Metabolic β-Hydroxylation and N-Oxidation of N'-Nitrosonornicotine[1]

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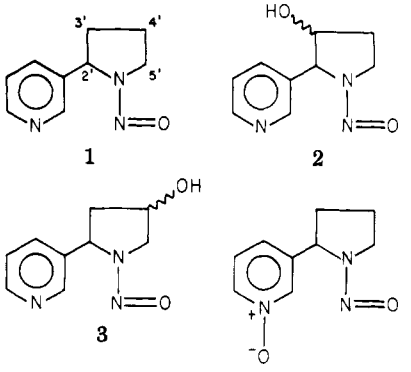
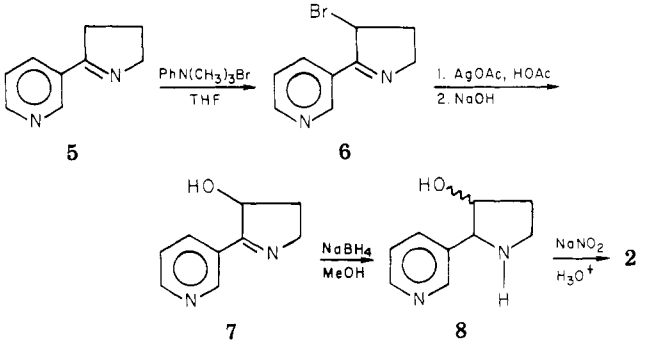
3'-Hydroxy-N'-nitrosonornicotine (2), 4-hydroxy-N'-nitrosonornicotine (3), and N'-nitrosonornicotine 1-N-oxide (4) were synthesized and identified as metabolites in the F-344 rat of the tobacco-specific carcinogen N'-nitrosonornicotine (1). For the synthesis of 2, myosmine (5) was converted to 3'-bromomyosmine (6). Displacement by acetate and hydrolysis gave 3'-hydroxymyosmine (7), which was reduced and nitrosated to give 2. 4'-Hydroxymyosmine (13), the precursor to 3, was prepared by ammonolysis of 1,2-epoxy-4-(N-morpholino)-4-(3-pyridyl)-4-cyanobutane (10). N'-Nitrosonornicotine 1-N-oxide (4) was prepared by m-chloroperbenzoic acid oxidation of 1. When 1 was incubated with liver microsomes from Aroclor-pretreated F-344 rats, trace amounts of 2 and 3 were produced and 4 was a major metabolite. The urine from rats treated with N'-nitrosonornicotine-2'-^{14}C contained only trace amounts of 2 and 3, whereas 4 accounted for 6.7–9.4% of the dose.

