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## Identification of Adducts Formed in the Reaction of 5'-Acetoxy-N'-Nitrosonornicotine with Deoxyguanosine and DNA

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### Abstract

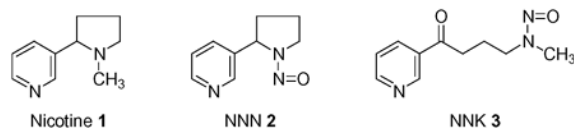
N'-Nitrosonornicotine (NNN) is believed to play an important role as a cause of cancer in people who use tobacco products and is considered to be a human carcinogen. NNN requires metabolism to form DNA adducts, which are absolutely critical to its carcinogenic properties. Previous studies have identified cytochrome P450-catalyzed 2'- and 5'-hydroxylation of NNN as potential DNA adduct forming metabolic pathways. 5'-Hydroxylation is the more prevalent of these in monkeys and humans, and is known to generate mutagenic intermediates, but the DNA adducts formed by this pathway have never been characterized. In this study, we used 5'-acetoxyNNN as a stable precursor to 5'-hydroxyNNN, and investigated its esterase-catalyzed reactions with deoxyguanosine (dGuo) and DNA. Adducts resulting from carbocation and oxonium ion intermediates, produced by the spontaneous decomposition of 5'-hydroxyNNN, were identified. The carbocation pathway resulted in the formation of 2-[2-hydroxy-5-(3-pyridyl)pyrrolidin-1-yl]deoxyinosine (**12**) which was characterized by comparison to an independently synthesized standard. Treatment of **12** with NaBH<sub>3</sub>CN produced two diastereomers of 2-[2-(3-pyridyl)pyrrolidin-1-yl]deoxyinosine (**14**), and their absolute configurations at the 2-position were determined by comparison to synthetic standards. The oxonium ion pathway produced diastereomers of N<sup>2</sup>[5-(3-pyridyl)tetrahydrofuran-2-yl]dGuo (**16**), identified by comparison to synthetic standards. The absolute configuration at the 5-position was determined by establishing the stereochemistry of the enantiomers of 5-(3-pyridyl)-2-hydroxytetrahydrofuran at the 5-position, and allowing these to react individually with dGuo. Treatment of **16** with NaBH<sub>3</sub>CN produced N<sup>2</sup>[4-hydroxy-4-(3-pyridyl)but-1-yl]dGuo (**18**) which was also synthesized independently. Using liquid chromatography-electrospray ionization-tandem mass spectrometry with selected reaction monitoring, adducts **12** and **16** were identified as products of the reactions of 5'-acetoxyNNN with dGuo. Similarly, adducts **14** and **18** were identified as products of the reaction of 5'-acetoxyNNN with DNA followed by NaBH<sub>3</sub>CN treatment and enzymatic hydrolysis. These results provide the first structural characterization of DNA adducts that can be formed by 5'-hydroxylation of NNN.

### Introduction

Two critical biological properties of tobacco products- addiction and carcinogenicity- are represented by the structurally related compounds nicotine (**1**) and N'-nitrosonornicotine (NNN, **2**). Nicotine is the principal known addictive agent in tobacco products and is the reason why most people cannot break their tobacco habit in spite of a strong desire to quit (1). Nicotine is not carcinogenic, but replacement of its methyl group by a nitroso- group produces the carcinogen NNN (2–4). NNN, which is present in both unburned tobacco and tobacco smoke,

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causes tumors of the esophagus and nasal cavity in rats, and the respiratory tract in mice, hamsters, and mink (2–4). NNN and the related nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK, **3**) elicit oral tumors when co-applied to the rat oral cavity (5). NNN is believed to play a significant role as a cause of cancers of the oral cavity, esophagus, nasal cavity, and perhaps other tissues in people who use tobacco products (2,6). The International Agency for Research on Cancer ranks NNN and NNK in Group 1, carcinogenic to humans (7).



NNN requires metabolism, catalyzed principally by cytochrome P450 enzymes, to produce its mutagenic and, presumably, its carcinogenic effects (4). DNA adduct formation, which results in miscoding and the formation of permanent mutations, is critical in this process (8). Two pathways of NNN metabolism, 2'-hydroxylation and 5'-hydroxylation, illustrated in Scheme 1, can produce electrophiles that react with DNA, forming adducts (4). 2'-Hydroxylation of NNN initially produces 2'-hydroxyNNN (**5**) which spontaneously ring opens yielding diazohydroxide **7**. This intermediate, which is also formed metabolically from NNK, is known to react with DNA, and several adducts have been characterized (9–11). 5'-Hydroxylation of NNN initially gives 5'-hydroxyNNN (**6**) which ring opens yielding diazohydroxide **8**. Although the mutagenicity of 5'-acetoxyNNN (**4**), a stable precursor to 5'-hydroxyNNN (**6**), has been known for nearly 30 years (12), and the 5'-hydroxylation pathway is a prevalent one in the metabolism of NNN in monkeys and in human tissues (13,14), there are no reports in the literature on DNA adduct formation by 5'-hydroxylation of NNN. In this paper, we describe the first characterization of deoxyguanosine (dGuo) and DNA adducts resulting from this pathway of NNN metabolism.

## Materials and Methods

### Caution

NNN and 5'-acetoxyNNN are carcinogenic and mutagenic and therefore should be handled with extreme care, using appropriate clothing and ventilation at all times.

All chemicals were obtained from Sigma-Aldrich Chemical Co. (Milwaukee, WI), unless noted otherwise.

**Apparatus and Assay Conditions**—HPLC was carried out with Waters Associates (Milford, MA) systems equipped with a model 996 photodiode array detector or a model 440 UV-visible detector set to 254 nm, unless noted otherwise. The following solvent elution programs were used, with linear solvent gradients: system 1, a 300 mm × 3.9 mm, 10 $\mu$  C-18 Bondclone column (Phenomenex, Torrance, CA) eluted at 1 mL/min with 0% CH<sub>3</sub>OH in H<sub>2</sub>O to 100% CH<sub>3</sub>OH over the course of 60 min; system 2, a 250 mm × 10 mm, 10 $\mu$ , C-18 Vydac 201TP column (Separations Group, Hesperia, CA) eluted at 3 mL/min with 0% CH<sub>3</sub>OH in H<sub>2</sub>O to 100% CH<sub>3</sub>OH over the course of 60 min; system 3, a 250 mm × 6 mm, 5 $\mu$  Luna C18 column (Phenomenex) eluted isocratically at 0.7  $\mu$ L/min with 70% CH<sub>3</sub>OH in H<sub>2</sub>O (20  $\mu$ L of 20 mM oxalic acid solution was injected immediately before each chromatographic run); system 4, a 0.46 cm × 25 cm Beckman Ultrasphere ODS 5 $\mu$  C-18 column (Beckman Coulter, Fullerton, CA) eluted with 20 to 60% CH<sub>3</sub>OH in H<sub>2</sub>O over the course of 30 min at a flow rate of 1 mL/min, and then held at 60% CH<sub>3</sub>OH for 10 min.

LC-ESI-MS was carried out in systems 5–7 as follows. System 5 used an Agilent 1100 Series capillary flow HPLC ion trap MS system (Agilent Technologies, Palo Alto, CA) with a 150 mm × 0.5 mm Zorbax Extend-C-18, 3.5 μm column (Agilent) eluted at 13.0 μL/min with 10% CH<sub>3</sub>OH in 15 mM NH<sub>4</sub>OAc (pH 5.8) to 60 % CH<sub>3</sub>OH over the course of 30 min. N<sub>2</sub> was used as drying gas (200 °C, 5 L/min) and as nebulizing gas (15 psi). The system also contained an in-line UV detector. The mass spectrometer was operated in the full scan mode (*m/z* 100–800), with target-ion abundance of 30,000, maximum accumulation time of 300 ms, and fragmentation amplitude of 0.9 V. MS/MS experiments were performed with a CID gas pressure of 1.5 mTorr and CID energy of 30 V. System 6 used a Thermo Finnigan LCQ Deca instrument (Thermo Finnigan LC-MS Division, San Jose, CA) interfaced with an Alliance 2690 HPLC multisolvent delivery system (Waters) equipped with an SPD-10A UV-Vis detector (Shimadzu Scientific Instruments, Columbia, MD) and a 300 mm × 3.9 mm, 10μ C-18 Bondclone column eluted at 0.5 mL/min with 5% CH<sub>3</sub>OH in 40 mM NH<sub>4</sub>OAc to 35% CH<sub>3</sub>OH over the course of 60 min, then from 35% to 70% CH<sub>3</sub>OH in 30 min, then from 70% CH<sub>3</sub>OH to 100% CH<sub>3</sub>OH in 5 min. The ESI source was set as follows: voltage, 2.0 kV; capillary temperature 275 °C. MS/MS data were acquired with the following parameters: parent mass, *m/z* 417, isolation width, 1.5 amu; activation amplitude, 30%; activation Q, 0.25; activation time 30 ms. System 7 used a TSQ Quantum Ultra instrument (Thermo Finnigan LC-MS Division, San Jose, CA) interfaced with an Agilent 1100 Series capillary flow HPLC system and a 150 × 0.5 mm, 5μ Zorbax SB-18 column (Agilent) eluted at 10.0 μL/min with 10% CH<sub>3</sub>OH in 15 mM NH<sub>4</sub>OAc (pH 5.8) to 70% CH<sub>3</sub>OH over the course of 40 min. The ESI source was set in the positive mode as follows: voltage, 4 kV; current, 20 μA; and ion transfer tube, 150 °C. MS/MS experiments used the selected reaction monitoring (SRM) mode, *m/z* 415 → *m/z* 299. The collision energy was 13 eV, and the argon collision gas pressure was 0.8 mTorr.

High resolution mass spectra (HRMS) were determined with a Finnigan-MAT 90/95 instrument (Thermo Finnigan MAT, Bremen, Germany).

NMR spectra were recorded in DMSO-d<sub>6</sub> or CDCl<sub>3</sub> using Varian Unity (Varian, Inc., Palo Alto, CA) spectrometers operated at either 600 or 800 MHz and standard 5 mm tubes or 3 mm Shigemi tubes (Shigemi, Inc., Allison Park, PA).

**Reaction of 5'-acetoxyNNN(4) with dGuo:** 5'-AcetoxyNNN (**4**, 59.0 mg, 0.25 mmol) (15) was allowed to react with dGuo (13.2 mg, 0.05 mmol) in 5 mL of 0.1M phosphate buffer, pH 7.0, in the presence of porcine liver esterase (90 units) at 37 °C for 1h. Products **12** and **16** were separated using system 6 and characterized by LC-ESI-MS using system 7, and by comparison to synthetic standards.

