Synthesis of 5,6-Dihydro-5-(α-thyminyl)thymine[1]

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5-(Hydroxymethyl)uracil (1) reacted with 6-aminothymine (3) in trifluoroacetic acid–trifluoroacetic anhydride to give 5,6-dihydro-6-imino-5-(α-thyminyl)thymine (5). Reduction of 5 with sodium cyanotrihydridoborate gave 5,6-dihydro-5-(α-thyminyl)thymine (6), which was identical spectroscopically and chromatographically to the principal photoproduct obtained from UV-irradiated dry DNA. Hydrolysis of 5 gave 5-methyl-5-(α-thyminyl)barbituric acid (4). 5-(Hydroxymethyl)uracil (1) reacted with 6-aminouracil (7) to give 6-amino-5-(α-thyminyl)uracil in trifluoroacetic acid.

Ultraviolet irradiation of calf thymus DNA in water–ethylene glycol at −78 °C has been shown to give one major thymine-derived photoproduct, tentatively identified as 5,6-dihydro-5-(α-thyminyl)thymine (6) on the basis of spectroscopic evidence.[2] The compound is isolated after trifluoroacetic acid degradation of the DNA. On the basis of chromatographic data, it is identical with the major photoproduct isolated from irradiated bacterial spores of *Bacillus megaterium*[3] or *Bacillus subtilis*.[4] Since the photoproduct was first observed from irradiated bacterial spores, it is commonly referred to as "spore photoproduct".

We have pursued the synthesis of 5,6-dihydro-5-(α-thyminyl)thymine in order to establish the structure of "spore photoproduct" beyond all reasonable doubt. Resolution of synthetic **6**, comparison of CD spectra to material isolated from irradiated DNA, and X-ray crystallography may establish stereochemistry at C-5 of the dihydrothymine ring. Furthermore, synthetic material provides a model for the investigation of the chemical mechanism underlying a DNA repair process which cleaves spore photoproduct into two thymines.[5]

The synthetic route which we devised requires only two steps from the readily available substituted pyrimidine, 5-(hydroxymethyl)uracil[6] and 6-aminothymine.[7] The rationale for our approach was based upon the recognition that bond formation between C-5 and the exocyclic methylene could potentially be achieved via simple electrophilic addition.

6-Aminouracil and the related N-protected derivative 1,3-dialkyl-6-aminouracil undergo electrophilic addition at C-5 by such carbon electrophiles as aldehydes,[8] dimethyl acetylenedicarboxylate,[9] isothiocyanates,[10] Vilsmeier reagents,[11] α-bromo ketones and esters,[12] and acyl chlorides.[13]

5-(Hydroxymethyl)uracil in acid solution reacts with carbon nucleophiles such as phenol via the apparent intermediacy of a pyrimidinylmethyl cation.[14]

When 5-(hydroxymethyl)uracil (1) and excess trifluoroacetic anhydride (TFAA) are dissolved in trifluoroacetic acid (TFA) (Scheme I), the methylene proton resonance shifts from δ 4.67 (s, 2 H) to 5.20 (s, 2 H). The latter signal we attribute to 5-(trifluoroacetoxymethyl)uracil (2). It was not necessary to include trifluoroacetic anhydride to observe the

