Reaction of 5-Chlorouracil Derivatives with Cysteine[1]

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Abstract: 5-Bromo-2′-deoxyuridine reacts with cysteine to produce 2′-deoxyuridine and 5-(cystein-*S*-yl)-2′-deoxyuridine in 2:1 and 1:2 molar ratio at pHs 7 and 12, respectively. 5-Chloro-2′-deoxyuridine as well as its 1-methyl analogue, on the other hand, does not react appreciably with cysteine at pH 7 but at pH 12 forms 5-(cystein-*S*-yl)-2′-deoxyuridine and its 1-methyl analogue, respectively, in better than 99% yield. A reaction mechanism is proposed to account for the role of halogen in influencing the course of this reaction. The first step is the addition of cysteine across the 5,6 double bond of the pyrimidine ring. In case of a good leaving group such as Br, attack of the cysteinyl anion on the 6 position leads to the formation of cystine, 2′-deoxyuridine, and bromide. Parallel to this reaction, the 5 halogen (either Cl or Br) may undergo nucleophilic substitution by cysteine and form 5-(cystein-*S*-yl)-2′-deoxyuridine after base-catalyzed elimination of cysteine at the 6 position. Synthesis and ultraviolet absorption data of 1-methyl-5-chlorouracil, 1-methyl-5-(cysteine-*S*-yl)uracil, and 5-(cystein-*S*-yl)-2′-deoxyuridine are reported.

Compared with 5-fluoro, 5-bromo, and 5-iodo derivatives of uracil, the 5-chloro derivatives have received scant attention in the literature. 5-FUra,[2,3] 5-IUra,[4] and 5-BrUra[5] are an anticancer agent, an antiviral agent, and a mutagen, respectively. However, the recent discovery of 5-ClUra[6] in chlorinated effluent from sewage treatment plants has spurred interest in this compound. Recent studies by Pal et al.[7] show that 5-ClUra is incorporated into the liver DNA of mice ingesting 5-ClUra-saturated water. Since 5-ClUra is probably subjected to reaction with nucleophiles in vivo, I decided to study its reaction with cysteine.

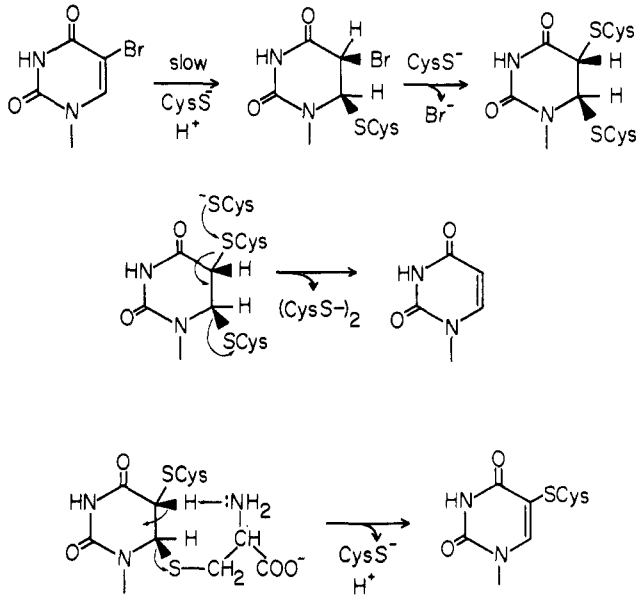
Results and Discussion

The dehalogenation of 5-chloro-, 5-bromo-, and 5-iodouracil by cysteine and bisulfite has been intensively studied.[8-13] The debromination of 5-BrdUrd by cysteine and bisulfite has been reported by Wataya et al.[14] and Hayatsu et al.,[15] respectively. The dehalogenation of 5-bromo- and 5-iodo-2′-deoxyuridylate by thymidylate synthetase has been investigated by Garrett et al.[16] One common feature of all these reactions is that the dehalogenation step is preceded by the addition of the nucleophile to the pyrimidine ring at C-6. Deuterium exchange studies by Szabo et al.[17] on the reaction of 1-Me-5-BrUra with NaSD provided experimental evidence in support of this mechanism. Two products were formed: 1-MeUra and 1-methyl-5-thiouracil, the former being labeled with deuterium in the 5 position. A similar mechanism was postulated by Wataya et al.[14] for the reaction of 5-BrdUrd with cysteine (Scheme I).