**Reaction of 5'-acetoxyNNN(4) with DNA:** 5'-AcetoxyNNN (**4**, 12.0 mg, 0.05 mmol) was allowed to react with calf thymus DNA (20 mg) in 5 mL of 0.1M phosphate buffer, pH 7.0, in the presence of porcine liver esterase (90 units) at 37 °C for 1h. The DNA was precipitated by addition of ethanol, then washed with 70% ethanol and 95% ethanol. The DNA (1.2 mg) was dissolved in 1 mL of 10 mM Tris HCl/5 mM MgCl<sub>2</sub> buffer, pH 7.0. NaBH<sub>3</sub>CN (10 mg) was added and the mixture was incubated at 37 °C for 30 min and the DNA was then hydrolyzed enzymatically (16). The hydrolysate was further purified by solid phase extraction on C18 Seppak Cartridges (Waters) as described (16). Products **14** and **18** were eluted with CH<sub>3</sub>OH and analyzed by LC-ESI-MS using system 7 (starting from 5% CH<sub>3</sub>OH in 15 mM NH<sub>4</sub>OAc, pH 5.8 for 10 min, then to 95% CH<sub>3</sub>OH over the course of 60 min, collision energy 1.3 mTorr) and by comparison to synthetic standards.

**5-(Benzhydrylidenamino)-5-(3-pyridyl)-1-pentene (20).** 2-Aza-1,1-diphenyl-3-(3-pyridyl)-1- propene (**19**) (17) (2.7 g, 9.9 mmol) was dissolved in anhydrous THF (50 mL)

under N<sub>2</sub> and the mixture was cooled to -78 °C. Lithium diisopropylamide (14 mL, 26 mmol) was added dropwise via syringe over a 15 min period. The reaction mixture turned a deep purple color as the temperature rose to -40 °C. A solution of 4-bromo-1-butene (1.24 mL, 12.2 mmol) in THF (15 mL) was added via syringe over a period of 20 min. The mixture was allowed to stir at 40 °C for 8 h. The pH was adjusted to 6 with 10% HCl and the mixture was extracted with CHCl<sub>3</sub> (5 × 30 mL). The organics were combined, dried (Na<sub>2</sub>SO<sub>4</sub>), filtered, and condensed. The resulting oil was purified by column chromatography with elution by hexanes/ethyl acetate (1:1) and ethyl acetate giving **20** (1.1 g, 3.4 mmol, 39%) as a yellow oil.

<sup>1</sup>H-NMR (CDCl<sub>3</sub>) δ 8.47 (ddd, J=4.8, 1.8 Hz, 1H, pyr-6H), 8.40 (d, J=1.8 Hz, 1H, pyr-2H), 7.73 (dd, J=1.82, 2.4 Hz, 1H, pyr-4H), 7.72 (dd, J=1.82, 2.4 Hz, 1H, pyr-4H), 7.67 (d, J=7 Hz, 2H, PhH), 7.48–7.32 (m, 6H, PhH), 7.23 (m, 1H, pyr-5H), 7.02 (d, J=3.6 Hz, 2H, PhH), 5.71 (m, 1H, 2H), 4.93 (dd, J=1.2, 16.8 Hz, 1H, 1H<sub>a</sub>), 4.88 (d, J=10.2 Hz, 1H, 1H<sub>b</sub>), 4.39 (dd, J=4.8, 5.4 Hz, 1H, 5H), 2.03 (m, 2H, 4H), 1.88 (m, 2H, 3H); ESI-MS *m/z* 327.1 (M + H)<sup>+</sup>.

**5-(Benzhydrylidenamino)-5-(3-pyridyl)-1,2-pentanediol (21):** Compound **20** (0.5 g, 1.5 mmol) was dissolved in anhydrous THF (25 mL) and treated with aqueous OsO<sub>4</sub> (0.08M, 1mL) and 4-methylmorpholine *N*-oxide (270 mg, 2.3 mmol). The reaction was allowed to stir at room temperature for 3 h and was quenched with 10% NaHSO<sub>3</sub> (15 mL). The volatiles were removed at reduced pressure and the remaining material was extracted with ethyl acetate (2 × 30 mL). The organics were combined, dried (Na<sub>2</sub>SO<sub>4</sub>), filtered, and condensed to give **21** as a brown oil (500 mg, 1.39 mmol, 93%).

<sup>1</sup>H-NMR (CDCl<sub>3</sub>) δ 8.40 (m, 1H, pyr-6H), 8.32 (m, 1H, pyr-2H), 7.77 (d, J=6.6Hz, 2H, Ph), 7.66 (dd, J=1.8, 2.4 Hz, 1H, pyr-4H), 7.62–7.23 (m, 6H, PhH), 7.18 (m, 1H, pyr-5H), 7.02 (d, J=3.6 Hz, 2H, PhH), 4.38 (dd, J=6.6, 6.6 Hz, 1H, 5H), 3.61 (m, 1H, 2H), 3.52 (m, 1H, 1H<sub>a</sub>), 3.34 (m, 1H, 1H<sub>b</sub>), 2.31–1.82 (m, 2H, 4H), 1.44–1.18 (m, 2H, 3H); ESI-MS *m/z* 361.1 (M + H)<sup>+</sup>.

**5-Amino-5-(3-pyridyl)-1,2-pentanediol (22):** Compound **21** (0.5 g, 1.4 mmol) was dissolved in 12 mL ethanol containing 0.5M NH<sub>2</sub>OH. HCl and the mixture was allowed to stir at room temperature overnight, condensed under reduced pressure, and the residue suspended in 4 mL H<sub>2</sub>O. Ion-exchange chromatography (Dowex 1X8-200, strongly basic) with elution by H<sub>2</sub>O gave **22** (250 mg, 1.28 mmol, 91%) as a clear purplish oil. <sup>1</sup>H-NMR (D<sub>2</sub>O) δ 8.34 (s, 1H, pyr-6H), 8.29 (dd, J=1.2, 3.6 Hz, 1H, pyr-2H), 7.71 (dd, J=1.8, 4.2 Hz, 1H, pyr-4H), 7.32 (dd, J=3.6, 4.2 Hz, 1H, pyr-5H), 3.82 (dd, J=7.2, 6.6 Hz, 1H, 5H), 3.52 (m, 1H, 2H), 3.38 (m, 1H, 1H<sub>a</sub>), 3.26 (m, 1H, 1H<sub>b</sub>), 1.80–1.58 (m, 2H, 4H), 1.38–1.04 (m, 2H, 3H); ESI-MS *m/z* 197.1 (M + H)<sup>+</sup> HRMS Calc'd for C<sub>10</sub>H<sub>16</sub>N<sub>2</sub>O<sub>2</sub> (M + H)<sup>+</sup> 197.1285, found 197.1278

***O*<sup>6</sup>[(Trimethylsilyl)ethyl]-N<sup>2</sup>[1,2-dihydroxy-5-(3-pyridyl)pent-5-yl]dGuo (24):**

Compound **22** (7.5 mg, 0.038 mmol) was added to a mixture of *O*<sup>6</sup>[(trimethylsilyl)ethyl]2-fluoroinosine (**23**, 9.5 mg, 0.25 mmol, ChemGene Corp, Wilmington, MA) and *N,N*-diisopropylethylamine (50 μL) in 200 μL DMSO. The mixture was stirred at 65 °C for 7 h, cooled, and the solvents were removed under vacuum. The residue was purified with HPLC system 2, retention time 42 min, giving **24** (4.5 mg 0.008 mmol, 22%). <sup>1</sup>H-NMR (DMSO-*d*<sub>6</sub>) δ 8.58 (m, 1H, pyr-2H), 8.39 (m, 1H, pyr-6H), 8.04 (s, 1H, dGuo-C8H), 7.77 (m, 1H, pyr-4H), 7.46 (bs, 1H, dGuo-N<sup>2</sup>H), 7.28 (m, 1H, pyr-5H), 6.20 (dd, J=7.2, 7.2 Hz, 1H, 1'H), 5.27 (bs, 1H, 3'OH), 4.93 (m, 2H, 5H and 1OH), 4.42–4.33 (m, 5H, 5'OH, 2OH, OCH<sub>2</sub>CH<sub>2</sub>Si and 3'H), 3.80 (m, 1H, 4'H), 3.53 (m, 1H, 5'Ha), 3.43–3.2 (m, 4H, 1H, 2H and 5'H<sub>b</sub>), 2.56 (m, 1H, 2' H<sub>b</sub>), 2.00–1.60 (m, 2H, 4H), 1.44–1.15 (m, 2H, 3H), 1.08 (m, 2H, OCH<sub>2</sub>CH<sub>2</sub>Si), 0.065 (s, 9H, Si(CH<sub>3</sub>)<sub>3</sub>); ESI-MS *m/z* 547.1 (M + H)<sup>+</sup>; HRMS Calc'd for C<sub>25</sub>H<sub>38</sub>N<sub>6</sub>O<sub>6</sub>Si (M + Na)<sup>+</sup> 569.2514, found 569.2506

***N*<sup>2</sup>[1,2-dihydroxy-5-(3-pyridyl)pent-5-yl]dGuo (25):** Compound **24** (4.5 mg, 0.008 mmol) was dissolved in 5% acetic acid (1 mL) and the mixture was stirred at room temp for 3 h, concentrated at reduced pressure and the residue purified using HPLC system 2, giving **25**, retention time 21 min (2 mg, 0.0045 mmol, 56%). <sup>1</sup>H-NMR (DMSO-*d*<sub>6</sub>) δ 8.59 (s, 0.5H, pyr-2H), 8.57 (s, 0.5 H, pyr-2H), 8.44 (m, 1H, pyr-6H), 7.88 (s, 0.5H, dGuo-C8H), 7.84 (s, 0.5H, dGuo-C8H), 7.76 (d, *J*=8Hz, 0.5H, pyr-4H), 7.74 (d, *J*=8Hz, 0.5H, pyr-4H), 7.36 (m, 1H, pyr-5H), 7.10 (bs, 1H, dGuo-*N*<sup>2</sup>H), 6.08 (dd, *J*=6.4, 5.6Hz, 0.5H, 1'H), 6.04 (dd, *J*=6.4, 5.6 Hz, 0.5H, 1'H), 4.94–4.85 (m, 2H, 5H and 1OH), 4.31 (m, 1H, 3'H), 3.78 (m, 1H, 4'H), 3.59 (m, 1H, 5'H<sub>a</sub>), 3.51–3.27 (m, 4H, 5'H<sub>a</sub>, 5'H<sub>b</sub>, 1H<sub>a</sub> and 2H), 3.20 (m, 1H, 1H<sub>b</sub>), 2.44 (m, 1H, 2'H), 2.13 (m, 0.5H, 2'H), 2.06 (m, 0.5H, 2'H), 2.02–1.77 (m, 2H, 4H), 1.57–1.16 (m, 2H, 3H); ESI-MS *m/z* 447.1 (M + H)<sup>+</sup>. MS/MS of *m/z* 447; 331[(M + H) – deoxyribose]<sup>+</sup>; HRMS Calc'd for C<sub>20</sub>H<sub>26</sub>N<sub>6</sub>O<sub>6</sub> (M + Na)<sup>+</sup> 469.1806, found 469.1822

