R_f^V 0.5; both contain material remaining at origin; amino acid ratios in acid hydrolysate, $Lys_{1.1}His_{2.0}Asp_{7.0}Thr_{3.1}Ser_{7.3}{}^{29}Glu_{5.1}Pro_{1.0}Gly_{5.0}Ala_{4.3}Cys_{2.5}Val_{1.9}Leu_{1.1}Tyr_{5.7}{}^{29}$ (80%); amino acid ratios in performic acid oxidized sample, $Lys_{1.0}His_{1.7}Asp_{7.0}Thr_{3.2}Ser_{6.8}{}^{29}Glu_{5.0}Pro_{1.0}Gly_{5.0}Ala_{4.2}Cys(SO_3H)_{3.0}Val_{2.0}Leu_{1.0}Tyr_{5.6}$;[29]

(29) Corrected for destruction during acid hydrolysis.

ratios of diagnostic amino acids, fragment A/fragment C (Cys-(SO_3H)/Leu) = 3.0; fragment A/fragment D (Cys(SO_3H)/Lys) = 3.0; fragment B contains no diagnostic residues.

Acknowledgments. The authors wish to express their appreciation to Judy Montibeller for the PCMB titrations and to Guirguis Rizk for the amino acid analyses.

Structure of the Borohydride Reduction Product of Photolinked 4-Thiouracil and Cytosine. Fluorescent Probe of Transfer Ribonucleic Acid Tertiary Structure

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Abstract: 5-(4-Pyrimidin-2-one)cytosine, Pyo(4–5)Cyt (**2**), a photoproduct which can be isolated from the irradiation (335 nm) of certain *E. coli* transfer RNAs and irradiation (254 nm) of polycytidylic acid, deoxycytidine, and cytidine, is reduced by sodium borohydride to a fluorescent compound, 5-(4-pyrimidin-2-one)-3,6-dihydrocytosine, Pyo(4–5)hCyt (**3**). Catalytic oxygenation (Pt, O_2) converted Pyo(4–5)hCyt back to Pyo(4–5)Cyt. Treatment of Pyo(4–5)hCyt with aqueous acid gave 5-(4-pyrimidin-2-one)-3,6-dihydrouracil, Pyo(4–5)hUra (**8**), which could also be obtained by the treatment of 5-(4-pyrimidin-2-one)uracil, Pyo(4–5)Ura (**7**), with sodium borohydride. 5-(4-Pyrimidin-2-one)-4-thiouracil, Pyo(4–5)Sur (**9**), a photoproduct from irradiation (335 nm) of 4-thiouracil in aqueous solution, is reduced by sodium borohydride to 5-(4-pyrimidin-2-one)-4-thio-3,6-dihydrouracil, Pyo(4–5)hSur (**10**). The fluorescent nature of Pyo(4–5)hCyt provides a useful monitor of the photoreaction of tRNAs containing proximate 4-thiouridine and cytidine moieties.

A specific intramolecular photoreaction has been shown to occur between 4-thiouridine and a cytidine in *E. coli* $tRNA_1^{Val}$ on irradiation at 335 nm.[1] Only those *E. coli* tRNAs known to possess a 4-thiouridine moiety in nucleoside position 8 and a cytidine in position 13 from the 5′-terminal end yield a photoproduct under these conditions. Evidence for the covalent cross-linking between the two nucleosides after photolysis of the intact *E. coli* $tRNA_1^{Val}$ at 335 nm was provided by enzymic fragmentation sequence studies.[1] Subsequently the photochemically cross-linked binucleotide unit was isolated by the complete enzymic digestion of the irradiated tRNA.[1b]. The structure of the corresponding binucleoside photoproduct has recently been determined as **1**.[2a] Compound **1**, 5-(1-β-D-ribofuranosyl-4-pyrimidin-2-one)cytidine, was obtained in fair yield by irradiating 4-thiouridine and cytidine in aqueous solution at 4° at 335 nm. The corresponding bipyrimidine product, 5-(4-pyrimidin-2-one)cytosine, Pyo(4–5)Cyt (**2**),[3] resulted on photolysis (335 nm) of 4-thiouracil in the presence of cytosine.[2]

The nature of the photoreaction and the structure of the photoproduct may lead to the acquisition of important structural and functional information about tRNA. It has been shown[4] that the photolytically cross-linked *E. coli* $tRNA_1^{Val}$ can be charged with valine in the presence of its corresponding aminoacyl synthetase, although the affinity for the synthetase is decreased. The Val-$tRNA_1^{Val}$ functions normally in a reconstructed *in vitro* protein-synthesizing system. Qualitatively similar results have been obtained in experiments with *E. coli* $tRNA^{Arg}$ and $tRNA_1^{Phe}$.[5]

The susceptibility of 4-thiouridine in tRNA to borohydride reduction[6] apparently led to an attempted borohydride reduction of the photolytically cross-linked tRNA. When the photoproduct was treated with sodium borohydride it was converted to a new, highly fluorescent compound with emission maximum 440 nm and excitation maximum 386 nm.[7] The degree and

(1) (a) M. Yaniv, A. Favre, and B. G. Barrell, *Nature (London)*, **223**, 1331 (1969); (b) A. Favre, A. M. Michelson, and M. Yaniv, *J. Mol. Biol.*, **58**, 367 (1971).

(2) (a) N. J. Leonard, D. E. Bergstrom, and G. L. Tolman, *Biochem. Biophys. Res. Commun.*, 44, 1524 (1971); (b) D. E. Bergstrom and N. J. Leonard, *Biochemistry*, **11**, 1 (1972).

