Peptide Synthesis. I. The Use of *p*-Toluenesulfonyl Chloride for Carboxyl Activation[1]

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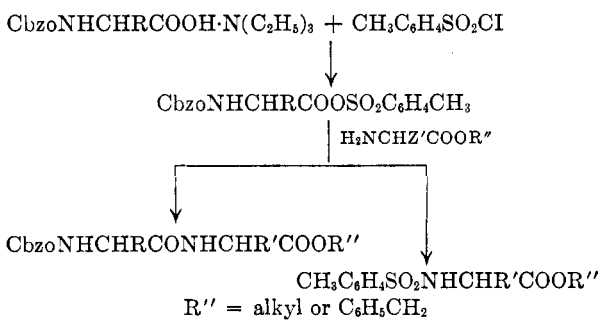
p-Toluenesulfonyl chloride (tosyl chloride) has been used for the carboxyl activation of carbobenzoxy amino acids and peptides *via* the formation of mixed *p*-toluenesulfonic-carboxylic anhydrides. Although carbobenzoxy L-amino acids, thus activated, do not racemize when condensed with amino acid and peptide esters, carbobenzoxyglycyl-L-phenylalanine, on the other hand, gives completely racemic carbobenzoxyglycyl-D,L-phenylalanylglycine ethyl ester, when coupled with glycine ethyl ester in tetrahydrofuran or acetonitrile as the solvent. The thionyl chloride method for carboxyl activation also resulted in extensive racemization.

In an attempt to develop methods which would minimize the danger of racemization during the synthesis of peptide bonds, we have investigated the use of mixed *p*-toluenesulfonic-carboxylic anhydrides as intermediates. Reaction between *p*-toluenesulfonyl chloride and a trialkyl ammonium salt of the appropriate carbobenzoxy amino acid furnishes the mixed anhydride to which the amino acid ester is then added.



Although a mixed anhydride of two acids, can, in principle, afford two products, the desired peptide derivatives have been readily isolated in most cases tested. The yield of these purified products varies between 40–50%, provided that there is no marked steric hindrance of the desired reaction, with consequently greater chance of *N*-tosylation. When carbobenzoxy-L-leucine was coupled with glycylglycine benzyl ester in the presence of *p*-toluenesulfonyl chloride, the only crystalline product isolated was tosylglycylglycine benzyl ester. The latter, m.p. 113–115°, was obtained in 32% yield, while the tripeptide, carbobenzoxy-L-leucylglycylglycine benzyl ester,[2] m.p. 115–117°, failed to crystallize in this case. Furthermore, when tosylglycine[3] was coupled under these conditions with glycine benzyl ester, it produced mainly tosylglycine benzyl ester instead of the desired acyldipeptide ester. In contrast, when carbobenzoxyglycine and carbobenzoxy-L-phenylalanine reacted under the same conditions, the corresponding peptide derivatives were isolated in satisfactory yield.

The effect of lengthening the peptide chain, either by *N*-terminal or C-terminal addition, on the degree of racemization of the final product has been examined. As examples of *N*-terminal addition, carbobenzoxy L-amino acids were used; when these were coupled with amino acid or peptide esters by the procedure described in this paper, the products obtained were found not to be racemized, as has also been found by the use of other methods.[2,4,5] The stringent test, however, was made by condensing α-carbethoxy(ε-carbobenzoxy)-L-lysine with L-serylglycine benzyl ester to produce α - carbethoxy(ε - carbobenzoxy) - L - lysyl - L-serylglycine benzyl ester with an $[\alpha]^{25}D$ value of − 17.5° as 1% solution in acetic acid. The same optical value was previously found for this compound[6] when prepared by the mixed carboxylic-carbonic anhydride procedure[4,7]; furthermore, this tripeptide derivative had been found to be completely digestible by trypsin,[6] when protecting groups were removed by catalytic hydrogenation.

