Experimental

Equipment and Reagents.—Mineral oil baths regulated to $\pm 0.05^\circ$ were used for the kinetics runs. All final determinations were carried out spectrophotometrically on a Cary Model 11 MS spectrophotometer. The adjustment and determinations of pH were made with a Beckman Zeromatic pH meter with an expanded scale.

Only reagent or analytical grade chemicals were used throughout. Aniline was freshly distilled and kept under nitrogen.

Procedure for Kinetic Runs on Succinic Acid–Aniline Reaction.—Reaction solutions containing varying amounts of aniline were prepared by mixing appropriate quantities of 2% aniline in 0.5 *M* succinate or citrate buffer with additional quantities of the buffer. Each solution was adjusted to the desired pH value, prior to mixing, with powdered sodium hydroxide and the mixtures were checked after mixing. They were then placed in thoroughly washed, dry neutral glass ampoules and sealed under nitrogen.

The ampoules were preheated to approximately the final temperature by vigorously shaking in a water bath for 2 min. and then quickly transferred to the oil bath. The zero hour sample was taken at this time and subsequent ampoules were normally removed at 0.5, 1, 2, 4, 8, 12, 24, 50, and 75 hr. These were immediately chilled in acetone–Dry Ice mixtures.

The residual aniline content of the ampoules was determined by spectrophotometric measurements at 287 mμ on a chloroform extract of the neutralized solutions (pH $\cong$ 7). Although a fairly adequate assay could be based on direct ultraviolet measurements on the reacted solutions, the method would be subject to varying errors if side reactions occurred.

The residual aniline concentration reported as A_∞ were largely based on runs made at low initial aniline concentration. Since these rapidly reached equilibrium states, determination of the amine concentration at effectively infinite time presented no problem.

Procedure for Determination of k_{-1} and k_2 on Succinic Anhydride.—These were determined in a conventional fashion in a thermostated Cary Model 11 spectrophotometer. The reactions were permitted to occur directly in the photometer cell. For the aniline reaction varying amounts of an alcoholic solution of the anhydride were first introduced into the cell followed by a buffered solution of aniline. The resulting rate constants were then extrapolated to zero alcohol concentration.

Procedure for Determination of the Dissociation Constants.—Dissociation constants were determined on systems comparable to that used for the rate studies except for aniline. The values for succinic acid were obtained, for example, from potentiometric titration of a 0.5 *M* solution, that for succinanilic acid by partition coefficient determination on the acid from succinate buffer solutions at several pH values. The pK_a' values for aniline were measured potentiometrically in 0.5 *M* sodium chloride.

Acknowledgment.—This study was supported in part by grants from the National Institutes of Health (RG-5830 (c2, c3) and A-03437 (c2, c3)) of Bethesda 14, Md., The Parke, Davis and Co. of Detroit, Mich., and the Research Committee of the Graduate School from funds furnished by the Wisconsin Alumni Research Foundation.

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

New Methods in Peptide Synthesis. I. Tritylsulfenyl and *o*-Nitrophenylsulfenyl Groups as N-Protecting Groups[1,2]

BY LEONIDAS ZERVAS, DIMITRIOS BOROVAS, AND EUTHYMIOS GAZIS

RECEIVED MAY 23, 1963

N-Tritylamino acid *p*-nitrophenyl esters, with the exception of the glycine derivative, do not couple with amino acid esters. On the other hand, N-tritylsulfenyl (TRS) or N-*o*-nitrophenylsulfenyl (NPS) amino acid *p*-nitrophenyl esters show no steric hindrance and have been used to synthesize N-protected dipeptide esters. The N-*o*-nitrophenylsulfenyl derivatives of L-amino acids themselves have also been prepared and isolated in the pure state as their dicyclohexylammonium (DCHA) salts. These salts have been used directly for peptide synthesis. The TRS or NPS group can be easily removed from N-protected peptides by means of two equivalents of hydrogen chloride in methanol or nonpolar solvents, with the generation of TRS or NPS chloride. The use of the N-sulfenyl protecting groups is advantageous for the lengthening of a peptide chain, especially when the peptide includes amino acids bearing protected functional groups in their side chain.

Introduction

The very progress of the synthesis of peptides depends upon finding suitable methods for (a) the protection of the α-amino group, the carboxyl group, and the side-chain functional groups; and (b) the coupling reaction.[3] This communication deals with the problem of the N-protection of the amino acids.

Thirty years after its introduction, the carbobenzoxy group[4] is still most commonly used for the N-protection of amino acids, especially since this method of N-protection[3,4] has been adapted to the peculiarities of some amino acids, such as lysine,[5] arginine,[6] and cysteine–cystine.[7–9] The great usefulness of this group may be attributed to the fact that the coupling of carbobenzoxyamino acids with other amino acids or peptides proceeds without racemization whatever method of coupling is employed; moreover, the removal of this N-protecting group can be easily brought about by treatment with hydrogen bromide–acetic acid,[10] by trifluoroacetic acid,[11] or under very mild conditions, *i.e.*, by catalytic hydrogenolysis.[4]

In spite of the wide application of the carbobenzoxy group, other groups have proved useful in some cases, for instance the toluenesulfonyl,[12] the phthaloyl,[13] the trifluoroacetyl,[14] the butyloxycarbonyl,[15] the di-

(1) A preliminary communication on this work was presented at the 5th European Peptide Symposium, Oxford, Eng., September, 1962; E. Gazis, B. Bezas, G. C. Stelakatos, and L. Zervas, "Proceedings of the 5th European Peptide Symposium," Pergamon Press, London, 1963, p. 17.

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

(3) A detailed description of the methods for peptide synthesis can be found in J. P. Greenstein and M. Winitz, "Chemistry of the Amino Acids," John Wiley and Sons, Inc., New York, N. Y., 1961.

(4) M. Bergmann and L. Zervas, German Patent 556,798 (1932); *Ber.*, **65**, 1192 (1932).

(5) M. Bergmann, L. Zervas, and W. F. Ross, *J. Biol. Chem.*, 111, 245 (1935); K. Hoffmann, E. Stutz, G. Spühler, H. Yajima, and E. T. Schwarz, *J. Am. Chem. Soc.*, **82**, 3727 (1960); B. Bezas and L. Zervas, *ibid.*, **83**, 719 (1961); R. Schwyzer and W. Rittel, *Helv. Chim. Acta*, **44**, 159 (1961).

(6) L. Zervas, M. Winitz, and J. P. Greenstein, *J. Org. Chem.*, **22**, 1515 (1957); L. Zervas, T. Otani, M. Winitz, and J. P. Greenstein, *J. Am. Chem. Soc.*, **81**, 2878 (1959); L. Zervas, M. Winitz, and J. P. Greenstein, *ibid.*, **83**, 3300 (1961); M. Bergmann, L. Zervas, and H. Rinke, *Z. physiol. Chem.*, **224**, 40 (1934); C. Gros, M. P. de Garilhe, A. Costopanagiotis, and R. Schwyzer, *Helv. Chim. Acta*, **44**, 2042 (1961).

(7) R. H. Sifferd and V. du Vigneaud, *J. Biol. Chem.*, **108**, 753 (1935).

(8) C. R. Harington and T. H. Mead, *Biochem. J.*, **29**, 1602 (1935).

(9) L. Zervas and I. Photaki, *Chimia*, **14**, 375 (1960); *J. Am. Chem. Soc.*, **84**, 3887 (1962); L. Zervas, I. Photaki, A. Cosmatos, and N. Ghelis, "Proceedings of the 5th European Peptide Symposium," Pergamon Press, London, 1963, p. 27; L. Zervas, I. Photaki, and N. Ghelis, *J. Am. Chem. Soc.*, **85**, 1337 (1963).

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

(11) F. Weygand and W. Steglich, *Z. Naturforsch.*, **14b**, 472 (1959).

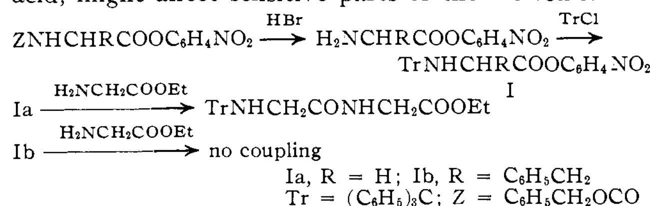
(12) R. Schönheimer, *Z. physiol. Chem.*, **154**, 203 (1926); V. du Vigneaud and O. K. Behrens, *J. Biol. Chem.*, **117**, 27 (1937); J. Honzl and J. Rudinger, *Collection Czechoslov. Chem. Commun.*, **20**, 1190 (1955).

(13) J. C. Sheehan and V. S. Frank, *J. Am. Chem. Soc.*, **71**, 1856 (1949); D. A. Kidd and F. E. King, *Nature*, **162**, 776 (1948).