(3) Abbreviations following the IUPAC-IUB Commission on Biochemical Nomenclature recommendations (*J. Mol. Biol.*, **55**, 299 (1971)) are used throughout. The photochemistry symbolism employed in ref 2 has been modified and improved after discussions with Dr. Waldo E. Cohn, Director of the Office of Biochemical Nomenclature. For example, the earlier abbreviation, Cyt-Sur, for the photoproduct **2**, which indicated the *source* of the two fragments, has been replaced by Pyo(4–5)Cyt, which represents the *actual* structure, now that it is known.[2] Pyo stands for pyrimidin-2-one and 4–5 indicates that it is attached by covalent linkage from the 4 position to the 5 position of cytosine. Based on the bipyrimidine system of nomenclature, which is less indicative of the biochemical connotation and interest, 5-(4-pyrimidin-2-one)cytosine is 4-amino-4′,5-bipyrimidine-2,2′(1*H*,1′*H*)-dione. Other abbreviations follow the new photochemistry symbolism, *e.g.*, Pdo(4–5)Cyd (in place of Cyd-Srd[2a]) for 5-(1-β-D-ribofuranosyl-4-pyrimidin-2-one)cytidine (**1**); Pyo(4–5)Ura for 5-(4-pyrimidin-2-one)-uracil (**7**); Pyo(4–5)Sur for 5-(4-pyrimidin-2-one)-4-thiouracil (**9**). The corresponding dihydro products obtained by treatment of **2**, **7**, and **9** with sodium borohydride are designated, respectively, as Pyo(4–5)-hCyt (**3**) (rather than $Cyt\text{-}Sur_{red}$), Pyo(4–5)hUra (**8**), and Pyo(4–5)hSur (**10**).

(4) M. Yaniv, A. Chestier, F. Gros, and A. Favre, *J. Mol. Biol.*, **58**, 381 (1971).

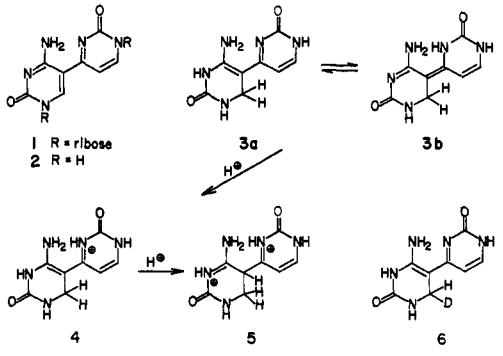
(5) L. Chaffin, D. R. Omilianowski, and R. M. Bock, *Science*, 172, 854 (1971).

(6) P. Cerutti, T. W. Holt, and N. Miller, *J. Mol. Biol.*, 34, 505 (1968).

(7) A. Favre and M. Yaniv, *FEBS (Fed. Eur. Biochem. Soc.) Lett.*, 17, 236 (1971).

rate of photochemical cross-linking can be followed conveniently by treating the irradiated tRNA with sodium borohydride and measuring the relative fluorescent intensity at 440 nm.

The reduced photoproduct takes on added significance with the recent discovery that Pdo(4–5)Cyd (**1**) is a major photoproduct of the irradiation of polycytidylic acid at pH 4.[8] Although the importance of the formation of the photoproduct in RNA, or of the related deoxyribose photoproduct in DNA, has yet to be determined, borohydride treatment of photolyzed RNA and DNA may allow detection of the cross-linked photoproduct at very low concentration levels.



We are now able to report the synthesis and structure of a fluorescent compound **3** identical with the product formed on borohydride reduction of cross-linked tRNA. The reduced photoproduct **3** was obtained by treatment of Pyo(4–5)Cyt (**2**) with sodium borohydride. In addition, the structures of the products formed on borohydride reduction of 5-(4-pyrimidin-2-one)uracil, Pyo(4–5)Ura (**7**), and 5-(4-pyrimidin-2-one)-4-thiouracil, Pyo(4–5)Sur (**9**), have been determined. The synthesis of Pyo(4–5)Ura by irradiation of a dilute aqueous solution of uracil and 4-thiouracil at 4° and of Pyo(4–5)Sur by irradiation (335 nm) of 4-thiouracil at 4° has been described.[2b] Although neither of these bipyrimidine photoproducts has yet been found in irradiated tRNA, they have served as valuable analogs in the study of Pyo(4–5)Cyt (**2**).

Results

Sufficient quantities of Pyo(4–5)Cyt (**2**), Pyo(4–5)Ura (**7**), and Pyo(4–5)Sur (**9**) were necessary in order to characterize their borohydride reduction products. Pyo(4–5)Cyt can be prepared in low yield by direct photolysis of an aqueous solution of cytosine and 4-thiouracil; however, it was more efficiently prepared (70% yield) by treatment of Pyo(4–5)Sur with sodium metaperiodate in an aqueous ammonium ion buffer solution at pH 9.8. If the periodate reaction was attempted in the normal two steps, as for the conversion of 2′-deoxy-4-thiouridine to 2′-deoxycytidine *via* an intermediate sulfonate,[9] it failed. Pyo(4–5)Sur (**9**) was easily obtained by photolysis of 4-thiouracil as previously described.[2b] When 4-thiouracil was photolyzed in aqueous solution with a twofold excess of uracil the major product was Pyo(4–5)Ura (**7**).[2b]

