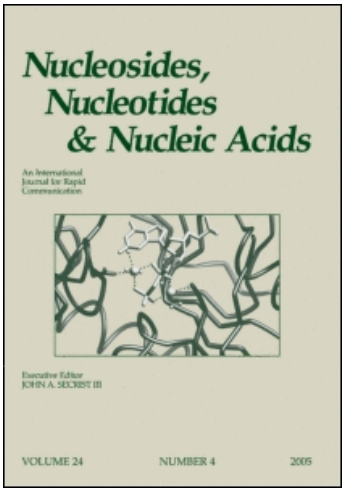

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REACTION OF 5-HALOCYTOSINE DERIVATIVES WITH CYSTEINE[1a,b]

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Abstract: 5-Bromo-(1) and 5-iodo-2'-deoxycytidine (2) undergo 100 percent dehalogenation to 2'-deoxycytidine on heating with cysteine in 1N aq K_2CO_3 in a nitrogen atmosphere at 50° for 72 h and 21 h, respectively. 5-Chloro-2'-deoxycytidine (3) and 1-methyl-5-chlorocytosine (3m) on the other hand undergo very little dehalogenation, forming instead two products, 4, 5 and 4m, 5m respectively, whose structures have been determined by mass spectrometry and proton magnetic resonance spectroscopy of the 3m products: 1-methyl-5-(cystein-S-yl)cytosine (4m) and 3-methyl-2,6,7,8-tetrahydro-2-oxo-3H-pyrimido [5,4-b][1,4]-thiazine-7-carboxylic acid (5m), mp. 236°C, 78 percent yield. Initially produced 4m undergoes a novel and facile cyclization to form 5m with loss of NH_3 in the presence of cysteine. The compound 3m, mp. 258°C, has been synthesized by treating 1-methylcytosine with N-chlorosuccinimide in acetic acid at 105° for 3 h in 56 percent yield. Ultraviolet absorption spectral properties of 1, 2, 3, 3m, 4, 4m, 5, and 5m are reported.

Introduction

An estimated 100,000 tons of chlorine are used annually for the treatment of sewage in the United States. Although 99 percent of the chlorine used is converted into the innocuous chloride ion, about 1 percent (1000 tons per year) is converted into stable chloro-organics and discharged into our aquatic environment. Jolley[2] had identified 5-chlorouracil and 5-chlorouridine among the major chloro-organics formed during chlorination of sewage, concentrations being 4.3 and 1.7 µg/L, respectively. Apparently, these two compounds can survive for a long time in the environment, since Crathorne et al.[3] have found both of them in drinking water from several sources. Although the

presence of 5-chlorocytosine or its derivatives in the environment has not yet been reported, the base has been found to be a constituent of salmon sperm DNA by Lis et al.[4,5] Recent studies by Pal et al.[6] indicate that 5-chlorouracil is heavily incorporated into mouse liver DNA (1/250 nucleotides) and RNA (1/100 nucleotides) when the animals ingest the base in their drinking water. These findings provided the impetus to study the chemistry of 5-chloropyrimidines.

It has been shown[7] that 5-I-dU undergoes 100 percent dehalogenation to form dU on treatment with cysteine. 5-Cl-dU, on the other hand, forms 5-cystein-S-yl-dU in practically quantitative yield, while 5-Br-dU forms both dU and 5-cystein-S-yl-dU on treatment with cysteine. The relative yield of the two products in this case depends on the pH of the reaction mixture. The reaction of 5-halocytosine derivatives with cysteine and characterization of the products is the subject of this report.

Results and Discussion

Compared with 5-I-dU, 5-I-dC has been found to be less reactive towards cysteine. Nevertheless, it undergoes 100 percent dehalogenation in aqueous K_2CO_3 in the presence of cysteine to form dC. 5-Br-dC, on the other hand, behaves quite differently from 5-Br-dU. It undergoes 100 percent dehalogenation to form dC. In contrast with 5-Br-dC, 5-Br-dU undergoes both dehalogenation to form dU and substitution to form 5-Cys-dU in 2:1 and 1:2 molar ratios at pH's 7 and 12 respectively.[7]

The reaction of 5-Cl-dC with cysteine in aqueous K_2CO_3 has turned out to be unique among the family of 5-halodeoxycytidines. In the initial phase of our investigations, we found that two major products 4 and 5 were formed as revealed by analysis of the reaction mixture on Aminex A-6 (Bio-Rad cation-exchanger) column using 0.1 M ammonium borate as eluant. A parallel experiment with ^{14}C-labelled cysteine indicated that both the products contained the label from cysteine (Fig. 1). To rule out the involvement of the 2'-deoxyribosyl group in this reaction and to facilitate identification by mass spectrometry and UV spectrophotometry, we decided to synthesize the 1-Me-5-Cl-Cyt and study its reaction with cysteine under similar conditions. 1-Me-5-Cl-Cyt was prepared by chlorination of 1-Me-Cyt with N-chlorosuccinimide. The chromatographic analysis of the reaction mixture of 1-Me-5-Cl-Cyt and cysteine in aqueous K_2CO_3 revealed the formation of two products (4m and 5m), with UV absorption spectra very similar to those of 4 and 5 respectively (Fig. 2). The UV absorption spectra of 5-Cl-dC and its 1-methyl analog

