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

N-Tritylamino Acids and Peptides. A New Method of Peptide Synthesis[1]

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A method of peptide synthesis is described in which the trityl group is used to protect the amino group of amino acids and peptides. These trityl derivatives are coupled with other amino acids by the usual methods. The trityl group is removed under mild conditions, *e.g.*, by acetic acid, by one equivalent of hydrochloric acid or by catalytic hydrogenation.

Introduction

Although the carbobenzoxy method[3] has been used successfully for the synthesis of peptides,[4] the development of alternate methods for the preparation of complex polypeptides is desirable. Recently several such methods have been proposed.[5] In this report we describe the trityl method of peptide synthesis, which has been discussed independently of our work[2] by Hillmann-Ellies, Hillmann and Jatzkewitz[6] and by Amiard, Heymes and Velluz.[7]

N-Tritylamino acids (II) and a N-trityl dipeptide (IV) were prepared by the action of trityl chloride on the hydrochlorides of esters of the corresponding amino acids and dipeptide in the presence of pyridine at 100°, followed by the saponification of the resulting products (I, III).[8] It was found[2,6,7] that tritylation proceeds more satisfactorily under the conditions described in the Experimental section; the sulfhydryl group of cysteine is also tritylated under these conditions.

$(C_6H_5)_3CNHCHRCOOR'$ I $\qquad$ $(C_6H_5)_3CNHCHRCOOH$ II

$(C_6H_5)_3CNHCHRCONHCHRCOOR'$ III

$(C_6H_5)_3CNHCHRCONHCHRCOOH$ IV

Since the $(C_6H_5)_3C$–N group is hydrolyzed by acid,[9] only the alkaline hydrolysis of the ester linkages of I and III needs to be considered. Under mild conditions (Table I) the glycyl derivatives hydrolyzed more rapidly than the alanyl derivatives, and the esters of the higher tritylamino acids hydrolyzed very slowly. Apparently a larger R group augments the steric hindrance which the trityl group exerts on the saponification of the esters of tritylamino acids.[10] This inhibitory effect was not observed in the case of the N-trityl peptide esters.

TABLE I

HYDROLYSIS OF ESTERS OF TRITYLAMINO ACIDS AND PEPTIDES

The ester (0.005 mole) was hydrolyzed in a mixture of 5 ml. of dioxane and 5 ml. of N methanolic KOH at 50°; in the case of the cystine derivative 0.0025 mole was used.

Methyl ester	Time, min.	Hydrolysis, %
N-Tritylglycine	5	63
N-Tritylglycine	10	80
N-Trityl-DL-alanine	10	13
N-Trityl-DL-alanine	30	23
N-Trityl-L-phenylalanine	30	9
N-Ditrityl-L-cystine di-	30	5
N-Tritylglycylglycine	5	93
N-Tritylglycyl-DL-alanine	5	88
N-Tritylglycyl-L-phenylalanine	5	85

Similarly, tritylglycine[11] and trityl peptide esters readily form hydrazides, while the esters of the higher tritylamino acids do not.[2,6]

The esters of tritylamino acids are saponified at high temperatures with excess alkali[6,7]; however, since hot alkali may cause racemization or at least partial destruction of the tritylated amino acids, we developed another method for the preparation of N-tritylamino acids.

In spite of the known sensitivity of trityl chloride toward hydrolyzing media and despite a controversial statement,[6] it is possible to tritylate amino acids directly in aqueous solutions.

$$H_2NCHRCOOH \xrightarrow[\text{water, isopropyl alcohol, 20–25°}]{(C_6H_5)_3CCl,\ (C_2H_5)_2NH} II$$

Most of the trityl chloride that does not react with the amino acid undergoes hydrolysis, while a small portion of it reacts with diethylamine to form trityldiethylamine. If a tertiary base (pyridine or triethylamine) is used instead of diethylamine, the yield of tritylamino acid is minute and practically all of the trityl chloride is hydrolyzed to triphenylcarbinol.

The trityl derivatives of the monoamino acids

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(2) The Rockefeller Institute for Medical Research, New York.

(3) M. Bergmann and L. Zervas, *Ber.*, **62**, 1192 (1932). For the adaption of the original carbobenzoxy method for the synthesis of peptides containing cystine, arginine or lysine see R. H. Siffert and V. du Vigneaud, *J. Biol. Chem.*, **108**, 753 (1935); C. R. Harington and T. H. Mead, *Biochem. J.*, **29**, 1602 (1935); M. Bergmann, L. Zervas and H. Rinke, *Z. physiol. Chem.*, **224**, 40 (1934); D. T. Gish and F. H. Carpenter, THIS JOURNAL, **75**, 5872 (1953); G. W. Anderson, *ibid.*, **75**, 6081 (1953); H. O. van Orden and E. L. Smith, *J. Biol. Chem.*, **208**, 753 (1954); M. Bergmann, L. Zervas and W. F. Ross, *ibid.*, **111**, 245 (1935).

