Preparation of Crystalline N^G-Tosylarginine Derivatives[1]

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N^α-Carbobenzoxy-N^G-tosyl-L-arginine, N^G-tosyl-L-arginine, N^α-*t*-butyloxycarbonyl-N^α-tosyl-L-arginine, and N^G-tosyl-L-arginine benzyl ester have been prepared in crystalline form. The synthesis of crystalline N^α-carbobenzoxy-N^G-tosyl-L-arginyl-L-proline benzyl ester also is described. The usefulness of these arginine derivatives for peptide synthesis is discussed.

The introduction of the arginine residue into a peptide chain has proved to be a quite formidable undertaking and became feasible only in the past decade.[2-4] In the synthesis of arginine peptides, the highly basic guanidino function is masked either with the nitro group[5] or by protonation.[2,3] For example, N^α-carbobenzoxy-N^G-nitroarginine is linked to an amino acid or peptide derivative by the mixed anhydride procedure[6-8] or by the dicyclohexylcarbodiimide[9-11] method; the resulting protected peptide is then hydrogenated and further synthesis is carried out with the hydrohalide salt. This procedure has been used in the synthesis of α-MSH analogs[12,13] and ACTH peptide.[14,15] For the syntheses[16-20] of peptides containing no tryptophan, the carbobenzoxy group was removed by treatment with hydrogen bromide and the nitro group was retained on the guanidino group until the end of the synthesis. Despite these achievements, the situation with regard to the protection of the guanidino group for the synthesis of long-chain peptides is far from satisfactory.[21-24]

The use of the carbobenzoxy group for the protection of the guanidino group has been investigated by Zervas, *et al.*,[25,26] who prepared N^α, N^ω, N^ω-tricarbobenzoxy-L-arginine and utilized it in the synthesis of N-terminal arginine peptides. Extending this application, Schwyzer and coworkers,[21] recently have prepared N^α-*t*-butyloxycarbonyl-N^ω, N^ω-dicarbobenzoxy-L-arginine and demonstrated its use in peptide synthesis. This derivative of arginine is quite useful since the *t*-butyloxycarbonyl group can be removed readily while the carbobenzoxy groups on the guanidino function are retained. However, this procedure precludes the use of the carbobenzoxy group at the N-terminus. The carbobenzoxy group is still the most useful protecting group and it would be advantageous to protect the guanidino function with some other group, thus permitting the extension of the peptide chain at the N-terminus by linking carbobenzoxy amino acids or peptides.

The tosyl group has been employed successfully for the protection of the ϵ-amino function of lysine in several syntheses.[27-29] The use of the tosyl group for masking the guanidino function of arginine was first conceived by Schwyzer and Li[30] who prepared N^α - *p* - (*p'* - methoxyphenylazo)-benzyloxycarbonyl - N^G - tosyl - L - arginine and used it in the synthesis of the pentapeptide L-histidyl - L - phenylalanyl - L - arginyl - L - tryptophylglycine. Subsequently, Schnabel and Li[31] described the preparation of N^α-carbobenzoxy-N^G-tosyl-L-arginine. Recently, Guttmann, *et al.*,[32] have used N^α-carbobenzoxy-N^G-tosyl-L-arginine in the synthesis of bradykinin. However, this deriva-

