molecular models reveals very low, if any, strain in the planar arrangements shown for the three donor groups of the ligand. Since resonance between the imine and aromatic ring would tend to keep the bonds adjacent to the imine nitrogens in the same plane, the planar arrangement for the ligands shown in B and C is favored over other possible structures.

Additional evidence for the proposed chelate structures may be obtained by a comparison of the infrared spectra in Fig. 1. The absence or presence of broad bands near 2500–2600 cm.$^{-1}$ indicates no intermolecular hydrogen bonding for the Cu(II) chelate, and increasing amounts of such bonding for the Fe(III) and Ni(II) chelates. The intensities of these bands correlate exactly with what would be expected for the presence of none, one, and two pyridinium protons capable of forming intermolecular hydrogens bonds as indicated in structures A, B and C, respectively. The alternative structures in which water is coördinated to the metal and the 3-pyridol group is engaged in intermolecular hydrogen bonding cannot be excluded for Fe(III) and Ni(II) on the basis of infrared evidence. On the other hand, such alternative structures are considered improbable on the basis of what is known about the affinities between metal ions and various types of donor groups in organic ligands.

---

[CONTRIBUTION FROM THE LABORATORY OF ORGANIC CHEMISTRY, UNIVERSITY OF ATHENS, GREECE]

# On Cysteine and Cystine Peptides. II. S-Acylcysteines in Peptide Synthesis[1,2]

BY LEONIDAS ZERVAS, IPHIGENIA PHOTAKI AND NICOLAOS GHELIS

RECEIVED OCTOBER 31, 1962

An approach to the synthesis of unsymmetrical cystine peptides containing at least two –S–S– bridges which entails the use of selectively removable S-protecting groups is discussed. In addition to S-trityl- and S-diphenylmethyl-, S-benzoyl-, S-acetyl- and S-carbobenzoxycysteines are shown to be very useful intermediates for the incorporation of cysteine residues into a peptide chain. The removal of these S-acyl groups which are, in a certain sense, "active esters" can easily be achieved by methanolysis in the presence of sodium methoxide. Furthermore, the S-benzoyl group is not attacked by the N-decarbobenzoxylating agents trifluoroacetic acid and hydrogen bromide in acetic acid. The S-acetyl group is resistant to trifluoroacetic acid but is removed by hydrogen bromide in acetic acid and the S-carbobenzoxy group is split off by trifluoroacetic acid but survives the treatment with 2 *N* hydrogen bromide in acetic acid to a very great extent. Consequently, these S-acylcysteine residues can be used for peptide synthesis by means of their N-carbobenzoxy derivatives. Using S-acylcysteine residues, several cysteine-containing peptides have been synthesized and converted to the corresponding cystine peptides. The use of the above-mentioned S-protecting groups for overcoming the unique difficulties inherent in establishing a disulfide bridge specifically between two of three cysteine residues of a peptide chain, as in fragment IX of insulin, has been explored.

## Introduction

Many methods for the incorporation of amino acid residues, including cysteine residues, into a peptide chain are now well established.[3a] In particular, the synthesis of symmetrical cystine peptides[3a,b] and cyclic peptides of the oxytocin type[3b] is, in principle, no longer a problem in peptide chemistry. On the other hand, the synthesis of unsymmetrical cystine peptides with two or more –S–S– bridges is an extremely difficult task.[3c] A random solution to this problem may be that of synthesizing the proper sequences of amino acids, including cysteine residues, as they are represented in a natural product, *e.g.*, insulin, in the hope that the oxidation of these synthetic cysteine peptides would lead to the formation of the natural product and its characteristic –S–S– bridge system. Indeed, it has recently been reported that when insulin is reduced and the sulfhydryl chains so obtained are reoxidized, some insulin activity is regenerated.[4] Many fragments of the insulin molecule, some of which contain S-benzylcysteine residues, have already been synthesized in different laboratories.[5]

Another, much more controlled approach to the synthesis of unsymmetrical cystine peptides containing at least two –S–S– bridges requires that the following conditions be fulfilled: (a) cysteine residues bearing different S-protecting groups which may be removed selectively must be available; and (b) procedures must be developed for preventing the rearrangement of cystine chains during synthesis, so that the desired multimembered ring system may be formed.[3c]

Concerning the first of the above requirements S-trityl-(Tr) and S-diphenylmethyl-(DPM) L-cysteine have recently been proposed as intermediates for the incorporation of cysteine residues into a peptide chain.[3c] It has been found that S-acylcysteines are also suitable for the above purposes since the S-acyl groups, like the S-trityl and S-DPM groups, can be very easily removed without affecting peptide bonds or other sensitive parts of the molecule. Very useful, in our opinion, are the S-benzoyl- (Ia) and S-acetyl-L-cysteine(Ib) and especially their N-carbobenzoxy derivatives (Ic,Id) which can be easily prepared by reduction of N,N′-dicarbobenzoxy-L-cystine with zinc–hydrochloric acid followed by acylation of the N-carbobenzoxy-L-cysteine

(1) (a) A summary of a part of this paper was presented at the 4th European Peptide Symposium, Moscow, August, 1961; L. Zervas, *Collection Czechoslov. Chem. Communs.*, **27**, 2229 (1962); (b) a summary of this paper was presented at the 5th European Peptide Symposium, Oxford, England, September, 1962; L. Zervas, I. Photaki, A. Cosmatos and N. Ghelis, Proceedings of the 5th European Peptide Symposium, Pergamon Press, London, in press.

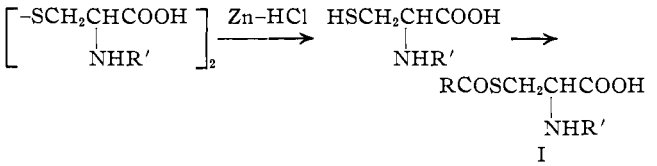
(2) This investigation was supported by the Royal Hellenic Research Foundation, to which we are greatly indebted.

(3) (a) M. Bergmann and L. Zervas, *Ber.*, **65**, 1192 (1932); J. P. Greenstein and M. Winitz, "Chemistry of the Amino Acids," John Wiley and Sons, Inc., New York, N. Y., 1961. (b) V. du Vigneaud and G. L. Miller, *J. Biol. Chem.*, **116**, 469 (1936); V. du Vigneaud, C. Ressler, J. M. Swan, C. W. Roberts and P. G. Katsoyannis, *J. Am. Chem. Soc.*, **76**, 3115 (1954). (c) *Cf.* I. Communication of this Series, L. Zervas and I. Photaki, *ibid.*, **84**, 3887 (1962).