(4) J. S. Fruton, "Advances in Protein Chemistry," Vol. V, ed. by M. Anson, J. Edsall and K. Bailey, Academic Press, Inc., New York, N. Y., 1949, p. 1.

(5) J. C. Sheehan and V. S. Frank, THIS JOURNAL, **71**, 1856 (1949); C. M. Stevens and R. Watanabe, *ibid.*, **72**, 725 (1950); D. A. Kidd and F. E. King, *Nature*, **162**, 776 (1948); V. du Vigneaud and O. K. Behrens, *J. Biol. Chem.*, **117**, 27 (1937); Th. Wieland and W. Schafer, *Ann.*, **576**, 104 (1952); F. Weygand and E. Leising, *Angew. Chem.*, **64**, 136 (1952); *Ber.*, **87**, 248 (1954).

(6) A. Hillmann-Elies, G. Hillmann and H. Jatzkewitz, *Z. Naturf.*, **86**, 445 (1953).

(7) G. Amiard, R. Heymes and L. Velluz, *Bull. soc. chim. France*, 191 (1955).

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

(9) K. Elbs, *ibid.*, **17**, 703, 741 (1884); **30**, 2044 (1897).

(10) The steric effect noted in Table I was utilized to prepare N-trityl-L-glutamic-α-ethyl ester through partial saponification of the corresponding diester.

(11) It was previously reported that the esters of tritylglycine do not give a hydrazide; ref. 6.

are weak acids[12] which give crystalline salts with diethylamine. However, the tritylation of the amino group does not fully deprive the amino acids and peptides or their esters of basic properties; these trityl derivatives are secondary amines and in non-aqueous solutions they form hydrochlorides (V) which undergo complete hydrolysis in cold water. When V is heated briefly with alcohol, it yields trityl ether and the hydrochloride ester of the respective amino acid quantitatively.

$$\text{I} \underset{\text{H}_2\text{O or OH}^-,\ 0^\circ}{\overset{\text{HCl(gas), chloroform}}{\rightleftharpoons}} [(\text{C}_6\text{H}_5)_3\text{CNH}_2\text{CHRCOOR}']^+\text{Cl}^- \quad \text{V}$$

$$\text{V} \xrightarrow[1\text{–}2\text{ min., }70^\circ]{\text{ROH}} \text{HCl}\cdot\text{H}_2\text{NCHRCOOR}' + (\text{C}_6\text{H}_5)_3\text{COR}$$

In order to split off the trityl group, it is not necessary to isolate V; detritylation occurs when the tritylamino acid or peptide ester is heated briefly with alcohol containing one equivalent of hydrogen chloride. Similarly, treatment of tritylamino acids or peptides with dilute acetic acid[2,6,7] or one equivalent of hydrochloric acid or catalytic hydrogenation also removes the trityl group. The peptide bond is not attacked by any of these procedures.

$$\text{II} \begin{cases} \xrightarrow[\text{HCl or CH}_3\text{COOH}]{\text{H}_2\text{O, 1 equiv.}} \text{H}_2\text{NCHRCOOH} + (\text{C}_6\text{H}_5)_3\text{COH} \\ \xrightarrow{\text{Pd, H}_2} \text{H}_2\text{NCHRCOOH} + (\text{C}_6\text{H}_5)_3\text{CH} \end{cases}$$

The ampholytic nature of the tritylamino acids is also indicated by the fact that they form chloride hydrochlorides VI, as

$$\text{II} \underset{\text{OH}^-}{\overset{\text{PCl}_5}{\rightleftharpoons}} [(\text{C}_6\text{H}_5)_3\text{CNH}_2\text{CHRCOCl}]^+\text{Cl}^- \quad \text{VI}$$

Owing to the work of Helferich,[13] the trityl group has been regarded as a protective group for the primary hydroxyl groups of carbohydrates. That it also constitutes an ideal protecting group for amino acids and peptides is illustrated by the use of N-tritylamino acids for the preparation of peptides.

N-Tritylglycine,[2,6,7] N-trityl-DL-alanine[2,7] and N-trityl-L-alanine can be coupled with the alkyl or benzyl esters of amino acids by the method of mixed anhydrides[14]; instead of the mixed anhydride of tritylglycine the chloride hydrochloride can be used. The resulting N-trityldipeptide esters III are easily converted to the corresponding hydrazides or detritylated to dipeptide esters. The latter reaction is important because it yields free, non-acylated di- and polypeptide benzyl esters which cannot be easily prepared at the present time by other means.[15] On the other hand, the N-trityldipeptide esters can be saponified and then detritylated to yield the free dipeptides.

By the mixed anhydride method we were not able to couple the higher tritylamino acids (*e.g.*, trityl-L-phenylalanine, trityl-L-leucine, trityl-L-asparagine) with esters of amino acids. This means that the steric hindrance previously mentioned either inhibits the formation of a mixed anhydride or, more probably, forces the anhydride to form a carbamate instead of a peptide. Perhaps other methods of coupling[16] would be successful.