I have repeated the debromination of 5-BrdUrd with cysteine and my results are in general agreement with those reported earlier by Wataya et al.[14] Relative yields of dUrd and 5-CysdUrd were pH dependent. At high pH, formation of 5-CysdUrd was more favored whereas at neutrality formation of dUrd was more favorable. Yields of dUrd and 5-CysdUrd at pH 11.7 were 38 and 62%, respectively (Figure 1). At neutral pH, yields of dUrd and 5-CysdUrd were 67 and 33%, respectively. There was no destruction of 5-BrdUrd and there was no other pyrimidine derivative formed in this reaction.

In order to find out if the deoxyribose moiety in 5-BrdUrd offers any anchimeric assistance, the reaction of 1-Me-5-BrUra with cysteine was also studied at pH 12. The yields of 1-Me-5-CysUra and 1-MeUra were 28 and 72%, respectively (Figure 2). Such a large disparity in relative yields of the two products from 5-BrdUrd and its methyl analogue indicates that anchimeric assistance of the deoxyribose moiety probably plays a role in this reaction. Experimental evidence has been presented for such anchimeric assistance of the 5′-OH group in

Scheme I



facilitating electrophilic substitution reactions of uracil furanosides.[18,19]

In contrast with 5-BrdUrd, the reaction of 5-CldUrd with cysteine in 1 N K_2CO_3 forms 5-CysdUrd in practically quantitative yield. The reaction mixture has been directly analyzed by chromatography, and formation of only a trace of dUrd (less than 0.1%) has been detected (Figure 3). The reaction of 1-Me-5-ClUra with cysteine also results in practically quantitative yield of 1-Me-5-CysUra. Since Wataya et al.[14] reported that the formation of dUrd from 5-BrdUrd and cysteine is more favorable at neutral pH, the reaction of 5-CldUrd with cysteine was carried out at pH 7.2 in 0.1 M phosphate buffer. There was no reaction, and 5-CldUrd was recovered almost quantitatively from the reaction mixture.

These results strongly suggest that at some point in the reaction mechanism the 5-bromouracil derivatives follow a route different from that followed by the 5-chloro analogues. The mechanism of Wataya et al.[14] (Scheme I) proposes the same intermediate 5,6-diCysdUrd to be the precursor for both the products, dUrd and 5-CysdUrd. The lack of formation of dUrd from 5-CldUrd and cysteine cannot be explained by this mechanism. This observation indicates that the nature of the halogen plays an important role in deciding the course of this reaction.

In constrast with these results, 5-bromo- and 5-iodo-2′-deoxyuridylate are dehalogenated to 2′-deoxyuridylate by

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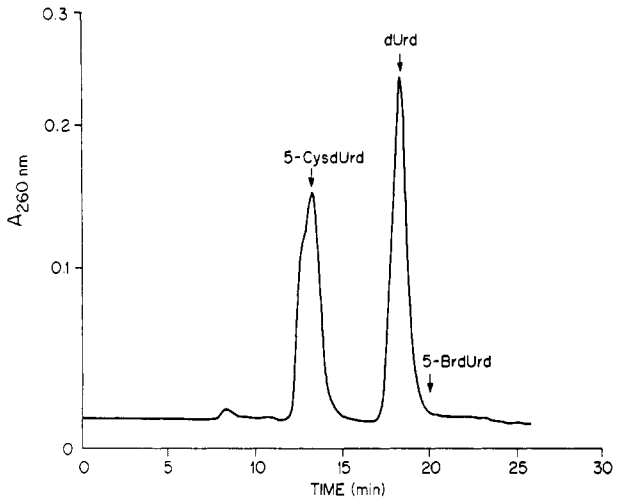


Figure 1. Chromatography of a reaction mixture of 5-BrdUrd, cysteine, and 1 N K_2CO_3 on an Aminex A-6 column (20 × 0.63 cm). A 5-μL sample (dilution 1:20) was injected into the column (maintained at 50 °C) and eluted with 0.1 M boric acid adjusted to pH 7.4 with 1 M ammonia at 0.31 mL/min (50 psi pressure).