When Pyo(4–5)Cyt (**2**) was reduced with excess sodium borohydride in aqueous solution a single major product was isolated ($\lambda_{max}^{H_2O}$ 374 nm) (Figure 1). On

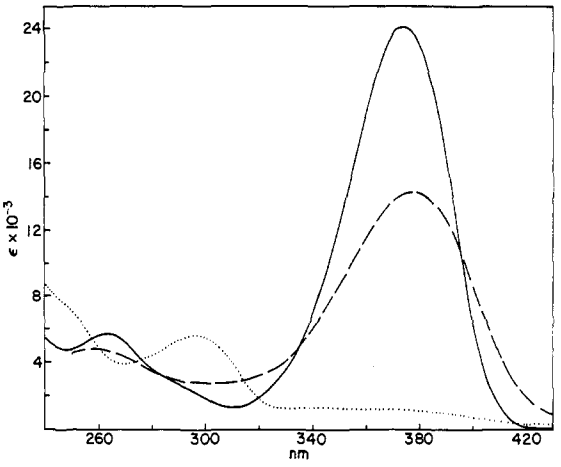
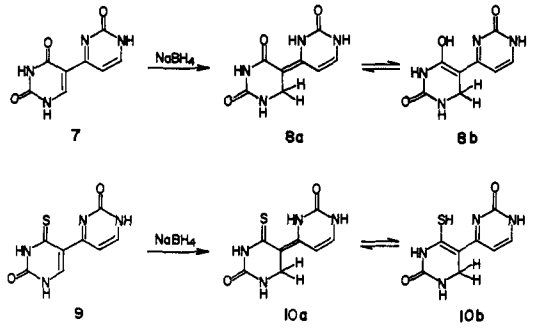


Figure 1. Ultraviolet spectrum of Pyo(4–5)hCyt (**3**) in 0.04 *M* HCl–10% DMSO–H_2O (– – –), 10% DMSO–H_2O (——), and 0.005*M* NaOH–10% DMSO–H_2O (· · ·); *cf.* Figure 1a in ref 7.



the basis of spectral data and elemental analysis the product was assigned structure **3**. If one can extrapolate from the stable tautomeric forms of cytosine and 2-pyrimidinone, either one, or both, of the tautomeric forms **3a** and **3b** can be present. This point could not be clarified readily from the first spectral data. The reduced product, Pyo(4–5)hCyt (**3**), was found to have the composition $C_8H_9N_5O_2$ by elemental analysis, confirmed by the molecular ion M^+ at *m/e* 207 observed in the low-resolution mass spectrum.

The nmr spectrum of **3** in trifluoroacetic acid was complex and unrewarding. However, the spectrum was somewhat simplified in trifluoroacetic acid-d_1, and peaks were observed which apparently arise from the presence of two different protonated forms of **3**. The minor component showed a pair of doublets at δ 7.19 and 8.43 (J = 6.5 Hz) assignable to the C-5 and C-6 protons of the 2-pyrimidinone moiety, and a pair of doublets at δ 4.04 and 4.24 (J = 14.5 Hz) indicative of nonequivalent geminal protons. The major component showed a singlet at δ 4.37, a doublet at 7.45 (J = 7.5 Hz), and an unresolved multiplet at 6.76. For comparison, the nmr spectrum of the unreduced photoproduct, Pyo(4–5)Cyt, showed a singlet at δ 8.99 due to the C-6 proton of the cytosine moiety and two doublets at 7.37 and 8.38 (J = 7.5 Hz) assignable to the C-5 and C-6 protons of the 2-pyrimidinone moiety. The disappearance of the C-6 proton resonance at δ 8.99, retention of low-field doublets[10] between 6.5 and 8.5, and

(8) D. F. Rhoades and S. Y. Wang, *Biochemistry*, **10**, 4603 (1971).
(9) E. B. Ziff and J. R. Fresco, *J. Amer. Chem. Soc.*, **90**, 7338 (1968).

(10) Although the peak at δ 6.76 appeared as a broad singlet in the 220-MHz spectrum, it sometimes appeared as a broad doublet (J = 7.5 Hz), especially in 60-MHz spectra. Such peak broadening has often been observed for the C-5 proton of cytosine derivatives. We have observed that the C-5 and C-6 protons of 4-methyl-2-pyrimidinone are

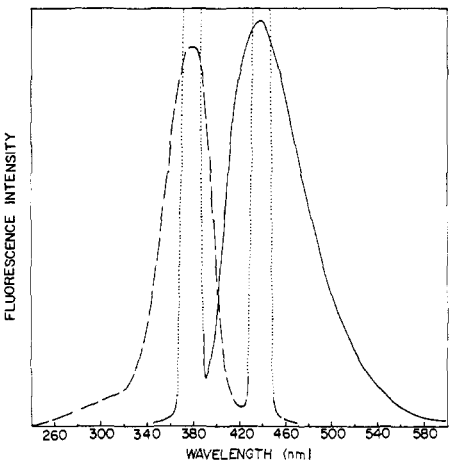


Figure 2. Technical fluorescence excitation (– – –) (λ_{em} 440 nm) and emission spectra (——) (λ_{ex} 386 nm) of Pyo(4–5)hCyt (**3**) in 1,2-propanediol at 20°. The peaks resulting from light scattering by the solvent are shown (· · ·); *cf.* Figure 2 in ref 7.