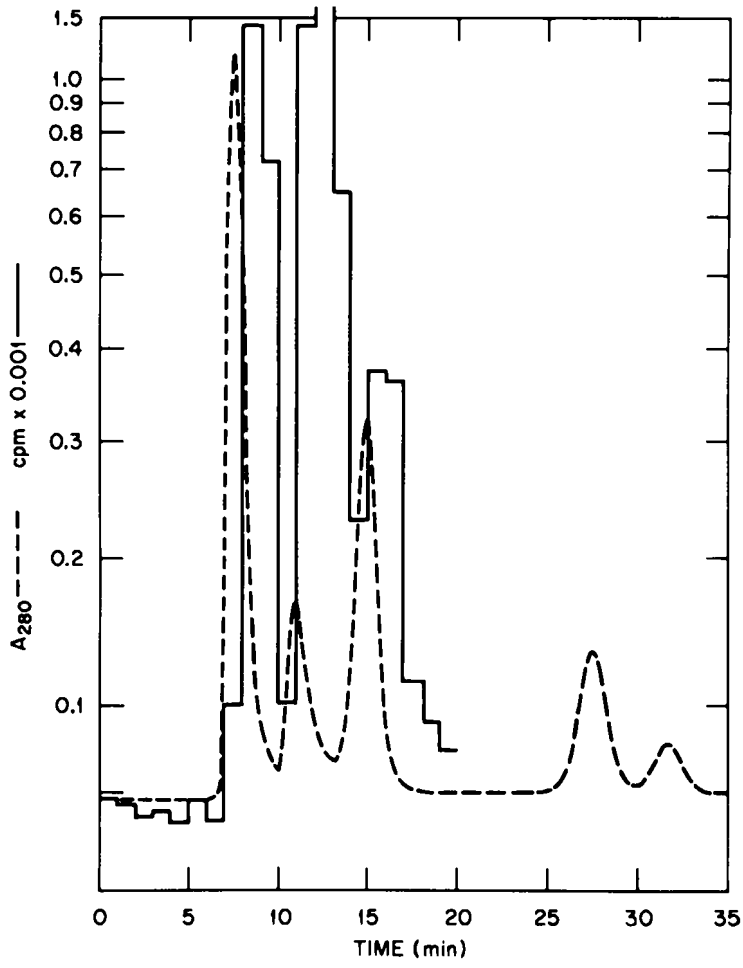


FIGURE 1. Chromatography of the reaction mixture of 5-Cl-dC, [^{14}C]cysteine, and aqueous 1 N K_2CO_3 after heating at 60° for 24 h, on Aminex A-6 column (20 x 0.63 cm dia.) using 0.1 N ammonium borate, pH 7.4, as eluant, flow rate 0.3 mL/min.

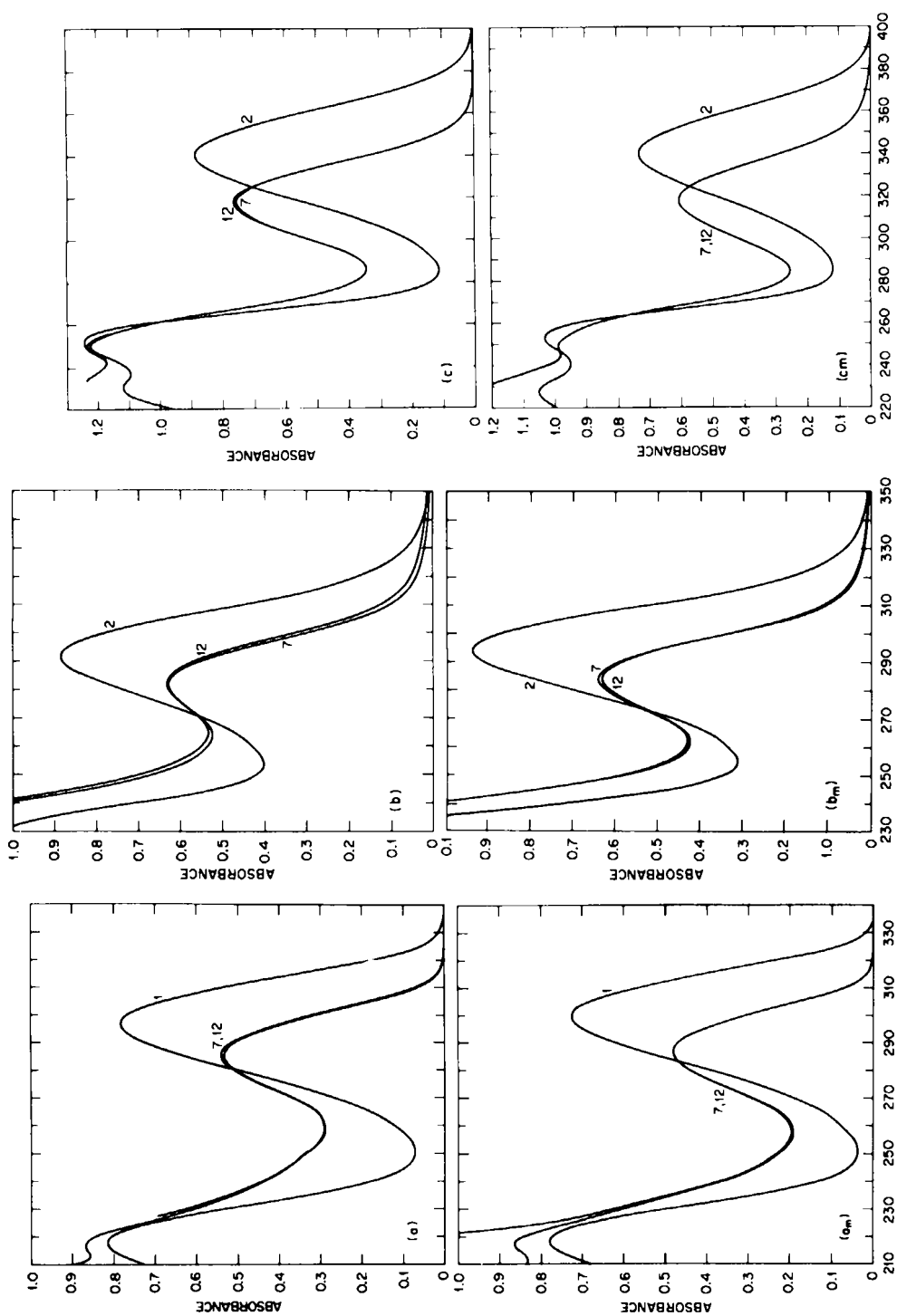


FIGURE 2. Ultraviolet absorption spectra of 5-Cl-dC (a), 1-Me-5-Cl-Cyt (a_m) at pH's 1, 7, and 12. Ultraviolet absorption spectra of 4 (b), 4_m(b_m), 5 (c), and 5_m(c_m) at pH's 2, 7, and 12.

are similar (Fig. 2). The similarity of the UV absorption spectra of 4, 4m and 5, 5m indicate the structural similarity of the two sets of compounds and the elucidation of the structures of 4m and 5m will lead to the identity of the nucleoside derivatives 4 and 5.