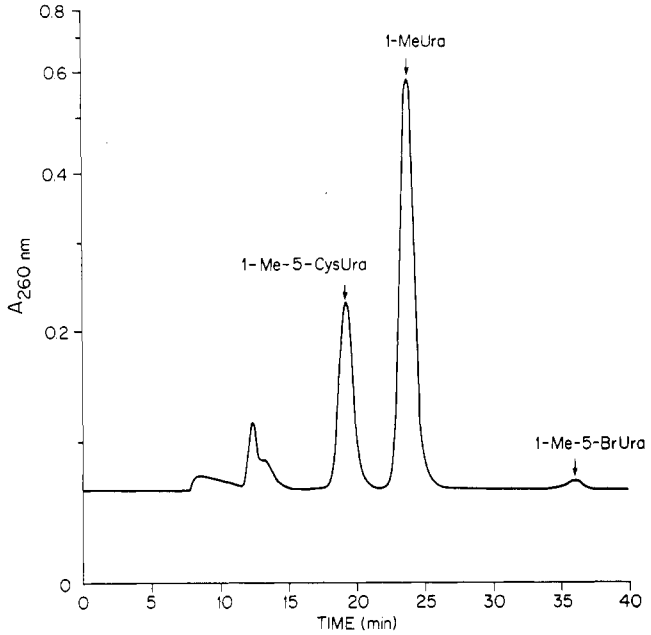


Figure 2. Chromatography of a reaction mixture of 1-Me-5-BrUra, cysteine, and 1 N K_2CO_3 on an Aminex A-6 column (20 × 0.63 cm). A 5-μL sample (dilution 1:7) was injected into the column (maintained at 50 °C) and eluted with 0.1 M boric acid adjusted to pH 7.4 with 1 M ammonia at 0.31 mL/min (50 psi pressure).

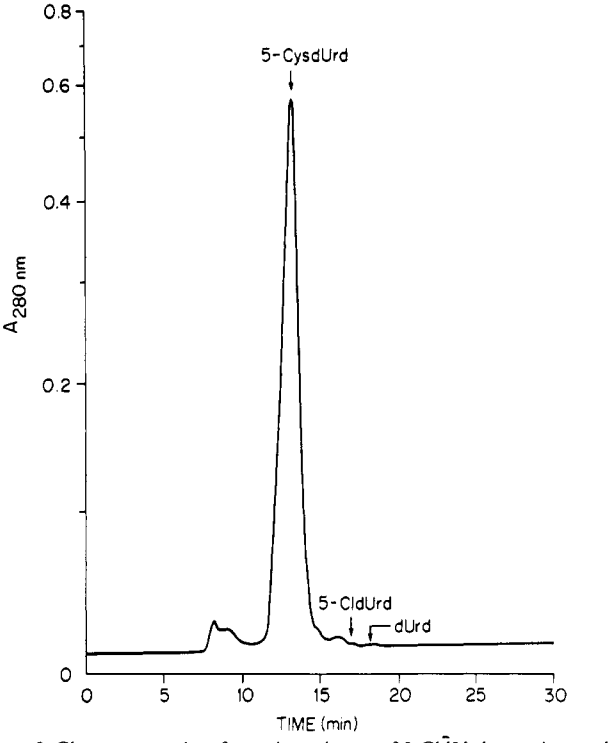
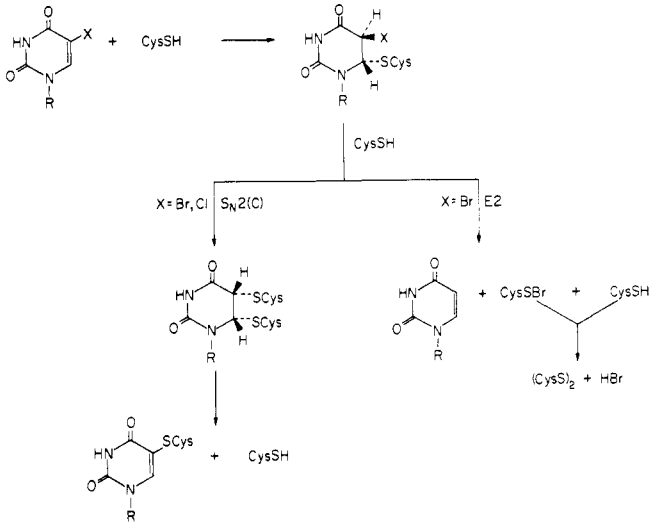


Figure 3. Chromatography of reaction mixture of 5-ClUrd, cysteine, and 1 N K_2CO_3 on an Aminex A-6 column (20 × 0.63 cm). A 5-μL sample (dilution 1:7) was injected into the column (maintained at 50 °C) and eluted with 0.1 M boric acid adjusted to pH 7.4 with 1 M ammonia at 0.31 mL/min (50 psi pressure).