Scheme I

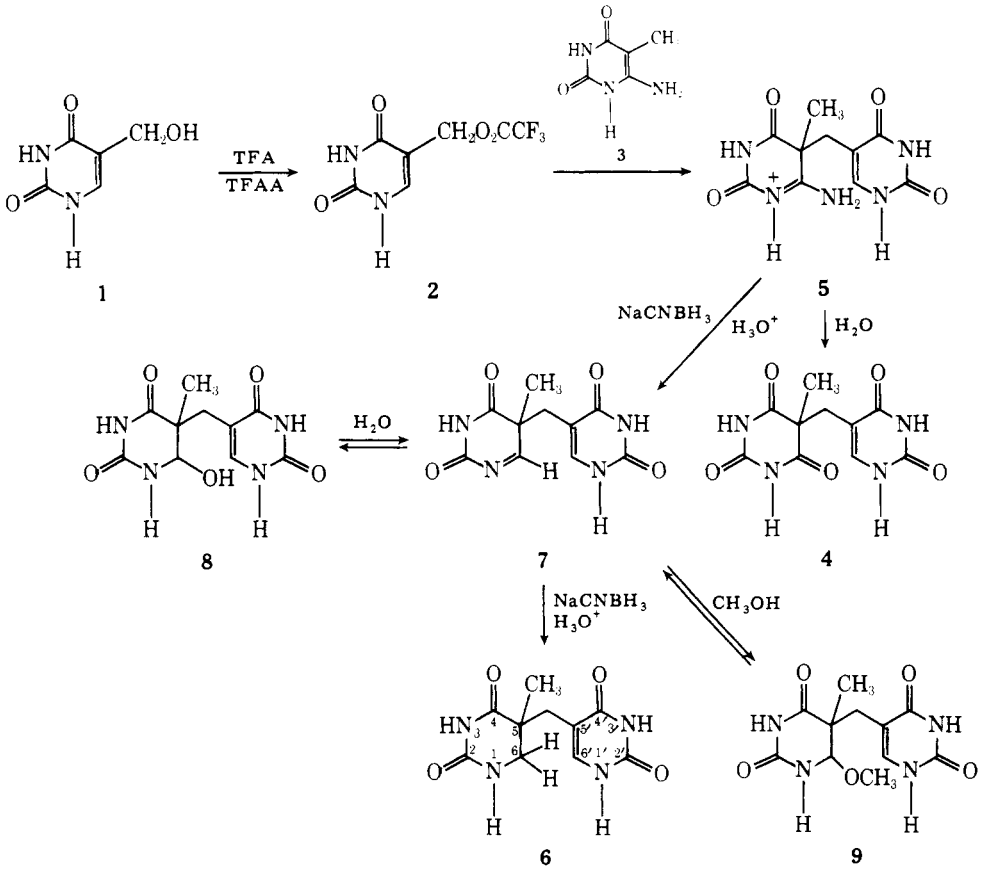

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Table I. Carbon-13 Chemical Shifts[a]

compd	carbon positions[b] C-2	C-4	C-5	C-6	C-5 CH_2	C-5 CH_3	C-2′	C-4′	C-5′	C-6′
thymine	151.32	164.74	107.56	137.55		11.73				
5,6-dihydrothymine	153.82	173.51	34.20	41.89		12.40				
4	153.23	174.91	40.95	45.28	30.03	20.01	151.05	164.65	106.86	140.32
6	150.93 (150.07)	172.61	50.00	172.61	35.22	20.71	150.07 (150.93)	163.91	106.08	140.40

[a] Spectra were run in perdeuterated dimethyl sulfoxide; shifts are given in parts per million relative to internal Me_4Si; spectra are completely decoupled. [b] Carbon positions are indicated in Scheme I.

appearance of **2**; however, the results of the subsequent steps were drastically altered depending upon the amount of water present in the reaction mixture. Addition of 1 equiv of 6-aminothymine (**3**) to a solution of **2** in TFA followed by refluxing for 4 h resulted in a shift of the methylene proton resonance from δ 5.20 to 3.51. A second product (CH_2 proton resonance at δ 3.31) was identified as 5-methyl-5-(α-thyminyl)barbituric acid (**4**) by ^1H and ^{13}C NMR mass spectra and elemental analysis. When water was rigorously excluded, **4** occurred in relatively minor amounts (<10% yield). If trifluoroacetic anhydride or phosphorus pentoxide was not added to remove the water, then compound **4** was obtained in high yield. The major product when water was excluded (δ 3.51 (2 H) resonance) has tentatively been assigned the structure 5,6-dihydro-6-imino-5-(α-thyminyl)thymine (**5**). Presumably, **5** exists as an imminium salt. Removal of the trifluoroacetic acid in vacuo gave **5** as a white powder, which was used directly in all subsequent synthetic transformations without purification.