(4) G. H. Dixon and A. C. Wardlaw, *Nature*, **188**, 721 (1960); Y. C. Du, Y. S. Zhang, Z. X. Lu, and C. L. Tsou, *Sci. Sinica* (Peking), **10**, 84 (1961); C. L. Tsou, Y. C. Du and G. J. Xü, *ibid.*, **10**, 332 (1961).

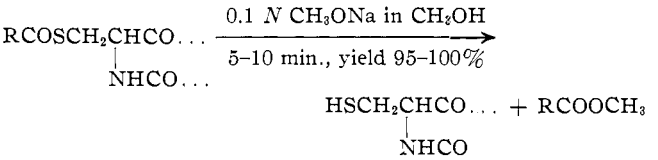
(5) (a) J. A. Maclaren, W. E. Savige and J. M. Swan, *Austral. J. Chem.*, **11**, 345 (1958); B. Milligan and J. W. Swan, *J. Chem. Soc.*, 2172 (1962); G. F. Holland and L. A. Cohen, *J. Am. Chem. Soc.*, **80**, 3765 (1958); G. L. Tritsch and D. W. Woolley, *ibid.*, **82**, 2787 (1960); H. Zahn and N. H. La France, *Ann.*, **630**, 37 (1960); H. Zahn and H. Schuesser, *ibid.*, **641**, 176 (1961); J. Kunde and H. Zahn, *ibid.*, **646**, 137 (1961); H. Zahn and M. Heinz, *ibid.*, **652**, 76 (1962); P. G. Katsoyannis and K. Suzuki, *J. Am. Chem. Soc.*, **83**, 4057 (1961); **84**, 1420 (1962); C. Berse, Th. Massiah and L. Piche, *J. Org. Chem.*, **26**, 4514 (1961); J. E. Shields and F. H. Carpenter, *J. Am. Chem. Soc.*, **83**, 3066 (1961); L. T. Ke, Y. T. Kung, W. T. Huang, K. Y. Chi, and C. I. Niu, *Sci. Sinica* (Peking), **11**, 337 (1962); W. T. Huang, C. S. Yang, K. Z. Wang and C. I. Niu, *ibid.*, **11**, 499 (1962). (b) P. G. Katsoyannis, *J. Am. Chem. Soc.*, **83**, 4053 (1961).

thus formed. S-Benzoyl-L-cysteine (Ia) can also be prepared by direct benzoylation of L-cysteine at *p*H 7–7.5 and may then be easily esterified and N-formylated.



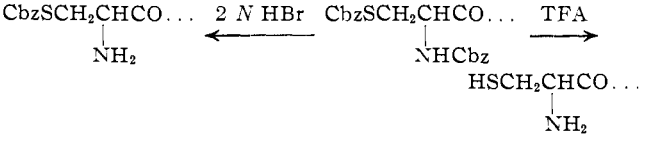
Ia, R = $C_6H_5$; R′ = H  Ic, R = $C_6H_5$; R′ = $C_6H_5CH_2OCO$
Ib, R = $CH_3$; R′ = H  Id, R = $CH_3$; R′ = $C_6H_5CH_2OCO$

As expected, the S-acyl groups being in a certain sense "active esters,"[6,7] are especially susceptible to dilute alkali; *e.g.*, the S-benzoyl group is completely removed by 0.2 *N* NaOH and 50% cleaved by 2 *N* ammonia in 20 minutes. However, we prefer to remove S-acyl groups by methanolysis in the presence of sodium methoxide in an atmosphere of hydrogen, since this reaction proceeds rapidly and almost quantitatively without causing $\beta$-elimination or racemization even when the S-acylcysteine residue constitutes the carboxyl end of the peptide chain.

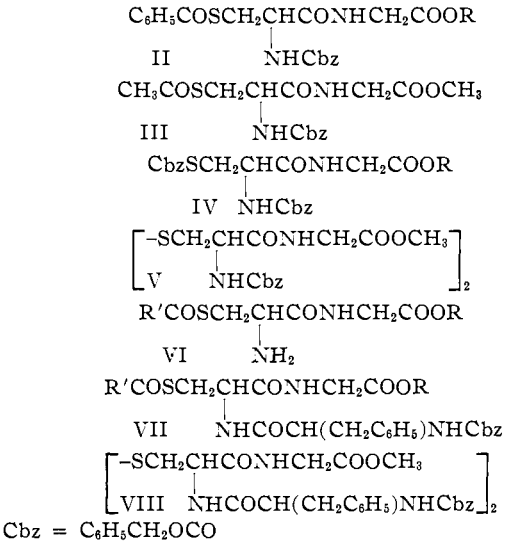


Further, the N-decarbobenzoxylating agents trifluoroacetic acid[8] and hydrogen bromide in acetic acid[9] do not attack S-benzoylcysteine and thus allow it to be incorporated into a peptide chain as the N-carbobenzoxy derivative. S-Acetylcysteine is practically resistant to trifluoroacetic acid but is cleaved to a great extent by hydrogen bromide.

S-Carbobenzoxy- and S,N-dicarbobenzoxy-L-cysteine[10] can also be used for our purposes, but they deserve special consideration. The alcoholysis of the S-carbobenzoxy group with 0.1 *N* sodium methoxide in methanol proceeds much more slowly than in the case of the corresponding S-benzoyl group and requires approx. 30 minutes to be practically complete.[11] Ammonolysis with 2 *N* ammonia also proceeds very slowly (20% within 30 minutes) but may, according to Katchalski,[10] be achieved with concd. ammonia. Furthermore, whereas boiling trifluoroacetic acid (TFA) does not differentiate between N- and S-carbobenzoxy groups, both of them being cleaved, the S-carbobenzoxy group survives treatment with 2 *N* hydrogen bromide to a very great extent despite statements to the contrary.[5b,10] Thus, S-carbobenzoxycysteine can also be used for peptide synthesis by means of the carbobenzoxy method.[12]



The following examples of the synthesis of some peptides are presented to illustrate the possibilities of the incorporation of cysteine into a peptide chain using S-acyl- and S-carbobenzoxy-L-cysteine. By coupling the N-carbobenzoxy derivatives of such S-protected cysteine residues with amino acid esters or by coupling of other carbobenzoxyamino acids with S-acylcysteine



