with 300 ml of water, and then with 200 ml of acetone. The product was collected and recrystallized by dissolving in 500 ml of 1% NaOH solution, heating to boiling, and acidifying slightly with HCl while hot. A small portion was recrystallized from a large volume of water for analysis. Even though the compound was dried in a vacuum oven at 100°, the analysis indicated that water of crystallization was present. All water of crystallization was eliminated by heating 24 hr at 160° in a vacuum oven.[11]

5-Substituted 6-Amino-4-carboxy-2-thiopyrimidine (III).—To a solution of 16 g (0.24 mole based on 85% purity) of KOH in 600 ml of 95% ethyl alcohol, 60 g (0.72 mole) of thiourea was added with stirring, followed by 0.24 mole of the potassium ethyl cyanopyruvate[9a] (I) and the reaction mixture was stirred at room temperature for 24 hr. The alcohol was evaporated under reduced pressure and then 500 ml of water was added. The resulting solution was made strongly acidic with concentrated HCl. After several hours the yellowish crystals were separated and purified as described above. When the melting point was determined by the capillary tube method, melting did not occur until that of the decarboxylated derivative. The crystals however did give a characteristic decomposition point on a Fisher-Johns melting point apparatus. After drying the product 8 hr in a vacuum oven at 100°, the analysis of some pyrimidines in this series showed that water of crystallization was still retained.

5-Substituted 6-Amino-4-carboxy-2-hydroxypyrimidine (IV).—Ten grams of the corresponding 6-amino-4-carboxy-2-thiopyrimidine (III) was mixed with 120 ml of a 10% H_2O_2 solution and 120 ml of a 5% NaOH solution was added with stirring. The resulting solution effervesced and a sharp rise in temperature occurred. After the effervescence had subsided, the solution was boiled for several minutes and then allowed to cool. It was made strongly acidic with concentrated HCl. Precipitation occurred slowly. After 3 days enough NaOH solution was added to make the mixture just slightly acidic. The product was separated by filtration, washed with water and acetone, and dried. The product was purified in the same manner as described above. Some members of this series retained water of crystallization after being dried.

Biological Testing. *In Vitro* Antiviral Tests.—Ten-day embryonated eggs were inoculated with four twofold dilutions of the test compound starting with the maximum tolerated dose. Immediately afterwards, the eggs were inoculated with $100EID_{50}$ of virus. For the influenza virus (PR-8) system, the allantoic fluid was harvested at 24 hr, pooled, and titered for hemagglutinating virus. The HA reduction factor was calculated dividing the titer of the controls by that of the treated groups. An HA reduction factor of 10 or greater was considered significant. For the herpes simplex virus system, the eggs were candled each day for 10 days and survivors were recorded. A survival rate of 50% or greater over the control is considered significant.

Antimicrobial Screen.—The test compounds were dissolved or suspended in a nutrient agar at a final concentration of 7.5, 12.5, 50, and 200 mμ/ml. A Steers replicator apparatus simultaneously introduced the microorganisms (*Staphylococcus aureus* (resistant strain), *Klebsiella pneumonia*, *Trichomona foetus*, *Candida albicans*, and *Trichophyton mentagrophytes*) onto the surface of the agar. After appropriate incubation, the plates were observed for inhibition of growth.

Antimalarial Screen.—Mice were infected with a lethal dose of *Plasmodium berghei* 3 days prior to administration of the chemical. Administration of the chemical in oil was made subcutaneously at concentrations of 4, 16, and 64 mg/kg of body weight. The mean survival time of the mice was 6.3 ± 0.5 days. An extension in survival time of chemically treated mice was interpreted as evidence of antimalarial activity.

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(11) On several occasions attempts to remove all water of crystallization resulted in decarboxylation to the corresponding 5-substituted 2,4-diaminopyrimidine.

Synthesis of a Nonapeptide Sequence of Chymotrypsin[1]

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The nonapeptide glycyl-L-aspartyl-L-serylglycylglycyl-L-prolyl-L-leucyl-L-valyl-S-benzyl-L-cysteine, a sequence from chymotrypsin which contains the enzymically active serine residue, has been synthesized by the Merrifield solid phase method.[3] The modifications described by Stewart and Woolley[4] were used. All amino acids were introduced as their *t*-butyloxycarbonyl (Boc) derivatives. The synthesis of a related octapeptide by classical methods has been described.[5] The purified synthetic peptide was found, as expected, not to catalyze the hydrolysis of acetylphenylalanine ethyl ester. The histidine residue which is also necessary for catalytic activity resides in a different peptide chain of the chymotrypsin molecule. The plan of the present project was to synthesize separate peptides containing the essential serine and histidine and to link these peptides by means of a disulfide bridge.