A number of routes were investigated to convert **5** to 5,6-dihydro-5-(α-thyminyl)thymine. 5,5-Dialkyl-substituted 5,6-dihydrouracils have been obtained previously from the corresponding 6-imino-5,6-dihydrouracils via thiation with H_2S followed by Raney nickel dethiation.[15] By this procedure we were never able to isolate and characterize 5,6-dihydro-6-thio-5-(α-thyminyl)thymine, but could only isolate **4**. This problem was not pursued in depth because of the success with reduction of **5** by sodium cyanotrihydridoborate. When **5** was treated with aqueous or methanolic sodium cyanotrihydridoborate at pH 2–4, a rapid initial reaction took place and a mixture of unidentified products was obtained. When the solution was heated on a steam bath, sodium cyanotrihydridoborate was added periodically, and the pH was maintained at 2–4 over 30 min, then a mixture of products was obtained which could be separated largely on the basis of solubility differences. The major water-soluble UV-absorbing product was 5,6-dihydro-5-(α-thyminyl)thymine (**6**), which was obtained analytically pure by chromatography on Bio-gel P-2 in aqueous solution (2.4% yield).[17] Although the crude yield is significantly greater and yields of purified product have been obtained in higher yield in a few runs, normally one can expect to obtain no more than a 3% yield of analytically pure product.

The signals in the ^1H NMR spectra of **6** in TFA and in 100% Me_2SO-d_6 (Figure 1) were assigned by comparison of the spectra with spectra of thymine and dihydrouracil. In the TFA spectrum the protons at C-6 appear as a singlet at δ 3.57; however, in 100% Me_2SO-d_6 they are nonequivalent and appear as doublets of doublets at δ 2.88 and 3.06. In addition to the 13-Hz geminal coupling constant, splitting by the adjacent N-1 proton ($J \geq 2$ Hz) was observed at 25 °C but not at 140 °C. 5,6-Dihydrouracil in 100% Me_2SO-d_6 showed a broad peak at δ 7.47 for the N-1 proton which is coupled to the magnetically equivalent C-6 protons centered at δ 3.21 with a constant of 2.5 Hz. The assignments for the C-6 protons were confirmed by reducing **5** with $NaCNBD_3$ to give **6** deuterated at C-6. The

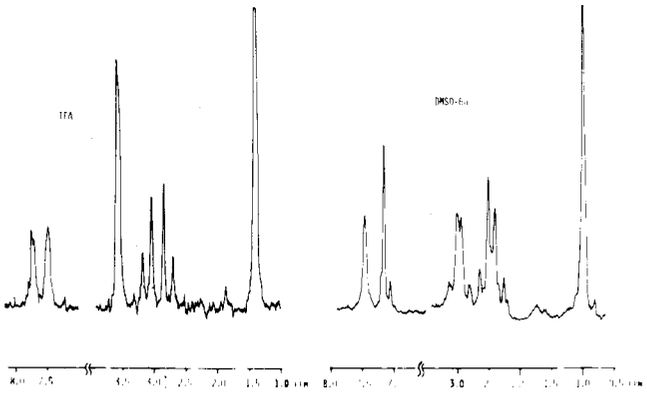


Figure 1. 100-MHz ^1H NMR spectra of **6** in trifluoroacetic acid (TFA) and perdeuterated dimethyl sulfoxide (Me_2SO-d_6) in parts per million (δ values) from tetramethylsilane.

proton resonance in TFA at δ 3.57 was absent.

The ^{13}C NMR spectrum of **6** was nearly a perfect fit for a combination of the spectra measured for the model compounds 5,6-dihydrothymine and thymine (Table I).[16] The downfield shifts for C-5 and C-6 (relative to dihydrothymine) can be attributed to the additional substituent at C-5. Consequently, on this basis the structure of the carbon skeleton may be considered established.

The ^{13}C NMR spectrum determined for **4** closely fits that predicted for the proposed structure on the basis of the ^{13}C NMR spectra of thymine.

Spectral data for synthetic **6** are virtually identical with those reported for "spore photoproduct" isolated from irradiated calf thymus DNA.[2] The ^1H NMR spectra in TFA are identical, and the mass spectra (see Experimental Section) show identical fragmentation patterns, differing only in relative intensity as might be expected from instrument differences.

