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THE AMINOMETHYLATION OF ADENINE, CYTOSINE AND GUANINE

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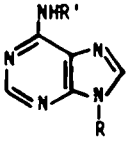
Abstract - Aminomethylated derivatives of adenine, cytosine and guanine have been isolated and characterized for the first time. These results are important because of the potential for similar adducts being formed transiently between nucleosides and nucleotides, and endogenous aldehydes and amines in vivo, and of the potential use of similar adducts for drug delivery. Mono-alkylated products obtained were from the reaction of adenine with one equivalent of aminomethylating agent derived from amines exhibiting lower basicity (e.g., morpholine and N-methylpiperazine); bis-alkylated products were obtained with agents derived from more basic amines regardless of the stoichiometry. On the other hand, only bis-alkylated products were obtained from the reaction of cytosine or guanine with the aminomethylating agent regardless of the basicity of the secondary amine used or the stoichiometry of the reaction. The mono-alkylated adenine products were alkylated on N-9 while the bis-alkylated cytosine products were alkylated on N-1 and N^4 and the bis-alkylated adenine products were alkylated on N-9 and N^6. The adenine and cytosine aminomethyl adducts hydrolyzed rapidly in dilute aqueous solution.

The reaction of formaldehyde with the primary exocyclic amino groups in purines and pyrimidines or in their corresponding nucleosides and nucleic acids has failed to yield any isolable and completely characterizable products[1,2] because the products are unstable and revert to the parent compounds in the absence of excess formaldehyde. However, a number of reports have appeared recently where the initial reaction products of formaldehyde with these primary exocyclic amino groups have been trapped by nucleophilic agents such as ethanol,[3] thiols[4,5] or bisulfite[6] to give stable products. On the other hand, no report on the isolation of aminomethyl adducts (trapping the formaldehyde adduct with secondary amines) has been published.

There are two reasons why the isolation and characterization of the aminomethyl adducts are of interest. First, although the existence of these adducts had been suggested previously,[7] they had resisted isolation because, like the formaldehyde adducts, they were unstable in aqueous solution in the absence of excess alkylating agent--formaldehyde and amine. Hence, their isolation would provide additional information about the regioselectivity of the reactions of aldehydes with purines and pyrimidines. Second, labile aminomethyl adducts of drugs containing amide and imide groups, i.e., 5-fluorouracil and theophylline,[8] have been shown to enhance the delivery of their parent drugs through skin. Thus, it was of interest to see whether similar adducts of drugs containing exocyclic amino groups (i.e., drugs based on adenine, cytosine or guanine) could be isolated, and whether the adducts would exhibit delivery enhancing properties similar to the aminomethyl adducts of amides and imides. In this paper the results obtained from the trapping of the formaldehyde adducts with adenine, cytosine, and guanine by secondary amines, and the stability of

the aminomethyl adducts will be described.

Tetranydrofuran (THF) was used as the solvent in all of the reactions. The miscibility of THF with water, its polar, yet aprotic nature, and its ease of evaporation appear to have made THF a unique solvent for these reactions because no other solvent (acetone, DMF, ether, chloroform, methanol) worked nearly as well if at all. In the case of the reactions of adenine with amines exhibiting low basicity such as morpholine (pKa 8.36)[9] and N-methylpiperazine (by analogy to piperazine pKas 5.68, 9.82),[9] if only one equivalent of secondary amine-formaldehyde[10] was used, a mono-adduct was isolated. If one equivalent of formaldehyde and the more basic amines such as piperidine (pKa 11.22),[9] and diethylamine (pKa 10.98)[9] was used, no mono-adducts were isolated from the reactions; only mixtures of adenine and bis-adducts were observed. In the case of the less basic amines the use of three to four equivalents of amine-formaldehyde were necessary to drive the reactions completely to bis-adducts, while in the case of the more basic amines, only two equivalents of the amine-formaldehyde mixture were needed.