the appearance of new signals at high field, integrating for two protons, suggested that hydride had added at C-6. The nmr spectrum of Pyo(4–5)hCyt in fluorosulfonic acid clarified the nature of the two components observed in the trifluoroacetic acid-d_1 spectrum. In fluorosulfonic acid two sharp doublets at δ 7.78 and 8.91 (J = 6.5 Hz) were assignable to the C-5 and C-6 protons, a broad singlet at 9.41 was assumed to be due to the two N-4 protons, and a broad singlet at 8.47 was assignable to the N-1 proton. An apparent doublet of doublets at δ 5.14 (J uncertain) had been observed previously in the nmr spectrum of Pyo(4–5)hCyt in trifluoroacetic acid as an unresolved multiplet at 4.91 which integrated for a single proton of the minor component. This peak was not observed in the nmr spectrum of Pyo(4–5)hCyt in trifluoroacetic acid-d_1. The final peak in the fluorosulfonic acid spectrum was a complex multiplet at δ 4.40, integrating for two protons, which is explicable as part of an ABX pattern assignable to the two C-6 protons.[11] Wec onclude that Pyo(4–5)-hCyt has the doubly protonated structure **5** in fluorosulfonic acid and that the singly protonated structure **4** is the major component of an equilibrium mixture of **4** and **5** in trifluoroacetic acid. Consistent with the singly protonated Pyo(4–5)hCyt structure **4**, which would exist in a time-averaged planar conformation with the positive charge distributed over both rings (one contributor to the resonance hybrid is shown), is the singlet in the nmr spectrum for nondifferentiated protons at C-6. By contrast, the nonequivalence of the C-6 protons in the doubly protonated form requires a structure (**5**, one contributor to the resonance hybrid shown) in which the two rings are noncoplanar.

The nmr spectroscopic assignments were checked by the reduction of Pyo(4–5)Cyt (**2**) with sodium borodeuteride, which yielded a product **6** that gave a molecular ion M^+ at m/e 208 in the low-resolution mass spectrum. Whereas the borohydride reduction product **3** showed, *inter alia*, two doublets in the nmr spectrum taken in trifluoroacetic acid-d_1 (δ 4.04 and 4.24, J = 14.5 Hz), corresponding to the doubly protonated species with deuterium at C-5, the borodeuteride reduction product **6** showed two singlets under the same conditions.

sharp doublets in $(CH_3)_2SO$-d_6, but in CF_3COOH are broadened to the extent that they appear as broad singlets similar to the δ 6.76 resonance for compound **3**.

(11) Resolution of the multiplet was too poor to obtain accurate coupling constants.

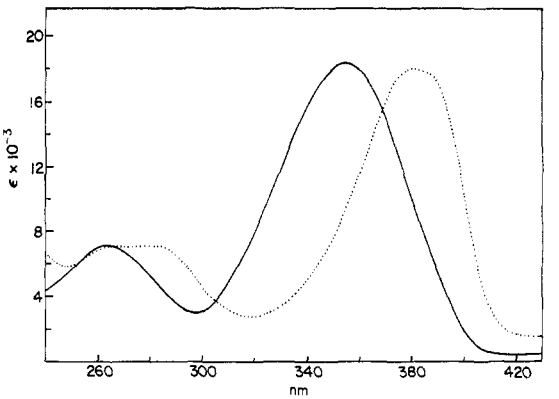


Figure 3. Ultraviolet spectrum of Pyo(4–5)hUra (**8**) in 0.04 M HCl–10% DMSO–H_2O and 10% DMSO–H_2O (——), and 0.005 M NaOH–10% DMSO–H_2O (· · ·).

Compounds Pyo(4–5)Cyt and Pyo(4–5)hCyt were found to be interconvertible. Thus, compound **3** could be oxidized back to **2** by platinum and oxygen in aqueous solution. Precedent for the oxidation was found in the catalytic oxygenation of the borohydride-reduced *cis-syn*-thymine photodimer back to the *cis-syn*-thymine dimer.[12]

Many of the difficulties encountered in studying the spectroscopic properties of Pyo(4–5)hCyt arose because of its low solubility in solvents in which it was stable. A neutral solvent for the observation of the nmr spectrum would have been especially desirable, but, for instance, the low solubility (0.5 mg/ml) of Pyo(4–5)hCyt in dimethyl sulfoxide was only sufficient to allow preparation of solutions for quantitative uv spectra. Although compound **3** was readily soluble in aqueous base or acid, decomposition was evident in both media. The product resulting from treatment of **3** with 1 N hydrochloric acid was characterized as 5-(4-pyrimidin-2-one)-3,6-dihydrouracil, Pyo(4–5)hUra (**8**), and was identical with the product obtained on borohydride reduction of Pyo(4–5)Ura (**7**).

The fluorescence emission of Pyo(4–5)hCyt at 440 nm was examined in different solvents and was found to increase in the order: aqueous solution at neutral and basic pH, 4% dimethyl sulfoxide–ethanol, 1,2-propanediol. In aqueous acid, Pyo(4–5)hCyt was not fluorescent. Even in 1,2-propanediol the intensity of the fluorescence was slight in comparison with the intensity of the solvent scattering at the wavelength of excitation (374 nm) (Figure 2). The absolute quantum efficiencies of Pyo(4–5)hCyt in 1,2-propanediol and water were determined to be 0.013 and approximately 0.001, respectively, by integration and comparison of the peak areas of the corrected emission spectra with the area of the emission peak for quinine sulfate[13] obtained with the same instrument settings.