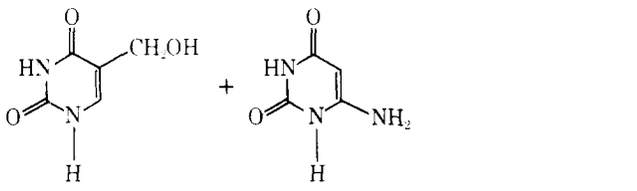
However, in order to establish the identity of synthetic **6** and the product isolated from irradiated DNA beyond all doubt, calf thymus DNA was irradiated and "spore photoproduct" isolated following the procedure of Varghese.[2] The synthetic and DNA isolated product ran at identical R_f values in solvent systems A–F (Experimental Section) on silica gel. It is of interest to note here that **6** is completely stable in trifluoroacetic acid at 175 °C (90 min), the conditions used for its isolation from DNA. Synthetic **6** is also stable to concentrated ammonium hydroxide, but rapidly reacts with 0.1 N NaOH at room temperature.

The two key reactions of the synthesis, coupling 5-(hydroxymethyl)uracil to 6-aminothymine and the reduction of the imino group to a methylene group by sodium cyanotrihydridoborate, are possibly more general than this specific application. The structural restrictions for the coupling reaction may be relatively narrow if unprotected pyrimidines are used. Most of the examples cited earlier[8–13] utilize 6-aminouracil, substituted at N-1, or N-1 and N-3 by alkyl

groups. At least in the case of acylation, when N-1 is unsubstituted, reaction occurs principally at N-6, possibly via the 1-acyl derivatives. Only when N-1 is substituted is the principal product the C-5 acylated product.[9]

We attempted to learn the fate of the majority of organic material from the cyanoborohydride reduction, but with little success. Shorter reduction times were employed in the belief that over reduction was occurring. However, the major isolable UV-absorbing product after a 15-min reduction followed by workup in methanol was 6-methoxy-5,6-dihydro-5-(α-thyminyl)thymine (**9**, 6.1% yield). The assigned structure was based upon ^{1}H NMR, UV, and mass spectra and the observation that purified **9** is reduced to **6** on treatment with $NaCNBH_3$ in water at pH 2–3. In aqueous solution **9** readily exchanges water for methanol to give **8**. When a mass spectrum of **9** was attempted, thermal decomposition resulted and methanol (*m/e* 32) was observed. Further heating in the direct inlet probe gave a mass spectrum with the highest molecular weight ion at *m/e* 250, which would correspond to structure **7**.

The coupling reaction between 6-aminouracil (**10**) and **2** leads to 6-amino-5-(α-thyminyl)uracil (**11**). The lack of an



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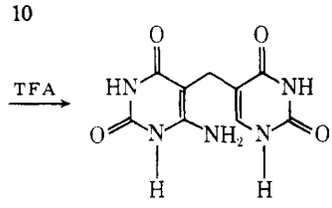

11

additional substituent at C-5 results in retention of the aromatic system of **11**, the 6-aminouracil moiety, and hence a hydrolytically stable compound. The ^{1}H NMR spectrum with two observable resonances at δ 3.60 (2 H) and 8.03 (1 H) fits structure **11**, better than any conceivable alternate structure. The absence of a C-5 proton resonance in the region δ 5.5–6 and the high field location of the two-proton singlet at δ 3.60 are highly indicative of the C-5 coupling.

Further studies directed toward investigation of the scope of the coupling reaction may be of interest. However, we have turned our attention toward further studies of synthetic spore photoproduct, which shall be the subject of a separate report.

Experimental Section

Proton magnetic resonance spectra (^{1}H NMR) were determined on either a Varian EM360 60-MHz instrument or a Fourier transform Jeol NMR Model PS100 with Me_4Si as an internal standard. Sodium 2,2,3,3-tetradeuterio-3-(trimethylsilyl)propionate was employed as an internal reference for spectra run in D_2O. ^{13}C NMR were also obtained on the latter instrument. Ultraviolet spectra were measured on a Cary 17 spectrometer. Infrared spectra were obtained on a Beckman IR-8 in solid KBr. Low-resolution mass spectra were obtained on a Model 3200 Finnigan mass spectrometer at 70 eV.

