The Phenol $C_{18}H_{16}O$ from Dehydrogenation of Rubijervine.—A sample of the material obtained by Jacobs and Craig[9] was used without further purification for the experiments reported in this paper. In our hands it melted at 130–132° (reported[9] m.p. 136–138°). Its infrared spectrum (KBr) was characterized by bands at 3240 cm.$^{-1}$ (OH stretching) and 859, 853, 844, 827, 817, 755sh. and 748 cm.$^{-1}$ (aromatic C–H).

Reduction of the Phenol $C_{18}H_{16}O$ with Zinc Dust.—A mixture of 55 mg. of the phenol and 2.1 g. of zinc dust was heated in a sealed tube at 350–360° for 2 hr. After cooling to room temperature, the tube was opened and the contents were extracted repeatedly with benzene. On evaporation to dryness *in vacuo*, the benzene solution gave 39 mg. of a dark brown gum. This material was chromatographed over alumina giving 8 mg. of brown oil on elution with benzene and an additional 30 mg. of brown oil on elution with ether and methanol. The former resisted attempts at further purification and the latter furnished, on rechromatography and recrystallization (Norit) from ether, colorless needles, m.p. 133–134°, undepressed on admixture with starting material.

A mixture of 51 mg. of the phenol and 1.0 g. of zinc dust was heated at 400 ± 10° for 6 hr. to give 18 mg. of crude product which on chromatography over alumina gave on elution with benzene 11.5 mg. of an amorphous colorless glass. This material was crystallized from ethanol to give irregular crystals subliming above 220°, with a melting point *ca.* 242–248°, much dependent on the rate of heating. The melting point of a mixture of this material and a sample of chrysene (m.p. 256–258°) was 252–256°. The ultraviolet absorption spectrum was identical with that reported for chrysene[55] within experimental error.

Reduction of 3-Phenanthrol to Phenanthrene *via* Its Diethyl Phosphate Ester.[43]—Solutions containing 0.28 mmole of diethyl phosphite[56] and of triethylamine in carbon tetrachloride were prepared. A 52-mg. (0.26 mmole) sample of 3-phenanthrol[44] was dissolved in a 1-ml. aliquot of the diethyl phosphite solution, and this solution was cooled in an ice-bath. A 1-ml. aliquot of the triethylamine solution was then added, and the solution was allowed to stand at room temperature overnight. An additional 10 ml. of carbon tetrachloride was added, and the solution was washed with dilute hydrochloric acid and cold dilute sodium hydroxide (four times) and finally water. The organic layer was evaporated to dryness *in vacuo* giving 67 mg. of light brown oil. This material was taken up in a minimum of ether and cooled in a Dry Ice–Cellosolve-bath. A large excess of liquid ammonia was then run in until only one liquid phase was present and a solid had separated. The solution was then removed from the bath, and when it had reached the boiling temperature of the liquid ammonia several drops of ether were added to bring the solid into solution. Small pieces of sodium were added until a permanent blue color developed. Several drops of alcohol were added, and the solution was allowed to evaporate overnight. The residue was taken up in ether, washed with sodium bicarbonate, sodium hydroxide and finally water and then dried and evaporated to dryness *in vacuo* to give 27 mg. of nearly colorless oil. This was chromatographed over alumina to give 14.5 mg. of white crystals, m.p. 83–91°. Recrystallization from ethanol gave colorless scales 95–98°, undepressed when mixed with authentic phenanthrene. The infrared absorption spectrum of this material in KBr was identical with that of an authentic sample.

Reduction of the Phenol $C_{18}H_{16}O$ *via* Its Diethyl Phosphate Ester.—A sample of 45 mg. of the phenol from the dehydrogenation of rubijervine was converted to its diethyl phosphate ester (54 mg.) by the above procedure. Reduction was accomplished as above, 11 mg. (2 moles) of sodium being added without a permanent blue color developing. The reaction mixture was worked up as before giving 28 mg. of crude petroleum ether-soluble product. This material on chromatography over alumina furnished 15 mg. of white crystals, m.p. 75.5–78.5°. Rechromatography and recrystallization from ethanol gave crystals of m.p. 79–81°, undepressed on admixture with authentic 1′-methyl-1,2-cyclopentenophenanthrene. The infrared (Fig. 3) and ultraviolet absorption spectra of this material are identical with those of 1′-methyl-1,2-cyclopentenophenanthrene.

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(55) R. A. Friedel and M. Orchin, "Ultraviolet Spectra of Aromatic Compounds," John Wiley and Sons, Inc., New York, N. Y., 1951, Fig. 447.

(56) H. McCombie, B. C. Saunders and G. J. Stacey, *J. Chem. Soc.*, 380 (1945).

[CONTRIBUTION FROM THE LABORATORY OF BIOCHEMISTRY, NATIONAL CANCER INSTITUTE, NATIONAL INSTITUTES OF HEALTH]

Preparation and Properties of the Isomeric Forms of Cystine and S-Benzylpenicillamine

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Racemization of bis-(acetyl)-L-cystine in glacial acetic solution was achieved readily through the action of acetic anhydride at room temperature, and the epimeric mixture of optically inactive bis-(acetyl)-cystine so derived isolated in high yield. The asymmetric hydrolytic action of the L-directed renal acylase I upon this latter derivative, at *p*H 7.5 and 37°, resulted in a mixture of free L-cystine, monoacetyl-*meso*-cystine (acetyl moiety at D-position), and bis-(acetyl)-D-cystine. The marked insolubility of the former compound permitted its direct isolation from the enzymic digest by filtration, while the two latter compounds were acid hydrolyzed to *meso*-cystine and D-cystine, respectively, after their separation and isolation from the filtrate on a Dowex-50 column in the acid phase. Specific rotation values were −211 and +208° (1% in *N* HCl) for the L- and D-enantiomorphs, respectively, whereas the *meso*-cystine was completely devoid of detectable optical activity. In addition, a resolution of S-benzyl-DL-penicillamine was effected *via* the hydrolytic action of hog kidney amidase on the amide derivative at *p*H 8.0. Subsequent separation of the liberated S-benzyl-L-penicillamine from the unhydrolyzed S-benzyl-D-penicillamine amide on an Amberlite XE-64 column, followed by acid hydrolysis of the latter compound, ultimately yielded the pure enantiomorphs with specific rotation values of +91.3 and −90.6° (1% in *N* HCl) for the L- and D-forms, respectively. Infrared spectra for each of the stereoisomeric forms of cystine, in addition to data on the susceptibility of these compounds or certain of their selected derivatives to the enzymic action of L- and D-amino acid oxidase and hog renal acylase I, are presented.