Scheme II



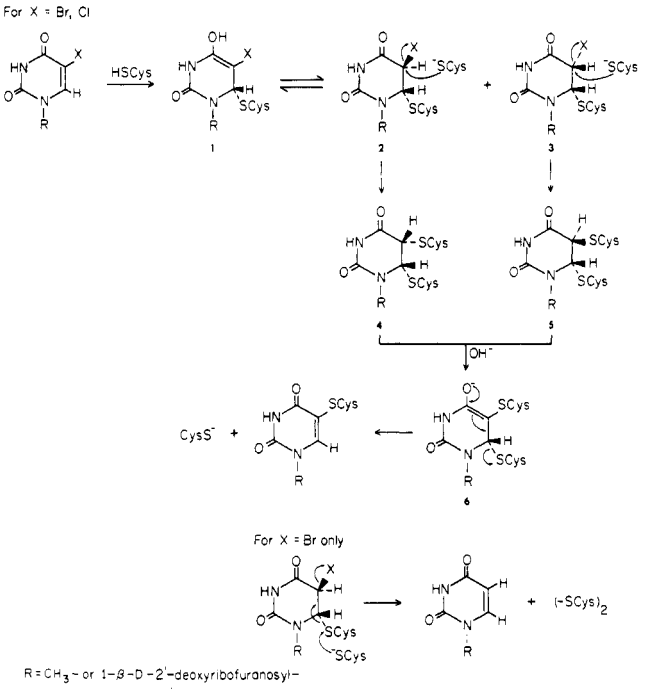
thymidylate synthetase in the presence of thiols.[16] Bellisario et al.[20] have reported strong evidence suggesting that 5-fluoro-2′-deoxyuridylate is linked to a cysteinyl residue in thymidylate synthetase that has been inactivated irreversibly by this nucleotide. Since 5-fluoro-2′-deoxyuridylate does not undergo dehalogenation, the enzyme is probably attached to the C-6 of the pyrimidine ring via a cysteine residue.

Dr. T.-L. Ho of Brookhaven National Laboratory has suggested an alternative mechanism for this reaction based on the principle of the hard and soft acids and bases.[21] There is a common pathway for both halo compounds leading to the cysteinyl nucleoside (Scheme II). Thus, the initial Michael addition would furnish *trans*-5-halo-6-CysdUrd. Subsequent displacement of the C_5 halogen would give *cis*-5,6-diCysdUrd, which can undergo elimination of cysteine.

Owing to the softness of Br, the Michael adduct from 5-BrdUrd may eliminate both Br^+ and $CysS^-$ groups under the influence of the soft base cysteine. However, a similar elimination from the 5-CldUrd adduct is not competitive with the S_N2 (C) reaction, because Cl is less polarizable.

This mechanism does successfully explain the behavior of 5-CldUrd vs. 5-BrdUrd toward cysteine, although it suffers from the following shortcomings: (1) The scheme proposes the formation of only the *trans*-5-haloCysdUrd. On theoretical grounds formation of both isomers is possible as shown in Scheme III. (2) Formation of both *cis*- and *trans*-5,6-diCysdUrd is possible as shown in Scheme III. (3) In case of dehalogenation of 5-bromo-6-methoxy-5,6-dihydrothymine by cysteine at pH 6–8, Sedor and Sander[22] have proposed the formation of CysBr as an intermediate which can react with

Scheme III



another mol of cysteine to form cystine or undergo solvolysis with water forming $CysSO_2H$, $CysSO_3H$, CysSH, and Br^-. Rork and Pitman[23] have proposed the formation of a halosulfonic acid during dehalogenation of 5-halo-5,6-dihydrouracils by sodium bisulfite at pH 4–8. These uracil derivatives are poor models for 5-halodUrd and further experimentation is clearly necessary to demonstrate that cysteine-mediated dehalogenation of 5-Br(I)-dUrd proceeds through the formation of the intermediate CysBr(I), particularly at high pH. An alternative pathway can be formulated for the formation of dUrd (cf. Scheme III) which does not require the formation of this intermediate.

We propose a somewhat different mechanism that is in harmony with the literature data and at the same time explains satisfactorily the anomalous behavior of 5-CldUrd vs. 5-BrdUrd toward cysteine (Scheme III). Essential features of this scheme are as follows.