Analytical thin-layer chromatography was carried out on precoated 3.5 × 11 cm plastic backed sheets coated with silica gel 60 (E. Merck A.G.) and cellulose (Eastman 13254) with a fluorescent indicator. Solvent systems used were (A) 1-butanol–water (86:14 v/v), (B) 2-propanol–concentrated ammonium hydroxide–water (7:2:1 v/v), (C) 1-butanol–methanol–concentrated ammonium hydroxide–water (60:20:1:20 v/v), (D) acetonitrile–0.1 M aqueous ammonium acetate–concentrated ammonium hydroxide (7:2:1 v/v), (E) acetonitrile–1-butanol–0.1 M aqueous ammonium acetate–concentrated ammonium hydroxide (10:60:20:10 v/v), and (F) chloroform–methanol–concentrated ammonium hydroxide (90:30:1 (v/v). All solvents and reagents were reagent grade. Water was deionized and then distilled through glass.

5-(Hydroxymethyl)uracil,[6] 6-aminouracil, and 6-aminothymine[7] were all prepared by literature procedures and recrystallized to chromatographic purity prior to use. Elemental analyses were performed by the microanalytical laboratory at the University of California, Berkeley. Melting points were taken on a Büchi 510 melting point apparatus and are uncorrected.

5,6-Dihydro-5-(α-thyminyl)thymine was isolated from UV-irradiated calf thymus DNA according to the procedure of Varghese.[2] Synthetic **6** and material isolated from irradiated DNA were cochromatographed on silica gel eluting with solvent systems D–F and ran at identical R_f values as given for synthetic **6** below.

5,6-Dihydro-6-imino-5-(α-thyminyl)thymine (5). 5-(Hydroxymethyl)uracil (960 mg, 6.74 mmol) was stirred in trifluoroacetic acid (TFA, 15 mL)–trifluoroacetic anhydride (TFAA, 2 mL) for 1.5 h at room temperature in a dry flask under argon. The solution was heated to reflux, trifluoroacetic anhydride (2 mL) followed by 6-aminothymine (960 mg, 6.8 mmol) were added, and refluxing was continued for 7 h. An outlet tube was attached to the cooled reaction mixture and the TFA–TFAA mixture was evaporated under a slow stream of dry argon. The partially crystalline mixture was fitted with a KOH drying tube and heated at 40–50 °C and 0.1 mmHg for 12 h to give a compact white mass which was characterized only by ^{1}H NMR: ^{1}H NMR (TFA) δ 2.01 (3 H, s), 3.51 (2 H, broad s), 7.80 (1 H, broad s). The product was 90% pure, the major side product (by ^{1}H NMR) being **4**. The instability of **5** to hydrolysis prevented us from further purification and characterization.

5-Methyl-5-(α-thyminyl)barbituric Acid (4). To 500 mg of **5** was added 25 mL of water and enough sodium bicarbonate to bring the pH of the solution to pH 2 (the amount varied depending upon how well **5** was dried to remove excess TFA). Stirring overnight gave a white precipitate which was collected by suction filtration. Thin-layer chromatography showed the presence of a single product [R_f 0.28, system D; R_f 0.42, system C (silica gel)] in both the solid and in the filtrate. The product was recrystallized from water to give white crystals (220 mg) which did not melt below 320 °C: ^{1}H NMR (TFA) δ 1.81 (3 H, s), 3.31 (2 H, s), 7.78 (1 H, broad singlet); ^{13}C NMR (Table I); mass spectrum, *m/e* (rel intensity) 266 (M^+, 3), 251 (6.5, M − CH_3), 179 (4), 165 (4), 142 (37), 125 (28), 114 (15). Anal. Calcd for $C_{10}H_{10}N_4O_5$: C, 45.12; H, 3.79; N, 21.05. Found: C, 45.28; H, 3.57; N, 21.20.

