Synthesis and Thiolytic Cleavage of S-Acyl Derivatives of 5-Mercaptouracil and 5-Mercapto-2′-deoxyuridine[1a]

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Several S-acyl derivatives of both 5-mercaptouracil (MU) and 5-mercapto-2′-deoxyuridine (MUdR) were synthesized for the purpose of temporarily protecting the parent compounds from oxidation to the disulfides. These acyl derivatives were found to be stable to hydrolysis under neutral conditions but to undergo facile transacylation with aliphatic thiols, thus releasing the antimetabolites in their active forms. The rate of this thiolytic cleavage depends on the structure of the acyl group as well as on the pK_a and concentration of the thiols.

Recently, we reported the synthesis of 5-mercapto-2′-deoxyuridine (MUdR), a structural analog of thymidine.[2,3] It was found that this compound is utilized as a substrate by thymidine kinase which phosphorylates it to the 5′-monophosphate,[4] and that the latter is a potent inhibitor of thymidylate synthetase.[5] Both the free base 5-mercaptouracil[6] (MU) and the deoxyriboside (MUdR) inhibit the growth of various microorganisms[7] and potentiate the carcinostatic effects of 5-fluorouracil, 5-fluorodeoxyuridine, or methotrexate in several transplanted mouse tumor tests.[7,8] However, in neutral aqueous buffer solutions both MU and MUdR are largely present in the form of their anions, I and II, respectively, which undergo very rapid, trace-iron-catalyzed autoxidation to the corresponding disulfides;[9] the latter can be reduced again to the mercapto compounds with aliphatic thiols.[10,11] Since the disulfide of MUdR is not phosphorylated by thymidine kinase, the intracellular reduction to the mercapto form is essential for the metabolic activation and subsequent inhibitory action of the analog.

It was thought that by blocking the SH groups of MU and MUdR with a hydrolyzable substituent, one could temporarily protect them from oxidation to the disulfides and, at the same time, make their inhibitory action also dependent on the intracellular cleavage of their S-blocking groups, thus introducing an additional cellular variable which could increase their selectivity.[12] For this reason, a series of S-acyl derivatives of both MU and MUdR were synthesized.

The S-acyl derivatives of MU were readily prepared by refluxing the free thiol with the appropriate acyl chloride or acid anhydride in anhydrous pyridine. In the case of MUdR, selective acylation of the SH group could be achieved by treatment of the nucleoside with the appropriate acid anhydride at near 0°, in the presence of H_2O.

Hydrolysis studies indicated that these thiolesters are quite stable at room temperature to hydrolysis in aqueous buffer solutions at, or below, pH 7.4. At alkaline pH, they are hydrolyzed with the liberation of MU or MUdR, respectively; the rate of hydrolysis becomes significant at pH 8.0–9.0 for the S-acetyl and S-butyryl derivatives, but only at pH >10.0 in the case of the S-isovaleryl compounds.

However, when equimolar concentrations of glutathione, dithiothreitol, or 2-mercaptoethanol were added to the solution (in order to protect the liberated MU or MUdR from oxidation), rapid release of the MU anion (I) or MUdR anion (II) was observed spectroscopically (see Experimental Section) even at neutral pH, indicating that the cleavage of the S-acyl bond in the thiolesters was dramatically accelerated by these thiols. Some of the spectrophotometer recordings of the reactions of III with various concentrations of glutathione and 2-mercaptoethanol at pH 7.4 are illustrated in Figure 1 which also includes control experiments with S-acetylglutathione and ethylene glycol. It is apparent that the initial reaction rates are increased proportionally with the concentration of the thiol, and that glutathione is more effective than 2-mercaptoethanol in converting III to I. The inability of S-acetylglutathione[13] and ethylene glycol (the oxoanalog of 2-mercaptoethanol) even at one order of magnitude higher concentrations to promote the liberation of I from III can be taken as evidence that the observed effect depends on the free SH group of the thiols and that the amino and hydroxyl groups are ineffective in this reaction. The pH dependence of the reaction and the difference in reactivity between glutathione and 2-mercaptoethanol (the pK_a of the SH groups being 9.17 and 9.5, respectively,) suggest that the active species is the ionized form of the SH group.