The first step is the 1,4 addition of cysteine to form the derivative **1** followed by 1,3 shift of the proton to form a pair of isomers **2** and **3**. A similar scheme has been proposed earlier by Szabo et al.[17] for reaction of 1-Me-5-BrUra with SD^-. Sedor et al.[24] have also proposed the 1,3 shift of the proton in the case of the addition of bisulfite to 5-fluorouracil.

The addition of cysteine across the 5,6 double bond of the pyrimidine ring of the 5-iodouracil by a concerted, intramolecular general-acid-catalyzed reaction, which involves proton transfer from cysteine's protonated amine function to C-5 of the pyrimidine ring concomitant with thiol anion attack at C-6, has been proposed by Sedor et al.[9] The large hydrogen-deuterium isotope effect observed in case of the addition of cysteine to 5-iodouracil ($k_2^{H_2O}/k_2^{D_2O} = 4.10$) compared to the much smaller ratio for the $Tris^+$–HCl catalyzed addition of 2-mercaptoethanol ($k_2^{H_2O}/k_2^{D_2O} = 1.13$) has provided the experimental basis for this postulate. It is possible that such a mechanism may operate at lower pH, but, when the reaction of 5-CldUrd with cysteine is carried out at a pH of 11.7 and at 50 °C, such a mechanism is highly unlikely on the following ground. The pK_a^3 of cysteine has been reported[9] to be 10.23 in water at 25 °C and ionic strength 1.0 M and represents the ionization of the following species: $-OOC(NH_3^+)CHCH_2S^-$ or $-OOC(NH_2)CHCH_2SH \rightleftarrows -OOC(NH_2)CHCH_2S^-$. Lowering of pK_a^3 of cysteine by about 0.42 pH units has been reported at 45 °C compared with that at 25 °C. If one assumes the pK_a^3 of cysteine to be (10.23 − 0.42) or 9.81 at 50 °C, then the concentration of the amino-protonated species of cysteine is calculated to be only about 1.2% at pH 11.7 ignoring the other ionic species $-OOC(NH_2)CHCH_2SH$. Contribution of such a small amount of $-OOC(NH_3^+)CHCH_2S^-$ to the overall mechanism at pH 11.7 is likely to be small. If this conclusion is correct, one should not expect a large hydrogen–deuterium isotope effect on the reaction of 5-CldUrd with cysteine at pH 11.7.

The next step is the nucleophilic attack of the $CysS^-$ on the 5 position of both **2** and **3** leading to the formation of 5,6-diCysdUrd **4** and **5** and X^-. This reaction takes place in the case of both 5-bromo and 5-chloro derivatives. Base-catalyzed deprotonation of **4** and **5** leads to **6**, which undergoes elimination of cysteine to form 5-CysdUrd. This is in agreement with the observation that when the reaction of 5-BrdUrd with cysteine was carried out at pH 7.2, dUrd was the major product whereas at pH 12,5-CysdUrd was the major product.

The parallel nucleophilic attack by $CysS^-$ on the 6 position takes place only when there is a good leaving group such as Br at the 5 position. Br is eliminated as Br^- and $CysS^+$ is eliminated as cystine resulting in the formation of dUrd. This reaction does not take place in case of 5-chloro derivatives since Cl is not a good enough leaving group under the reaction conditions.

Parallel formation of 5-CysdUrd and dUrd from 5-BrdUrd and cysteine reported by Wataya et al.[14] can thus be accounted for. At the same time lack of formation of dUrd from 5-CldUrd and cysteine reported herein is also explained on the basis of Cl being a poor leaving group under the reaction conditions. This scheme predicts that, in the reaction of 5-IdUrd with cysteine, dUrd will be the major product, since I is even a better leaving group than Br. Our experimental results with dehalogenation of 5-IdUrd with cysteine (not reported) support this prediction.

In conclusion, the proposed reaction mechanism has eliminated the deficiency in earlier schemes and has delineated the role of halogen in deciding the course of the dehalogenation of 5-halopyrimidines by cysteine.

5-CysdUrd has been previously prepared from 5-BrdUrd and cysteine by Wataya et al.[14] The yield was low and purification was difficult because of the simultaneous formation of dUrd. On the other hand, its preparation from 5-CldUrd is straightforward; the yield is high and the purification is easy because no dUrd is formed.

Since 5-CldUrd does not undergo dehalogenation, its reaction with thymidylate synthetase may shed some light on the enzymatic mechanism. We have shown that 5-ClUra is incorporated into mouse liver DNA when the animals are kept on 5-ClUra-saturated water. The relative unreactivity of 5-CldUrd compared with 5-BrdUrd may cause a persistent alteration of the DNA[7] as a result of continuous exposure to this environmental mutagen at a low level.

