Conversion of the L-Serine Residue to an L-Cysteine Residue in Peptides*

Christine Zioudrou,† Meir Wilchek, and Abraham Patchornik

ABSTRACT: The reaction of thioacetate ion with *O*-*p*-toluenesulfonyl-L-serine peptides in *N*,*N*-dimethylformamide or aqueous media (*p*H 7) results in the quantitative formation of *optically active* *S*-acetyl-L-cysteine peptides via an SN2 displacement mechanism. The method has been applied to the syntheses, in high yields, of several L-cysteine peptides and of a derivative of glutathione from the corresponding serine analogs. Similarly the anions of tritylthiocarbinol, benzyl mercaptan, 2-mercaptoacetic acid, and cysteamine. when treated with *O*-tosylated L-serine peptides, yield the corresponding *S*-alkyl-L-cysteine peptides. The reaction of *N*-carbobenzoxy-*O*-tosyl-L-serine methyl ester with thiols and thio acids is discussed. Several analytical methods for the determinations of *O*-tosylserine peptides by nonaqueous titration as well as for the determination of *S*-acetyl- and *S*-alkylcysteine peptides by bromine oxidation are described.

The chemical problems encountered in the synthesis of cysteine and unsymmetrical cystine peptides have been described by Zervas (Zervas and Photaki, 1962; Zervas *et al.*, 1963). Considerable progress in this field has been achieved by the use of a number of blocking groups for the protection of the sulfhydryl group of cysteine and their selective removal. Recently Photaki (1963) has converted *N*-carbobenzoxy-*O*-tosyl-L-serine methyl ester to *N*-carbobenzoxy-*S*-trityl-DL-cysteine by reaction with the sodium salt of triphenylmethylthiocarbinol. The mechanism proposed for this reaction involves a β-elimination process leading to the formation of a dehydroalanine derivative and the subsequent addition of the thiol to the double bond. The author also suggested that the method can be used to convert serine residues into cysteine residues in peptides. Farlow (1948) and Behringer (1948) reported the formation of DL-cysteine derivatives by addition of hydrogen sulfide or thioacetic acid to α-acetamidoacrylic acid. Hydrolysis of thiazoline derivatives (Crawhall and Elliott, 1951) also gives rise to cysteine derivatives. Optically active *N*,*S*-dibenzoyl-L-cysteine has been synthesized (Fry, 1950) by treating L-2-phenyl-4-carboxy-Δ^2-oxazoline methyl ester with thiobenzoic acid.

It has been known that sulfonate esters, when allowed to react with thio acid or thiol anions, can be converted to thio esters or *S*-alkylthiols via an SN2 displacement mechanism. For example, *O*-*p*-toluenesulfonylated sugars (Raymond, 1934; Chapman and Owen, 1950; Michelson, 1962) are converted to thio sugars. Winstein *et al.* (1956), Bunnett and Merritt (1957), and Eliel and Ro (1957) have studied the reaction of substituted cyclohexyl-*p*-toluenesulfonates and of other aliphatic *p*-toluenesulfonates with nucleophiles such as mercaptide, alkoxide, and halide anions. The literature related to the nature of the displaced group and the nucleophile in nucleophilic displacement reactions has been recently reviewed by Bunton (1963) and Hudson (1962).

Nucleophilic displacement reactions on carbon by thioanions have recently been discussed by Swan (1960) and Zahn *et al.* (1960). Thionucleophiles such as thioanions of cysteine residues, produced by the action of sulfite or cyanide on wool, could attack the β-carbon atom of *S*-sulfo or *S*-cyano derivatives by an SN2 process, resulting in the formation of monosulfide linkages such as in lanthionine.

The present work describes the results of a study of the reaction of the anions of thioacetic acid, thioglycolic acid, triphenylthiocarbinol, benzyl mercaptan, and cysteamine with *O*-*p*-toluenesulfonylated serine peptides as well as the displacement of diphenyl phosphate ion from *N*-*p*-nitrobenzoyl-*O*-diphenylphosphorylethanolamine by thioacetate to yield the corresponding cysteine peptides and cysteamine derivatives. This study was carried out in connection with the synthesis of cysteine peptides from L-serine peptides and the possibility of converting the "active serine" in esterases into a cysteine residue.

Results and Discussion

The reaction of N-carbobenzoxy-O-tosyl-L-serylglycine ethyl ester (**1**) in *N*,*N*-dimethylformamide[1] with a

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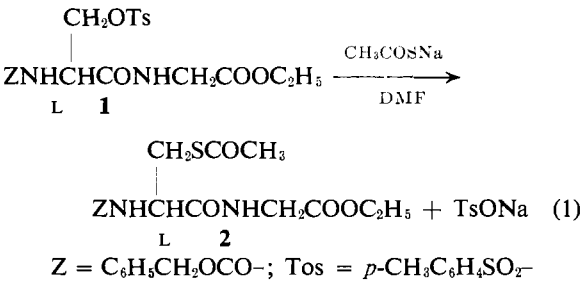
† Visiting scientist at the Weizmann Institute, Department of Biophysics, 1964. Permanent address: Nuclear Research Center "Democritus," Aghia Paraskevi Attikis, Athens, Greece.

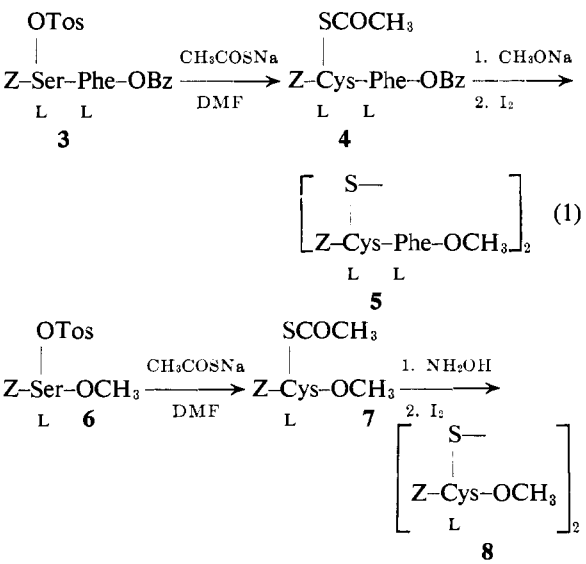
stoichiometric amount of sodium thioacetate at 50° results in the quantitative formation of *N-carbobenzoxy-S-acetyl-*L*-cysteinylglycine ethyl ester* (**2**) according to equation (1)



(1)

Z = $C_6H_5CH_2OCO$–; Tos = *p*-$CH_3C_6H_4SO_2$–

The same product (**2**) was obtained in high yield (84%) when the reaction was carried out in 50% DMF–phosphate buffer, *p*H 6.8. The product, compound **2**, is optically active and has the same specific rotation ($[\alpha]_D^{21}$ −47°) as that reported by Zervas *et al.* (1963). Similarly, *N*-carbobenzoxy-*O*-tosyl-L-seryl-phenylalanine benzyl ester (**3**) and *N*-carbobenzoxy-*O*-tosyl-L-serine methyl ester (**6**) were converted in high yields to the corresponding *S*-acetyl-L-cysteine derivatives **4** and **7** by treatment with sodium thioacetate in DMF (Scheme I).



Bz = $C_6H_5CH_2$–; Ser = serine; Cys = cysteine
Phe = phenylalanine

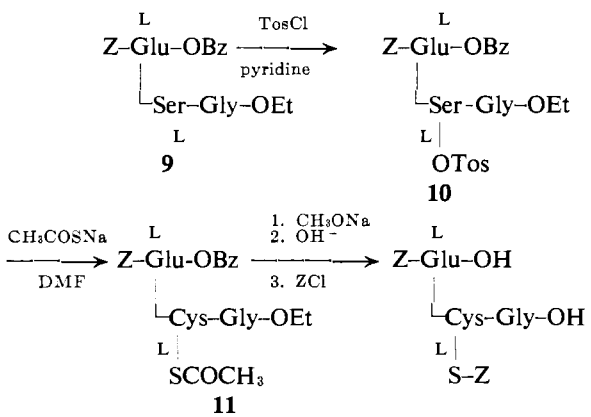
SCHEME I

To prove the optical purity of compounds **4** and **7**, these were transformed into the corresponding cystine derivatives **5** and **8** by removal of the acetyl groups with an alkoxide solution (Zervas *et al.*, 1963; Sokolovsky *et al.*, 1964a,b) and hydroxylamine (Baddiley and Thain, 1951; Schwyzer, 1952), respectively, followed by oxidation with iodine. For the removal of the *S*-acetyl group from compound **7**, hydroxylamine was used because the use of sodium methoxide gave unexpectedly low yields of compound **8**.[2] The products obtained, **5** and **8**, had the same melting point and optical rotation as the ones prepared from L-cysteine. Thus, compound **5** was prepared from *N,S*-dicarbobenzoxy-L-cysteinyl-L-phenylalanine benzyl ester (Sokolovsky, *et al.*, 1964a,b) by removal of the *S*-carbobenzoxy group and concomitant transesterification with sodium methoxide and subsequent oxidation with iodine.