N'-Nitrosonornicotine (NNN, 1) is a tobacco-specific

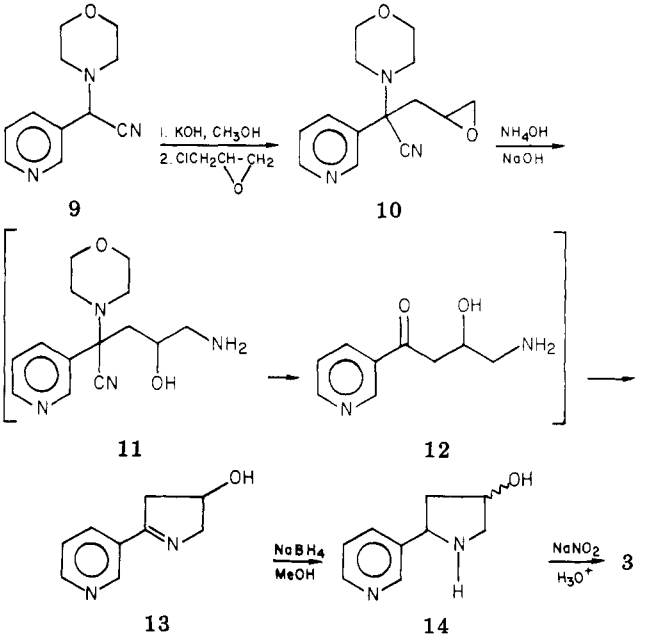


carcinogen derived principally from the major tobacco alkaloid nicotine. Smokers and chewers are exposed to NNN, which occurs in mainstream and sidestream tobacco smoke and in unburned tobacco.[2] Since NNN causes tumors in rats, hamsters, and mice, its metabolism is of considerable interest.[3,4] Nitrosamines such as NNN are metabolically converted to electrophilic intermediates which react with cellular macromolecules. Considerable data indicate that α-hydroxylation is a major activation pathway for cyclic nitrosamines.[5-13] However, activation of nitrosamines by β-hydroxylation has also been proposed.[14] In a previous report, we described the metabolism of NNN by α-hydroxylation (2'-hydroxylation and 5'-hydroxylation).[5] In the current study, we have examined the metabolism of NNN by β-hydroxylation to 2 and 3 and by pyridine N-oxidation to 4.

Scheme I



Scheme II



Synthesis. There have been no previous syntheses of 3'-hydroxynornicotine (8) or 4'-hydroxynornicotine (14), which are the necessary precursors to 2 and 3. Scheme I outlines the synthesis of 8. Bromination of myosmine (5) with phenyltrimethylammonium tribromide gave 3'-bromomyosmine (6). The structure of 6 was assigned by MS, IR, and NMR. We did not obtain 6 by acid-catalyzed bromination of 5. The bromo compound 6 was unstable and was converted without purification to 3'-hydroxymyosmine (7) by sequential treatment with silver acetate and aqueous NaOH. Reduction of 7 with $NaBH_4$ gave 8, which was nitrosated to yield 2. The overall yield of pu-

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Table I. Rates of Formation of NNN Metabolites by F-344 Rat Liver Microsomes[a]

product	source	rate, $nmol^{-1}$ min^{-1} (mg of protein)$^{-1}$
4-hydroxy-1-(3-pyridyl)-1-butanone[b]	2′-hydroxylation	0.40 ± 0.02
3′-hydroxy-*N*′-nitrosonornicotine (2)	3′-hydroxylation	<0.01
4′-hydroxy-*N*′-nitrosonornicotine (3)	4′-hydroxylation	<0.01
5-(3-pyridyl)-2-hydroxytetrahydrofuran[b]	5′-hydroxylation	0.76 ± 0.02
N′-nitrosonornicotine 1-*N*-oxide (4)	N-oxidation	0.57

[a] Liver microsomes from Aroclor 1254 pretreated rats were used. [b] Reference 17.

rified **2** from **5** was 8%. There are four possible isomers of **2**; the 2′-(3-pyridyl) and 3′-hydroxy groups can be either cis or trans and, in each of these isomers, the *N*-nitroso group can be either syn or anti to the 2′-(3-pyridyl) group. The cis and trans isomers of **2** were separable by TLC and high-pressure LC or by GLC after silylation. The syn and anti forms of each isomer were observed by NMR and were partially resolved by high-pressure LC.

Scheme II summarizes the synthesis of 4′-hydroxynornicotine (**14**). The potassium salt of α-morpholino-α-(3-pyridyl)acetonitrile (**9**), an acyl anion equivalent,[15,16] was allowed to react with epichlorohydrin to give the condensation product **10**. Reaction of **10** with ammonium hydroxide gave **13** in 10% overall yield from **9**. Formation of **13** probably occurred by way of the intermediates **11** and **12**, which resulted from ring opening of the epoxide and loss of the protecting group. The structure of **13** was assigned by MS, IR, and NMR and by comparison to **7**. Reduction of **13** and nitrosation gave **3**. The cis and trans isomers of **3** were separable by high-pressure LC, and each isomer existed as a syn and anti mixture, as observed for **2**.

NNN 1-*N*-oxide (**4**) was prepared by *m*-chloroperbenzoic acid oxidation of **1** (0–5 °C, 3 h). When the reaction was allowed to proceed for 18 h at 20 °C, *N*′-nitronornicotine 1-*N*-oxide was also formed. The latter was separable from **4** by high-pressure LC.