Experimental Section

Materials and Methods. 5-CldUrd from Calbiochem and 5-BrdUrd from Sigma Chemical Co. were used as obtained. 1-Me-5-BrUra was prepared by the method of Benitez et al.[26] 1-MeUra was synthesized by the method of Hilbert and Johnson.[27] The procedure of West and Barrett[28] was modified for the preparation of 1-Me-5-ClUra. *N*-Chlorosuccinimide was purified by recrystallization from a mixture of chloroform (ethanol-free)–carbon tetrachloride (19:10); about 22 mL of solvent mixture was required per g of *N*-chlorosuccinimide. Ethanol-free chloroform was prepared by distilling chloroform over phosphorus pentoxide. Cysteine solutions were freshly prepared before use. A sample of synthetic 5-CysdUrd was kindly provided by Dr. Hikoya Hayatsu, University of Tokyo, Japan, for comparison. All other chemicals were reagent grade.

Table I. Ultraviolet Absorption Data for Some Uracil Derivatives

compd	pH	λ_{max} ($\epsilon \times 10^{-3}$)	λ_{min} ($\epsilon \times 10^{-3}$)	A_{250}/A_{260}	A_{280}/A_{260}	A_{290}/A_{260}
1-Me-5-ClUra	2	281 (8.8) 210 (8.7)	241 (0.76)	0.44	2.18	1.84
	12	277 (6.2)	248 (2.4)	0.68	1.71	1.11
1-Me-5-CysUra	2	282 (7.3)	248 (3.5)	0.86	1.74	1.54
	12	278 (4.8)	261 (3.9)	1.15	1.20	0.96
5-CysdUrd[a]	2	278.5 (7.3)	247 (4.4)	0.86	1.40	1.16
	12	277 (4.7)	265 (4.3)	1.24	1.06	0.86

[a] Reported λ_{max} 278 nm (pH 2), 276 nm (pH 12); ϵ_{278} (pH 2) 7550.[14]

1-Me-5-ClUra. 1-MeUra (1.26 g, 10 mmol) was dissolved in a mixture of 10 mL of acetic acid and 0.2 mL of acetic anhydride by heating to 80 °C, and 1.67 g (12.5 mmol) of *N*-chlorosuccinimide was added after the material cooled to 50–55 °C. Heating was continued until a weak test for *N*-chlorosuccinimide was obtained with starch–iodide paper. The solution was then poured into ice water to precipitate the product. It was collected by filtration and recrystallized twice from hot water, yield 0.92 g (57% of theory), mp 285 °C dec. Anal. ($C_5H_5N_2O_2Cl$) C, H, N. Spectral data are recorded in Table I.

1-Me-5-CysUra. A mixture of 160.6 mg (1 mmol) of 1-Me-5-ClUra, 724 mg (6 mmol) of cysteine, and 1 N aqueous K_2CO_3 (20 mL) was magnetically stirred and heated in a stoppered flask in a nitrogen atmosphere at 50 °C for 36 h. At the end of the period, the solution was adjusted carefully to pH 5 with Dowex-50 (H^+), then filtered. The filtrate was evaporated to dryness in a rotary evaporator, and the residue was recrystallized from water, yield 130 mg (53% of theory), mp 213–214 °C dec. Anal. ($C_8H_{11}N_3O_4S$) C, H, N. Yield of 1-Me-5-CysUra was 95% of theory as indicated by column analysis of the original reaction mixture. Spectral data are recorded in Table I.

Reaction of 1-Me-5-ClUra with Cysteine at pH 7.2. The reaction described above has been carried out in 0.1 M phosphate buffer, pH 7.2 (200 mL). There was no reaction as evident from the almost quantitative recovery of 1-Me-5-ClUra from the reaction mixture by column analysis.

Reaction of 5-BrdUrd with Cysteine. A. At pH 11.7. A mixture of 15.4 mg (0.05 mmol) of 5-BrdUrd, 36.3 mg (0.3 mmol) of cysteine, and 0.7 mL of 1 N aqueous K_2CO_3 (pH 11.7) was magnetically stirred and heated in a stoppered flask in a nitrogen atmosphere at 50 °C for 2 h. Yields of 5-CysdUrd and dUrd were 62.3 and 37.7 mol %, respectively, as determined by column analysis. There was no destruction of 5-Br-dUrd and there was no other pyrimidine derivative formed. 5-CysdUrd and dUrd were identified by UV spectra and by elution position in column chromatography (Figure 1).