Cystine is a symmetrical diaminodicarboxylic acid which, although hitherto found in nature only as the L-form, may in fact exist as two racemic modifications, one an internally compensated *meso*-form, and the other a mixture of externally compensated isomerides. Since L-cystine may be relatively inexpensively isolated from acid hydrolysates of certain natural materials, *e.g.*, hair or wool, such source has provided the major supply of commercially available cystine. However, the drastic hydrolytic conditions employed in its manufacture might also lead to rather extensive racemization, and commercial samples of "L-cystine" whose specific rotation values differ by as much as thirty or forty degrees from the generally accepted value of −212° (in *N* HCl) have not been uncommon. With the view in mind that certain biochemical studies demand not only L-cystine, but also the D-

and *meso*-forms, in a high degree of stereochemical purity, the preparation of these isomers *via* the general enzymic resolution procedures developed in this Laboratory[1] is here presented. In addition, an enzymic resolution procedure for preparing the optical antipodes of S-benzylpenicillamine is also described.

Results and Discussion

Racemization and Resolution of Cystine.— The preparation of inactive cystine by Mörner,[2] in 1899, subsequently led to a series of investigations[3-5] concerned with the question of its stereochemical identity. Such question was resolved when du Vigneaud, in collaboration with Hollander[4] and Loring,[5] demonstrated that the racemization of L-cystine, upon boiling for five days in hydrochloric acid, led to an epimeric mixture of DL- and *meso*-cystine. Separation of the *meso*- from the DL-form could be effected through a series of fractional crystallizations of the hydrochloride salts. Treatment of the bis-(acetyl) derivative of the epimeric mixture with brucine resulted in a separation of the alkaloid salt of bis-(acetyl)-D-cystine, which, after some twelve crystallizations, yielded D-cystine with $[\alpha]D^{20}$ +212° (in HCl).[4] Preparation of D-cystine with $[\alpha]^{26}D$ +208° could be achieved, in addition, from the insoluble salt which arose from the treatment of bis-(formyl)-DL-cystine with strychnine.[6] Alternative preparations of D-cystine with $[\alpha]^{20}D$ +224°[7] and +221.2°[8,9] (in HCl) resulted from the debenzylation of S-benzyl-D-cysteine with the sodium–liquid ammonia procedure, followed by oxidation of the D-cysteine so liberated.[10]

The direct resolution of DL-cystine was handicapped by virtue of the prolonged refluxing (5 days) of L-cystine with concentrated HCl required for its conversion to the epimeric starting material, as well as by the tedious fractional crystallizations necessitated for the separation of the DL- and the *meso*-forms.[5] A search for methods whereby these difficulties could be obviated was therefore undertaken. The former difficulty was resolved with the observation that the action of a glacial acetic acid–acetic anhydride mixture[11] on bis-(acetyl)-L-cystine, at room temperature, readily proceeded with the formation of an epimeric mixture of bis-(acetyl)-DL- and *meso*-cystine, which could be isolated in high yield (84% of theory).[12] With an eye toward the previous success achieved in the separation of the D-, L- and *meso*-forms of α,ϵ-diaminopimelic acid *via* the asymmetric enzymic hydrolysis of a mixture of their amide derivatives,[13] it appeared particularly tempting to subject the epimeric mixture of acetylcystine to an analogous scheme. Such scheme, if successful, would lead to an easily separable mixture of L-cystine, bis-(acetyl)-D-cystine and monoacetyl-*meso*-cystine (acetyl moiety at the D-position). Although a previous report from this Laboratory[9] had indicated that the N-acylated cystines were highly resistant to the hydrolytic action of hog renal acylase I, under the conditions given, re-investigation of the hydrolytic reaction with varying concentrations of enzyme revealed that it could be induced to proceed at a rate which was of sufficiently large magnitude to make the resolution of inactive bis-(acetyl)-cystine practicable.

Racemization of bis-(acetyl)-L-cystine was effected upon treatment of a glacial acetic acid solution thereof with an excess of acetic anhydride at room temperature. The course of the racemization was followed polarimetrically. As is revealed in Fig. 1, a plot of observed rotation *vs.* time

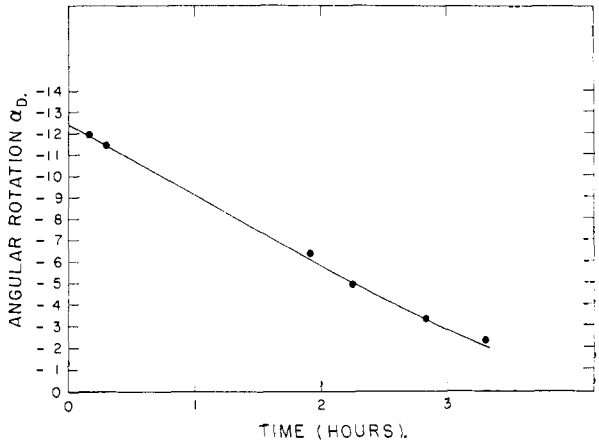

Fig. 1.—Change in optical rotation, with time, of a solution of 20 g. of bis-(acetyl)-L-cystine in 150 ml. of glacial acetic acid upon the addition of 12 moles of acetic anhydride at room temperature (about 27°). A 2 dm. tube was employed.

revealed a linear relationship over approximately the first three hours, during which time the observed rotation fell to about 25% of its initial value. In order to ensure complete optical inactivation, the reaction mixture was permitted to stand for some 16 hours prior to the isolation of the epimeric substance. No detectable desulfuration of the cystine residue was apparent during the entire course of the reaction under the conditions employed herein.

(1) J. P. Greenstein, *Adv. Protein Chem.*, **9**, 122 (1954); J. P. Greenstein, S. M. Birnbaum and M. C. Otey, *J. Biol. Chem.*, **204**, 307 (1953).

(2) K. A. H. Mörner, *Z. physiol. Chem.*, **28**, 595 (1899).

(3) W. F. Hoffman and R. A. Gortner, THIS JOURNAL, **44**, 341 (1922); R. A. Gortner and W. F. Hoffman, *J. Biol. Chem.*, **72**, 433 (1927).

(4) L. Hollander and V. du Vigneaud, *ibid.*, **94**, 243 (1931–1932).

(5) H. S. Loring and V. du Vigneaud, *ibid.*, **102**, 287 (1933).

(6) V. du Vigneaud, R. Dorfmann and H. S. Loring, *ibid.*, **98**, 577 (1932).

(7) J. L. Wood and V. du Vigneaud, *ibid.*, **130**, 109 (1939).

(8) L. Levintow, V. E. Price, and J. P. Greenstein, *ibid.*, **184**, 55 (1950).

(9) S. M. Birnbaum and J. P. Greenstein, *Arch. Biochem. Biophys.*, **39**, 108 (1952).

(10) The S-benzyl-D-cysteine here employed was secured from the resolution of formyl-S-benzyl-DL-cysteine with brucine,[7] and from the asymmetric enzymic hydrolyses of S-benzyl-DL-cysteine amide[8] and acetyl-S-benzyl-DL-cysteine[9] with hog kidney amidase and acylase I, respectively.

(11) M. Bergmann and L. Zervas, *Biochem. Z.*, **203**, 280 (1928).

(12) The racemization of bis-(acetyl)-L-cystine with acetic anhydride in an aqueous medium at 37° was previously reported (V. du Vigneaud and C. E. Meyer, *J. Biol. Chem.*, **98**, 295 (1932)). However, an appreciable degree of desulfuration was noted by these investigators during the course of the reaction.