On the other hand, compounds of the general type XNHCHRCONHCHR'COOH, when activated by the present procedure, afforded extensively racemized peptides during coupling with amino acid esters. Characteristically, when carbobenzoxyglycyl-L-phenylalanine was condensed with glycine ethyl ester, in the presence of *p*-toluenesulfonyl chloride, fully racemic carbobenzoxyglycyl-D,L-phenylalanylglycine ethyl ester[8] was obtained; this occurred whether a polar (acetonitrile) or nonpolar (tetrahydrofuran) solvent was used.

(1) This investigation was partly supported by the Royal Hellenic Research Foundation.

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(3) D. Theodoropoulos and L. C. Craig, *J. Org. Chem.*, **21**, 1376 (1956).

(4) J. Vaughan, *J. Am. Chem. Soc.*, **74**, 6137 (1952).

(5) H. Schwarz and F. Bumpus, *ibid.*, **81**, 890 (1959).

(6) D. Theodoropoulos, H. Bennick, G. Fölsch, and O. Mellander, *Nature*, **184**, 187 (1959).

(7) R. Boissonnas, *Helv. Chim. Acta*, **34**, 874 (1951).

(8) G. Anderson and F. Callahan, *J. Am. Chem. Soc.*, **80**, 2902 (1958).

Extensive racemization also was observed when the thionyl chloride method of Wieland[9] was used for coupling carbobenzoxyglycyl-L-phenylalanine with glycine ethyl ester. The tripeptide ester, thus synthesized, was separated by Anderson's test[8] in two fractions. The first fraction, which crystallized from a 2% solution in ethanol, m.p. 126–128°, was almost completely racemized. The second fraction, also obtained by evaporation of the solvent, showed an $[\alpha]^{25}D$ value of −1.2°, as 2% solution in ethanol, as compared to $[\alpha]^{25}D$ −13.2° value, reported for the optically pure product.[8]

It should be emphasized that the crude carbobenzoxyglycyl-L-phenylalanine, which was recovered from the reaction mixture, following Wieland's method,[9] exhibited $[\alpha]^{25}D$ −33° value, as compared to $[\alpha]^{25}D$ +38.2°, the value of the pure product.

We are aware of the fact that racemization may vary depending on the acyldipeptide used. We are further aware that coupling of an amino acid ester produced from the hydrochloride with a molar equivalent of triethylamine *in situ* leads to increased racemization.[10] It nevertheless appears safe to conclude that mixed sulfuric anhydrides of carbobenzoxy dipeptides, as those described in this paper, show a great tendency to produce racemic peptides, when condensed with amino acid esters.

Experimental

Carbobenzoxy-L-phenylalanylglycine Benzyl Ester.—Unless otherwise stated, the following procedure was employed: To a solution of 2.99 g. (0.01 mole) of carbobenzoxy-L-phenylalanine in 10 ml. of tetrahydrofuran, cooled to −10°, were added 1.01 g. (0.01 mole) of triethylamine and 1.9 g. (0.01 mole) of tosyl chloride, m.p. 69°, dissolved and cooled in 5 ml. of tetrahydrofuran. After 10 min. a solution of 3.37 g. (0.01 mole) of glycine benzl ester *p*-toluenesulfonate and 1.01 g. (0.01 mole) of triethylamine in 10 ml. of tetrahydrofuran was added to the anhydride with vigorous shaking. The reaction mixture was permitted to remain at room temperature overnight, then triethylamine salts were removed by filtration and the filtrate evaporated to dryness. The remaining residue was taken up in chloroform and this solution was washed successively with 10% bicarbonate solution and water, dried over sodium sulfate, and finally evaporated to dryness. Upon adding ether the residue crystallized; yield 2.3 g. (52%), recrystallized from ethanol-ether), m.p. 130–131°.

Anal. Calcd. for $C_{26}H_{26}N_2O_5$: C, 69.93; H, 5.87; N, 6.27. Found: C, 69.90; H, 5.75; N, 6.12.

