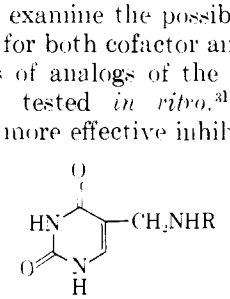
interest has been focused on the possibility of inhibiting this enzyme with analogs of N^5,N^{10}-methylenetetrahydrofolic acid (CH_2-THFA).[3] This stems from studies on the enzyme that demonstrate a sequential order in binding; the initial complex formed between the enzyme and the cofactor CH_2-THFA is followed by formation of a ternary complex with dUMP. Stepwise dissociation leads to the products TMP and 7,8-dihydrofolic acid (DHFA).[4] The source of the methyl group is firmly established as coming from formaldehyde *via* the cofactor. Reduction of the carbon transferred to dUMP is accomplished by oxidation of tetrahydrofolic acid to 7,8-dihydrofolic acid. Although there is a question of the position of label (tritium at C_6 or C_7) on the isotopic reduction of DHFA to THFA by enzymatic and chemical methods, it has been established that the hydrogen (tritium) introduced in reduction of DHFA is transferred to the methyl group of TMP.

Several folate analogs have been studied as thymidylate synthetase inhibitors and found to be effective agents. Kisliuk examined the relative inhibitory effect of reduced aminopterins on both dihydrofolate reductase and thymidylate synthetase.[3d] Reduced homofolate derivatives also have been shown to inhibit thymidylate synthetase.[3f]

The nature of the intermediate in the transfer of the carbon unit from the enzymatically bound cofactor to the bound substrate necessitates the proper spatial positioning of these units on the enzyme. Although an intermediate has been proposed for the methyl transfer,[4d] several questions have arisen regarding the linkage from N_5 of the cofactor to the substrate and the position of loss of the hydrogen from the cofactor (C_6 or C_7) to the product.[5]

In an effort to examine the possibility of combining the binding sites for both cofactor and substrate in one inhibitor a series of analogs of the intermediate were synthesized and tested *in vitro*.[3l] Although these compounds were more effective inhibitors of thymidylate synthetase than dihydrofolate reductase, the anticipated "bridging of the binding sites" was not realized. Two additional compounds in this series were synthesized and tested: N-thymidylglycine (**1**) and ethyl *p*-(N-thymidyl)aminobenzoate (**2**).



1. R = CH_2COOH
2. R = *p*-$C_6H_4COOC_2H_5$

Cline and co-workers[6] reported the detection of a product giving a positive ninhydrin test by treating 5-hydroxymethyluracil with glycine. Examples of uracil undergoing the Mannich reaction at C-5 have been reported.[7] Under these conditions the condensation of 5-hydroxymethyluracil with glycine gave **1** and with ethyl *p*-aminobenzoate gave **2**.

The *in vitro* testing was carried out according to reported procedures on thymidylate synthetase and dihydrofolate reductase.[3l] Compounds **1** and **2** failed to inhibit the former enzyme in a concentration ratio of (inhibitor)/(deoxyuridine 5′-monophosphate) of 30.

Against dihydrofolate reductase in an (inhibitor)/(dihydrofolic acid) ratio of 10 compound **1** showed 20% inhibition and **2** was less than 10% inhibitory.

Experimental Section

Melting points were obtained on a calibrated Thomas–Hoover Unimelt and are corrected. Ultraviolet spectra were recorded on a Cary 14 spectrophotometer. Microanalyses were carried by Drs. G. Weiler and F. B. Strauss, Oxford, England, and using an F and M Model 185 CHN chromatograph.

N-Thymidylglycine (**1**).—5-Hydroxymethyluracil[6] (2.01 g, 0.015 mole) and 3.1 g of glycine hydrochloride (0.03 mole) in 25 ml of water were heated at 90° for 24 hr. After cooling, a precipitate was collected which was purified by repeated precipitation from base by titration to pH 6.8–7.1 and recrystallized from water to give 0.2 g (7%) of product that did not melt below 340°, $\lambda_{max}^{0.1\ M\ HCl}$ 262 mμ (ε 12,000), $\lambda_{max}^{0.1\ M\ NaOH}$ 286 mμ (ε 9200).