(13) E. Work, S. M. Birnbaum, M. Winitz and J. P. Greenstein, THIS JOURNAL, **77**, 1916 (1955); R. Wade, S. M. Birnbaum, M. Winitz, R. J. Koegel and J. P. Greenstein, *ibid.*, **79**, 648 (1957).

The bis-(acetyl) derivative of inactive cystine was subjected to the asymmetric hydrolytic action of the L-directed renal acylase I. Description of the preparation of this enzyme from hog kidney homogenates has appeared elsewhere.[14] Digestion of a 0.1 *M* solution of the epimeric substrate with the lyophilized enzyme, at *p*H 7.5, was effected at 37°. An initial rate of hydrolysis, as measured by the Van Slyke manometric ninhydrin–CO_2 procedure, was 10 micromoles per hr. per mg. of protein nitrogen. Upon completion of the reaction, the liberated L-cystine, by virtue of its extreme insolubility, could be isolated directly from the enzymic digest by simple filtration. The mixture of bis-(acetyl)-D-cystine and monoacetyl-*meso*-cystine, which remained in the filtrate, could ultimately be separated upon passage through a Dowex-50 column in the acid phase. Acid hydrolysis of each of the latter two compounds subsequently yielded pure D- and *meso*-cystine, respectively, which could be crystallized from water through the hydrochloride salts.[15] Optical rotation values for the enantiomorphic D- and L-forms were equal and opposite within the limits of experimental error, while the *meso*-form was devoid of any detectable optical activity, an indication of its freedom from optical contamination. The D-cystine, when subjected to the action of L-amino acid oxidase (*Crotalus adamanteus*) in the usual fashion,[16] revealed an optical purity which was greater than 99.9%. A time–rate comparison of the susceptibility of *meso*-cystine and L-cystine to the oxidative action of L-amino acid oxidase has appeared elsewhere.[17,18]

Effect of N-Acyl Substituents and Co^{++} Concentration on Hydrolytic Rates.—In view of earlier communications[19,20] which demonstrated that the hydrolytic activity of renal acylase I toward certain acetyl-L-amino acids was accelerated in some instances, and depressed in others, by the addition of Co^{++} to the enzymic digest, it became of interest to determine the behavior of bis-(acetyl)-L-cystine under comparable circumstances. Thus, although the initial hydrolytic rate of a digest, which was 0.017 *M* in this substrate, was 10 micromoles per hr. per mg. of protein nitrogen, such rate was progressively increased to a value of 19.6 at a Co^{++} concentration of 10^{-3} *M*. An even greater increase in activation was shown with bis-(chloroacetyl)-L-cystine, with the rate of hydrolysis in the presence of a 10^{-3} *M* concentration of Co^{++} increasing some 186% over the digest devoid of added cobalt. Such results indicated that the degree of activation of acylase I by Co^{++}, in addition to being dependent upon the nature of the amino acid residue, as hitherto observed,[20] was also highly dependent upon the nature of the N-acyl substituent. Substantiation of this latter view was obtained with digests containing acetyl-L-alanine and D-chloropropionyl-L-alanine[21] as substrates, with a 10^{-3} *M* Co^{++} concentration leading to a 60% activation in the former instance and a 147% activation in the latter. The results secured with various levels of Co^{++} are revealed in Table I.

TABLE I

EFFECT OF Co^{++} ON THE SUSCEPTIBILITY OF N-ACYLAMINO ACIDS TO THE HYDROLYTIC ACTION OF RENAL ACYLASE I

Substrate	Rate[a] without added Co^{++}	Increase in rate on addn. of Co^{++} at concn., % 1×10^{-4} *M*	1×10^{-3} *M*	1×10^{-2} *M*	4×10^{-2} *M*
Bis-(acetyl)-L-cystine	10.2	+ 14	+ 96	+81	−75
Bis-(chloroacetyl)-L-cystine	36.6	+109	+186	+87	−59
D-Chloropropionyl-L-alanine	32.3	+ 63	+147	+80	−61
Acetyl-L-alanine	5800	+ 12	+ 60	+25	− 9

[a] Rates expressed as micromoles of substrate hydrolyzed per hr. per mg. of protein N in the reaction mixture at *p*H 7.0 and 37°; substrate at 0.017 *M* concentration; hydrolysis figures taken within 30% of complete cleavage so as to approximate a zero order reaction.

It should perhaps be noted, in addition, that preliminary studies on the hydrolytic susceptibility of various acylated cystines to acylase I indicated that while the monocarbobenzoxy derivative of L-cystine[22,23] was completely resistant to enzymic cleavage, the bis-(chloroacetyl) and bis-(glycyl) derivatives were hydrolyzed at rates of 37 and 60 micromoles per hr. per mg. of protein nitrogen, respectively. This finding was of interest in the light of previous data[19] that, with the exception of the tryptophan and tyrosine derivatives, the acylase I induced hydrolysis of a given chloroacetyl-L-amino acid generally proceeded at a greater rate than that of the corresponding glycyl-L-amino acid. The anomalous behavior of the part of the tyrosine and tryptophan derivatives was attributed to the presence of one or more as yet unidentified highly specific acylases in hog kidney. On the basis of the data here obtained, the association of the hydrolytic activity toward the acylated cystines with an unidentified acylase is likewise suggested.[24]

(14) S. M. Birnbaum, L. Levintow, R. B. Kingsley and J. P. Greenstein, *J. Biol. Chem.*, **194**, 455 (1952).

(15) E. Fischer and K. Raske, *Ber.*, **41**, 893 (1908).

(16) A. Meister, L. Levintow, R. B. Kingsley and J. P. Greenstein, *J. Biol. Chem.*, **192**, 535 (1951).

(17) N. Izumiya and J. P. Greenstein, *Arch. Biochem. Biophys.*, **52**, 203 (1954).

(18) The initial rates of oxidation for L- and *meso*-cystine by L-amino acid oxidase (*Crotalus adamanteus*) here observed were 59 and 28 micromoles of oxygen consumed per hr. per mg. of protein nitrogen, respectively. The former value is in full accord with that previously observed,[17] while the latter, although still considerably less than that of L-cystine, is somewhat larger than the value recorded earlier.[17]

(19) K. R. Rao, S. M. Birnbaum, R. B. Kingsley and J. P. Greenstein, *J. Biol. Chem.*, **198**, 507 (1952).

(20) R. Marshall, S. M. Birnbaum and J. P. Greenstein, THIS JOURNAL, **78**, 4636 (1956).

(21) S. C. J. Fu, S. M. Birnbaum and J. P. Greenstein, *ibid.*, **76**, 6054 (1954).

(22) Carbobenzoxy-*cyclo*-L-cystinyl was also resistant to enzymic cleavage. The only carbobenzoxy-amino acid which has thus far proved susceptible to hydrolysis by acylase I was the L-glutamic acid derivative (L. Levintow, J. P. Greenstein and R. B. Kingsley, *Arch. Biochem. Biophys.*, **31**, 77 (1951)).

