Reactions with Deoxyguanosine of 4-(Carbethoxynitrosamino)-1-(3-pyridyl)-1-butanone, a Model Compound for α -Hydroxylation of Tobacco-Specific Nitrosamines^{1,2}

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Abstract: 4-(3-Pyridyl)-4-oxobutanediazohydroxide (10) is a likely product of metabolic α -hydroxylation of the tobacco nitrosamines N'-nitrosonornicotine (2) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (1). The reactions of 4-(carbethoxynitrosamino)-1-(3-pyridyl)-1-butanone (12), a stable precursor to 10, with deoxyguanosine and H_2O at pH 8 were investigated. Three products of the reaction of 12 with deoxyguanosine were characterized as 2'-deoxy-N-[1-methyl-3-oxo-3-(3-pyridyl)propyl]guanosine (18), 2'-deoxyguanosine 3'-(ethyl carbonate), and 2'-deoxyguanosine 5'-(ethyl carbonate). The adduct 18 was also formed upon reaction of 1-(3-pyridyl)-2-buten-1-one (17) with deoxyguanosine. Whereas the branched chain adduct 18 was the major product formed upon reaction of 12 with deoxyguanosine, the solvolysis of 12 gave mainly the nonrearranged keto alcohol 4-hydroxy-1-(3-pyridyl)-1-butanone (21), with lesser amounts of 3-hydroxy-1-(3-pyridyl)-1-butanone (22) and 17. Possible mechanisms for the formation of 18 are discussed.

Nicotine can be nitrosated to form 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK, 1) and N'-nitrosonornicotine $(NNN, 2).^{3}$ These nitrosamines are present in tobacco and tobacco smoke in relatively high concentrations and are strong carcinogens in mice, rats, and hamsters.⁴ They are likely to be causative factors in tobacco-related cancer in man.4-6

Modification of DNA is considered to be important in initiation of carcinogenesis. Compounds 1 and 2 do not react directly with DNA but are metabolized in vitro and in vivo to intermediates which do react with DNA.^{7,8} Metabolic methylene hydroxylation of 1 gives the unstable α -hydroxynitrosamine 3, which spontaneously decomposes yielding keto aldehyde 7 and the unstable electrophile methanediazohydroxide (8, Scheme I).^{4,8} Methanediazohydroxide reacts with DNA, yielding 7-methylguanine and O^6 -methylguanine which have been detected in the liver, lung, and nasal mucosa of rats treated with 1.⁹ The analogous pathways of α -hydroxylation at the methyl carbon of 1 or the 2'- or 5'carbons of 2 would yield α -hydroxynitrosamines 4-6. These, in turn, are expected to give diazohydroxides 10 and 11. Solvolyses of the acetates of 5 and 6 support this assumption.¹⁰ The structures of the adducts formed upon reaction of 10 and 11 with DNA are not known. Such adducts should be formed in animals or humans exposed to 1 and 2. Therefore, we have carried out studies aimed at elucidating their structures. Our initial investigations have focused on diazohydroxide 10 since it can be formed from either 1 or 2. We have used 4-(carbethoxynitrosamino)-1-(3-pyridyl)-1-butanone (12) as a stable precursor to 10. Since deoxyguanosine is often the most reactive of the DNA bases toward electrophiles, we have studied its reaction with 10 under mildly basic conditions.

Results and Discussion

The nitrosourethane 12 was allowed to react with $[^{14}C]$ deoxyguanosine for 4 days at 37 °C in pH 8 phosphate buffer. Analysis by TLC showed that 12 had reacted completely. Analysis

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of the mixture by HPLC-scintillation counting gave the radiochromatogram illustrated in Figure 1. In addition to unreacted $[^{14}C]$ deoxyguanosine which eluted at 32 min, three major radioactive peaks, I-III, were detected. Their UV spectra are illustrated in Figure 2. These spectra were similar to those of the diastereomeric adducts 15 and 16 which we had previously characterized as the major products formed upon reaction of nitrosourethane 13 or crotonaldehyde (14) with deoxyguanosine.¹¹ The spectra suggested a 1- and/or N^2 -substitution pattern and excluded reaction at the 7- or O⁶-position of deoxyguanosine.