Experimental Section

Synthesis of the Peptide on the Resin Support.—Boc-S-Benzyl-L-cysteine (2.54 g), 0.83 g of triethylamine, and 6.0 g of chloromethylated copolystyrene–divinylbenzene (2% cross-linked) (Biobeads, Bio-Rad Co., Richmond, Calif., 1.36 mequiv of Cl/g) were stirred and refluxed in 30 ml of absolute ethanol for 48 hr. The Boc-S-benzylcysteinyl resin was collected by filtration, washed thoroughly with ethanol, and dried, 6.8 g. The amount of Boc-S-benzylcysteine esterified to the resin was determined by hydrolysis of an aliquot of the product by refluxing for 24 hr with 1:1 dioxane–concentrated HCl. The liberated amino acids were measured with the Spinco amino acid analyzer; the sum of half-cystine and S-benzylcysteine was found to be 0.385 mmole/g of resin.

The coupling of the remaining eight amino acids to the S-benzylcysteinyl resin was carried out in the vessel previously described.[3a] The batch of resin (3.0 g, 1.16 mmoles of cysteine) was rocked with dioxane to swell the resin and was then rocked for 30 min with 4.0 *M* anhydrous HCl in dioxane to remove the Boc protecting group.[6] The resin was washed well (three times each) with dioxane and chloroform, and the hydrochloride of the cysteine was converted to the free base by rocking the resin for 10 min with 10% triethylamine in $CHCl_3$. After thorough washing with $CHCl_3$ and CH_2Cl_2, the resin was treated with a solution of 755 mg (3.48 mmoles) of Boc-L-valine in 10 ml of CH_2Cl_2 and rocked for 5 min to allow the amino acid to penetrate the resin. A solution of 720 mg (3.50 mmoles) of dicyclohexylcarbodiimide (Aldrich Chemical Co.) in CH_2Cl_2 (50% w/v, 1.44 ml) was then added and the vessel was rocked for 2 hr to allow the coupling reaction to go to completion. The resin was then washed thoroughly with CH_2Cl_2 and dioxane. In the same manner, the peptide resin was then acylated successively with the Boc derivatives[7] of leucine, proline, glycine, glycine, O-benzylserine, aspartic acid β-benzyl ester, and glycine. Following the

(1) Supported in part by Grant A1260 from the U. S. Public Health Service.

(2) Deceased July 1966. Address inquiries to J. M. Stewart, Rockefeller University.

(3) (a) R. B. Merrifield, *J. Am. Chem. Soc.*, **85**, 2149 (1963); (b) R. B. Merrifield, *Science*, **150**, 178 (1965).

(4) J. M. Stewart and D. W. Woolley, *Nature*, **206**, 614 (1965).

(5) H. T. Cheung, T. S. Murthy, and E. R. Blout, *J. Am. Chem. Soc.*, **86**, 4200 (1964).

(6) All dioxane was freed of peroxide before use by passing it through a short column of alumina (Merck No. 71707).

(7) Boc-L-amino acids were obtained from Cyclo Chemical Corp., Los Angeles, Calif.

last coupling reaction, the resin was washed thoroughly with CH_2Cl_2, transferred to a Büchner funnel with ethanol, and dried thoroughly *in vacuo*.

Cleavage and Purification of the Nonapeptide.—A 3.0-g portion of the protected nonapeptide resin was suspended in 15 ml of trifluoroacetic acid, and anhydrous HBr was bubbled slowly through the suspension for 90 min. The resin was filtered and washed three times with trifluoroacetic acid. The combined filtrates were evaporated under reduced pressure below 30°, and trifluoroacetic acid was again added and evaporated several times to remove excess HBr. The product was then dissolved in acetic acid and lyophilized to yield 735 mg of crude peptide. Hydrolysis and amino acid analysis of the crude nonapeptide gave approximately the expected amino acid ratios.

The crude nonapeptide (100 mg) was purified by a 99-transfer countercurrent distribution in 1-butanol–water–formic acid (50:50:1 by vol.). The principal component (k = 1.22) was isolated by combination and evaporation of the contents of tubes 40–70 and was subjected to a further 99-tube distribution in the system 1-butanol–pyridine–acetic acid–water (150:2:0.2:150 by vol.). The principal component (k = 0.11) was isolated by combination and evaporation of the contents of tubes 3–20. An aqueous solution of the product was lyophilized to yield 39 mg of a white solid.

The glycylaspartylserylglycylglycylprolylleucylvalyl-S-benzylcysteine was shown to be homogeneous by paper chromatography and paper electrophoresis. The peptide had R_f 0.54 in 2-propanol–water (2:1 by vol.), R_f 0.84 in liquid phenol–water (3:1), and R_f 0.58 in 1-butanol–acetic acid–water (4:1:5). In paper electrophoresis (0.6 M pyridine–acetic acid, pH 6.5) the peptide moved as an anion with R_{asp} 0.28. Hydrolysis and amino acid analysis gave the ratios Gly 3.4, Ser 1.0, Val 1.0, Pro 1.0, Leu 1.1, Asp 1.1, Cys + S-Bzyl Cys 0.94. The nonapeptide exhibited no catalytic activity for the hydrolysis of acetyl-L-phenylalanine ethyl ester when tested by the method of Woolley[8] at 0.15 mg/ml at any pH between 8.5 and 3.0.