Results

To study the formation of **2–4** in vitro, we incubated NNN with microsomes prepared from the livers of rats which had been pretreated with Aroclor 1254. NNN 1-*N*-oxide (**4**) was isolated by TLC and identified by its MS. The rate of formation of **4** was determined by high-pressure LC. The β-hydroxy compounds **2** and **3** were identified by GLC–MS as the corresponding trimethylsilyl ethers, after enrichment from the incubation mixtures by preparative TLC. Compounds **2–4** were not observed in control incubations in which heat-treated microsomes GLC–MS. used. The rates of formation of **2–4** are compared in Table I, which also presents data on the rates of 2′-hydroxylation and 5′-hydroxylation of NNN.[17]

When rats were treated with NNN, **2–4** were identified in the urine. NNN 1-*N*-oxide was isolated by high-pressure LC and was one of the major metabolites. The formation of NNN 1-*N*-oxide did not vary significantly (6.7–9.4%) over a dose range of 3–300 mg/kg of NNN.[18] The β-hydroxy derivatives **2** and **3** were only trace urinary metabolites (<0.1% after a dose of NNN of 300 mg/kg). They were identified by extraction of the urine with $CHCl_3$, silylation, and GLC-MS. When the extracted urine was incubated with β-glucuronidase and sulfatase, an additional 1.7% of the dose was recovered as organic-soluble metabolites. Analysis of this fraction by high-pressure LC indicated that the maximum amount of **2** and **3** present as glucuronides or sulfates was 0.2% of the dose.

Discussion

β-Hydroxylation of cyclic nitrosamines has been reported in studies on *N*-nitrosopyrrolidine, *N*-nitrosomorpholine, and *N*-nitroso-2,6-dimethylmorpholine.[19–21] In these studies, the relative rates of β- and α-hydroxylation were not measured. The data in Table I indicate that liver microsomes from Aroclor-treated rats metabolized NNN more rapidly by α-hydroxylation than by β-hydroxylation. Similar results were obtained when *N*-nitrosopyrrolidine was incubated with rat liver or lung microsomes; α-hydroxylation but not β-hydroxylation was observed.[22] The rates of β-hydroxylation of NNN in vitro and in vivo were so low that it seems unlikely that this is an important activation mechanism for NNN. However, rat liver is not a major target organ for NNN and data on rates of β-hydroxylation in target tissues are not yet available.

Metabolic *N*-oxidation has been observed for a number of pyridine-containing compounds, including the tobacco alkaloids.[23–25] However, the major mammalian N-oxidation product of nicotine is nicotine 1′-*N*-oxide, resulting from N-oxidation of the pyrrolidine nitrogen.[25] Cotinine [5-(3′-pyridyl)-1-methyl-2-pyrrolidinone], the major metabolite of nicotine, is metabolically oxidized by pyridine N-oxidation to cotinine 1-*N*-oxide.[26] N-Oxidation of NNN to NNN 1-*N*-oxide is an important metabolic pathway in vitro and in vivo. Since NNN 1-*N*-oxide is the only major metabolite of NNN which is not likely to be also formed from nicotine,[25] it may be a useful marker for uptake and metabolism of NNN in smokers. Bioassays of NNN 1-*N*-oxide for carcinogenicity are currently in progress.

Experimental Section

Infrared spectra were run on a Perkin-Elmer Model 267 spectrometer as liquid films. ^1H NMR spectra were determined with a Hitachi Perkin-Elmer Model R-24 spectrometer in $CDCl_3$ with Me_4Si as internal reference. Mass spectra and combined GLC–mass spectra were recorded with a Hewlett-Packard Model 5982A mass spectrometer. High-resolution mass spectra were determined by Shrader Analytical and Consulting Laboratories, Detroit, Mich. Gas–liquid chromatography was carried out using a Hewlett-Packard Model 5711 instrument with a flame-ionization detector and a 6 ft × $^1/_8$ in. column filled with 10% UCW-98 on gas chrom Q. A flow rate of 40 mL/min of He was used. TLC was performed on 0.25-mm silica gel 60 F_{254} (Merck) glass plates. High-pressure liquid chromatography was performed with a