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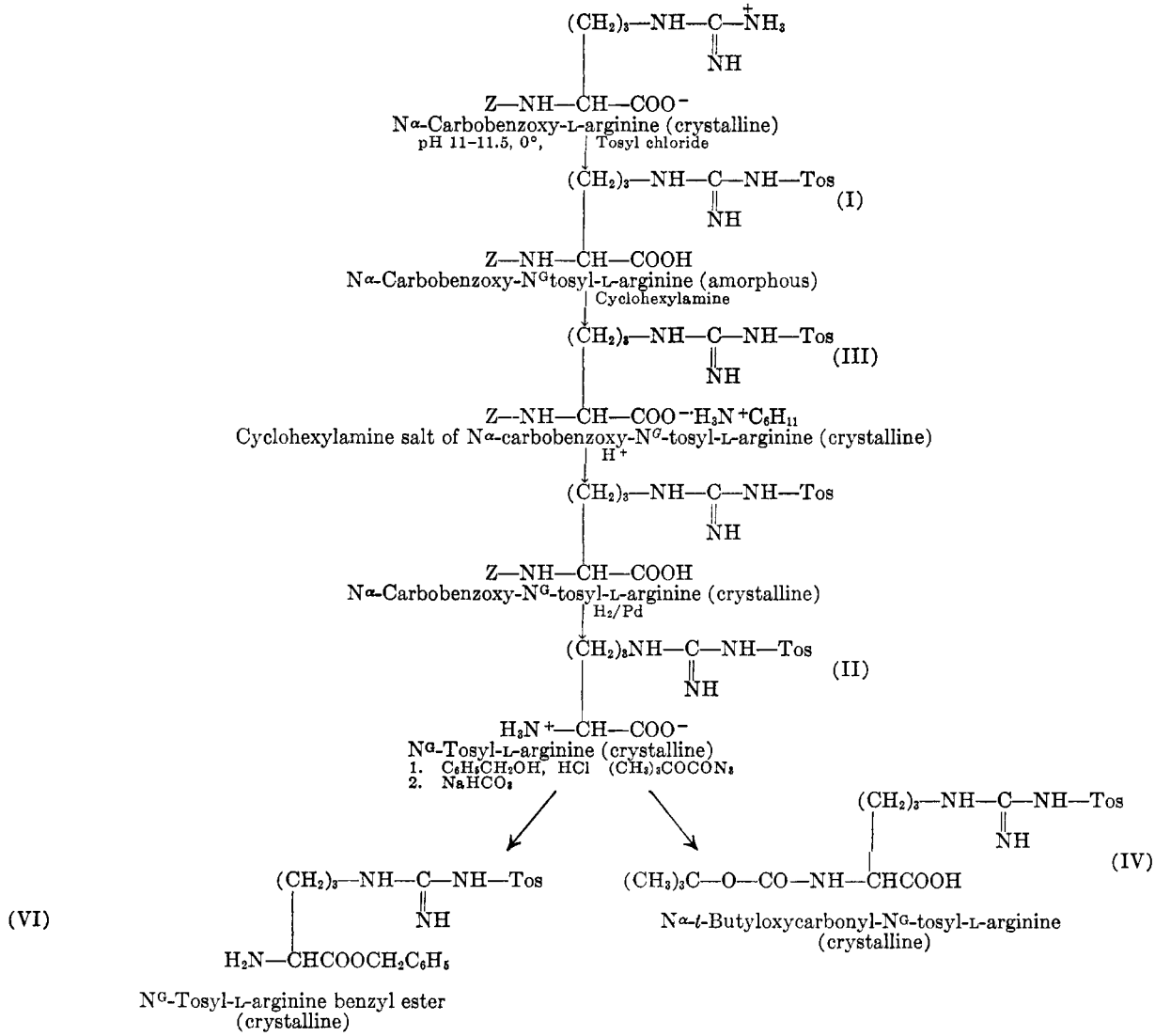

Fig. 1.—Synthetic scheme of various arginine derivatives. Z, Carbobenzoxy-; Tos, tosyl.

tive could not be obtained in crystalline form, and the amorphous material was used in these syntheses. The use of the tosyl group for the protection of the guanidino group now has been reinvestigated, and this paper describes the preparation of several crystalline derivatives of arginine and their use in peptide synthesis.

The tosylation of N^{α}-carbobenzoxy-L-arginine[25] was carried out in acetone–water solution by a procedure similar to that of Schnabel and Li,[31] with some modifications. The pH was maintained at pH 11–11.5 during the reaction and an excess of tosyl chloride was used. The product N^{α}-carbobenzoxy-N^G-tosyl-L-arginine (I) was obtained in amorphous form.