Cbz = $C_6H_5CH_2OCO$
IIa, R = $CH_3$; b, R = *p*-$NO_2C_6H_4$
IVa, R = $CH_3$; b, R = *p*-$NO_2C_6H_4$; c, R = *p*-$NO_2C_6H_4CH_2$
VIa, VIIa, R = $CH_3$, R′ = $C_6H_5$
VIb, VIIb, R = *p*-$NO_2C_6H_4$, R′ = $C_6H_5$
VIc, VIIc, R = *p*-$NO_2C_6H_4CH_2$, R′ = $C_6H_5CH_2O$

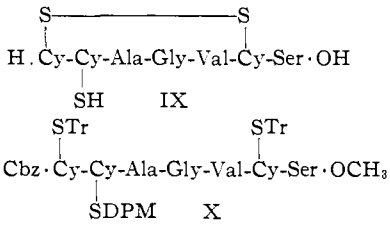
esters by known methods, the methyl (IIa) and nitrophenyl (IIb) esters of S-benzoyl-N-carbobenzoxy-L-cysteinylglycine, the S-acetyl-N-carbobenzoxy-L-cysteinylglycine methyl ester (III), and the methyl (IVa), *p*-nitrophenyl (IVb) and *p*-nitrobenzyl (IVc) esters of N,S-dicarbobenzoxy-L-cysteinylglycine were obtained. Methanolysis of compounds IIa, III and IVa in the presence of sodium methoxide followed by oxidation by iodine led to the formation of N,N′-bis-carbobenzoxy-L-cystinylglycine methyl ester (V) in good yield.

N-Decarbobenzoxylation of IIa with trifluoroacetic acid and of IIb, IVb and IVc with 2 *N* HBr, followed by coupling of the salts of VIa, VIb and VIc thus formed with N-carbobenzoxy-L-phenylalanine, yielded the corresponding tripeptide esters VIIa, VIIb and VIIc. Each of these esters upon methanolysis in the presence of sodium methoxide and subsequent oxidation by iodine afforded the same pentapeptide ester VIII. The use of nitrophenyl or nitrobenzyl esters offers in many cases some advantages, since they are much more resistant to the action of the N-decarbobenzoxylating agents than the corresponding alkyl esters; also being "active esters" they can be used directly for lengthening the peptide chain. Moreover the alcoholysis of the nitrophenyl and nitrobenzyl esters presents no difficulties.

It is evident that S-acyl- and S-carbobenzoxycysteines are useful intermediates for the synthesis of cysteine, symmetrical cystine and oxytocin-like peptides. Furthermore, since each of the above groups and also the S-trityl and S-DPM groups can be removed selectively,[3c] their introduction into peptide chemistry may be considered to fulfil the first requirement mentioned above for the synthesis of unsymmetrical cystine peptides containing at least two –S–S– bridges. The synthesis of a key fragment of the A-chain of sheep insulin may serve as an approach to this goal.[1b] This fragment (IX) consists of a 20-membered disulfide ring, *i.e.*, the same size ring as that found in oxytocine, in which is included an additional cysteine residue bearing a free SH group. Its precursor, the corresponding protected heptapeptide

(6) F. Lynen, E. Reichert and L. Rueff, *Ann.*, **574**, 1 (1951).
(7) Th. Wieland, W. Schaefer and E. Bockelmann, *ibid.*, **573**, 99 (1951); R. Schwyzer, *Helv. Chim. Acta*, **36**, 414 (1953).
(8) F. Weygand and W. Steglich, *Z. Naturforsch.*, **14b**, 472 (1959).
(9) D. Ben-Ischai and A. Berger, *J. Org. Chem.*, **17**, 1564 (1952).
(10) A. Berger, J. Niguchi and E. Katchalski, *J. Am. Chem. Soc.*, **78**, 4483 (1956).
(11) The reductive cleavage by means of sodium in liquid ammonia (ref. 10) is not applicable to our purposes.
(12) For the incorporation of S-carbobenzoxycysteine into a peptide chain by means of its N-formyl derivative compare Katsoyannis (ref. 5b).

N-carbobenzoxy-S-trityl-L-cysteinyl-S-diphenylmethyl-L-cysteinyl-L-alanyl-glycyl-L-valyl-S-trityl-L-cysteinyl-L-serine methyl ester (X, m.p. 235°, $[\alpha]$ −19°, *c* 3, dimethylformamide) has already been synthesized in such a way that racemization was avoided.[1b] A full description of the synthesis of X and its transformation to IX will be included in one of our next communications.




## Experimental

For the coupling reactions anhydrous reactants and dry solvents were used; the ether used was free from peroxides. Freshly prepared solutions of pure hydrogen bromide, free of bromine, were always used. Evaporations were carried out *in vacuo* at 35–40°. The melting points are not corrected.

Prior to analysis[13] the compounds were dried at 56° under high vacuum over phosphorus pentoxide. The derivatives of cysteine were determined by titration with 0.1 *N* iodine at *p*H 5–6; the method was sufficiently accurate for the purposes of this work. For these titrations with iodine, it was found that the maximum volume of solvent in which 1 mmole of SH compound could be accurately determined must not exceed 20 ml.

**S-Benzoyl-L-cysteine (Ia).**—To a mixture of 50 ml. of *N* sodium hydroxide and 20 ml. of ether, cooled to 0°, 6.2 ml. of benzoyl chloride and 8.75 g. (0.05 mole) of L-cysteine hydrochloride monohydrate were added, and while the temperature of the mixture was kept between 0 and 5°, 6 g. of potassium hydrogen carbonate was added in 4 portions over a period of 10 minutes with vigorous stirring. The mixture was stirred for 20 minutes more at room temperature. Upon addition of 15 ml. of 5 *N* HCl the precipitate was dissolved and the solution was successively extracted with ethyl acetate–ether (1:1) and ether. Addition of sodium acetate to the aqueous solution until it became neutral to congo red paper caused Ia to separate. The mixture was cooled and then the precipitate was collected by filtration, washed with cold water and dried in a desiccator. The yield was 5.7 g. (50%), m.p. 142°. For analytical purposes the product was dissolved in dilute hydrochloric acid and reprecipitated by addition of sodium acetate; $[\alpha]^{25}D$ −27.8° (*c* 5, 1 *N* HCl).

*Anal.* Calcd. for $C_{10}H_{11}NO_3S$: C, 53.34; H, 4.88; N, 6.21; S, 14.24. Found: C, 53.45; H, 4.95; N, 6.12; S, 14.37.