Therefore, we suspected that they might have similar structures to those of 15 and 16 and that larger quantities, sufficient for further characterization, might be obtained by reaction of ketone 17 with deoxyguanosine. The unstable ketone 17 was synthesized



by reaction of pyridine-3-carboxaldehyde with allylmagnesium

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Scheme I

DPM



RETENTION TIME (min)





Figure 2. UV spectra of A, peak I, B, peak II, and C, peak III, of Figure 1 at pH 7 (-), 1 (---), and 13 (...).

bromide, followed by oxidation with CrO_3 in H_2SO_4 . It was purified and identified by its NMR and MS. It is presumed to be predominantly trans. Compound 17 was allowed to react with [14C]deoxyguanosine under conditions identical with those used for reaction of 12 with [14C]deoxyguanosine. Only one product other than [14C] deoxyguanosine was detected, and it eluted at the same position as peak II of Figure 1. Its UV spectrum was identical with that of peak II.

The 500-MHz PMR spectrum of the product together with the UV data cited above provides strong evidence that the structure of the adduct is 2'-deoxy-N-[1-methyl-3-oxo-3-(3-pyridyl)propyl]guanosine (18), as a mixture of two diastereomers. Several features of the PMR spectrum are noteworthy. The methyl resonances appear as two doublets at 1.27 and 1.28 ppm, corre-

Figure 3. Analysis by HPLC of the products formed by reduction of 18

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sponding to the two diastereomers; the diastereomeric 1'-protons and C-8 protons are also resolved. The exchangeable N²-H and 1-NH protons appeared at 6.6 and 10.5 ppm, respectively, which is in agreement with the proposed N²-substitution pattern. These data alone would not exclude a cyclic 1,N²-adduct analogous to 15 and 16. However the position of the 2-hydrogen of the pyridine ring at 9.13 ppm is consistent with 18 rather than the cyclic structure. Pyridines substituted at the 3-position with a carbonyl group show a downfield shift of the 2-hydrogen from 8.5-8.7 ppm, where it is observed when the 3-position is substituted by -CHOH or -CH₂, to 9.1-9.4 ppm, as in 18. The relatively downfield position, 3.2 ppm, of the protons on the carbon adjacent to the carbonyl are also in agreement with structure 18. The protons at position 7 of 15 or 16 are observed at 1.4 and 2.0 ppm.

The FAB spectrum of 18 showed an M + 1 peak at m/e 415and a peak at 437.15593, corresponding to C₁₉H₂₂N₆O₅Na (calculated, 437.15496).

We then sought further evidence for the identity of peak II of Figure 1, formed in the reaction of 12 with deoxyguanosine, with the identified diastereomers 18, formed in the reaction of 17 with deoxyguanosine. HPLC with UV detection allowed partial separation of the two diastereomers. The retention times of 18 formed in the two reactions were identical, but the ratios of the two peaks were slightly different: 48:52 from 12 and 54:46 from 17. Reduction of 18 with NaBH₄ would be expected to give four diastereomeric alcohols since a new asymmetric center is created. As illustrated in Figure 3, four peaks were observed upon reduction of the diastereomers obtained from 17. From the ratios of the peak heights, it is probable that peaks A and C of Figure 3 are formed from the minor diastereomer and peaks B and D from the major diastereomer of 18, as obtained from reaction of 17 with deoxyguanosine. Reduction of 18, obtained from the reaction of



12 with deoxyguanosine, gave the same four peaks as illustrated in Figure 3 except that the relative proportions of the peaks corresponded to the 48:52 ratio of the diastereomers of 18 in this mixture. These results provide further confirmation of the identities of the diastereomeric adducts 18 formed by reaction of deoxyguanosine with either 12 or 17. Inspection of space-filling models suggests that approach of borohydride from one side of each of the diastereomers is hindered. This could explain the preferential formation of peaks A and B from each of the two diastereomers.