1a R'=R=H, adenine

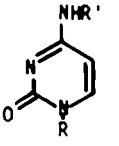
1b R'=H, R=CH_2-morpholine

1c R'=H, R=CH_2-N-NCH_3

1d R'=R=CH_2-morpholine

1e R'=R=CH_2-piperidine

1f R'=R=$CH_2N(C_2H_5)_2$



2a R'=R=H, cytosine

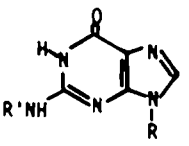
2b R'=H, R=CH_2-morpholine

2c R'=H, R=$CH_2N(C_2H_5)_2$

2d R'=R=CH_2-morpholine

2e R'=R=CH_2-piperidine

2f R'=R=$CH_2N(C_2H_5)_2$



3a R'=R=H, guanine

3b R'=R=$CH_2N(CH_3)_2$

The NMR spectra of the bis-adducts of adenine showed two different N-$C\underline{H}_2$-N absorptions. The upfield absorptions were doublets which collapsed to singlets on D_2O exchange. At the same time, triplets in each spectrum that integrated for one proton disappeared. Thus, the upfield N-$C\underline{H}_2$-N absorption was coupled to an N$\underline{H}$ absorption. The only possibility was that the upfield absorption was due to the product of the aminomethylation of the exocyclic amino group in adenine. The downfield absorption could then be due to alkylation at a number of different sites: 1, 3, 7, or 9. However, the UV spectrum of 1d was more similar to the N^6,9-dibenzyladenine isomer and to adenine itself than to the other dibenzyl isomers (a bathochromic shift of UV max in going from neutral to either basic solution or acidic solution).[11] In addition, the positions of the carbon absorptions in the ^{13}C NMR spectrum of the bis-adduct 1d were identical with the positions of the ring carbon absorptions in the ^{13}C NMR spectra of the 9-substituted adenines reported by Chenon, et al.[12] This clearly excluded the 7-position as an alternate alkylation site to account for the substitution pattern in 1d because of the dependence of the position of the ring carbon absorptions on substitution patterns.[12] Thus, the adenine adducts have been assigned the N^6,9-substituted structure on the basis of the structure of 1d.

The structures of the two mono-adducts that were isolated from the reaction of one equivalent of lower basicity amine-formaldehyde with adenine were derived from their NMR spectra. In each case the N-$C\underline{H}_2$-N absorption was at the same position as the downfield N-$C\underline{H}_2$-N absorption in the bis-adduct. In addition, the UV spectra of the mono-adducts were essentially identical with adenine which has an 9-H structure.[12] Thus, the mono-adducts have been assigned a 9-alkylated structure.

There was no change in the type of product isolated from the reaction of amine-formaldehyde with cytosine that resulted from a change in either the basicity of the amine used or the number of equivalents used; no 2b or 2c were observed under any conditions from the reaction of cytosine with formaldehyde, and morpholine or diethylamine. If only one equivalent was used, mixtures of bis-adduct and unreacted cytosine were isolated. Usually the use of four equivalents of aminomethylating agent was necessary to drive the reactions completely to bis-adducts.

The NMR spectra of the bis-adducts of cytosine were very similar to that of the bis-adducts of adenine. The upfield absorptions due to N-CH_2-N were again doublets in most cases. The doublets collapsed to singlets on D_2O exchange in all cases, and were coupled to an exchangeable absorption which integrated for one proton. Thus, the upfield N-CH_2-N absorptions were due to aminomethylation of the exocyclic amino group. The remaining downfield N-CH_2-N absorptions in the spectra of the bis-adducts could be due to alkylation on either the 1- or the 3-positions. However, the UV spectra of the bis-adducts were similar to the UV spectra of 1-, N^4,N^4-, or 1,N^4-substituted cytosines or cytosine itself; they exhibited bathochromic shifts of their UV max on going from neutral to acid or basic solution whereas 3-substituted cytosines without a 1-substituent exhibited a large hypsochromic shift of their UV max on going from neutral to acid solution.[13,14] In addition, the ^{13}C NMR spectrum of the bis-adduct 2d was essentially identical with that reported for cytidine.[15] Thus, the cytosine bis-adducts have been assigned N^4,1-substituted structures.