Anal. Calcd for $C_7H_9N_3O_4$: C, 42.21; H, 4.55; N, 21.10. Found: C, 42.48; H, 4.39; N, 21.19.

Ethyl *p*-(N-thymidyl)aminobenzoate (**2**) was prepared by the procedure used for **1** from 2.8 g (0.02 mole) of 5-hydroxymethyluracil and 4.0 g (0.2 mole) of ethyl *p*-aminobenzoate in a solution of 1% HCl in 85 ml of 30% aqueous ethanol. After refluxing for 19 hr the hot suspension was filtered and the solid was recrystallized several times from ethanol to give 2.1 g (36%) of product melting at 261–262°; $\lambda^{0.1\ M\ HCl}$ 267 mμ (ε 10,000), 303 mμ (ε 7200); $\lambda_{max}^{0.1\ M\ NaOH}$ 287 mμ (ε 20,000).

Anal. Calcd for $C_{14}H_{15}N_3O_4$: C, 58.12; H, 5.23; N, 14.53. Found: C, 57.88; H, 5.14; N, 14.20.

The authors are indebted to Mrs. William Riggs for the enzyme inhibition studies.

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Chemistry of Cephalosporin Antibiotics. X.[1] Synthesis of Methyl 3-Formyl-7-(thiophene-2-acetamido)-3-cephem-4-carboxylate, a New Cephalosporin Derivative

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Received May 16, 1967

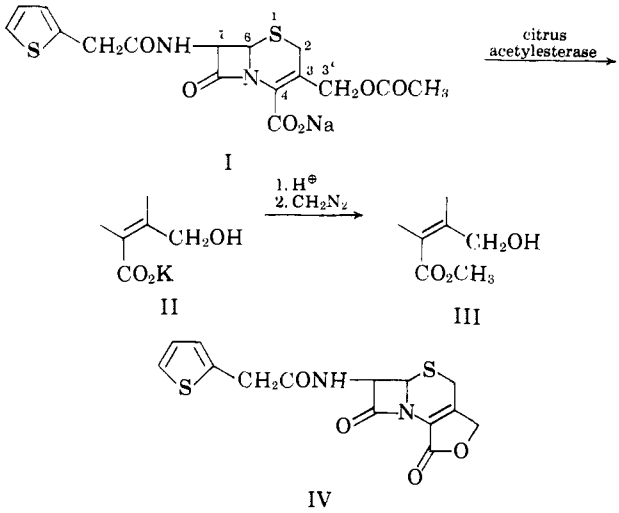
The 3-acetoxymethyl side chain of the cephalosporin nucleus has been subjected to modification in the search

(1) Paper IX: J. L. Spencer, F. Y. Siu, B. G. Jackson, H. M. Higgens, and W. H. Flynn, *J. Org. Chem.*, **32**, 500 (1967).

for new and useful analogs. Displacement of the acetoxy group by nucleophiles[2] has led to the preparation of a large number of new compounds. Hydrogenolysis of the acetoxy group to yield the corresponding deacetoxycephalosporins has been accomplished in several instances.[3] Removal of the acetyl group of cephalosporin C by means of citrus acetylesterase[4] to give the corresponding 3-hydroxymethyl compound was reported by Jeffery, *et al.*[5] This method has been used to prepare other deacetyl cephalosporins.[6] Acylation of deacetylcephalosporins with aroyl halides has been reported by Van Heyningen.[7] The purpose of the present report is to describe the transformation of the 3-acetoxymethyl side chain to the 3-formyl group.

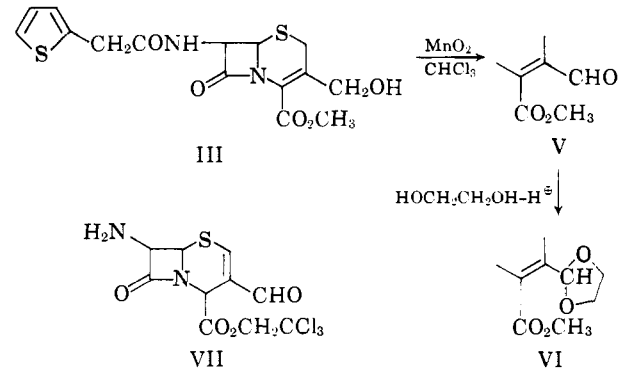
The sodium salt of 7-(thiophene-2-acetamido)cephalosporanic acid[8] (I) was deacetylated by the use of citrus acetylesterase,[4] and the product was isolated in the manner described by Van Heyningen[7] as the potassium salt of 7-(thiophene-2-acetamido)cephalosporadesic acid (II).[9] Acidification of an aqueous solution of II, followed by extraction of the free acid into organic solvent and treatment of the solution with diazomethane, furnished the methyl ester (III) (Scheme I).