As expected, this steric effect was not observed in the case of trityldi- or polypeptides. Thus, tritylglycyl-L-phenylalanine couples easily with glycine ester by the mixed anhydride procedure yielding finally the glycyl-L-phenylalanylglycine.

The catalytic hydrogenation of the carbobenzoxy polypeptides often proceeds at a very slow rate and their reduction by other methods is frequently unsatisfactory. On the other hand, the removal of the trityl group from tritylpolypeptides proceeds rapidly under mild conditions. We, therefore, recommend that the carbobenzoxy method be used for the synthesis of the smaller peptides and that the trityl method be used for lengthening of the peptide chain.

Experimental

Prior to analysis, the trityl derivatives were dried at 40° in high vacuum; other compounds were dried at 78°.

L-Cysteine Methyl Ester Hydrochloride.—A solution of 3.4 g. (0.01 mole) of L-cystine dimethyl ester dihydrochloride in 50 ml. of methanol was hydrogenated in the presence of 1 g. of palladium black; in 4 hours 250 ml. of hydrogen (24°, 756 mm.) was absorbed. The methanol was evaporated *in vacuo* and upon the addition of chloroform to the residue the methyl ester was obtained; yield 3.2 g., m.p. 140° (reported[17] m.p. 140–141°), $[\alpha]^{20}\text{D}$ −2.9° (10% in methanol), reported $[\alpha]^{20}\text{D}$ −2.9° (in methanol).

Esters of Tritylamino Acids and Peptides.—The following procedure is typical for the tritylation of amino acid and peptide esters. Some of these trityl derivatives are listed in Table II.

To a suspension of 1.25 g. (0.01 mole) of glycine methyl ester hydrochloride in 15 ml. of dry chloroform was added 2.2 g. (0.022 mole) of triethylamine followed by 2.8 g. (0.01 mole) of triphenylchloromethane; the mixture was allowed to react for 6 hours at room temperature. The solution was washed twice with water and dried with sodium sulfate. The solvent was evaporated *in vacuo*. Complete removal of the chloroform was ensured by the addition of a few ml. of methanol and reconcentration *in vacuo*. The residue was recrystallized from methanol.

N-Trityl-L-glutamic-α-ethyl Ester.—The corresponding oily diester which was prepared from 0.01 mole of L-glutamic diethyl ester as described above was dissolved in 20 ml. of 0.5 *N* alcoholic potassium hydroxide. The solution was stored for 30 minutes at room temperature, diluted with 150 ml. of water and extracted with ethyl ether. The water layer was acidified with acetic acid and cooled. The precipitate was dissolved in aqueous diethylamine solution and precipitated with acetic acid; yield 55%, m.p. 65–66°.

Anal. Calcd. for $\text{C}_{26}\text{H}_{27}\text{O}_4\text{N}$: C, 74.8; H, 6.5; N, 3.3. Found: C, 74.9; H, 6.7; N, 3.5.

N-Tritylglycine Hydrazide.—Tritylglycine methyl ester (1.65 g., 0.005 mole) was dissolved in a mixture of 3 ml. of

(12) The *p*H of a 0.1 *M* aqueous solution of the sodium salt of tritylglycine is 9.5.

(13) B. Helferich, "Advances in Carbohydrate Chemistry," Vol. 3, ed. by W. W. Pigman and W. L. Wolfrom, Academic Press, Inc., New York, N. Y., 1948, p. 79.

(14) Th. Wieland and H. Bernhard, *Ann.*, **572**, 190 (1951); R. H. Boissonas, *Helv. Chim. Acta*, **34**, 874 (1951); J. R. Vaughan and R. L. Osato, THIS JOURNAL, **73**, 553 (1951).

(15) If free dipeptide or polypeptide benzyl esters are coupled with carbobenzoxy- (or trityl) amino acids and peptides, saponification of the carbobenzoxy (or trityl) polypeptide esters is unnecessary, since the benzyl group is split off by hydrogenolysis.

(16) O. Suss, *Ann.*, **572**, 96 (1951); S. Goldschmidt and H. Lautenschlager, *ibid.*, **580**, 68 (1953); S. Goldschmidt and Ch. Jutz, *Ber.*, **86**, 116 (1953); G. W. Anderson, J. Blondinger and A. D. Welcher, THIS JOURNAL, **74**, 5309 (1952); R. Schwyzer, M. Feurer and B. Iselin, *Helv. Chim. Acta*, **38**, 83 (1955); J. C. Sheehan and G. P. Hess, THIS JOURNAL, **75**, 1068 (1955).

(17) M. Bergman and G. Michalis, *Ber.*, **63**, 987 (1930).