In the case of the reaction of guanine with formaldehyde-amine the products from the reactions were solids which exhibited high melting points (>270°), and which were insoluble in solvents used for NMR spectroscopy. In one case the reaction product was submitted for elemental analysis. The elemental analyses of 3b were consistent with a bis-adduct. Analogy to previous work with formaldehyde adducts of guanosine[7] and the thiophenol-formaldehyde adducts[4] of guanosine, suggested that the exocyclic amine was alklated, and analogy to the remainder of the present work suggested that the 9-position was alkylated. Thus, the bis-alkylated guanine derivative 3b has been tentatively assigned the N^2,9-substituted structure.

The formation of the aminomethyl adducts with cytosine and adenine was reversible in dilute aqueous solution. Although UV spectroscopy was not capable of determining whether both aminomethyl groups were being lost in the hydrolyses, ^1H NMR spectroscopy definitely showed that they were, because new equilibria between N-aminomethylated product and parent purine or pyrimidine were established with each addition of D_2O to their DMSO-d_6 solutions until all of the N-CH_2-N absorptions were gone. However, it was also clear on the basis of the integration of the absorptions due to the two types of N-CH_2-N that the aminomethyl adduct with the exocyclic amino group was more easily reversible because the absorption due to NH-CH_2-N decreased in intensity faster. No N-CH_2-OH type absorptions[7] were observed by NMR spectroscopy during the hydrolyses.

It was possible to use UV spectroscopy to determine the rates at which the exocyclic adducts hydrolyzed.[16] It was found that the basicity of the secondary amine influenced the rate significantly as might be expected from the similarity of the chemistry of aminomethyl adducts to Mannich base chemistry.[17] The morpholine adduct 1d was much slower ($t_{\frac{1}{2}}$=30 min) than the piperidine adduct 1e ($t_{\frac{1}{2}}$=about 5 min). The cytosine adduct was much slower yet; 2d exhibited a $t_{\frac{1}{2}}$ of about 30 hr. The order of these rates of hydrolyses for the aminomethylated adducts were comparable to the rates of hydrolyses observed by Bridson et al.,[3] for the ethoxymethyl adducts of the protected nucleosides i.e., the cytidine adduct hydrolyzed more slowly than the adenosine adduct.

EXPERIMENTAL

The UV spectra were obtained using a Beckman model 25 spectrophotometer; the low pH spectra were obtained by adding 1 drop of 12 N HCl, and the high pH spectra were obtained by adding 1 drop of 10% NaOH to the CH_3OH solutions.[11] TLC were run on Brinkman Polygram Sil G/UV 254. MP (corrected) were taken with a Thomas-Hoover Capillary apparatus. ^1H NMR spectra were recorded on a Varian T-60 spectrometer; IR spectra on a Beckman Accu Lab 1 spectrophotometer. Microanalyses were obtained from Atlantic Microlab Inc., Atlanta, GA. Adenine, guanine, and cytosine were obtained from Sigma, while the amines and formaldehyde were obtained from Aldrich, and the bulk solvents were obtained from Fisher. ^{13}C NMR spectra were run on a Nicolet NB-300 spectrometer.

The Reaction of Adenine with One Equivalent of Secondary Amine and Formaldehyde. To a THF (4ml) solution of 0.29g (0.0033 mole) of morpholine and 0.27g (0.0033 mole) of 37% H_2C=O in water was added 0.45g (0.0033 mole) of adenine. The suspension was stirred at room temperature for 4 days; then it was filtered to give 0.50g [foamed at 99-104°, > 240° (d)] of the 9-(4'-morpholinyl)-methyladenine (1b): 67% yield; IR (KBr) 3520, 3400, 3270 and 3080 cm^{-1} (M) (N-H and OH), and 1675 and 1605 cm^{-1}(S); ^1H NMR (DMSO-d_6) δ 8.07 (s,1, C_2-H), 7.97 (s,1, C_8-H), 7.3-6.8 (m, 2, N-H, exchangeable with D_2O), 4.97 (s,2, N-CH_2-N), 3.8-3.3 (m,4, C-CH_2-O), 3.33 (s,2, H_2O) and 2.7-2.35 (m,4, C-CH_2-N); UV (CH_3OH) max 261 nm (ε = 1.29 x 10^4 l/mole), OH⊖ 271 nm (ε = 1.19 x 10^4 l/mole) and H⊕ 264 nm (ε = 1.27 x 10^4 l/mole). (Found: C, 47.60; H, 6.42; N, 33.34. Calc. for $C_{10}H_{14}N_6O$ H_2O: C, 47.61; H, 6.39; N, 33.32).

