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Synthesis of (+) [5-3H] N'-Nitrosoanatabine, a Tobacco-Specific Nitrosamine

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Summary

Tobacco-specific N'-nitrosamines (TSNA) are a unique class of systemic organ-specific carcinogens. The TSNA are formed by N-nitrosation of nicotine and of the minor tobacco alkaloids after harvesting of tobacco and during smoking. The N-nitrosation of anatabine leads to N'-nitrosoanatabine (NAT; 1-nitroso-1,2,3,4-tetrahydro-2,3’-bipyridyl) which requires in-depth assays in laboratory animals other than the rat. Furthermore, delineation of its tissue distribution and metabolism is needed for structure:activity comparisons with other TSNA and for the assessment of potential human risk from this TSNA. We have, therefore, synthesized (+)[5-3H]NAT. 5-Bromo-3-pyridine-carboxaldehyde was condensed with ethyl carbamate prior to Diels-Alder reaction with 1,4-butadiene to give the racemic anatabine ring system. Hydrolysis followed by reduction with LiAlT4 and nitrosation, led to (+)[5-3H]NAT (60% yield, specific activity 266 mCi/mmol, radiochemical purity of >99%).

Key Words: Tobacco-specific N-nitrosamine, synthesis, (+)[5-3H]N'-nitrosoanatabine.

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Introduction

Chemical analytical studies with processed tobacco, chewing tobacco, snuff, mainstream and sidestream smoke of cigarettes and cigars, and tobacco smoke-polluted environments have revealed the presence of tobacco-specific N'-nitrosamines (TSNA) in these matrices.\textsuperscript{1,2} The highly carcinogenic N'-nitrosonornicotine (NNN) elicits benign and malignant tumors in the lungs of mice and in the upper aerodigestive tract of rats, hamsters, and minks. The ketone, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) induces primarily adenomas and adenocarcinomas of the lung in mice, rats, and hamsters.\textsuperscript{1} So far NAT has not been found to be carcinogenic in rats.\textsuperscript{3} In vitro assays with oral cavity and lung tissues have shown that these nicotine-derived TSNA are activated in humans to the same reactive species that are formed \textit{in vivo} in rodents.\textsuperscript{4,5} The mainstream smoke (MS) of U.S. cigarettes with or without filter tips contains [per cigarette] 9-180 ng NNK, 50-500 ng NNN, 3-25 ng N'-nitrosoanabasine (NAB) and 55-300 ng N'-nitrosoanatabine (NAT). The levels of these TSNA in sidestream smoke of cigarettes are 5- to 10-fold above those in mainstream smoke (MS).\textsuperscript{6} In moist and dry snuff tobaccos, TSNA concentrations range from 8.4-166.3 µg/g (based on dry weight). The level of NAT in snuff is similar to that of NNN, both occur in higher concentration than other TSNA.\textsuperscript{7}

In general, freshly harvested tobacco leaves do not contain TSNA. These compounds are formed during tobacco curing and fermentation, and, under certain conditions, even during the storage of tobacco products such as snuff.\textsuperscript{6} During smoking, some of the TSNA from the processed tobacco transfer into the smoke while additional TSNA are being pyrosynthesized.\textsuperscript{6} Physiological fluids contain nitrite; in smokers, this can lead to endogenous nitrosation of amines such as proline and thioproline;\textsuperscript{8,9} thus it is probable that, upon smoke inhalation, and in the presence of a catalyst, TSNA are also formed endogenously from amine precursors. As a matter
of fact, Carmella et al. demonstrated that endogenous formation of TSNA such as NNN occurs in rats treated with either (S)-nicotine or synthetic (R,S) nicotine in the presence of NaNO₂.¹⁰

The first large scale epidemiological study in the U.S.A. in 1950 on cigarette smoking and lung cancer recorded mainly bronchiogenic carcinoma, which included squamous cell carcinoma (SCC) and small cell carcinoma. Adenocarcinoma (AC) of the lung occurred far less frequently.¹¹ However, the SCC:AC ratios for lung cancer cases changed from 3.1:1.0 in men and 1.0:1.64 in women for the period between 1964 and 1971 to 1.4:1.0 in men and 1.0:1.8 in women in the years 1984-1986.⁶,¹²-¹⁸ This change in trend may possibly be attributed to the changing nitrate content in the U.S. blended cigarette that increased gradually from an average of <0.5% in the 1950s to between 1.2 and 1.5% since the 1980's. The more intense smoking of cigarettes with low nicotine yield may be a contributing factor as well. It has been shown that the higher nitrate content of tobacco blends is an underlying factor for the increased formation of TSNA during tobacco processing and during smoking.⁶,¹⁹,²⁰ These results suggest that TSNA are important contributors to the development of lung adenocarcinoma in smokers. Moreover, NNK, a representative TSNA, is known to induce adenocarcinoma in the lungs of mice and rats independent of the route of administration.²¹,²² However, the role of NAT as a cigarette smoke constituent that may induce adenocarcinoma in the lung needs to be investigated.