(23) Preparation of this derivative was effected by the treatment of a several-fold excess of L-cystine with carbobenzoxy chloride under Schotten-Baumann conditions. Removal of the excess L-cystine was achieved by adjustment of the *p*H to 6, followed by filtration. Subsequent adjustment of the *p*H of the filtrate to 3.2 led to the precipitation of the desired monocarbobenzoxy derivative which, upon being copiously washed with ethanol, was freed from any contaminating dicarbobenzoxy derivative. The compound should prove of value for the synthesis of unsymmetrical cystine peptides.

(24) The peptidase action of an 87% glycerol extract of hog intestinal mucosa has been earlier employed to cleave L-cystinylglycine and L-cystinylglycylglycine, and the L-cystine thus liberated isolated in pure, crystalline form (J. P. Greenstein, *J. Biol. Chem.*, **124**, 255 (1938)).

Resolution of S-Benzylpenicillamine.—The synthetic work related to penicillin during the early 1940's stimulated considerable interest in the general preparation and chemistry of the penicillamines. Resolution of DL-penicillamine was generally effected *via* the action of alkaloids on N-formyl-DL-penicillamine,[25] N-formylisopropylidene-DL-penicillamine[25] and various derivatives of S-benzylpenicillamine.[25,26] Application of the general enzymic procedures developed in this Laboratory to the resolution of DL-penicillamine assumed especial interest in view of the previous behavior exhibited toward hydrolytic enzymes by derivatives of the structurally related *t*-leucine. Thus, although the complete resistance of chloroacetyl-DL-*t*-leucine to the hydrolytic action of renal acylase I could be conceivably attributed to the high degree of steric hindrance arising from complete substitution about the β-carbon atom of the substrate, no such absolute resistance was encountered upon subjecting *t*-leucine amide to the hydrolytic action of hog kidney amidase.[27] That the same general pattern was followed for the N-acetyl derivatives of S-benzyl- and S-methylpenicillamine was indicated by the inability of renal acylase I to effect their cleavage. However, the ability of the L-directed hog kidney amidase[28] to induce an asymmetric cleavage of S-benzyl-DL-penicillamine amide, albeit slow, nonetheless made an enzymic resolution feasible here.

The S-benzyl-DL-penicillamine amide employed in the present investigation was prepared by the conversion of carbobenzoxy-S-benzyl-DL-penicillamine to the corresponding amide derivative, *via* the mixed carbonic–carboxylic anhydride procedure,[29] followed by decarbobenzoxylation of this latter compound with hydrogen bromide in glacial acetic acid.[30] Asymmetric cleavage of the amide with a hog kidney amidase preparation[28] proceeded at a rate of 5 micromoles per hr. per mg. protein nitrogen at *p*H 8.0, 38°, and a substrate concentration of 0.01 to 0.02 *M*. Isolation of the liberated S-benzyl-L-penicillamine and S-benzyl-D-penicillamine amide was achieved through the utilization of an Amberlite XE-64 resin in the acid phase.[31] It is especially noteworthy that the steric hindrance imposed by the groups about the β-carbon atom presumably made the D-amide extremely resistant to acid hydrolysis, for less than 10% cleavage occurred upon refluxing the compound with ten volumes of 2 *N* HCl for 2 hr.[32] Conversion of the D-amide to S-benzyl-D-penicillamine was achieved upon its treatment with 5 *N* HCl, under reflux, for some 6 hr. An $[\alpha]^{24}D$ −90.6° (1% in *N* HCl) was revealed for the D-amino acid so procured, a value which was equal and opposite, within the limits of experimental error, to the $[\alpha]^{24}D$ +91.3° (1% in *N* HCl) secured for the L-antipode. Unfortunately, the complete resistance of these enantiomers to D-amino acid oxidase and L-amino acid oxidase (*Crotalus adamanteus*), respectively, did not permit measurements of their optical purity.[16]

Infrared Spectra.—The absorption spectra of L-cystine and of such peptides as cyclo-L-cystinyl and of L-cystinyl-L-cystine have been described.[33] L-Cystine and D-cystine were mixed in equal amounts and the mixture recrystallized as described above. The solid state spectrum in the infrared region of the resulting DL-cystine was found to be identical with that of L-cystine which in turn is identical with that of D-cystine The spectra of *meso*-cystine was found to be almost indistinguishable from that of L-, D- or DL-cystine.

Experimental

I. Resolution and Derivatives of Cystine. Monocarbobenzoxy-L-cystine.—One hundred grams (0.42 mole) of L-cystine was dissolved in 1 l. of cold 1.65 *N* NaOH. The solution was placed in an ice-bath and 25 ml. of carbobenzoxy chloride added thereto over a period of 1 hr. with vigorous stirring. After an additional 20 min., the reaction mixture was carefully adjusted to *p*H 6 with 6 *N* HCl and the stirring and cooling continued for 20 min. longer. The precipitate of excess L-cystine was filtered over suction and washed with 100 ml. of water. The combined filtrate and washings were adjusted to exactly *p*H 3.2 with 6 *N* HCl and, after cooling at 4° for several hours, was filtered and washed copiously with alcohol, then ether. A yield of 37.5 g. of pure monocarbobenzoxy-L-cystine was so secured. Recrystallization could be effected by suspending the material in 1 l. of water, bringing the *p*H to 6.5 by the addition of 2 *N* NaOH with stirring to effect solution filtering off any undissolved material, and then adjusting the *p*H downward to 3.2 by the addition of 6 *N* HCl. After cooling, the precipitate was filtered, and washed successively with a little cold water, ethanol and ether; $[\alpha]^{23}D$ −126° (1% in 5 *N* HCl) and −120° (1% in 1 *N* NaOH).

Anal. Calcd. for $C_{14}H_{18}O_6N_2S_2$: C, 44.9; H, 4.8; N, 7.5; S, 17.1. Found: C, 44.6; H, 4.9; N, 7.4; S, 16.7.

Carbobenzoxycyclo-L-cystinyl.—This compound was prepared by the carbobenzoxylation of cyclo-L-cystinyl[34] according to the usual Schotten–Baumann procedure. The precipitate secured after acidification of the reaction mixture was recrystallized from ethyl acetate–petroleum ether; dec. p. 170–172° with preshrinking at 141°, $[\alpha]^{24}D$ −52.5° (1% in methanol).

Anal. Calcd. for $C_{14}H_{18}O_5N_2S_2$: C, 47.2; H, 4.5; N, 7.9; S, 18.0. Found: C, 47.6; H, 4.8; N, 7.6; S, 17.6.

Bis-(glycyl)-L-cystine.—Preparation of this compound has been reported by several investigators.[35–38] The material here employed was prepared by amination of the precursor bis-(chloroacetyl)-L-cystine[39] according to a modification of the procedure of Abderhalden and Wybert[35]; $[\alpha]^{23}D$ −118° (1% in water) for the monohydrate, and −114° (1% in *N* HCl).