Treatment of Pyo(4–5)Ura (**7**) with sodium borohydride in aqueous solution gave a white solid with $\lambda_{max}^{H_2O}$ 355 nm (Figure 3) and composition $C_8H_8N_4O_3$ by ele-

(12) T. Kunieda and B. Witkop, *J. Amer. Chem. Soc.*, **93**, 3493 (1971).

(13) T. G. Scott, R. D. Spencer, N. J. Leonard, and G. Weber, *ibid.*, **92**, 687 (1970).

mental analysis. A low-resolution mass spectrum showed a molecular ion M^+ at m/e 208, which was also the base peak in the 9-eV mass spectrum. These data, along with an unambiguous nmr spectrum (CF_3COOH), which showed a singlet at δ 4.36 for the C-6 methylene protons, doublets at 5.96 and 7.38 (J = 7.5 Hz) assignable to the C-5 and C-6 protons at the 2-pyrimidinone moiety, and two broad singlets at 7.40 and 9.32 assignable to the N-1 and N-3 protons of the uracil portion of the molecule,[14] led us to assign structure **8** to the borohydride reduction product of Pyo(4–5)Ura.

Reduction of Pyo(4–5)Sur (**9**) with sodium borohydride gave 5-(4-pyrimidin-2-one)-4-thio-3,6-dihydrouracil, Pyo(4–5)hSur (**10**). The assigned structure followed from spectral data and elemental analysis. Neither Pyo(4–5)hUra nor Pyo(4–5)hSur was fluorescent in aqueous solution. Although we have not distinguished between the two most likely tautomeric forms for Pyo(4–5)hUra (**8a** and **8b**) and Pyo(4–5)hSur (**10a** and **10b**) by spectroscopic means, tautomers **8a** and **10a** are favored for two reasons. First, on the basis of past experience, the keto and thioketo forms of the oxygen- and sulfur-substituted pyrimidines are favored over the enol and thioenol forms. Second, the large hypsochromic shift (61 nm) in the uv maximum of Pyo(4–5)-hSur (λ_{max} 427.5 nm, Figure 4) when it is oxidized to a disulfide by aqueous iodine suggests that thioketone conjugation was responsible for the long-wavelength absorption of **10**. In analogy the uv maxima of 4-thiouridine and its disulfide are 328 and 309 nm, respectively.[15] 1,4-Dithiothreitol (Cleland's reagent) regenerates **10** from its disulfide.

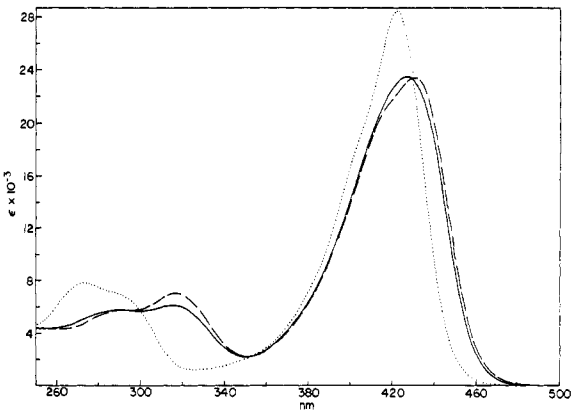


Figure 4. Ultraviolet spectrum of Pyo(4–5)hSur (**10**) in 0.04 *M* HCl–10% DMSO–H_2O (– – –), 10% DMSO–H_2O (——), and 0.005 *M* NaOH–10% DMSO–H_2O (· · ·).

Discussion

5-(4-Pyrimidin-2-one)cytosine, Pyo(4–5)Cyt (**2**), has been identified as the major photoproduct (at the base level) from photolysis of *E. coli* tRNA at 335 nm[1,2] and irradiation of polycytidylic acid, deoxycytidine, and cytidine at pH 4 and 254 nm.[8] The formation of **1**, and of **2** by subsequent hydrolysis, by a photoreaction between the 4-thiouridine in position 8 and the cytidine in position 13 from the 5′-terminal end of *E. coli* $tRNA_1^{Val}$, $tRNA_{2A}^{Val}$, $tRNA_{2B}^{Val}$, $tRNA^{Phe}$, $tRNA_m^{Met}$, $tRNA_f^{Met}$,[1] and $tRNA^{Arg}$ [5] is of interest because of the detailed information it provides concerning the tertiary structure in the dihydrouridine-arm region of the molecule.[2b] Moreover, an added dividend is the facility with which Pyo(4–5)Cyt can be converted to a fluorescent derivative, Pyo(4–5)hCyt (**3**), by treatment with sodium borohydride. The structure proof of 5-(4-pyrimidin-2-one)-3,6-dihydrocytosine (**3**) implies a similar structure, ribose-substituted at both 1 positions, for the sodium borohydride reduction product of 5-(1-β-D-ribofuranosyl-4-pyrimidin-2-one)cytidine, Pdo(4–5)Cyd (**1**), and thus settles the question of the structure of the reduced cross-linked moiety in tRNA. We recognize, of course, that the reactivity of Pyo(4–5)Cyt may be altered by change in environment from base to nucleoside to polynucleotide levels.