5,6-Dihydro-5-(α-thyminyl)thymine (6). To the solid **5** prepared as described above (from 6.74 mmol of **1**) was added aqueous $NaCNBH_3$ (2.13 g in 10 mL of water) in small portions over a period of 30 min. After addition of the first portion (3 mL), the reaction mixture was diluted with water (15 mL) and kept on a steam bath throughout the addition of the remaining $NaCNBH_3$ solution. The pH was maintained in the range 2–4 by the periodic addition of 10% HCl. The solution was then allowed to stir at room temperature for 11 h and filtered to give a white solid and clear filtrate. The filtrate was lyophilized to give an off-white powder which was stirred in warm methanol (20 mL) and filtered to give 1.28 g of a white solid which by ^{1}H NMR was **6**. However, this material is principally inorganic salts (borates) which were eliminated by chromatography on Bio-gel P-2 with water eluent to give 40.5 mg (2.4%) of analytically pure **6** (mp >320 °C): ^{1}H NMR (TFA) δ 1.42 (3 H, s, $-CH_3$), 2.77 (1 H, d, J = 14 Hz), 3.12 (1 H, d, J = 14 Hz), 3.57 (2 H, broad s, H_2C-6), 7.52 (1 H, broad s, HN-1), 7.77 (1 H, d, J = 5 Hz, HC-6′); ^{1}H NMR (25 °C, Me_2SO-d_6) δ 1.01 (3 H, s, $-CH_3$), 2.35 (1 H, d, J = 14 Hz), 2.59 (1 H, d, J = 14 Hz), 2.88 (1 H, dd, J = 13 and ≤2 Hz), 3.06 (1 H, dd, J = 13 and ≤2 Hz), 7.16 (1 H, s, HC-6′), 7.46 (1 H, broad s, HN-1), 9.89 (1 H, broad s, HN-3); ^{13}C NMR (See Table I); mass spectrum, *m/e* (rel intensity) 252 (M^+, 3), 250 (2), 235 (2), 179 (6), 164 (2), 151 (3), 137 (2), 129 (2), 128 (29), 127 (100), 126 (56), 125 (33), in addition to numerous peaks below *m/e* 115; λ_{max} (H_2O, pH 6) 265 nm (ε 8000); λ_{max} (H_2O, pH 10) 290 nm (ε 8000); IR (KBr) 3190, 2875, 3075, 1660, 1750, 1480, 1430, 1390, 1315, 1230, 830, 765 cm^{-1}. Thin-layer chromatography: cellulose–system A, R_f 0.08; cellulose–system B, R_f 0.55; cellulose–system C, R_f 0.43; silica gel–system D, R_f 0.53; silica gel–system E, R_f 0.31; silica gel–system F, R_f 0.21. Anal. Calcd for $C_{10}H_{12}N_4O_4$: C, 46.72; H, 4.80; N, 22.22. Found: C, 47.01; H, 4.89; N, 21.97.

Compound **6** deuterated at C-6 was prepared by an identical procedure with $NaCNBD_3$ used in place of $NaCNBH_3$.

6-Methoxy-5,6-dihydro-5-(α-thyminyl)thymine (9). The procedure described above for the preparation of **6** was followed except on a 1.9-mmol scale and with 4 equiv instead of 5 equiv of $NaCNBH_3$. Also, the reaction mixture was heated on the steam bath a shorter

period of time (15 min). The resulting aqueous solution was immediately acidified to pH 1 with 1.0 M HCl and lyophilized to dryness. The resulting solid was suspended in 10 mL of methanol and filtered washing with 20 mL more of methanol. The filtrate was evaporated to dryness in vacuo three times with 20 mL of methanol. The resulting solid was suspended in a mixture of of 0.5 mL of water, 2 mL of methanol, and 10 mL of ethanol. The solid precipitate was collected by filtration and recrystallized from methanol to give 32.6 mg of **9** (6.1%). The amorphous crystals do not melt, but are transformed to long needles at 198–200 °C and then to a viscous glass between 243 and 267 °C: ^{1}H NMR (TFA) δ 1.54 (1 H, s), 3.06 (1 H, d, J = 17 Hz), 3.50 (1 H, d, J = 17 Hz), 4.09 (3 H, s, OCH_3), 5.23 (1 H, br s, $-CHOCH_3$), 7.95 (1 H, br s); mass spectrum, m/e (rel intensity) 250 ($M - OCH_3$, 4), 141 (17), 140 (3), 127 (58), 126 (100), 125 (100); λ_{max} (H_2O, pH 6) 264 mm. Thin-layer chromatography: silica gel–system C, R_f 0.38; system D, R_f 0.44. An analytically pure sample of **9** could not be obtained because of the ease with which it decomposes. Treatment of **9** with aqueous 1 N HCl gave **8** as a white solid. The ^{1}H NMR spectrum of **8** was virtually identical with that of **9** except the methoxyl protons were absent.