Product I was hydrogenated in the presence of freshly prepared palladium to yield N^G-tosyl-L-arginine (II). II could be crystallized from hot water. Recarbobenzoxylation of II in bicarbonate buffer still yielded I in an amorphous state. However, the cyclohexylamine salt (III) of I was prepared[33] and could be crystallized from methanol–ether. Regeneration of I from III yielded a product which resisted all attempts at crystallization.

By reaction with *t*-butyloxycarbonyl azide,[34,35] II was converted to the N^{α}-*t*-butyloxycarbonyl-N^G-tosyl-L-arginine (IV) which was crystallized from ethyl acetate. Upon cross-seeding a solution of I in ethyl acetate with crystals of IV, crystallization of I was induced. Thus, a standard procedure for the synthesis of *crystalline* N^{α}-carbobenzoxy-N^G-tosyl-L-arginine from N^{α}-carbobenzoxy-L-arginine through the cyclohexylamine salt has been developed; it has been found that it is preferable to proceed through III, since the crystallization of

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I prior to purification by salt formation is rather difficult. I has been used for the synthesis of crystalline N^{α}-carbobenzoxy-N^{G}-tosyl-L-arginyl-L-proline benzyl ester in 85% yield, with Woodward's reagent[36] used as the coupling agent.

Product I was obtained in an overall yield of 42–48%, whereas IV could be prepared in 86% yield from II. Esterification of II with benzyl alcohol and hydrogen chloride yielded a hydrochloride salt (V) of N^{G}-tosyl-L-arginine benzyl ester (VI) in the form of a hygroscopic oil. V was directly treated with ice-cold sodium bicarbonate and VI was extracted into ethyl acetate. VI also was obtained in crystalline form from ethyl acetate. Figure 1 summarizes the various reactions involving arginine that are mentioned herein.

In order to establish that the conditions of tosylation did not result in partial racemization of the product I, crystalline I was reduced with sodium in liquid ammonia.[37] Paper chromatograms of the reduced material in the solvent system consisting of butanol–pyridine–acetic acid–water (30–20–6–24 by volume; designated BPAW) revealed a single spot corresponding to arginine. This spot gave positive reactions with ninhydrin and the Sakaguchi reagent. Analysis of an aliquot in the Amino Acid Analyzer[38] showed a single peak corresponding to arginine with no trace of II.[39] The optical rotation of the product was $[\alpha]^{25}D$ + 27.3° (c I, 1.7, 6 *N* HCl). Arginine used in the preparation of I exhibited a rotation of $[\alpha]^{25}D$ + 27.2° (c 2, 5 *N* HCl). Thus, it is clear that the guanidino group of arginine can be safely protected with the tosyl group and the protecting group can be removed readily at the end of the synthesis without racemization by reduction with sodium in liquid ammonia.

The removal of the tosyl group by treatment with sodium in liquid ammonia is incomplete only when there are several such groups to be removed.[40,41] Hence, the use of a group such as the *t*-butyloxycarbonyl group for the protection of basic functions other than those of arginine, in conjunction with the use of the tosyl group for masking the guanidine group of arginine, might well prove to be the desirable approach for the protection of side chain functions in long peptides. This also will permit the selective removal of one or other of the groups protecting the basic functions, thus allowing evaluation of the relative importance of lysine and arginine residues in biologically active polypeptides.

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Experimental[42]