In all cases, the rate of reaction of thioacetate with the *O*-tosyl-L-serine derivatives was followed by iodimetric titration of acidified aliquots, as a measure of the amount of thioacetate left. Linear plots (up to 95% reaction) were obtained when the reciprocal of the concentrations of thioacetate ($1/c$) was plotted versus time, where c equals the concentration of thioacetate remaining in the solution at time t. For instance, from kinetic data for the reaction (equation 1) in dimethylformamide at 50° and with each reactant at 0.16 M, it was estimated that the second-order rate constant is 1.2 ± 0.2 M^{-1} min^{-1}. This value is similar to the value of the second-order rate constant (0.49 M^{-1} min^{-1}) for the displacement of tosylate anion from *n*-butyl tosylate by the thiophenolate anion in 87% ethanol–H_2O (Eliel and Ro, 1957). When the same reaction was carried out in 50% dimethylformamide-phosphate buffer (*p*H 6.8) at 50° and with the reactants at 0.06 M, the second-order rate constant was 6×10^{-2} M^{-1} $min.^{-1}$.

The retention of optical configuration, as well as the kinetic data, support the hypothesis that the reaction proceeds by nucleophilic attack of thioacetate anion on the β-carbon of the L-serine residue with displacement of tosylate anion to yield an *S*-acetyl-L-cysteine peptide.

The method was used to synthesize a derivative of glutathione, *N,S*-dicarbobenzoxyglutathione, from the serine tripeptide *N*-Z-γ-L-glutamyl-(α-benzyl ester)-L-serylglycine ethyl ester (**9**) in an over-all yield of 40% (Scheme II). The tripeptide **9** was synthesized by coupling



GluOBz = α-benzyl glutamate; Gly = glycine

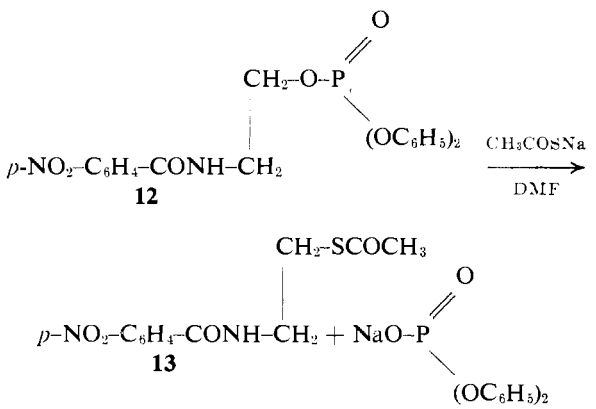
SCHEME II

[1] Abbreviations used in this work: DMF, *N,N*-dimethylformamide; Z, carbobenzoxy.

[2] This might be due to a base-catalyzed β-elimination reaction leading to the dehydroalanine derivative and thioacetate. Base-catalyzed β-elimination reactions are reported to occur in cysteine peptides (Swan, 1957; Sokolovsky *et al.*, 1964a,b).

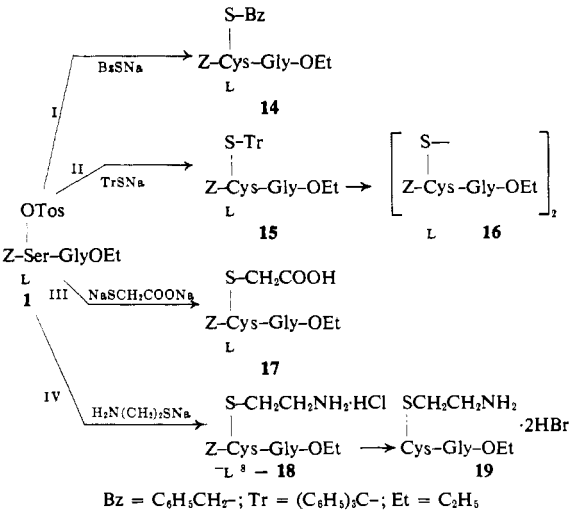
N-carbobenzoxy-L-glutamic acid α-benzyl ester (Losse *et al.*, 1963) with L-serylglycine ethyl ester using *N,N'*-dicyclohexylcarbodiimide. The tosylation of **9** was performed in pyridine in the presence of a threefold excess of tosyl chloride. It has been reported (Schmir and Zioudrou, 1963; Benoiton *et al.*, 1964) that *O*-tosylated or *O*-diphenylphosphorylated serine peptides cyclize easily to yield Δ^2-oxazoline derivatives even under very mild conditions. This property introduces difficulties in the phosphorylation and tosylation of a nonterminal serine residue in a peptide chain (see Benoiton *et al.*, 1964). By working at −10° in anhydrous pyridine and especially by maintaining low temperatures during the isolation stages, the cyclization reaction could be avoided, and the tosylation of **9** proceeded smoothly. The purity of the tosylated tripeptide (**10**) and in general of *O*-tosylated serine peptides can be determined by titration with sodium methoxide (see Experimental).

N-*p*-Nitrobenzoyl-*O*-diphenylphosphorylethanolamine (**12**) also reacts with thioacetate in DMF. The ethanolamine derivative is converted quantitatively to the *S*-acetylcysteamine derivative **13**. In this case the



rate of the displacement of diphenyl phosphate anion by thioacetate at 50° is approximately 40 times slower than that of tosylate anion in peptide **1**. The ratio in rates is similar to that observed in the displacement of diphenyl phosphate and tosylate anion by the unionized peptide bond to form Δ^2-oxazolines in *N*-acyl-*O*-phosphorylated and -*O*-tosylated ethanolamine derivatives (Schmir and Zioudrou, 1963). The reaction as applied to *O*-phosphorylated serine peptides is now being studied.

The thioanions of benzyl mercaptan, triphenylmethylthiocarbinol, 2-mercaptoacetic acid, and cysteamine react with *O*-tosylated L-serine peptides in DMF to yield *S*-benzyl-, *S*-trityl, *S*-carboxymethyl-, and *S*-β-aminoethyl-L-cysteine peptides, respectively, via an SN2 displacement mechanism. The reaction of peptide **1** with the different thiol anions at room temperature (equations I–IV, Scheme III) is faster than the one with thioacetate.



Bz = $C_6H_5CH_2$–; Tr = $(C_6H_5)_3C$–; Et = C_2H_5

SCHEME III

The amount of the thiol consumed can be followed by iodimetric titration (see Experimental). The products **14–19** were optically active L-cysteine derivatives. The specific rotation of the product **14** (equation I) is identical with that reported for *N*-carbobenzoxy-*S*-benzyl-L-cysteinylglycine ethyl ester by Maclaren *et al.* (1958). The reaction of peptide **1** with the sodium salt of tritylthiol (equation II) yielded *N*-carbobenzoxy-*S*-trityl-L-cysteinylglycine ethyl ester (**15**) with optical properties identical with those reported by Zervas and Photaki (1962). In addition its structure was confirmed by transforming it to the known L-cystine peptide **16** (Zervas and Photaki, 1962).

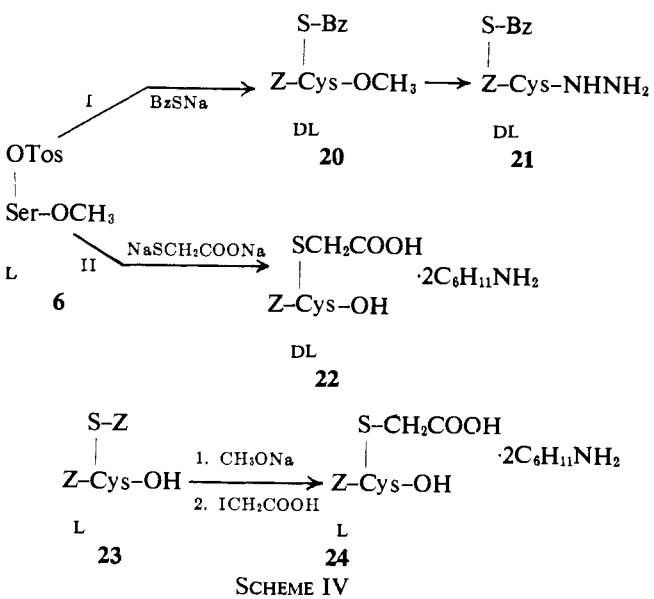
The reaction of peptide **1** in DMF or ethanol with 1–2 eq of disodium thioglycolate (equation III) at room temperature results in the formation of *N*-carbobenzoxy-*S*-carboxymethyl-L-cysteinylglycine ethyl ester (**17**), with a specific rotation of $[\alpha]_D^{23}$ −29.6° in DMF and $[\alpha]_D^{25}$ −8.3° and $[\alpha]_{314m\mu}^{25}$ −44° in dioxane. The change in optical rotation of a mixture of 0.1 M solution of *N*-carbobenzoxy-*O*-tosyl-L-serylglycine ethyl ester (**1**) with an equimolar amount of disodium thioglycolate in DMF was followed in the polarimetric tube (length 10 cm). The negative value of $\alpha = -0.130°$ measured after 2 minutes increased with time to reach the value of $\alpha = -0.985°$ after 30 minutes, indicating a complete reaction.