The availability of the nicotine-derived TSNA, NNK, and NNN in radiolabeled form has stimulated research with these TSNA. Even though anatabine is the most abundant of the minor alkaloids in the Nicotiana tabacum varieties grown in North America,²³ data on metabolism of NAT and its possible genotoxic effects in laboratory animals are scarce. While NAT appears to lack carcinogenic activity in the rat,³ its potential carcinogenicity in other laboratory animals remains undefined. Furthermore, nothing is known about its metabolic pathways and disposition.
in several species and at various dose levels. To generate such information requires initially the synthesis of radiolabeled NAT. This communication focuses on the synthesis of (±) [5-^3H]NAT.

Results and Discussion

Several literature procedures describe the synthesis of (±) anatabine. The report by Ohno et al. on the synthesis of (±) anatabine lacked experimental details; not even the yield was provided.\textsuperscript{25} A regioselective alkylation afforded an efficient synthesis of minor alkaloids and related compounds, as reported by Deo et al.,\textsuperscript{26} but the synthesis sequence was not suitable for the synthesis of radiolabeled anatabine. Felpin et al. achieved an efficient enantiomeric synthesis of tobacco alkaloids.\textsuperscript{27} Syntheses of non-radiolabeled (±) anatabine were also reported by Quan et al.\textsuperscript{28} and by Hoffmann et al.\textsuperscript{29} We have followed a similar approach for the synthesis of (±) [5-^3H]NAT, as shown in Scheme 1. The key intermediate in this procedure was 5-bromoanatabine, 4. The starting material, 5-bromo-3-pyridinecarboxaldehyde, was condensed with ethylcarbamate to give compound 2 in quantitative yield. Diels-Alder reaction of compound 2 with 1,3-butadiene led to a complex mixture which, on column chromatography, gave the N’-substituted anatabine 3 in 10% yield. Hydrolysis, followed by decarboxylation of 3 under basic conditions, produced the desired key intermediate 4 in 63% yield. Compound 4 was reduced with LiAlT\textsubscript{4} to give (±)[5-^3H]-anatabine [5] in 94% yield, with a specific activity of 7.5 Ci/mmol. (±)[5-^3H]-Anatabine [5] having high specific activity was diluted with non-labeled anatabine and then nitrosated to the final product (±)[5-^3H]NAT [6]. After final purification using normal-phase HPLC, the radiochemical yield of (±)[5-^3H]NAT was 2.2 mCi (60%) and the specific activity was 266 mCi/mmol.
Experimental

General

$^1$H NMR spectra were obtained on a Bruker AM 360 WB spectrometer using CDCl$_3$. Chemical shifts are expressed in ppm downfield from tetramethylsilane. MS were run on a Hewlett-Packard Model 5988A instrument and high resolution MS with a Finnigan Mat 95 instrument. Thin-layer chromatographic separations were done on aluminum-supported pre-coated silica gel plates from EM Industries, Gibbstown, NJ. Most starting materials were obtained from Aldrich Chemical Co., Milwaukee, WI.

5-Bromo-3-pyridinecarboxaldehyde [1]