The NMR spectra of peaks I and III of Figure 1 showed no pyridine protons but did display resonances corresponding to $-CH_3$ and $-CH_2$ of a $-CO_2CH_2CH_3$ group. By comparison of their spectra to that of deoxyguanosine, the structures of peaks I and III were tentatively assigned as 2'-deoxyguanosine 5'-(ethyl carbonate) and 2'-deoxyguanosine 3'-(ethyl carbonate), respectively. Their identities were not further investigated.

Thus, the major pyridine-containing adduct produced by reaction of 12 with deoxyguanosine at pH 8 was 18. To examine the basis for formation of this rearranged product, we reinvestigated the solvolysis products of 12, formed in the same reaction from which 18 was isolated. As in our previous study,¹⁰ the major product was the nonrearranged keto alcohol 21 (66%). The rearranged keto alcohol 22 was produced in 7% yield and the ketone 17 in 2% yield. The formation of these products is rationalized in Scheme II. The nonrearranged keto alcohol 21 can be formed either by solvolysis of diazohydroxide 10 or via carbonium ion 20; the latter can rearrange to 19 which reacts with solvent, yielding 22, or eliminates to give 17. The formation of the nonrearranged keto alcohol 21 as a major product in the solvolysis of 12 contrasts to the formation of the rearranged adduct 18 in the reaction of 12 with deoxyguanosine. Although 18 appears to be the major deoxyguanosine adduct, we cannot exclude the possibility that some nonrearranged adduct is also produced.

The simplest explanation for the apparent preferential formation of the rearranged adduct 18 is that deoxyguanosine reacted with the small amount of 17 produced in solvolysis of 12 (Scheme II). However, considering the 2% yield of 17 from 12, and the 30% yield in reaction of 17 with deoxyguanosine, this may not account for 18, which was formed in 7% yield from 12. Alternatively, 18 could be produced by selective reaction of the carbonium ion 19 with deoxyguanosine. However, preferential reaction of deoxyguanosine with 19, as opposed to 20, seems unlikely. Another proposal for selective formation of the rearranged adduct 18 is presented in Scheme III. The carbonyl group of diazohydroxide 10 or nitrosourethane 12 may react with N-1 of deoxyguanosine, yielding intermediate 23. This postulate is based on our studies with nitrosourethane 13, in which the aldehyde carbonyl group Scheme III



has a strong directing effect, resulting in preferential formation of N-1,N² cyclic adducts, rather than the 7-alkylated products which are generally formed from alkanediazohydroxides. In the case of 10 or 12, the carbonyl group is a ketone and is less reactive than in 13. Thus, we expect that the equilibrium would not favor 23. Hydrolysis of 23 (R = CO₂Et) or spontaneous decomposition (R = H) would give 24. Rearrangement to 25 would be favored for production of the six-membered ring in 26. Compound 26 is analogous to the adducts 15 and 16 formed upon reaction of 13 with deoxyguanosine. As in 23, the ring-opened tautomer 18 would be favored due to the relative stability of the carbonyl group of 18. Scheme III would suggest that some 26 might be present in the reaction mixture; we did not detect it in this study.

We are presently using 18 as a marker for studies of in vivo DNA adduct formation from tritiated NNN and NNK, with the label in the pyridine ring.