Bis-(acetyl)-L-cystine.—To a solution of 24.0 g. of L-cystine in 100 ml. of ice-cold 2 *N* NaOH were added alter-

(25) "The Chemistry of Penicillin," Princeton University Press, Princeton, N. J., 1949, p. 464.

(26) L. H. Werner, A. Wettstein and K. Miescher, *Helv. Chim. Acta*, **30**, 432 (1947).

(27) N. Izumiya, S.-C. J. Fu, S. M. Birnbaum and J. P. Greenstein, *J. Biol. Chem.*, **205**, 221 (1953).

(28) D. Hamer and J. P. Greenstein, *ibid.*, **193**, 8 (1951); S. M. Birnbaum in "Methods in Enzymology," Vol. II, Academic Press, Inc., New York, N. Y., 1955, p. 397.

(29) J. R. Vaughan and R. Osata, THIS JOURNAL, **74**, 676 (1952); R. A. Boissonas, *Helv. Chim. Acta*, **34**, 874 (1951); T. Wieland and H. Bernhard, *Ann.*, **572**, 190 (1951).

(30) D. Ben-Ishai and A. Berger, *J. Org. Chem.*, **17**, 1564 (1952).

(31) *Cf.* C. G. Baker and H. A. Sober, THIS JOURNAL, **75**, 4058 (1953).

(32) Determinations with a 0.03 *M* solution of S-benzyl-DL-penicillamine amide in 5 *N* HCl revealed that after reflux periods of 1, 1.5 2.5 and 4.8 hours, the degree of hydrolysis was 19, 34, 70 and 100%, respectively.

(33) R. J. Koegel, J. P. Greenstein, M. Winitz, S. M. Birnbaum and R. A. McCallum, THIS JOURNAL, **77**, 5708 (1955); M. C. Otey and J. P. Greenstein, *Arch. Biochem. Biophys.*, **53**, 501 (1954).

(34) R. Wade, M. Winitz and J. P. Greenstein, THIS JOURNAL, **78**, 373 (1956).

(35) E. Abderhalden and E. Wybert, *Ber.*, **49**, 2449, 2838 (1916).

(36) E. Fischer and U. Suzuki, *ibid.*, **37**, 4575 (1904).

(37) N. W. Pirie, *Biochem. J.*, **25**, 614 (1931).

(38) J. P. Greenstein, *J. Biol. Chem.*, **128**, 241 (1939).

(39) E. Fischer and O. Gerngross, *Ber.*, **42**, 1485 (1909).

nately, and in small portions, 500 ml. of 2 *N* NaOH and 50 ml. of acetic anhydride, with vigorous cooling and shaking. The reaction mixture was shaken for an additional 30 min. and then acidified to congo red with 6 *N* HCl. The mixture was exhaustively extracted with ethyl acetate and the combined ethyl acetate fractions dried over anhydrous sodium sulfate and concentrated to dryness. The residual oil was taken up in hot acetone and a white solid precipitated upon the addition of petroleum ether. This was filtered off, again dissolved in hot acetone, filtered and the solution permitted to stand for 2 hr. at 10°. Microscopic examination revealed white, prism-shaped crystals which were highly hygroscopic upon exposure to air. The bis-(acetyl)-L-cystine was therefore separated from the acetone supernatant by centrifugation and subsequent decantation, and immediately placed in a vacuum desiccator where the residual solvent was removed. After drying over phosphorus pentoxide at 80° for some 12 hr., the compound appeared no longer hygroscopic; yield 19 g., m.p. 121–122°, $[\alpha]^{23}D$ −118° (1% in water). This compound was previously prepared[4] as a non-crystalline solid for which no physical constants were given, and as a white solid with m.p. 120° (J. P. Greenstein and F. M. Leuthardt, *Archiv. Biochem.*, **18**, 377 (1948)).

Anal. Calcd. for $C_{10}H_{16}O_6N_2S_2$: C, 37.0; H, 4.9; N, 8.6; S, 19.8. Found: C, 37.1; H, 5.1; N, 8.6; S, 19.5.

Racemization of Bis-(acetyl)-L-cystine.—To a solution of 20 g. of bis-(acetyl)-L-cystine in 150 ml. of glacial acetic acid, at room temperature (*ca.* 27°), was added 30 ml. of acetic anhydride. A very small quantity of crystalline material appeared within a few minutes but did not change noticeably in amount during the next 16 hours. An aliquot of the solution was removed and its change in optical rotation followed polarimetrically. A plot of the optical rotation value *versus* time is given in Fig. 1. After some 16 hr., the optically inactive solution was filtered and the filtrate evaporated to dryness under a jet of air. A white, sticky residue remained which began to crystallize upon rubbing under ether and which appeared to be completely crystalline after standing for two days in the cold. Since the material was highly hygroscopic, it was separated from the ethereal layer by centrifugation and decantation, and then immediately placed in a vacuum desiccator where the residual ether was drawn off. After drying over potassium hydroxide *in vacuo*, the yield of the epimeric mixture of bis-(acetyl)-cystine was 16.8 g. (84%), dec. point 78°, $[\alpha]^{23}D$ 0° (in water). The compound exhibited a slight hygroscopicity and was analyzed after drying *in vacuo* at 60°.

Anal. Calcd. for $C_{10}H_{16}O_6N_2S_2$: C, 37.0; H, 4.9; N, 8.6. Found: C, 37.2; H, 5.2; N, 8.2.

One gram of the epimeric mixture was hydrolyzed by refluxing with 100 ml. of 2 *N* HCl for 2 hr. The solution was concentrated to dryness *in vacuo* at 37°, and the evaporation repeated several times subsequent to the addition of water. The residue was taken up in water, the solution filtered, and the *p*H adjusted to 5.5 with aqueous ammonia. After standing for several hours in the cold, the optically inactive cystine was recovered by filtration over suction; yield 0.43 g. (58%), $[\alpha]^{23}D$ 0° (1% in 1 *N* HCl).

Anal. Calcd. for $C_6H_{12}O_4N_2S_2$: C, 30.0; H, 5.0; N, 11.7; S, 26.7. Found: C, 29.9; H, 5.0; N, 11.6; S, 26.5.