TABLE II

Esters of tritylamino acids	Yield, %	M.p., °C.	Formula	Carbon, % Calcd.	Carbon, % Found	Hydrogen, % Calcd.	Hydrogen, % Found	Nitrogen, % Calcd.	Nitrogen, % Found
N-Tritylglycine methyl	70	106–107	$C_{22}H_{21}O_2N$	79.7	79.5	6.4	6.8	4.2	4.1
N-Tritylglycine ethyl	86	114[a]							
N-Trityl-DL-alanine methyl	78	101	$C_{23}H_{23}O_2N$	80.0	79.8	6.7	6.8	4.05	4.1
N-Trityl-L-alanine methyl[b]	80		$C_{23}H_{23}O_2N$	80.0	79.9	6.7	6.8	4.05	3.8
N-Trityl-L-phenylalanine methyl	60	95	$C_{29}H_{27}O_2N$	82.6	82.3	6.4	6.6	3.3	3.4
N,N'-Bistrityl-L-cystine dimethyl[c]	80	146	$C_{46}H_{44}O_4N_2S_2$			8.5[e]	8.4[e]	3.7	3.6
N,S-Ditrityl-L-cysteine methyl[c]	75	90–92	$C_{42}H_{37}O_2NS$			5.2[e]	5.3[e]	2.2	2.3
N-Tritylglycylglycine methyl	75	165	$C_{24}H_{24}O_3N_2$					7.2	7.3
N-Tritylglycylglycine ethyl	75	162[d]							

[a] M.p. 114°, ref. 8. [b] Sirup. [c] For the tritylation of the corresponding ester hydrochloride (0.01 mole) were added 0.044 mole of triethylamine and 0.02 mole of trityl chloride. The product was recrystallized by slow evaporation of a solution in ether–methanol (1:4). [d] M.p. 161°, ref. 8. [e] Sulfur.

TABLE III

Tritylamino acids	Yield, %	M.p., °C.	Formula	Carbon, % Calcd.	Carbon, % Found	Hydrogen, % Calcd.	Hydrogen, % Found	Nitrogen, % Calcd.	Nitrogen, % Found
N-Tritylglycine	92,[a] 47[b]	178–179[c]	$C_{21}H_{19}O_2N$	317[k]	321[d,k]				
N-Trityl-DL-alanine	85,[a,e] 46[b]	170[f]	$C_{22}H_{21}O_2N$	331[k]	335[d,k]				
N-Trityl-L-alanine	85,[a,e] 46[b]	200–205 d.[g]	$C_{22}H_{21}O_2N$	331[k]	335[k]			4.2	4.2
N-Trityl-L-asparagine[h]	43[b]	173–174[i]	$C_{22}H_{22}O_3N_2$	73.8	73.6	5.9	5.9	7.5	7.5
N-Trityl-L-leucine	35[b,j]	160–165 d.[g]	$C_{25}H_{27}O_2N$	63.4	63.2	5.7	5.9	3.75	3.6
N-Trityl-L-phenylalanine	35[b,j]	185–188 d.[g]	$C_{28}H_{25}O_2N$	82.5	82.5	6.2	6.3	3.4	3.6

[a] By procedure A. [b] By procedure B; about one-fifth of the original amino acid was recovered unchanged. [c] M.p. 168° (dec. 180°) ref. 8; m.p. 179–180°, ref. 7. [d] By titration with 0.1 *N* potassium hydroxide with thymolphthalein as the indicator. [e] Prior to the acidification, the solution was kept at room temperature for 24 hours. [f] M.p. 170°, ref. 7. [g] After recrystallization from alcohol–water and adding one drop of acetic acid in order to avoid formation of emulsion. The substance begins to soften at 80–90°. [h] $[\alpha]^{20}D$ −6.1° (*c* 3.5% in methanol). [i] After recrystallization from alcohol or ethyl acetate. [j] Since the diethylammonium salt of this acid is sparingly soluble in water, instead of water dilute alkali was added at the end of the tritylation. The precipitated carbinol was filtered; prior to acidification, the diethylamine was removed from the filtrate by warming at 25–30° *in vacuo* for a few minutes. [k] Neutralization equivalent.

dioxane and 3 ml. of methanol. Hydrazine (1 ml.) was added and the solution stored for 2 days at room temperature. The reaction mixture was heated at 60° for ten minutes. The hydrazide, which precipitated upon the addition of water, was recrystallized from alcohol; yield 1.3 g. (78%), m.p. 164–165°.

Anal. Calcd. for $C_{21}H_{21}ON_3$: N, 12.7. Found: N, 12.5.

N-Tritylglycine Ethyl Ester Hydrochloride.—To 3.45 g. (0.01 mole) of N-tritylglycine ethyl ester dissolved in a few ml. of dry chloroform was added anhydrous ethyl ether saturated with dry hydrogen chloride. The salt precipitated at once; it was redissolved in chloroform and precipitated with anhydrous ethyl ether; yield 3 g. (78%), m.p. 95–96°.