Experimental Section

PMR spectra (90MHz) were determined with a Jeol Model FX90Q spectrometer; 500-MHz spectra were kindly provided by Dr. Dwight Miller of the National Center for Toxicological Research, Jefferson, AR. All spectra are reported as parts per million (ppm) downfield from Me4Si as the internal reference. UV spectra were determined with a Cary Model 118 instrument. MS were run with a Hewlett-Packard Model 5982A dual-source instrument. High-resolution FAB MS were kindly provided by Dr. Pamela Crain, Department of Medicinal Chemistry, University of Utah, using a Varian MAT 731 instrument operated at 8 kV with fast-atom bombardment, 6 keV of Xe. Conventional high-resolution MS were obtained at the Rockefeller University Mass Spectrometric Biotechnology Resource. HPLC was carried out with a Waters Assoc. Model ALC/GPC-204 high-speed liquid chromatograph equipped with a Model 660 solvent programmer and a Model LC-25 UV/visible detector. Scintillation counting was performed with a Beckman LS-9000 Liquid Scintillation System. GC was performed with a Hewlett-Packard Model 5830A instrument.

4-(Carbethoxynitrosamino)-1-(3-pyridyl)-1-butanone (12) and 4hydroxy-1-(3-pyridyl)-1-butanone (21) were prepared as described previously.^{10,12} [¹⁴C]Deoxyguanosine (56 mCi/mmol) was procured from Moravek Biochemicals, Brea, CA. Deoxyguanosine, 3-pyridinecarboxaldehyde, allylmagnesium bromide, 3-acetylpyridine, and acetaldehyde were obtained from Aldrich Chemical Co., Milwaukee, WI. Regisil RC-2 [bis(trimethylsilyl)trifluoroacetamide plus 1% trimethylchlorosilane] was obtained from Regis Chemical Co., Morton Grove, IL.

1-(3-Pyridyl)-2-buten-1-one (17). To a solution of 3-pyridinecarboxaldehyde (5.35 g, 0.05 mol) in 100 mL of anhydrous ether was added ally lmagnesium bromide (0.05 mol, 1 M in ether). The addition was carried out under a stream of N₂, dropwise at -5 °C over a 20-min period. After the addition was complete, 20 mL of saturated NH₄Cl was gradually added while the temperature was maintained at 0 °C. After 20 min of stirring, the mixture was extracted 4 times with 40-mL portions of ether. The combined ether extracts were dried (Na₂SO₄) and concentrated, giving 5.2 g of a brownish-red oil. The crude product was purified by chromatography on a column of basic alumina with elution by 1.5% MeOH in CH₂Cl₂, yielding a yellow oil, 3.1 g, 42%: NMR (CDCl₃) δ 8.8 (m, 2, pyr-2H, 6 H), 7.7 (d, pyr-4H), 7.2 (m, pyr-5H), 5.7 (m, 1, CH₂-CH=CH₂), 5.1 (d, 2, =CH₂), 4.7 (t, 1, CHOH), 4.6 (s, 1, CHOH), 2.5 (dd, 2, $-CH_2-$); MS, m/e (rel intensity) 108 (100), 85 (37), 83 (59), 80 (46). This material (2 g, 0.013 mol) was used without further purification. It was dissolved in 19 mL of acetone, and 3 g (0.03 mol) of CrO₃ in 63 mL of 10 N H₂SO₄ was added dropwise at 0 °C. After stirring at 0 °C for 3.5 h, the mixture was washed with hexane, neutralized with NaHCO₃, and extracted with ether (3×25) mL) and CH_2Cl_2 (4 × 30 mL). The combined organic layers were dried (Na₂SO₄) and concentrated to give 1.6 g of crude product containing 17. It was purified by column chromatography on silica gel with elution by 0.5% MeOH in CHCl₃, giving 0.7 g (10%) of 17, which darkens rapidly on standing in air: NMR (CDCl₃) δ 9.4-9.0 (br s, 1, pyr-2H), 9.0-8.7 (m, 1, pyr-6H), 8.25 (d, 1, pyr-4H), 7.7-7.0 (m, 3, pyr-5H, CH=CH), 2.05 (d, 3, CH₃); MS, m/e (rel intensity) 147 (32), 146 (39), 145 (21), 132 (15), 118 (18), 106 (28), 89 (15), 78 (100), 52 (25), 51 (45), 50 (37); high-resolution MS calcd for C₉H₉NO 147.0684, found 147.0710.