Enzymic Resolution.—A solution of 9.72 g. (0.03 mole) of optically inactive bis-(acetyl)-cystine in 150 ml. of water was prepared, the *p*H adjusted to 7.5 with 2 *N* NaOH, and the volume brought to 300 ml. with distilled water. After the addition of 1 g. of hog renal acylase I powder,[14] the digest was incubated at 37° for some 16 hr., at which point the degree of hydrolysis, as measured by the van Slyke manometric-CO_2 procedure,[40] had proceeded to only 18.5% of theory. The *p*H of the digest was adjusted to 7.5 and an additional 8 g. of "crude" acylase I[41] was added. After some 24 hr., a 50% hydrolysis of the total substrate was indicated. This value remained unchanged upon the further addition of 1 g. of crude acylase I and subsequent incubation for an additional 16 hr. The hydrolysate was then adjusted to *p*H 5.5 with glacial acetic acid and concentrated to 100 ml. *in vacuo* at 35–40° in the presence of capryl alcohol to eliminate foaming. The concentrate was warmed over the steam-bath for some 10 min. (to denature the protein), allowed to stand in the cold overnight, and the precipitate of L-cystine plus coagulated protein filtered and washed with 100 ml. of cold water. The combined filtrate and washings, which contained both the bis-(acetyl)-D-cystine and monoacetyl-*meso*-cystine, was concentrated to about 50 ml. *in vacuo* at 40°, and the concentrate then run onto a Dowex 50-X[4] column (30 × 2.5 cm.) in the acid phase which had been previously washed with water. Fractions were collected in 30–35-ml. cuts, with water as the eluent, and the presence of bis-(acetyl)-D-cystine was indicated in tubes 2 to 16 by the drop in *p*H of the eluate. The column was further eluted with 1 l. of water, which was discarded, and then with 1 *N* HCl, which was collected in 30–35-ml. cuts and examined for the presence of monoacetyl-*meso*-cystine with ninhydrin as the indicator. All "positive" fractions were combined.

L-Cystine.—The combined precipitate of L-cystine and denatured protein, secured above, was extracted twice with 250-ml. portions of warm (70°) 1 *N* HCl, the extracts treated with Norite, filtered and the filtrate adjusted to *p*H 5.5 with aqueous ammonia. After standing for 6 hr. in the cold, the L-cystine was filtered, washed with water and recrystallized from water *via* the hydrochloride derivative according to the procedure of Fischer and Raske.[15] An over-all yield of 0.8 g. of L-cystine was obtained, $[\alpha]^{23}D$ −211° (1% in *N* HCl).

Anal. Calcd. for $C_6H_{12}O_4N_2S_2$: C, 30.0; H, 5.0; N, 11.7; S, 26.7. Found: C, 29.9; H, 5.3; N, 11.7; S, 26.5.

D-Cystine.—The combined eluates of tubes 2 to 16, secured above, were concentrated to dryness *in vacuo* and the residual oil was dissolved in 100 ml. of 2 *N* HCl. After refluxing for 2 hr., the hydrolysate was decolorized with Norite and the *p*H brought to 5.5 with aqueous ammonia. The precipitated D-cystine was recrystallized in the same manner employed for the L-antipode; yield 0.95 g., $[\alpha]^{23}D$ +208° (1% in *N* HCl).

Anal. Found: C, 30.1; H, 5.1; N, 11.6; S, 26.5.

Mesocystine.—The combined 1 *N* HCl eluates, secured above, were concentrated to dryness and the residual monoacetyl-*meso*-cystine taken up in 100 ml. of 2 *N* HCl and refluxed for 2 hr. Recovery of the *meso*-cystine from the acid hydrolysate, as well as its recrystallization, was effected as given for the D-stereoisomer; yield 0.8 g., $[\alpha]^{23}D$ 0° (1% in *N* HCl).

Anal. Found: C, 29.8; H, 5.0; N, 11.7; S, 26.5.

II. Resolution, Derivatives and Analogs of S-Benzyl-penicillamine. N-Acetyl-S-benzyl-DL-penicillamine.—To a solution of 19.1 g. (0.1 mole) of acetyl-DL-penicillamine[25] in 500 ml. of liquid ammonia was added small pieces of sodium metal, with stirring, until a slight excess was present (as indicated by a permanent light blue color). S-Benzylation[12] was effected by the dropwise addition of benzyl chloride until the blue color just disappeared, and then the further dropwise addition of an additional 12.7 ml. (0.11 mole) of benzyl chloride. The mixture was stirred for 3 hr. after the addition was complete, the liquid ammonia then allowed to evaporate spontaneously, and the resulting residue extracted into water. Following filtration of a slight amount of insoluble residue, the *p*H of the solution was adjusted to 1.7 with 6 *N* HCl and the mixture extracted exhaustively with ethyl acetate. The ethyl acetate extracts were pooled, dried over anhydrous sodium sulfate, filtered and the filtrate concentrated to dryness. After recrystallization of the crystalline residue from 50% ethanol, a yield of 21.9 g. (78%) of N-acetyl-S-benzyl-DL-penicillamine was secured, m.p. 174°.

Anal. Calcd. for $C_{14}H_{19}O_3NS$: C, 59.8; H, 6.8; N, 5.0; S, 11.4. Found: C, 60.0; H, 6.9; N, 5.0; S, 11.4.

N-Acetyl-S-methyl-DL-penicillamine.—This compound was prepared in the same general manner as employed for the S-benzyl analog through the use of methyl iodide *in lieu* of benzyl chloride.[43] A 76% yield of the desired product

(40) Although the precipitation of free L-cystine accompanied the hydrolysis, a representative aliquot could be removed successfully from the well-shaken reaction mixture and the degree of hydrolysis measured with a high degree of precision.

(41) This acylase I preparation has not been carried as far as the acetone fractionation step, but rather is a lyophilized powder of a dialysate prepared just subsequent to the ammonium sulfate precipitation stage (*cf.* ref. 14).

(42) *Cf.* V. du Vigneaud, L. F. Audrieth and H. S. Loring, THIS JOURNAL, **52**, 4500 (1930).

(43) *Cf.* V. du Vigneaud, H. M. Dyer and J. Harmon, *J. Biol. Chem.*, **101**, 719 (1933); J. E. Wilson and V. du Vigneaud, *ibid.*, **184**, 63 (1950).

was secured after recrystallization from water; m.p. 196–197°.

Anal. Calcd. for $C_8H_{15}O_3NS$: C, 46.8; H, 7.3; N, 6.8; S, 15.6. Found: C, 46.7; H, 7.2; N, 6.7; S, 15.6.

N-Carbobenzoxy-S-methyl-DL-penicillamine.—N-Acetyl-S-methyl-DL-penicillamine was hydrolyzed to S-methyl-DL-penicillamine upon refluxing with 2 *N* HCl (10 ml. per g. of acetyl derivative) for 2 hr. After evaporation of the hydrolysate to dryness *in vacuo*, the residue was extracted into water, the *p*H adjusted to *p*H 5.5 with aqueous ammonia, and the resulting crystals of S-methyl-DL-penicillamine filtered, washed with water, and finally recrystallized from water. This compound has been previously prepared[43] *via* methylation of penicillamine with methyl iodide in the sodium–liquid ammonia system.

Anal. Calcd. for $C_6H_{13}O_2NS$: C, 44.2; H, 8.0; N, 8.6; S, 19.6. Found: C, 44.1; H, 8.0; N, 8.2; S, 19.4.

Preparation of the carbobenzoxy derivative was effected in a manner comparable to that given below for the S-benzyl analog. A 67% yield was obtained after recrystallization from benzene–petroleum ether; m.p. 89°.

Anal. Calcd. for $C_{14}H_{19}O_4NS$: C, 56.6; H, 6.5; N, 4.7; S, 10.8. Found: C, 57.0; H, 6.5; N, 4.6; S, 10.3.