B. At pH 7.2. A solution of 15.4 mg (0.05 mmol) of 5-BrdUrd and 36.3 mg (0.3 mmol) of cysteine in 8 mL of oxygen-free 0.1 N potassium phosphate buffer, pH 7.2, was heated in a stoppered flask in a nitrogen atmosphere at 37 °C for 18 h. Yields of 5- CysdUrd and dUrd were 33 and 67 mol %, respectively. Aliquots, 5 μL, were taken at the beginning and end of the reaction for chromatographic analysis on the column (vide infra).

Reaction of 1-Me-5-BrUra with Cysteine. A mixture of 10.3 mg (0.05 mmol) of 1-Me-5-BrUra, 36.3 mg (0.3 mmol) of cysteine, and 1 mL of 1 N aqueous K_2CO_3 (pH 11.7) was magnetically stirred and heated in a stoppered flask in a nitrogen atmosphere at 50 °C for 2 h. Yields of 1-Me-5-CysUra and 1-MeUra were 28 and 72%, respectively, as determined by column analysis. They were identified by UV spectra and by elution position in column chromatography (Figure 2).

5-CysdUrd. A mixture of 132 mg (0.5 mmol) of 5-CldUrd, 362 mg (3 mmol) of cysteine, and 10 mL of 1 N aqueous K_2CO_3 was magnetically stirred and heated in a stoppered flask in a nitrogen atmosphere at 50 °C for 36 h. At the end of this period, the solution was carefully adjusted to pH 6 with Dowex-50 (H^+), then filtered. The filtrate was evaporated to dryness in a rotary evaporator, and the residue was recrystallized from ethanol–water (80:20), yield 60 mg (34.6% of theory), mp 195–196 °C dec. A sample for analysis was dried at 100 °C under high vacuum for 1 h. Anal. ($C_{12}H_{17}N_3O_7S$) C, H. N. Spectral data are recorded in Table I. Column analysis of the reaction mixture indicated practically 100% conversion into 5-CysdUrd and there was no destruction of 5-CldUrd (Figure 3). Yield of 5-CysdUrd was 99.3% of theory. Deoxyuridine formed in the reaction was found to be 0.08%.

Reaction of 5-CldUrd with Cysteine at pH 7.2. A mixture of 5-CldUrd (13.2 mg, 0.05 mmol), cysteine (36.2 mg, 0.3 mmol), and 0.1 M oxygen-free potassium phosphate buffer, pH 7.2 (8 mL), was heated in a nitrogen atmosphere at 37 °C for 20 h. Aliquots, 5 μL, were taken at the beginning and end of the reaction for column analysis. Recovery of 5-CldUrd from the reaction mixture at the end of the heating period was 97.6%.

Analysis of the Reaction Products by Liquid Chromatography. An aliquot of the reaction mixture (5–25 μL) was diluted (if necessary) with oxygen-free water to 200 μL, and 5 μL of the diluted solution was injected into a column (20 × 0.63 cm) of cation exchanger (Bio-Rad A-6) maintained at 50 °C and eluted with 0.1 M ammonium borate (pH 7.4) as described previously.[29] Samples were taken at the beginning and end of the reaction. The amount of material represented by each peak area was calculated according to Uziel et al.[30]

For estimation of traces of dUrd formed in the reaction, 25 μL of the undiluted reaction mixture was run through the same column using the same buffer system. The fraction (0.9 mL) containing dUrd was collected, evaporated to dryness, and taken up in 50 μL of water and a 40-μL aliquot was run through the column again for complete separation and quantitation of dUrd. This was necessary because, owing to overloading of the column, the dUrd peak was not completely separated from the 5-CysdUrd peak in the first run. 5-CldUrd used in the reaction was found to be free from dUrd by similar column analysis.