(14) F. Weygand and E. Csendes, *Angew. Chem.*, **64**, 136 (1952); F. Weygand and E. Leising, *Chem. Ber.*, **87**, 248 (1954); F. Weygand and G. Adermann, *ibid.*, **93**, 2334 (1960).

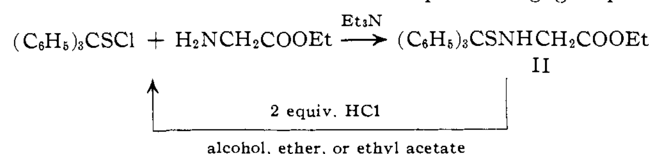
(15) F. C. McKay and N. F. Albertson, *J. Am. Chem. Soc.*, **79**, 4686 (1957); L. A. Carpino, *ibid.*, **79**, 4427 (1957); G. W. Anderson and A. C.

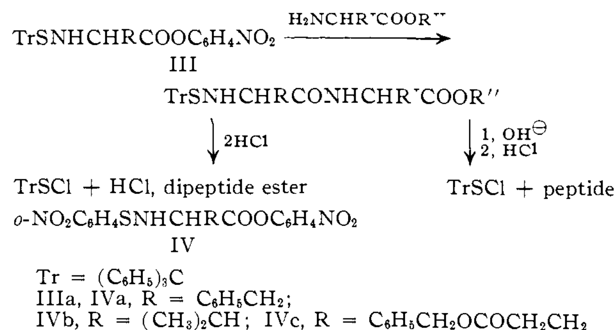
benzylphosphoryl,[16a] the formyl,[16b] and the trityl group[1,17–19]. However, the preparation of N-tritylamino acids does not proceed in good yield. In the hope that this difficulty could be overcome, the carbobenzoxy group of N-carbobenzoxyamino acid *p*-nitrophenyl esters,[20] which can be easily prepared, was exchanged for the trityl group. This was accomplished by removing the carbobenzoxy group with hydrogen bromide–acetic acid[21] and tritylating the free *p*-nitrophenyl esters thus obtained. The N-tritylamino acid *p*-nitrophenyl esters would be expected to behave as "active esters"[20] which could be used directly for coupling with other amino acids. Since the N-trityl group can be removed more easily than the N-carbobenzoxy group by acid hydrolysis or alcoholysis,[17–19] this type of ester could be useful in those cases where the removal of the N-carbobenzoxy group either by catalytic hydrogenation, or by hydrogen bromide or trifluoroacetic acid, might affect sensitive parts of the molecule.



The use of N-tritylamino acid *p*-nitrophenyl esters for coupling purposes with other amino acid esters has been successful in the case of the glycine derivative Ia but, unfortunately, it was not feasible for other amino acid *p*-nitrophenyl esters, for instance the L-phenylalanine derivative Ib. Apparently, this behavior may be attributed to steric factors. It is worthwhile to recall that N-tritylamino acids, again with the exception of tritylglycine, do not couple with other amino acids by the mixed carboxylic–carbonic anhydride method,[22] but only *via* the carbodiimide[23] method or the diphenylphosphoryl method.[16]

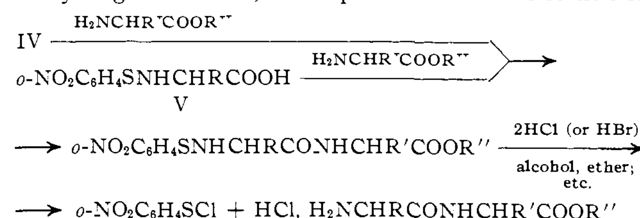
The aforementioned problem of steric hindrance has been overcome by introducing as N-protecting group the tritylsulfenyl (TRS) group instead of the trityl group; TRS chloride[24] reacts very readily with amino acid esters, *e.g.*, glycine ester, forming the corresponding N-TRS derivative II. The TRS protecting group is split off almost instantaneously under very mild conditions even with the theoretical amount of hydrogen chloride in alcohol or nonpolar solvents; TRS chloride is regenerated, and the hydrochloride of the corresponding amino acid ester is formed. This is not surprising, since it has long been known that the sulfenyl–nitrogen bond (RS–N) is cleaved very easily with hydrogen chloride under these conditions.[25] It is surprising, though, that the reaction follows this course exclusively, the yield of TRS chloride thus regenerated being almost quantitative; S-detritylation[9] was not observed even when a higher concentration of hydrogen chloride was used.





By the interaction of TRS chloride and amino acid *p*-nitrophenyl esters, for instance of L-phenylalanine, the corresponding N-TRS derivative IIIa was obtained in good yield. These derivatives can be coupled with amino acid esters forming the N-protected dipeptide esters which upon treatment with hydrogen chloride, as described above, yield the hydrochlorides of the corresponding dipeptide esters. On the other hand, saponification of the TRS dipeptide esters followed by treatment with hydrogen chloride leads to the formation of the free dipeptides in good yield.

Better yields of N-protected dipeptide esters were obtained using N-*o*-nitrophenylsulfenyl (NPS) amino acid *p*-nitrophenyl esters (IV) instead of the corresponding N-TRS esters. These active esters IV can also be prepared easily by the interaction of NPS chloride[25,26] and amino acid *p*-nitrophenyl esters. The NPS derivatives of amino acids and peptides are yellow substances which, as a rule, crystallize easily. The cleavage of the nitrophenylsulfenyl group prior to, or after, the saponification of the peptide esters proceeds very easily with acids, even with acetic acid, in aqueous alcohol (or acetone, dioxane, etc.) solutions, or, preferably, as described above with hydrogen chloride,[25,27] or hydrogen bromide, in nonpolar or alcoholic solutions.



Besides the "active ester" method, several other methods of coupling are in use in the field of peptide chemistry. Most of these, however, require the availability of free N-TPS- or N-NPS-amino acids. Saponification of the easily prepared N-TRS- and N-NPS-amino acid alkyl esters, again with the exception of glycine esters, proceeds very slowly; an excess of alkali

MacGregor, *ibid.*, **79**, 6180 (1957); L. A. Carpino, C. A. Giza, and B. A. Carpino, *ibid.*, **81**, 955 (1959); R. Schwyzer, H. Kappeler, B. Iselin, W. Rittel, and H. Zuber, *Helv. Chim. Acta*, **42**, 1702 (1959); R. Schwyzer, P. Sieber, and H. Kappeler, *ibid.*, **42**, 2622 (1959).

(16) (a) A. Cosmatos, I. Photaki, and L. Zervas, *Chem. Ber.*, **94**, 2644 (1961); (b) S. G. Waley and J. Watson, *Biochem. J.*, **57**, 529 (1954).

(17) A. Hillmann-Elies, G. Hillmann, and H. Jatzkewitz, *Z. Naturforsch.*, **8b**, 445 (1953).

(18) L. Velluz, G. Amiard, and R. Heymes, *Bull. soc. chim. France*, 1283 (1955); G. Amiard, R. Heymes, and L. Velluz, *ibid.*, 97, 698 (1956); 191 (1955); L. Velluz, G. Amiard, J. Bartos, B. Coffinet, and R. Heymes, *ibid.*, 1464 (1956).

(19) L. Zervas, Communications du XIV Congres International de Chimie Pure et Appliquée, Zürich, 1955, p. 224; D. Theodoropoulos, Thesis, University of Athens, 1953; G. C. Stelakatos, Thesis, University of Athens, 1954; G. C. Stelakatos, D. M. Theodoropoulos, and L. Zervas, *J. Am. Chem. Soc.*, **81**, 2884 (1959); L. Zervas and D. M. Theodoropoulos, *ibid.*, **78**, 1359 (1956).

(20) M. Bodanszky, *Nature*, **175**, 685 (1955); D. F. Elliott and D. W. Russell, *Biochem. J.*, **66**, 49P (1957); M. Bodanszky and V. du Vigneaud, *Nature*, **183**, 1324 (1959); *J. Am. Chem. Soc.*, **81**, 5688 (1959).

(21) M. Goodman and K. C. Stueben, *ibid.*, **81**, 3980 (1959).

(22) R. A. Boissonnas, *Helv. Chim. Acta*, **34**, 874 (1951); J. R. Vaughan and R. L. Osato, *J. Am. Chem. Soc.*, **73**, 5553 (1951).

(23) J. C. Sheehan and G. P. Hess, *ibid.*, **77**, 1067 (1955).

(24) D. Vorländer and E. Mittag, *Ber.*, **52**, 413 (1919).

(25) Th. Zincke, *Ber.*, **44**, 769 (1911); Th. Zincke and F. Farr, *Ann.*, **391**, 57 (1912); H. Lecher and F. Hohlschneider, *Ber.*, **57**, 755 (1924); J. H. Billman and E. O'Mahony, *J. Am. Chem. Soc.*, **61**, 2340 (1939).