N-Carbobenzoxy-S-benzyl-DL-penicillamine.—A solution of 105.5 g. of S-benzyl-DL-penicillamine in 450 ml. of 1 *N* NaOH was cooled to about 5° and a total of 95.4 ml. of carbobenzoxy chloride and 650 ml. of 1 *N* NaOH added alternately thereto, in small portions, with vigorous shaking and cooling. Separation of an oil was noted at this stage. The reaction mixture was shaken for an additional 2 hr. in the cold and then acidified to *p*H 1.7 with 6 *N* HCl. An oil was precipitated which was taken up in half-saturated sodium bicarbonate solution, filtered, and reprecipitated with 6 *N* HCl. Although an oil was initially formed, crystallization soon commenced. The crystals were dissolved in ethyl acetate and the solution dried with anhydrous sodium sulfate, filtered and concentrated to dryness in a stream of dry air. An oil was secured which readily crystallized upon rubbing with water; yield 99 g., m.p. 81°.

Anal. Calcd. for $C_{20}H_{23}O_4NS$: C, 64.3; H, 6.2; N, 3.8; S, 8.6. Found: C, 64.0; H, 6.2; N, 3.7; S, 8.4.

N-Carbobenzoxy-S-benzyl-DL-penicillamine Amide.—A solution of 98.5 g. of carbobenzoxy-S-benzyl-DL-penicillamine and 37.1 ml. of triethylamine in 1.5 l. of dry dioxane was cooled to 10°, and 35.2 ml. of isobutyl chloroformate added thereto. The reaction mixture was maintained at 10° for 1 hr., with occasional shaking, and 34.4 ml. of concentrated aqueous ammonia then added. As no carbon dioxide was evolved at this temperature, the mixture was warmed to 80° over a steam-bath and was maintained at this temperature for 10–15 minutes, during which time vigorous gas evolution was observed. An additional 34.4 ml. of concentrated ammonia was then added, the mixture allowed to stand at room temperature overnight, the precipitate of triethylamine hydrochloride removed by filtration, and the filtrate concentrated to dryness *in vacuo*. The residue was dissolved in chloroform and the chloroform layer washed successively with half-saturated sodium bicarbonate, water, 1 *N* HCl and water. The chloroform layer was evaporated to dryness *in vacuo*, benzene added, and the evaporation repeated. Crystallization of the residue was effected from ether–petroleum ether. Since the precipitate was slightly colored, it was taken up in ethyl acetate, the solution decolorized with Norite, dried over anhydrous sodium sulfate, and evaporated to dryness. The white crystals were recrystallized from absolute ethanol; yield 63.5 g. (65%), m.p. 123–124°.

Anal. Calcd. for $C_{20}H_{25}O_3N_2S$: C, 64.3; H, 6.7; N, 7.5; S, 8.6. Found: C, 64.2; H, 6.6; N, 7.4; S, 8.4.

S-Benzyl-DL-pencillamine Amide Hydrobromide.—Decarbobenzoxylation of carbobenzoxy-S-benzyl-DL-penicillamine amide was effected according to the general procedure of Ben-Ishai and Berger.[30] A stream of dry hydrogen bromide was passed through a solution of 60 g. of the carbobenzoxy-amide in 1 l. of glacial acetic acid to saturation, the temperature being maintained below 35°. After 0.5 hr. at room temperature, the mixture was concentrated to 500 ml. and 3 l. of dry ether added thereto. After standing overnight in the cold, the mixture was filtered and the precipitate washed with ether, dried *in vacuo* over potassium hydroxide and recrystallized from 95% ethanol; yield 44 g. (86%), m.p. 227–228°.

Anal. Calcd. for $C_{12}H_{25}ON_2SBr$: C, 45.1; H, 6.0; N, 8.8; S, 10.0; Br, 25.0. Found: C, 45.0; H, 6.0; N, 8.6; S, 9.7; Br, 25.0.

Enzymic Resolution.—The *p*H of a solution of 4 g. of S-benzyl-DL-penicillamine amide hydrobromide and 50 mg. of $MnCl_2 \cdot 4H_2O$, in water, was adjusted to 8.0 with 2 *N* LiOH; 30 ml. of a hog kidney amidase[44] solution containing 5 mg. of nitrogen per ml. was added thereto, and the final volume diluted to 250 ml. The mixture was subsequently digested at 38° for 18 hr., at which point the manometric ninhydrin-CO_2 procedure revealed 50% hydrolysis of the total substrate. No change in the degree of hydrolysis was revealed upon digestion for an additional 24 hr. after the addition of 7 ml. more of amidase solution. The digest was adjusted to *p*H 5.2 with glacial acetic acid, deproteinized by filtration in the presence of Norite, the precipitate washed with water and the filtrate and washings combined. Since the precipitate contained some adsorbed substrate, this was extracted twice with hot methanol, filtered, the filtrate evaporated to dryness *in vacuo* at 35° and the resulting residue taken up in the pooled aqueous filtrate. This latter was concentrated to about 250 ml. *in vacuo* and the concentrate run onto an Amberlite XE-64 column (28 × 2.5 cm.) in the acid phase. With water as the eluent, fractions were collected every 8 ml. and examined for ninhydrin-positive material. The presence of S-benzyl-L-penicillamine in tubes 10 to 170 was so indicated. After emergence of the L-amino acid, the column was washed with 2 l. of distilled water and the S-benzyl-D-penicillamine amide then eluted with 0.3 *N* HCl (fractions tested for ninhydrin positive material, as previously).

S-Benzyl-L-penicillamine.—The combined eluate of tubes 10 to 170 was concentrated to dryness *in vacuo* at 40°. The residue was dissolved in 1 *N* HCl and the *p*H brought to 5.5 with aqueous ammonia. A crystalline precipitate of S-benzyl-L-penicillamine was so obtained in 96% yield. Recrystallization was effected from water; $[\alpha]^{24}D$ +87.5° (1% in NaOH) and $[\alpha]^{24}D$ +91.3° (1% in *N* HCl).

Anal. Calcd. for $C_{12}H_{17}O_2NS$: C, 60.3; H, 7.1; N, 5.9; S, 13.4. Found: C, 60.2; H, 7.3; N, 5.8; S, 13.1.

S-Benzyl-D-penicillamine Amide Hydrochloride.—The 0.3 *N* HCl eluate from the column, which contained the D-amide, was brought to *p*H 4.5 with silver carbonate and filtered. Adsorbed material on the silver chloride precipitate was eluted off with hot methanol, the methanol extracts filtered, concentrated to dryness and the residue taken up in the aqueous filtrate. Hydrogen sulfide was passed through the latter to remove silver ions, the suspension filtered, and the filtrate concentrated to dryness *in vacuo*. The residue was dissolved in approximately 100 ml. of methanol, the solution filtered, the filtrate concentrated to dryness, and the residue crystallized from 80% ethanol to yield S-benzyl-D-penicillamine amide hydrochloride. This material contained approximately one molecule of water of crystallization which could be removed upon drying *in vacuo* at 80°; yield 1.4 g. (81%) of anhydrous product, m.p. 182–183°, $[\alpha]^{24}D$ −73.0 (1% in ethanol).