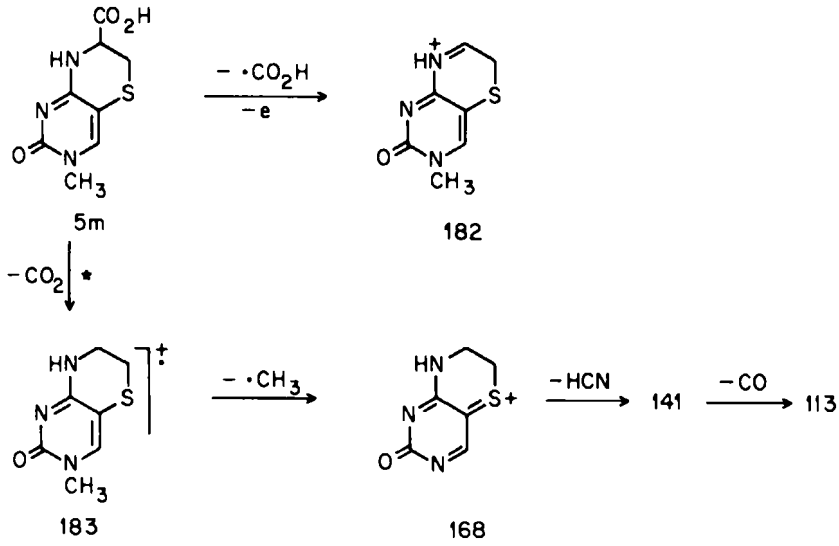
Structure of 5m. The elemental analysis agreed with the structure proposed for 5m (Scheme 1). An attempt to deaminate 5m with nitrous acid was not successful.

The electron ionization mass spectrum of 5m following thermal vaporization is shown in Fig. 3. The molecular ion pattern at m/z 227-229 supports the presence of sulfur and unambiguously demonstrates the absence of chlorine. Confirmation of the molecular weight as 227 was gained by fast atom bombardment (FAB) mass spectrometry. The positive ion FAB spectrum showed MH^+, m/z 228, and M·glycerol·H^+, m/z 320 (36% intensity of m/z 228) and m/z 226 for $(M-H)^-$ in the negative ion spectrum. The exact mass of the MH^+ ion was measured as m/z 228.0442, corresponding to a molecular weight of 227.0370. The elemental composition of 5m was thereby established as $C_8H_9N_3O_3S$ (calc. 227.0365), a value requiring loss of HCl and NH_3 from a combination of one molecule each of cysteine and 1-Me-5-Cl-Cyt.

The number of active hydrogens was determined from the FAB mass spectrum following hydrogen-deuterium exchange.[8] The molecular species MD^+ was observed at m/z 231, revealing the presence of 2 active hydrogens in 5m.

Trimethylsilylation of 5m yielded a mixture of products of molecular weights 371 and 299 (Fig. 4). These values correspond to the mono- and bis-silyl derivatives, as determined from mass shifts of 9 and 18 units, resulting from use of $Si(CD_3)_3$ blocking groups[9] in a separate experiment. Incomplete silylation under the conditions employed is often observed with derivatives of cytosine.[10] The silyl derivative further supports the molecular weight of 227, and provides independent corroboration of the presence of a free carboxyl group in 5m, through loss of 117 mass units (m/z 371→254 and 299→182, Fig. 4), assigned as CO_2SiMe_3, and supported by deuterium mass shifts as shown in Fig. 4. The presence of a CO_2 moiety in 5m is also shown by loss of CO_2 (m/z 183) and CO_2H (m/z 182) in Fig. 3, supported by measurements of exact mass. The peak at m/z 162 is due primarily to an impurity, as determined from repetitive scans of the spectrum.

The molecular composition $C_8H_9N_3O_3S$ requires a total of 6 rings and double bonds, which is two more than cytosine or 1-Me-5-Cl-Cyt. One is



Scheme 1

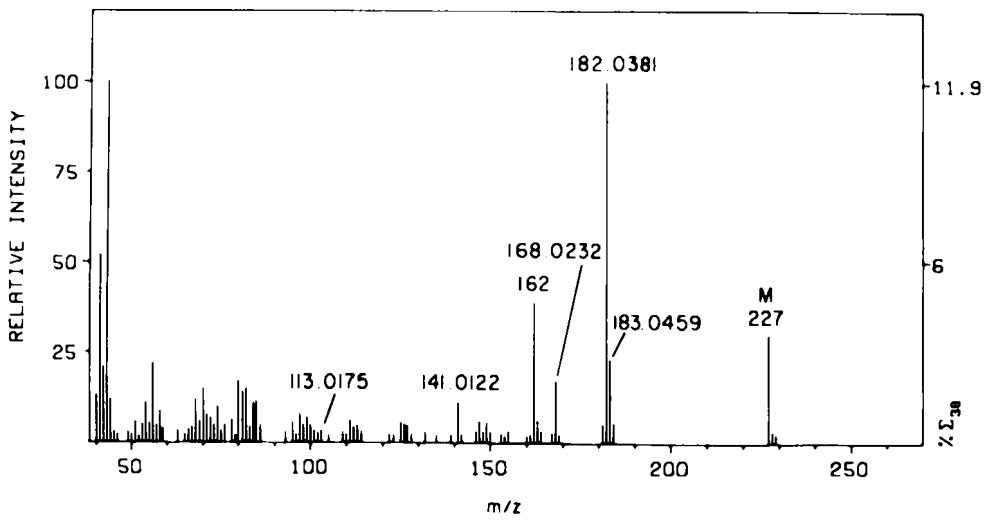


FIGURE 3. Electron ionization mass spectrum of reaction product 5m.

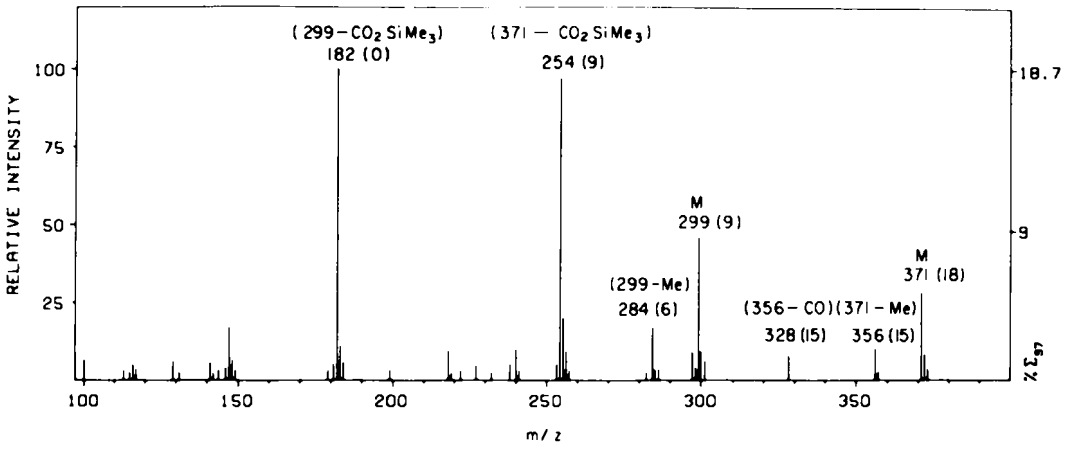


FIGURE 4. Electron ionization mass spectrum of 5m following trimethylsilylation. Values in parentheses refer to mass shifts from a separate experiment in which [$^2H_9$]trimethylsilyl blocking groups were introduced.

present as a carboxyl group; the other is judged to be a ring, based on the presence of only two active hydrogens in the molecule. If one hydrogen is in the carboxyl group, the remaining one must be at N^4. All of these results and conclusions, and earlier results which defined the site of cysteine substitution,[7] result in the structure shown in Scheme 1. The major ions from the mass spectrum shown in Fig. 3 are readily rationalized in terms of the bicyclic structure, as indicated above, with all assignments supported by measurements of exact mass. Pathways supported by metastable transitions are denoted by asterisks. Losses of CO and HCN are characteristically observed in the mass spectra of nucleic acid bases[7,10] and confer no structural details.