The introduction of a covalently bonded fluorescent probe in tRNA is clearly of interest for studying tRNA conformation and interaction.[7] In addition, the borohydride reduction of Pdo(4–5)Cyd (**1**) or Pyo(4–5)Cyt (**2**) provides a sensitive detection method for the photoproduct. The excitation and emission spectra of reduced photoproduct in $tRNA_1^{Val}$ have been reported[7] and the quantum yield and fluorescent lifetime measured (22 ± 5% and <5 nsec). When the photoproduct was first isolated from $tRNA_1^{Val}$ as a binucleotide and then reduced, the fluorescence quantum yield in aqueous solution was estimated to have decreased by a factor of 400.[1b,7] The absolute quantum efficiencies of 0.013 and approximately 0.001 for Pyo(4–5)hCyt which we observed in 1,2-propanediol and water, respectively, indicating that the fluorescence intensity of Pyo(4–5)hCyt increases with decreasing solvent polarity, are consistent with Favre and Yaniv's conclusion[7] that Pyo(4–5)hCyt must lie within a hydrophobic region in the transfer RNA. The fluorescence of Pyo(4–5)hCyt spotted on cellulose tlc plates and viewed under long-wavelength uv light is intense enough to allow ready visual detection of as little as 2–5 ng (10 pmol).

Sodium borotritide reduction has been used for the quantitative determination of dihydrouridine, 4-thiouridine, and N^4-acetylcytidine in tRNA,[6] and of cyclobutane photodimers in polynucleotides and deoxyribonucleic acid.[16] For the detection of small amounts of Pyo(4–5)Cyt quantitatively, reduction with sodium borotritide would give Pyo(4–5)hCyt tritiated at C-6. One may speculate that the amount of photoproduct could then be determined from the ^3H activity of any of a number of isolated products: Pyo(4–5)hUra by direct degradation of the irradiated nucleic acid with 1 *N* HCl, Pdo(4–5)hCyd by an enzymic isolation procedure, or Pyo(4–5) Cyt by the catalytic oxygenation (O_2,Pt) of Pyo(4–5)hCyt in the nucleic acid followed by acid degradation. Since it is of further interest to determine the importance of Pyo(4–5)Cyt to the photobiology of DNA,[8] the fluorescent product of borohydride reduction should also aid in these studies.

Experimental Section[17]

5-(4-Pyrimidin-2-one)cytosine, Pyo(4–5)Cyt (2). A buffer solution, pH 9.8, was prepared by combining equal volumes of 7.4 *M*

(14) For comparison, the N-1 and N-3 protons of dihydrouracil (CF_3COOH) fall at δ 7.34 and 9.36, respectively.

(15) J. J. Fox, D. Van Praag, I. Wempen, I. L. Doerr, L. Cheong, J. E. Knoll, M. L. Eidinoff, A. Bendich, and G. B. Brown, *J. Amer. Chem. Soc.*, **81**, 178 (1959).

(16) (a) T. Kunieda and B. Witkop, *ibid.*, **89**, 4232 (1967); (b) T. Kuneida, L. Grossman, and B. Witkop, *Biochem. Biophys. Res. Commun.*, **33**, 453 (1968); (c) B. Witkop, *Photochem. Photobiol.*, **7**, 813 (1968).

(17) Melting points, determined using a Büchi melting point apparatus, are uncorrected. Ultraviolet spectra were taken in dimethyl sulfoxide–water (1:9, v/v) with a Cary 15 spectrophotometer and technical

ammonium hydroxide and 8.0 *M* ammonium chloride. 5-(4-Pyrimidin-2-one)-4-thiouracil (**9**) (58.5 mg, 0.264 mmol) was dissolved in a solution of concentrated ammonium hydroxide (1 ml) and water (10 ml) and the solution was diluted with pH 9.8 buffer (100 ml). To a solution of pH 9.8 buffer (100 ml), water (25 ml), and 0.3 *M* sodium periodate (10 ml), the buffered solution of Pyo(4–5)Sur (**9**) was added dropwise over a period of 1 hr. After stirring an additional hour at room temperature the reaction mixture was stored overnight at 5° and filtered, and the solid product was collected and washed thoroughly with water. The crude product was dissolved in 0.1 *M* HCl (18 ml), filtered, and reprecipitated by neutralization of the solution with ammonium hydroxide. Filtration and drying *in vacuo* gave 41.1 mg of Pyo(4–5)Cyt·H_2O (70%). The identity of Pyo(4–5)Cyt (**2**) prepared in this way with that obtained photochemically from cytosine and 4-thiouracil[2] was shown by thin-layer chromatography in three different solvent systems, ultraviolet spectra in acidic, basic, and neutral aqueous solution, and direct comparison of physical properties and solubility behavior.