(26) M. H. Hubacher in "Organic Syntheses," Coll. Vol. II, John Wiley and Sons, Inc., New York, N. Y., 1943, p. 455.

(27) H. Goerdeler and A. Holst, *Angew. Chem.*, **71**, 775 (1959).

is required and, at the same time, extensive oxidation of the TRS and especially of the *o*-NPS derivatives takes place. The tendency for oxidation is much more pronounced in the case of N-*p*-nitrophenylsulfenyl derivatives.[28] Consequently, the preparation of pure TRS- and NPS-amino acids in reasonable yields in this way is rather tedious. On the other hand, N-TRS and N-NPS dipeptide esters are quickly saponified with the theoretical amount of alkali to give the corresponding N-protected dipeptides in good yield.

Another route to the preparation of TRS- and NPS-amino acids is provided by the interaction of TRS or NPS chloride and amino acids in aqueous alkaline solution. However, the rate of reaction of TRS chloride with amino acids in aqueous alkaline solutions is so slow and the yields are so low that the preparation of N-TRS derivatives in this way is not feasible on a preparative scale. The reverse is the case with *o*-NPS chloride. The N-NPS derivatives V can easily be prepared from *o*-NPS chloride and the various amino acids in aqueous alkaline solution but they are usually contaminated to a small extent with di-*o*-nitrophenyl disulfide and other undesirable products. For this reason, we prefer to isolate and store the N-NPS-amino acids in the form of their dicyclohexylammonium (DCHA) salts which, as a rule, do not need any further purification and are more stable than the corresponding free acids. Accordingly, we have prepared pure N-NPS derivatives of glycine, L-alanine, L-phenylalanine, L-valine, L-isoleucine, L-leucine, L-threonine, L-proline, L-hydroxyproline, L-tyrosine, L-glutamine, L-tryptophan, L-asparagine, γ-benzyl L-glutamate,[29] S-benzyl-L-cysteine, S-trityl-L-cysteine, L-methionine, and N^{ϵ}-carbobenzoxy-L-lysine. The preparation of NPS derivatives of a few amino acids (glycine, DL-alanine, DL-phenylalanine, DL-serine) has been briefly described by Goerdeler and Holst.[27]

The coupling of N-NPS-amino acids with amino acid esters is accomplished very easily *via* the mixed carboxylic–carbonic anhydride,[22] and, in much better yields, *via* the dicyclohexylcarbodiimide[23] or the diphenylphosphoryl method.[16] When applying the first two of these methods of coupling, it is necessary to use *o*-NPS-amino acids which have been freshly prepared either by the interaction of NPS chloride and amino acids as described or by liberation from their corresponding DCHA salts. In the case of the carbodiimide method it is particularly convenient to generate the NPS-amino acids from their DCHA salts simply by adding the hydrochloride of the amino acid ester. When using the diphenylphosphoryl method, it is not at all necessary to use free NPS-amino acids since their DCHA salts can be directly transformed to the corresponding mixed carboxylic–phosphoric anhydrides.[16] No racemization was observed during the coupling step and, moreover, the yields were good. Several peptides have been synthesized in this way (*cf.* Experimental part). The optical purity of the peptides was established by comparing their optical rotations with those of the same peptides which had been prepared by the carbobenzoxy method.

In our experience, the "sulfenyl method" seems to possess the advantages of the carbobenzoxy method. Furthermore, the TRS and NPS protecting groups are cleaved more easily than the carbobenzoxy or even the trityl group. Although we consider it advisable to continue to prepare oligopeptides by the well-attested carbobenzoxy method, the use of these new N-protecting groups may, nevertheless, have certain advantages in lengthening a peptide chain. The removal of the commonly used N-protecting groups, *e.g.*, the carbobenzoxy group, from a long peptide chain is still—in most cases—a difficult task, whereas the TRS and the NPS groups can be removed rapidly under very mild conditions without any danger to sensitive parts of the peptide chain. Therefore, the N-carbobenzoxy or the N-trityl group of an oligopeptide may be exchanged for a TRS or a NPS protecting group prior to lengthening the chain through its carboxyl group. On the other hand, a peptide chain can be lengthened at its amino end by coupling with N-NPS-protected amino acids or peptides. The usefulness of the new N-protecting groups becomes most apparent in the case of the incorporation into a peptide chain of amino acids bearing functional groups in their side chain. These functional groups can be protected, for instance, with benzyl groups, as in the case of glutamic acid, aspartic acid, serine, etc., or with carbobenzoxy or trityl groups as in the case of lysine[5] and arginine.[1,6] An example of such a synthesis by the sulfenyl method is the preparation of L-prolyl-N^{ϵ}-carbobenzoxy-L-lysyl-L-alanine. The free peptide could be obtained by splitting off the remaining protecting group in the usual way.

Experimental

For the coupling reactions anhydrous reactants and dry solvents were used; the ether used was free of peroxides. Evaporations were carried out *in vacuo* at 35–40°. The melting points are not corrected.

Prior to analysis[30] the free peptides were dried at 78° under high vacuum over phosphorus pentoxide; other compounds were dried at room temperature.

γ-Benzyl α-*p*-Nitrophenyl L-Glutamate Hydrobromide.—A solution of 2.4 g. of γ-benzyl-N-carbobenzoxy-L-glutamate *p*-nitrophenyl ester[31] in 5.2 ml. of warm acetic acid was allowed to cool to room temperature. Then 2.3 ml. of 6.6 *N* HBr in acetic acid was added and the solution was stirred for 3 min. at room temperature. The hydrobromide precipitated out upon addition of 45 ml. of dry ether. After allowing the mixture to stand for 1 hr. in the refrigerator it was filtered and the precipitate was washed repeatedly with ether. After recrystallization from chloroform-ether the yield was 1.4 g. (63%), m.p. 120° (reported[31] 120–120.5°).

Alkyl and *p*-Nitrophenyl Esters of N-Trityl-, N-Tritylsulfenyl-, and N-*o*-Nitrophenylsulfenylamino Acids.—These compounds were prepared from the hydrohalides of the alkyl or *p*-nitrophenyl esters of amino acids or of amino acid esters bearing a protected functional group, for instance of γ-benzyl α-nitrophenyl L-glutamate.[31] The N-trityl, N-tritylsulfenyl, and N-*o*-nitrophenylsulfenyl esters thus prepared are listed in Table I. The following is a general procedure.

To a solution of 0.01 mole of an amino acid ester hydrochloride or hydrobromide in 25 ml. of chloroform and 2.8 ml. of triethylamine, 0.01 mole of trityl chloride, tritylsulfenyl chloride,[24] or *o*-nitrophenylsulfenyl chloride[26] was added. After being left to stand at room temperature for 6 hr. the solution was successively washed with water, dilute acetic acid, aqueous potassium hydrogen carbonate solution, and again with water, dried with sodium sulfate, and evaporated to dryness. The crude products were usually recrystallized from ethanol, methanol, or ethyl acetate-petroleum ether.

N-*o*-Nitrophenylsulfenylamino acids were prepared by the interaction of *o*-nitrophenylsulfenyl chloride[26] and α-amino acids (or α-amino acids bearing a protected side-chain functional group, for instance S-benzyl-L-cysteine,[7] S-trityl-L-cysteine,[9] γ-benzyl L-glutamate,[32] and N^{ϵ}-carbobenzoxy-L-lysine[5,33]) in aqueous alkaline solution. The *o*-nitrophenylsulfenylamino acids thus prepared were isolated either as free acids (procedure A) or in the form of their dicyclohexylammonium (DCHA) salts (procedure B and C), and are listed in Table II.

A. The amino acid (0.02 mole) was dissolved in a mixture of 10 ml. of 2 *N* NaOH and 25 ml. of dioxane. During a period of

(28) H. Baganz and H. Peissker, *Arch. Pharm.*, **289**, 262 (1956).

(29) The *o*-nitrophenylsulfenyl derivative of this amino acid as well as compound IVc have been prepared by Mr. Ch. Chamalidis in this Laboratory.

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

(31) M. Goodman and K. O. Stueben, *J. Am. Chem. Soc.*, **81**, 3980 (1959).

(32) St. Guttmann and R. A. Boissonnas, *Helv. Chim. Acta*, **41**, 1864 (1958).

(33) F. Sanger and A. Neuberger, *Biochem. J.*, **37**, 515 (1943).