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Waters Associates Model ALC/GPC-202 high-speed liquid chromatograph equipped with a Model 6000A solvent delivery system, a Model 660 solvent programmer, a Model U6K septumless injector, a Model 440 UV/visible detector, and two 6 mm × 30 cm μ-Bondapak C_{18} columns in series. A gradient was used as follows: solvent A for 10 min and then linear to 60% B in 60 min at 1 mL/min. Solvent A was 30 mL of 1 M HOAc, 12 mL of 1 M NaOH, and 18 mL of 1 M NaCl in a total volume of 0.5 L of H_2O, pH 4.5. Solvent B was CH_3OH/H_2O, 1:1. Liquid scintillation counting was performed with a Nuclear-Chicago Isocap 300 scintillation system.

3'-Bromomyosmine (6). A solution of phenyltrimethylammonium tribromide[27] (Aldrich; 21.4 g, 57 mmol) in 150 mL of dry THF was added dropwise with stirring at 0 °C to a solution of myosmine (5)[28] (5.0 g, 34.2 mmol) in 200 mL of dry THF. After the addition was complete, the orange mixture was stirred at 0 °C for 4 h. A solution of K_2CO_3 (21.5 g in 100 mL of H_2O) was added, and the resulting mixture was extracted with $CHCl_3$ and dried (Na_2SO_4). An aliquot was concentrated to dryness to give crude **6** (75%) and purified by TLC: ($CHCl_3$/MeOH, 15:1) R_f 0.32. Spectral properties: NMR ($CDCl_3$) δ 2.50 (m, 2 H, CH_2CH_2N), 4.13 (m, 2 H, CH_2N=), 5.22 (t, 1 H, CHBr), 7.33 (m, 1 H), 8.19 (d, 1 H), 8.65 (d, 1 H), 9.13 (s, 1 H, pyr H); IR (film) 1609, 1590 cm^{-1}; MS, *m/e* (relative intensity) 226 (M^+, 26), 225 (28), 224 (27), 223 (25), 146 (27), 145 (30), 118 (100). Since **6** was prone to spontaneous decomposition, especially in the absence of solvent, most of the $CHCl_3$ extracts were concentrated to 200 mL, and 200 mL of glacial HOAc was added. The remainder of the $CHCl_3$ was then removed and the product, in HOAc, was used directly in the next step.

3'-Hydroxymyosmine (7). To a solution of crude **6** (5.8 g, 25.8 mmol) in 350 mL of HOAc was added AgOAc (4.30 g, 25.8 mmol), in one portion with stirring at 20 °C. The mixture was stirred under reflux for 2 h. After cooling, the mixture was adjusted to pH 12 with 10 N NaOH and extracted with $CHCl_3$. The $CHCl_3$ extracts were dried (Na_2SO_4) and concentrated, giving 2.9 g (69%) of crude 3'-hydroxymyosmine (7): NMR ($CDCl_3$) δ 1.7–2.7 (m, 2 H, CH_2CH_2N), 3.8–4.2 (m, 2 H, CH_2N=), 5.1–5.6 (m, 2 H, CHOH), 7.1–7.4 (m, 1 H), 8.1–8.6 (m, 2 H), 9.05 (s, 1 H pyr H); IR (film) 3500–3100 (br), 1620, 1594 cm^{-1}; MS, *m/e* (relative intensity) 162 (M^+, 44), 118 (68), 105 (100). Because of ease of dehydration, **7** was used in the next step without further purification.