That the thiols act as acyl "acceptors" rather than

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(13) The very slow reaction observed (curve 6, Figure 1) could be accounted for by a trace amount of glutathione which contaminated the commercial sample of S-acetylglutathione.

Table I
Paper Chromatographic Analysis of the Reaction between S-Acetyl-5-mercaptouracil (III) and Glutathione[a]

No.	Reactants, M		Chromatography of the reaction mixture				
	III	Glutathione	R_f	Uv	Ninhydrin	NH_2OH	Identity[b]
1	0	2×10^{-2}	0.37		+++		GSH
2	2×10^{-2}	0	0.87	+++		+++	III
3	2×10^{-2}	2×10^{-2}	0.87	++		++	III
			0.67	++			MU
			0.57		+++	++	GS-Ac
			0.37		++		GSH
			0.13		+		GSSG

[a] III and glutathione were dissolved in 0.1 M sodium EDTA, pH 8.0, and allowed to react at room temperature (25°), at the indicated concentrations. After 1 hr, aliquots were removed, chromatographed, and analyzed as described under the Experimental Section. [b] Identity was established by parallel chromatography of authentic reference compounds; the number of + signs designates relative intensities of the spots; GSH = glutathione, GS-Ac = S-acetylglutathione, GSSG = oxidized glutathione.

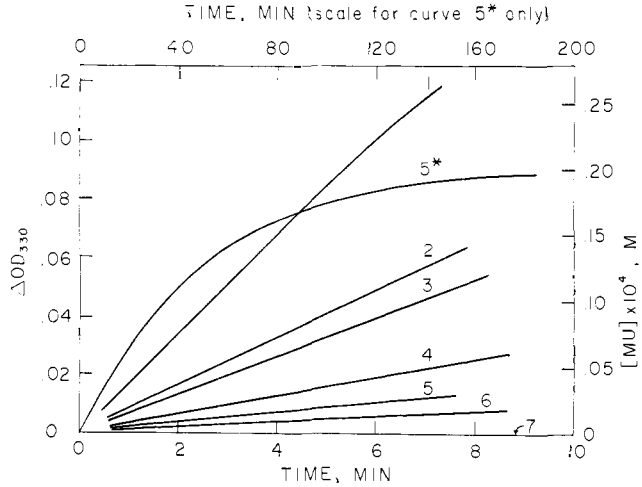


Figure 1.—Conversion of S-acetyl-5-mercaptouracil (III) to MU in the presence of thiols. III, 10^{-4} M, was dissolved in 0.1 M EDTA (containing 10% ethanol, v/v), pH 7.4, and the change of absorbance at 330 mμ was followed in the presence of the following compounds: glutathione, 2×10^{-4} M (curve 1), 10^{-4} M (curve 2), 2×10^{-5} M (curves 5 and 5*); 2-mercaptoethanol, 2×10^{-4} M (curve 3), 10^{-4} M (curve 4); S-acetylglutathione, 10^{-3} M (curve 6); none, or ethylene glycol, 10^{-3} M (curve 7).

catalysts promoting hydrolysis of the thiolesters is suggested by the results of an experiment (curve 5* in Figure 1) in which the reaction of 10^{-4} M III with 2×10^{-5} M glutathione was followed until completion. It is apparent that the glutathione present liberated only a stoichiometric amount of I during a 200-min period, after which no further change in absorbance at 330 mμ could be observed. This indicates that glutathione was consumed while it reacted with a stoichiometric amount of III. Conclusive proof for the actual participation of glutathione as an acyl acceptor in the reaction with III was obtained by paper chromatographic separation and identification of both reaction products, MU and S-acetylglutathione (see Table I). Thus, the reaction of the acyl derivatives of MU and MUdR with aliphatic thiols may be represented by Scheme I.