**S-Benzoyl-L-cysteine Methyl Ester Hydrochloride.**—A solution of 4.5 g. (0.02 mole) of dry Ia in 45 ml. of 3.5 *N* HCl in methanol was kept for 48 hours at room temperature and then 24 hours at 0–4°. The ester hydrochloride which separated (2.5 g.) was collected by filtration and was washed with ethyl acetate and finally with ether. An additional amount of the product (0.5 g.) was obtained by evaporating the filtrate to dryness, redissolving the residue in 15 ml. of the above methanolic HCl and keeping the solution first for 24 hours at room temperature and then several hours in the ice-box. The total yield was 55%, m.p. 165–166°, $[\alpha]^{25}D$ −4.2° (*c* 5, methanol).

*Anal.* Calcd. for $C_{11}H_{14}NO_3SCl$: N, 5.08; S, 11.63; Cl, 12.86. Found: N, 4.98; S, 11.58; Cl, 12.76.

**N-Formyl-S-benzoyl-L-cysteine.**—Compound Ia was formylated in exactly the same manner as described for the formylation of S-diphenylmethyl-L-cysteine.[3c] The yield was 2.2 g. (87%), m.p. 163°. Recrystallization from acetone raised the m.p. to 165°, $[\alpha]^{25}D$ −40° (*c* 1, ethanol).

*Anal.* Calcd. for $C_{11}H_{11}NO_4S$: C, 52.17; H, 4.37; N, 5.52. Found: C, 52.14; H, 4.53; N, 5.69.

**N-Carbobenzoxy-S-benzoyl-L-cysteine** (Ic).—N,N'-Bis-carbobenzoxy-L-cystine (20.4 g., 0.04 mole) was reduced with zinc–hydrochloric acid as described[3c] and the oily N-carbobenzoxy-L-cysteine thus formed was extracted into ether. The ether solution was washed with water and then was repeatedly extracted with cold potassium hydrogen carbonate solution immediately after, and while the temperature of the aqueous layer was kept between 0–5°, 12 ml. of benzoyl chloride was added and the solution was stirred for 15 minutes. The stirring was continued for an additional 15 minutes at room temperature and then the solution was acidified with hydrochloric acid. An oil precipitated which crystallized after being seeded and allowed to stand in the ice-box. The precipitate was collected by filtration and dried in a desiccator. Contaminating benzoic acid was removed by treatment of the product with warm ether–petroleum ether (1:1) followed by repeated extraction with boiling petroleum ether. Finally the product (16.5 g.) was dissolved in 55 ml. of methanol. Water (approx. 20 ml.) was added until the solution became opaque and the mixture was left in the ice-box. Pure Ic precipitated; the yield was 12.5 g. (43%), m.p. 135°, and 137° after recrystallization from methanol–water; $[\alpha]^{52}D$ −36.6° (*c* 5, ethanol).

*Anal.* Calcd. for $C_{18}H_{17}NO_5S$: C, 60.18; H, 4.73; N, 3.89; S, 8.92. Found: C, 60.27; H, 4.82; N, 4.02; S, 8.75.

Removal of the S-benzoyl group by (A) saponification, (B) ammonolysis, (C) treatment with HBr in acetic acid and (D) trifluoroacetic acid: In each case the removal of the S-benzoyl group was followed by titration with 0.1 *N* iodine and in some cases by subsequent isolation of the corresponding cystine derivative thus formed.

A. Compound Ia (0.7183 g., 0.002 mole) was dissolved in 30 ml. of 0.2 *N* sodium hydroxide and the solution was kept under nitrogen. At time intervals aliquots of the solution were acidified with acetic acid and titrated with iodine. Five minutes after dissolution 66% of the S-benzoyl group was removed; after 15 minutes almost 100%. The rest of the solution was acidified with sulfuric acid and after oxidation with iodine the solution was extracted with ethyl acetate. The ethyl acetate layer was washed with water until the water extract became neutral to congo red paper, dried over sodium sulfate and concentrated to a small volume *in vacuo*. Upon the addition of cyclohexylamine to the solution, N,N'-biscarbobenzoxy-L-cystine cyclohexylamine salt separated. The yield of product recrystallized from ethanol was 35% calculated on the basis of the amount of Ia used; m.p. 183° (reported[3c] 183°).

B. Compound Ia (0.001 mole) was dissolved in 20 ml. of aqueous 2 *N* ammonia. The solution was kept under nitrogen and was worked up as in case A. Fifteen minutes after dissolution 51% of the S-benzoyl group was split off; after 30 minutes, 95% was removed.

C. Compound Ia (0.001 mole) was dissolved in 8.5 ml. of 2 *N* HBr in acetic acid. Concentrated aqueous sodium acetate solution was added to the reaction solution, after it had stood for 30 minutes at room temperature. Iodine titration showed that 5% of the S-benzoyl group had been split off.

D. A solution of 0.001 mole of Ia and 0.2 g. of phenol in 1.5 ml. of trifluoroacetic acid was refluxed for 30 minutes on a steam-bath and was then diluted with 10 ml. of saturated sodium acetate solution. Iodine titration revealed 2% removal of the S-benzoyl group.

**N-Carbobenzoxy-S-acetyl-L-cysteine (Id)** was prepared in the same manner as Ia by reduction of biscarbobenzoxy-L-cystine (0.01 mole) with zinc–hydrochloric acid and acetylation of the potassium hydrogen carbonate solution of the N-carbobenzoxy-L-cysteine thus formed with 12 ml. of acetic anhydride. Upon acidification with dilute sulfuric acid, Id separated as an oil which crystallized after being seeded and allowed to stand in the ice-box. The yield was 55%, m.p. 115–116°, and 116–117° after recrystallization from benzene; $[\alpha]^{25}D$ −52.4° (*c* 3, ethanol).

*Anal.* Calcd. for $C_{13}H_{15}NO_5S$: C, 52.54; H, 5.04; N, 4.71; S, 10.78. Found: C, 52.45; H, 5.20; N, 4.85; S, 10.62.

Saponification of the S-acetyl group of Id by procedure A as described for Ia was almost complete after 2 minutes; for a complete ammonolysis of the S-acetyl group (procedure B) approx. 12 minutes was required.

Treatment of Id with HBr in acetic acid (procedure C) caused substantial splitting of the S-acetyl group, whereas treatment of Id with trifluoroacetic acid (procedure D) afforded only a 5% cleavage.