**2[2-Hydroxy-5-(3-pyridyl)pyrrolidin-1-yl]deoxyinosine (12):** Sodium periodate (200 μL, 80 mM in 0.01 M phosphate buffer, pH 7) was added to a solution of **25** (2 mg, 0.0045 mmol) in phosphate buffer (200 μL, 0.01M, pH 7). The mixture was stirred at room temperature for 25 min; HPLC showed complete disappearance of starting material. HPLC purification using system 2, retention time 20–24 min, gave a mixture of diastereomers of **12** (1 mg, 0.0024 mmol, 54%). In HPLC system 1 (instead of H<sub>2</sub>O, 15 mM NH<sub>4</sub>OAc pH 5.0 was used), retention times were 27.5, 28.3, 28.9, and 29.6 min. UV (λ<sub>max</sub>) of the 4 diastereomers: 27.5 min, 209.8, 258.0, 285.0 (sh); 28.3 min 211.0, 260.3, 285.0 (sh); 28.9 min 212.2, 260.3, 285.0 (sh); 29.6 min 209.8, 258.0, 285.0 (sh).

<sup>1</sup>H-NMR (DMSO-*d*<sub>6</sub>) δ 8.63 (d, *J*=2.4 Hz, 0.5H, pyr-2H), 8.59 (d, *J*=2.4 Hz, 0.5H, pyr-2H), 8.41 (m, 1H, pyr-6H), 7.89 (s, 0.5H, dGuo-C8H), 7.82 (s, 0.5H, dGuo-C8H), 7.78 (m, 0.5H, pyr-4H), 7.73 (m, 0.5H, pyr-4H), 7.37–7.28 (m, 1H, pyr-5H), 6.57 (s, 1H, 2-OH), 6.02–5.93 (m, 1H, 1'H), 5.69 (m, 1H, 2H), 5.13 (dd, *J*=7.2, 8.0 Hz, 0.5H, 5H), 5.10 (dd, *J*=7.2, 8.0 Hz, 0.5H, 5H), 4.26–4.21 (m, 1H, 3'H), 3.80–3.71 (m, 1H, 4'H), 3.57 (m, 0.5H, 5'H<sub>a</sub>), 3.49–3.38 (m, 1.5H, 5'H<sub>a</sub> and 5'H<sub>b</sub>), 2.67 (m, 2'H), 2.42 (m, 1H, 4H<sub>a</sub>), 2.34 (m, 2'H), 2.21 (m, 2'H), 2.09 (m, 2'H and 3H<sub>a</sub>), 2.07–1.86 (m, 2'H, 3H<sub>b</sub> and 4H<sub>b</sub>), 1.81 (m, 2'H), 1.71(m, 2'H). LC-ESI-MS and HPLC-UV analysis were carried out using system 5, retention time 17–21 min; MS (positive ion) *m/z* 415 (M + H)<sup>+</sup>; MS/MS of *m/z* 415; *m/z* 299; SRM analysis (*m/z* 415→299) was carried out using system 7, retention time 23.3–25.6 min; HRMS calculated for C<sub>19</sub>H<sub>22</sub>NaN<sub>6</sub>O<sub>5</sub>, (M + Na)<sup>+</sup>; 437.1544 found, 437.1536.

**2-(*R*)- and 2-(*S*)-2[2-(3-Pyridyl)pyrrolidin-1-yl]deoxyinosine (14).** (*R*)- or (*S*)-Nornicotine (18) (1.8 mg, 0.012 mmol), *O*<sup>6</sup>[(trimethylsilyl)ethyl]2-fluoroinosine (**23**, 3.0 mg, 0.008 mmol), and Et<sub>3</sub>N (60 μL) were dissolved in DMSO (400 μL) and heated at 60 °C for 36 h. The mixture was condensed under reduced pressure, and purified with HPLC system 2 (eluted at 3 mL/min with 10% CH<sub>3</sub>OH in H<sub>2</sub>O to 100% CH<sub>3</sub>OH in 60 min), giving 2-(*R*)-**14**, retention time 19.7 min (800 μg, 0.002 mmol, 25%) and 2-(*S*)-**14**, retention time 21.2 min (800 μg, 0.002 mmol, 25%). <sup>1</sup>H-NMR (DMSO-*d*<sub>6</sub>) 2-(*R*)-**14** δ 8.44 (d, *J*=2.4Hz, 1H, pyr-2H), 8.42 (dd, *J*=1.2, 4.8Hz, 1H, pyr- 6H), 7.80 (s, 1H, dGuo-C8H), 7.56 (d, *J*=7.8Hz, 1H, pyr-4H), 7.30 (dd, *J*=4.8, 7.8 Hz, 1H, pyr- 5H), 5.94 (m, *J*=6.0 Hz, 1H, 1'H), 5.24 (d, *J*=4.8Hz, 1H, 2H), 5.17 (d, *J*=4.2 Hz, 1H, 3'-OH), 4.83 (t, *J*=5.4 Hz, 1H, 5'-OH), 4.19 (br, 1H, 3'H), 3.90 (m, 1H, 5H<sub>a</sub>), 3.74 (m, 1H, 4' H), 3.61 (m, 1H, 5H<sub>b</sub>), 3.54 (m, 1H, 5'H<sub>a</sub>), 3.47 (m, 1H, 5'H<sub>b</sub>), 2.39 (m, 2H, 3H), 1.97 (m, 2H, 2'H), 1.81–1.94 (m, 2H, 4H). LC-ESI-MS and HPLC-UV analysis were carried out using system 5, 2-(*R*)-**14** retention time 19.1 min; MS (positive ion) *m/z* 399 (M + H)<sup>+</sup>; MS/MS of *m/z* 399; *m/z* 283; HRMS calculated for C<sub>19</sub>H<sub>22</sub>NaN<sub>6</sub>O<sub>4</sub>, 421.1595 (M + Na)<sup>+</sup>; found, 421.1616.

<sup>1</sup>H-NMR (DMSO-*d*<sub>6</sub>) 2-(*S*)-**14** δ 8.45 (d, *J*=1.8 Hz, 1H, pyr-2H), 8.42 (dd, *J*=1.8, 4.8Hz, 1H, pyr- 6H), 7.85 (s, 1H, dGuo-C8H), 7.58 (d, *J*=8.4 Hz, 1H, pyr-4H), 7.31 (dd, *J*=4.8, 7.8 Hz,



1H, pyr- 5H), 5.97 (m, J=6.0 Hz, 1H, 1'H), 5.31 (d, J=6.6 Hz, 1H, 2H), 5.21 (d, J=3.6 Hz, 1H, 3'OH), 4.79 (t, J=6.0 Hz, 1H, 5'OH), 4.22 (br, 1H, 3'H), 3.90 (m, 1H, 5H<sub>a</sub>), 3.72 (dd, J=4.8, 7.8 Hz, 1H, 4'H), 3.58 (m, 1H, 4'H<sub>b</sub>), 3.41 (m, 1H, 5'H<sub>a</sub>), 3.31 (m, 1H, 5'H<sub>b</sub>), 2.37 (m, 2H, 3H), 1.99 (m, 2H, 2'H), 1.84–1.94 (m, 2H, 4H).

LC-ESI-MS and HPLC-UV analysis were carried out using system 5, 2-(*S*)-**14** retention time 20.7 min. MS (positive ion)  $m/z$  399 ( $M + H$ )<sup>+</sup>; MS/MS of  $m/z$  399;  $m/z$  283; HRMS calculated for C<sub>19</sub>H<sub>22</sub>NaN<sub>6</sub>O<sub>4</sub>, 421.1595 ( $M + Na$ )<sup>+</sup>; found, 421.1616.

***N*<sup>2</sup>[5-(3-pyridyl)tetrahydrofuran-2-yl]dGuo (16):** 5-(3-Pyridyl)-2-hydroxytetrahydrofuran (**27**) (19) (5.8 mg, 0.035 mmol) was dissolved in 0.5 mL dry DMF under N<sub>2</sub>. Trifluoroacetic acid (1 μL) and dGuo (10 mg, 0.035 mmol) were added and the mixture was allowed to stir at room temperature for 4 days. Reaction progress was monitored by HPLC system 1. Retention times were: dGuo (16.2 min); **27** (23.7 min); diastereomers of **16** (peak 1, 29.2; peak 2, 30.8; peak 3, 32.2; peak 4, 32.8 min). Material eluting from 27 to 34 min was collected and the solvent was removed to give **16** (3.5 mg, 0.008 mmol, 22%). <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>) δ 10.5 (bs, 1H, dGuo-N1- H), 8.54 (d, J=6.3 Hz, 1H, pyr-2H), 8.46 (m, 1H, pyr-6H), 7.95 (s, 1H, dGuo-C8H), 7.76 (dd, J=1.6, 6.4 Hz, 1H, pyr-4H), 7.35 (m, 2H, pyr-5H and dGuo-*N*<sup>2</sup>H), 6.16 (dd, J=8.3, 8.3 Hz, 1H, 1'H), 6.05 (d, J=6.4 Hz, 0.5H, 2H), 5.91 (d, J=6.4 Hz, 0.5H, 2H), 5.28 (d, J=4.4 Hz, 1H, 3'OH), 5.04 (dd, J=7.1, 7.1 Hz, 0.5H, 5H), 4.96 (dd, J=7.1, 7.1 Hz, 0.5H, 5H), 4.87 (dd, J=5.4, 5.4 Hz, 1H, 5'OH), 4.35 (m, 2H, 3'H), 3.79 (m, 1H, 4'H), 3.53 (m, 1H, 5'H<sub>a</sub>), 3.47 (m, 1H, 5'H<sub>b</sub>), 2.57 (m, 2'H<sub>a</sub>), 2.41 (m, 1H, 3H<sub>a</sub>), 2.35 (m, 1H, 4H<sub>a</sub>), 2.18 (m, 1H, 2'H<sub>b</sub>), 1.92–1.77 (m, 2H, 3H<sub>b</sub> and 4H<sub>b</sub>); UV (CH<sub>3</sub>OH/15mM NH<sub>4</sub>OAc) λ<sub>max</sub> peaks 1–4 207.7, 255.8. LC-ESI-MS and HPLC-UV analysis were carried out using system 5 (see Figure 5), retention times: peak 1, 19.0; peak 2, 19.5, peak 3, 20.9, peak 4, 22.1 min; MS (positive ion)  $m/z$  415 ( $M + H$ )<sup>+</sup>; MS/MS of  $m/z$  415;  $m/z$  299; SRM analysis ( $m/z$  415→ $m/z$  299) was carried out using system 7, retention time 26–28 min; HRMS calculated for C<sub>19</sub>H<sub>22</sub>NaN<sub>6</sub>O<sub>5</sub>, 437.1544 ( $M + Na$ )<sup>+</sup>; found, 437.1549.