3-Hydroxy-1-(3-pyridyl)-1-butanone (22). A mixture of 3-acetylpyridine (5.0 g, 0.04 mol) and acetaldehyde (1.8 g, 0.04 mol) was added at 0 °C to a stirred suspension of NaOCH₃ (2.3 g, 0.04 mol) in 50 mL of toluene. After 3 min, the ice bath was removed and solid formed. Stirring was continued for 30 min at room temperature. After addition of H₂O, the mixture was extracted twice with Et₂O and twice with CH₂Cl₂. The combined extracts were dried and concentrated, giving a residue which was purified by silica gel chromatography with elution by 0.5% MeOH in CH₂Cl₂. Fractions containing **22** were detected by TLC on silica ($R_f = 0.28$, 15/1; CHCl₃/MeOH) and were repurified by preparative TLC. The R_f of **21** under these conditions is 0.20: NMR (CDCl₃) δ 9.05 (br s, 1, pyr-2H), 8.68 (m, 1, pyr-6H), 8.12 (m, 1, pyr-4H), 7.32 (m, 1, pyr-5H), 4.35 (sextuplet, 1, CH₂CH(OH)CH₃), 3.44 (brs, 1, OH), 3.03 (d, 2, CH₂CH(OH)CH₃), 1.21 (d, 3, CH₂CH-(OH)CH₃); MS, m/e (rel intensity) 165 (M⁺, 5.9), 150 (17.5), 148 (11.1), 121 (29.1), 106 (100), 78 (37.9); high-resolution MS calcd for C₉H₁/NO₂ 165.0786, found 165.0789.

Reaction of 4-(Carbethoxynitrosamino)-1-(3-pyridyl)-1-butanone (12) with Deoxyguanosine. A mixture of 12 (0.7 g, 2.9 mmol), [14C]deoxyguanosine (5 μ Ci, 0.27 g, 1 mmol) and pH 8 sodium phosphate buffer (53 mL, 0.1 M) was incubated in a shaking water bath at 37 °C for 4 days. The mixture was analyzed by HPLC using two Waters μ -Bondapak-C₁₈ columns in series with a gradient from 0 to 50% MeOH/H₂O in 100 min at 1 mL/min. Fractions were collected for scintillation counting; the radiochromatogram is illustrated in Figure 1. Yields of peaks I-III (percent of deoxyguanosine) were I, 4, II, 7, III, 2. The reaction mixture was concentrated under reduced pressure and the residue partially purified by column chromatography on silica gel with elution by 5-10% MeOH in CH₂Cl₂. Peaks I-III of Figure 1 were then isolated by HPLC and characterized by their spectral properties. These are described in the text and below for peak II (18): PMR (90 MHz) of peak I (Me₂SO- d_6) δ 10.6 (s, 1, 1-NH), 7.80 (s, 1, 8-H), 6.4 (s, 2, NH₂), 6.15 (t, 1, 1'-H), 5.45 (d, 1, 3'-OH), 4.4-3.9 (m, 7, CO₂CH₂CH₃ + 5'-CH₂ + 4'-H + 3'-H), 2.8-2.2 (m, 2, 2'-H), 1.20 (t, 3, CO₂CH₂,CH₃); PMR (500 MHz) of peak III (Me₂SO-d₆) δ 10.65 (s, 1, 1-NH), 7.95 (s, 1, 8-H), 6.48 (s, 2, NH₂), 5.25 (d, 1, 3'-H), 5.16 (t, 1, 5'-OH), 4.18 (q, 2, CO₂CH₂CH₃), 4.07 (t, 1, 4'-H), 3.60 (m, 2, 5'-CH₂), 2.81 (m, 1, 2'- β H), 2.5 (m, 1, 2'- α H), 1.25 (t, 3, CO₂CH₂, CH₃).