N-Carbobenzoxy-*S*-β-aminoethyl-L-cysteinylglycine ethyl ester (**18**) was formed when a solution (0.1–0.2 M) of the sodium salt of cysteamine in DMF reacted with an equivalent quantity of peptide **1**. The structure of the *N*-carbobenzoxy-L-4-thialysylglycine ethyl ester hydrochloride (**18**) is supported by its behavior on paper electrophoresis and by amino acid analysis of a hydrolyzed sample and measurement of its specific rotation. The acid hydrolysate of **18** on paper electrophoresis at *p*H 3.5 (3 kv) revealed, besides glycine, an amino acid with electrophoretic mobility identical with that of authentic L-4-thialysine (obtained by acid

hydrolysis of ε-carbobenzoxy-L-4-thialysine[3]). Analysis of the hydrolysate on the standard amino acid analyzer revealed equal quantities of glycine and L-4-thialysine. Compound **18** was converted to L-4-thialysylglycine ethyl ester dihydrobromide (**19**) by decarbobenzoxylation with HBr-CH_3COOH. The specific rotation in water, $[\alpha]_D^{23}$ +13°, is similar to that of L-4-thialysylglycine dihydrobromide, $[\alpha]_D^{20}$ +13 ± 0.5° in water (Shiota *et al.*, 1961). The optical configuration of compound **18** was established by the action of L-lysine decarboxylase (Gale and Epps, 1944). When solutions of L-4-thialysine and the hydrolysate of **18** were treated with L-lysine decarboxylase and the reaction mixtures examined on paper electrophoresis, it was found that in both cases all of the material which originally moved to the position of L-4-thialysine had disappeared. Instead a new substance, with the mobility of cadaverine, had been formed.

The products of the displacement of tosyl and phosphoryl anions in L-serine peptides by cysteamine to form *S*-β-aminoethylcysteine (L-4-thialysine) can serve as substrates for trypsin (Lindley, 1956; Tesser and Nivard, 1964) for specific cleavage at serine residues. The hydrochloride of *N*-carbobenzoxy-L-4-thialysylglycine ethyl ester (**18**) was converted to the corresponding amide and subjected to tryptic hydrolysis. Glycinamide (97%) was recovered from the hydrolysate. This finding also supports the L-configuration of compound **18**. Tryptic cleavage at thialysine residues, after modification of cysteinyl residues, has been applied to study the structure of *Clostridium pasteurianum* ferrodoxin (Raftery and Cole, 1964).

In connection with the synthesis of L-cysteine peptides, other amino acid derivatives such as β-chloroalanine can react with the anions of thioacids and of thiols by an SN2 mechanism.



SCHEME IV

In contrast to these straightforward bimolecular substitution reactions, when a solution of *N*-carbobenzoxy-*O*-tosyl-L-serine methyl ester (**6**) in dimethylformamide or methanol was treated with the sodium salt of benzyl mercaptan (equation I, Scheme IV) or disodium mercaptoacetic acid (equation II) at room temperature, it was found that the isolated products **20** and **22** were *optically inactive*. The *N*-carbobenzoxy-*S*-benzyl-DL-cysteine methyl ester (**20**) obtained as an oil showed no optical rotation, and neither did the solid hydrazide **21** obtained from it. The properties of the dicyclohexylammonium salt of *N*-carbobenzoxy-*S*-carboxymethyl-L-cysteine (**24**), synthesized independently by removing the carbobenzoxy group from the sulfhydryl group of *N,S*-dicarbobenzoxy-L-cysteine (**23**) and reaction with iodoacetic acid, were different from those of compound **22**.

The reactions described by equations I and II (Scheme IV) apparently proceed via a β-elimination process and subsequent addition of the thiol to the acrylic acid intermediate and not according to the SN2 mechanism proposed above. This is in agreement with the results of Photaki (1963), who obtained racemic *N*-carbobenzoxy-*S*-trityl-DL-cysteine from the reaction of *N*-carbobenzoxy-*O*-tosyl-L-serine methyl ester with the sodium salt of tritylthiocarbinol in anhydrous acetone.

The different route by which the reactions of Scheme IV proceed, i.e., preference of the base-catalyzed β-elimination over the substitution mechanism, could be due to several factors. (a) Structural features of the substrate may influence the acidity of the C_α-H: The methoxycarbonyl group of compound **6**, being a stronger electron-withdrawing group than the peptide bond of compound **1**, favors the base-catalyzed β-elimination and facilitates the addition of the thiolate to the double bond. It has been observed (Sokolovsky *et al.*, 1964a,b) that the β-elimination reaction of *S*-dinitrophenylated L-cysteine derivatives proceeds faster with the esters than with the corresponding amides. The same structural features favor the formation of dehydroalanine in *N*-acyl-*O*-tosylserine esters, whereas the amide group promotes the cyclization to Δ^2-oxazolines (Ginsburg and Wilson, 1964). (b) The basicity of the nucleophile: Thiolate ions are stronger bases than thioacetate ion [*pK* of SH, for thioglycolic acid 10.4 (Danehy and Noel, 1960); for benzyl mercaptan 10.5; for thioacetic acid 3.33] and therefore promote the abstraction of the proton from C_α. Thus the reaction of *N*-carbobenzoxy-*O*-tosyl-L-serine methyl ester with thioacetate (Scheme I) leads quantitatively to an optically active substitution product, whereas the reaction with benzyl mercaptan and thioglycolate leads to a racemic product, probably via a β-elimination process.

An alternative mechanism leading to the racemization of the products **20** and **22** could be the direct base-catalyzed racemization of the substrate *N*-carbobenzoxy-*O*-tosyl-L-serine methyl ester, or of any optically active product (L-**20** and L-**22**). It is known that serine peptides (Schnabel, 1959) as well as *S*-benzyl-L-

[3] Carbobenzoxy-L-4-thialysine was prepared by Mr. Y. Degani by treating cysteine with ethylenimine and subsequently carbobenzoxylating the ε-amino group (see also Lindley, 1956; Shiota *et al.*, 1961).

cysteine peptides (Maclaren, 1958) racemize easily under mild conditions.

We have found that *N*-carbobenzoxy-L-serine methyl ester (**25**) racemizes rapidly in the presence of dilute solutions of sodium methoxide and that the rate of racemization is proportional to the concentration of the alkoxide. Similarly, when a 0.15 M solution of *N*-carbobenzoxy-L-serine methyl ester (**25**) in DMF was treated with a 0.25 M solution of sodium benzyl mercaptide at 23°, it was found that the serine derivative racemized rapidly. The pseudo-first-order rate constant of the racemization is 8.8×10^{-2} min^{-1} (half-life 7.8 minutes). However, when *N*-carbobenzoxy-L-seryl-glycine ethyl ester (**26**) was treated with benzyl mercaptan for 70 minutes under the same conditions, the re-isolated peptide was found to have racemized only to an extent of 5%. Similar results were obtained when the ester **25** and peptide **26** were treated with thioglycolate solution.

The reactions of thioacetate and of the other thiol anions with *O*-tosyl- and *O*-phosphorylserine peptides may be applied to specific chemical modification of proteins at serine residues. At present these reactions are being used for the modification of tosyl- and diisopropylphosphoryl-inhibited chymotrypsin (Strumeyer *et al.*, 1963; Kallos and Rizok, 1963; Fahrney and Gold, 1964).

During the course of this work, several analytical methods have been developed or adapted from the literature to provide a facile and reasonably accurate means of differentiating between derivatives of serine and cysteine peptides.

Nonaqueous titrations have been used for the determination of free carboxyl groups or of strong acids such as *p*-toluenesulfonic acid released from the *O*-tosylserine peptides after treatment with standard alkoxide solution (Fritz, 1952). For the sulfur-containing compounds (thiols, thio acids, *S*-acyl- and *S*-alkylcysteine derivatives) oxidative methods with iodine or bromine were used.

In the present work all free thiols, as well as thioacetic acid, consumed 1 eq of iodine and were oxidized to the corresponding disulfides. Tritylthiocarbinol is oxidized by 2 eq of iodine/mole as described for other tertiary mercaptans (Kolthoff and Harris, 1949). *S*-Acetyl derivatives of cysteine and thioacetic acid are oxidized by bromine solution to their cysteic acid analogs and to sulfuric acid, respectively. The stoichiometry for the *S*-acylcysteine derivatives is 3 moles of bromine (6 eq)/mole of compound and that for thioacetic acid is 4 moles of bromine/mole of acid. *S*-Alkylcysteine derivatives are oxidized with bromine to the sulfoxides (Sampey *et al.*, 1932). The *S*-carboxymethyl and S-β-aminoethyl derivatives of cysteine in 50% acetic acid react with 2 eq of bromine to yield probably the corresponding sulfoxides. The stoichiometry of the oxidation of the *S*-benzylcysteine derivatives proved to be complicated, presumably due to the facile formation of the stable $C_6H_5CH_2^+$ ion which yields benzyl bromide by reacting with the bromide ions present in the solution.