The concentration dependence of the initial rate of the reaction of the various thiolesters with a fixed (4×10^{-4} M) concentration of glutathione, at pH 7.18, is illustrated in Figure 2. The linearity of the plots indicates that the reaction is first order with respect to the thiolesters. It is also apparent from Figure 2 that the acetyl derivatives (III and VII) react faster than the butyryl analogs (IV and VIII), the nucleosides being somewhat more reactive than the corresponding bases. The slowest rates were obtained in the case of the isovaleryl and pivaloyl derivatives (V, VI, and IX), indicating that the rate of transacylation is influenced by steric hindrance.

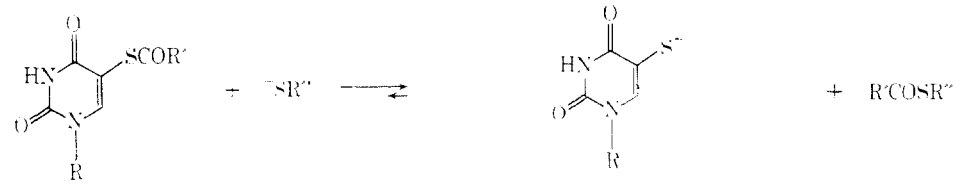
It is noteworthy that the over-all reaction equilibrium of the transacylation of these S-acyl derivatives (Scheme I) is displaced far to the right. This fact is obviously related to the four orders of magnitude difference in the ionization constants of the SH groups of the 5-mercaptopyrimidines (pK_a = 5.3 for MU and 5.0 for MUdR)[10] as compared to those of the aliphatic thiols (pK_a = 9.17 for glutathione). As a consequence, the S-acyl derivatives of MU and MUdR appear to be unique in their effectiveness (as well as versatility) as acylating agents for aliphatic sulfhydryl groups. Results of our preliminary studies using thymidine kinase[14] indicated that these compounds can act as irreversible enzyme inhibitors *via* acylation of the essential sulfhydryl groups of the enzyme, and that the rate of the inactivation of the enzyme is dependent on the structure of the acyl group. The possible uses of these and other S-acyl derivatives of MU and MUdR, as a novel type of sulfhydryl inhibitors in chemotherapy, or as versatile S-acylating agents in the study of enzyme structure, are presently under investigation.

From the point of view of our original objective, it is of interest that this new series of compounds contains MU and MUdR in "protected" forms from which the antimetabolites are released in their active forms (I and II, respectively) *at different rates*, depending on the nature of the acyl group as well as the intracellular concentration of free sulfhydryl groups. By varying the acyl groups, we should be able to establish the optimal conditions for the *in vivo* release of I and II, for maximum chemotherapeutic effectiveness of these antimetabolites.

In the *Lactobacillus leichmannii* assay system (which contains sufficient sulfhydryl groups to reduce the disulfides of MU and MUdR to the free thiols),[3] the S-acetyl derivatives III and VII showed the same activities as MU and MUdR, respectively, while the growth-inhibitory activities of the other S-acyl derivatives showed some variations (ID_{50} = 10^{-6}–10^{-3} M) in spite of the relatively long (18 hr) incubation time.

(14) T. I. Kalman and T. J. Bardos, 158th National Meeting of the American Chemical Society, New York, N. Y., Sept 1969, Abstract BIOL-302.

SCHEME I



III, R = H; R′ = CH_3
IV, R = H; R′ = $(CH_2)_2CH_3$
V, R = H; R′ = $CH_2CH(CH_3)_2$
VI, R = H; R′ = $C(CH_3)_3$
VII, R = 1-(β-D-2-deoxyribofuranosyl); R′ = CH_3
VIII, R = 1-(β-D-2-deoxyribofuranosyl); R′ = $(CH_2)_2CH_3$
IX, R = 1-(β-D-2-deoxyribofuranosyl); R′ = $CH_2CH(CH_3)_2$

I, R = H
II, R = 1-(β-D-2-deoxyribofuranosyl)