3'-Hydroxynornicotine (8). A solution of **7** (2.9 g, 18 mmol) in 35 mL of MeOH containing 1.5 mL of glacial HOAc was cooled to 0 °C in an N_2 atmosphere, and $NaBH_4$ (1.9 g, 50 mmol) was added in small portions with vigorous stirring. After the addition was complete, the mixture was allowed to stir at 20 °C for 2 h and at 50 °C for 1 h. The reaction was quenched by the addition of 5–10 mL of H_2O. The MeOH was removed and the remaining mixture was adjusted to pH 12 and extracted 10 times with 25 mL of $CHCl_3$. The $CHCl_3$ extracts were combined, dried (Na_2SO_4), and concentrated to give 1.7 g (58%) of crude **8**. This was purified by column chromatography on silica gel with elution by $CHCl_3$, $CHCl_3$/MeOH (15:1), and finally $CHCl_3$/MeOH/NH_4OH (85:15:2) to give 0.9 g of pure **8**: NMR ($CDCl_3$) δ 1.4–2.4 (m, 2 H, CH_2CH_2N–), 2.7–3.3 (m, 2 H, CH_2N–), 3.53 (s, 2 H, NH and OH), 3.7–4.5 (m, 2 H, CHOH and pyr CHN), 6.8–7.3 (m, 1 H), 7.5–7.8 (m, 1 H), 8.0–8.5 (m, 2 H, pyr H); IR (film) 3600–3100 (br), 1580 cm^{-1}; MS, *m/e* (relative intensity) 164 (M^+, 4), 120 (50), 119 (100).

3'-Hydroxy-N'-nitrosonornicotine (2). A solution of **8** (0.9 g, 5.5 mmol) in H_2O (10 mL) was adjusted to pH 4 and $NaNO_2$ (0.48 g, 7.0 mmol) was added in one portion. The mixture was allowed to stir for 16 h at 20 °C. The pH was adjusted to 12 and the mixture was extracted 10 times with 25 mL of $CHCl_3$. Drying (Na_2SO_4) and concentration gave 0.7 g of crude **2**, which was purified on a silica gel column with elution by $CHCl_3$ and 15:1 $CHCl_3$/MeOH to give 0.53 g (50%) of pure **2** as an oil. The purity of **2**, a mixture of cis and trans and syn and anti isomers, was greater than 99% as established by TLC [(silica; $CHCl_3$/MeOH, 10:1) R_f 0.25, 0.31], high-pressure LC [retention volumes of the cis/trans isomers: 38 (30%), 49 mL (70%)], and GLC following silylation, as described below. Spectral properties of the mixture: NMR ($CDCl_3$) δ 1.7–2.4 (m, 2 H, CH_2CH_2N), 3.6–4.0 (m, 1.5 H, *syn*-CH_2NN=O), 4.1–4.7 (m, 1.5 H, *anti*-CH_2NN=O + CHOH), 4.9–5.7 (m, 2 H, *syn*- + *anti*-pyr-CHNN=O), 7.1–7.6 (m, 2 H), 8.1–8.6 (m, 2 H, pyr H); IR ($CHCl_3$) 3600–3100, 1580 cm^{-1}; High-resolution MS 193.0818 (M^+, $C_9H_{11}N_3O_2$), 163.0856 ($C_9H_{11}N_2O$), 119.0606 ($C_7H_7N_2$), 92.0498 (C_6H_6N).

1,2-Epoxy-4-(N-morpholino)-4-(3-pyridyl)-4-cyanobutane (10). Methanolic KOH (30%, 5.8 mL) was added, with stirring at 20 °C, to a solution of 1-(*N*-morpholino)-1-(3-pyridyl)acetonitrile (9)[16] (5.7 g, 28 mmol) in 125 mL of dry DMF. After the addition was complete, the mixture was stirred for 15–20 min and epichlorohydrin (28.4 g, 300 mmol) was added dropwise. The reaction mixture was allowed to stir for 65 h and was then quenched by the addition of 150 mL of H_2O. The resulting mixture was extracted with $CHCl_3$ (5 × 150 mL), and the combined $CHCl_3$ layers were concentrated to about 250 mL, washed with H_2O (5 × 250 mL) to remove DMF, dried (Na_2SO_4), and concentrated to give 7.0 g of an oil, 70% **10** and 30% **9** by NMR ($CDCl_3$): δ 2.1–3.0 (m, 6.1 H, CH_2NCH_2 + $CHOCH_2$), 3.3–3.9 (m, 5.4 H, CH_2OCH_2 + 3-pyr-C-CH_2CHO), 4.83 (s, 0.3 H, pyr CH of **9**), 7.1–7.4 (m, 1 H), 7.7–8.1 (m, 1 H), 8.5–8.8 (m, 2 H, pyr H); MS, *m/e* (relative intensity) 259 (M^+, 0.6), 228 (2), 203 (42, from **9**), 202 (100), 172 (43), 117 (54), 56 (46). Since **10** decomposed when chromatography on Florisil was attempted, it was used in the next step without further purification.