5-(4-Pyrimidin-2-one)-3,6-dihydrocytosine, Pyo(4–5)hCyt (3). 5-(4-Pyrimidin-2-one)cytosine (**2**) (16.8 mg, 0.075 mmol) was dissolved in 0.14 *N* HCl (7 ml) and diluted to 150 ml with water (distilled under nitrogen). To the vigorously stirred solution under nitrogen was added 1 *M* NaOH (1.0 ml) followed immediately by aqueous 1 *M* $NaBH_4$ (1.0 ml). The reaction mixture was stirred for 45 min at room temperature and cooled for 30 min in an ice bath, and 1 ml of acetone was added to decompose the unreacted borohydride. The cold reaction mixture was filtered, and the pale yellow precipitate was washed thoroughly with cold water to give, after drying, 11.8 mg of **3** (76%): mp >340° dec; nmr (CF_3COOD) showed two components to be present, minor with δ 4.04 (d 1, *J* = 14.5 Hz), 4.24 (d, 1, *J* = 14.5 Hz), 7.19 (d, 1, *J* = 6.5 Hz), 8.43 (d, 1, *J* = 6.5 Hz); major with δ 4.37 (s, 2), 6.76 (br m, 1), 7.45 (d, 1, *J* = 7.5 Hz); nmr (FSO_3H) δ 4.40 (m, 2), 5.14 (d of d, 1, *J* uncertain), 7.78 (d, 1, *J* = 6.5 Hz), 8.47 (br s, 1), 8.91 (d 1, *J* = 6.5 Hz), 9.41 (br s, 2); tlc, R_f in system A, 0.20; B, 0.21; C, 0.29; λ_{max} 374 nm (ε 24,300), 264 (5760); $\lambda_{max}^{0.04\ N\ HCl}$ 377 (14,300), 259 (4820); $\lambda_{max}^{0.005\ M\ NaOH}$ 368 (1260), 344 (1340), 297 (5690). The mass spectrum (70 eV) showed prominent peaks at *m/e* (rel intensity) 207 (5), 150 (7), 149 (36), 148 (22), 135 (6), 124 (8), 123 (9), 122 (11), 121 (15), 120 (8), 105 (7), 96 (12), 95 (8), 94 (9), 80 (5), 79 (5), 69 (5), 68 (14), 67 (6), 66 (5), 55 (5), 54 (6), 53 (8) 52 (13), 51 (6), 44 (22), 43 (100), 42 (33), 41 (8), 40 (8), 39 (6), 32 (5), 29 (17), 28 (44), 27 (10), 26 (6); (8.7 eV) *m/e* (rel intensity) 207 (47), 178 (22), 176 (17), 167 (15), 166 (79), 152 (70), 150 (26), 149 (100).

Anal. Calcd for $C_8H_9N_5O_2$: C, 46.38; H, 4.38; N, 33.80. Found: C, 46.12; H, 4.43; N, 33.45.

5-(4-Pyrimidin-2-one)-3,6-dihydrocytosine-6-d_1 (6). 5-(4-Pyrimidin-2-one)cytosine (**2**) (34.5 mg, 0.168 mmol) was reduced with sodium borodeuteride (70.2 mg, 1.68 mmol) essentially as described above for the sodium borohydride reduction, to give **6** (23.9 mg, 68% yield): mp >340° dec; λ_{max} 374 nm; nmr (CF_3COOD) showed two components to be present, minor with δ 4.04 (s, 0.5), 4.24 (s, 0.5), 7.20 (d, 1, *J* = 6.5 Hz), 8.44 (d, 1, *J* = 6.5 Hz); major with δ 4.36 (s, 1), 6.84 (d, 1, *J* = 7.5 Hz), 7.46 (d, 1, *J* = 7.5 Hz); R_f values in three solvent systems were identical with the R_f values of Pyo(4–5)hCyt (**3**). The mass spectrum (70 eV) showed prominent peaks at *m/e* (rel intensity) 208 (3), 152 (5), 151 (20), 150 (41), 149 (32), 148 (14), 125 (8), 124 (10), 123 (14), 122 (15), 121 (12), 107 (5), 106 (6), 97 (6), 96 (15), 95 (13), 94 (5), 81 (5), 80 (6), 79 (6), 69 (6), 68 (16), 67 (7), 55 (7), 54 (7), 53 (10), 52 (12), 44 (9), 43 (100), 42 (20), 41 (8), 40 (10), 29 (14) 28 (23), 27 (5); mass spectrum (8.7 eV) *m/e* (rel intensity) 208 (14), 168 (13), 167 (22), 152 (22), 151 (42), 150 (100), 149 (29).

5-(4-Pyrimidin-2-one)-3,6-dihydrouracil, Pyo(4–5)hUra (8). To a solution of 5-(4-pyrimidin-2-one)uracil (**7**) (90.5 mg, 0.44 mmol) in 300 ml of 0.007 *M* NaOH was added sodium borohydride (100 mg, 2.64 mmol). The reaction mixture was buffered to pH 9 with 0.5 *M* KH_2PO_4 and stirred for 30 min at room temperature, and a further portion of sodium borohydride (20 mg, 0.53 mmol) was added. After stirring 15 min more at room temperature the reaction mixture was buffered to pH 7 with 0.5 *M* KH_2PO_4 and the excess $NaBH_4$ was decomposed by adding 1 ml of acetone. The light yellow precipitate which formed on storing the solution overnight at 5° was collected and dried *in vacuo* to give 66.4 mg (73%) of **8**. The compound was obtained analytically pure by recrystallization from CF_3COOH-CH_3COOH: mp >340°; nmr (CF_3COOH) δ 4.36 (s, 2), 5.96 (d, 1, *J* = 7.5 Hz), 7.38 (d, 1, *J* = 7.5 Hz), 7.40 (br s, 1), 9.32 (s, 1); R_f in system A, 0.43; B, 0.41; C, 0.48; λ_{max} 355 nm (ε 17,600), 263 (7100); $\lambda_{max}^{0.04\ M\ HCl}$ 355 (18,300), 263 (7100); $\lambda_{max}^{0.005\ M\ NaOH}$ 380 (16,700), 280 (5830), 266 (5830). The mass spectrum (70 eV) showed prominent peaks at *m/e* (rel intensity) 208 (42), 207 (100), 164 (48), 137 (25), 136 (34), 135 (17), 122 (12), 121 (33), 113 (22), 108 (16), 96 (30), 95 (12), 94 (12), 93 (12), 82 (14), 68 (27), 67 (23), 66 (23), 65 (11), 54 (10), 53 (16), 52 (26), 51 (11), 44 (25), 43 (31), 42 (13), 41 (15), 40 (19), 39 (15), 32 (13), 29 (10), 28 (74), 27 (11); mass spectrum (9 eV) *m/e* (rel intensity) 209 (13), 208 (100), 207 (38), 206 (11), 113 (4), 96 (5).