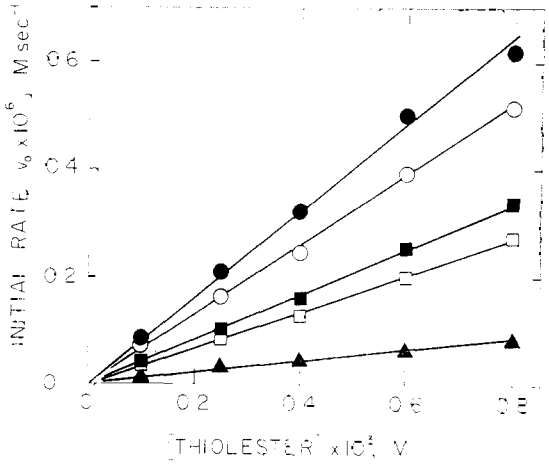


Figure 2.—Initial rates (v_0) of the reaction of glutathione with various S-acyl derivatives of MU and MUdR, at pH 7.18. Reaction rates were determined spectrophotometrically, as described in the Experimental Section. The following thiolesters were used: (●) VII, (○) III, (■) VIII, (□) IV, (▲) IX.

Preliminary animal experiments with III, against leukemia L1210 in mice, indicated that the S-acetyl derivative III is considerably more active than the corresponding free thiol (MU) in potentiating the antitumor effect of 5-fluorodeoxyuridine.[8]

Experimental Section[15]

5-Mercaptouracil[6,16] **(MU), 5-mercapto-2′-deoxyuridine**[2] **(MUdR), and S-acetyl-5-mercaptouracil**[2] **(III)** were prepared by the previously described methods.

S-Butyryl-5-mercaptouracil (IV).—A solution of MU (2.88 g, 0.020 mole) and $(PrCO)_2O$ (3.15 ml, 0.022 mole), in pyridine (25 ml), was refluxed under anhydrous conditions with stirring for 2 hr. After cooling, the solution was evaporated to dryness and the residue crystallized from EtOAc (650 ml), with filtration to remove the unreacted MU. Cooling of the filtrate overnight at 5° gave IV, 2.64 g, mp 212–214°. Concentration of the mother liquors gave additional IV, 0.92 g, mp 211–213°, total yield 83.2%. A sample for analysis was recrystallized from EtOAc, mp 212.5–214°, λ_{max}^{EtOH} 270 mμ (ε 7560), λ_{min}^{EtOH} 242 mμ (ε 2070). *Anal.* ($C_8H_{10}N_2O_3S$) C, H, N.

S-Isovaleryl-5-mercaptouracil (V) was prepared in the same manner as IV, except using isovaleric anhydride.[17] The product was crystallized (MeAc) in two crops (as above), total yield 85%; after recrystallization from MeAc, mp 250–252°, λ_{max}^{EtOH} 271 mμ (ε 7330), λ_{min}^{EtOH} 242 mμ (ε 3290). *Anal.* ($C_9H_{12}N_2O_3S$) C, H, N.

S-Pivaloyl-5-mercaptouracil (VI).—To a solution of MU (2.0 g, 0.138 mole) in pyridine (50 ml) was added pivaloyl chloride (1.83 g, 0.0153 mole), dropwise, at room temperature. After stirring for 0.5 hr, the solution was refluxed ($CaCl_2$ tube) for 1 hr. After evaporation to dryness, the residue was taken up in boiling EtOAc and, after filtration, VI was crystallized from the filtrate (as above), yield 82.2%; after recrystallization from EtOAc, mp 292–294°, λ_{max}^{EtOH} 269 mμ (ε 7980), λ_{min}^{EtOH} 242 mμ (ε 3780). *Anal.* ($C_9H_{12}N_2O_3S$) C, H, N.