**[1-(1,3-Dioxan-2-yl)-4-(3-pyridyl)but-4-yl]α-methoxy-α-(trifluoromethyl)phenyl acetate (32):** A mixture of 1-(1,3-dioxan-2-yl)-4-oxo-4-(3-pyridyl)butane (**30**) (19) (8.0 mg, 0.036 mmol) and NaBH<sub>4</sub> (100 mg) in CH<sub>3</sub>OH (5 mL) was stirred for 6 h at room temperature. HPLC analysis (system 2) indicated complete disappearance of starting material. The pH of the reaction mixture was adjusted to 7.0 with 1 N HCl, and **31** was purified using HPLC system 2, retention time 27 min (7.0 mg, 0.031 mmol, 86%). Compound **31** (6.6 mg, 0.030 mmol) was allowed to react with (*R*)-(-)-α-methoxy-α-(trifluoromethyl)phenylacetic acid chloride (22.3 mg, 0.088 mmol) in CH<sub>2</sub>Cl<sub>2</sub> as described (20). The mixed diastereomers of **32** were collected in HPLC system 2 (6.45 mg, 0.015 mmol, 50%), and separated with HPLC system 3; retention times 23.1 and 24.6 min. <sup>1</sup>H NMR (23.1 min peak, carbinol (*S*)-**32**) δ 8.60 (s, 1H, pyr-2H), 8.55 (d, J=3.5 Hz, 1H, pyr-6H), 7.64 (d, J=6.0, 1H, pyr-4H), 7.38–7.30 (m, 5H, aromatic), 7.26 (dd, J=5.0, 8.0 Hz, 1H, pyr-5H), 5.99 (dd, J=6.0, 8.0 Hz, 1H, butyl-4H), 4.47 (t, J=5.0 Hz, 1H, butyl-1H), 4.04 (m, 2H, OCH<sub>2</sub>), 3.69 (m, 2H, OCH<sub>2</sub>), 3.42 (d, J=1.0 Hz, 3H, OCH<sub>3</sub>), 2.08–1.92 (m, 2H, butyl-3H), 1.67 (m, 1H, OCH<sub>2</sub>CH), 1.56–1.47 (m, 2H, butyl-2H), 1.31–1.28 (dd, J=1.0, 14Hz, 1H, OCH<sub>2</sub>CH); MS (positive ion ESI)  $m/z$  440 ( $M + 1$ ), MS/MS of  $m/z$  440;  $m/z$  206 (loss of O(CO)C(CF<sub>3</sub>)(Ph)(OCH<sub>3</sub>)).

<sup>1</sup>H-NMR (24.6 min peak, carbinol (*R*)-**32**) δ 8.53 (s, 1H, pyr-2H or pyr-6H), 8.49 (s, 1H, pyr-2H or pyr-6H), 7.46 (d, J=8.0 Hz, 1H, pyr-4H), 7.38–7.30 (m, 5H, PhH), 7.20 (m, 1H, pyr-5H), 5.94 (dd, J=6.0, 8.0 Hz, 1H, butyl-4H), 4.52 (t, J=5.0 Hz, 1H, butyl-1H), 4.07 (dd, J=4.0Hz, 2H, OCH<sub>2</sub>), 3.71 (m, 2H, OCH<sub>2</sub>), 3.53 (d, J=1.0Hz, 3H, OCH<sub>3</sub>), 2.14–1.92 (m, 2H, butyl-3H), 1.7–1.56 (m, 3H, OCH<sub>2</sub>CH + butyl-2H), 1.31 (m, 1H, OCH<sub>2</sub>CH); MS (positive ion ESI)  $m/z$  440, MS/MS of  $m/z$  440;  $m/z$  206.

**5-(R)-(+)** and **5-(S)-(-)** **5-(3-Pyridyl)-2-hydroxytetrahydrofuran (27)**: Each diastereomer of **32** (4.4 mg, 0.010 mmol) was treated with 0.1N NaOH, 37 °C, 12 h and the reaction mixture was adjusted to pH 7.0 with 1 N HCl, to give (R)-(+)- and (S)-(-)-**31** (1.6 mg, 0.010 mmol, 98%), purified by HPLC system 2. Each of (R)-(+)- and (S)-(-)-**31** was then treated with 10% aq H<sub>2</sub>SO<sub>4</sub> as described (19) to give 5-(R)-(+)- and 5-(S)-(-)- **27**. Products were collected using HPLC system 2: 5-(R)-(+)-**27**, retention time 23 min (1.0 mg, 0.006 mmol, 61 %,  $[\alpha]_D^{25} +42.0^\circ$ ) and 5-(S)-(-)-**27**, retention time 23 min (1.0 mg, 0.006 mmol, 61 %,  $[\alpha]_D^{25} - 59.7^\circ$ ).

**Determination of absolute configuration of 5-(R)-(+)** **5-(3-pyridyl)-2-**

**hydroxytetrahydrofuran (27)** 5-(R)-(+)-**27** (3.2 mg, 0.019 mmol) was heated under reflux in xylene (5 mL) in the presence of Ag<sub>2</sub>CO<sub>3</sub> on Celite (5.9 mg, 0.1 mmol) for 2 h at 160 °C as described (19). Lactone **33** (2.0 mg, 0.012 mmol, 63%) was purified using HPLC system 2. It was then heated under reflux with 6N HCl as described (21) for 1 h, cooled and the mixture neutralized to pH 7.0 with 6N NaOH. This gave hydroxy acid **34** which was purified with HPLC system 4 (1.0 mg, 0.006 mmol, 50%). Hydroxy acid **34** was allowed to react with acidic methanol followed by (S)- (-)- $\alpha$ -methylbenzyl isocyanate to give methyl 4-[ $\alpha$ -methylbenzylcarbamoyl]-4-(3- pyridyl)butanoate (MMPB, **35**) as described (22,23). The mixture was purified with HPLC system 4, and LC-ESI-MS and HPLC-UV analysis were carried out using system 5, except that there was a hold at 60% CH<sub>3</sub>OH for an additional 10 min. MS (positive ion ESI)  $m/z$  343 (M + H)<sup>+</sup>; MS/MS of  $m/z$  343;  $m/z$  178. The single diastereomer co-eluted with standard methyl 4-(R)- [(S)-  $\alpha$ -methylbenzylcarbamoyl]-4-(3-pyridyl)butanoate ((R,S)-MMPB, **35**).

**N<sup>2</sup>[5-(R)-(3-Pyridyl)tetrahydrofuran-2-yl]dGuo and N<sup>2</sup>[5-(S)-(3-pyridyl)**

**tetrahydrofuran-2- yl]dGuo (16)** 5-(R)-(+)- or 5-(S)-(-)-**27** (1.45 mg, 0.009 mmol) was each allowed to react with dGuo (2.5 mg, 0.009 mmol) in DMF (0.5 mL) and trifluoroacetic acid (1  $\mu$ L) for 4 days at room temperature, as described above. The mixture was purified using HPLC system 1. LC-ESI-MS and HPLC-UV analysis were carried out using system 5: retention time of the 5-(R)-isomers 19.7 min (peak 2) and 22.7 min (peak 4); MS (positive ion ESI)  $m/z$  415 (M + H)<sup>+</sup>, MS/MS of  $m/z$  415,  $m/z$  299 [(M – deoxyribose) + H]<sup>+</sup>; retention time of the 5-(S)-isomers 19.0min (peak 1) and 21.4 min (peak 3); MS (positive ion ESI)  $m/z$  415, MS/MS of  $m/z$  415,  $m/z$  299.

**N<sup>2</sup>[4-Hydroxy-4-(3-pyridyl)but-1-yl]dGuo (18)**: Racemic adduct **16** (1.2 mg, 0.003 mmol) and NaBH<sub>3</sub>CN (10 mg) in methanol:H<sub>2</sub>O (1:1) (5 mL) were stirred overnight at room temperature. The pH of the mixture was adjusted to 7.0 with 0.1N HCl, and it was extracted with CHCl<sub>3</sub>. The extracts were concentrated and **18** was purified using HPLC system 1 (retention time 29.8 min). Standard **18** was prepared by treating adduct **29** (24) (1.0 mg, 0.0024 mmol) with NaBH<sub>4</sub> (10 mg) in CH<sub>3</sub>OH (5 mL). The HPLC retention time, UV, MS (system 7), and <sup>1</sup>H-NMR spectra of **18** prepared from **16** matched those of **18** prepared from **29**.

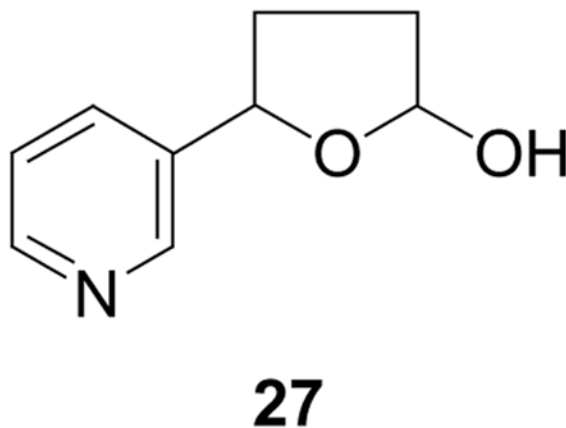
## Results

Scheme 2 shows that diazohydroxide **8**, formed from 5'-hydroxylation of NNN as illustrated in Scheme 1, could decompose to produce a series of electrophiles including diazonium ion **9**, carbocation **10**, and oxonium ion **15**. Based on previous work on DNA adduct formation from  $\alpha$ -hydroxylation of *N*-nitrosopyrrolidine (16), we hypothesized that two dGuo adducts resulting from 5'-hydroxylation of NNN would be **12** and **16**. We investigated the reactions of 5'-acetoxyNNN (**4**, Scheme 1) with dGuo and DNA to test this hypothesis. The data described below demonstrate that stereoisomers of **12** and **16** were indeed formed in these reactions.