TABLE I

ESTERS OF N-TRITYL-(TR), N-TRITYLSULFENYL-(TRS), AND N-*o*-NITROPHENYLSULFENYL-(NPS) AMINO ACIDS

No.	Esters	Yield, %	M.p., °C.	[α]D, deg.	Formula	Nitrogen, % Calcd.	Nitrogen, % Found	Sulfur, % Calcd.	Sulfur, % Found
Ia	N-Tr-glycine *p*-nitrophenyl ester[a]	80	153–154		$C_{27}H_{22}N_2O_4$	6.38	6.32		
Ib	N-Tr-L-phenylalanine *p*-nitrophenyl ester[b]	56	72–82	+7.36[c]	$C_{34}H_{28}N_2O_4$	5.29	5.24		
II	N-TRS-glycine ethyl ester	79	120–123		$C_{23}H_{23}NO_2S$	3.71	3.72	8.49	8.39
	N-TRS-glycine methyl ester	75	95–97		$C_{22}H_{21}NO_2S$	3.85	3.77	8.82	8.82
	N-NPS-glycine ethyl ester	72	81–83		$C_{10}H_{12}N_2O_4S$	10.93	10.92	12.51	12.78
IIIa	N-TRS-L-phenylalanine *p*-nitrophenyl ester	71	154–156	−67.2[c]	$C_{34}H_{28}N_2O_4S$	4.99	5.11	5.71	5.88
	N-NPS-L-phenylalanine methyl ester	68	93–95	−32.6[c]	$C_{16}H_{16}N_2O_4S$	8.42	8.42	9.64	9.33
IVa	N-NPS-L-phenylalanine *p*-nitrophenyl ester	61	116–118	−115.5[c]	$C_{21}H_{17}N_3O_6S$	9.56	9.53	7.29	7.69
	N-NPS-L-valine methyl ester	89	98	− 85.5[c]	$C_{12}H_{16}N_2O_4S$	9.86	9.95	11.27	11.12
IVb	N-NPS-L-valine *p*-nitrophenyl ester	72	75–77	−189.2[c]	$C_{17}H_{17}N_3O_6S$	10.73	10.70	8.19	7.98
IVc	N-NPS-γ-benzyl-α-nitrophenyl-L-glutamate	80	77–78	−121.0[d]	$C_{24}H_{21}N_3O_8S$	8.21	8.19	6.27	6.16

[a] Calcd. for $C_{27}H_{22}N_2O_4$: C, 73.95; H, 5.05. Found: C, 73.92; H, 5.32. [b] Calcd. for $C_{34}H_{28}N_2O_4$: C, 77.25; H, 5.33. Found: C, 77.10; H, 5.62. [c] *c* 3, in dimethylformamide. [d] *c* 3, in chloroform.

TABLE II

N-*o*-NITROPHENYLSULFENYL (NPS) DERIVATIVES OF AMINO ACIDS

N-*o*-Nitrophenylsulfenyl derivatives of	Yield, %	M.p., °C.	[α]D, deg.	Formula	Nitrogen, % Calcd.	Nitrogen, % Found	Sulfur, % Calcd.	Sulfur, % Found
L-Alanine	83[a]	128–130	−101.8[d]	$C_9H_{10}N_2O_4S$	11.56	11.54	13.23	13.50
L-Alanine dicyclohexylammonium (DCHA) salt	83[b]	176–178[m]	−56.5[e]	$C_{21}H_{33}N_3O_4S$	9.92	9.79	7.57	7.66
Glycine DCHA salt	60[b]	190–191		$C_{20}H_{31}N_3O_4S$	10.25	10.26	7.83	7.65
L-Valine	65[a]	105	−127.8[d]	$C_{11}H_{14}N_2O_4S$	10.36	10.07	11.84	11.65
L-Valine DCHA salt	64[b]	191–193[n]		$C_{23}H_{37}N_3O_4S$	9.30	9.00	7.08	6.94
L-Phenylalanine	90[a]	134–135	−47.6[f]	$C_{15}H_{14}N_2O_4S$	8.80	8.78	10.07	10.26
L-Leucine	70[a]	102–106	−99.7[d]	$C_{12}H_{16}N_2O_4S$	9.85	9.66	11.26	11.36
L-Leucine DCHA salt	90[b]	182–183[n]	−76.1[g]	$C_{24}H_{39}N_3O_4S$	9.02	8.87	6.87	6.73
L-Asparagine	78[a]	165–166[l]	−119.3[f]	$C_{10}H_{11}N_3O_5S$	14.73	14.62	11.24	11.20
L-Asparagine DCHA salt[p]		185–186	−86.7[d]	$C_{22}H_{34}N_4O_5S$	12.00	11.93	6.87	6.82
L-Glutamine	87[a]	165	−74.3[d]	$C_{11}H_{13}N_3O_5S$	14.04	13.95	10.71	10.57
L-Threonine	60[a]	138–141	−111.6[d]	$C_{10}H_{12}N_2O_5S$	10.29	10.11	11.76	11.76
L-Threonine DCHA salt	60[b]	181–182[m]	−103.0[g]	$C_{22}H_{35}N_3O_5S$	9.26	9.27	7.05	7.05
L-Proline DCHA salt	45[b]	151–154[r]	−43.2[g]	$C_{23}H_{35}N_3O_4S$	9.32	9.10	7.12	7.50
L-Hydroxyproline DCHA salt	50[b]	169–171[q]	−39.0[h]	$C_{23}H_{35}N_3O_5S$	9.02	8.89	6.86	6.68
L-Tryptophan DCHA salt	50[b]	168–169[q]	−20.4[d]	$C_{29}H_{38}N_4O_4S$	10.40	10.24	5.95	5.75
L-Tyrosine DCHA salt	62[b]	173–175[m]	+41.8[e]	$C_{27}H_{37}N_3O_5S$	8.15	8.18	6.21	6.38
L-Isoleucine	70[a]	90–93	−102.2[d]	$C_{12}H_{16}N_2O_4S$	9.85	9.96	11.27	11.16
L-Isoleucine DCHA salt	85[b]	188–189[l]	−53.8[e]	$C_{24}H_{39}N_3O_4S$	9.02	8.92	6.88	6.80
L-Methionine DCHA salt	54[b]	196–197[l]	−34.4[i]	$C_{23}H_{37}N_3O_4S_2$	8.68	8.55	13.25	13.25
L-Glutamic acid γ-benzyl ester DCHA salt	57[c]	168[s]	−34.0[k]	$C_{30}H_{41}N_3O_6S$	7.36	7.52	5.60	5.74
S-Trityl-L-cysteine	67[a]	146	−42.0[d]	$C_{28}H_{24}N_2O_4S$	5.42	5.45	12.41	12.57
S-Benzyl-L-cysteine DCHA salt	73[b]	168–169	−43.5[g]	$C_{25}H_{39}N_3O_4S_2$	7.70	7.87	11.75	11.62
N^ε^-Carbobenzoxy-L-lysine DCHA salt	70[b]	184–187	−29.1[g]	$C_{32}H_{46}N_4O_6S$	9.11	9.27	5.20	5.43

[a] Yield by method A. [b] Yield by method B. [c] Yield by method C. [d] *c* 2, in dimethylformamide. [e] *c* 2, in methanol. [f] *c* 4, in dimethylformamide. [g] *c* 0.7, in dimethylformamide. [h] *c* 1, in ethanol. [i] *c* 0.7, in methanol. [k] *c* 3.5, in chloroform. [l] After recrystallization from methanol. [m] After recrystallization from ethanol. [n] After recrystallization from tetrahydrofuran. [p] Prepared by addition of DCHA to the alcoholic solution of the corresponding free acid and recrystallized from methanol–ether. [q] After recrystallization from tetrahydrofuran–ether. [r] After recrystallization from ethyl acetate. [s] After recrystallization from acetone or acetone–ethyl acetate.

15 min. *o*-nitrophenylsulfenyl chloride[26] (0.022 mole) was added in 10 equal portions as 2 *N* NaOH (12 ml.) was added dropwise, with vigorous shaking. The solution was diluted with 200 ml. of water, filtered, and acidified with 1 *N* sulfuric acid. The sirupy precipitate usually crystallized on scratching and cooling. The product was filtered off, washed with water, dried, dissolved in ethyl acetate or ether, and precipitated again by addition of petroleum ether.

B. The amino acid was treated with *o*-nitrophenylsulfenyl chloride as described under procedure A. After the acidification with sulfuric acid the *o*-nitrophenylsulfenyl derivatives were extracted with ethyl acetate or ether, or with a mixture of both of these solvents (1:1). The extract was repeatedly washed with water until the aqueous layer became neutral to congo red paper and then dried with sodium sulfate; upon addition of 4 ml. of dicyclohexylamine the corresponding salt separated out in most cases in the form of needles.

C. The amino acid (0.02 mole) was dissolved (or suspended) in a mixture of 10 ml. of water and 25 ml. of dioxane. During a period of 15 min. *o*-nitrophenylsulfenyl chloride (0.022 mole) was added in 10 equal portions as 2 *N* sodium hydroxide (12 ml.) was added dropwise, with vigorous shaking. The solution was then acidified with dilute sulfuric acid and was worked up as described under procedure B.