**N-Formyl-S-carbobenzoxy-L-cysteine**[1a] was prepared by formylation of S-carbobenzoxy-L-cysteine in the same manner as was the corresponding S-benzoyl derivative. The crude product was recrystallized from ethyl acetate; the yield was 76%, m.p. 140–141° (reported[5b] 141–142°), $[\alpha]^{25}D$ −43.1° (*c* 1, dimethylformamide), reported[5b] $[\alpha]^{25}D$ −41.6° (in dimethylformamide).

*Anal.* Calcd. for $C_{12}H_{13}NO_5S$: C, 50.87; H, 4.55; N, 4.92. Found: C, 50.87; H, 4.74; N, 5.05.

Treatment of this compound with trifluoroacetic acid as described for Ic (procedure D) afforded an almost quantitative removal of the S-carbobenzoxy group.

**Removal of the S-Carbobenzoxy Group from N,S-Dicarbobenzoxy-L-cysteine.**—Saponification of this compound by procedure A as described for Ic led to 50% removal of S-carbobenzoxy group within 30 minutes. For an almost 100% removal approx. 2–3 hours was required; the yield of cyclohexylammonium N,N'-biscarbobenzoxy-L-cystinate was 40%. Ammonolysis of the same compound by procedure B as described for Ic led to 20% removal of S-carbobenzoxy group within 30 minutes.

Upon treatment of N,S-dicarbobenzoxy-L-cysteine[10] (0.001 mole) with 3 ml. of 2 *N* HBr in acetic acid for 30 minutes at room temperature approx. 10–14% of the sulfhydryl compound was

(13) Microanalyses were carried out by Mr. H. Mantzos in the Analytical Laboratory of the Royal Hellenic Research Foundation.

detected by iodine titration (procedure C as described above). Boiling trifluoroacetic acid (procedure D) removed the S-carbobenzoxy group to the extent of 85–90% within 30 minutes.

**S-Carbobenzoxy-L-cysteine Methyl Ester Hydrochloride.**—Pure S-carbobenzoxy-L-cysteine[10] was esterified by the thionyl chloride method.[14] The crude ester hydrochloride was recrystallized by dissolving it in methanol and precipitating it again with ethyl acetate. The yield was 72%, m.p. 146–147°, $[\alpha]^{20}D$ −14.9° (*c* 5, methanol).

*Anal.* Calcd. for $C_{12}H_{16}NO_4SCl$: C, 47.13; H, 5.27; N, 4.58. Found: C, 46.54; H, 5.26; N, 4.58.

**N,S-Dicarbobenzoxy-L-cysteine Methyl Ester.**—A solution of 3.9 g. (0.01 mole) of N,S-dicarbobenzoxy-L-cysteine[10] in 15 ml. of methanolic *N* HCl was kept for 2 days at room temperature and then was poured into dilute potassium hydrogen carbonate solution. An oil precipitated which crystallized upon trituration several times with water; the yield was 3.2 g. (75%), m.p. 60–61° after recrystallization from ether acetate–petroleum ether, $[\alpha]^{20}D$ −68.5° (*c* 1, dimethylformamide).

*Anal.* Calcd. for $C_{20}H_{21}NO_6S$: C, 59.57; H, 5.20; N, 3.47; S, 7.95. Found: C, 59.72; H, 5.35; N, 3.59; S, 8.19.

**N,S-Dicarbobenzoxy-L-cysteine *p*-Nitrophenyl Ester.**—To a solution of 11.7 g. (0.03 mole) of N,S-dicarbobenzoxy-L-cysteine[10] in 40 ml. of anhydrous tetrahydrofuran cooled to 0° 4.2 g. of *p*-nitrophenol and 6.6 g. of N,N′-dicyclohexylcarbodiimide[15] were added. After the reaction mixture had been allowed to stand for several hours at room temperature the N,N′-dicyclohexylurea which separated was filtered off and washed with tetrahydrofuran. The filtrate was evaporated to dryness and the crystalline residue was recrystallized from 100 ml. of hot ethanol. The yield was 10 g. (71%), m.p. 90–92° and 93–94° after a second recrystallization from alcohol (recovery 90%), $[\alpha]^{25}D$ −50.3° (*c* 1, dimethylformamide).

*Anal.* Calcd. for $C_{25}H_{22}N_2O_8S$: C, 58.84; H, 4.31; N, 5.48; S, 6.28. Found: C, 58.92; H, 4.43; N, 5.61; S, 6.40.

**S-Carbobenzoxy-L-cysteine *p*-Nitrophenyl Ester Hydrobromide.**—A solution of 5.1 g. (0.01 mole) of N,S-dicarbobenzoxy-L-cysteine *p*-nitrophenyl ester in 17 ml. of warm acetic acid was allowed to cool to room temperature; 11 ml. of 5 *N* HBr in acetic acid was added and the solution was kept for 20 minutes at room temperature. Addition of ether and cooling caused the above hydrobromide to separate. The yield was 3.8 g. (83%), m.p. 140–141° and 148–149° after recrystallization from ethanol–ethyl acetate (recovery 65%); $[\alpha]^{25}D$ −6.2° (*c* 2.5, methanol).

*Anal.* Calcd. for $C_{17}H_{17}N_2O_6SBr$: C, 44.65; H, 3.75; N, 6.12; S, 6.99; Br, 17.47. Found: C, 44.45; H, 3.84; N, 6.06; S, 6.98; Br, 17.38.

**N,S-Dibenzoyl-L-cysteine Methyl Ester (DBCME).**—To a solution of 3.4 g. (0.02 mole) of pure L-cysteine methyl ester hydrochloride[3c] in 15 ml. of pyridine precooled at 0°, 5 ml. of benzoyl chloride was added. After being kept for 1 hour at room temperature the mixture was poured onto ice. The precipitate was collected by filtration and was recrystallized from methanol. The yield was 6 g. (88%), m.p. 145–146° (reported[3c] m.p. 140–141°), $[\alpha]^{25}D$ +56.8° (*c* 2, chloroform), reported[3c] $[\alpha]^{25}D$ +55.5° (chloroform).