6-Amino-5-(α-thyminyl)uracil (11). 5-(Hydroxymethyl)uracil (170 mg, 1.20 mmol) was dissolved in 5 mL of TFA, and the mixture was stirred at room temperature for 2 h. 6-Aminouracil (150 mg, 1.18 mmol) was added, and the mixture was refluxed for 5.5 h and then condensed to dryness by rotary evaporation. The solid which was isolated was recrystallized by dissolution in 2 N ammonium hydroxide followed by reprecipitation by dropwise addition of concentrated hydrochloric acid, yielding 219 mg (70.1%) of white solid which did not melt on heating to 220 °C: ^{1}H NMR (TFA) δ 3.60 (2 H, s), 8.03 (1 H, broad d); IR (KBr) 3350, 3190, 3050, 2820, 1745, 1660, 1400, 1205, 1020, 830, 760 cm^{-1}. Anal. Calcd for $C_9H_9N_5O_4$: C, 43.0; H, 3.6; N, 27.9. Found: C, 43.7; H, 3.8; N, 27.7.

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Registry No.—**4**, 67513-78-4; **5**, 69155-20-0; **6**, 28100-77-8; **8**, 69155-21-1; **9**, 69155-22-2; **11**, 69188-74-5; thymine, 65-71-4; 5,6-dihydrothymine, 696-04-8; 5-(hydroxymethyl)uracil, 4433-40-3; 6-aminothymine, 15828-63-4; 6-aminouracil, 873-83-6.

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(17) Previously we reported a yield of 38%;[1] however, this was incompletely purified product.

Reaction of Alkanals and Amino Acids or Primary Amines. Synthesis of 1,2,3,5- and 1,3,4,5-Substituted Quaternary Pyridinium Salts

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The condensation of alkanals and amino acids or primary amines was studied under neutral condition at room temperature. From the reaction products of the glycine–propanal and L-α-leucine–propanal system, for example, the 1-(1-carboxymethyl)-2-ethyl-3,5-dimethylpyridinium betaine and the 1-(1-carboxy-3-methylpropyl)-4-ethyl-3,5-dimethylpyridinium betaine were isolated and identified. From the reaction products of the ethanolamine–alkanal system, the pyridinium salt with four substituents located at the 1,2,3,5 positions was also isolated. The substitution patterns of these pyridinium betaines are similar to that of the amino acids isodesmosine and desmosine. A mechanism involving the intermediacy of α,β-unsaturated aldimines is proposed, and evidence is presented that 2-ethyl-1,3,5-trimethyl- and 4-ethyl-1,3,5-trimethylpyridinium salts are formed by condensation of *N*-2-methyl-2-pentenilidenemethylamine with propanal in the presence of acetic acid. It may be concluded from the results that addition of alkanal to α,β-unsaturated aldimine occurred regiospecifically and was followed by ring closure and oxidation to give pyridinium salts.

The reaction of alkanals with amino acids or primary amines under neutral conditions is of interest in connection with the study of foodstuff deterioration.[1] In the course of our research on the reaction between alkanals and amino acids or primary amines under neutral condition at room temperature, quarternary pyridinium betaines **1** and salts **2** with four substituents located at the 1,2,3,5 and 1,3,4,5 positions were obtained.

Recently, two polyfunctional amino acids, desmosine and isodesmosine, derived from the cross-linkages in elastin have been isolated.[2,3] These isomers contain a pyridinium ring with four substituents located at the 1,3,4,5 and 1,2,3,5 positions. The structures suggested that the compounds may be formed from ring closure of four lysine residues.

While the reaction of pyridine ring formation, the "Chichibabin Pyridine Condensation", between carbonyl compounds and ammonia has been extensively investigated,[4] the formation of quarternary pyridinium salts by the reaction of carbonyls with primary amines has received little attention. In this paper we wish to report the identification and synthesis of quarternary pyridinium betaines **1** and pyridinium salts **2** which are condensation products of alkanals with amino acids

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