L-Phenylalanylglycine.—A solution of the above ester (2.2 g., 0.005 mole) in acetic acid–water was hydrogenated in the presence of 0.5 g. of palladized charcoal. The catalyst was removed by filtration, the filtrate evaporated *in vacuo*, and upon adding acetone the peptide crystallized; yield I g. (90%), m.p. 249–250° (decomp.), $[\alpha]^{22}D$ +95° (*c*, 2 in water). The compound was dried at 78° over phosphorus pentoxide in high vacuum before analysis.

Anal. Calcd. for $C_{11}H_{14}N_2O_3$: C, 59.45; H, 6.35; N, 12.60. Found: C, 59.60; H, 6.38; N, 12.56.

(9) T. Wieland and H. Bernhard, *Ann.*, **527**, 190 (1951).

(10) N. Smart, G. Young, and M. Williams, *J. Chem. Soc.*, **776**, 3902 (1960).

Carbobenzoxyglycylglycine Benzyl Ester.—This compound was prepared in similar manner to that used in the preparation of the carbobenzoxy-L-phenylalanylglycine derivative; yield 1.7 g. (50%), m.p. 111–112° (reported[11,12] 110°, 111–112°).

Glycyl-L-leucine.—Carbobenzoxyglycyl-L-leucine benzyl ester, obtained in oily form was hydrogenated over palladized charcoal and the free peptide isolated by addition of acetone; yield 0.64 g. (36%), m.p. 231–232°, $[\alpha]^{25}D$ −34.9° (*c*, 1 in water) [(reported 231–232°, 234–237°, $[\alpha]^{25}D$ −36° (*c*, 1 in water)].[13]

Carbobenzoxy-L-serylglycine Benzyl Ester.—This peptide derivative was prepared by the carbodiimide method.[14]

To a solution of 6.8 g. (0.02 mole) of glycine benzyl ester *p*-toluenesulfonate in 25 ml. of methylene chloride was added 2.02 g. (0.02 mole) of triethylanine and mixed with another solution of 2.78 g. (0.02 mole) of carbobenzoxy-L-serine in 10 ml. of methylene chloride. Then 4 g. of dicyclohexylcarbodiimide was added and the solution stirred overnight at room temperature. Dicyclohexylurea was removed by filtration and the filtrate washed with dilute hydrochloric acid, bicarbonate solution, water, and finally dried over sodium sulfate. The solvent was evaporated *in vacuo* and the remaining solid residue recrystallized by dissolving in ethyl acetate and precipitating with petroleum ether; yield 5.5 g. (71%), m.p. 105–106°, reported[15] 105–106° for the same product prepared by the azide method.

L-Serylglycine.—Hydrogenation of 3.68 g. (0.01 mole) of the above dipeptide derivative over 0.5 g. of palladized charcoal, in ethanol–water (1:1), afforded 1.4 g. (80%) of the free dipeptide, $[\alpha]^{24}D$ +30.8° (*c*, 6 in *N* HCl), reported[15] $[\alpha]^{26}D$ +30.2° (*c*, 6 in *N* HCl).

L-Serylglycine Benzyl Ester Benzenesulfonate.—A mixture of 1.75 g. (0.01 mole) of L-serylglycine, 1.78 g. (10% excess) of benzenesulfonic acid monohydrate, 5 ml. of benzyl alcohol, and 20 ml. of carbon tetrachloride was heated in a steam bath, the liberated water being removed azeotropically. A thick mass was formed after 5 min., but in spite of that an additional amount of 20 ml. of carbon tetrachloride was added. This process was repeated four times more and, when most of the carbon tetrachloride had distilled, the residue was treated with ether and the ether layer was then decanted. Upon addition of a new portion of ether the residue solidified, filtered, washed well with ether, and dried in a desiccator ($CaCl_2$). It was recrystallized by dissolving in isopropyl alcohol and precipitating with ether; yield 3.7 g. (90%), m.p. 170° (decomp.), $[\alpha]^{25}D$ +7.4 (*c*, 1 in glacial acetic acid).