**Methanolysis and Transformation to N,N-Bisbenzoyl-L-cystine Dimethyl Ester.**—To a suspension of 0.686 g. (0.002 mole) of the above ester in 16 ml. of absolute methanol 4.1 ml. of methanolic 0.5 *N* sodium methoxide was added in an atmosphere of hydrogen with stirring at approx. 20°. The ester dissolved completely during the first few minutes of stirring which was maintained for 10 minutes. On acidification with 3 ml. of acetic acid and titration with 0.1 *N* iodine, 19 ml. of iodine solution (95% of the theoretical amount) was consumed. The mixture was concentrated *in vacuo* until most of the methanol was removed and the N,N′-bisbenzoyl-L-cystine dimethyl ester (BBCDE) which precipitated was taken up in chloroform. The chloroform solution was washed with potassium hydrogen carbonate and with water, dried over sodium sulfate and evaporated to dryness *in vacuo*. The crystalline residue (BBCDE) was triturated with petroleum ether and was filtered off. The yield was 0.41 g. (87%), m.p. 170°, $[\alpha]^{25}D$ −232° (*c* 1, dimethylformamide); after recrystallization from methanol (recovery 70%) the m.p. was raised to 177–179°, but the specific rotation changed slightly to the value of −233°. Authentic samples of BBCDE[3c] melted at 177–179° and exhibited $[\alpha]^{25}D$ −233° (*c* 1, dimethylformamide).

**N-Carbobenzoxy-S-benzoyl-L-cysteinylglycine Methyl Ester (IIa).**—To a solution of 2.5 g. (0.02 mole) of glycine methyl ester hydrochloride and 2.8 ml. of triethylamine in 60 ml. of chloroform 7.6 g. (5% excess) of Ic and 4.4 g. of N,N′-dicyclohexylcarbodiimide were added. After the solution was stored at room temperature overnight, a few drops of 50% acetic acid were added and the insoluble precipitate of dicyclohexylurea (4.4 g.) was removed by filtration. The filtrate was washed successively with dilute hydrochloric acid, potassium hydrogen carbonate and water, dried over sodium sulfate and evaporated to dryness. The residue (IIa) was recrystallized from methanol; the yield was 5.4 g. (62%), m.p. 154°, $[\alpha]^{25}D$ −59.1° (*c* 1, dimethylformamide).

*Anal.* Calcd. for $C_{21}H_{22}N_2O_6S$: C, 58.65; H, 5.11; N, 6.51; S, 7.45. Found: C, 58.73; H, 5.17; N, 6.74; S, 7.75.

**Methanolysis and Transformation to N,N′-Biscarbobenzoxy-L-cystinyldiglycine Dimethyl Ester (V).**—The methanolysis of IIa (0.002 mole) and its transformation to the corresponding cystine derivative V was carried out in the same manner as described for DBCME except that a mixture of 10 ml. of methanol and 6 ml. of anhydrous dimethylformamide was used as solvent instead of 15 ml. of methanol. The iodine titration revealed 99% methanolysis; the yield of V thus formed was 95%, m.p. 171–172°, $[\alpha]^{25}D$ −141° (*c* 1, dimethylformamide). After recrystallization from methanol or ethyl acetate (recovery 60%) the substance melted at 172–172.5° and exhibited $[\alpha]^{25}D$ −146° (*c* 1, dimethylformamide).

For comparative purposes V has also been prepared from N,N′-dicarbobenzoxy-L-cystine and glycine methyl ester in the same manner as described for IIa. The yield was 70%, m.p. 171°, $[\alpha]^{25}D$ −147° (*c* 1, dimethylformamide). Mixture m.p. with the above product was undepressed.

**N-Carbobenzoxy-S-benzoyl-L-cysteinylglycine ethyl ester** was prepared in the same manner as was IIa; the yield was 70%, m.p. 153°, $[\alpha]^{25}D$ −58.5° (*c* 1, dimethylformamide).

*Anal.* Calcd. for $C_{22}H_{24}N_2O_6S$: C, 59.47; H, 5.40; N, 6.30; S, 7.21. Found: C, 59.55; H, 5.55; N, 6.17; S, 7.35.

**N-Carbobenzoxy-S-benzoyl-L-cysteinylglycine *p*-Nitrophenyl Ester (IIb).** To a solution of 1.95 g. (0.0055 mole) of Ic and 1.4 g. of glycine *p*-nitrophenyl ester hydrobromide[16] in 50 ml. of chloroform and 0.7 ml. of triethylamine 1.1 g. of N,N′-dicyclohexylcarbodiimide was added and the mixture was shaken for 4 hours. The reaction mixture was allowed to stand for an additional 12 hours at room temperature and then the solvent was removed by evaporation. The residue was extracted with 30 ml. of boiling tetrahydrofuran, the undissolved material (triethylamine hydrobromide and dicyclohexylurea) was filtered while warm, and washed with a small amount of hot tetrahydrofuran. The filtrate was evaporated to dryness and the residue was recrystallized from ethanol; the yield of IIb was 2.3 g. (88%), m.p. 174–176°, unchanged by further recrystallization, $[\alpha]^{25}D$ −42° (*c* 1, dimethylformamide).

*Anal.* Calcd. for $C_{26}H_{23}N_3O_8S$: C, 58.12; H, 4.28; N, 7.81. Found: C, 58.15; H, 4.41; N, 7.97.

**N-Carbobenzoxy-S-acetyl-L-cysteinylglycine methyl ester** (III) was prepared by coupling Id with glycine methyl ester in the same manner as described for IIa. The yield was 73%, m.p. 125–126°, $[\alpha]^{25}D$ −46.7° (*c* 1, dimethylformamide).

*Anal.* Calcd. for $C_{16}H_{20}N_2O_6S$: C, 52.17; H, 5.43; N, 7.60; S, 8.70. Found: C, 52.35; H, 5.44; N, 7.56; S, 8.49.

Methanolysis and transformation to V was carried out in the same manner as described for DBCME. The iodine titration revealed a 98% methanolysis; the yield of V thus formed was 92%, m.p. 170–172°, $[\alpha]^{25}D$ −148° (*c* 1, dimethylformamide).

**N-Carbobenzoxy-S-acetyl-L-cysteinylglycine ethyl ester**, was prepared in the same manner as the corresponding methyl ester III. The yield was 80%, m.p. 135–136°, $[\alpha]^{25}D$ −48.4° (*c* 1, dimethylformamide).

*Anal.* Calcd. for $C_{17}H_{22}N_2O_6S$: N, 7.32; S, 8.38. Found: N, 7.48; S, 8.66.