Anal. Calcd. for $C_{12}H_{19}ON_2SCl$: C, 52.4; H, 6.9; N, 10.2; S, 11.7; Cl, 12.9. Found: C, 52.4; H, 7.0; N, 10.0; S, 12.0; Cl, 13.0.

S-Benzyl-D-penicillamine.—A solution of 0.7 g. of S-benzyl-D-penicillamine amide hydrochloride in 20 ml. of 5 *N* HCl was refluxed for 6 hr. and the hydrolysate then concentrated to dryness *in vacuo* at 38°. After the addition of a little water, the evaporation was repeated. The residue was dissolved in a few ml. of water and the *p*H adjusted to 5.5 with 2 *N* LiOH. The D-isomer, which crystallized out on standing, was filtered, washed with water and dried; yield 0.58 g. Recrystallization was effected from water; $[\alpha]^{24}D$ −90.6 (1% in 1 *N* HCl).

(44) The amidase preparation employed was prepared as described previously,[28] but without prior adsorption on calcium phosphate gel. Its rate of hydrolysis toward L-leucine amide, under the same conditions as utilized for the S-benzylpenicillamine amide, was 2880 μmoles per hr. per mg. of N.

Anal. Calcd. for $C_{12}H_{17}O_2NS$: C, 60.3; H, 7.1; N, 5.9; S, 13.4. Found: C, 60.1; H, 7.0; N, 5.8; S, 13.2.

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BETHESDA, MD.

[CONTRIBUTION FROM THE LABORATORY OF ORGANIC CHEMISTRY OF THE UNIVERSITY OF WISCONSIN]

Synthesis of Ethyl β,β-Diethoxyalanyl-L-cysteinate Hydrochloride[1]

BY HOMER ADKINS[2] AND BURRIS D. TIFFANY[3]

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Ethyl β,β-diethoxyalanyl-L-cysteinate hydrochloride (XIX) has been synthesized through the coupling of N-carbobenzoxy-β,β-diethoxyalanine azide (XI) with S-benzyl-L-cysteine, followed by selective removal of the protecting N-carbobenzoxy and S-benzyl groups by mild reduction. The azide XI was prepared by the carbobenzoxylation of ethyl β,β-diethoxyalanate (IV) followed by conversion of the ester IX to the hydrazide X and then to the azide. The primary coupling product, N-carbobenzoxy-β,β-diethoxyalanyl-S-benzyl-L-cysteine (XIV) and (XV), was separated into its component D,L- and L,L-diastereoisomers by fractional crystallization of its methyl esters and sodium salts. D,L-N-Carbobenzoxy-β,β-diethoxyalanylglycine (XII) resulted from a preliminary investigation of the synthetic route using glycine. An attempt to couple the unprotected β,β-diethoxyalanine azide (VI) with glycine yielded 3-diethoxymethyl-2,5-piperazinedione (VIII).

In connection with a study of the structure of benzylpenicillin,[4] it was of interest to synthesize the stereoisomeric ester hydrochlorides (I) of β,β-diethoxyalanylpenicillamine, a dipeptide incorporating certain structural features known to be present in benzylpenicillin itself.

$(CH_3CH_2O)_2CHCHCONHCHCO_2CH_2CH_3$ I, R = CH_3; II, R = H
$H_2N{\cdot}HCl$ CR_2SH

This paper describes the synthesis of one of the stereoisomers of the lower homolog, ethyl β,β-diethoxyalanyl-L-cysteinate hydrochloride (II), derived from L-cysteine, as a model for the synthesis of the dipeptide ester hydrochlorides I, which require the less available penicillamine.

The first approach studied was the azide coupling of β,β-diethoxyalanine (III) with ethyl glycinate. The latter amino acid was employed in developing the method to avoid difficulties due to the sensitive sulfhydryl group and the complications of diastereoisomeric dipeptides.

Ethyl β,β-diethoxyalanate (IV), prepared from ethyl glycinate,[5] was converted to the hydrazide V in good yield, using anhydrous hydrazine. The hydrazide was surprisingly stable, considering the functional groups present, and could be distilled at reduced pressure without change.

We were encouraged by the unusual stability of the hydrazide to attempt direct conversion to the azide VI and coupling with glycine without protecting the amino group. When this unconventional series of reactions was tried, the product, obtained in 10% yield, was not the dipeptide ester VII, however, but a white water-soluble solid which gave correct carbon and hydrogen values for 3-diethoxymethyl-2,5-piperazinedione (VIII) *i.e.*, the compound to be expected upon cyclization of the coupled product VII. The failure, therefore, appeared not to be in the coupling, although of low yield, but rather in preventing the subsequent cyclization. Herein is implied the existence of a moderately stable aliphatic primary amino acid azide, which seems to be unprecedented.[6]

For a successful coupling procedure we turned to Bergmann's method for protection of the amino group. Carbobenzoxylation of ethyl β,β-diethoxyalanate (IV) was accomplished in 81% yield, and the resulting ethyl N-carbobenzoxy-β,β-diethoxyalanate (IX) was converted readily to the hydrazide X with 85% hydrazine hydrate in ethanol. Before treatment with nitrous acid this acetal-containing hydrazide was tested for stability in aqueous acid. It could be dissolved in three equivalents of cold 1 *N* hydrochloric acid and largely recovered unchanged upon neutralization of the acid. The azide XI was prepared from the hydrazide using nitrous acid and coupled with glycine in potassium carbonate solution, giving N-carbobenzoxy-β,β-diethoxyalanylglycine (XII) as a white crystalline solid in good yield. The crystalline methyl ester XIII was prepared readily from the acid upon treatment with diazomethane.

Having demonstrated that the acetal system would survive in the coupling reaction, attention was next turned to the dipeptide containing the L-cysteine residue. The sensitive sulfhydryl group was protected with a benzyl group as described by Wood and du Vigneaud.[7] When the coupling procedure was carried out with S-benzyl-L-cysteine, the product, obtained in 80% yield, was a solid with a broad melting range and presumably was a mixture of the D,L- and L,L-diastereoisomers of the dipeptide XIV and XV.

For separation of the diastereoisomers the mixture of acids was converted to the methyl esters

(1) From a thesis submitted by Burris D. Tiffany to the Graduate School of the University of Wisconsin in partial fulfillment of the requirements for the degree of Doctor of Philosophy, 1949.

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(4) *Cf.* H. Adkins, R. M. Ross and D. C. Schroeder, THIS JOURNAL, **72**, 5401 (1950).

(5) "The Chemistry of Penicillin," Clarke, Johnson and Robinson, Editors, Princeton University Press, Princeton, N. J., 1949, p. 512.

(6) *Cf.* P. A. S. Smith's review of the Curtius reaction in "Organic Reactions," Vol. III, John Wiley and Sons, Inc., New York, N. Y., 1946, p. 353.

(7) J. L. Wood and V. du Vigneaud, *J. Biol. Chem.*, **130**, 110 (1939).