SCHEME I



A likely contaminant of the methyl ester was the lactone (IV)[10] since it is known that the cephalosporadesic acids undergo lactonization with facility.[7] The lactone IV may be formed also by cyclization of III. In the oxidation of III,[11] after 18 hr the ultraviolet absorption maximum at 265 mμ had shifted to 275 mμ, and after 24 hr to 280 mμ. No appreciable change occurred in the spectrum during an additional 16 hr of reaction time. Chromatography of the product on silicic acid yielded a crystalline compound which was assigned structure V on the basis of spectral data (see Experimental Section and Scheme II). The aldehyde

SCHEME II



V was converted to the ethylene acetal derivative (VI). Attempts to hydrolyze V to the free acid were unsuccessful.

The aldehyde V inhibited the growth of *Staphylococcus aureus* 3055 at a concentration of 25 μg/ml (agar dilution test). Against *Escherichia coli* (N-26), the MIC was 30 μg/ml (gradient plate assay). In the mouse protection test against *Streptococcus pyogenes* (1000 LD_{50}), the ED_{50} was greater than 41.5 mg/kg × 2 (oral and subcutaneous).[12]

Experimental Section[13]

Methyl 7-(Thiophene-2-acetamido)cephalosporadesate (III).—To a solution of 1.50 g of the potassium salt (II) prepared from I as described by Van Heyningen,[7] in 75 ml of H_2O was added 50 ml of ethyl acetate. The mixture was cooled in ice, stirred vigorously, and the pH was adjusted to 2.0 by the dropwise addition of 1 *N* HCl. The ethyl acetate layer was separated and treated at once with ethereal CH_2N_2. After standing for several minutes, the solution was evaporated to dryness under reduced pressure. The solid residue amounted to 1.07 g. As estimated from the nmr spectrum of this material, it contained 65–75% of III. The lactone (IV) appeared to be an impurity (see nmr data below). Attempts to purify III by recrystallization or chromatography were not successful, and therefore the crude product was used for the next step. The crude product exhibited λ_{max} 235 mμ (ε 13,500) and 260 mμ (ε 7180); ν_{max} 2.75 (OH), 3.00 (NH), 5.60 (β-lactam C=O), 5.82 (ester C=O), 6.00 (amide I), and 6.51 μ (amide II); nmr (DMF-d_7) peaks at δ 3.70, 3.82, 3.92 (C-2 CH_2, ester methyl, and side-chain CH_2), 4.38 (doublet, J = 5.0 cps, C-3′ CH_2), 5.17 (doublet, J = 4.5 cps, H-6; superimposed on this doublet were peaks assigned to H-6 and the CH_2 of the lactone ring of IV), 5.81 (quartet, J = 4.5 and 8.5 cps, H-7), and 6.95, 7.37 (aromatic protons).

Attempts to purify the methyl ester (III) by recrystallization from aqueous acetone yielded a crystalline product which was identified as the lactone IV: mp 235–238° dec (lit.[10] 230–232°); λ_{max} 236 mμ (ε 12,800) and 260 mμ (ε 7020); ν_{max} 3.10, 3.25 (NH), 5.56 (β-lactam C=O), 5.69 (lactone C=O), 6.00 (amide I) and 6.35 μ (amide II); nmr (DMF-d_7) peaks at δ 3.88 (partially resolved doublet, C-2 CH_2 and side-chain CH_2), 5.12 (singlet,

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(9) Deacetylcephalosporins have been assigned the trivial name of cephalosporadesic acids.[7]

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(13) Melting points were determined on a Fisher-Johns apparatus and are uncorrected. The ultraviolet spectra were obtained in ethanol solution on a Cary Model 14 recording spectrometer. The nmr spectra were taken on a Varian Associates Model HR-60 spectrometer. The chemical shifts are reported as δ values in parts per million relative to TMS = 0. The infrared spectra were determined in Nujol.

lactone CH_2), 5.22 (doublet, J = 5.0 cps, H-6), 6.00 (quartet, J = 5.0 and 9.0 cps, H-7), and 7.00, 7.40 (aromatic protons).