**N,S-Dicarbobenzoxy-L-cysteinylglycine Methyl Ester (IVa).**—To a solution of 2.75 g. (0.022 mole) of glycine methyl ester hydrochloride in 50 ml. of chloroform and 3 ml. of triethylamine 10.2 g. (0.02 mole) of N,S-dicarbobenzoxy-L-cysteine *p*-nitrophenyl ester was added. After being allowed to stand for 12 hours at room temperature the solution was evaporated to dryness. The residue was dissolved in ethyl acetate and the solution was washed many times with sodium carbonate until the aqueous layer was practically colorless, then with dilute hydrochloric acid and finally with water. The solution was dried and evaporated to dryness and the residue was dissolved in hot ethanol. After the solution was allowed to cool to room temperature for 48 hours the precipitate (11 g.) was collected by filtration and was washed successively with ethanol and petroleum ether; the yield was 4.5 g. (50%), m.p. 103°, $[\alpha]^{25}D$ −51.1° (*c* 1, dimethylformamide).

*Anal.* Calcd. for $C_{22}H_{24}N_2O_7S$: C, 57.38; H, 5.25; N, 6.08; S, 6.96. Found: C, 57.48; H, 5.21; N, 6.10; S, 6.93.

The methanolysis of IVa and its transformation to V were carried out in the same manner as described for DBCME except that in this case the solution was stirred for about 30 minutes. The iodine titration revealed an 88% methanolysis; the yield of V was 81%, m.p. 168–170°, $[\alpha]^{25}D$ −143° (*c* 1, dimethylformamide).

(14) M. Brenner and W. Huber, *Helv. Chim. Acta*, **36**, 1109 (1953).

(15) J. C. Sheehan and G. P. Hess, *J. Am. Chem. Soc.*, **77**, 1067 (1955).

(16) M. Goodmann and K. Stueben, *J. Am. Chem. Soc.*, **81**, 3980 (1959).

After recrystallization from methanol (recovery 65%) the m.p. was raised to 171° and $[\alpha]^{25}D$ −148° (in dimethylformamide).

**N,S-Dicarbobenzoxy-L-cysteinylglycine *p*-nitrobenzyl ester** (IVc) was prepared by coupling N,S-dicarbobenzoxy-L-cysteine *p*-nitrophenyl ester with glycine *p*-nitrobenzyl ester[17] in the same manner as described for the preparation of IVa. The yield was 73%, m.p. 146°, $[\alpha]^{25}D$ −39.8° (*c* 1, dimethylformamide).

*Anal.* Calcd. for $C_{28}H_{27}N_3O_9S$: C, 57.82; H, 4.68; N, 7.23; S, 5.51. Found: C, 57.86; H, 4.75; N, 7.09; S, 5.71.

**N,S-Dicarbobenzoxy-L-cysteinylglycine *p*-Nitrophenyl Ester** (IVb).—To a solution of 4.3 g. (0.011 mole, *i.e.* 10% excess) of N,S-dicarbobenzoxy-L-cysteine and 2.8 g. (0.01 mole) of glycine nitrophenyl ester hydrobromide[16] in 100 ml. of chloroform and 1.4 ml. of triethylamine 2.2 g. of N,N′-dicyclohexylcarbodiimide was added and the mixture was shaken for 4 hours. After the mixture had been allowed to stand for an additional 12 hours at room temperature, more chloroform (500 ml.) was added and the solution was successively washed with hydrochloric acid, potassium hydrogen carbonate solution and water, dried and evaporated to dryness. The residue was heated with 40 ml. of tetrahydrofuran on the steam-bath. The mixture was allowed to cool and after standing several hours at room temperature the precipitate (N,N′-dicyclohexylurea, 3.3 g.) was removed by filtration. Upon concentrating the filtrate and crystallizing the residue from ethanol, 3.4 g. (60%) of IVb was obtained, m.p. 168° and 169–170° after recrystallization from ethanol; $[\alpha]^{25}D$ −46.5° (*c* 1, dimethylformamide).

*Anal.* Calcd. for $C_{27}H_{25}N_3O_9S$: N, 7.40; S, 5.65. Found: N, 7.35; S, 5.90.

The methanolysis of IVb and transformation to V were carried out in the same manner as described for DBCME except that 2 equiv. of sodium methoxide was used and stirring was continued for approximately 30 minutes. Iodine titration revealed a 85% conversion to V. The yield of V was 75%, m.p. 163°, $[\alpha]^{25}D$ −128° (*c* 1, dimethylformamide); after recrystallization from methanol (recovery 65%) the m.p. was 170° and $[\alpha]^{25}D$ −147° (dimethylformamide).

**N-Formyl-S-carbobenzoxy-L-cysteinylglycine ethyl ester** was prepared by coupling of N-formyl-S-carbobenzoxy-L-cysteine with glycine ethyl ester in the same manner as described for the preparation of IIa. The crude product was recrystallized from hot acetone; the yield was 3 g. (81%), m.p. 126–127°, $[\alpha]^{25}D$ −36.2° (*c* 1, dimethylformamide).

*Anal.* Calcd. for $C_{16}H_{20}N_2O_6S$: C, 52.31; H, 5.21; N, 7.62. Found: C, 52.50; H, 5.42; N, 7.55.

**S-Benzoyl-L-cysteinylglycine Methyl Ester Hydrochloride** (VIa).—A solution of 0.86 g. (0.002 mole) of IIa and 0.4 g. of phenol in 3 ml. of trifluoroacetic acid was refluxed on a steam-bath for 30 minutes and then was evaporated to dryness. Upon dissolving the residue in ether and adding ether saturated with hydrogen chloride, VIa separated as an oil which crystallized upon trituration with ether and finally with hot ethyl acetate. The product was recrystallized by solution in a small amount of methanol and precipitation with ethyl acetate. The yield was 0.4 g. (60%), m.p. 163–164°, $[\alpha]^{25}D$ +29° (*c* 5, methanol).

*Anal.* Calcd. for $C_{13}H_{17}N_2O_4SCl$: N, 8.41; S, 9.63; Cl, 10.65. Found: N, 8.28; S, 9.62; Cl, 10.42.

**S-Benzoyl-L-cysteinylglycine *p*-Nitrophenyl Ester Hydrobromide** (VIb).—A suspension of 2.1 g. (0.004 mole) of IIb in 16 ml. of 2 *N* HBr in acetic acid was shaken for about 35 minutes at room temperature. During this time IIb dissolved completely. Addition of ether to the solution caused 1.6 g. (84%) of VIb (m.p. 158–160°) to separate. The product was recrystallized by solution in the minimum amount of hot ethanol and precipitation by addition of ethyl acetate (recovery 81%); m.p. 162–163°, $[\alpha]^{25}D$ +20.3° (*c* 2, dimethylformamide).