Anal. Calcd. for $C_{23}H_{24}O_2NCl$: N, 3.6; Cl, 9.3. Found: N, 3.3; Cl, 9.4.

The above salt is hydrolyzed rapidly and almost quantitatively either with cold water (1–2°) or with a dilute solution of sodium bicarbonate to give tritylglycine ethyl ester, m.p. 114°. The hydrochloride salts of the other tritylamino acid esters were prepared in the same way or by bubbling dry hydrogen chloride through the ether solution of the ester. Some of these salts were obtained as oils.

Detritylation of Tritylamino Acid and Peptide Esters.—The following is a general procedure for the detritylation of amino acid and peptide esters.

A solution of 1.72 g. (0.005 mole) of tritylglycine ethyl ester in 5 ml. of 1 *N* HCl in absolute ethanol (or 1.9 g. of tritylglycine ethyl ester hydrochloride in 5 ml. of absolute ethanol) was heated for 1–2 minutes in a water-bath. Evaporation of the ethanol *in vacuo* and trituration of the residue with ether resulted in crystallization of the glycine ethyl ester hydrochloride; yield 0.7 g. (95%), m.p. 144°; admixture with an authentic sample gave no depression of the melting point.

Trityl ethyl ether was obtained from the ether filtrate; yield 1.32 g. (91%), m.p. 80–82°.

Anal. Calcd. for $C_{21}H_{20}O$: C, 87.4; H, 7.0. Found: C, 87.15; H, 7.1.

N-Tritylamino Acids.—The tritylamino acids were prepared by saponification of the corresponding esters or by tritylation of the corresponding amino acids. The following procedures are typical; the tritylamino acids thus prepared are listed in Table III.

A.—Tritylglycine ethyl ester (3.45 g., 0.01 mole) was dissolved on warming in 11 ml. (10% excess) of *N* alcoholic potassium hydroxide and 6 ml. of alcohol. After the solution had stood at room temperature for one hour, it was diluted to 3 times its volume with water, cooled and then acidified with acetic acid. The precipitated tritylglycine was washed several times with water and recrystallized from alcohol.

B.—To a solution of glycine (0.75 g., 0.01 mole) in a mixture of 4 ml. of water, 3 ml. (0.03 mole) of diethylamine and 8 ml. of isopropyl alcohol was added trityl chloride (3.6 g., 0.013 mole) with continuous shaking; the addition was accomplished in twelve portions within one hour at room temperature. When the reaction was complete, 25 ml. of water was added[18]; the mixture of triphenylcarbinol and trityldiethylamine which precipitated was washed thoroughly with water. Acidification of the combined filtrate with acetic acid brought about the precipitation of N-tritylglycine

TABLE IV

Diethylammonium salts[a] of N-trityl-	Yield, %	M.p., °C.	Formula	Nitrogen, % Calcd.	Nitrogen, % Found
Glycine	98	132	$C_{25}H_{30}O_2N_2$	7.2	7.1
DL-Alanine	98	151–152	$C_{26}H_{32}O_2N_2$	6.9	7.1
L-Alanine[b]	95	157	$C_{26}H_{32}O_2N_2$	6.9	7.15
L-Asparagine	95	150–151	$C_{27}H_{33}O_3N_3$	9.4	9.3
L-Leucine[c]	94	154–155	$C_{29}H_{38}O_2N_2$	6.3	6.2
L-Phenylalanine[d]	95	150–151	$C_{32}H_{36}O_2N_2$	5.8[e]	5.9[e]

[a] Recrystallized from acetone. [b] $[\alpha]^{22}D$ −18.9° (*c* 4.4% in methanol). [c] $[\alpha]^{24}D$ +2.8° (*c* 10%, in methanol). [d] $[\alpha]^{24}D$ +12.3° (*c* 5% in methanol). This compound can be obtained more readily as follows: at the end of the tritylation of L-phenylalanine by procedure B, chloroform was added; the chloroform layer was washed with water, dried over sodium sulfate and distilled to dryness *in vacuo*. Ether was added to the residue; upon standing in the icebox the diethylammonium salt precipitated; yield 38%, m.p. 150–151°, $[\alpha]^{22}D$ +12.2° (in methanol). [e] Calcd.: C, 79.9; H, 7.55. Found: C, 80.1; H, 7.5.

(18) Instead of isopropyl alcohol, tetrahydrofuran may be used; in this case, the tetrahydrofuran was removed by warming at 30° *in vacuo* for a few minutes before the water was added.