Experimental

All melting points are uncorrected. Prior to analysis, the compounds were dried *in vacuo* over phosphorus pentoxide at 45°. Microanalyses were performed by the Microanalytical Laboratory of the Weizmann Institute (Rehovoth, Israel).

N-Carbobenzoxy-S-acetyl-L-cysteinylglycine Ethyl Ester (**2**). METHOD A. To a solution of 240 mg (0.5 mmole) of *N*-carbobenzoxy-*O*-tosyl-L-serylglycine ethyl ester (Photaki, 1963), mp 96–98°, in 2 ml of anhydrous DMF was added 50 mg (approx 0.7 mmole) of freshly distilled thioacetic acid followed by 1.1 ml of 0.6 M sodium ethoxide. The solution was mixed and a 0.1-ml sample was titrated, after acidification, with 0.01 N iodine solution. The solution was found to contain 0.675 mmole of sulfhydryl group. The solution was then kept at 50° and the reaction was followed by iodine titration of 0.1-ml samples. After 70 minutes only 0.170 mmole of sulfhydryl group remained in the solution. The reaction mixture (2.5 ml) was acidified by addition of 2 ml of acetic acid and the solvents were removed *in vacuo*. The oily residue crystallized on addition of ice-cold water. The crude material (125 mg, 84% yield) melted at 128–130°. After recrystallization from ethyl acetate–petroleum ether it melted at 134–135°, $[\alpha]_D^{21}$ −47° (*c* 1, DMF); reported (Zervas *et al.*, 1963) mp 134–135°, $[\alpha]_D^{25}$ −48.8° (*c* 1, DMF).

Anal. Calcd for $C_{17}H_{22}N_2O_6S$ (382.41): C, 53.50; H, 5.80; N, 7.32; S, 8.38. Found: C, 55.00; H, 5.8; N, 7.14; S, 8.18.

Alcoholysis of the *S*-acetyl group followed by iodine titration revealed 98% of free thiol group. Oxidation of the compound with bromine to its cysteic acid analog gave a molecular weight of 405. Hydrolysis of the oxidized product with 6 N HCl and subsequent quantitative paper electrophoresis at *p*H 6.5 (pyridine-acetate) gave cysteic acid and glycine in a molar ratio 1:1, as determined by the ninhydrin method.

METHOD B. *N*-Carbobenzoxy-*O*-tosyl-L-serylglycine ethyl ester (480 mg, 1 mmole) was dissolved in 8 ml of DMF and treated with a solution of 0.1 ml of thioacetic acid in 8 ml of phosphate buffer (0.2 M, *p*H 6.8). The reaction was kept at 50° and followed iodometrically. At the end of the reaction (10 hours) the theoretical amount of thioacetate was consumed. The solution (11 ml) was chilled in ice and crystalline material precipitated; 260 mg (83%), mp 134–135°, was collected. Recrystallization from ethyl acetate–petroleum ether did not change the melting point. Mixture melting point with the material obtained by method A showed no depression.

N-Carbobenzoxy-L-seryl-L-phenylalanine Benzyl Ester. To a solution of 2.9 g of L-phenylalanine benzyl ester hydrochloride (10 mmole) and 3 ml of triethylamine (10 mmole) in 30 ml of chloroform were added a solution of 2.4 g of *N*-carbobenzoxy-L-serine (10 mmole) in 30 ml of chloroform and 2.1 g of dicyclohexylcarbodiimide (10 mmole); the mixture was stirred overnight at room temperature. After removal of dicyclohexylurea the filtrate was washed with 0.5 N hydrochloric

acid, water, 5% sodium bicarbonate solution, and water. Removal of the solvent yielded a crystalline residue which was recrystallized from ethyl acetate–petroleum ether or methanol; yield, 89%, m.p. 137°, $[\alpha]_D^{24}$ −19° (*c* 1, methanol).

Anal. Calcd for $C_{27}H_{28}O_6N_2$: C, 68.05; H, 5.92; N, 5.88. Found: C, 67.88; H, 6.02; N, 6.12.

*N-Carbobenzoxy-O-tosyl-*L*-seryl-*L*-phenylalanine Benzyl Ester* (**3**). To an ice-cold solution of *N*-carbobenzoxy-L-seryl-L-phenylalanine benzyl ester (4.7 g, 10 mmoles) in 20 ml of pyridine was added 2.1 g of tosyl chloride, and the solution was kept at −10° for 1.5 hour and an additional hour at room temperature. The solvent was removed under high vacuum at 30° and the residue solidified after treatment with ice and water. The compound was recrystallized from hot methanol; yield 4.9 g (75%), m.p. 119°, $[\alpha]_D^{26}$ −7° (*c* 5, DMF).

Anal. Calcd for $C_{34}H_{34}N_2O_8S$ (630.68): C, 64 76; H, 5.43; N, 4.45; S, 5.05. Found: C, 64.73; H, 5.33; N, 5.00; S, 4.98. A neutral equivalent of 650 was found by nonaqueous titration of the tosyl group with 0.1 N sodium methoxide.

*N-Carbobenzoxy-S-acetyl-*L*-cysteinyl-*L*-phenylalanine Benzyl Ester* (**4**). Thioacetic acid (0.5 ml) was treated with 12 ml of 0.58 M sodium methoxide and the solvent was removed *in vacuo*. The residue, sodium thioacetate, was dissolved in 4 ml of anhydrous DMF. The molarity of this solution (0.82 M) was estimated by iodine titration of an acidified sample. *N*-Carbobenzoxy-*O*-tosyl-L-seryl-L-phenylalanine benzyl ester (630 mg, 1 mmole) was dissolved in 4.4 ml of DMF and treated with 1.6 ml (1.3 mmoles) of the thioacetate solution. The reaction mixture was kept at 50° and the reaction was followed by iodine titration. After 60 minutes the theoretical amount of thioacetate has been consumed. The solution was then treated with 10 ml of ice-cold water and the crystalline material was collected by filtration and dried. The crude material (450 mg, 94% yield) melted at 145°. It was recrystallized from ethyl acetate–petroleum ether and melted at 150–152°; $[\alpha]_D^{23}$ −39° (*c* 1, DMF).

Anal. Calcd for $C_{29}H_{30}N_2O_8S$ (534.60): C, 65.15; H, 5.65; N, 5.25; S, 6.00. Found: C, 64.85; H, 5.34; N, 5.30; S, 5.92.

A neutralization equivalent of 540 was determined by iodine titration after alcoholysis of the *S*-acetyl group. The molecular weight estimated by bromine oxidation was found to be 535. Hydrolysis of the product with 6 N HCl and quantitative paper electrophoresis (*p*H 6.5) indicated equimolar amounts of cysteic acid and phenylalanine, as determined by the ninhydrin method.

*N,N′-Biscarbobenzoxy-*L*-cystyl-*L*-phenylalanine Dimethyl Ester* (**5**). Peptide **4** (106 mg, 0.2 mmole) was suspended in 1.5 ml of methanol and treated with 1.5 ml of sodium methoxide (1.08 M). The mixture was kept for 10 minutes at room temperature until all material dissolved. It was then acidified with dilute acetic acid and oxidized with iodine. The solid precipitate (75 mg) sintered at 147° and melted at 160°; $[\alpha]_D^{24}$ −99° (*c* 1, DMF).

Anal. Calcd for $C_{42}H_{46}N_4O_{10}S_2$ (830.94): C, 60.70; H, 5.45; N, 6.75; S, 7.70. Found: C, 60.68; H, 5.61; N, 6.33; S, 7.10.

*Conversion of N,S-Dicarbobenzoxy-*L*-cysteinyl-*L*-phenylalanine Benzyl Ester to Compound* **5**. *N,S*-Dicarbobenzoxy-L-cysteinyl-L-phenylalanine benzyl ester (1.25 g, 2 mmoles) (Sokolovsky *et al.*, 1964a,b) in 15 ml of methanol was treated with 10 ml of sodium methoxide (1.08 M) and stirred at room temperature until a clear solution was obtained. Acidification with dilute acetic acid and oxidation with iodine yielded 1 g of material which, after recrystallization from methanol-water, sintered at 147° and melted at 160°. The melting point was not depressed on admixture with **5**; $[\alpha]_D^{24}$ −102° (*c* 1, DMF).

*N-Carbobenzoxy-S-acetyl-*L*-cysteine Methyl Ester* (**7**). To a solution of *N*-carbobenzoxy-*O*-tosyl-L-serine methyl ester (410 mg, 1 mmole) (Photaki, 1963) in 4 ml of DMF was added 1 ml of 1.2 M sodium thioacetate in DMF. The flask was kept at 50° and the reaction was followed by iodine titration of 0.1-ml samples. After the end of the reaction, the solution (4.3 ml) was treated with ice and water. The crystalline material (260 mg, 82% yield) melted at 71°. It was recrystallized from a small quantity of ethyl acetate and hot petroleum ether and melted at 77–78°; $[\alpha]_D^{21}$ −47° (*c* 1, methanol).

Anal. Calcd for $C_{14}H_{17}NSO_5$ (311.33): C, 54.02; H, 5.50; N, 4.50; S, 10.30. Found: C, 53.98; H, 5.55; N, 4.75; S, 10.10.