A 1-L three-neck flask was charged with glacial acetic acid (300 mL) and 3-pyridinecarboxaldehyde (30 g, 0.278 mmol). To this solution, bromine (90 g, 0.5 mmol) was added dropwise over 30 min at room temperature. The reaction mixture was heated under reflux for 2 h while fumes of HBr were exhausted. The mixture was cooled, poured into 600 mL of H$_2$O, and extracted 3 times with 200 mL each of CH$_2$Cl$_2$. The combined organic layers were washed with brine, and then with 2N NaOH, before being dried over MgSO$_4$. Removal of the solvent produced a dark brown product which was purified by column chromatography on a silica gel column, eluted first with CH$_2$Cl$_2$, then with CH$_2$Cl$_2$:EtOAc (9:1) to give 5-bromo-3-pyridinecarboxaldehyde (19 g, 36%) as light yellow crystals, mp 94-96 °C (Lit.$^{24}$, 95-96 ºC; $^1$H NMR: $\delta$ 8.28-8.32 (m, 1H, pyr H4), 8.92 (d, 1H, pyr H2, J = 2.6 Hz), 8.99 (d, 1H, pyr H6, J = 1.94 Hz), 10.10 (s, 1H, CHO); MS m/z (rel intensity) 185 and 187 (M$^+$, 20), 156 and 158 (10), 138 and 140 (40).
To a 2-L three-neck flask fitted with a reflux condenser and a Dean Stark trap with 475 mL of benzene, 5-bromo-3-pyridinecarboxaldehyde (29.3g, 18 mmol), ethyl carbamate (32.04g, 36 mmol), and p-toluenesulfonic acid (0.57g) were added. The solution was heated under reflux for 5 days. After cooling to room temperature the reaction mixture was filtered by suction to yield 2 (41.36g, 75%), mp 168-170°C. $^1$H NMR $\delta$ 1.26 (t, 6H, OCH$_2$CH$_3$, J = 7.1 Hz), 4.15 (q, 4H, OCH$_2$, J = 7.1 Hz), 6.2 (bs, 3H, CH and NH), 7.92 (s, 1H, pyr H4), 8.62 (bs, 1H, pyr H2), 8.66 (bs, 1H, pyr H6). $^{13}$C NMR $\delta$ 150.1, 145.78, 145.71, 136.7, 128.3, 113.0, 61.7, 59.4, 14.4. High-resolution positive ion CI-MS (M$^+$+1): calcd. for C$_{12}$H$_{17}$BrN$_3$O$_4$, 346.0403; found: 346.0402.

2-(5′-Bromopyridyl-3′-yl)-1-ethoxycarbonyl-1,2,3,6-tetrahydropyridine

A 1-L steel bomb was charged with 2 (20 g, 57 mmol) and glacial acetic acid (200 mL) and then cooled to -78°C. To this mixture, butadiene (15.4 g, 285 mmol), and BF$_3$ acetic acid complex (24 mL, 171 mmol) were added. The reaction vessel was kept at 85°C for 15h; the reaction mixture was then cooled and poured into 200 mL of H$_2$O, neutralized with NaOH, extracted with CH$_2$Cl$_2$ (4 x 300 mL), and dried over MgSO$_4$. Upon removing the solvent, 40 g of a dark brown viscous mixture was obtained. This mixture was chromatographed on a silica gel column with elution by hexane, and hexane:ethyl acetate (6:1) to yield 3 (2.30 g, 10%). $^1$H NMR $\delta$ 2.35 (s, 1H, 3′-CH) and 2.60 (s, 1H, 3′-CH), 3.25 (s, 1H, 2′-CH), 3.95-4.20 (m, 3H, 6′-CH and OCH$_2$CH$_3$), 5.40 (s, 1H, 5′-CH or 4′-CH), 5.60 (d, 1H, 5′-CH or 4′-CH), 5.75 (d, 1H, 2′-CH), 7.87 (bs, 1H, pyr H4), 8.30 (s, 1H, pyr H2), 8.40 (s, 1H, pyr H6). $^{13}$C NMR $\delta$ 149.1, 146.4, 137.7, 124.0, 122.2, 120.1, 61.3, 48.1, 39.8, 26.9, 14.2; MS m/z (rel. intensity) 310 and 312 (M$^+$, 80), 281 and 283 (90), 237 and 239 (60), 210 (20), 185 (30), 154 (40), 130 (20), 105 (10).
5-Bromoanatabine [4]