TABLE V

	Yield, %	M.p., °C.	Formula	Carbon, % Calcd.	Found	Hydrogen, % Calcd.	Found	Nitrogen, % Calcd.	Found
N-Tritylglycylglycine ethyl ester	72	162[a]							
N-Tritylglycylglycine benzyl ester	70	153	$C_{30}H_{28}O_3N_2$	77.6	77.5	6.1	6.3	6.0	5.95
Glycylglycine benzyl ester hydrochloride	95	160	$C_{11}H_{15}O_3N_2Cl$			13.7[m]	13.55[m]	10.8	10.75
N-Tritylglycylglycine	96	180[b]	$C_{23}H_{22}O_3N_2$	374[n]	377[c,n]				
Glycylglycine	94	215 d.	$C_4H_8O_3N_2$					18.7	18.6
N-Tritylglycyl-DL-alanine methyl ester	72	135	$C_{25}H_{26}O_3N_2$					7.0	6.9
N-Tritylglycyl-L-phenylalanine methyl ester[d]	67								
N-Tritylglycyl-L-phenylalanine[e,f]	90		$C_{30}H_{28}O_3N_2$	464[n]	468[c,n]			6.0	5.9
Glycyl-L-phenylalanine[g]	95		$C_{11}H_{14}O_3$					12.6	12.4
N,N'-Bistritylglycyl-L-cystyl dihydrazide	58	138–140	$C_{48}H_{50}O_4N_8S_2$			7.4[o]	7.3[o]	12.9	12.7
N-Trityl-L-alanylglycine ethyl ester[h]	65	155–165	$C_{26}H_{28}O_3N_2$	75.0	75.1	6.8	7.0	6.7	6.6
N-Trityl-L-alanylglycine[i]	87		$C_{24}H_{24}O_3N_2$					7.2	7.05
L-Alanylglycine[j]	95		$C_5H_{10}O_3N_2$					19.2	19.3
N-Tritylglycyl-L-phenylalanylglycine ethyl ester[d]	65								
N-Tritylglycyl-L-phenylalanylglycine[e,k]	90		$C_{32}H_{31}O_4N_3$	521[n]	525[c,n]			8.05	7.9
Glycyl-L-phenylalanylglycine[i]	95		$C_{13}H_{17}O_4N_3$	55.9	55.6	6.1	6.1	15.0	14.9
								NH_2-N, 5.0	5.4

[a] M.p. 163–164°, ref. 7; m.p. 161°, ref. 8. [b] M.p. 180°, ref. 8. [c] By titration with 0.1 *N* potassium hydroxide with thymolphthalein as the indicator. [d] Sirup. [e] Purified by dissolving in dilute aqueous diethylamine solution and acidifying with acetic acid after cooling. [f] The substance softened at 80–90° and decomposed at 210–215°. [g] $[\alpha]^{24}D$ 42.7° (*c* 2%, water), $[\alpha]D$ 42.0° (water); E. Fischer and W. Schoeller, *Ann.*, **357**, 1 (1907). [h] The same yield of peptide derivative was obtained, when instead of trityl-L-alanine its diethylammonium salt was used as the starting material; in this case, 0.04 mole of triethylamine and 0.02 mole of ethyl chloroformate were added for the preparation of the mixed anhydride. [i] The substance softened at 115–120° and decomposed over 180°. [j] $[\alpha]^{23}D$ 51.3° (*c* 2.5%, water), $[\alpha]D$ 50.2° (water); E. Fischer, *Ber.*, **38**, 2914 (1905); **41**, 850 (1908). [k] The substance softened at 90° and decomposed at 205–210°. [l] $[\alpha]^{25}D$ 30.9° (*c* 5.6%, 0.2 *N* HCl). [m] Chloride. [n] Neutralization equivalent. [o] Sulfur.

which was recrystallized from ethanol. Glycine (0.15 g.) was recovered from the acetic acid filtrate by evaporation followed by addition of ethanol.

Since some tritylamino acids do not have a characteristic melting point, the diethylammonium salts, which have better physical properties, were prepared by the addition of diethylamine to the ether or acetone solutions of the pure or crude tritylamino acids; these salts are listed in Table IV.

N-Tritylglycyl Chloride Hydrochloride.—To a suspension of 3.2 g. (0.01 mole) of tritylglycine in 25 ml. of dry chloroform, precooled to 0°, was added 2.1 g. (0.01 mole) of phosphorus pentachloride. After 1–2 minutes stirring the reagents dissolved and the chloride began to precipitate; the precipitation was completed by the addition of 60 ml. of cold dry petroleum ether. The chloride hydrochloride was collected, washed with petroleum ether and dried in a vacuum desiccator over phosphorus pentoxide; yield 2.9 g. (80%), m.p. 114–115°.

Anal. Calcd. for $C_{21}H_{19}ONCl_2$: N, 3.75; Cl, 19.05. Found: N, 3.65; Cl, 18.85.

To above chloride (0.37 g., 0.001 mole) was added 4 ml. of absolute ethanol, precooled to 0°. Triethylamine (0.3 ml.) was then added and the solution allowed to stand for 30 minutes at room temperature. Addition of water brought about the precipitation of tritylglycine ethyl ester; yield 0.24 g. (69%), m.p. 114°.