(8) D. W. Woolley, *J. Am. Chem. Soc.*, **88**, 2309 (1966).

The Synthesis of the 2α-Methylthio Derivatives of Cortisone, Hydrocortisone, and Progesterone. The Reaction of Methanesulfenyl Chloride with Alkoxalylated Steroid Ketones

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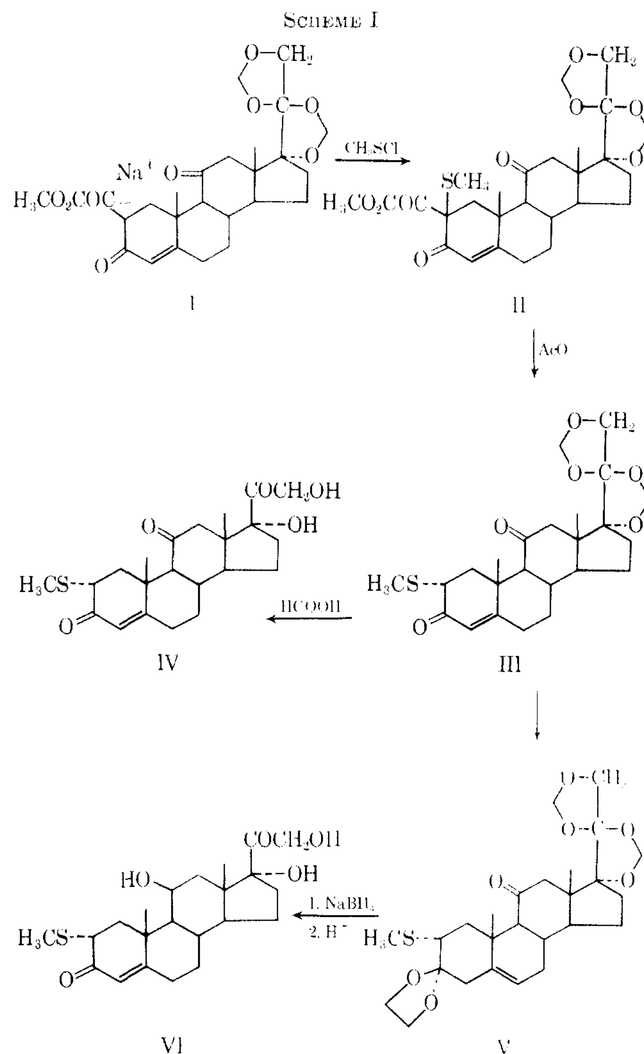
In continuation of our program in the steroid hormone analog field we wish to report the preparation of the 2α-methylthio derivatives of cortisone, hydrocortisone, and progesterone.[1] Introduction of the methylthio group was accomplished by the reaction of appropriate alkoxalylated steroids with methanesulfenyl chloride[7] and this reaction represents a further demonstration of the synthetic versatility of these steroid derivatives. The utility of alkoxalylated steroid ketones and/or the related hydroxymethylene ketones for the introduction of alkyl, aryl,[8] cyano, benzoyloxy, and hydroxyimino[9] groups as well as for the halogens, including fluorine, has been demonstrated.[10]

2α-Methylthiocortisone was prepared by the sequence I → IV (Scheme I). Reaction of the sodium



salt (I)[11] of the bismethylenedioxy derivative[12] of 2-methoxalylcortisone with methanesulfenyl chloride gave the 2-methylthio-2-methoxalyl derivative II in 75% crude yield. Acetate-induced demethoxalylation afforded 57% of the 2α-methyl thio-Δ^4-3-ketone III, and BMD hydrolysis with formic acid[13] furnished the desired 2α-methylthiocortisone (IV).

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(5) R. E. Schaub and M. J. Weiss, *J. Org. Chem.*, **26**, 3915 (1961).

(6) R. E. Schaub and M. J. Weiss, *ibid.*, **26**, 1223 (1961).

(7) The successful condensation of 2-hydroxymethylenecholestan-3-one with trimethylene dithiotoluene-*p*-thiosulfonate has been reported [J. L. Beton, T. G. Halsall, E. R. H. Jones, and P. C. Phillips, *J. Chem. Soc.*, 753 (1957); see also R. L. Autrey and P. W. Scullard, *J. Am. Chem. Soc.*, **87**, 3284 (1965)]. For a discussion of the reaction of sulfenyl halides with various nucleophiles see E. E. Reid, "Organic Chemistry of Bivalent Sulfur," Vol. I, Chemical Publishing Co., Inc., New York, N. Y., 1958, p 273 ff.

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(10) See G. R. Allen, Jr., and M. J. Weiss, *J. Org. Chem.*, **27**, 4681 (1962), and references cited therein.

(11) The lithium salt gave similar results (see Experimental Section).

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