S-Acetyl-5-mercapto-2′-deoxyuridine (VII).—A solution of MUdR (100 mg) in water (30 ml) was adjusted to pH 7 with 0.5 *M* $KHCO_3$ (2.5 ml) and cooled in an ice bath with stirring under N_2. Ac_2O (4.7 ml in 50 ml of H_2O, cooled on an ice bath) was added and stirring was continued in the ice bath under N_2 for 0.5 hr. The solution was extracted with Et_2O (four 40-ml portions) and treated with Dowex 50 W resin (H^+ form, 10 ml suspended in H_2O). After standing for several minutes, the solution was filtered from the resin, the resin was washed with H_2O, and the combined filtrates were evaporated to dryness *in vacuo* (bath 50°). After extensive drying of the residue, the material was taken up in hot MeOH (10 ml) and filtered. The filtrate, after standing for several days at −15°, gave white crystals which were collected by filtration and dried to give VII, 45 mg, mp 198–198.5°. Concentration of the mother liquors gave additional VII, mp 195–196°, total yield 57.2%. Recrystallization from MeOH gave an analytical sample, mp 198–199°, λ_{max}^{EtOH} 270 mμ (ε 8640), λ_{min}^{EtOH} 243 mμ (ε 3460). *Anal.* ($C_{11}H_{14}N_2O_6S$) C, H, N, S.

S-Butyryl-5-mercapto-2′-deoxyuridine (VIII) was prepared in a similar manner as VII, but using a solution of $(PrCO)_2O$ in dioxane. The product, crystallized from H_2O, was obtained in 71% yield, mp 193.5–194.5°. *Anal.* ($C_{13}H_{18}N_2O_6S$) C, H, N, S.

S-Isovaleryl-5-mercapto-2′-deoxyuridine (IX).—Acylation of MUdR, in aqueous dioxane, with a solution of isovaleric anhydride[17] in dioxane, gave IX in 59% yield. After recrystallization from H_2O, mp 175–176°. *Anal.* ($C_{14}H_{20}N_2O_6S$) C, H, N, S.

Kinetic Measurements.—The transacylation rate studies were performed at 25.0 ± 0.2° in a Beckman DU monochromator, equipped with a Gilford 2000 multiple-absorbance recorder, at a wavelength of 330 mμ (1.0-cm light path). Stock solutions of the thiolesters were made up with absolute EtOH. The reaction mixture contained varying amounts of a thiolester and glutathione in 0.1 *M* sodium EDTA,[18] pH 7.18, containing 10% EtOH (v/v). The increase of absorbance at 330 mμ which is directly related to the liberation of the thiolate anion of MU (I) (ϵ_{330} 4.5×10^3) or MUdR (II) (ϵ_{330} 5.0×10^3) was recorded, and the transacylation rates (r_0) were calculated from the slopes of the initial linear portions of the curves, corrected for simple hydrolysis in the absence of added acyl acceptor. The pH of the reaction mixtures was determined at 25°, using a Radiometer Type 26 pH meter.

Paper Chromatographic Analysis.—Aliquots of the reaction mixture or the reference solutions were applied on Whatman No. 1 paper sheets and ascending chromatograms were developed overnight with 80% aqueous phenol solution. Three methods of identification were employed: (1) uv light was employed to

(15) Melting points were taken in open capillary tubes on a Mel-Temp apparatus and are uncorrected. Uv spectra were obtained on a Beckman DB recording spectrophotometer, and the molar extinction coefficients were determined with a Gilford multiple sample recorder. Elemental analyses were performed by Galbraith Laboratories, Knoxville, Tenn.

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detect the pyrimidine compounds MU and S-acetyl MU (III) by visual examination (Chromato-Vue, Ultra-Violet Products, Inc.); (2) for the detection of the peptides, glutathione, oxidized glutathione, and S-acetylglutathione, the ninhydrin dipping reagent was used as described by Toennies and Kolb;[19] (3) for the identification of the thiolester compounds the hydroxamate test was employed as described by Stadtman.[20] The R_f values obtained for glutathione, oxidized glutathione, and S-acetylglutathione agree within ±0.06 with those reported.[21]

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Cofactor Inhibition of Thymidylate Synthetase. Tetrahydrofolic Acid Analogs[1,2]