N-Trityl-L-phenylalanyl Chloride Hydrochloride.—This was prepared from trityl-L-phenylalanine[19] as described above; the chloroform solution was distilled to dryness *in vacuo* at 20–25° and then ether was added; yield 80%, dec. 190°.

Anal. Calcd. for $C_{28}H_{25}ONCl_2$: N, 3.0; Cl, 15.3. Found: N, 2.8; Cl, 15.05.

Detritylation of Tritylamino Acids and Tritylpeptides.—The following procedures given for tritylglycine were used for the detritylation of other tritylamino acids and peptides.

A.—When tritylglycine (1.6 g., 0.005 mole), suspended in 5 ml. of 50% acetic acid was heated for 1–2 minutes[20] on a steam-bath, it dissolved and triphenylcarbinol was precipitated. Water was added and the carbinol filtered; yield 1.2 g. (95%), m.p. 161–162°. The filtrate was evaporated to dryness *in vacuo*. When ethanol was added to the residue, glycine crystallized; yield 0.35 g. (93%).

Anal. Calcd. for $C_2H_5O_2N$: N, 18.6. Found: N, 18.5.

If acetone instead of water was added at the end of the reaction, the glycine precipitated at once; the yield was the same.

B.—To a suspension of 1.6 g. of tritylglycine in 5 ml. of acetone (or alcohol) was added 0.5 ml. of aqueous 10 *N* HCl. The compound went into solution immediately presumably due to the formation of the hydrochloride. The solution was boiled for one minute[21] and the precipitation of the glycine hydrochloride completed by the addition of ether; 0.3 g. (80%) of glycine was obtained from the hydrochloride. Triphenylcarbinol was obtained from the ether filtrate; yield 1.2 g. (92%), m.p. 161–162°. When alcohol was used as the solvent for the tritylglycine, trityl ether was formed as well as the carbinol and glycine hydrochloride.

C.—Tritylglycine (1.6 g.) dissolved in 50 ml. of ethanol, was hydrogenated in the presence of palladium black; 130 ml. of hydrogen (25°, 757 mm.) was absorbed in 4–5 hours. The catalyst was filtered and washed with water. The precipitated triphenylmethane was then filtered and washed with water; yield 92%, m.p. 92°; the mixed m.p. (authentic sample[22]) showed no depression.

Syntheses of Peptides.—Tritylglycine, trityl-L-alanine[19] and tritylglycyl-L-phenylalanine[19] were coupled with esters of amino acids by the mixed anhydride method. The resulting trityl peptide esters were converted to hydrazides, de-

(19) Prior to use the trityl compound was dried at 40° in high vacuum; the last traces of water were removed by dissolving the substance in anhydrous benzene and distilling to dryness *in vacuo*.

(20) Detritylation also occurs within 2 hours at room temperature.

(21) Detritylation also takes place within a few minutes at room temperature.

(22) Prepared according to "Organic Syntheses," Coll. Vol. 1, ed. H. Gilman, John Wiley and Sons, Inc., New York, N. Y., 1947, p. 548.

tritylated to peptide esters, or first saponified and then detritylated to give the free peptides. Examples of these procedures are given below; the peptides and intermediates prepared are listed in Table V.

A solution containing 3.2 g. (0.01 mole) of tritylglycine, 30 ml. of dry chloroform and 2 g. (0.02 mole) of dry triethylamine was cooled to 0°, and 1.08 g. (0.01 mole) of ethyl chloroformate was added. After 15 minutes 2.0 g. (0.01 mole) of glycine benzyl ester hydrochloride dissolved in 15 ml. of chloroform with 1 g. (0.01 mole) of triethylamine was added. Coupling proceeded with the evolution of CO_2. The solution was kept at room temperature for 30 minutes and then washed successively with dilute acetic acid, twice with dilute aqueous diethylamine solution and with water. It was dried with sodium sulfate and evaporated to dryness *in vacuo*. To the residue ethanol was added and the mixture distilled *in vacuo*, yielding tritylglycylglycine benzyl ester.[23] This ester was (a) detritylated with alcoholic hydrogen chloride, as described previously, to give glycylglycine benzyl ester hydrochloride; or (b) saponified with alcoholic potassium hydroxide. After 30 minutes, 80 ml. of water was added and the solution acidified with acetic acid. The precipitated tritylglycylglycine was recrystallized from alcohol. It was detritylated with acetic acid or by catalytic hydrogenation to yield glycylglycine.

N,N-Bistritylglycyl-L-cystine dimethyl ester[23,24] (sirup), dissolved in 50 ml. of methanol and 4 ml. of hydrazine hydrate, was stored in the ice-box for 48 hours. The precipitated dihydrazide was recrystallized from methanol.