9-(4'-Methyl-1'-piperazinyl)methyladenine (1c) was prepared in a similar manner: 68% yield, foam

132-146°, decomposed 226-248°; IR(KBr) 3450 and 3100 cm^{-1} (M) (N-H and OH), 1640 and 1660 cm^{-1} (S); ^1H NMR (DMSO-d_6) δ 8.13 (s,1, C_2-H), 8.08 (s,1, C_8-H), 7.4-6.9 (m,2, N-H), 5.0 (s,2, N-CH_2-N), 3.6-3.0 (m,2, OH), 2.7-2.0 (m,8, CH_2-N) and 2.08 (s,3, CH_3N). Although the spectral data were consistent with a mono-hydrate of the mono-adduct, correct elemental analyses could not be obtained for the product.

The Reaction of Adenine with two or more Equivalents of Secondary Amines and Formaldehyde. Generally 3 to 4 equivalents of a 1:1 mixture of the secondary amine and 37% aqueous formaldehyde in tetrahydrofuran (10 ml for 0.01 mole scale) were allowed to react with adenine at room temperature overnight in order to drive the reaction to completion. The solutions that resulted were concentrated and those concentrates were triturated with ether for 2-5 hr, then filtered. The residues were usually analytically pure. However, in those cases where the secondary amine was diethylamine or other amines where there were more degrees of vibrational freedom available to the alkyl substituents, the concentrates were oils and the excess alkylating agent could not be easily separated from the product. Then, only 2 equivalents of secondary amine and formaldehyde were used. Any unreacted adenine was filtered from the reaction mixture at the trituration stage and the oils were characterized as such. Thus, the following bis-adducts were obtained: Bis-N^6,9-(4'-morpholinyl)methyladenine (1d): mp 142-144°, 91% yield; IR (KBr) 1630 cm^{-1}(S), and 3260 and 3200 cm^{-1} (W) (N-H); ^1H NMR ($CDCl_3$) δ8.33 (s,1, C_2-H), 7.8 (s,1, C_8-H), 6.53 (t,1, J = 6 Hz, N-H, D_2O exchangeable), 5.0 (s,2, N-CH_2-N), 4.55 (d,2, J = 6 Hz, NH-CH_2N, collapses to a singlet on D_2O exchange), 3.8-3.55 (m,8, C-CH_2-O) and 2.8-2.45 (m,8, NCH_2-C); ^{13}C NMR (DMSO-d_6)δ152.47 (C_2), 150.30 (C_4), 118.46 (C_5), 155.20 (C_6) and 141.56 (C_8); UV (CH_3OH) max 269 nm (ε = 1.59 x 10^4 l/mole), OH⁰ 274 nm (ε = 1.51 x 10^4 l/mole) and H⊕ 270 nm (ε = 1.30 x 10^4 l/mole). (Found: C, 53.97; H, 6.99; N, 29.35. Calc. for $C_{15}H_{23}N_7O_2$: C, 54.04; H, 6.95; N, 29.41).