Anal. Calcd. for $C_{18}H_{22}N_2O_7S$: C, 52.67; H, 5.40; N, 6.82. Found: C, 52.56; H, 5.45; N, 6.79.

α-Carbethoxy(α-carbobenzoxy)-L-lysyl-L-serylglycine Benzyl Ester.—A solution of 5.6 g. (0.02 mole) of ε-carbobenzoxy-L-lysine[16] in 22 ml of 2 *N* sodium hydroxide, cooled to 0°, was treated with 2.16 g. (0.02 mole) of ethyl chlorocarbonate. Washing with ether, followed by acidification of the water layer with *N* hydrochloric acid liberated the acid, α-carbethoxy(ε-carbobenzoxy)-L-lysine, which was immediately extracted with ether. The solvent was well washed with water, dried with sodium sulfate, and evaporated to dryness. The remaining oily product (5.6 g., 80%), was dried over phosphorus pentoxide in vacuum before using for the next synthetic step.

To a solution of 1.75 g. (0.005 mole) of the above acid and 0.5 g. (0.005 mole) of triethylamine in 10 ml. of tetrahydrofuran, cooled to −10°, was added 0.95 g. (0.005 mole) of tosyl chloride. After 10 min. it was mixed with a solution of 4.1 g. (0.01 mole) of L-serylglycine benzyl ester benzene-

(11) D. Ben-Ishai, *J. Org. Chem.*, **19**, 62 (1954).

(12) L. Zervas and D. Theodoropoulos, *J. Am. Chem. Soc.*, **78**, 1359 (1956).

(13) H. Carpenter and T. Gish, *ibid.*, **74**, 3818 (1952).

(14) J. Sheehan and G. Hess, *ibid.*, **77**, 1067 (1955).

(15) J. S. Fruton, *J. Biol. Chem.*, **146**, 463 (1942).

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

sulfonate, 2.02 g. (0.02 mole) of triethylamine, and 2 ml. of water in 10 ml. of tetrahydrofuran.[17] The reaction mixture was kept at room temperature for 30 min. and then diluted by addition of 500 ml. of water. Upon scratching with a glass rod, the product precipitated; it was cooled, filtered, washed with bicarbonate solution and water, and dried (P_2O_5). Finally it was purified by dissolving in 90% ethanol and precipitating with an equal amount of ether; yield 1 g. (35%), m.p. 185–186°, $[\alpha^{25}D$ −17.5° (*c*, 1 in glacial acetic acid).

Anal. Calcd. for $C_{29}H_{38}N_4O_9$: C, 59.36; H, 6.52; N, 9.55. Found: C, 59.50; H, 6.57; N, 9.64.

Following exactly the same directions and employing the mixed carboxylic-carbonic anhydride procedure[4,7] for coupling, the tripeptide derivative was obtained in 50% yield,[18] m.p. 185–186°, $[\alpha]^{25}D$ −17.6 (*c*, I in glacial acetic acid).

Carbobenzoxyglycyl-L-phenylalanine.—Carbobenzoxyglycine (2 g., 0.01 mole), 2.0 g. (0.01 mole) of L-phenylalanine methyl ester hydrochloride, and 1.01 g. (0.01 mole) of triethylamine were coupled in tetrahydrofuran with 1.9 g. (0.01 mole) of tosyl chloride. Working up as usual gave 2.8 g. oily ester. Saponification with 3.8 ml of 2 *N* sodium hydroxide, extraction of unhydrolyzed material with ether, and acidification of the water layer with *N* hydrochloric acid precipitated the acid; over-all yield 1.2 g. (34%) m.p. 127°, $[\alpha]^{25}D$ +38.2° (*c*, 5 in ethanol), reported[8] m.p. 127.5° $[\alpha]^{24}D$ +38.8° ± 0.5 (*c*, 5 in ethanol).