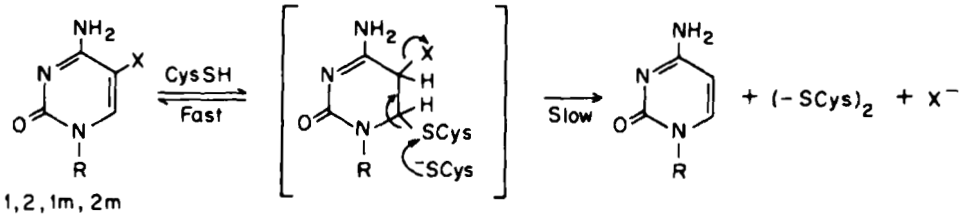
The structure assignment of 5m is also supported by the ^{1}H-NMR spectral data. The H-5 and H-6 of the pyrimidine ring are distinguishable by their ^{1}H-NMR spectrum (e.g., 1-methylcytosine, δ 5.63, H-5, 7.56, H-6). The ^{1}H-NMR spectrum of 1-methyl-5-chlorocytosine shows the presence of H-6 at δ, 8.01. The ^{1}H-NMR spectrum of 5m also shows the presence of H-4 at δ, 7.49 which is equivalent to H-6 of 1-methyl-5-chlorocytosine. This rules out the possible substitution of the cysteine moiety at C-6 of the pyrimidine ring of 3m in its reaction with cysteine.

The ultraviolet absorption spectra of 4, 4m (Fig. 2b, 2bm), are expected to be similar to those of 5, 5m (Fig. 2c, 2cm) respectively since they have similar substituents in the 4 and 5-position of the pyrimidine ring; but they are not; the most striking difference is the unusually large shift of ~48 nm in the position of λmax at pH 2. This probably suggests that the electronic structures of 5, 5m are not as shown in Scheme 2. We propose a zwitterion structure for 5, 5m (Scheme 2) which is supported by the X-ray crystallographic data obtained by C. H. Wei of Oak Ridge National Laboratory from crystalline 5m.

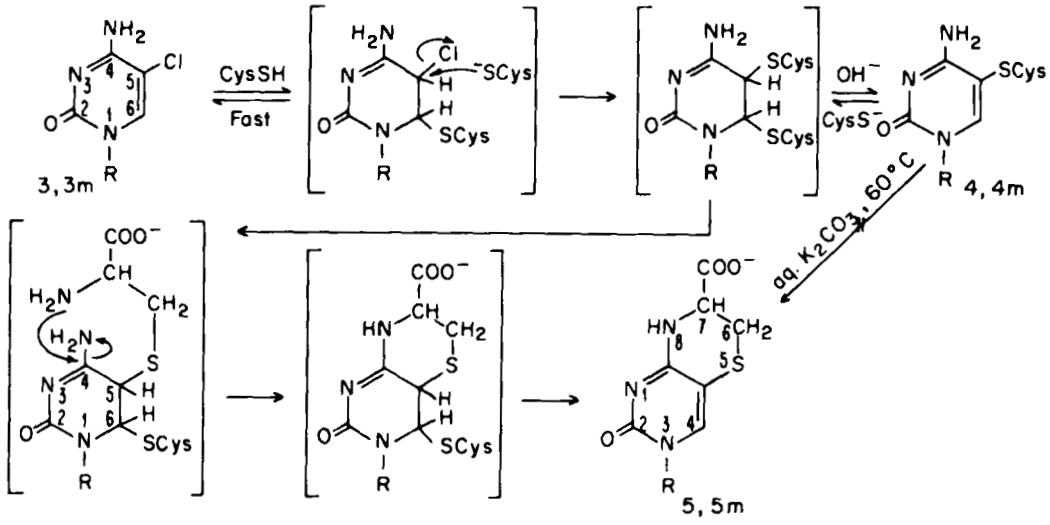
Structure of 4m. The high resolution FAB mass spectrum of 4m showed MH^+ = m/z 245.0713, indicating an exact molecular mass of 244.0634. The isotope pattern supports the presence of sulfur and demonstrates the absence of chlorine. These data support the molecular composition $C_8H_{12}N_4O_3S$ (calc. 244.0630), corresponding to one molecule each of 1-Me-5-Cl-Cyt and cysteine, minus HCl. The mass spectrum of the trimethylsilyl derivative of 4m, shown in Fig. 5, shows a molecular weight of 460 as expected for the tris(trimethylsilyl) derivative of a compound of molecular weight 244. The presence of the free α-amino acid function in 4m is shown by the m/z 218 fragment ion,[11,12] while m/z 229 and the silyl rearrangement[13] product m/z 301 and their daughter ions demonstrate the attachment of sulfur directly to the cytosine ring. From the assignments made in Scheme 3, and the precedent[7] of dehalogenation with cysteine substitution at C-5, the structure of 4m is assigned as 1-methyl-5-(cysteine-S-yl)cytosine.

The structure assignments for 5m and 4m, which differ only by the elements of NH_3, were further supported by demonstrating the conversion of 4m to 5m by heating the former in aqueous K_2CO_3 containing cysteine. The identity of 5m obtained by this conversion was verified by FAB mass spectrometry, ultraviolet absorption spectra, and chromatographic analysis.