Bis-N^6,9-(1'-piperidinyl)methyladenine (1e): mp 139-141°, 68% yield; IR (KBr) 1620 cm^{-1}(S), and 3240 and 3160 cm^{-1}(W) (N-H); ^1H NMR ($CDCl_3$) δ8.33 (s,1, C_2-H), 7.77 (s,1, C_8-H), 6.3-6.0 (m,1, N-H, D_2O exchangeable), 5.0 (s,2, N-CH_2N) 4.55 (d,2, J = 6 Hz, NHCH_2-N, collapses to a singlet on D_2O exchange), 2.75-2.4 (m,8, C-CH_2N) and 1.85-1.0 (m, 12, CH_2-CH_2-CH_2); UV (CH_3OH) max 267 nm (ε = 1.65 x 10^4 l/mole), OH⁰ 274 nm (ε = 1.54 x 10^4 l/mole) and H⊕ 270nm (ε=1.44x10^4 l/mole). (Found: C, 61.89; H, 8.30; N, 29.72. Calc. for $C_{17}H_{27}N_7$: C, 61.98; H, 8.26; N, 29.76).

Bis-N^6,9-(diethylamino)methyladenine (1f): an oil, 67% yield; ^1H NMR ($CDCl_3$) δ8.30 (s,1, C_2-H), 7.77 (s,1, C_8-H), 6.2-5.8 (m,1, N-H, D_2O exchangeable), 5.07 (s,2, N-CH_2-N), 4.70 (d,2, J= 6 Hz, NH-CH_2-N, collapses to a singlet on D_2O exchange), 2.8 (q,4, J= 7 Hz, CH_2N), 2.83 (q, 4, J = 7 Hz, CH_2-N) and 1.13 (t, 12, J = 7 Hz, CH_3-CH_2-N). (Found: C, 57.62; H, 8.70; N, 31.32. Calc. for $C_{15}H_{27}N_7 \cdot 0.5 \cdot H_2O$: C, 57.29; H, 8.98; N, 31.19).

The Reaction of Cytosine with two or more Equivalents of Secondary Amines and Formaldehyde. Generally 3 to 4 equivalents of a 1:1 mixture of secondary amine and 37% aqueous formaldehyde in tetrahydrofuran (10 ml for 0.01 mole scale) were allowed to react at room temperature with cytosine overnight. The suspensions were kept well stirred; then the suspensions were filtered. These residues were analytically pure products. With diethylamine and similar secondary amines, clear solutions were obtained after reaction overnight. The solutions were concentrated and the residues were triturated with ether or hexane to give the desired compound as suspended white solids which were isolated by filtration. Thus, the following bis-adducts were obtained:

Bis-N^4,1-(4'-morpholinyl)methylcytosine (2d): mp 192-193°, 83% yield; IR (KBr) 3260 cm^{-1}(M) (N-H), and 1655 and 1630 cm^{-1}(S); ^1H NMR (DMSO-d_6) δ7.75 (t,1, J = 5 Hz, NH, D_2O exchangeable), 7.41 (d,1, J = 8 Hz, CH = C), 5.65 (d,1, J = 8 Hz, CH = C), 4.43 (s,2, N-CH_2-N), 4.08 (d,2, J= 6 Hz, NH-CH_2N, collapses to a singlet on D_2O exchange), 3.7-3.4 (m,8, C-CH_2-O) and 2.65-2.3 (m,8, C-CH_2-N); ^{13}C NMR (DMSO-d_6) δ156.26 (C_2), 164.54 (C_4), 93.48 (C_5) and 145.45 (C_6); UV (CH_3OH) max 270 nm (ε = 7.77 x 10^3 l/mole), OH⁰ 276 nm (ε = 7.25 x 10^3 l/mole) and H⊕ 282 nm (ε = 10.8 x 10^3 l/mole). (Found: C, 54.42; H, 7.50; N, 26.63. Calc. for $C_{14}H_{23}N_5O_3$: C, 54.35 H, 7.49; N, 22.64).