Oxidation of the compound with 6 eq of bromine to its cysteic acid analog gave a molecular weight of 350. Hydrolysis of the product with 6 N HCl and quantitative paper electrophoresis indicated 95% of cysteic acid. The same product (**7**) has been isolated when the reaction was carried out in 50% DMF–buffer, *p*H 6.8.

*N,N′-Biscarbobenzoxy-*L*-cystine Dimethyl Ester* (**8**). N,N′-Biscarbobenzoxy-L-cystine (2 g, 4 mmoles) (Bergmann and Zervas, 1932) was dissolved in 10 ml of anhydrous methanol and treated with 2 ml of concentrated H_2SO_4 for 10 minutes at room temperature. The esterification of the carboxyl groups was followed by titration of an aliquot with a standard solution of sodium methoxide in the presence of thymol blue. No free carboxyl group could be detected after 5 minutes. The solution was triturated several times with ice and water which was decanted; the syrup was then dissolved in ethyl acetate, washed with bicarbonate and water, and dried over Na_2SO_4. Addition of petroleum ether induced crystallization. The crystals (2 g, 88% yield) melted at 69–71°. Recrystallization from ethyl acetate–petroleum ether raised the melting point to 71–73°; $[\alpha]_D^{22}$ −105° (*c* 2, methanol); $[\alpha]_D^{24}$ +58° (*c* 3, chloroform); reported (Zervas and Photaki, 1962) m.p. 73–75°, $[\alpha]_D^{20}$ +59° (*c* 5, chloroform).

Anal. Calcd for $C_{24}H_{28}N_2O_8S_2$ (536.60): C, 53.70; H, 5.27; N, 5.23; S, 11.95. Found: C, 53.79; H, 5.18; N, 5.12; S, 12.03.

*Conversion of N-Carbobenzoxy-S-acetyl-*L*-cysteine Methyl Ester to* **8**. A solution of **7** (150 mg, 0.5 mmole) in 1 ml of methanol was treated with 2.5 ml of meth-

anolic hydroxylamine[4] (0.22 M) and kept for 10 minutes at room temperature. It was then oxidized with 0.1 N iodine solution. The syrup which separated was chilled, washed several times with water, dissolved in methanol, and crystallized by addition of drops of water. After recrystallization from ethyl acetate–petroleum ether, the product (110 mg, 80% yield) melted at 69–71°. Mixture melting point with authentic **8** showed no depression; $[\alpha]_D^{24}$ −105° (*c* 1, methanol).

N-Carbobenzoxy-γ-L-glutamyl-(α-benzyl ester)-L-serylglycine Ethyl Ester (**9**). A solution of *N*-carbobenzoxy-L-serylglycine ethyl ester (2.5 g, 10 mmoles) in 20 ml of methanol was hydrogenated in a Parr apparatus in the presence of 10 mmoles of HCl and palladium on charcoal. After removal of the catalyst and evaporation of the solvent, the dried syrup was dissolved in chloroform and coupled by the dicyclohexylcarbodiimide method with 3.7 g (10 mmoles) of *N*-carbobenzoxy-L-glutamic acid α-benzyl ester (Losse *et al.*, 1963) in the presence of 1.5 ml of triethylamine. The *N,N*-dicyclohexylurea was removed and the filtrate was washed with bicarbonate solution, 1 N HCl, and water, and dried over Na_2SO_4, and the chloroform was removed *in vacuo*. Upon addition of petroleum ether the residue crystallized, yielding 4 g (85%) of peptide which melted at 106–108°. After recrystallization from ethyl acetate–petroleum ether, the product melted at 138–139°. The tripeptide of m.p. 138° and 106–108° gave identical elemental analyses.

Anal. Calcd for $C_{27}H_{33}N_3O_9$ (543.56): C, 59.66; H, 6.12; N, 7.73. Found: C, 59.68; H, 6.19; N, 7.84; $[\alpha]_D^{24}$ −23° (*c* 1, methanol).

N-Carbobenzoxy-γ-L-glutamyl-(α-benzyl ester)-O-tosyl-L-serylglycine Ethyl Ester (**10**). The tripeptide **9** (1.08 g, 2 mmoles) was dissolved in 7 ml of anhydrous pyridine and the solution was cooled at −10°. The tosylation was performed by adding 1.3 g (6 mmoles) of tosyl chloride (recrystallized from petroleum ether), and the reaction mixture was kept at −10° for 2 hours and then allowed to reach room temperature. The pyridine was removed under high vacuum at 25–30° and the oily residue was triturated several times with ice and water. The aqueous layer was decanted and addition of ice-cold ethanol and scratching induced crystallization of the hard oily residue. The crude product (1 g, 80%) melted at 87°; when recrystallized from lukewarm methanol it melted at 92–94°. Determination of the neutralization equivalent of the tosyl tripeptide gave a molecular weight of 696.

Anal. Calcd for $C_{34}H_{39}N_3O_{11}S$ (697.76): C, 58.53; H, 5.7; N, 6.02; S, 4.59. Found: C, 58.05; H, 6.00; N, 6.5; S, 4.36.

From mother liquors as well as from preparations where low temperatures (0 to 5°) had not been maintained during the isolation, impure tosyl tripeptide (70–90% pure) was isolated as determined by nonaqueous titration with sodium methoxide and subsequently by iodine titration during the reaction with thioacetate. The same tripeptide (**10**), m.p. 87°, was obtained by coupling solid amorphous *O*-tosyl-L-serylglycine ethyl ester hydrobromide (5 mmoles) with *N*-carbobenzoxy-L-glutamic acid α-benzyl ester (5 mmoles) in chloroform in the presence of triethylamine (5 mmoles) by the carbodiimide method; yield 50%.

Reaction of Tosyl Tripeptide with Thioacetate. To a solution of *O*-tosyl tripeptide **10** (500 mg, 0.72 mmole) in 10 ml of DMF was added 1 ml of 1 M thioacetate in DMF, and the reaction mixture was kept at 45°. After 2 hours the theoretical amount of thioacetate was consumed as determined by iodine titration of an aliquot. The solution was triturated with ice and water and acidified with HCl to Congo red. The crude product (370 mg, 85%) melted at 125–130°. Recrystallization from ethyl acetate–petroleum ether and ether yielded 280 mg of *N-carbobenzoxy-γ-L-glutamyl-(α-benzyl ester)-S-acetyl-L-cysteinylglycine ethyl ester* (**11**), which melted at 133–135°.

Anal. Calcd for $C_{29}H_{35}N_3O_9S$ (601.65): C, 57.90; H, 5.87; N, 7.00; S, 5.32. Found: C, 58.2; H, 5.58; N, 6.93; S, 5.10; $[\alpha]_D^{24}$ −28° (*c* 1, DMF).

N,S-Dicarbobenzoxyglutathione. The above cysteinyl tripeptide **11** (150 mg, 0.25 mmole) was dissolved in 2 ml of methanol and treated with 2 ml of sodium methoxide (1 M) to remove the acetyl group from the thiol (Zervas *et al.*, 1963). After 5 minutes at room temperature 2 ml of water was added, and the solution was kept for 30 minutes at room temperature in order to accomplish the hydrolysis of the terminal glycine ethyl ester. The *p*H of the solution was then adjusted to 8 and 0.3 ml of carbobenzoxy chloride was added followed by solid K_2CO_3. The mixture was shaken in the cold for 5–10 minutes and then extracted with ether and petroleum ether. Upon acidification with 5 N HCl and cooling, crystalline material separated which after recrystallization from methanol-water melted at 105–107°; yield, 90 mg (65%). Mixture melting point with authentic *N,S*-dicarbobenzoxyglutathione (Sokolovsky *et al.*, 1964) showed no depression; $[\alpha]_D^{23}$ −30° (*c* 1.1, methanol); reported (Sokolovsky *et al.*, 1964a,b) $[\alpha]_D^{25}$ −32° (*c* 1, methanol).

Reaction of N-p-Nitrobenzoyl-O-diphenylphosphoryl-ethanolamine with Thioacetate. To a solution of 440 mg (1 mmole) of *N-p*-nitrobenzoyl-*O*-diphenylphosphorylethanolamine (Zioudrou and Schmir, 1963) in 3.8 ml of DMF was added 1.2 ml of 1.13 M sodium thioacetate in dimethylformamide, and the reaction mixture was kept at 50°. The reaction was followed by iodine titration of acidified aliquots. When the theoretical amount of thioacetate has been consumed the solvent was removed under high vacuum at 40–50° and the residue was dissolved in 50 ml of ethyl acetate. The solution was washed twice with 1 N HCl, bicarbonate, and H_2O, dried over Na_2SO_4, and decolorized with charcoal. Addition of petroleum ether to the colorless filtrate induced crystallization. The product, *N-p*-nitrobenzoyl-*S*-acetylcysteamine (200 mg, 75%), melted at 100–102°. Recrystallization from ethyl acetate–

[4] The hydroxylamine solution was prepared by addition of 1 eq of sodium methoxide to a methanolic solution of hydroxylamine hydrochloride.

petroleum ether did not raise the melting point; E_{max} at 262 mμ 13,100. Iodine titration after removal of the acetyl group by treatment with sodium methoxide and acidification indicated a molecular weight of 270.