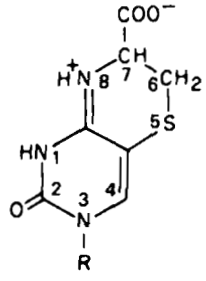
Isolation of 5m. The separation of 5m and 4m could not be achieved by chromatography on DEAE-Sephadex, Dowex-1, or silica gel. To avoid this problem, the reaction mixture was treated again with another lot of cysteine so as to completely convert 4m into 5m. Potassium was removed from the reaction mixture by treatment with Dowex-50-(H^+). It was filtered and the filtrate was treated with H_2O_2 in the presence of a catalytic amount of $FeCl_2$ to oxidize cysteine to cystine, which precipitated and was removed by filtration. The filtrate was subjected to silicic acid chromatography to obtain analytically pure 5m in



1, X = Br, R = 1-β-D-2'-deoxyribofuranosyl- 1m, X = Br, R = CH_3
2, X = I, R = 1-β-D-2'-deoxyribofuranosyl- 2m, X = I, R = CH_3



3, R = 1-β-D-2'-deoxyribofuranosyl- 3m, R = CH_3-
4, R = 1-β-D-2'-deoxyribofuranosyl- 4m, R = CH_3-
5, R = 3-β-D-2'-deoxyribofuranosyl- 5m, R = CH_3-



5, R = 3-β-D-2'-deoxyribofuranosyl-
5m, R = CH_3-

Zwitterion form of 5 and 5m

Scheme 2

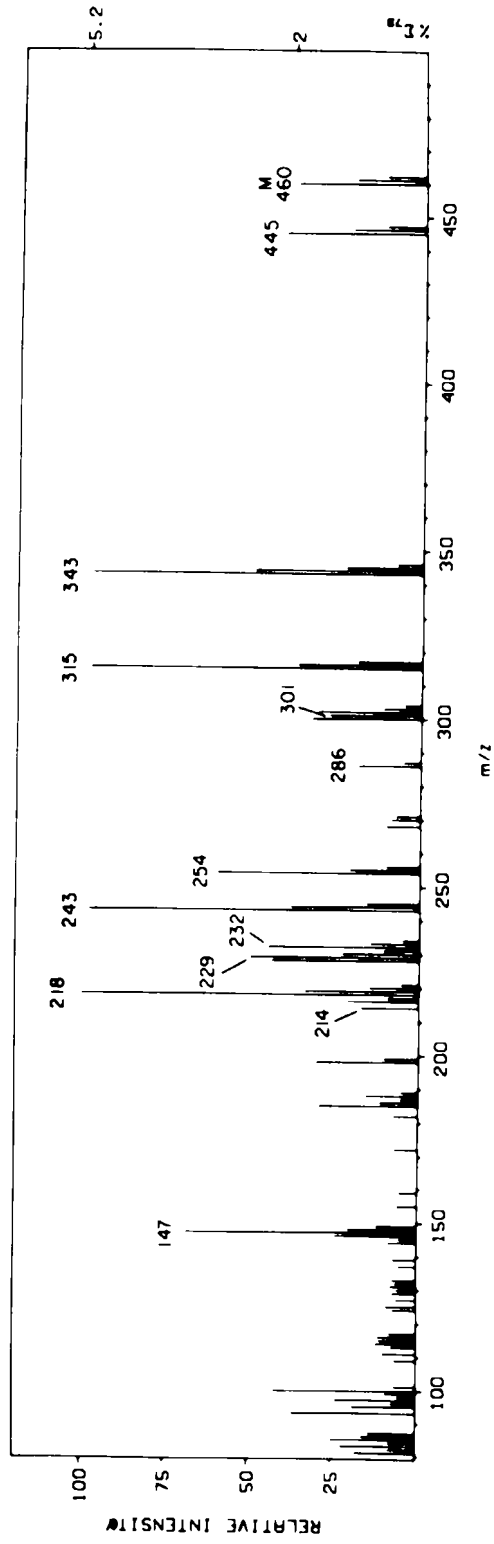
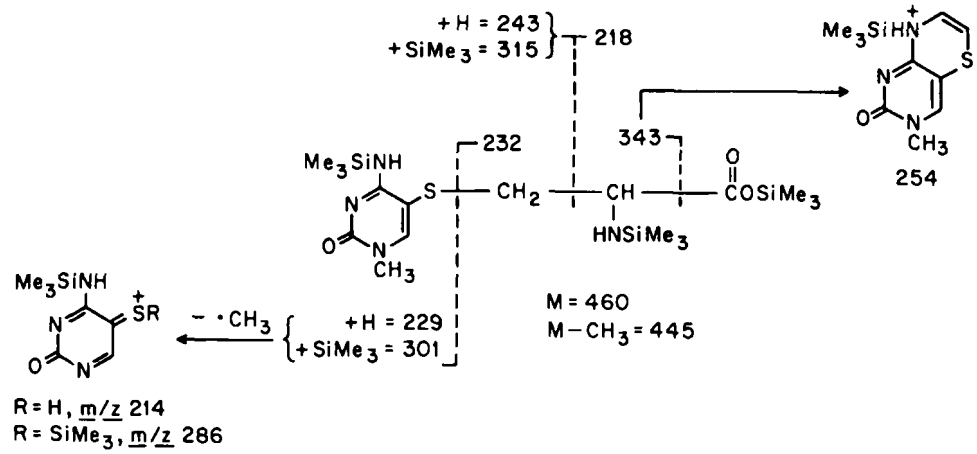


FIGURE 5. Electron ionization mass spectrum of 4m following trimethylsilylation.