Cyclohexylamine Salt of N^{α}-Carbobenzoxy-N^{G}-tosyl-L-arginine.—N^{α}-Carbobenzoxy-L-arginine[25] (25 g., 81 mmoles) was suspended in a mixture of 100 cc. of water and 400 cc. of acetone, cooled to 0°, and stirred vigorously. Sufficient 4 *N* sodium hydroxide (percooled to 0°) was added to maintain the pH at 11–11.5 (checked by a pH meter). The suspension was completely dissolved in 1.5–2 hr. *p*-Toluenesulfonyl chloride (38 g., 200 mmoles) dissolved in 60 cc. of acetone was added dropwise during a period of 30 min. Stirring was continued and the pH was maintained at 11–11.5 by the addition of pre-cooled 4 *N* sodium hydroxide. The clear solution was stirred at 0° for another 3 hr. The pH then was adjusted to 7 with 1 *N* hydrochloric acid. Acetone was removed *in vacuo* at room temperature. Water (200 cc.) was added to the residual solution and the resulting aqueous solution was extracted with ether (3 × 150 cc.). The aqueous layer then was cooled to 0° and acidified to pH 3 with 6 *N* hydrochloric acid. A thick white oil separated. The supernatant was removed, saturated with sodium chloride, and extracted with ethyl acetate (3 × 150 cc.). The oily residue was dissolved in the combined ethyl acetate extracts and the ethyl acetate layer was washed repeatedly with cold 0.1 *N* hydrochloric acid until the acid wash gave a negative reaction to the Sakaguchi test. The organic phase then was washed with water until neutral, dried over anhydrous sodium sulfate, and evaporated to dryness *in vacuo* at room temperature to yield 30 g. of a thick oily residue. This was dissolved in 100 cc. of methanol, the solution was cooled to 0°, and 7 cc. of cyclohexylamine (approximately 70 mmoles—the amount of cyclohexylamine is determined by the weight of the oily residue) was added. Ether was added until the solution became faintly cloudy, and the solution was cleared by the addition of a few drops of methanol. The cyclohexylamine salt of N^{α}-carbobenzoxy-N^{G}-tosyl-L-arginine began to crystallize on scratching. The salt was filtered after being kept at 4° for 2 days, and the filtrate was washed with ice-cold methanol and dried; yield: 22.1 g. (48.6%); m.p. 152–154°; $[\alpha]^{25}D$ 6.1° (c 3.2, methanol). A sample was recrystallized for analysis from methanol–ether, m.p. 152–154°.

Anal. Calcd. for $C_{27}H_{30}N_6O_6S$ (561.7); 57.7 C, H, 7.00; N, 12.5; S, 5.71. Found: C, 57.8; H, 6.93; N, 12.5; S, 5.91.

N^{α}-Carbobenzoxy-N^{G}-tosyl-L-arginine.—The above cyclohexylamine salt (20.7 g., 35 mmoles) was dissolved in 150 cc. of methanol by warming. The solution was cooled in ice and stirred with 30 cc. of 2 *N* hydrochloric acid (60 mmoles) for 1 hr. Methanol then was removed *in vacuo* at 20° and 200 cc. of water was added. The aqueous solution was extracted with ethyl acetate (4 × 150 cc.); the ethyl acetate extract was washed with water until neutral, dried over anhydrous sodium sulfate, and evaporated to dryness *in vacuo*. The residue was again dissolved in 150 cc. of warm ethyl acetate and crystallized upon cooling; yield: 15.5 g. (95.8%) based on the cyclohexylamine salt; overall yield, 46.6%. In several preparations the overall yield varied from 40–50%; m.p. 86–89°; $[\alpha]^{25}D$ −0.5° (c 7.5, methanol); $[\alpha]^{25}D$ −1.3° (c 4, dimethylformamide); R_{fBAW} 0.83.

Anal. Calcd. for $C_{21}H_{26}N_4O_6S$ (462.5): C, 54.5; H, 5.67; N, 12.1; S, 6.93. Found: C, 54.8; H, 5.91; N, 12.2; S, 6.94.

(42) Melting points were performed on a Fisher-Johns melting point apparatus and are uncorrected. Microanalyses were performed by the Microanalytical Laboratory of Department of Chemistry in this University. All samples for microanalyses were dried in an Abderhalden drying pistol with P_2O_5 at 77° for 16 hours at 0.3-mm. pressure. Paper chromatography was carried out on Whatman no. 1 filter paper at room temperature; the solvents used were 1-butanol–acetic acid–water (BAW) in a ratio of 4:1:1, 2-butanol–10% NH_3 (SBA) in a ratio of 85:15; 1-butanol–pyridine–acetic acid–water (BPAW) in a ratio of 30:20:6:24, and 3% NH_3–2-butanol (ASB) in a ratio of 44:100.