Anal. Calcd for $C_{11}H_{12}N_2O_4S$ (268.27): C, 49.22; H, 4.50; N, 10.44; S, 11.95. Found: C, 49.07; H, 4.47; N, 10.17; S, 11.36.

Preparation of Triphenylmethylthiol. Triphenylmethylthiol was prepared by treating a solution of triphenylmethyl thioacetate[5] in methanol with sodium methoxide for 1 hour. Upon acidification and addition of cold water the thiocarbinol crystallized. It was recrystallized from methanol and melted at 105–106°; reported mp 106° (Vorländer and Mittag, 1913).

Anal. Calcd for $C_{19}H_{16}S$ (276.37): C, 82.58; H, 5.84; S, 11.58. Found: C, 82.81; H, 5.83; S, 11.82.

Conversion of N-Carbobenzoxy-O-tosyl-L-serylglycyl Ethyl Ester to N-Carbobenzoxy-S-trityl-L-cysteinylglycine Ethyl Ester (**15**). To a solution of triphenylthiocarbinol (350 mg, 1.25 mmoles) in 2 ml of DMF was added 1.25 ml of 1 M sodium methoxide. The methanol was removed in the flash evaporator and the remaining solution was mixed with a solution of *N*-carbobenzoxy-*O*-tosyl-L-serylglycine ethyl ester (480 mg, 1 mmole) in 2 ml of dimethylformamide. The solution was kept for 1 hour at 40° and then was triturated with ice and water and a few drops of 2 N HCl. The precipitate was filtered, dried, and recrystallized from ethyl acetate–petroleum ether; yield 500 mg (86%), m.p. 108–110°; $[\alpha]_D^{23}$ +8.6° (*c* 1, methanol); reported (Zervas and Photaki, 1962) m.p. 112–113°, $[\alpha]_D^{28}$ +9.5° (*c* 3, methanol). The rate of the reaction was followed by iodine titration of the thiocarbinol in solution.

Anal. Calcd for $C_{34}H_{34}N_2O_5S$ (582.68): C, 70.08; H, 5.88; N, 4.80; S, 5.50. Found: C, 69.76; H, 5.55; N, 5.20; S, 5.73.

Conversion of Compound **15** *to the Cystine Peptide* **16**. For the conversion of compound **15** to the corresponding cystine peptide **16** the method described by Zervas and Photaki (1962) was followed, i.e., removal of the trityl group by $AgNO_3$ in pyridine, conversion of the silver mercaptide to the free thiol, and subsequent oxidation with iodine. Peptide **16** melted at 165–166°; $[\alpha]_D^{24}$ −145° (*c* 0.6, DMF); reported mp 167–168°, $[\alpha]_D^{28}$ −141.6° (*c* 0.6, DMF).

Anal. Calcd for $C_{30}H_{38}N_4O_{10}S_2$ (678.76): N, 8.26; S, 9.45. Found: N, 8.32; S, 9.10.

N-Carbobenzoxy-S-benzyl-L-cysteinylglycine Ethyl Ester (**14**). To 6.5 ml of a solution of benzyl mercaptan (0.5 M) in ethanol was added 2.5 ml of ethoxide solution (2.2 M), and the solvent was removed *in vacuo*. The residue (sodium benzyl mercaptide) was dissolved in 10 ml of DMF and the thiol content was estimated by iodine titration of an acidified sample. To this solution was added 1.44 g (3 mmoles) of peptide **1**, and the reaction was followed iodometrically. After 20 minutes at room temperature the solution was treated with ice water and 0.1 N HCl. Upon cooling, the *S*-benzylcysteine peptide crystallized; yield 1.05 g (82%). It was recrystallized from hot ether and petroleum ether and melted at 99–100°; $[\alpha]_D^{24}$ −39.2° (*c* 2.08, dioxane); $[\alpha]_D^{24}$ −27° (*c* 3.25, glacial acetic acid); reported (Maclaren, 1958) mp 99–100°; $[\alpha]_D^{22}$ −39.7° (*c* 4.32, dioxane; $[\alpha]_D^{20}$ −28.7° (*c* 5.83, glacial acetic acid).

Anal. Calcd for $C_{22}H_{26}N_2O_5S$ (430.44): C, 61.38; H, 6.09; N, 6.51; S, 7.45. Found: C, 60.84; H, 5.95; N, 6.37; S, 7.72.

Reaction of Peptide **1** *with Disodium Thioglycolate.* Six ml of a solution of 2-mercaptoacetic acid (1.28 M) in ethanol was treated with 5 ml of sodium ethoxide solution (1.9 M) and the solvent removed *in vacuo*. The residue was dissolved in 20 ml of ethanol and 10 ml of DMF, and to this was added 1.44 g (3 mmoles) of peptide **1**. The reaction was followed iodometrically. After 60 minutes at room temperature the solution was acidified with glacial acetic acid, and the solvents were removed *in vacuo* at 30°. The residue was triturated with water, acidified to Congo red with 1 N HCl, and extracted with ethyl acetate. The ethyl acetate extract was washed several times with small quantities of bicarbonate solution (1 M). The bicarbonate extract was acidified with concentrated HCl in the cold and the solution was re-extracted with ethyl acetate. The ethyl acetate extracts were washed with water and dried over Na_2SO_4, and solvent was removed *in vacuo*. The residual syrupy *N-carbobenzoxy-S-carboxymethyl-L-cysteinylglycine ethyl ester* (**17**) crystallized after trituration with ether. It was recrystallized from ethanol-ether and addition of a small quantity of petroleum ether and melted at 112–114°. The neutralization equivalent of **17** determined by nonaqueous titration of the free carboxyl group with sodium methoxide in the presence of thymol blue was found to be 382. Oxidation of **17** with bromine (probably to the sulfoxide) gave a molecular weight of 400; $[\alpha]_D^{23}$ −29.6° (*c* 2.12, DMF); $[\alpha]_D^{25}$ −8.3° (*c* 1.8, dioxane). The specific rotation in dioxane (1.8% solution) was measured at lower wavelengths: $[\alpha]_{313m\mu}^{25}$ −44°; $[\alpha]_{366m\mu}^{25}$ −27.1°; $[\alpha]_{436m\mu}^{25}$ −18°; and $[\alpha]_{546m\mu}^{25}$ −10°.

Anal. Calcd for $C_{17}H_{22}N_2O_7S$ (398.11): C, 51.25; H, 5.53; N, 7.03; S, 8.03. Found; C, 51.02; H, 5.47; N, 6.92; S, 8.30.

Reaction of Peptide **1** *with Cysteamine.* To a solution of cysteamine hydrochloride (1.08 g, 9.5 mmoles) in ethanol was added 4.8 ml of sodium ethoxide solution (2.5 M), and the solvent was removed *in vacuo*. The residue was suspended (NaCl was not removed) in 10 ml of DMF and 1.24 g of peptide **1** (2.6 mmoles) was added. After 30 minutes at room temperature, the mixture was treated with a large quantity of ice and water. The clear solution (*p*H 10.5) was extracted several times with ethyl acetate. The extracts were washed with water and dried over Na_2SO_4, and the ethyl acetate was removed *in vacuo*. The residue was dissolved in dioxane and saturated with dry hydrogen chloride, and the hydrochloride of the *N-carbobenzoxy-S-β-aminoethyl-*

[5] Triphenylmethyl thioacetate (mp 145°) was obtained from the reaction of sodium thioacetate with trityl chloride in anhydrous dioxane; reported mp 139–141° (Morse and Tarbell, 1952).

L-*cysteinylglycine ethyl ester* (**18**) was precipitated by addition of dry ether; yield 0.75 g (68%). The material is very hygroscopic. Neutral equivalent for $C_{17}H_{25}N_3O_5S \cdot HCl$ (419.5) by titration with standard methoxide solution (indicator thymol blue) was found to be 420; $[\alpha]_D^{23}$ −15.4° (*c* 2.05, DMF). The compound when oxidized with 0.1 N bromine solution consumed 1 mole of bromine/mole and gave a molecular weight of 415.

L-**4**-*Thialysylglycine Ethyl Ester Dihydrobromide* (**19**). Compound **18** (150 mg) was treated with 3 ml of glacial acetic acid–HBr solution for 20 minutes at 50°. The solvent was evaporated *in vacuo* and the residue was triturated with dry ether. The dihydrobromide **19** was isolated as a highly hygroscopic powder. It was purified by dissolving it in ethanol and reprecipitating with ether. Neutralization equivalent gave a molecular weight of 400; theory 411.1, calculated for $C_9H_{19}N_3O_3S \cdot 2\,HBr$.

Anal. Calcd: Br, 39; S, 7.6. Found: Br, 41.5; S, 7.9; $[\alpha]_D^{23}$ +13° (*c* 1.95, water).