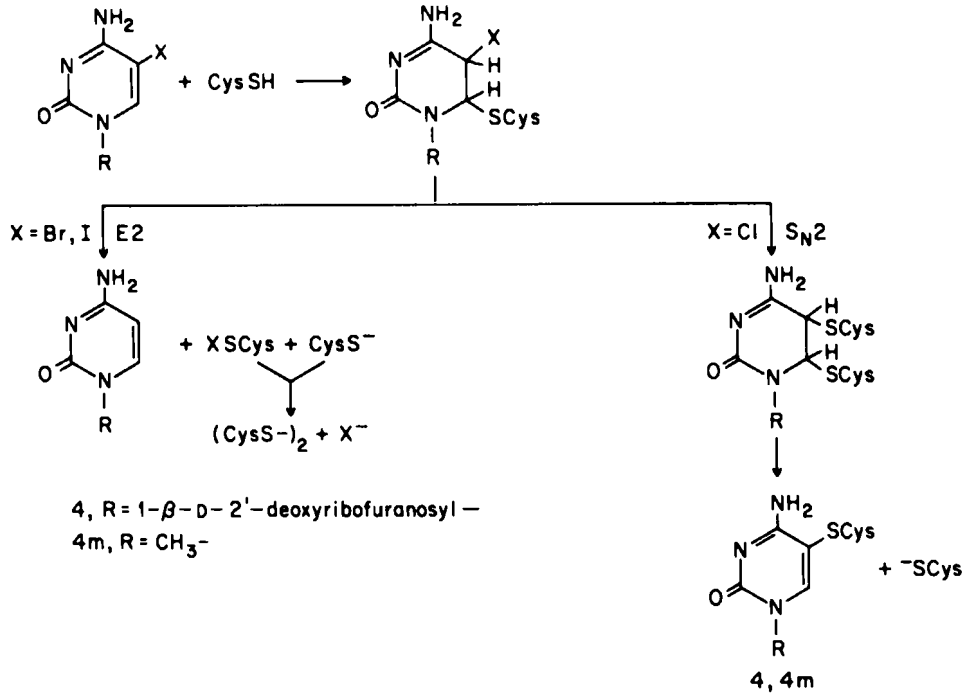


Scheme 3

25 percent yield. The yield of 5m as judged from the chromatographic analysis of the reaction mixture was 78 percent of theory based on 1-Me-5-Cl-Cyt. Various attempts were made either to improve the yield or shorten the reaction time without success. When the reaction was carried out at 100° for 24 h, there was considerable decomposition of 5m. The use of catalytic amounts of 4-dimethylaminopyridine and ethanolic NaOEt or NEt_3 in 50 percent EtOH:H_2O in place of aq K_2CO_3 in the reaction of 1-Me-5-Cl-Cyt with cysteine proved abortive.

As a result of these investigations, we propose the following schemes (Scheme 2, 4) for the reaction of 5-halo-2'-deoxycytidine and its 1-methyl analog with cysteine.

These results strongly suggest that at some point in the reaction mechanism the 5-chlorocytosine derivatives follow a route different from 5-bromo- and 5-iodo analogs (Scheme 2). It is noteworthy that in the 5-halodeoxyuridine series, 5-bromo-2'-deoxyuridine follow a dual mechanism of reaction forming two different products, 2'-deoxyuridine and 5-cysteine-S-yl-2'-deoxyuridine,[7] but in the 5-halodeoxycytidine series, 5-bromo-2'-deoxycytidine follows a single mechanism of reaction forming only one product, 2'-deoxycytidine. The first step in the reaction of 5-Cl-dC with cysteine parallels the reaction of 5-Cl-dU with cysteine[7] leading to the formation of 5-Cys-dC and 5-Cys-dU respectively; but in presence of cysteine, 5-Cys-dC undergoes reversible addition of cysteine which labilizes the 4-NH_2 group. The nucleophilic attack of the amino group of the cysteine residue on the 4-C of the



Scheme 4

pyrimidine moiety results in the formation of the thiazine ring. There are two pieces of evidence supporting this mechanism. It has been found that neither 1-Me-5-CysC (4m) nor 5-Cys-dC (4) can be directly converted into the thiazine derivatives (5m, 5) by heating in aq K_2CO_3. The presence of cysteine in the reaction medium is essential for this conversion. The bisulfite catalyzed deamination of cytosine derivatives follows a similar course.[14]

We have carried out the reaction of 1-methyl-5-chlorocytosine labeled with N-15 in the $-NH_2$ group with cysteine. The product 5m did not contain any N-15 as revealed by the mass spectrum of its TMS derivative which was identical with that of 5m obtained from unlabeled 1-methyl-5-chlorocytosine (Fig. 4). This conclusively proves that the exocyclic $-NH_2$ of the pyrimidine ring is lost in the cyclization step (Scheme 2).

The concept of the hard and soft acids and bases[15] can also be used to postulate an alternate mechanism of the formation of the products. The first step is common in both the reaction schemes and involves addition of cysteine across the 5,6-double bond. Subsequent displace-

ment of C5 chlorine will give the 5,6-diCys-dC or 1-methyl-5,6-diCys-cytosine which can undergo elimination of cysteine forming 4 and 4m respectively. Owing to the softness of Br and I, the Michael adduct may eliminate both $Br^+(I^+)$ and $CysS^-$ groups under the influence of the soft base cysteine. However, a similar elimination is not competitive with the S_N2 reaction because Cl is less polarizable.

This novel and facile reaction of 5-chlorocytosine derivatives with cysteine should be generally applicable and provide an easy route to the pyrimido-thiazine derivatives. Investigations are under way to synthesize the arabinosyl analog of 5 and to find out if it retains the anticancer activity of the parent compound arabinosylcytosine (ara-C). A positive anticancer response of this derivative would be of potential interest, since unlike ara-C, the arabinosyl analog of 5 will not undergo inactivation *in vivo* by deamination[16] because the imino function is sequestered in the ring.

Experimental Section

Materials and Methods. N,O-Bis(trimethylsilyl)acetamide and trimethylchlorosilane were from Pierce Chemical Co., Rockford, IL. N,O-Bis([$^2H_9$]trimethylsilyl)acetamide, and [$^2H_9$]trimethylchlorosilane, [hydroxy-$^2H_3$]glycerol and ^2H_2O were purchased from Merck Isotopes, St. Louis, MO. Glycerol, Ultra Pure Reagent grade, was from Bethesda Research Laboratories, Gaithersburg, MD. 5-Bromo-, 5-iodo- and 5-chloro-2'-deoxycytidine were obtained from Calbiochem. L-[U-^{14}C]-cysteine hydrochloride, sp. act. 47 mCi/mmol, was obtained from Amersham. 1-Methylcytosine was prepared by treating 1-methyl-2-oxo-4-methoxyprimidine with ammonia after the method of Flynn et al.[17] 1-Methylcytosine ($^{15}NH_2$) was prepared similarly using $^{15}NH_3$, 99%, obtained from KOR Inc., Cambridge, MA. N-Chlorosuccinimide was purified by recrystallization from $CHCl_3/CCl_4$ as described before.[7] All reactions with cysteine were carried out in a nitrogen atmosphere in vessels sealed with rubber septa. Air in the vessels was replaced by nitrogen using the double needle technic. Aqueous K_2CO_3 solution was rendered oxygen-free by bubbling water-saturated nitrogen through the solution. Silicic acid and cellulose thin-layer plastic sheets were obtained from Eastman Kodak. Silicic acid for column chromatography was obtained from E. Merck and used as such. Melting points were determined on a Thomas-Hoover apparatus and are uncorrected. Elemental analyses were carried out by Galbraith Laboratories, Knoxville, TN. ^{1}H-NMR

spectra were recorded with the use of a Varian Associates FT-80 or Nicolet NT-200 instrument. Chemical shifts are reported on the scale in parts per million downfield from internal 2,2-dimethylsilapentane-5-sulfonate (DSS), and apparent coupling constants (J) are given in hertz (Hz).

Mass Spectrometry

Mass spectra were acquired using a MAT 731 instrument with samples introduced by direct probe, under the following conditions: electron ionization, 70 eV ionizing energy, ion source 240°C; FAB, Xe energy 6 keV, ion source 250°C, using glycerol matrix. An Ion Tech FAB 11N saddle field-type gun was used, closely following the modification previously described.[18] Exact mass measurements were made by peak matching at resolution 12,000.