Liquid chromatography-electrospray ionization-tandem mass spectrometry with selected reaction monitoring (LC-ESI-MS/MS-SRM) analysis of  $m/z$  415 $\rightarrow$ 299, corresponding to the transition  $[M + H]^+ \rightarrow [(M - \text{deoxyribose}) + H]^+$ , of the products of reaction of 5'-acetoxyNNN with dGuo produced the chromatogram illustrated in Figure 1. We proposed that the peaks eluting at 23.6 min corresponded to adduct **12** (Scheme 2). Standard adduct **12** was synthesized as summarized in Scheme 3. Aminodiol **22** was prepared by alkylation of **19** with 4-bromo-1-butene followed by OsO<sub>4</sub> oxidation and deprotection. Reaction of **22** with protected 2-fluoroinosine **23** gave **24**, which was deprotected and treated with NaIO<sub>4</sub> to yield the desired adduct **12**, as a mixture of 4 diastereomers, characterized by <sup>1</sup>H-NMR, UV, and LC-ESI-MS. In our hands, this material was somewhat unstable and prone to decomposition, possibly because of equilibrium with Schiff base **13** and open-chain amino aldehyde **11** (Scheme 2). However, its retention time in LC-ESI-MS/MS System 7 was the same as that of the 23.6 min peak in Figure 1. Treatment of synthetic adduct **12** with NaBH<sub>3</sub>CN produced 2 diastereomers of **14** (Scheme 2), as indicated in Figure 2. Two major peaks were observed by HPLC with UV detection and LCESI- MS/MS. The same 2 peaks were cleanly produced upon NaBH<sub>3</sub>CN treatment of the reaction mixture of 5'-acetoxyNNN and dGuo. Their structures were confirmed by reaction of nornicotine (**26**) with protected 2-fluoroinosine **23** (shown for (*R*)-**26** in Scheme 4), producing 2 diastereomers of **14**, which were characterized by UV, <sup>1</sup>H-NMR, and MS. The 2 diastereomers of **14** were identical to those formed in the NaBH<sub>3</sub>CN reduction of synthetic **12** or in the reduction of **12** produced in the reaction of 5'-acetoxyNNN with dGuo. The absolute configuration of carbon 2 of the pyrrolidine ring of **14** (bearing the pyridine ring) was assigned by separate reactions of 2-(*R*)- and 2-(*S*)-nornicotine with protected 2-fluoroinosine **23** (shown for (*R*)-**26** in Scheme 4). Thus, peak 1 in Figure 2 was assigned as the 2-(*R*)-isomer while peak 2 was assigned as the 2-(*S*)-isomer. Collectively, these results established the structure of the 23.6 min peak of Figure 1 as diastereomers of adduct **12**.

We then turned our attention to the identity of the four peaks eluting from 26.3–28.8 min in Figure 1, proposed to correspond to adduct **16** (Scheme 2). The UV spectra of these peaks are shown in Figure 3A-D. All were essentially identical and characteristic of *N*<sup>2</sup>-substituted dGuo derivatives. Standard adduct **16** was prepared by reaction of 5-(3-pyridyl)-2-hydroxytetrahydrofuran (**27**) with dGuo. LC-ESI-MS analysis of the product produced



4 main peaks similar to those eluting from 26.3–28.8 min in Figure 1. The UV spectra of these peaks were the same as those shown in Figure 3A-D, from the reaction of 5'-acetoxyNNN with dGuo. The <sup>1</sup>H-NMR spectrum of the four peaks was completely consistent with the overall structural assignment as **16**.

Further evidence for the structural assignment of **16** was obtained by NaBH<sub>3</sub>CN treatment of this adduct, which was expected to be in equilibrium with Schiff base **17** (Scheme 2). This reaction produced **18**, which was identical to a standard prepared by the reaction of keto aldehyde **28** with dGuo in the presence of NaBH<sub>3</sub>CN to give **29** (24), followed by reduction (Scheme 5). The identity of the products was established by LC-ESI-MS-selected ion monitoring (SIM) at  $m/z$  417 ( $M + H$ )<sup>+</sup>, and by SRM of the transition  $m/z$  417 →  $m/z$  301. These results conclusively established the overall structure of adduct **16**.

The stereochemistry of **16** was then investigated by synthesizing chiral lactols **27**, as illustrated in Scheme 6. Acetal **30** was reduced with NaBH<sub>4</sub> providing **31**, which was allowed to react with (*R*)-(-)- $\alpha$ -methoxy- $\alpha$ -(trifluoromethyl)phenylacetic acid chloride. The resulting diastereomeric Mosher esters **32** were separated by HPLC. They were subjected to stepwise hydrolysis, first by base to remove the ester group and then by acid to remove the acetal, yielding hydroxy aldehydes which spontaneously cyclized to the enantiomeric lactols **27**.

The absolute configurations of the enantiomers of lactol **27** were determined (Scheme 6). One enantiomer was oxidized with Ag<sub>2</sub>CO<sub>3</sub> to give chiral lactone **33** which was hydrolyzed with acid yielding hydroxy acid **34**. This was esterified and derivatized with (*S*)- $\alpha$ -methylbenzyl isocyanate, yielding a single diastereomer of methyl[ $\alpha$ -methylbenzylcarbamoyl]-4-(3-pyridyl) butanoate (MMPB, **35**). The HPLC retention time, UV, and MS of this diastereomer were compared to those of the previously characterized (*R,S*)-MMPB and (*S,S*)-MMPB (**35**) (22, 23). As shown in Figure 4, standard (*R,S*)-MMPB elutes before (*S,S*)-MMPB. The diastereomer formed from this enantiomer of lactol **27** co-eluted with the first eluting standard, and was therefore assigned as (*R,S*)-MMPB, formed from 5-(*R*)-lactol **27**.

Each chiral lactol **27**, of known absolute configuration, was allowed to react with dGuo as shown in Scheme 6, giving adducts **16** with known absolute configuration at the 5-position of the tetrahydrofuran ring. Using these standards and optimized conditions for separation, peaks 1 and 3 of Figure 5 corresponded to the 5-(*S*)-diastereomers while peaks 2 and 4 corresponded to the 5-(*R*)-diastereomers. Peaks 1 and 3 and peaks 2 and 4 were interconvertible. Thus, collection of peak 1 from HPLC system 1 and re-injection within 2 h at room temperature produced a mixture of peaks 1 and 3. Similarly, collected peak 2, when re-injected, gave a mixture of peaks 2 and 4. The corresponding peaks in Figure 1 behaved similarly. These results are consistent with the equilibrium involving Schiff base **17**, as shown in Scheme 2, and impedes determination of the absolute configuration at the 2-position of adduct **16**.

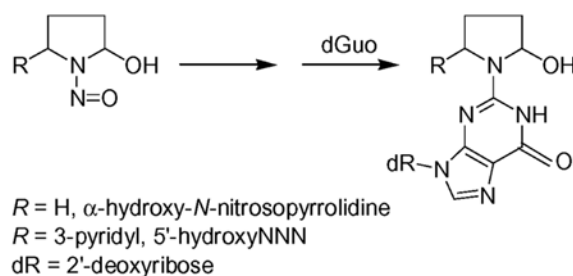
The formation of adducts **12** and **16** in DNA reacted with 5'-acetoxyNNN was then investigated. The DNA was treated with NaBH<sub>3</sub>CN and enzymatically hydrolyzed. The hydrolysates were analyzed by LC-ESI-MS/MS-SRM. As shown in Figure 6, clear peaks were observed in the chromatograms corresponding to **14** and **18**, the reduced forms of adducts **12** and **16**, respectively.

## Discussion

This paper reports the first structural characterization of dGuo and DNA adducts formed from NNN 5'-hydroxylation, a known major metabolic pathway of this tobacco-specific carcinogen in primates and humans. Adducts **12** and **16** are formed by reactions of intermediates derived from NNN 5'-hydroxylation- oxonium ions, carbocations, or related species- with the exocyclic amino group of dGuo. Since adducts **12** and **16** introduce a relatively bulky residue into the base pairing region of DNA, it appears likely that they will lead to miscoding or related negative consequences during DNA replication. Such changes are the critical initial steps in the cascade of events leading to tumor formation.

The absolute configurations of the pyridyl-substituted carbon atom in adduct **16** and in **14**, the reduced form of adduct **12**, were determined in this study. In the case of **16**, this was accomplished by establishing the absolute configuration of the pyridyl-substituted carbon in lactol **27**, by conversion to MMPB (**35**) of known configuration, followed by reaction of **27** with dGuo. For **14**, the absolute configuration of the pyridyl-substituted carbon was determined by reaction of chiral nornicotine with protected 2-fluorinosine **23**. While few previous studies of nitrosamine-DNA adducts have elaborated absolute stereochemistry (16,25,26), this has been an area of extensive research in the study of DNA adducts of polycyclic aromatic hydrocarbons (27–29). The results of those studies clearly demonstrate that the absolute stereochemistry of DNA adducts is critical in determining their mutagenic and carcinogenic effects. NNN itself is a chiral molecule, and it is known that (*S*)-NNN is more prevalent in tobacco products than (*R*)-NNN (30). Further, there are clear differences in cytochrome P450-catalyzed 5'-hydroxylation of the NNN enantiomers (31) as well as in their metabolism in vitro and in vivo in rats (32). Thus, the stereochemical assignments determined here will be important in elucidating the relationship between exposure to NNN enantiomers, their metabolism, and consequent DNA adduct formation.

The structures of adducts **12** and **16** are consistent with observations in our previous studies of *N*-nitrosopyrrolidine, in which the corresponding adducts, lacking the pyridine ring, were identified in reactions with dGuo and DNA of  $\alpha$ -acetoxy-*N*-nitrosopyrrolidine (16). Adduct **12** and the corresponding adduct of  $\alpha$ -acetoxy-*N*-nitrosopyrrolidine are structurally interesting because they represent the formal replacement of the *N*-nitroso- group of the  $\alpha$ -hydroxy metabolite with deoxyinosine (although the ring nitrogen in the adduct is provided by dGuo). While adducts such as these which are in equilibrium with Schiff bases can be unstable at the nucleoside level, some studies show that Schiff base adducts are quite stable in DNA (25,33). The chemical stability in DNA and persistence of the adducts isolated here will be evaluated in future studies.