4'-Hydroxymyosmine (13). A mixture of **10** (and 30% **9**) (7.0 g), concentrated NH_4OH (120 mL), and 5 pellets of NaOH was stirred at 40 °C for 5 h. The reaction mixture was saturated with NaCl and extracted three times with equal volumes of $CHCl_3$. The $CHCl_3$ layers were combined, washed with saturated aqueous NaCl, dried (Na_2SO_4), and concentrated to give a residue of 4.6 g, which was purified on 50 g of Florisil with elution of $CHCl_3$, 1% MeOH in $CHCl_3$, 5% MeOH in $CHCl_3$, and 10% MeOH in $CHCl_3$. Elution of **13** was followed by TLC (silica; $CHCl_3$/MeOH/NH_4OH, 85:15:2), R_f 0.4. The spot corresponding to **13** turned orange on standing owing to dehydration to 2-(3-pyridyl)pyrrole: NMR ($CDCl_3$) δ 4.95 (br s, 1 H, NH), 6.2 (m, 1 H), 6.5 (m, 1 H), 6.8 (m, 1 H, pyrrole H), 7.2 (m, 1 H), 7.7 (m, 1 H), 8.2 (m, 1 H), 8.7 (m, 1 H, pyridyl H); MS, *m/e* (relative intensity) 144 (100), 117 (26), 90 (17), 89 (16). The latter was also isolated when purification of **13** was attempted on silica. Pure **13** (0.45 g, 10% from **9**) had the following spectral properties: NMR ($CDCl_3$) δ 3.00 (d, 2 H, CH_2C=N), 4.09 (d, 2 H, CH_2N=), 4.60 (quintet, 1 H, CHOH), 5.0 (s, 1 H, OH), 7.2 (m, 1 H), 8.05 (m, 1 H), 8.5 (m, 1 H), 8.85 (m, 1 H, pyr H); IR (film) 3600–3100 (br), 1615 cm^{-1}; MS, *m/e* (relative intensity) 162 (M^+, 12), 161 (22), 144 (100), 143 (26), 118 (44).

4'-Hydroxynornicotine (14). A mixture of **13** (0.9 g, 5.4 mmol) and $NaBH_4$ (0.52 g, 13.7 mmol) in 72 mL of 3:1 H_2O/EtOH was stirred at 20 °C for 5 days. The EtOH was removed and the aqueous residue was extracted four times with $CHCl_3$. The $CHCl_3$ layers were combined, dried (Na_2SO_4), and purified on silica gel with elution by $CHCl_3$/MeOH (15:1) and $CHCl_3$/MeOH (10:1), giving 0.66 g (74%) of **14**: greater than 95% pure by TLC (silica; $CHCl_3$/MeOH/NH_4OH, 85:15:2), R_f 0.3; NMR ($CDCl_3$) δ 2.2–2.7 (m, 2 H, $CHCH_2CHOH$), 3.0–3.4 (m, 2 H, CH_2N), 3.5–3.9 (m, 1 H, CHOH), 3.9–4.4 (m, 3 H, pyr CHN, OH, NH), 7.21 (m, 1 H), 7.75 (m, 1 H), 8.50 (m, 2 H, pyr H); IR (neat) 3600–3100 (br), 1577 cm^{-1}; MS, *m/e* (relative intensity) 164 (M^+, 7), 120 (52), 119 (100).