Carbobenzoxyglycyl-L-phenylalanylglycine Ethyl Ester. A. By the Tosyl Chloride Method.—Carbobenzoxyglycyl-L-phenylalanine (3.56 g., 0.01 mole) and 1.4 g. (0.01 mole) of glycine ethyl ester hydrochloride were coupled in tetrahydrofuran by using 1.9 g. (0.01 mole) of tosyl chloride. The tripeptide ester was isolated in the usual fashion, and washed well with ether; yield 2 g. (47%), m.p. 132–133°, $[\alpha]^{25}D$ 0.0° (*c*, 2 in ethanol), reported[8] m.p. 132–133° for the pure D,L-form.

Acetonitrile replaced tetrahydrofuran in the above experiment and the reaction mixture upon dilution with water precipitated the product, which was washed with ether; yield 2.1 g. (50%), m.p. 132–133°.

B. By the Thionyl Chloride Method.—Carbobenzoxyglycyl-L-phenylalanine (3.54 g., 0.01 mole) with 1.01 g. (0.01 mole) of triethylamine was dissolved in 10 ml. of tetrahydrofuran, the solution was cooled to −10° and 0.59 g. (0.005 mole) of thionyl chloride was added with shaking. After 1 min. a solution of 0.7 g. (0.005 mole) of glycine ethyl ester[19] and 0.5 g. (0.005 mole) of triethylamine in tetrahydrofuran was added and the temperature was allowed to rise. After 1 hr. the ester was isolated as described above.; yield,[20] 1 g. (45%), m.p. 106–109°. A 2% solution in ethanol, after cooling overnight, deposited 0.43 g. of white product, m.p. 126–128°, $[\alpha]^{25}D$ −0.7° (*c*, 2 in ethanol). Evaporation of the filtrate yielded 0.52 g. of ester, m.p. 116–118°, $[\alpha]^{25}D$ −1.2° (*c*, 2 in ethanol), reported[8] for the optically pure substance, m.p. 120–120.5°, $[\alpha]^{25}D$ −13.2° (*c*, 2 in ethanol).

Tosylglycylglycine Benzyl Ester.—Glycine benzyl ester *p*-toluenesulfonate (3.37 g., 0.01 mole), 1.01 g. (0.01 mole) of triethylamine, and 2 g. (0.01 mole) of tosylglycine were coupled by means of 2 g. of carbodiimide. Treatment as usual produced an oily product which crystallized under ether; yield, 1.5 g. (40%), m.p. 113–115°.

Anal. Calcd. for $C_{18}H_{20}N_2O_5S$: C, 57.43; H, 5.35; N, 7.43. Found: C, 57.21; H, 5.40; N, 7.59.

Tosylglycine Benzyl Ester.—To a solution of 1.68 g. (0.005 mole) of glycine benzyl ester *p*-toluenesulfonate and 1.01 g. (0.01 mole) of triethylamine in 10 ml. of chloroform, added, in the cold, 0.95 g. (0.005 mole) of tosyl chloride. After 12 hr. the solution was washed as usual, then evaporated to dryness and the residue crystallized under petroleum ether; yield 1.2 g. (75%), m.p. 78° (crude product). When recrystallized from ether–petroleum ether melted at 80–81°.

Anal. Calcd. for $C_{16}H_{17}N\ O_4S$: C, 60.17; H, 5.36; N, 4.39. Found: C, 60.25; H, 5.15; N, 4.20.

(17) Excess of ester in tetrahydrofuran–water eliminates the side reaction with the hydroxyl group of serine.

(18) Yield of tripeptide derivative calculated on basis of α-carbethoxy-(ε-carbobenzoxy)-L-lysine used.

(19) Use of 0.01 mole of ester does not affect the yield in tripeptide ester. The same was experienced with the synthesis of carbobenzoxyglycylglycine benzyl ester by this method.

(20) Calculated on basis of ester used.