N^G-Tosyl-L-arginine.—N^α-carbobenzoxy-N^G-tosyl-L-arginine (9.25 g, 20 mmoles) was dissolved in 100 cc. of methanol and hydrogenated in the presence of palladium freshly prepared[43] from 1 g. of palladium chloride. A Vibro-mixer[44] was used for stirring. When carbon dioxide evolution stopped, the solution was filtered from the catalyst, the catalyst was washed with methanol, and the combined filtrates were evaporated to dryness. When the residue was dissolved in 80 cc. of hot water and filtered, crystals of N^G-tosyl-L-arginine began to form. After the filtrate had been kept overnight at room temperature, the crystals were filtered and dried; yield: 5.6 g. (85.3%); m.p. 146–150°. Recrystallization from hot water did not change the melting point. $[\alpha]^{25}D$ −5.5° (c 1.3, methanol); $[\alpha]^{25}D$ +5° (c 2, dimethylformamide); R_{fBAW} 0.34; R_{fSBA} 0.07; R_{fBPAW} 0.62; R_{fASB} 0.47, (ninhydrin, chlorine positive; Sakaguchi negative).

Anal. Calcd. for $C_{13}H_{20}N_4O_4S$ (328.4): C, 47.5; H, 6.11; N, 17.1; S, 9.77. Found: C, 47.5; H, 6.18; N, 17.2; S, 10.0.

N^α-*t*-Butyloxycarbonyl-N^G-tosyl-L-arginine.—N^G-Tosyl-L-arginine (9.85 g., 30 mmoles) was mixed with 2.42 g. (60 mmoles) of magnesium oxide and the mixture was stirred at 45–50° for 1 hr. in 200 cc. of 50° aqueous dioxane. *t*-Butyloxycarbonyl azide (8.6 g., 60 mmoles), freshly prepared from *t*-butyloxycarbonyl hydrazide by the procedure of Carpino,[34] was added and the stirring was continued for 30 hr. at 45–50°. The solution then was diluted with 300 cc. of water, filtered free of traces of insoluble material, and extracted with ethyl acetate (2 × 60 cc.). The aqueous phase was cooled in ice and acidified to pH 4 with ice-cold 10% citric acid. The aqueous solution was saturated with sodium chloride and extracted with ethyl acetate (3 × 150 cc.). The ethyl acetate extracts were washed with saturated sodium chloride solution, dried over anhydrous sodium sulfate, and evaporated to dryness. The residue was redissolved in 200 cc. of ethyl acetate. Crystals appeared on scratching. The ethyl acetate solution was kept at 4° for 24 hours, filtered, and dried to give 11 g. (86%) of N^α-*t*-butyloxycarbonyl-N^G-tosyl-L-arginine, m.p. 98–99°. A sample was recrystallized from ethyl acetate; m.p. 99–100°; $[\alpha]^{26}D$ −3.3° (c 4, dimethylformamide); R_{fBAW} 0.83.

Anal. Calcd. for $C_{18}H_{28}N_4O_6S$ (428.5): C, 50.5; H, 6.59; N, 13.1; S, 7.48. Found: C, 50.3; H, 6.75; N, 13.0; S, 7.19.

N^G-Tosyl-L-arginine Benzyl Ester.—N^G-Tosyl-L-arginine (4.92 g., 15 mmoles) was added to 70 cc. benzyl alcohol saturated with dry hydrogen chloride, and stirred at room temperature. The solution again was saturated with dry hydrogen chloride after 6 hr. The excess hydrogen chloride was removed after 24 hr. by means of a water pump and the residual solution was treated with 200 cc. of dry ether. The oily residue was washed twice with ether by decantation. The sirup then was dissolved in 50 cc. of water, the solution was cooled to 0°, and the pH was brought to 10 with ice-cold 50% potassium carbonate. The benzyl ester was extracted into ethyl acetate (3 × 50 cc.). The organic phase was washed with water, dried over anhydrous sodium sulfate, and evaporated to dryness *in vacuo* at room temperature. The residue was dissolved in 50 cc. of ethyl acetate, and petroleum ether was added until the solution became faintly cloudy. The solution was cleared by the addition of a few drops of ethyl acetate. Crystals appeared on scratching. After they were kept for 2 days at 4°, N^G-tosyl-L-arginine benzyl ester was isolated in 56% yield (3.5 g.), m.p. 72–73°. A sample was recrystallized from ethyl acetate–petroleum ether, m.p. 74–75°. The m.p. was unaltered on further recrystallization. $[\alpha]^{25}D$ +3.3° (c 2, dimethylformamide); R_{fBAW} 0.68; R_{fSBA} 0.70.