N-Trityldiethylamine.—To a solution containing 2.8 g. (0.01 mole) of trityl chloride in 20 ml. of chloroform, was added 4 ml. of diethylamine. After one hour the solution was washed twice with water and dried with sodium sulfate. The crystalline residue, which was obtained after distillation of the solvent *in vacuo*, was recrystallized from petroleum ether; yield 2.2 g. (70%) needles, m.p. 107°.

Anal. Calcd. for $C_{23}H_{25}N$: C, 87.6; H, 8.0; N, 4.4. Found: C, 87.4; H, 8.3; N, 4.3.

(23) It was obtained in the same yield when tritylglycyl chloride-hydrochloride (0.01 mole) was added to a chloroform solution containing at least 0.03 mole of amino acid ester.

(24) This ester was prepared by coupling tritylglycine with 1.7 g. (0.005 mole) of L-cystine dimethyl ester hydrochloride dissolved in 15 ml. of chloroform with 1 g. of triethylamine as described above.

ATHENS, GREECE

[CONTRIBUTION FROM THE CHEMISTRY DEPARTMENT, UNIVERSITY OF MARYLAND]

The Purification of Hog Kidney D-Amino Acid Oxidase

BY F. P. VEITCH AND ROBERT MCCOMB[1]

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This paper describes the purification of D-amino acid oxidase by application of the principle of heat stabilization of enzyme by a competitive inhibitor. D-Amino acid oxidase is exceptionally stable to temperatures of 57° for 15 minutes in the presence of the ammonium salt of *m*-toluic acid (0.0178 *M*) at *p*H 5.2. In the absence of inhibitor 81% of the enzyme is destroyed under these same conditions. By the use of this technique a one hundred-fold enrichment, with a 67% yield, is accomplished in two steps. The product is pure as judged by several tests of homogeneity.

The stabilizing influence of substrates, coenzymes and inhibitors upon enzymes is well known. Advantage has been taken of this effect in the differential heat denaturation of contaminating proteins during the course of enzyme purification.[2]

In the research reported here, *m*-toluic acid, a competitive inhibitor of D-amino acid oxidase[3] has been found to stabilize the enzyme to the effect of temperature to a very marked degree (Table I).

TABLE I

EFFECT OF TIME OF HEATING, TEMPERATURE AND *p*H OF HEATING ON THE STABILITY OF D-AMINO ACID OXIDASE IN THE PRESENCE OF 0.0178 *M* AMMONIUM TOLUATE

Temp., °C.	Time, min.	*p*H	Specific activity, μl. O_2/10 min./mg. protein	Yield, %
45–50	5	5.1	134	..
55–60	5	5.1	188	..
67–70	5	5.1	177	..
57	10	5.1	930	79
57	15	5.1	1000	77
57	20	5.1	850	41
57	15	4.6	..	4
57	15	5.0	..	42
57	15	5.1	1250	50
57	15	5.15	1500	70
57	15	5.2	1390	67
57	15	5.6	..	63
57	15	7.5	..	6

(1) Monsanto Fellow, 1953–1954.

(2) (a) K. Burton, *Biochem. J.*, **48**, 458 (1951); (b) T. P. Singer and E. B. Kearney, *Arch. Biochem.*, **29**, 190 (1950).

(3) G. R. Bartlett, THIS JOURNAL, **70**, 1010 (1948).

We ascribe this stabilizing effect to the binding of the functional groups of the enzyme by the inhibitor in a manner similar to, but more pronounced than, the binding of normal substrates. This belief is strengthened by a comparison of the Michaelis constants of the enzyme–inhibitor and enzyme–substrate complexes (Table II). Further, we have found that weaker competitive inhibitors are less effective in protecting the enzyme to heat denaturation (Table II).

TABLE II

PROTECTION TO HEAT DENATURATION OFFERED BY VARIOUS COMPETITIVE INHIBITORS

Inhibitor	K_i/K_m[a]	% of original activity after heating and removal of inhibitor
m-Aminobenzoic acid	29×10^{-2}	66
Benzoic acid	3.47×10^{-2}	68
m-Toluic acid	1.15×10^{-2}	73
None	.....	19

[a] Bartlett[3] reported the effectiveness of various substituted benzoic acids as inhibitors of D-amino acid oxidase as molar concentrations giving 50% inhibition. Using the value of $K_m = 6.1 \times 10^{-3}$ for alanine obtained by Hellerman,[4] and substituting the inhibitor and substrate concentrations used by Bartlett in the equation $V/V_i = 1 + K_m/K_i\ (I/K_m + S)$ we have calculated the K_i values used above. Terms are defined as: V = velocity of uninhibited reaction (μl. O_2/10 min./mg. protein); V_i = velocity of inhibited reaction (μl. O_2/10 min./mg. protein); K_m = Michaelis constant; K_i = inhibitor constant; S = concentration of substrate in moles; I = concentration of inhibitor in moles.

(4) L. Hellerman, A. Lindsay and M. R. Bovarnick, *J. Biol. Chem.*, **163**, 553 (1946).