Samples were trimethylsilylated for mass spectrometry by heating approximately 50 µg of dried material dissolved in N,O-bis(trimethylsilyl)acetamide, trimethylchlorosilane and pyridine (100:1:10) for 1 h at 100°C. [$^2H_9$]Trimethylsilyl derivatives were prepared in analogous fashion using deuterated silylation reagents. Trimethylsilylated 5m evaporated from the direct probe at 125°C, and 4m at 100°C. In both cases additional more volatile material was observed in the range 30-100°C, the principal component of which was identified by its mass spectrum[12] as the tetra(trimethylsilyl) derivative of cystine (M = m/z 528).

Exchange of active hydrogen by deuterium in 5m prior to FAB mass spectrometry was effected using [hydroxy-$^2H_3$]glycerol and ^2H_2O (1:1) following an earlier described method.[8]

Ultraviolet Spectrophotometry

When a reaction mixture was analyzed by chromatography on an Aminex A-6 column, eluting with 0.1 M ammonium borate buffer, the appropriate fractions containing the UV-absorbing materials were collected and evaporated to dryness in a rotary evaporator. The evaporation was repeated three times, adding three small portions of methanol to remove boric acid. The final residue was dissolved in water and the spectrum was recorded. Ten µL of 10 N HCl was added to the cuvette and the UV absorption spectrum was recorded and reported as the spectrum at pH 2. Twenty-five µL of 10 N NaOH was then added to the same cuvette and the UV absorption spectrum was recorded and reported as the spectrum at pH 12. All spectra were recorded on a Cary Model 14 recording spectrophotometer.

1-Methyl-5-chlorocytosine

The reaction mixture, containing 1-MeCyt, 8.5 g (67.9 mmol) and N-chlorosuccinimide, 13.54 g (101.9 mmol), dissolved in 125 mL of glacial acetic acid, was heated for 3 h at 105°. On cooling to room temperature, a solid precipitated. It was filtered and the filtrate was concentrated by evaporation in a rotary evaporator when some more precipitate was obtained. It was filtered and mixed with the first crop. The mixed solids were recrystallized from ethanol containing a little 6N aqueous ammonia (95:5 v/v). 1-Me-5-Cl-Cyt crystallized in needles, m.p. 258-9° (dec), yield 6.1g (56% of theory); chromatographically homogenous as judged by TLC on cellulose and silica gel in n-BuOH:H_2O (86:14 v/v). Ultraviolet spectral data are recorded in Table 1 and Fig. 2; ^{1}H-NMR (D_2O) δ 3.23 (3H,s,N-CH_3), 8.01 (1H,s,H-6). Anal. Calcd. for $C_5H_6N_3OCl$: C, 37.63; H, 3.79; N, 26.33; Cl, 22.21. Found: C, 37.49; H, 3.80; N, 26.29; Cl, 22.45.

^{1}H-NMR spectrum of 1-methylcytosine

^{1}H-NMR (D_2O) δ 3.19 (3H,s,N-CH_3), 5.63 (1H,d,J=6.8, H-5), 7.56 (1H,d,J=6.7, H-6).

Reaction of 5-iodo-2'-deoxycytidine with cysteine

A mixture of 5-iodo-2'-deoxycytidine, 17.7 mg (0.05 mmol), cysteine, 36.3 mg (0.3 mmol), and oxygen-free aq 1 N K_2CO_3, 1 mL, was heated in a Reactivial fitted with a serum stopper at 50° for 21 h. Prior to heating, air in the Reactivial was replaced by nitrogen by using the double needle technic described by Shriver.[19] The content of the Reactivial was stirred magnetically. Both cysteine and the nucleoside dissolved in about 8 minutes. Aliquots of 25 µL were withdrawn with a syringe at the beginning and at the end of the reaction and diluted with 175 µL of water. Ten µL of the diluted sample was analyzed on an Aminex A-6 (Bio-Rad Cation Exchanger) column (20 cm × 0.63 cm dia.) using 0.1 M ammonium borate, pH 7.4 as eluant. The column was monitored at 280 nm essentially as described by Uziel et al.[20] The completely separated 2'-deoxycytidine (dC) and 5-iodo-2'-deoxycytidine (5-I-dC) peaks were integrated, using the following extinction coefficients: ε_{280} (dC), 6840 and ε_{280} (5-I-dC), 5040. Ten µL of the diluted reaction mixture initially contained 54.2 nmol of 5-I-dC and no dC. At the end of the reaction, 10 µL of the diluted reaction mixture contained 56.2 nmol of dC and no 5-I-dC. Thus the conversion was practically quantitative. The 2'-deoxycytidine formed in the reaction was identified chromatog-

raphically and spectrophotometrically. The dC is easily distinguishable spectrophotometrically from 5-I-dC (see Table 1).

Reaction of 5-bromo-2'-deoxycytidine (5-Br-dC) with cysteine

This reaction was carried out in a manner similar to the reaction with the 5-iodo-2'-deoxycytidine (vide supra) except that heating was continued for 72 h to complete the reaction. The conversion of 5-Br-dC to dC was practically quantitative as judged chromatographically by integrating the completely separated peaks of dC and 5-Br-dC. The 5-Br-dC peak was integrated using ε_{280} (6300). The dC is easily distinguishable spectrophotometrically from 5-Br-dC (see Table 1).

Reaction of 5-chloro-2'-deoxycytidine with cysteine

A mixture of 5-chloro-2'-deoxycytidine, 13.08 mg (0.05 mmol), cysteine, 36.3 mg (0.3 mmol) and 2 N oxygen-free aq K_2CO_3, 0.5 mL, was heated in a Reactivial fitted with a serum stopper at 60° for 96 h as described for the reaction of 5-iodo-2'-deoxycytidine with cysteine (vide supra). At the end of the reaction, the mixture was analyzed on the Aminex-A6 column using 0.1 M ammonium borate buffer, pH 7.4 (this system does not separate 5-Cl-dC from dC), or 0.4 M ammonium formate, pH 6 (this system does separate 5-Cl-dC from dC) as eluants. These two different chromatographic systems are complementary. Compared with the ammonium formate system, it was easier to recover material from the fractions containing ammonium borate by removing borate with methanol in a rotary evaporator. There were traces of 5-Cl-dC and dC. No dU or 5-cystein-S-yl-dU was detected. There were four major peaks in the chromatogram of the 24 h sample, peak I (5), peak II (unreacted cysteine possibly some cystine), peak III (4), and unreacted 5-Cl-dC, eluting at 7.5, 11, 15, and 27.5 minutes respectively (Fig. 1). The ultraviolet absorption spectra of 4 and 5 are shown in Fig. 2. Another reaction carried out with ^{14}C-labelled cysteine was also analyzed in a similar manner on A-6 column using 0.1 M ammonium borate, pH 7.4, collecting 1 minute fractions. The radioactivity profile superimposed on the chromatogram indicated that both peak I and peak III materials contained cysteine (Fig. 1). The offset in the two traces was due to the lag between the time the material ran through the UV monitor and the time the material was collected in the fraction collector for counting the radioactivity. No attempt was made to correct for this offset. The peak II had the highest reactivity because it was mostly composed of unreacted cysteine along with possibly some cystine formed as a result of oxidation of cysteine.