Methyl 3-Formyl-7-(thiophene-2-acetamido)-3-cephem-4-carboxylate (V).—A mixture of 0.300 g of impure III, 0.900 g of activated MnO_2,[11] and $CHCl_3$ (30 ml) was stirred at room temperature for 20 hr. An additional 0.450 g of MnO_2 was added and the stirring was continued for another 20 hr. Aliquot samples were taken after 18, 24, and 40 hr. The samples exhibited uv absorption (in $CHCl_3$) at 275, 280, and 280 mμ, respectively, due to the cephalosporin nucleus, and at 241 mμ due to the thiophene side chain. The mixture was filtered and the filtrate was evaporated to dryness. The residue was dissolved in $CHCl_3$ and chromatographed on SiO_2 (Mallinckrodt, 15 g). Elution with EtOAc–$CHCl_3$ (1:3) afforded 0.050 g of V, mp 165–170° dec. Recrystallization from 2-propanol gave material with mp 167–170° dec; λ_{max} 233 mμ (ε 10,550), 296 (10,750), and 340 (shoulder);[14] ν_{max} 3.06 (NH), 5.59 (β-lactam C=O), 5.81 (ester C=O), 6.00 (amide I), 6.23 (double bond), and 6.53 μ (amide II); nmr (DMF-d_7) peaks at δ 3.95, 4.03 (C-2 CH_2, ester CH_3, side-chain CH_2), 5.44 (doublet, J = 6.0 cps, H-6), 6.15 (quartet, J = 6.0 and 9.0 cps, H-7), 7.07, 7.47 (aromatic protons), 9.20 (doublet, J = 9.0 cps, NH), and 9.88 (singlet, CHO proton). A satisfactory mass spectrum was not obtained for this compound due to thermal decomposition of the sample in the mass spectrometer.

Anal. Calcd for $C_{15}H_{14}N_2O_5S_2$: C, 49.16; H, 3.85; N, 7.65; S, 17.50. Found: C, 49.15; H, 4.03; N, 7.18; S, 18.03.

Ethylene Acetal of V (VI).—A mixture of 0.090 g of V, 0.20 g of ethylene carbonate, 0.5 ml of ethylene glycol, 0.002 g of *p*-toluenesulfonic acid monohydrate, and 2.5 ml of tetrahydrofuran was allowed to stand at room temperature for 19.5 hr.[15] The mixture was then poured into water and extracted twice (Et_2O–EtOAc). The combined extracts were washed (1 *M* $NaHCO_3$, twice with H_2O, once with saturated NaCl), and then dried (Na_2SO_4). Evaporation of the solvent gave 0.071 g of solid residue which was crystallized from 2-propanol to give 0.036 g of the ethylene acetal (VI): mp 200–205° dec; λ_{max} 236 mμ (ε 12,600) and 260 mμ (ε 7370); ν_{max} 2.97 (NH), 5.60 (β-lactam C=O), 5.77 (ester C=O), 6.04 (amide I), 6.20 (double bond), and 6.50 μ (amide II); nmr ($CDCl_3$) peaks at δ 3.45 (C-2 CH_2), 3.85, 3.97 (acetal CH_2, ester CH_3 and side-chain CH_2), 4.95 (doublet, J = 5.0 cps, H-6), 5.74 (singlet, H-3′), 5.80 (quartet, J = 5.0 and 8.0 cps, H-7), 6.41 (doublet, J = 8.0 cps, NH), and 6.95, 7.22 (aromatic protons). The molecular weight and formula of VI were determined by high-resolution mass spectrometry.

Anal. Calcd for $C_{17}H_{18}N_2O_6S_2$: mol wt, 410.06220. Found: mol wt, 410.06064.[16]

Acknowledgment.—The authors wish to express their gratitude to H. Boaz, W. Hargrove, G. Maciak, D. O. Woolf, L. G. Howard, and their associates for spectral data; to D. L. Cline, C. Ashbrook, and W. L. Brown for microanalyses; to W. E. Wick and C. W. Godzeski for microbiological data; and to C. P. Walters for the enzymatic deacetylations.