4'-Hydroxy-N'-nitrosonornicotine (3). Nitrosation of **14** was carried out as described for the synthesis of **2** to give **3** as an oil, 87%, purified by silica gel chromatography with elution by $CHCl_3$ and $CHCl_3$/MeOH, 15:1. The purity of **3** (a mixture of cis and trans and syn and anti isomers) was greater than 99%, as established by TLC [(silica; 10:1 $CHCl_3$/MeOH) R_f 0.26], high-pressure LC [retention volumes of the cis/trans isomers, 43 (43%) and 48 mL (57%)], and GLC following silylation as described below. Spectral properties of the mixture: NMR ($CDCl_3$) δ 1.9–2.8 (m, 2 H, $CHCH_2CHOH$), 3.6–4.0 (m, 1 H, *anti*-CH_2NN=O), 4.0–4.9 (m, 3 H, *syn*-CH_2NN=O + CHOH), 5.0–5.4 (m, 0.5 H, *syn*-pyr-CHNN=O), 5.5–5.9 (m, 0.5 H, *anti*-pyr-CHNN=O), 7.1–7.7 (m, 2 H), 8.1-8.7 (m, 2 H, pyr H); IR (film) 3600–3100 (br) cm^{-1}; high-resolution MS, 193.0818 ($C_9H_{11}N_3O_2$), 163.0856 (C_9-

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$H_{11}N_2O$), 119.0606 ($C_7H_7N_2$), 92.0498 (C_6H_6N).

***N'*-Nitrosonornicotine 1-*N*-Oxide (4).** A mixture of **1**[28] (1.68 g, 9.5 mmol) and *m*-chloroperbenzoic acid (1.9 g, 11 mmol) in 50 mL of $CHCl_3$ was stirred at 0–5 °C for 3 h. The resulting mixture was washed with saturated aqueous $NaHCO_3$ (2 × 25 mL), and the $CHCl_3$ solution was dried (Na_2SO_4) and concentrated to give 1.5 g of crude **4** as a yellow oil. Silica gel chromatography with elution by $CHCl_3$ and 15:1 $CHCl_3$/MeOH gave 1.3 g (70%) of **4** as an oil, a mixture of anti (55%) and syn (45%) isomers, pure by TLC [(silica; 10:1 $CHCl_3$/MeOH) R_f 0.25] and high-pressure LC (retention volume, 45 mL). Spectral properties of **4**: NMR ($CDCl_3$) δ 1.7–2.7 (4 H, m, CH_2CH_2), 3.5–3.8 (m, 1 H, *anti*-$CH_2NN{=}O$), 4.2–4.8 (m, 0.9 H, *syn*-$CH_2NN{=}O$), 4.8–5.2 (m, 0.45 H, *syn*-$CHNN{=}O$), 5.4–5.7 (m, 0.55 H, *anti*-$CHNN{=}O$), 6.8–7.5 (m, 2 H), 7.8–8.2 (m, 2 H, pyr H); IR (film) 1602, 1560, 1420, 1270, 1160, 1012 cm^{-1}; MS, *m/e* (relative intensity) 193 (M^+, 70), 163 (48), 145 (100), 118 (93). Anal ($C_9H_{11}N_3O_2$) C, H, N.

When the above reaction was carried out at 20 °C for 18 h, approximately 10% of *N'*-nitronornicotine 1-*N*-oxide was also formed: high-pressure LC retention volume, 47 mL; MS, *m/e* (relative intensity) 209 (69), 146 (68), 145 (100), 118 (83).

Analysis for 2–4 in Vitro. Liver microsomes from Aroclor 1254 pretreated rats were prepared as described previously.[29] Incubations were carried out at 37 °C for 60 min in five 25-mL Erlenmeyer flasks. Each flask contained NNN (18.4 mg, 0.104 mmol), NADPH (2.5 mg), glucose-6-phosphate dehydrogenase (50 units), glucose 6-phosphate (0.05 mmol), $MgCl_2$ (0.05 mmol), and microsomal suspension (2 mL; 9 mg/mL protein) brought to a total volume of 10 mL with 0.1 M pH 7.4 Tris-HCl buffer. In control incubations, heat-treated microsomes were used. Incubations were quenched by the addition of 10 mL of EtOH. Protein was removed by centrifugation and one-third of the supernatant was used for analysis of **4**. The remainder was analyzed for **2** and **3**.