The physical constants of the free *o*-nitrophenylsulfenylamino acids reported in Table II refer to freshly prepared compounds. Upon keeping these compounds even in a vacuum desiccator, a slight change of their physical constants (*e.g.*, a depression of the melting point by 3–5°) was observed. This is most probably due to the formation of small amounts of di-*o*-nitrophenyl disulfide. In this case purification can be easily achieved by dissolving the impure acids in ethyl acetate or ether, filtering off the sparingly soluble di-*o*-nitrophenyl disulfide (m.p. 192–195°), and precipitating again by the addition of petroleum ether.

The dicyclohexylammonium salts of *o*-nitrophenylsulfenylamino acids are more stable and do not exhibit any change of their physical constants upon storage in a vacuum desiccator.

N-Tritylsulfenylglycine.—To a suspension of 0.3 g. (0.0008 mole) of the corresponding ethyl ester (Table I) in boiling ethanol, 1 ml. of 2 *N* sodium hydroxide was added dropwise. Upon dilution with water and acidification with acetic acid the product precipitated in the form of needles. The yield was 0.2 g. (73%), m.p. 148–150° and 149–151° after recrystallization from ethanol.

Anal. Calcd. for $C_{21}H_{19}O_2NS$: N, 4.00; S, 9.17. Found: N, 4.17; S, 9.22.

Saponification of N-*o*-Nitrophenylsulfenyl-L-phenylalanine Methyl Ester.—A solution of 1.7 g. (0.005 mole) of this ester (Table I) in 10 ml. of dioxane and 6.5 ml. of 1 *N* sodium hydroxide was stirred for 30 min. It was then diluted with water, extracted twice with ether and acidified with diluted sulfuric acid. The acidified solution was extracted with ether. The ethereal

extract was repeatedly washed with water, dried, and evaporated *in vacuo*. Upon addition of petroleum ether N-*o*-nitrophenylsulfenyl-L-phenylalanine crystallized. After repeated recrystallization from ethyl acetate–petroleum ether the yield was 0.36 g. (24%), m.p. 133–135°, $[\alpha]_D$ −47.6° (*c* 4, dimethylformamide).

Anal. Calcd. for $C_{15}H_{14}O_4N_2S$: N, 8.80; S, 10.07. Found: N, 8.88; S, 10.16.

Removal of the N-Tritylsulfenyl Group.—To a solution of 0.94 g. (0.0025 mole) of N-tritylsulfenylglycine ethyl ester in 30 ml. of dry ethyl acetate, 3 ml. (0.0075 mole) of 2.5 *N* hydrogen chloride in ether was added. Upon scratching, glycine ethyl ester hydrochloride precipitated out at once. After allowing the mixture to stand in the refrigerator for 1 hr. it was filtered and the precipitate was washed with ethyl acetate and then with ether. The yield was quantitative (0.35 g.), m.p. 143–145°; a mixture melting point with glycine ethyl ester hydrochloride showed no depression.

The mother liquors and the washings were concentrated *in vacuo* and the residue was dissolved in a small amount of chloroform. Upon addition of ethanol, tritylsulfenyl chloride precipitated. The yield was 0.7 g. (90%), m.p. 137–138°.

Removal of the N-*o*-Nitrophenylsulfenyl Group.—(a) N-*o*-Nitrophenylsulfenylglycine ethyl ester was treated with ether containing hydrogen chloride in the same manner as described above for the N-tritylsulfenyl derivative. The yield of glycine ethyl ester hydrochloride was 95%, m.p. 143°.

(b) The N-*o*-nitrophenylsulfenylglycine ethyl ester was dissolved in methanol containing hydrogen chloride.[34] Upon addition of ether[35] the hydrochloride precipitated out almost instantaneously. The yield was 95%.

Upon concentration of the mother liquors and addition of alcohol, *o*-nitrophenylsulfenyl chloride separated out. The yield was 90%, m.p. 75°.

N-*o*-Nitrophenylsulfenyl-L-valyl-L-phenylalanine Methyl Ester (VI).—(a) To a solution of 2.15 g. (0.01 mole) of L-phenylalanine methyl ester hydrochloride in 30 ml. of chloroform and 1.4 ml. of triethylamine, 2.7 g. (0.01 mole) of *o*-nitrophenylsulfenyl-L-valine and 2.2 g. of N,N′-dicyclohexylcarbodiimide were added. After being left to stand at room temperature overnight a few drops of 50% acetic acid were added and the insoluble precipitate of dicyclohexylurea (2 g.) was removed by filtration. The filtrate was washed successively with water, dilute sulfuric acid, aqueous potassium hydrogen carbonate solution, and again with water, dried over sodium sulfate, and evaporated to dryness. Ethyl acetate was added to the residue. Some undissolved dicyclohexylurea was filtered off and the filtrate was evaporated again to dryness. The residue was triturated with a small amount of methanol and allowed to stand in the refrigerator whereupon crystalline nitrophenylsulfenyl dipeptide ester VI separated out; it was recrystallized from methanol or ethyl acetate–petroleum ether. The yield was 3 g. (70%), m.p. 123–124°, $[\alpha]^{15}_D$ −6.8° (*c* 5, dimethylformamide).

Anal. Calcd. for $C_{21}H_{25}N_3O_5S$: N, 9.73; S, 7.42. Found: N, 9.90; S, 7.24.

(b) A suspension of 2.15 g. (0.01 mole) of L-phenylalanine methyl ester hydrochloride and 4.5 g. (0.01 mole) of *o*-nitrophenylsulfenyl-L-valine dicyclohexylammonium salt in 60 ml. of chloroform was shaken for several hours at room temperature until an almost clear solution resulted. N,N′-Dicyclohexylcarbodiimide (2.2 g.) was then added and the mixture was kept for several hours at room temperature. The precipitate consisting of dicyclohexylurea contaminated with dicyclohexylammonium hydrochloride was filtered off and the filtrate was worked up as described above (case a). The yield was 3.2 g. (75%), m.p. 123–124°.

(c) To a suspension of 2.25 g. (0.005 mole) of *o*-nitrophenylsulfenyl-L-valine dicyclohexylammonium salt in 30 ml. of tetrahydrofuran, precooled to 0°, 1.32 g. of diphenylphosphoryl chloride[16] was added. The mixture was shaken for 15–20 min. at 0° and was then added to a solution of 1.05 g. (0.005 mole) of L-phenylalanine methyl ester hydrochloride in 15 ml. of chloroform and 1.4 ml. of triethylamine. After being allowed to stand for 15 min. at room temperature the mixture was evaporated to dryness and the residue was triturated with ethyl acetate. Undissolved material consisting mostly of dicyclohexylammonium and triethylammonium chlorides was filtered off and the filtrate was washed as usual and evaporated again to dryness. After recrystallization from methanol the yield of VI was 1.3 g. (60%), m.p. 121–122°.

If isobutyl chloroformate was used instead of diphenylphosphoryl chloride the yield of pure dipeptide derivative VI was reduced to 25%.

N-Carbobenzoxy-L-valyl-L-phenylalanine methyl ester was prepared from carbobenzoxy-L-valine and L-phenylalanine methyl ester by the carbodiimide method in the same manner as that described for the corresponding *o*-nitrophenylsulfenyl derivative. The yield was 78%, m.p. 139–140° after recrystallization from methanol; $[\alpha]^{17}_D$ −5° (*c* 6, dimethylformamide).

Anal. Calcd. for $C_{23}H_{28}N_2O_5$: C, 66.98; H, 6.84; N, 6.79. Found: C, 67.13; H, 6.93; N, 6.82.

L-Valyl-L-phenylalanine Methyl Ester Hydrochloride.—(a) The N-carbobenzoxy derivative of the dipeptide ester (2.06 g., 0.005 mole) was catalytically (Pd) hydrogenolyzed in methanol solution containing 1 equiv. of hydrogen chloride. The mixture was filtered and the filtrate was evaporated to dryness. The crystalline residue was recrystallized from methanol–ether, the yield being 1.2 g. (80%), m.p. 193–195°, $[\alpha]^{15}_D$ +21.4° (*c* 2.8, water); reported[36] m.p. 196–196.5°, $[\alpha]_D$ +26.6° (33.6 mg. in 1.2 ml. of water).

(b) Upon adding ether to a solution of 2.15 g. (0.005 mole) of the N-*o*-nitrophenylsulfenyl dipeptide ester in 15 ml. of 1 *N* hydrogen chloride in methanol the above hydrochloride separated out. It was filtered off, washed with ether, and finally recrystallized from methanol–ether. The yield was 1.45 g. (92%), m.p. 193–195°, $[\alpha]^{15}_D$ +21.4° (*c* 2.8, water).