Electrophoresis of the Acid Hydrolysate of Compound **18** *and Treatment with* L-*Lysine Decarboxylase.* ε-*N*-Carbobenzoxy-L-4-thialysine (16 mg, 50 μmoles) and compound **18** (42 mg, 100 μmoles) were separately hydrolyzed with 6 N HCl. Electrophoresis of the samples at *p*H 3.5 for 45 minutes (3 kv) revealed the presence of L-4-thialysine (19 cm from start) for the first hydrolysate and two spots for the second hydrolysate corresponding to glycine (5 cm) and L-4-thialysine (19 cm). Under these conditions lysine moves to 18 cm from the origin. Samples of the hydrolysates containing 1–2 μmoles of compound were diluted to 2 ml, the *p*H was adjusted to 6.5, and the solutions were incubated at 37° for 30 hours with 6 mg of L-lysine decarboxylase (Worthington, lot 6163). Electrophoresis of aliquots at *p*H 3.5 showed complete disappearance of the L-4-thialysine spots and revealed the presence of a faster moving material containing two positive charges (probably the analog of cadaverine). L-Lysine treated under the same conditions shows the same electrophoretic pattern. When the hydrolysates of compound **18** and of ε-*N*-carbobenzoxy-L-4-thialysine were analyzed on the short column of the amino acid analyzer the L-4-thialysine appeared at a position very close to lysine (Raftery and Cole, 1963). Equal quantities of L-4-thialysine and glycine were found to be present.

*Tryptic Hydrolysis of N-Carbobenzoxy-*L-**4**-*thialysylglycinamide.* A solution of *N*-carbobenzoxy-L-4-thialysylglycine ethyl ester hydrochloride (**18**) (210 mg, 0.5 mmole) in 15 ml of ethanol was saturated with ammonia and kept at room temperature for 2 days. Ammonium chloride was removed and the filtrate evaporated to dryness. By addition of ether a very hygroscopic material was obtained. The *N*-carbobenzoxy-L-4-thialysylglycinamide (160 mg, 0.45 mM) was dissolved in 10 ml of water. To 1 ml of this solution, 1 ml of Tris buffer (*p*H 7.8) and 1 mg of trypsin (Worthington 2× crystallized) were added. The solution (2 ml) was incubated at 37° for 10 hours.

Aliquots (10 and 20μl) were examined on paper electrophoresis (butanol-NH_3-H_2O) and the amount of glycinamide produced during the hydrolysis was determined by the quantitative ninhydrin method. The yield in glycin amide was 97% of the theory.

*Reaction of N-Carbobenzoxy-O-tosyl-*L-*serine Methyl Ester with Mercaptoacetic Acid.* A solution of thioglycolic acid (0.14 ml, 2 mmoles) in 3.5 ml of anhydrous methanol was neutralized by addition of 1.5 ml of methanolic sodium methoxide (2.6 M). To this was added a solution of compound **6** (820 mg, 2 mmoles) in 10 ml of methanol and the reaction was followed by iodometric titration of acidified samples. After 5 minutes (at room temperature) titration of a sample revealed that 87% of the thioglycolic acid had been consumed. Thirty minutes later the solvent was removed *in vacuo*, and the residue was triturated with water, acidified with HCl, and extracted with ethyl acetate. The extract was washed exhaustively with water and dried over Na_2SO_4, and the solvent was removed. The residue was then dissolved in a small volume of dioxane; cyclohexylamine (0.8 ml) was added, followed by addition of petroleum ether. The precipitate (0.8 g) was collected by filtration. The solid *dicyclohexylammonium salt of N-carbobenzoxy-S-carboxymethyl-*L-*cysteine* (**22**) was recrystallized from chloroform–petroleum ether and melted at 168–172°. Nonaqueous titration (Fritz, 1950) with a standard solution of sodium methoxide and standard perchloric acid (thymol blue indicator) revealed the presence of two carboxyl and two amino groups, giving an equivalent weight of 260 and a molecular weight of 520. The same product was obtained when the reaction was carried out in DMF with an equivalent amount of disodium thioglycolate.[6]

Anal. Calcd for $(C_{13}H_5NO_6S + 2C_6H_{11}NH_2)$ (511.65): C, 58.70; H, 8.08; N, 8.22; S, 6.27. Found: C, 58.23; H, 7.85; N, 8.10; S, 6.25; $[\alpha]_D^{22}$ −0.8° (*c* 2, H_2O).

*Preparation of the Dicyclohexylammonium Salt of N-Carbobenzoxy-S-carboxymethyl-*L-*cysteine* (**24**). A solution of *N*,*S*-dicarbobenzoxy-L-cysteine (Berger *et al.*, 1956) (1.3 g, 3.3 mmoles) in 3 ml of methanol was treated with 3 ml of sodium methoxide solution (2.6 M). Titration with iodine of a sample after 15 minutes revealed the presence of 90% free sulfhydryl groups. The solvent was removed *in vacuo*, the residue was treated with 10 ml of water, and the solution was allowed to react with 700 mg of iodoacetic acid. No free thiol could be found after 2 minutes. The solution, acidified with 6 N HCl, was extracted with ethyl acetate. The extract was washed with water and dried over Na_2SO_4, and the filtrate was evaporated to dryness. The residue was dissolved in dioxane and treated with 1 ml of cyclohexylamine, and the salt was precipitated after addition of petroleum ether. After recrystallization from chloroform–petroleum ether the salt melted at 170–175°; $[\alpha]_D^{25}$ −20° (*c* 2, water). Estimation of the neutralization equivalent by nonaqueous titration with 0.1 N $HClO_4$ and 0.1 N $NaOCH_3$ gave 270 and a molec-

[6] The unexpected solvolysis of the methyl ester of compound **22** may have occurred during the isolation steps.

ular weight of 540. Oxidation with bromine gave a molecular weight of 500.

Anal. Calcd for $C_{25}H_{41}N_3O_6S$ (511.65): N, 8.22; S, 6.27. Found: N, 8.0; S, 6.7.

Reaction of Compound **6** *with Benzyl Mercaptan.* To 5.4 ml (5 mmoles) of benzyl mercaptan solution (0.92 M) in ethanol was added 1.57 ml (4 mmoles) of sodium methoxide solution (2.5 M). The solvent was removed by evaporation and the residue was dissolved in 10 ml of DMF. Peptide **1** (2 mmoles, 0.82 g) was added, and the reaction was followed by iodine titration. After 30 minutes the solution was acidified, treated with ice-cold water, and extracted with ethyl acetate. The ethyl acetate extract was washed with $NaHCO_3$ solution, HCl, and water, dried over Na_2SO_4, and evaporated to dryness. The residual syrup *N-carbobenzoxy-S-benzyl-*DL*-cysteinyl methyl ester* (**20**) did not crystallize. The rotation of the syrup in dioxane was measured and the material was found to be *optically inactive*. *N*-Carbobenzoxy-*S*-benzyl-L-cysteinyl methyl ester melts at 65° and has a specific rotation of $[\alpha]_D^{23}$ −38.4° (*c* 1.97, dioxane).

*N-Carbobenzoxy-S-benzyl-*DL*-cysteine Hydrazide* (**21**). The oily compound **20** was treated with hydrazine hydrate in methanol for 12 hours at room temperature. The hydrazide **21** was recrystallized from methanol and melted at 115°; $[\alpha]_D^{25}$ 0° (*c* 1, ethanol). The specific rotation of L-**21** (mp 133°) is $[\alpha]_D^{23}$ −13.5° (*c* 1, ethanol); reported (Maclaren *et al.*, 1958) $[\alpha]_D^{19}$ −14.8° (*c* 0.96, ethanol).

*Racemization of the N-Carbobenzoxy-*L*-serine Methyl Ester* (**25**) *in the Presence of Sodium Benzyl Mercaptide.* The specific rotation of compound **25** in DMF is $[\alpha]_D^{23}$ −12.6° (*c* 3.8, DMF). A solution (3.25 ml, 3 mmoles) of benzyl mercaptan (0.92 M) in ethanol was treated with 1 ml of sodium methoxide solution (2.5 M). The solvent was removed and the residual sodium benzyl mercaptide was dissolved in 10 ml of DMF solution containing 380 mg (1.57 mmoles) of compound **25**. The solution was introduced immediately into a thermostated tube and the change in optical rotation was followed at 23°. After 30 minutes, the initial rotation of $\alpha = -0.480°$ had reached the value of $\alpha = -0.025°$ (95% racemization). The half-life of the racemization is 7.8 minutes.

*Treatment of N-Carbobenzoxy-*L*-serylglycine Ethyl Ester* (**26**) *with Sodium Benzyl Mercaptide in DMF.* The specific rotation of compound **26** in DMF is $[\alpha]_D^{23}$ +4° (*c* 4.8, DMF); 6 ml of a solution of benzyl mercaptan (1 M) was treated with 2 ml of sodium methoxide solution (2.5 M) and the solvent was removed *in vacuo*. The residue was dissolved in 10 ml of a solution of compound **26** in DMF (0.3 M) and the mixture was kept at 23° for 70 minutes. After addition of 2 ml of CH_3COOH the solvents were removed *in vacuo* and the residue was triturated with cold $NaHCO_3$ solution. The crystalline material was collected, washed several times with $NaHCO_3$ and water, dried, and recrystallized twice from ethyl acetate–petroleum ether. It melted at 99–100° and showed no depression of the melting point when mixed with starting material; $[\alpha]_D^{23}$ +3.8° (*c* 5, DMF). Compound **26** seems to have racemized to the extent of 5%.