(14) The Δ²-aldehyde (VII) was reported to have λ_{max}^{EtOH} 292 mμ (ε 13,600): R. B. Woodward, K. Heusler, J. Gosteli, P. Naegeli, W. Oppolzer, R. Ramage, S. Ranganathan, and H. Vorbrüggen, *J. Am. Chem. Soc.*, **88**, 852 (1966).

(15) The authors wish to thank Dr. E. Fornefeld for this procedure in which ethylene carbonate acts as a water scavenger.

(16) The mass spectrum was taken on a CEC Model 21-110A-1 spectrometer; ionizing potential 70 ev, temperature of the ion source 180°.

Synthesis of α-Methyl-DL$_g$-glutathione

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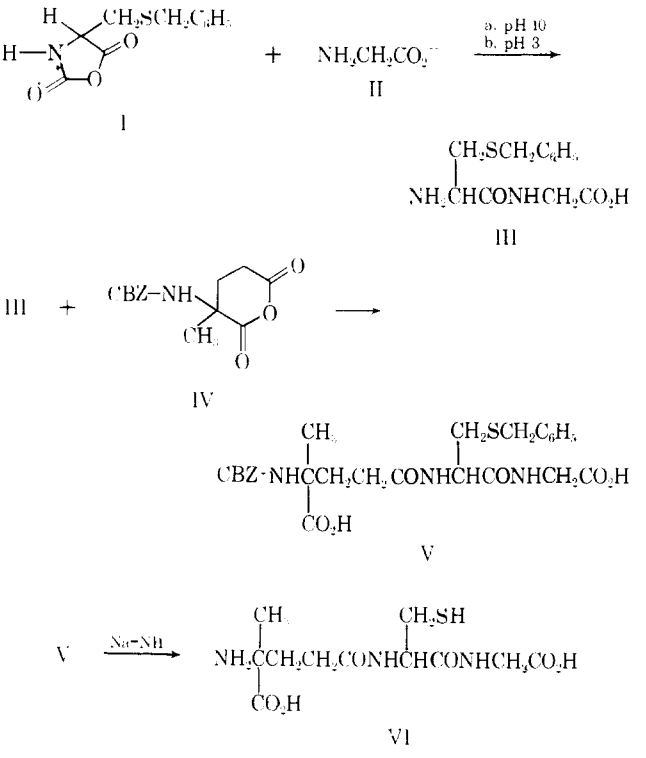
Received April 6, 1967

In recent years α-methylamino acids have proven to be of considerable interest in the study of enzyme mechanisms[1] and in medicinal chemistry.[2,3] However, to our knowledge these derivatives have not been introduced into the sequence of a biologically active peptide. We have carried out such an alteration at the glutamic acid residue of glutathione.

α-Methyl-DL$_g$-glutathione (VI) was prepared by the route outlined in Scheme I. S-Benzyl-L-cysteinyl-

SCHEME I



glycine (III) was prepared by the N-carboxyanhydride procedure.[4] In our initial preparations, III was isolated and purified. It was then allowed to react with CBZ-DL-α-methylglutamic anhydride[5] in aqueous dioxane at pH 8. However, the purification was later found to be unnecessary. On adjusting the pH to 2 and extracting with ethyl acetate, CBZ-α-methyl-DL-glutamyl-S-benzyl-L-cysteinylglycine (V) was obtained as a noncrystalline solid having a trace of CBZ-α-methylglutamic acid as impurity as shown by thin layer chromatography. The blocking groups of V were removed by sodium in liquid ammonia and the resulting thiol VI was purified through the copper(I) salt. The gross structure of VI was established by its elemental analysis and iodometric and base titration. The N-ethylmaleimide derivative moved as a single component by electrophoresis (pH 7.5 and 4.2) and by paper chromatography in several solvent systems.

The nature of the glutamyl peptide link (α *vs.* γ) is an important one. The use of glutamic anhydride generally leads to the formation of an α-peptide as the

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(5) CBZ = carbobenzoxy.