco for these components is currently in progress.

ZUSAMMENFASSUNG

Es wurden verschiedene Faktoren untersucht, die zu dem Auftreten von N'-Nitrosonornikotin (NNN) oder verwandter Nitrosamine im Tabak beitragen könnten oder über deren Bildung Aufschluß geben könnten. Unter Einsatz leistungsfähiger Flüssig-Chromatographie (HPLC) wurden die E- und Z-Isomere des N'-Nitrosonornikotins klar voneinander getrennt und die Umwandlungsgeschwindigkeiten bestimmt. Das Verhältnis der E- und Z-

Isomere zueinander entsprach im Tabak annähernd demjenigen, das in Lösung bei ähnlichem pH-Wert und ähnlicher Temperatur zu beobachten ist. Es wurde der Einfluß der Trocknung und der Blattposition auf den Gehalt des Tabaks an N'-Nitrosonornikotin bestimmt. N'-Nitrosonornikotin wurde im grünen Blatt nicht nachgewiesen, wohl aber in luftgetrockneten (air-cured) Blättern der gleichen Ernte. Zwischen der Blattposition und dem N'-Nitrosonornikotingehalt einer bestimmten Sorte von Bright-Tabak war keine signifikante Korrelation zu beobachten. Da N'-Nitrosonornikotin sich aus dem Nikotin bilden könnte, wurden zwei neue Nitrosamine, ein Nitrosaminketon und ein Nitrosaminaldehyd, welche theoretisch aus dem Nikotin entstehen könnten, synthetisiert. Gegenwärtig wird Tabak auf den Gehalt an diesen Verbindungen untersucht.

RÉSUMÉ

On a étudié divers facteurs qui pourraient contribuer à la présence ou rendre plus claire la formation de la N'-nitrosonornicotine (NNN) ou de nitrosamines de même famille dans le tabac. En utilisant la chromatographie liquide à haute performance (HPLC), on a pu séparer clairement les isomères E et Z de la NNN, et déterminer les vitesses d'interconversion. Le rapport E/Z dans le tabac est similaire à celui observé en solution de même pH et température. On a déterminé l'influence du séchage et de la position de feuille sur la tige, sur les teneurs en NNN du tabac. On n'a pas trouvé de la NNN dans le tabac vert, mais bien dans du tabac «air-cured» provenant de la même récolte. On n'a pas trouvé de corrélation significative des teneurs en NNN par rapport à la position de feuille sur la tige (pour une variété de tabac Bright). Comme la NNN pourrait dériver de la nicotine, deux nouvelles nitrosamines, une nitrosamino-cétone et une nitrosamino-aldéhyde, pouvant théoriquement se former à partir de la nicotine, ont été synthétisées. L'analyse permettant de retrouver ces composés dans le tabac est actuellement en cours.

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