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References and Notes

(1) Research sponsored by the Department of Energy under contract with the Union Carbide Corp.
(2) Abbreviations: 1-MeUra, 1-methyluracil; 1-Me-5-ClUra, 1-methyl-5-chlorouracil; 1-Me-5-BrUra, 1-methyl-5-bromouracil; 1-Me-5-CysUra, 1-methyl-5-(cystein-*S*-yl)uracil; 5-CysdUrd, 1-β-D-5-(cystein-S-yl)-2′-deoxyuridine. All other notations are according to IUPAC–IUB rules of nomenclature.
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Total Synthesis of Ovine β-Lipotropin by the Solid-Phase Method

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Abstract: Ovine β-lipotropin with a linear structure of 91 amino acid residues has been synthesized by the solid-phase method. The synthetic material was purified by gel filtration, chromatography on carboxymethylcellulose, and partition chromatography on agarose. The final product has been found to be indistinguishable from the natural hormone in its R_f value on partition chromatography, mobility in paper electrophoresis, behavior on thin layer chromatography, amino acid composition of both acid and enzymic hydrolysates, NH_2-terminal residue, behavior on peptide mapping, isoelectric focusing, circular dichroism spectra, optical rotation, lipolytic activity, and immunoreactivity.

The lipotropic hormone, β-lipotropin (β-LPH),[1] was isolated from sheep pituitary glands[2,3] and its primary structure determined[4-6] (Figure 1). Its sequence from residues 41 to 58 was noted to be identical with that of β-melanotropin,[7] and residues 61–91 correspond to the sequence of the opioid peptide β-endorphin.[8] Solid-phase synthesis[9] of a peptide corresponding to residues 42–91 has been reported.[10] Subsequent syntheses of β-melanotropins,[11-13] β-endorphins,[14,15] and β_s-LPH-(41–91)[16] have been accomplished in relatively good yields. It was therefore decided to undertake total synthesis of β-lipotropin.

Recently, a second form of β_s-LPH has been isolated.[17] Partition chromatography of a hormone preparation on agarose gel cleanly separated this form from β_s-LPH and its structure was shown to be identical with that of the hormone with the sole exception that the glutamic acid residue in position 1 was replaced by a pyroglutamyl residue. This result afforded a new and effective means of purification while at the same time providing a highly purified preparation of natural β_s-LPH.

Since use of liquid HF[18,19] was planned to be the penultimate synthetic step, preliminary experiments were carried out with the natural hormone. The susceptibility of glumatic acid containing peptides to side reactions with anisole in HF[20,21] posed a potential hazard for this hormone since it contains 12 such residues. The best results were obtained by use of 2,6-dimethylanisole as scavenger. The hormone was recovered in 34% yield after chromatography on carboxymethylcellulose (CMC). Partition chromatography of this material on agarose gel gave practically the identical pattern as found with untreated hormone. These results suggested that a synthetic preparation would be able to survive a standard treatment with HF.

For solid-phase synthesis of β_s-LPH, the anchoring linkage of the COOH-terminal glutamine residue was to brominated styrene–1% divinylbenzene polymer. This linkage when used in conjunction with N^{α}-*tert*-butyloxycarbonyl protection has been shown to be cleaved only 0.03% per cycle and is approximately 15–50 times as stable as the standard linkage.[15] Coupling was performed by a symmetrical anhydride technique[22] which has been used to advantage for the β-LPH sequence.[10] Trifluoroethanol was employed in the second stage of anhydride couplings to enhance the efficiency of this reaction.[23] The side chain of tryptophan was protected with the formyl group[24-26] in view of its susceptibility to destruction under conditions of repeated acidolysis of Boc groups.[25-28] For histidine, the benzyloxycarbonyl group was used since it has been employed in synthesis of peptide analogues of the carboxyl terminal plasmin fragment of human somatotropin.[29] For threonine, serine, and glutamic acid, the very stable *p*-halobenzyl protecting groups[30,31] were used through residue 66 and benzyl protection thereafter. Selection of the other protecting groups has been discussed previously.[32-34] Since methionine was not protected, the last Boc group was removed with TFA to reduce *tert*-butylation that occurs in HF.[35]

The scheme for isolation is shown in Figure 2 with recoveries for various steps indicated. The first purification step on CMC gave a sharply defined peak close to the position previously reported for the natural hormone.[4] It is evident from the recovery of material that this procedure constituted the major purification step in the scheme. The slower moving materials which represent the major side products of synthesis apparently consist of shorter sequences as shown by both amino acid analysis and peptide mapping. The formyl group on tryptophan was then removed by brief treatment at pH 11.5 with NaOH.[12] Rechromatography on CMC gave a sharply defined peak as does natural β_s-LPH. The ultraviolet spectrum of this material was identical with that of the natural hormone in the region of 245–360 nm, indicating that deformylation was complete.[24]

Final purification was effected by partition chromatography in a biphasic solvent system on agarose. The R_f value of the major peak (0.275) which is a reflection of its distribution constant in the solvent system was, within experimental error,

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