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Analogs of N^5,N^{10}-methylenetetrahydrofolic acid were designed and synthesized in an effort to define the essential features of cofactor binding and inhibition of the enzyme, thymidylate synthetase. Ethyl 3-methyl-2-pyrazinecarboxylate (1), prepared by condensation of ethylenediamine and ethyl 2,3-dioxobutyrate, was reduced (LAH) to the aldehyde **2**. Formation of the Schiff base **3** from **2** and ethyl *p*-aminobenzoate was followed by $NaBH_4$ reduction to give N-(*p*-carbethoxyphenyl)-3-methyl-2-aminomethylpyrazine (**4**). Treatment of **4** with 5-bromomethyluracil gave the N-thyminyl derivative **5**; reduction of **4** gave the piperazine derivative **6**. Condensation of **6** with 5-formyluracil gave 2-*p*-carbethoxyphenyl-3-(5′-uracil)-7-methyloctahydroimidazo-[1,5-*a*]pyrazine (**7**). By the same procedure used in the 3-methylpyrazine series, quinoxaline (**8a**–**10a**), 2-methylquinoxaline (**8b**–**10b**, **22**), and 2-methyl-1,2,3,4-tetrahydroquinoxaline (**11b**, **23**) analogs were prepared. An interesting ring enlargement to the symmetrical seven-membered diaza ketone was observed when 1,2,3,4-tetrahydro-2-hydroxymethyl-1,4-di-*p*-toluenesulfonylquinoxaline was oxidized using the DCC–DMSO method. The results of inhibition of thymidylate synthetase and dihydrofolate reductase are discussed.

Thymidylate synthetase, in the presence of the cofactor N^5,N^{10}-methylenetetrahydrofolic acid, catalyzes the conversion of 2′-deoxyuridine 5′-monophosphate to thymidine 5′-monophosphate.[3] The mechanism proposed for the one-carbon transfer is by a reductive methylation to give the product thymidine 5′-monophosphate and 7,8-dihydrofolic acid.[3]

Folate analogs have been studied as inhibitors of thymidylate synthetase.[4] In addition to the reduced aminopterin derivatives, tetrahydrohomofolate is an effective inhibitor of this enzyme. The rationale for this approach to inhibition is derived from kinetic studies on the enzyme.[3c] A sequential order of binding is noted; the initial complex of enzyme–cofactor is followed by formation of a ternary complex with the substrate. Stepwise dissociation leads to the products.

The nature of the intermediate proposed for the transfer of the carbon unit from the cofactor to the substrate necessitates the proper spatial positioning of these units on the enzyme. Attempts to bridge the binding sites of the cofactor and substrate in a single inhibitor have been unsuccessful.[2,4p] To achieve this additional studies have been undertaken to determine the essential structural features for cofactor binding to the enzyme. Previous results have suggested that the pyrimidine moiety of the pteridine ring of folic acid, important for dihydrofolate reductase binding, may not be essential for binding to thymidylate synthetase. In addition, relatively basic nitrogens corresponding to N^5 and N^8 of the folic acid model are essential since the piperazine ring is more inhibitory than the pyrazine ring in model compounds.[2a] Further studies in this series have been made to examine the effects of a CH_3 in a position corresponding to C-7 of folic acid since Zakrzewski[5] has involved C-7 of tetrahydrofolic acid as the source of H in the reductive methylation of deoxyuridine 5′-monophosphate. Substitution of a benzene ring for the pyrimidyl moiety of folic acid was also undertaken to access the effect of the aromatic ring on the binding affinity of N^5 and N^8 positions of folic acid analogs.

2,3-Dimethylpyrazine (Scheme I) was synthesized by condensation of ethylenediamine with 2,3-butanedione followed by aromatization; selective oxidation (K-

(1) This work was supported by Grant CA 7522 and IK3-CA-10739 from the National Cancer Institutes, National Institutes of Health. Taken in part from the dissertation presented by A. J. Lin to the Graduate School, University of Kansas, in partial fulfillment of the requirements for the Doctor of Philosophy Degree.

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