To analyze for **4**, the supernatant was extracted five times with $CHCl_3$, and the combined $CHCl_3$ layers were dried (Na_2SO_4), concentrated and applied to two 0.5-mm silica gel TLC plates with elution by $CHCl_3$/MeOH, 10:1. The band corresponding to **4** (R_f 0.25) was eluted from the silica with MeOH and analyzed by MS. For quantitation of **4**, an aliquot of the $CHCl_3$ extract was analyzed by high-pressure LC.

To analyze for **2** and **3** the supernatant was concentrated and applied to three 0.5-mm silica gel TLC plates with elution by 10:1 $CHCl_3$/MeOH. The bands corresponding in R_f to standards were eluted from the silica with MeOH, concentrated to dryness, and silylated with bis(trimethylsilyl)trifluoracetamide and 1% trimethylchlorosilane (Regisil, Regis Chemical Co.). An aliquot was analyzed by GLC and combined GLC–MS with a program of 150 °C for 8 min and then 4 °C/min to 240 °C. Under these conditions, the retention time of the cis/trans isomers of the trimethylsilyl ethers of **2** were 21.8 and 22.3 min, and the retention time of the trimethylsilyl ethers of **3** was 22.8 min.

Analysis for 2–4 in Vivo. Male F-344 rats were given a sc injection of [2'-^{14}C]NNN[28] (1×10^7 dpm; 3, 10, 30, 100 or 300 mg/kg body weight) in 2 mL of 0.9% NaCl solution.[18] Urine was collected for 48 h. To assay for **4**, urine was lyophilized and then sonically dispersed with CH_3OH. An aliquot of the CH_3OH solution was analyzed by high-pressure LC. The fraction with the same retention time as **4**, obtained after a dose of 300 mg/kg, was collected and identified as **4** by its MS. Quantitation was accomplished by scintillation counting (all doses) and by comparison of UV detector response with reference **4** (100 and 300 mg/kg doses). To assay for **2** and **3**, urine from rats treated with 300 mg/kg NNN was extracted four times with $CHCl_3$. The $CHCl_3$ extracts were combined, dried, concentrated, and silylated with Regisil. An aliquot was analyzed by combined GLC–MS as described in the in vitro assay.

To analyze for glucuronides or sulfates of **2** and **3**, urine was extracted with $CHCl_3$, and the aqueous portion was incubated with β-glucuronidase (type IX, from *Escherichia coli*, 17 000 units) and sulfatase (type H-1, from *Helix pomatia*, 2460 units) for 16 h at 37 °C. The resulting mixture was extracted with $CHCl_3$, and the $CHCl_3$ extracts were dried, concentrated, and analyzed by high-pressure LC.

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Urinary Metabolites of Timolol from Humans and Laboratory Animals. Syntheses and β-Adrenergic Blocking Activities

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Syntheses are reported for three metabolites (**2–4**) of timolol (**1**) formed by oxidative metabolism of the morpholine ring. GLC–MS comparisons are presented which establish that the two metabolites whose structures were previously in question are identical with their synthetic counterparts **2** and **3**. In **2**, metabolic oxidation of the 4-morpholinyl group of **1** has occurred at the carbon next to oxygen to give the 2-hydroxy-4-morpholinyl moiety, whereas in **3**, the morpholine of **1** has been oxidized one step further and then ring opened to produce the *N*-(2-hydroxyethyl)glycine substituent. Biological testing of synthetic samples of the three major metabolites from human urine (**3**, **4**, and **6**) indicated that only **4**, in which the morpholine moiety has been degraded to a 2-hydroxyethylamino group, had significant β-adrenergic blocking activity (one-seventh that of timolol in anesthetized dogs).

The β-adrenergic antagonist drug timolol (**1**), like propranolol and a number of other β-blockers, has been shown to be effective in humans for the treatment of hypertension and angina pectoris.[1] In addition, timolol has been

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