The relative importance of 2'-hydroxylation versus 5'-hydroxylation of NNN has been discussed previously (4). Studies in rats favor 2'-hydroxylation as the major metabolic activation pathway of NNN. Thus, 2'-hydroxylation exceeds 5'-hydroxylation in target tissues of NNN in the rat- esophagus and nasal mucosa- while in liver, a non-target tissue, 5'-hydroxylation exceeds 2'-hydroxylation. Hemoglobin and DNA adducts resulting from 2'-hydroxylation have been detected in NNN-treated rats. However, in mouse lung and hamster trachea, target tissues of NNN carcinogenicity, 5'-hydroxylation exceeds or is comparable to 2'-hydroxylation. 5'-Hydroxylation of NNN exceeds 2'-hydroxylation in human liver and in several other human tissues. The relative importance of these two pathways can be more definitively established by determining the levels, persistence, and miscoding potential of specific NNN-DNA adducts. The results presented here are an important step in that direction.

LC-ESI-MS/MS was critical for the isolation and identification of adducts **12** and **16**. Reactions of 5'-acetoxyNNN with dGuo and DNA proceed in low yield and it is difficult to identify the products using LC with UV detection because they are overwhelmed by even minor solvolysis

products of 5'-acetoxyNNN, all of which have UV absorption of similar intensities to those of the dGuo adducts. LC-ESI-MS/MS on the other hand provides both the required sensitivity and specificity. As currently available instruments routinely have detection limits for DNA adducts in the amol range, it seems likely that we will be able to investigate the presence of adducts **12** and **16** in DNA from tissues of people who use tobacco products and are exposed to several micrograms of NNN daily (7,34). We envision development of a comprehensive MS method for analysis of DNA adducts of both NNN and NNK. Adducts **12** and **16** will be important in this analysis, as they are the only adducts known to date which specifically arise from NNN. All other known adducts can be formed by both methyl hydroxylation of NNK and 2'-hydroxylation of NNN (9,11).

In summary, DNA adducts resulting from the major pathway of NNN metabolism observed in human tissues- 5'-hydroxylation- were characterized for the first time, and the absolute configurations of the pyridyl-substituted carbons were determined. Adducts **12** and **16** are formed by reaction of intermediates derived from NNN 5'-hydroxylation- oxonium ions, carbocations, or related species- with the exocyclic amino group of dGuo. These DNA adducts, which are unique to the tobacco-specific carcinogen NNN, will be important probes for understanding mechanisms of cancer induction by NNN and as potential biomarkers for metabolic activation of NNN in people who use tobacco products.

#### Acknowledgements

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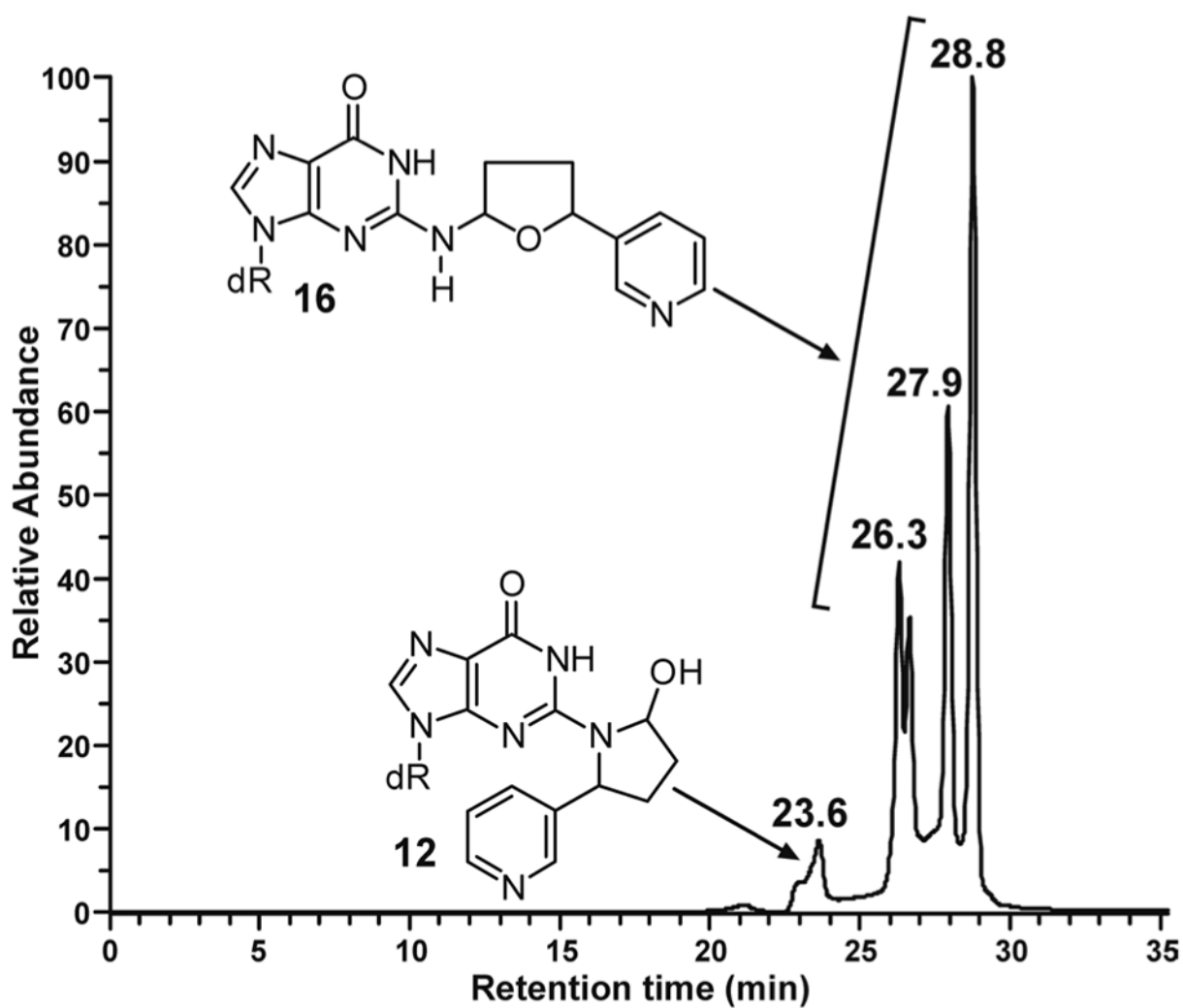
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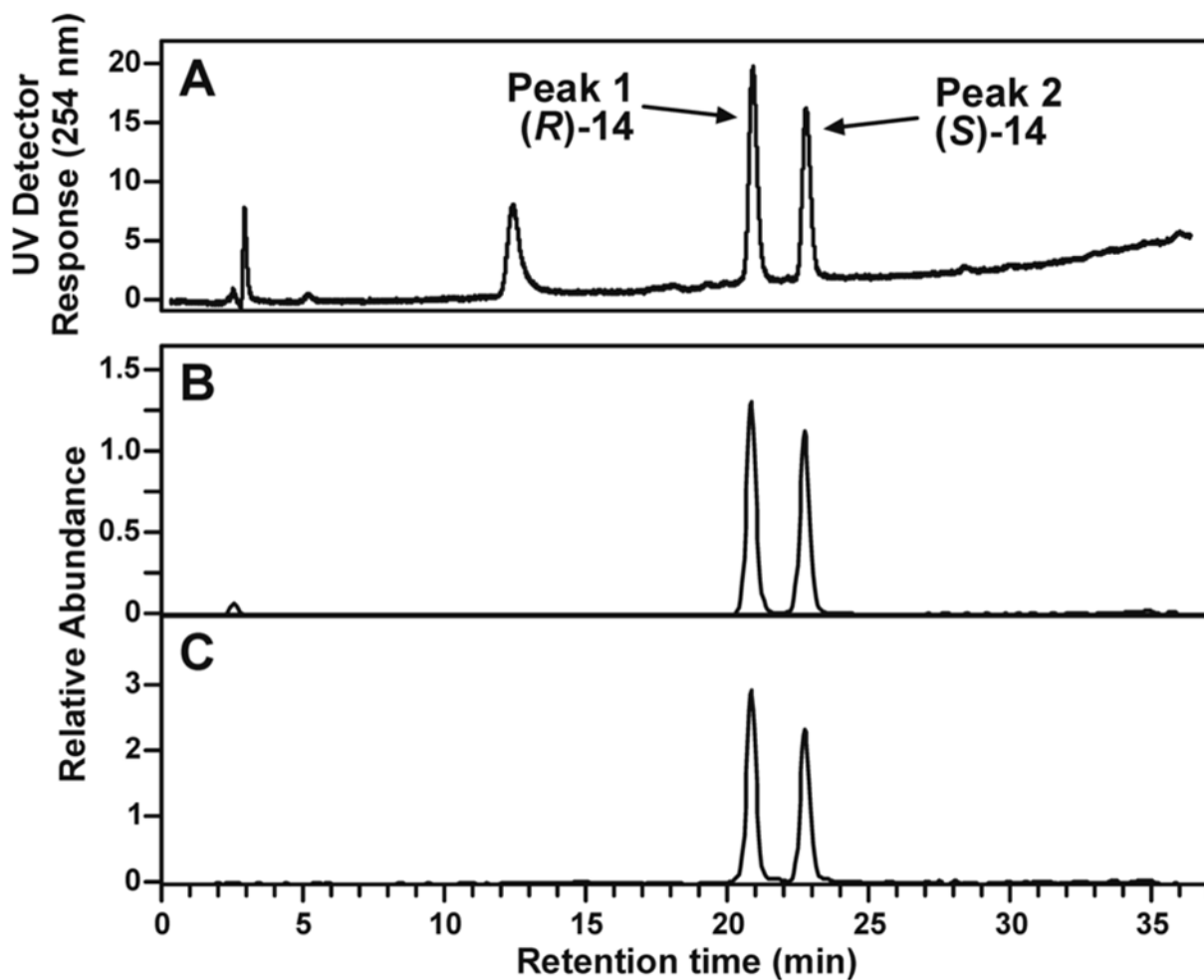
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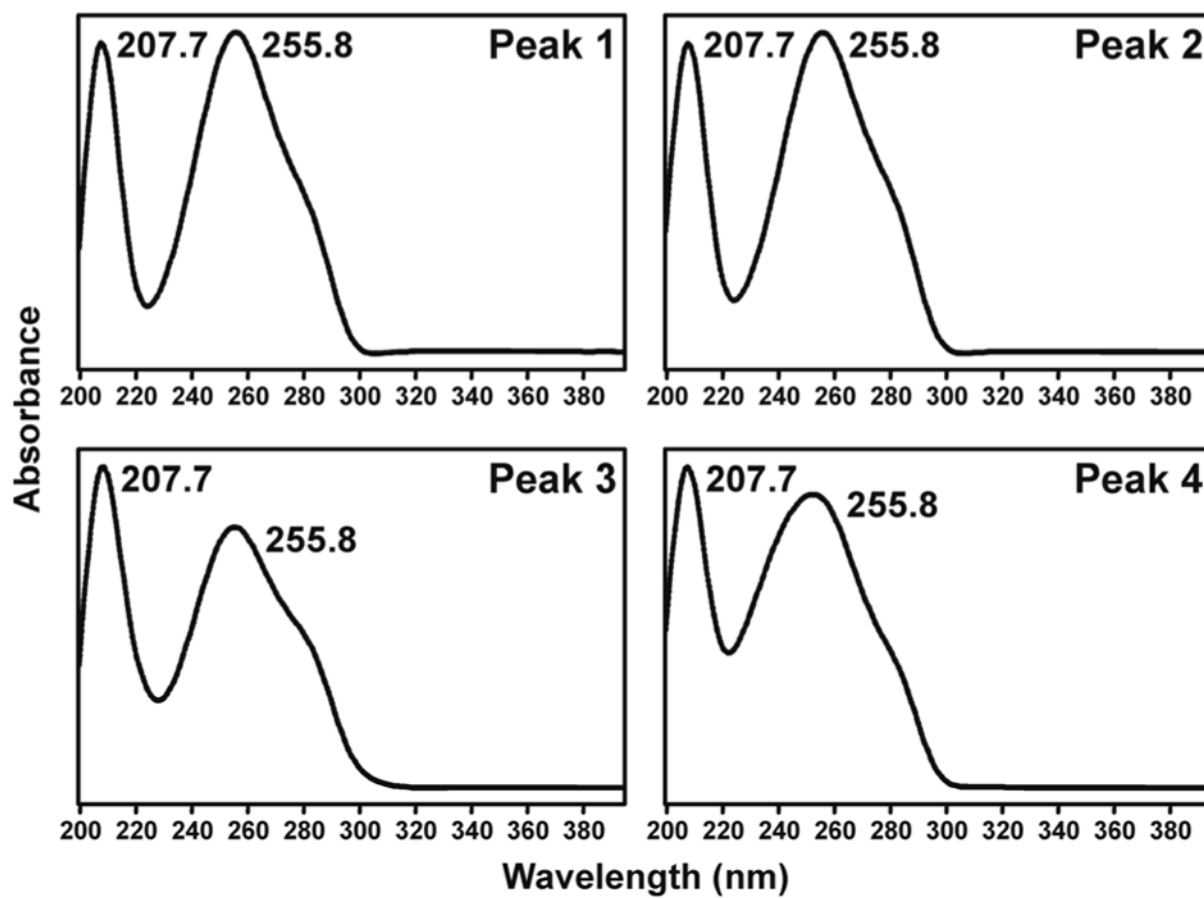
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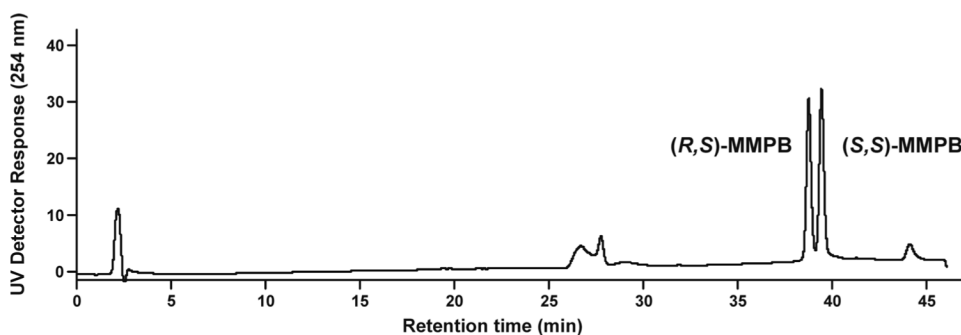
**Figure 1.** Chromatogram obtained upon LC-ESI-MS/MS-SRM analysis, at  $m/z$  415→299 (system 7), of the products of reaction of 5'-acetoxyNNN (**4**) and dGuo