Anal. Calcd for $C_8H_8N_4O_2$: C, 46.16; H, 3.87; N, 26.91. Found: C, 46.25; H, 3.94; N, 27.10.

5-(4-Pyrimidin-2-one)-4-thio-3,6-dihydrouracil, Pyo(4–5)hSur (10). To a solution of 5-(4-pyrimidin-2-one)-4-thiouracil (**9**) (41.3 mg, 0.186 mmol) in 20 ml of 0.1 *M* ammonium bicarbonate (pH 9.1) was added sodium borohydride (32.2 mg, 0.85 mmol). The reaction mixture was stirred for 10 min at room temperature, then quenched by adding consecutively 1 ml of acetone and 5 ml of 0.5 *M* H_3PO_4, and placed in an ice bath for 2 hr. The precipitate was collected and dried to give 29.5 mg (71%) of **10**. Analytically pure **10** was obtained by chromatography on Sephadex LH-20 and elution with *N,N*-dimethylformamide: mp >340° dec; nmr (CF_3COOH) δ 4.39 (s, 2), 6.13 (d, 1, *J* = 7.5 Hz), 7.30 (br s, 1), 7.47 (d, 1, *J* = 7.5 Hz), 9.32 (s, 1); R_f in system A, 0.59; B, 0.50; C, 0.59; λ_{max} 427.5 nm (ε 23,500), 316 (6110), 289.5 (5770); $\lambda_{max}^{0.04\ N\ HCl}$ 431 (23,300), 316 (6950), 291 (5650); $\lambda_{max}^{0.005\ M\ NaOH}$ 423 (28,400), 273 (7860); mass spectrum (70 eV) *m/e* (rel intensity) 224 (17), 223 (6), 191 (5), 73 (11), 68 (9), 62 (6), 60 (100), 59 (5), 44 (21), 43 (87), 42 (19), 34 (18), 33 (8), 32 (45), 30 (6), 29 (15), 28 (65), 27 (8), 26 (5).

Anal. Calcd for $C_8H_8N_4O_2S$: C, 42.85; H, 3.60; N, 24.98. Found: C, 43.13; H, 3.64; N, 24.73.

Oxidation of Pyo(4–5)hCyt (3) to Pyo(4–5)Cyt (2). Active platinum was prepared by reducing platinum oxide (18.6 mg) with H_2 in 25 ml of water. A suspension of the active platinum and compound **3** (3.4 mg, 0.016 mmol) was prepared in 25 ml of water. After sparging with oxygen for 1 min at room temperature, 2.0 ml of 1 *N* HCl was added to the reaction mixture. The oxygen sparging was discontinued after 15 min, the reaction mixture was filtered, and the water was removed *in vacuo*. The white residue was collected, washed thoroughly with ethanol, and dried to give 3.5 mg (89%) of **2** as the hydrochloride salt. The quantitative uv spectra of the product at acidic, neutral and basic pH were identical with those of authentic Pyo(4–5)Cyt.[2] The identity of the compound was further established by comparative tlc in three solvent systems and by its rereduction to Pyo(4–5)hCyt with sodium borohydride.

Hydrolysis of Pyo(4–5)hCyt (3) to Pyo(4–5)hUra (8). A solution of Pyo(4–5)hCyt (1.1 mg) in 1 *N* HCl (0.8 ml) was allowed to stand at room temperature overnight. The white precipitate which formed was collected, washed with water, and dried *in vacuo* yielding 1.0 mg of Pyo(4–5)hUra. The identity of Pyo(4–5)hUra prepared in this way with that obtained from the sodium borohydride reduction of Pyo(4–5)Ura was shown by tlc in three different solvent systems, uv spectra in acidic, basic, and neutral aqueous solution, and by the mass spectrum.

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fluorescence emission spectra with a Hitachi Perkin-Elmer MPF-2A fluorescence spectrophotometer. Absolute quantum efficiencies were determined by integration of the corrected spectra obtained with a digital spectrofluorometer as previously described.[13] As a reference the absolute quantum efficiency of quinine sulfate was taken as 0.70.[13] Proton magnetic resonance (pmr) spectra were determined on a Varian HA-100 or HR-220 spectrometer with tetramethylsilane (TMS) or tetramethylammonium fluoroborate as the internal standard. The tetramethylammonium fluoroborate resonance in fluorosulfonic acid was taken as δ 3.10.[18] The low-resolution mass spectral data were obtained on a MAT CH-5 spectrometer. Thin-layer chromatography (tlc) was carried out on 200 × 40 × 0.16 mm Eastman Chromagram sheets, cellulose without fluorescent indicator, in the following solvent systems: A, *n*-propyl alcohol–water (7:3, v/v); B, ethanol–1.0 *M* ammonium acetate (7:3, v/v), buffered to pH 7.95 with concentrated NH_4OH; C, *n*-propyl alcohol–concentrated NH_4OH–water–formic acid (60:29:10:1), v/v). Spots were visualized by long-wavelength uv light. Elemental microanalyses were performed by Mr. Josef Nemeth and his associates at the University of Illinois and by Midwest Microlab, Inc., Indianapolis, Ind.

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