Anal. Calcd. for $C_{15}H_{23}N_2O_3Cl$: N, 8.89; Cl, 11.27. Found: N, 8.97; Cl, 11.41.

N-*o*-Nitrophenylsulfenyl-L-alanylglycine methyl ester was prepared from N-*o*-nitrophenylsulfenyl-L-alanine dicyclohexylammonium salt and glycine methyl ester hydrochloride in the same manner as described for the preparation of VI (procedure b). The crude product was recrystallized from methanol. The yield was 70%, m.p. 123–125°, $[\alpha]^{17}_D$ −42.4° (*c* 5, dimethylformamide).

Anal. Calcd. for $C_{12}H_{15}O_5N_3S$: N, 13.41; S, 10.23. Found: N, 13.25; S, 10.12.

N-Carbobenzoxy-L-alanylglycine methyl ester was prepared[37] from carbobenzoxy-L-alanine and glycine methyl ester by the carbodiimide method in the usual manner. The crude product was recrystallized from ethyl acetate–petroleum ether. The yield was 77%, m.p. 98–99°, $[\alpha]^{15}_D$ −25.1° (*c* 5, methanol); reported[38] m.p. 94–96°, $[\alpha]^{20}_D$ −25° (in methanol).

Anal. Calcd. for $C_{14}H_{18}O_5N_2$: C, 57.13; H, 6.16; N, 9.51. Found: C, 56.88; H, 5.95; N, 9.42.

L-Alanylglycine Methyl Ester Hydrobromide.[37]—(a) The *o*-nitrophenylsulfenyl dipeptide ester was treated with hydrogen bromide in methanol as described above. Upon adding ether crystalline dipeptide ester hydrobromide separated out. It was recrystallized from methanol–ether. The yield was 83%, m.p. 162°, $[\alpha]^{22}_D$ +7.32° (*c* 5, methanol).

Anal. Calcd. for $C_6H_{13}O_3N_2Br$: N, 11.62; Br, 33.14. Found: N, 11.45; Br, 33.34.

(b) The corresponding carbobenzoxy derivative was catalytically (Pd) hydrogenated in methanolic solution in the presence of 1 equiv. of hydrogen bromide. After concentration and addition of ether, the above hydrobromide crystallized out. The yield was 95%, m.p. 163°.

N-*o*-Nitrophenylsulfenyl-L-isoleucylglycine ethyl ester was prepared (a) from N-*o*-nitrophenylsulfenyl-L-isoleucine dicyclohexylammonium salt and glycine ethyl ester hydrochloride in the same manner as described for the preparation of VI (procedure b). The crude product was recrystallized from ethyl acetate–petroleum ether. The yield was 56%, m.p. 114°, $[\alpha]^{15}_D$ −46.0° (*c* 5, dimethylformamide).

Anal. Calcd. for $C_{16}H_{23}O_5N_3S$: N, 11.37; S, 8.67. Found: N, 11.25; S, 8.41.

(b) A suspension of 2.45 g. (0.0052 mole) of N-*o*-nitrophenylsulfenyl-L-isoleucine dicyclohexylammonium salt in ether was shaken in a separatory funnel with 30 ml. of 0.2 *N* sulfuric acid until it dissolved. The ethereal layer was separated and washed repeatedly with water until the aqueous layer was neutral to congo red paper. It was then dried over sodium sulfate and evaporated to dryness. The residue, consisting of *o*-nitrophenylsulfenyl-L-isoleucine, was coupled with glycine ethyl ester by the carbodiimide method as described for the preparation of VI (procedure a). After recrystallization of the crude product from ethyl acetate–petroleum ether the yield was 1.35 g. (70%), m.p. 114°.

N-*o*-Nitrophenylsulfenyl-L-isoleucylglycine.—A suspension of 1 g. (0.0025 mole) of the corresponding ethyl ester in 10 ml. of

(34) Methanol containing hydrogen bromide which is free from bromine can also be used. In this case the hydrobromide is isolated.

(35) When the removal of the N-protecting group is carried out with hydrogen chloride or hydrogen bromide in methanol the ether must be added very soon, *e.g.*, 1–2 min., after the addition of the reagent; otherwise the generated *o*-nitrophenylsulfenyl halogenide is transformed into other insoluble products.

(36) J. C. Sheehan and D. D. H. Yang, *J. Am. Chem. Soc.*, **80**, 1154 (1958).

(37) This compound has been prepared by Dr. N. Ghelis in our Laboratory.

(38) K. T. Poroshin, V. G. Debabov, V. A. Shibnev, and T. D. Kozarenko, *Zh. Obshch. Khim.*, **31**, 3006 (1961); *Chem. Abstr.*, **56**, 15604*c* (1962).

ethanol and 1.6 ml. of 2 *N* sodium hydroxide was shaken until complete solution was obtained (about 10 min.). After being diluted with water the solution was extracted with ethyl acetate and the ethyl acetate layer discarded. The aqueous layer was acidified with dilute sulfuric acid and extracted with ethyl acetate, which was washed repeatedly with water until it became neutral to congo red paper, and then dried over sodium sulfate. Upon addition of dicyclohexylamine the corresponding dicyclohexylammonium salt precipitated. The yield was 1.1 g. (85%), m.p. 190–192° unchanged after recrystallization from ethanol; $[\alpha]^{20}D$ −57.2° (*c* 3, methanol).

Anal. Calcd. for $C_{26}H_{42}O_5N_4S$: N, 10.71; S, 6.13. Found: N, 10.60; S, 6.16.

The free acid was easily obtained by generation from its dicyclohexylammonium salt in the same manner as described for nitrophenylsulfenyl-L-isoleucine. After recrystallization from ethanol-water the acid melted at 135–138°.

Anal. Calcd. for $C_{14}H_{19}O_5N_3S$: N, 12.30; S, 9.39. Found: N, 12.21; S, 9.52.

L-Isoleucylglycine.—To a solution of 1.7 g. (0.005 mole) of N-*o*-nitrophenylsulfenyl-L-isoleucylglycine in 20 ml. of ethyl acetate, ethyl acetate containing hydrogen chloride was added. Immediately, the dipeptide hydrochloride precipitated. The mixture was shaken for 10 min. and then evaporated to dryness. The removal of the excess hydrogen chloride was achieved by repeated addition of ethyl acetate and evaporation to dryness. Finally the residue after addition of ethyl acetate was extracted several times with water. The combined aqueous extracts (25 ml.) were extracted with ethyl acetate, then with ether, and were passed through a column of Amberlite IR 4B (OH form). Upon evaporation of the HCl-free eluate and addition of alcohol, 0.83 g. (92%) of the product was obtained in the form of long needles, m.p. above 240° dec., $[\alpha]^{22}D$ +100° (*c* 1.5, water) after recrystallization from water–alcohol; R_f 0.48 (ascending chromatography on Whatman No. 1 paper (in 1-butanol–acetic acid–water–pyridine[39]).

Anal. Calcd. for $C_8H_{16}O_3N_2$: C, 51.04; H, 8.56; N, 14.88. Found: C, 51.30; H, 8.50; N, 14.55.

The compound referred to in the literature[40] as L-isoleucylglycine was soluble in chloroform and had $[\alpha]^{20}D$ +33.59° (in water). It seems probable that this compound did not have the structure of the dipeptide; no elementary analysis was given.

N-Tritylsulfenyl-L-phenylalanylglycine Ethyl Ester.—To a suspension of 0.92 g. (0.0066 mole) of glycine ethyl ester hydrochloride in 20 ml. of chloroform and 1.7 ml. of triethylamine 3.4 g. (0.006 mole) of IIIa was added and the mixture was left at room temperature for 2 days. The solution was washed successively with water, twice with 1 *N* ammonia,[41] once with sodium carbonate solution, and finally with water. The solution was dried and evaporated to dryness. The residue was dissolved in hot ethanol and the solution was allowed to stand for a few hours in the refrigerator. It was then filtered from a small amount of a solid and the filtrate was evaporated to a small volume. Cooling and scratching of the solution led to the precipitation of the compound (1.9 g., 64%), m.p. 113–114°, $[\alpha]^{20}D$ −54.1° (*c* 4, dimethylformamide).

Anal. Calcd. for $C_{32}H_{32}O_3N_2S$: N, 5.33; S, 6.11. Found: N, 5.41; S, 6.14.

N-*o*-Nitrophenylsulfenyl-L-phenylalanylglycine Ethyl Ester.—(a) Glycine ethyl ester and IVa were allowed to react in the same manner as described for the corresponding N-tritylsulfenyl derivative. The yield was 60%, m.p. 121–122°, $[\alpha]^{18}D$ +26.5° (*c* 2, dimethylformamide).