**Figure 2.** LC-ESI-MS/MS chromatograms (system 5) obtained upon analysis of the reaction of adduct **12** with  $\text{NaBH}_3\text{CN}$ : A, detection by UV at 254 nm; B, extracted ion chromatogram of  $m/z$  399 ( $M + H$ )<sup>+</sup>; C, extracted ion chromatogram of  $m/z$  399  $\rightarrow$  283 [ $M\text{-deoxyribose}$ ] +  $H$ )<sup>+</sup>



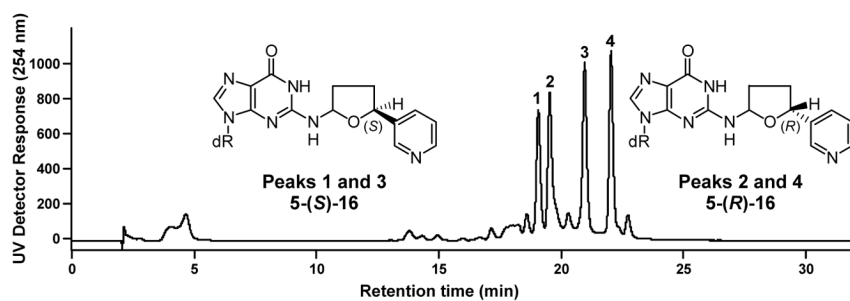
**Figure 3.**  
UV spectra of 4 diastereomers of adduct **16**, corresponding to the 4 peaks eluting from 26.3–28.8 min in Figure 1



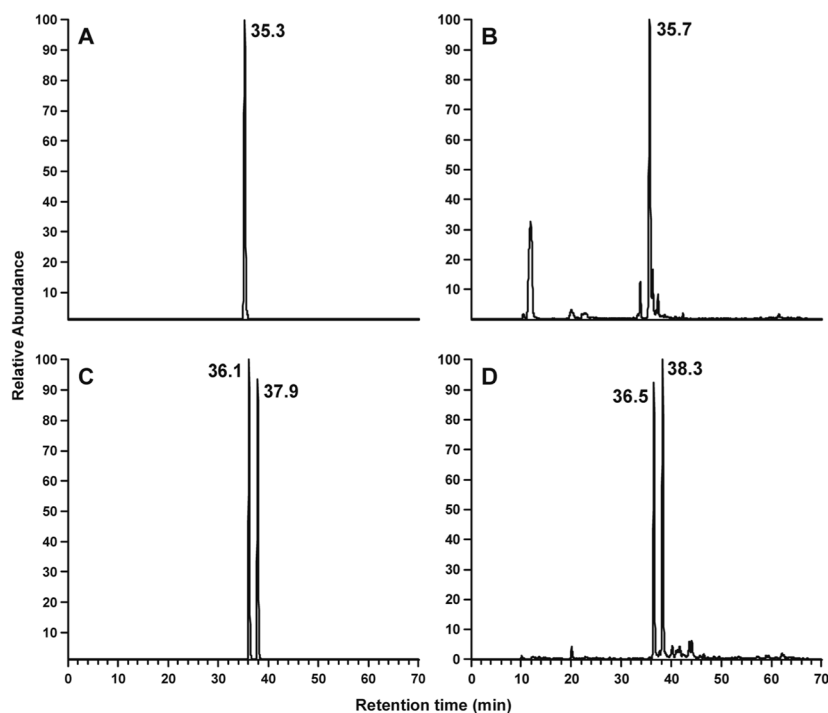
**Figure 4.**

Chromatogram obtained upon HPLC analysis (using the in-line UV detector of LC-ESI-MS system 5) of standard (*R,S*)- and (*S,S*)-MMPB (**35**). (Note: the assignments of (*R,S*)-MMPB and (*S,S*)-MMPB in references (22,23) are reversed because they are based on the incorrect assignment of the absolute configuration of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (20,35))