Nonaqueous Titration of O-Tosylseryl Peptides. The *O*-tosylseryl peptide (0.1–0.2 mmole) is treated with a known volume of standardized 0.1 M sodium methoxide solution in methanol-benzene (1:3) in the presence of a drop of thymol blue (0.5% in ethanol). The mixture is stirred until all peptide dissolves. Sometimes light heating is recommended to facilitate the reaction. The solution is then back-titrated to the red end point of the indicator with a standard solution of 0.1 M perchloric acid in dioxane (Fritz, 1950). The molecular weight of the compound is calculated according to the formula: mw = mg × 10/ml; mg = weight of sample, ml = milliliters of 0.1 M sodium methoxide consumed. The accuracy of the method is 2–5%. The method is based on the formation of sodium tosylate during the β-elimination reaction which *O*-tosylserine peptides undergo when treated with sodium methoxide.

Bromine Titration of Thioacetic Acid. Solutions of thioacetic acid or sodium thioacetate can be titrated by a standardized bromine solution in 50% acetic acid. A known volume of thioacetate solution is treated with a known excess of standardized 0.01 N bromine solution until the bromine color remains. The excess of the bromine is estimated after addition of an excess of a 10% potassium iodide solution and titration of the iodine with thiosulfate. Four molecules of bromine is required to oxidize 1 molecule of thioacetic acid to sulfuric acid according to the equation

$$CH_3COSH + 4Br_2 + 5H_2O \longrightarrow 8HBr + CH_3COOH + H_2SO_4$$

The method is accurate to within 2–4%. The amount of sulfuric acid produced was estimated on the same sample by titration with 7.7×10^{-3} M barium perchlorate solution in 80% ethanol (Thorin method, Fritz and Yammamura, 1955) and gave 96–98% of the expected amount.

The titration of the sulfuric acid was performed as follows: A solution of thioacetic acid was oxidized using the exact amount of bromine. The traces of free bromine were destroyed by addition of a drop of hydrogen peroxide and the sulfuric acid was titrated with the barium perchlorate solution in the presence of thorin indicator. The acetic acid and hydrogen bromine produced according to the equation do not interfere in the titration. Only the free bromine affects the color of the indicator. Triphenylmethylthiocarbinol is similarly oxidized with 4 moles of bromine to sulfuric acid and triphenylcarbinol. The sulfuric acid produced has been estimated as described above and the triphenylcarbinol melting at 160–162° was isolated quantitatively.

Bromine Titration of S-Acetylcysteinyl Peptides. Bromine oxidizes *S*-acetylcysteinyl peptides to their cysteic acid analogs. The *S*-acetylcysteinyl peptide (20–40 μmoles) is dissolved in glacial acetic acid and treated with a known excess of 0.01 N bromine solution in 50% acetic acid–water until the bromine color remains. Determination of the excess bromine is per-

formed as described above. It has been found that 3 molecules of bromine are required to oxidize 1 molecule of *S*-acetylcysteinyl peptide to its cysteic acid analog according to the equation

$$\begin{array}{l} \mathrm{CH_2SCOCH_3} \\ | \\ \mathrm{ZNHCH\text{-}COOR} + 3\mathrm{Br_2} + 4\mathrm{H_2O} \longrightarrow \\ \qquad\qquad \mathrm{CH_2SO_3H} \\ \qquad\qquad | \\ \qquad\qquad \mathrm{ZNHCH\text{-}COOR} + 6\mathrm{HBr} + \mathrm{CH_3COOH} \end{array}$$

The peptides **2**, **4**, and **7** had been oxidized with bromine. The products were evaporated to dryness and the residue after hydrolysis with 6 N HCl was chromatographed. Peptide **2** gave equal quantities of glycine and cysteic acid, **4** equal quantities of phenylalanine and cysteic acid, and **7** cysteic acid.

Iodine Titration of Thioacetic Acid. Each molecule of thioacetic acid requires 1 atom of iodine for oxidation. Thioacetic acid is probably oxidized to its disulfide derivative by iodine. All reactions of *O*-tosylseryl peptides with thioacetate are followed by iodine titration as described in the particular experiments. Triphenylmethylthiocarbinol consumes 1 mole of iodine and is probably oxidized to triphenylmethyl sulfenyl iodide (Kolthoff and Harris, 1949). The molecular weight determined by this method was found to be 285; theory 276. Tritylcarbinol (mp 162°) was isolated in quantitative yield from the reaction. Presumably the triphenylmethyl sulfenyl iodide hydrolyzes to give the carbinol and sulfur iodide.

Bromine Titration of S-Carboxymethyl- and S-p-Aminoethylcysteine Peptides. The S-alkyl peptide (40–60 μmoles) was dissolved in 50% glacial acetic acid and oxidized with a known excess of 0.1 N bromine solution according to the equation

$$\mathrm{R\text{-}S\text{-}R'} + \mathrm{Br_2} + \mathrm{H_2O} \longrightarrow \mathrm{R\text{-}\overset{\overset{\mathrm{O}}{|}}{S}\text{-}R'} + 2\mathrm{HBr}$$

The excess of bromine is estimated iodometrically.

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Specific Growth of a Soil Microorganism on the Natural Isomer of α-Tocopherol*

C. T. Goodhue†

ABSTRACT: A microorganism with the ability to metabolize *d*-α-tocopheryl acetate has been isolated from soil. This organism grows selectively on *d*-α-tocopheryl acetate in the presence of various other optical isomers of *d*-α-tocopheryl acetate. A growth test based on this property was developed in which the following mean responses were observed: *d*-α-tocopheryl acetate, 100%; 2*dl*-α-tocopheryl acetate, 48%; racemic α-tocopheryl acetate, 28%; *l*-α-tocopheryl acetate, 3.0%.

A rapid and sensitive method for determination of the optical isomers of α-tocopherol is needed. Assays based on biological functions such as gestation-resorption, encephalomalacia, and nutritional muscular dystrophy in rats and chicks respond selectively to *d*-α-tocopherol, the naturally occurring isomer, but these assays are rather time consuming. In these assays, the unnatural isomer, *l*-α-tocopherol is only about 20% as active as *d*-α-tocopherol (Ames *et al.*, 1963; Dam and Sondergaard, 1964; Scott and Desai, 1964; Witting and Horwitt, 1964). Presently available evidence suggests that the epimeric configuration at the 2 position[1] is dominant in determining biological activity in higher organisms (Ames *et al.*, 1963).

Using enrichment culturing methods in a medium containing *d*-α-tocopheryl acetate, we have obtained soil bacteria that grow selectively on *d*-α-tocopheryl acetate in the presence of other optical isomers of α-tocopheryl acetate. A diagnostic test for the presence of *d*-α-tocopherol based on this property has been developed in which the calculated weight of bacteria at maximum growth is proportional to the amount of *d*-α-tocopheryl acetate initially in the culture medium. This relationship holds true even in the presence of optical isomers of α-tocopherol not found in nature.

Experimental Methods

Isolation of Bacteria. Conventional enrichment culturing methods were used (Hayaishi, 1955). Mud samples from a local pond were incubated without shaking at 30° in a medium containing, per liter, 0.35 g KH_2PO_4, 2.0 g $(NH_2)_2SO_4$, 1.0 g *d*-α-tocopheryl acetate, and 10.0 ml mineral salt solution. The *p*H value of the medium was adjusted to 7.0 with KOH. At first, the α-tocopheryl acetate was suspended in acacia. Later this was found to be unnecessary. The mineral salt solution contained, per 100 ml, 2.5 g $MgSO_4 \cdot 7\ H_2O$, 0.01 g $CaCl_2 \cdot 2\ H_2O$, 0.28 g $FeSO_4 \cdot 7\ H_2O$, 0.17 g $MnSO_4 \cdot H_2O$, and 0.006 g $ZnSO_4$. After 1 week, a portion of the culture was used to inoculate shake flasks containing the same medium. After three transfers at 1-week intervals, a pure culture was isolated on agar plates of the same medium.

The organism was an acid-fast, nonmotile rod (usually paired) averaging 0.3 × 1 μ after cultivation 4

* From the Research Laboratories of Distillation Products Industries, Division of Eastman Kodak Company, Rochester, N.Y. *Received April 30, 1965.* Communication No. 323.

† Present address: Research Laboratories, Eastman Kodak Company, Rochester, N.Y. 14650.

[1] We will use the following designations for configuration of the optically active centers of α-tocopherol:

Trivial	Configuration
d-α-tocopherol	2D,4′D,8′D-α-tocopherol
l-α-tocopherol	2L,4′D,8′D-α-tocopherol
2*dl*-α-tocopherol	2DL,4′D,8′D-α-tocopherol
racemic α-tocopherol	2DL,4′DL,8′DL-α-tocopherol

The configurations of the optically active centers of *d*-α-tocopherol were established by Mayer *et al.* (1963).