Anal. Calcd. for $C_{19}H_{21}O_5N_3S$: N, 10.41; S, 7.94. Found: N, 10.46; S, 7.62.

(b) N-*o*-Nitrophenylsulfenyl-L-phenylalanine was coupled with glycine ethyl ester by the carbodiimide method in the same manner as that described for the preparation of VI (procedure a). The crude product was recrystallized from ethyl acetate–petroleum ether. The yield was 70%, m.p. 121°.

(c) To a solution of 1.65 g. (0.005 mole) of *o*-nitrophenylsulfenyl-L-phenylalanine in 20 ml. of chloroform and 0.7 ml. of triethylamine, which had been precooled to −10°, 0.65 ml. of isobutyl chloroformate was added. After being allowed to stand for 15 min. at −10°, the solution of the mixed anhydride was added to a solution of 0.7 g. (0.005 mole) of glycine ethyl ester hydrochloride in 15 ml. of chloroform and 0.7 ml. of triethylamine. The mixture was worked up as usual (*cf.* above). The yield was 1.4 g. (70%), m.p. 121–122°.

N-*o*-Nitrophenylsulfenyl-L-phenylalanylglycine was prepared by stirring a suspension of 0.8 g. (0.002 mole) of the corresponding ethyl ester in 8 ml. of ethanol and 1.5 ml. of 2 *N* sodium hydroxide for 10 min. to give a solution which was diluted with water and extracted with ethyl acetate. The aqueous layer was acidified with dilute sulfuric acid. The precipitate thus formed was extracted with ethyl acetate which was washed repeatedly with water and concentrated to dryness. The crude product thus obtained was purified by trituration with petroleum ether. The yield was 0.6 g. (82%), m.p. 185°. After recrystallization from ethanol the m.p. was raised to 188–190°, $[\alpha]^{20}D$ +32.0° (*c* 3, dimethylformamide).

Anal. Calcd. for $C_{17}H_{17}O_5N_3S$: N, 11.19; S, 8.54. Found: N, 11.22; S, 8.41.

L-Phenylalanylglycine.—(a) To a warm solution of 0.005 mole of N-*o*-nitrophenylsulfenyl-L-phenylalanylglycine in a mixture of ethyl acetate–acetone (1:1) anhydrous ether containing hydrogen chloride was added. Immediately the hydrochloride of the peptide separated out. The mixture was left to stand for 1 hr. in the refrigerator before the supernatant liquid was decanted. The precipitate was repeatedly washed with ether, dissolved in water, and the solution was passed through a column of Amberlite IR 4B (OH form). Upon evaporation of the HCl-free eluate to dryness and adding absolute ethanol to the residue, 0.77 g. (70%) of the free dipeptide was obtained, m.p. 258° dec., $[\alpha]^{20}D$ +98.2° (*c* 2, water); reported[42] m.p. 250° dec., $[\alpha]^{15}D$ +95.2° (*c* 2, water).

(b) N-Tritylsulfenyl-L-phenylalanylglycine ethyl ester (1.04 g., 0.002 mole) was saponified in the same manner as described for the corresponding nitrophenylsulfenyl derivative. The crude free acid thus obtained was transformed to L-phenylalanylglycine in the same way as described above (case a). The yield was 60%, m.p. 256° dec., $[\alpha]^{20}D$ +99.0° (*c* 2, water).

L-Phenylalanyl-L-leucine Benzyl Ester Hydrochloride.—(a) N-*o*-Nitrophenylsulfenyl-L-phenylalanine was coupled with leucine benzyl ester by the carbodiimide method in the manner described for the preparation of VI (procedure a). The sirupy N-*o*-nitrophenylsulfenyl-L-phenylalanyl-L-leucine benzyl ester thus obtained was dissolved in ether. Upon addition of an ethereal solution of hydrogen chloride and scratching, the required hydrochloride precipitated. The yield was 75%, m.p. 159–160°, reported[43] m.p. 161°.

(b) N-*o*-Nitrophenylsulfenyl-L-phenylalanine was coupled with L-leucine benzyl ester by the mixed carboxylic–carbonic anhydride method in the manner described above for the preparation of nitrophenylsulfenyl-L-phenylalanylglycine ester. The sirupy N-*o*-nitrophenylsulfenyl-L-phenylalanyl-L-leucine benzyl ester thus obtained was converted to the ester hydrochloride as described above (case a). The yield was 75%, m.p. 160°.

N-*o*-Nitrophenylsulfenyl-L-threonyl-L-phenylalanine methyl ester was prepared by the interaction of *o*-nitrophenylsulfenyl-L-threonine dicyclohexylammonium salt (4.5 g., 0.01 mole), 2.1 g. of L-phenylalanine methyl ester hydrochloride (0.01 mole), and 2.2 g. of N,N′-dicyclohexylcarbodiimide in chloroform solution in the manner described for the preparation of VI (procedure b). The sirupy product crystallized upon addition of a small amount of ether and after being allowed to stand in the refrigerator for several hours. The yield was 2.5 g. (60%), m.p. 110°, $[\alpha]^{15}D$ −17.0° (*c* 2, dimethylformamide).

Anal. Calcd. for $C_{20}H_{23}N_3O_6S$: N, 9.70; S, 7.33. Found: N, 9.92; S, 7.22.

L-Threonyl-L-proline Methyl Ester Hydrochloride.—(a) The corresponding N-*o*-nitrophenylsulfenyl derivative was first prepared by the interaction of N-*o*-nitrophenylsulfenyl-L-threonine dicyclohexylammonium salt (2.25 g., 0.005 mole), 0.83 g. of L-proline methyl ester hydrochloride, and 1.1 g. of N,N′-dicyclohexylcarbodiimide in chloroform solution in the manner described for the preparation of VI (procedure b). The sirupy nitrophenylsulfenyl-L-threonyl-L-proline methyl ester thus obtained was dissolved in ethyl acetate. Upon addition of anhydrous ether containing hydrogen chloride, the above ester hydrochloride separated out as a sirup, which was washed thoroughly with ether and dissolved in a small amount of dry methanol. Ethyl acetate was added and the solution was left to stand in the refrigerator whereupon the ester hydrochloride crystallized out. The yield was 0.5 g. (45%), m.p. 189–190° and 190–191° after recrystallization from methanol–ethyl acetate, $[\alpha]^{17}D$ −95.3° (*c* 3, water).

Anal. Calcd. for $C_{10}H_{19}N_2O_4Cl$: N, 10.50; Cl, 13.32. Found: N, 10.33; Cl, 13.22.

(b) N-Carbobenzoxy-L-threonine[3] (5.1 g., 0.02 mole) was coupled with L-proline methyl ester (3.3 g., 0.02 mole) by the carbodiimide method and the sirupy N-carbobenzoxy-L-threonyl-L-proline methyl ester thus obtained was catalytically (Pd) hydrogenated. After recrystallization from methanol–ethyl acetate 2.8 g. (53%) of the product was obtained, m.p. 191–192°, $[\alpha]^{17}D$ −95.3° (*c* 3, water).

(39) Y. Levin, A. Berger, and E. Katchalski, *Biochem. J.*, **63**, 308 (1956).

(40) E. Abderhalden, P. Hirsch, and J. Schuler, *Ber.*, **42**, 3394 (1909).

(41) Each of the ammonia washings was carried out with stirring for 1 hr.

(42) A. Cosmatos, I. Photaki, and L. Zervas, *Chem. Ber.*, **94**, 2644 (1961).

(43) G. C. Stelakatos, D. M. Theodoropoulos, and L. Zervas, *J. Am. Chem. Soc.*, **81**, 2884 (1959).

Anal. Calcd. for $C_{10}H_{19}N_2O_4Cl$: N, 10.50; Cl, 13.32. Found: N, 10.74; Cl, 13.08.

N-*o*-Nitrophenylsulfenyl-L-prolyl-L-phenylalanine methyl ester was prepared (a) by interaction of *o*-nitrophenylsulfenyl-L-proline dicyclohexylammonium salt (4.5 g., 0.01 mole), 2.1 g. (0.01 mole) of L-phenylalanine methyl ester hydrochloride, and 2.2 g. of N,N′-dicyclohexylcarbodiimide in the manner described above for the preparation of VI (procedure b). Treatment with isopropyl ether and a few ml. of ethyl acetate followed by cooling in the refrigerator caused the sirupy product to crystallize. After recrystallization from isopropyl ether containing a small amount of ethyl acetate the yield was 3 g. (70%) and the m.p. 91–92°, $[\alpha]^{18}D$ −7.6° (*c* 5, dimethylformamide).

Anal. Calcd. for $C_{21}H_{23}N_3O_5S$: N, 9.78; S, 7.44. Found: N, 9.88; S, 7.33.