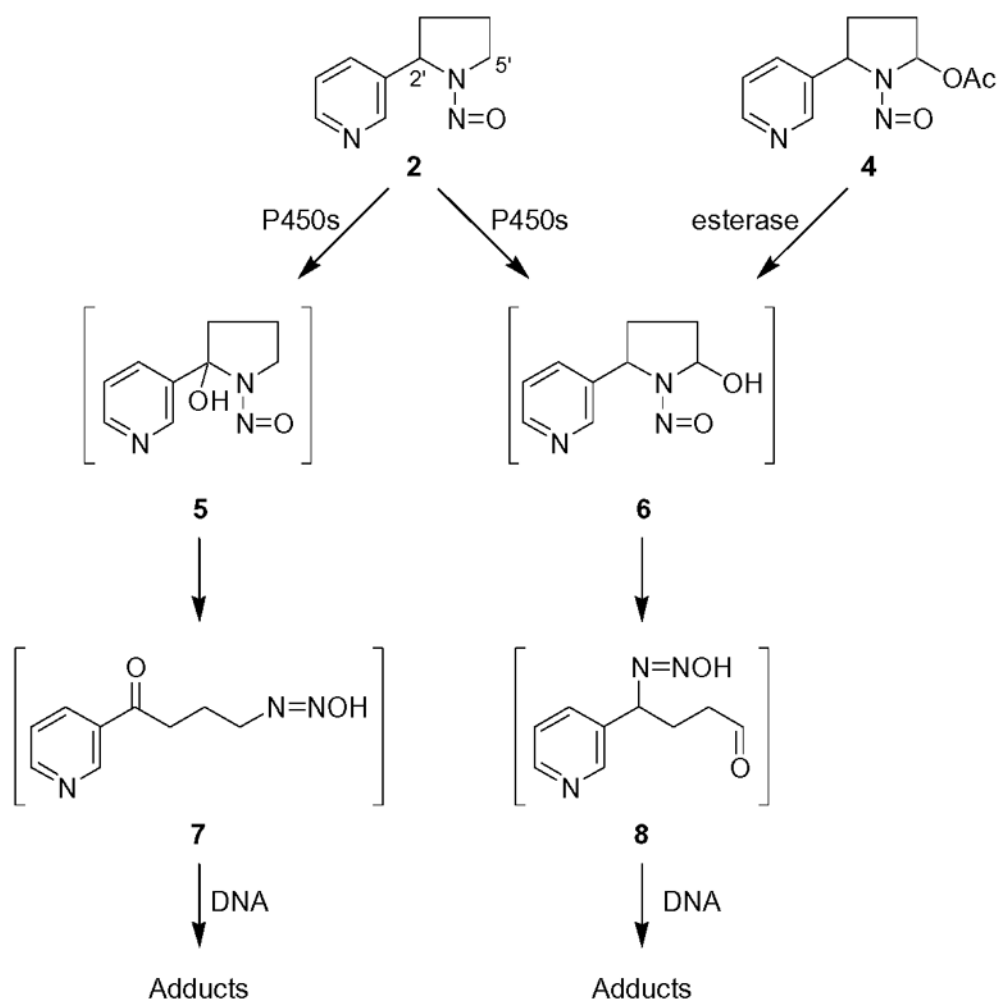




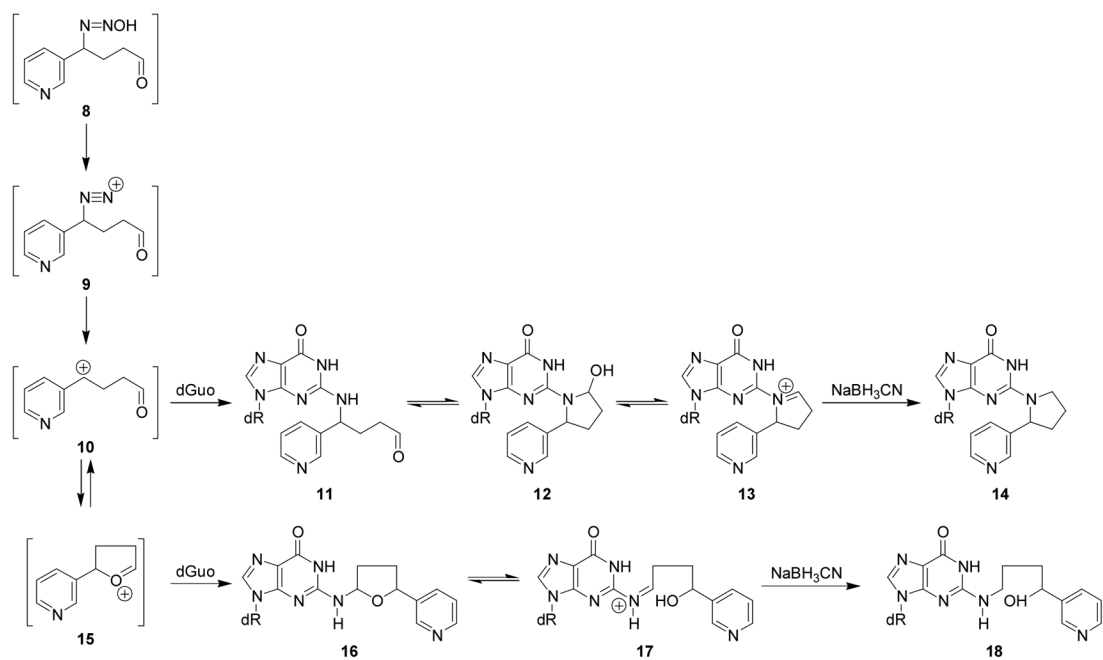
**Figure 5.** Chromatogram obtained upon HPLC analysis (using the in-line UV detector of LC-ESI-MS system 5) of adduct **16**.

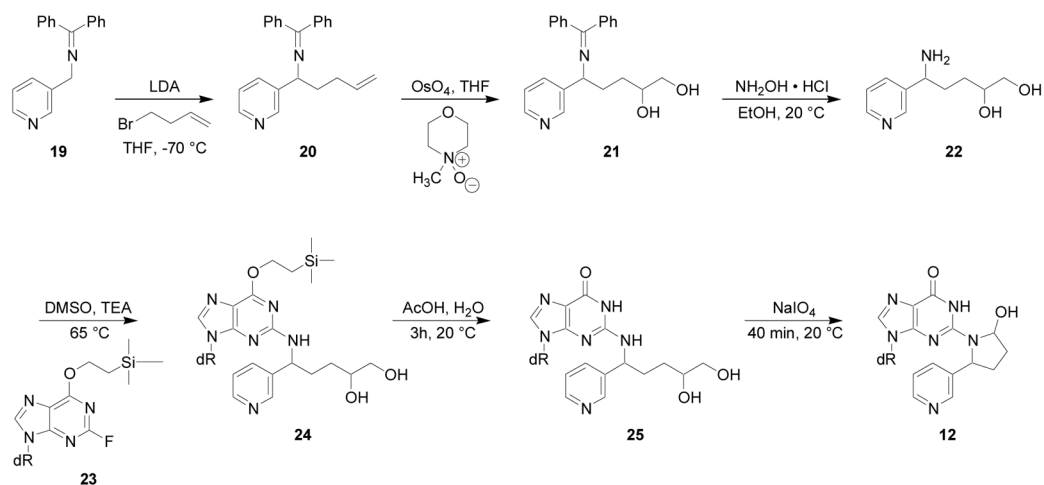


**Figure 6.** Chromatograms obtained upon LC-ESI-MS/MS-SRM analysis (system 7) of A, standard adduct **18**,  $m/z$  417→301; C, standard adduct **14**,  $m/z$  399→283; enzymatic hydrolysates of DNA that had been reacted with 5'-acetoxyNNN and treated with  $\text{NaBH}_3\text{CN}$ , analyzed for B,  $m/z$  417→301 (adduct **18**) and D,  $m/z$  399→283 (adduct **14**)

**Scheme 1.**

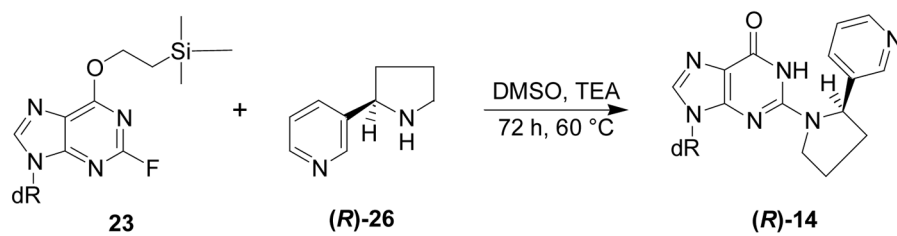
Metabolic activation of NNN (2) by cytochrome P450s and esterase catalyzed hydrolysis of 5'-acetoxyNNN (4)

**Scheme 2.**Adduct formation from diazohydroxide **8** and derived intermediates

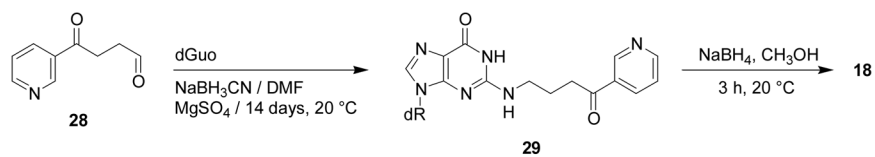


**Scheme 3.**  
Synthesis of adduct **12**

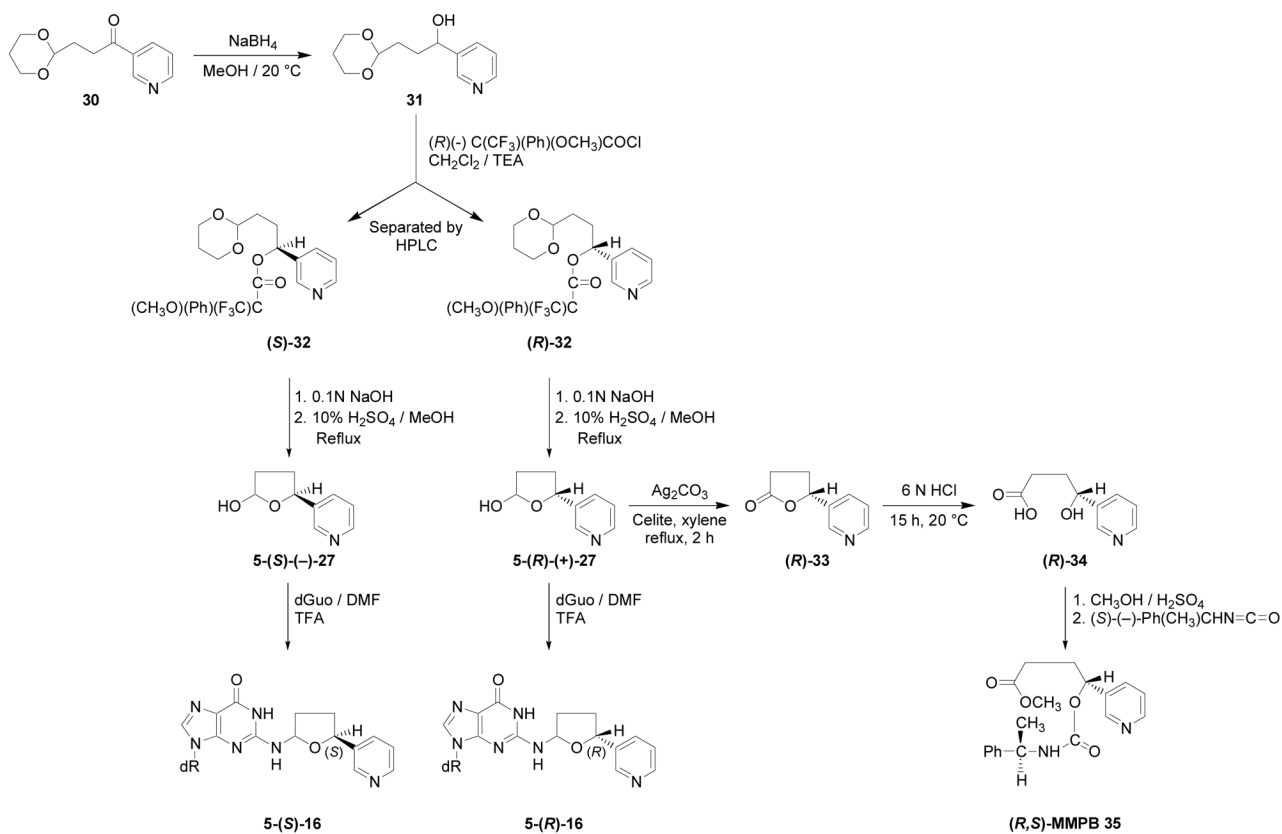




**Scheme 4.**  
Synthesis of (R)-14



**Scheme 5.**  
Synthesis of adduct **18**



**Scheme 6.**  
Synthesis and identification of diastereomers of **16**