Anal. Calcd. for $C_{20}H_{26}N_4O_4S$ (418.5): C, 57.4; H, 6.26; N, 13.4; S, 7.66. Found: C, 57.2; H, 6.44; N, 13.4; S, 7.52.

Sodium–Liquid Ammonia Reduction of N^α-Carbobenzoxy-N^G-tosyl-L-arginine.—N^α - Carbobenzoxy - N^G - tosyl - L-arginine (0.463 g., 1 mmole) was dissolved in 100 cc. of liquid ammonia freshly distilled from sodium, and small pieces of sodium were added with stirring until a blue color persisted for 30 min. Ammonia was allowed to evaporate. The residue was dried overnight over phosphorus pentoxide and concentrated sulfuric acid. It was dissolved in cold water and brought to a volume of 10 cc. An aliquot was analyzed on the short column of the Amino Acid Analyzer.[37] A single peak corresponding to arginine and a trace of ammonia were seen. No evidence of the presence of unreduced N^G-tosyl-L-arginine was found.

The optical rotation of the arginine obtained by reduction was found to be $[\alpha]^{25}D$ +27.3° (c 1.7, 6 *N* HCl). The rotation of the arginine used in the preparation of N^α-carbobenzoxy-N^G-tosyl-L-arginine was $[\alpha]^{25}D$ +27.2 (c 2, 5 *N* HCl). Lit.,[45] $[\alpha]^{23}D$ +26.9° (c 1.65, 6 *N* HCl).

The reduced product was found to be homogeneous in paper chromatography in two solvent systems and gave positive reactions with ninhydrin and in the Sakaguchi test. R_{fBPAW} 0.10; R_{fBAW} 0.08.

N^α-Carbobenzoxy-N^G-tosyl-L-arginyl-L-proline Benzyl Ester.—N^α-Carbobenzoxy-N^G-tosyl-L-arginine, 0.462 g. (1 mmole) was dissolved in 10 cc. of acetonitrile and then cooled to 0° in an ice water bath. Triethylamine, 0.101 g., (1 mmole), and Woodward's reagent K, 0.260 g. (1 mmole), were added and the mixture stirred at 0° for 1 hr. Then 0.25 g. of L-proline benzyl ester hydrochloride[46] (1 mmole) and 0.101 g. triethylamine (1 mmole) were added and the mixture was stirred overnight at room temperature. A clear solution resulted, which was then evaporated to dryness *in vacuo*, and the residue was dissolved in ethyl acetate (40 cc.). The ethyl acetate solution was then washed successively with water, 0.1 *N* hydrochloric acid, water, 7.5% sodium bicarbonate, and water. The ethyl acetate solution then was dried over anhydrous sodium sulfate, concentrated *in vacuo*, and then precipitated from petroleum ether, wt. 0.55 g. (85%). The product was homogeneous in paper chromatography. $R_{fBAW} = 0.86$ $R_{fSBA} = 0.85$.

The peptide ultimately crystallized from ethanol in the refrigerator at 0°, m.p. = 78–80° (recrystallized from ethanol, m.p. 80–82°); $[\alpha]^{25}D$ −39.3° (c 1, MeOH).

Anal. Calcd. for $C_{33}H_{40}N_6O_7S$ (649.8): C, 61.0; H, 6.22; N, 10.8; S, 4.82. Found: C, 60.4; H, 6.31; N, 10.4; S, 5.22.

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