TABLE 1. UV Spectral Data of the Pyrimidine Derivatives.

Compound	pH	λ_{max} ($\varepsilon \times 10^{-3}$)	λ_{min} ($\varepsilon \times 10^{-3}$)	A_{250}/A_{260}	A_{280}/A_{260}	A_{290}/A_{260}
1-Me-5-Cl-Cyt	1	299 (10.2)	251 (0.56)	0.37	4.88	7.75
	7,12	287 (6.8)	257 (2.7)	1.15	2.2	2.35
5-Cl-dC	1	297 (10.8)	251.5 (0.97)	0.63	4.25	6.00
	7,12	285 (7.32)	258 (4.0)	1.16	1.76	1.76
4m	1	293	255	1.04	2.09	2.73
	7,12	284	262	1.37	1.42	1.37
4	1	292	254	0.98	1.72	2.05
	7,12	282	265	1.24	1.17	1.02
5m	1	340 (7.27)	284 (0.47)	1.12	0.07	0.07
	7,12	318 (5.9)	284 (1.99)	1.14	0.28	0.31
5	1	340	285	1.13	0.13	0.11
	7,12	318	286	1.06	0.36	0.34
5-Br-dC	1	298.5 (9.87)	254.5 (1.3)	0.94	3.88	5.82
	7,12	287 (6.95)	260 (3.5)	1.28	1.80	1.95
5-I-dC	1	308 (8.92)	260 (1.87)	1.53	2.30	3.47
	7,12	293 (6.10)	263 (1.99)	1.32	1.47	1.76

Reaction of 1-methyl-5-chlorocytosine with cysteine

A reaction mixture containing 1-methyl-5-chlorocytosine, 8 mg (0.05 mmol), cysteine, 36.3 mg (0.3 mmol) and 2N oxygen-free, aq K_2CO_3 was heated at 60° in a manner similar to that described for the reaction of 5-Cl-dC with cysteine (vide supra). Chromatographic analysis of the reaction mixture on an A-6 column using 0.1 M ammonium borate at pH 7.4 revealed the formation of two products, 4m and 5m. The UV spectra of 4m and 5m were very similar to the UV spectra of 4 and 5 respectively (Fig. 2).

Conversion of 4m to 5m

About 5-10 A_{260} units[21] of 4m were collected by repeated injections of 5 µL aliquots of the reaction mixture described above into an A-6 column using 0.1 M ammonium borate as eluant. The combined fractions of 4m was evaporated to dryness in a rotary evaporator. The evaporation was repeated three times with three small additions of methanol. The final residue was heated at 60° for 72 h in 1 N K_2CO_3 (0.3 mL) containing 11 mg of cysteine. The final reaction mixture was analyzed on the A-6 column using 0.1 M ammonium borate as eluant. There was no 4m left in the reaction mixture and the only UV-absorbing product was chromatographically and spectophotometrically identical with 5m. Analysis of the product by FAB mass spectrometry also agreed with this conclusion.

A similar reaction carried out with aqueous 1 N K_2CO_3 without the addition of cysteine indicated no formation of 5m when analyzed chromatographically on the A-6 column using 0.1 M ammonium borate, pH 7.4 as eluant.

Conversion of 4 to 5

This conversion was attempted in the same manner as described above with similar results.

3-Methyl-2,6,7,8-tetrahydro-2-oxo-3H-pyrimido[5,4-b][1,4]thiazine-7-carboxylic acid (5m)

A reaction mixture containing 1-Me-5-Cl-Cyt, 500 mg (4 mmol), cysteine, 1.135g (9.4 mmol) and oxygen-free aq 2 N K_2CO_3, 15.5 mL, was heated for 72 h at 60° in a nitrogen atmosphere in a round-bottomed flask fitted with a serum stopper. A second portion of cysteine, 1.135g (9.4 mmol), was added and the heating was continued for another 72 h at 60° in a nitrogen atmosphere. The reaction mixture was cooled to room

temperature and treated with Dowex-50 (H^+) to lower the pH to 6.5. The resin and any precipitate formed was filtered. A stoichiometric amount of H_2O_2 (~12.1 mmol, 30 percent H_2O_2 solution) and a small crystal of $FeCl_2$ were added to the filtrate. An excess of H_2O_2 was indicated when an aliquot on treatment with 0.1 N $K_2Cr_2O_7$ and 0.1 N H_2SO_4 showed a transient blue color. Any excess H_2O_2 was destroyed by adding sodium sulfite. The precipitated cystine was filtered and the filtrate was evaporated to dryness in a rotary evaporator. The residue was dissolved in a small volume of aqueous methanol and absorbed on to 1g of silicic acid. It was applied to the top of a column of silicic acid (10 cm × 1 cm dia.) and eluted with 50 mL of 10% CH_3OH-$CHCl_3$ (v/v) and 100 mL of 40% CH_3OH:$CHCl_3$ (v/v) successively, collecting 10 mL fractions. The appropriate fractions were pooled and evaporated to dryness; yield, 200 mg. A sample was recrystallized from water for analysis; m.p. 236° (dec), pK_a 4.7 (spectrophotometric). The UV spectral data are reported in Table I and Fig. 2. ^{1}H-NMR (D_2O) δ 3.07 (2H,d,J=4.2, H_2-6), 3.34 (3H,s,N-CH_3), 4.31 (1H,t,J=4.9, H-7), 7.49 (1H,s,H-4). Anal. Calcd for $C_8H_9N_3O_3S \cdot 1/2H_2O$: C, 40.66; H, 4.27; N, 17.99. Found: C, 40.28; H, 4.37; N, 17.77.

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REFERENCES

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21. One absorbance unit represents that amount of material in 1 mL of a solution that has an absorbance of 1.0 when it is measured with a 1.0 cm optical path at a particular wavelength.

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