A 50-mL round-bottom flask was charged with compound 3 (0.85 g, 0.27 mmol) and aqueous 15% KOH solution (30 mL) containing 10% of ethoxyethanol. This reaction mixture was heated under reflux for 16 h, cooled, and extracted with CHCl₃ (3 x 75 mL). The combined organic layers were washed with H₂O and brine, and dried over MgSO₄. After removing the solvent, 0.45 g of crude oil was obtained. It was purified on a silica gel column by elution with hexane:ethyl acetate (9:1) to yield 4 (0.41 g, 63%) as a yellow oil. H NMR δ 2.06 (bs, 1H, NH), 2.14-2.19 (m, 2H, 3′-CH₂), 3.39 (d, 1H, 6′-CH, J = 5.7 Hz), 3.53 (dd, 1H, 6′-CH, J = 5.7 Hz), 3.81 (dd, 1H, 2′-CH, J = 4.8 Hz), 5.75 (m, 2H, 4′-CH, 5′-CH), 7.84 (d, 1H, pyr H4, J₂,₄ = 1.69, J₄,₆ = 2.0 Hz), 8.44 (d, 1H, pyr H2, J₂,₄ = 1.69 Hz), 8.49 (d, 1H, pyr H6, J₄,₆ = 2.18 Hz). C NMR δ 149.5, 146.5, 141.6, 136.7, 126.1, 124.5, 120.8, 54.38, 45.55, 33.48. High-resolution positive ion CI-MS: calcd. for C₁₀H₁₂BrN₂ for (M⁺+1), 239.0185; found: 239.0184.

(±) [5-³H]Anatabine [5]

At room temperature, 5-bromoanatabine [4] (5 mg, 0.021 mmol) in THF (500 µL) was added dropwise to LiAlT₄ (0.24 mmol) in THF (250 µL), and the reaction was continued while stirring for 1 h. The reaction was quenched with MeOH (500 µL) and the solvent was evaporated. The residual paste was extracted with EtOAc, washed with H₂O, and dried over MgSO₄ to yield 235 mCi of crude product. Preparative HPLC was performed on a 4.6 x 250 mm LC 18 Vydac column with a linear gradient of 0-30% acetonitrile in H₂O over 30 min. The purified (±) [5-³H]anatabine (175 mCi, 3.7 mg, 94%) was collected and lyophilized. Analytical HPLC on a 4.6
x 250 mm LC 18 Vydac column showed a single radioactive peak coincident in retention time with that of unlabelled anatabine. The specific activity was 7.5 Ci/mmol.

\((\pm) [5^-3\text{H}]N'-\text{Nitrosoanatabine} \ [6]\)

A 25-mL round-bottom flask was charged with compound 5 (3.68 mCi, 0.5 µmol, 7.5 Ci/mmol) and diluted with non-radiolabeled anatabine (2.6 mg, 16.2 µmol). To this mixture, 1 mL of 0.2 mM NaNO₂ was added at 0°C and the solution was adjusted to pH 4 with 5% HCl. Upon stirring at 0° for 4 h, the mixture was adjusted with NaHCO₃ to pH 8.0 and extracted with CH₂Cl₂. The combined organic layers were dried over MgSO₄ and filtered. The solvent was removed with a gentle N₂ stream and the residue was redissolved in 5 mL of CH₂Cl₂. Compound 6 was purified by normal phase HPLC using a Gilson UV detector (Middleton, WI) at 254 nm in conjunction with a Lichrosorb Si 60 (E. Merck, Darmstadt, Germany) silica gel column (5 µm) that was eluted with hexane:ethyl acetate (1:1). \((\pm) [5^-3\text{H}]\text{NAT} \ [6]\) was collected and the solvent was removed in a N₂ stream to yield 2.2 m Ci (60% radiochemical yield, 1.57 mg, 266 mCi/mmol). The compound’s purity was further examined by reverse-phase HPLC with detection by a Flow One/β-ram radioactive flow detector (In/Us Systems, Inc., Tampa, FL). A Phenomenex C-18 column (Phenomenex, Torrance, CA), with elution by a 10-mM sodium phosphate, pH=7.0 (Solvent A), and MeOH (Solvent B) gradient was used. The elution program was as follows: from 0 to 20 min, MeOH increased linearly to 20%; from 10 to 20 min, linear to 50% MeOH; from 30 to 40 min, held at 50% MeOH; then, for the next 10 min, MeOH was decreased linearly to 40%. The flow rate was 1 mL/min. The retention time of 6 was 40 min. After purification, compound 6 was homogeneous as confirmed by scanning TLC plates with a Bioscan System 200 image scanning counter (Bioscan Inc., Washington, D.C.).
Conclusion

Radiolabeled NAT is not available commercially, therefore, the synthesis described here is an important step toward our studies on the metabolism and disposition of NAT that are requisite to the design of carcinogenicity assays with this TSNA.

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Scheme I: Synthesis of [5-${}^3$H]nitrosoanatabine (6)
Graphical Abstract:

4 \[\xrightarrow{1) \text{LiAl} \cdot \text{H}_4 \quad 2) \text{NaNO}_2}\] \[\rightarrow\] 6, * = $^3$H