In a separate experiment, 12 was allowed to react with deoxyguanosine under conditions identical with those described above. The mixture was analyzed for 17 by HPLC using the conditions described above. The retention time of 17 was 104 min. The structure of 17 was confirmed by GC-MS of the collected HPLC peak, using a 25 m \times 0.25 mm SE-54 fused silica capillary column, programmed from 30 to 200 °C at 4 °C/min in the splitless mode, at a flow rate of 1 mL/min of He. The retention time of 17 was 25.8 min. For quantitation of 21 and 22, the reaction mixture was extracted 4 times with EtOAc. An aliquot was dried and concentrated and the residue silvlated with Regisil-RC-2. The resulting mixture was analyzed by GC using a 10 m \times 0.25 mm crosslinked methylsilicone-fused silica capillary column, programmed from 80 to 220 °C at 4 °C/min. The flow rate was 1 mL/min of He, and the split ratio was 20:1. Retention times of the trimethylsilyl ethers of 21 and 22 were 17.48 and 14.56 min, respectively. Their structures were confirmed by GC-MS.

Reaction of 1-(3-Pyridyl)-2-buten-1-one (17) with Deoxyguanosine. A mixture of 17 (0.12 g, 0.8 mmol) in 0.2 mL of Me₂SO and [^{14}C]deoxyguanosine (0.5 µCi, 0.22 g, 0.8 mmol) in pH 8 sodium phosphate buffer (27 mL, 0.1 M) was incubated with shaking for 4 days at 37 °C. The mixture was extracted with CH_2Cl_2 (5 × 30 mL), and the aqueous layer was concentrated. HPLC analysis of the aqueous layer showed that the yield of 18 was 30% based on deoxyguanosine. The residue was passed through a column of silica gel with elution by 5-10% MeOH in CH_2Cl_2 to remove most of the deoxyguanosine. The adduct 18 was isolated by HPLC on a Whatman Magnum 9, ODS-3 column with elution by a 0-50% MeOH/H₂O gradient in 100 min at 4 mL/min: PMR (500 MHz) (Me₂SO-d₆) δ 10.5 (brs, 1, 1-NH), 9.13 (m, 1, pyr-2H), 8.78 (m, 1, pyr-6H), 8.31 (m, 1, pyr-4H), 7.91 (s, 0.6, C-8H), 7.90 (s, 0.4, C-8H), 7.56 (m, 1, pyr-5H), 6.6 (brs, 1, N²-H), 6.11 (dd, J = 7, 7 Hz, 0.5, 1'-H), 6.07 (dd, J = 8, 7 Hz, 0.5, 1'-H), 5.27 (brs, 1, -OH), 4.85 (brs, 1, OH), 4.45 (m, 1, CH₃CH), 4.10 (m, 1, 3'-H), 3.79 (m, 1, 4'-H), 3.48 (m, 1, 5'-H₂), 3.23 (m, 2, CH₂C=O), 2.56 (m, 1, 2'-H), 2.18 (m, 1, 2'-H), 1.28 (d, J = 6.6 Hz, 1.5, CH_3CH), 1.27 (d, J= 6.6 Hz, 1.5, CH₁CH).

Registry No. 12, 68743-68-0; **17**, 100021-45-2; **18** (diastereomer 1), 100021-48-5; **18** (diastereomer 2), 100021-49-6; **21**, 59578-62-0; **22**, 100021-46-3; 3-pyridinecarboxaldehyde, 500-22-1; allyl bromide, 106-95-6; 3-acetylpyridine, 350-03-8; acetaldehyde, 75-07-0; deoxyguanosine, 961-07-9; 2'-deoxyguanosine 5'-(ethyl carbonate), 100021-47-4; 2'-deoxyguanosine 3'-(ethyl carbonate), 100044-90-4.

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