Bis-N^4,1-(1'-piperidinyl)methylcytosine (2e): mp 195-196°, 94% yield; IR (KBr) 3260 cm^{-1}(M) (N-H), and 1650 and 1630 cm^{-1}(S); ^1H NMR ($CDCl_3$) δ7.37 (d,1, J = 8 Hz, CH = C), 5.70 (d,1, J = 8 Hz, CH = C), 6.4-5.4 (broad m, 1, N-H), 4.47 (s,2, N-CH_2-N), 4.15 (m, 2, NH-CH_2N), 2.65 - 2.2 (m,8, C-CH_2-N) and 1.8-1.2 (m,12, C-CH_2-C); UV (CH_3OH) max 272 nm (ε = 6.34x10^3 l/mole). (Found: C, 63.08; H, 8.95; N, 22.87. Calc. for $C_{16}H_{27}N_5O$: C, 62.92; H, 8.91; N, 22.93).

Bis-N^4,1-(diethylamino)methylcytosine (2f): mp 134-136°, 75% yield; IR (KBr) 3230 cm^{-1} (M) (N-H), 1640 and 1620 cm^{-1} (S); ^1H NMR ($CDCl_3$) δ7.47 (d,1, J = 8 Hz, CH=C), 5.73 (d,1, J= 8 Hz, CH=C), 6.2-5.6 (m,1, N-H), 4.53 (s,2, N-CH_2-N), 4.6-4.0 (m,2, NH-CH_2N), 2.7 (q,4, J = 7 Hz, CH_2-N), 2.50 (q,4, J = 7 Hz, N-CH_2) and 1.03 (t,12, J = 7 Hz, CH_3CH_2N); UV (CH_3OH) max 270 nm (ε = 7.10 x 10^3 l/mole), OH⁰ 279 nm (ε = 7.18 x 10^3 l/mole) and H⊕ 281 nm (ε = 1.31 x 10^4 l/mole). (Found: C, 59.55; H, 9.59; N, 24.84. Calc. for $C_{14}H_{27}N_5O$: C, 59.75; H, 9.67; N, 24.89).

The Reaction of Guanine with an Excess of Formaldehyde and Dimethylamine. Guanine(0.38g,0.0025 mole) was suspended in 2.2g (0.02 mole) of 40% dimethylamine in water. Then, 1.62g (0.02 mole) of 37% CH_2=O was added and the suspension was stirred at room temperature for 1 day. The suspension was diluted with THF. The THF-water suspension was stirred for 2 hr then filtered, and the residue was dried to give 0.62g (mp > 260°) of white solid which was the desired bis-adduct, 3b: 94% yield; IR (KBr) 3250 cm^{-1} (S) 3600-2200 cm^{-1}(M) (N-H), 1695 cm^{-1}(shoulder), 1685 cm^{-1}(S) (C=O). (Found: C, 49.58; H, 7.25; N, 36.91. Calc. for $C_{11}H_{19}N_7O$: C, 49.79; H, 7.22; N, 36.96).

Stability and Rates of Hydrolysis of Adducts: The methanol UV spectra of the adducts of cytosine and adenine were stable for up to 2 hr. The water UV spectrum of the mono-adduct of adenine 1b was essentially identical with adenine itself under the same conditions. The water (pH 7.6) UV spectra of the bis-adducts of cytosine and adenine exhibited UV max shifted to longer wavelengths (UV max identical with the UV max of the adducts in methanol) which were more intense than the parent compounds. The rates of hydrolysis of three of the adducts (1d, 1e and 2d) were followed by UV at about 1 x 10-4 mole/l by following the decrease in intensity of their respective UV max with time. Spectra were monitored until stable spectra were produced which in all cases were essentially identical with the parent purine or pyrimidine. Plots of $A_t - A^\infty$ versus time were

used to determine $t_{\frac{1}{2}}$.[16] The hydrolyses of the adducts were also followed by ^{1}H NMR spectroscopy. Solutions (DMSO-d_6) of the adducts at about 30 mg/ml, were diluted with D_2O in a dropwise fashion until a 1:1 ratio of the two solvents was reached. Integrations (5 times) of the spectra were taken after each addition of D_2O. Comparisons of the averages of the integrations of the N-C$\underline{H}_2$-N absorptions and the integrations of the C_2-$\underline{H}$, C_8-$\underline{H}$, C_5-$\underline{H}$ or C_6-$\underline{H}$ absorptions were used to quantitate the amount of decomposition.

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