*Anal.* Calcd. for $C_{18}H_{18}N_3O_6SBr$: N, 8.69; S, 6.63; Br, 16.53. Found: N, 8.82; S, 6.42; Br, 16.34.

**S-Carbobenzoxy-L-cysteinylglycine *p*-nitrobenzyl ester hydrobromide** (VIc) was prepared by N-decarbobenzoxylation of IVc with 2 *N* HBr in acetic acid as described for the preparation of VIb from IIb. The yield was 45–50%, m.p. 141–142° after recrystallization from hot ethanol–ethyl acetate, $[\alpha]^{25}D$ +13.3° (*c* 3, dimethylformamide).

*Anal.* Calcd. for $C_{20}H_{22}N_3O_7SBr$: N, 7.95; S, 6.07; Br, 15.12. Found: N, 7.85; S, 6.24; Br, 15.33.

(17) H. Schwarz and K. Arakawa, *J. Am. Chem. Soc.*, **81**, 5691 (1959).

**S-Carbobenzoxy-L-cysteinylglycine *p*-Nitrophenyl Ester Hydrobromide.**—To a hot solution of 1.13 g. (0.002 mole) of N,S-dicarbobenzoxy-L-cysteinylglycine *p*-nitrophenyl ester in 12 ml. of acetic acid, 6 ml. of 6 *N* HBr in acetic acid was added. After being allowed to stand for 20 minutes at room temperature the solution was evaporated to dryness. Almost complete removal of the excess HBr was ensured by the addition of ethyl acetate and evaporation again to dryness. After trituration with ether the yield of the product was 0.9 g. (90%), m.p. 124°. After recrystallization from ethanol (recovery 50%) the m.p. was raised to the constant value of 132–133°, $[\alpha]^{25}D$ +12.3° (*c* 5, methanol).

*Anal.* Calcd. for $C_{19}H_{20}N_3O_7SBr$: C, 44.38; H, 3.89; N, 8.16; S, 6.23; Br, 15.54. Found: C, 44.25; H, 4.05; N, 8.27; S, 6.35; Br, 15.68.

**N-Carbobenzoxy-L-phenylalanyl-S-benzoyl-L-cysteinylglycine Methyl Ester** (VIIa).—A solution containing 3 g. (0.01 mole) of carbobenzoxy-L-phenylalanine, 40 ml. of chloroform and 1.4 ml. of triethylamine was cooled to 0° and 1.3 ml. of isobutyl chloroformate was added. After 10 minutes 3.3 g. (0.01 mole) of VIa and 1.4 ml. of triethylamine were added. After being allowed to stand for several hours at room temperature the solution was washed as usual, dried over sodium sulfate and evaporated to dryness. The crystalline residue was recrystallized from methanol; the yield was 3.6 g. (62%), m.p. 178–179°, $[\alpha]^{25}D$ −62.4° (*c* 1, dimethylformamide).

*Anal.* Calcd. for $C_{30}H_{31}N_3O_7S$: N, 7.27; S, 5.55. Found: N, 7.12; S, 5.75.

**Methanolysis and Transformation to N,N′-Biscarbobenzoxy-L-phenylalanyl-L-cystinyldiglycine Dimethyl Ester** (VIII).—The methanolysis of VIIa and its conversion to VIII as well as the isolation of the product VIII was carried out in the same manner as described for DBCME except that VIIa was suspended in a mixture of 6 ml. of dimethylformamide and 12 ml. of methanol. Iodine titration revealed 100% conversion to VIII and the yield of the product was 98%, m.p. 209–210°, $[\alpha]^{25}D$ −86° (*c* 1, dimethylformamide). After recrystallization from dimethylformamide–water the m.p. was raised to 223–224°, and the optical rotation was $[\alpha]^{25}D$ −87° (*c* 1, dimethylformamide).

*Anal.* Calcd. for $C_{48}H_{52}N_6O_{12}S_2$: C, 58.48; H, 5.50; N, 8.89; S, 6.78. Found: C, 58.66; H, 5.71; N, 8.67; S, 6.94.

**N-Carbobenzoxy-L-phenylalanyl-S-benzoyl-L-cysteinylglycine *p*-Nitrophenyl Ester** (VIIb).—Compound VIb (2.4 g., 0.005 mole) was dissolved in 40 ml. of chloroform and 0.7 ml. of triethylamine and immediately thereafter 1.65 g. (10% excess) of carbobenzoxy-L-phenylalanine and 1.1 g. of N,N′-dicyclohexylcarbodiimide were added. The mixture was shaken for several hours at room temperature and then it was worked up as described for the preparation of IIb. The yield of VIIb was 1.7 g. (70%), m.p. 195°, $[\alpha]^{25}D$ −44° (*c* 1.5, dimethylformamide).

*Anal.* Calcd. for $C_{35}H_{32}N_4O_9S$: N, 8.18; S, 4.68. Found: N, 8.13; S, 4.56.

Methanolysis of the product and transformation to VIII was carried out in the same manner as described except that 2 equiv. of sodium methoxide was used and stirring was continued for 30 minutes. Iodine titration revealed 85% conversion to VIII; the yield of VIII was 80%, m.p. 224°, $[\alpha]^{25}D$ −87° (*c* 1, dimethylformamide).

**N-Carbobenzoxy-L-phenylalanyl-S-carbobenzoxy-L-cysteinylglycine *p*-nitrobenzyl ester** (VIIc) was prepared by coupling carbobenzoxy-L-phenylalanine with VIc in the same manner as described for VIIa. After recrystallization from ethanol the yield of VIIc was 4 g. (55%), m.p. 173–174°, $[\alpha]^{25}D$ −37.6° (*c* 1, dimethylformamide).

*Anal.* Calcd. for $C_{37}H_{36}N_4O_{10}S$: N, 7.68; S, 4.40. Found: N, 7.57; S, 4.62.

The methanolysis and transformation to VIII and the isolation of VIII were carried out in the same manner as described except that 1.1 equiv. of sodium methoxide was used and stirring was continued for 30 minutes. Iodine titration revealed 90% conversion to VIII; the yield of VIII was 87%, m.p. 210°, $[\alpha]^{25}D$ −83.4° (*c* 1, dimethylformamide) and 60% after further recrystallization from dimethylformamide–water, m.p. 224°, $[\alpha]^{25}D$ −86.8° (*c* 1, dimethylformamide).

**Acknowledgment.**—The authors wish to acknowledge the assistance of Dr. G. C. Stelakatos.