(b) *o*-Nitrophenylsulfenyl-L-proline in the form of its dicyclohexylammonium salt was coupled with L-phenylalanine methyl ester by the diphenylphosphoryl method in the manner described for the preparation of VI (procedure c). Upon working up the mixture as described above 80% of the pure product was obtained, m.p. 91–92°.

L-Prolyl-L-phenylalanine Methyl Ester Hydrochloride.—Addition of hydrogen chloride in ether to an ethereal solution of 2.15 g. (0.005 mole) of the corresponding nitrophenylsulfenyl derivative and scratching led to the crystallization of the required product. After recrystallization from acetone–ether the yield was 1.4 g. (93%), m.p. 159°, $[\alpha]^{18}D$ −41.9° (*c* 2.5, water); reported[44] m.p. 157–158°, $[\alpha]D$ −41° (in water); reported[45] m.p. 162.5–163.5°, $[\alpha]D$ −41.8° (water).

Anal. Calcd. for $C_{15}H_{21}N_2O_3Cl$: N, 8.95; Cl, 11.33. Found: N, 8.95; Cl, 11.16.

N^{α}-*o*-Nitrophenylsulfenyl-N^{ϵ}-carbobenzoxy-L-lysyl-L-alanine methyl ester was prepared by interaction of N^{α}-nitrophenylsulfenyl-N^{ϵ}-carbobenzoxy-L-lysine dicyclohexylammonium salt (6.15 g., 0.01 mole), 1.4 g. of L-alanine methyl ester hydrochloride (0.01 mole), and 2.2 g. of N,N′-dicyclohexylcarbodiimide in the manner described for the preparation of VI (procedure b). The sirupy product was treated with petroleum ether and left to stand in the refrigerator, whereupon it solidified. The material was suspended in warm diisopropyl ether, the necessary amount of warm ethyl acetate required to give a solution was added, and the solution was cooled in the refrigerator. The amorphous product was filtered off and was washed with a small amount of diisopropyl ether. The yield was 4.6 g. (88%), m.p. 74–77°, $[\alpha]^{20}D$ −36.6° (*c* 3, dioxane).

Anal. Calcd. for $C_{24}H_{30}N_4O_7S$: N, 10.79; S, 6.17. Found: N, 11.35; S, 5.76.

N-*o*-Nitrophenylsulfenyl-L-prolyl-N^{ϵ}-carbobenzoxy-L-lysyl-L-alanine Methyl Ester.—Ether containing hydrogen chloride was added to a suspension of 2.6 g. (0.005 mole) of N^{α}-nitrophenylsulfenyl-N^{ϵ}-carbobenzoxy-L-lysyl-L-alanine methyl ester in a mixture of ethyl acetate–ether (1:1). The solvent was decanted from the precipitated sirupy hydrochloride of the dipeptide ester, which was washed with decantation with ether several times. The residue, which after evaporation and drying weighed 1.88 g., was dissolved in chloroform together with 2.15 g. of *o*-nitrophenylsulfenyl-L-proline dicyclohexylammonium salt and 1.1 g. of N,N′-dicyclohexylcarbodiimide. The mixture was allowed to stand overnight at room temperature and was worked up as described for the preparation of VI (procedure b). The crude product was dissolved in tetrahydrofuran, the solution was filtered, and the filtrate was evaporated to dryness. The product crystallized upon the addition of petroleum ether and was recrystallized from methanol. The yield was 1.5 g. (50%), m.p. 175–176°, $[\alpha]^{20}D$ −44.2° (*c* 4, dimethylformamide).

Anal. Calcd. for $C_{29}H_{37}N_5O_8S$: N, 11.37; S, 5.20. Found: N, 11.32; S, 5.47.

L-Prolyl-N^{ϵ}-carbobenzoxy-L-lysyl-L-alanine Methyl Ester Hydrochloride.—Ether was added to a solution of 3.1 g. (0.005 mole) of the nitrophenylsulfenyl derivative of the tripeptide ester in 8 ml. of 1.4 *N* hydrogen chloride in methanol. The precipitated sirup was triturated with several portions of ether. Finally, it was suspended in warm ethyl acetate and the necessary amount of methanol required to give a solution was added. Upon addition of ether and cooling, the above ester hydrochloride crystallized. It was collected by filtration, washed with ether, and was kept in a desiccator over phosphorus pentoxide. The yield was 1.5 g. (60%), m.p. 133–135°, $[\alpha]^{20}D$ −60.6° (*c* 3.5, water).

Anal. Calcd. for $C_{23}H_{35}N_4O_6Cl$: N, 11.22; Cl, 7.11. Found: N, 11.01; Cl, 7.29.

S-Trityl-L-cysteinylglycine *p*-Nitrophenyl Ester Hydrochloride.—To a solution of 2.8 g. (0.01 mole) of glycine *p*-nitrophenyl ester hydrobromide, 5.2 g. of S-trityl-N-nitrophenylsulfenyl-L-cysteine, and 1.4 ml. of triethylamine in 30 ml. of chloroform, 2.2 g. of dicyclohexylcarbodiimide was added. The mixture was allowed to stand at room temperature overnight and was worked up as described for the preparation of VI (procedure a). The protected dipeptide ester thus obtained was a sirup, which was dissolved in ethyl acetate. Addition of ether containing hydrogen chloride to this solution gave the required dipeptide ester hydrochloride as an amorphous powder. The yield was 4 g. (68%), $[\alpha]^{20}D$ +39.6° (*c* 3, ethanol); reported[46] $[\alpha]D$ +39.6° (ethanol).

Anal. Calcd. for $C_{30}H_{28}N_3O_5SCl$: N, 7.27; S, 5.55; Cl, 6.13. Found: N, 7.23; S, 5.12; Cl, 6.28.

N-Tritylglycylglycine ethyl ester was prepared by interaction of Ia with glycine ethyl ester in the manner described above for the preparation of N-tritylsulfenyl-L-phenylalanylglycine ethyl ester. The crude product was recrystallized from ethanol. The yield was 70%, m.p. 161° (reported[47] 161°).

(44) W. Rittel, B. Iselin, H. Kappeler, B. Riniker, and R. Schwyzer, *Helv. Chim. Acta*, **40**, 614 (1957).

(45) H. Schwarz, F. M. Bumpus, and I. H. Page, *J. Am. Chem. Soc.*, **79**, 5697 (1957).

(46) L. Zervas and I. Photaki, *ibid.*, **84**, 3887 (1962).

(47) B. Helferich, L. Moog, and A. Juenger, *Ber.*, **58**, 883 (1925).

[CONTRIBUTION FROM THE SCHOOL OF CHEMISTRY, UNIVERSITY OF MINNESOTA, MINNEAPOLIS 14, MINN.]

The Biosynthesis of the Alkaloids of *Colchicum*. III. The Incorporation of Phenylalanine-2-C^{14} into Colchicine and Demecolcine[1]

BY EDWARD LEETE[2]

RECEIVED JUNE 3, 1963

DL-Phenylalanine-3-C^{14} was fed to *Colchicum byzantinum* corms, alone and in the presence of relatively large amounts of inactive gallic, protocatechuic, and *p*-hydroxybenzoic acids. The two former compounds partially inhibited the incorporation of phenylalanine-3-C^{14} into colchicine and demecolcine. However, the alkaloids isolated from corms which had been fed gallic acid-carboxyl-C^{14} were devoid of radioactivity. When DL-phenylalanine-2-C^{14} was fed to *Colchicum* corms, radioactive alkaloids were obtained and systematic degradation established that they had more than 90% of their activity located in ring B at C_6.

It has been previously shown[3] that the administration of phenylalanine-3-C^{14} to *Colchicum byzantinum* corms led to the formation of radioactive colchicine (I) which had essentially all its activity located in ring B at C_5. However, the radioactive colchicine obtained when tyrosine-2-C^{14} was fed to *Colchicum autumnale* plants was not labeled at one specific position.[4] No activity was located at C_6 and considerable activity was detected in the N-acetyl group, indicating that the tyrosine-2-C^{14} had undergone metabolic breakdown in the plant, the resultant fragments being then incorporated into colchicine. These results led Battersby[5]

(1) An account of this work was presented at the I.U.P.A.C. Meeting, London, July 10–17, 1963. This investigation was supported by a research grant (MY-02662) from the National Institutes of Health, U. S. Public Health Service.

(2) Alfred P. Sloan Fellow, 1962–1964.

(3) E. Leete and P. E. Németh, *J. Am. Chem. Soc.*, **82**, 6055 (1960).

(4) A. R. Battersby and J. J. Reynolds, *Proc. Chem. Soc.*, 346 (1960).

(5) A. R. Battersby, *Quart. Rev.* (London), **15**, 259 (1961).