TOSYLATED PEPTIDES AND *p*-NITROPHENYL ESTERS[1]

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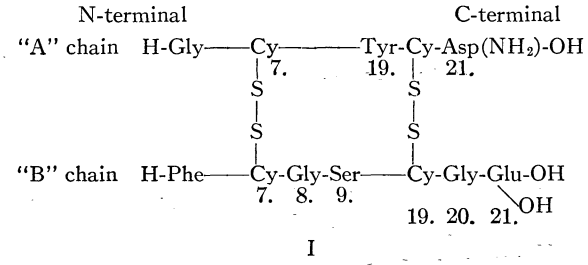
ABSTRACT

The tosyl group causes interference with mixed carbonic anhydride and *p*-nitrophenyl ester formation in certain instances. Several protected peptide sequences, occurring in the insulin molecule, and some tosylated *p*-nitrophenyl esters, have been prepared in the course of investigating this interference.

INTRODUCTION

At the inception of this work, we were interested in preparing certain peptide sequences which occur in the insulin molecule (1). Specifically, we were interested in preparing three peptides which occur in the vicinity of the interchain disulphide bridge of insulin, namely tyrosyl-cysteinyl-asparagine, cysteinyl-glycyl-glutamic acid, and cysteinyl-glycyl-serine, hereinafter abbreviated respectively as H-Tyr-Cy(SH)-Asp(NH_2)-OH, H-Cy(SH)-Gly-Glu(OH)-OH, H-Cy(SH)-Gly-Ser-OH, according to the system of Brand (2). A portion of the insulin molecule is reproduced in I. We were particularly interested in



I

these peptides, because we believed, for reasons which will not be delineated here, that the site of the hypoglycemic activity of insulin might be those peptides sequences in the vicinity of the interchain disulphide bridge (3).

In our search for a suitable method of "blocking" the amino group of the various amino acids, we were attracted to the tosyl blocking group, because of the general ease of preparation of tosylamino acids, and the ready crystallinity of such derivatives (4), particularly N-tosylated glutamic acid (5). Furthermore, it seemed desirable to attempt to limit ourselves to one amino blocking group and one method of forming the peptide bond. Indeed such an objective has been achieved in at least two syntheses of oxytocin (6, 7). For our purpose, we chose to use the tosyl blocking group, and to form the peptide bond by the mixed carbonic anhydride method (8). Later, we were forced to modify our original plan, such that we had to use other blocking groups, such as the well known "carbobenzoxy" (9) group, and other methods of forming peptide bonds, such as the N,N′-dicyclohexylcarbodiimide (10) method, because of the failure of the mixed anhydride method with N^{α}-tosylamino acids in certain instances, reported previously (11).

[1]*This paper was presented in abridged form at the 29th meeting of l'ACFAS at Ottawa (Canada), October 27–29, 1961.*

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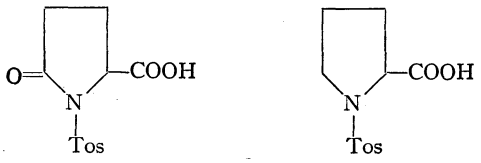
Ultimately, therefore, our main interest became the investigation of the factors influencing peptide bond formation, by various methods, with N^{α}-tosylamino acids.

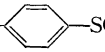
In this paper, we report on the formation of peptide bonds and *p*-nitrophenyl esters of N-tosylamino acids and peptides, and propose a general basis for predicting when either of the foregoing reactions will occur.

DISCUSSION

In Table I, we have tabulated the results of our own work and those of other workers, in the formation of certain peptide sequences and derivatives, using various amino blocking groups. It can be seen that in the case of the tosyl blocking group, as contrasted with the carbobenzoxy and phthaloyl groups, peptide bond or *p*-nitrophenyl ester formation fails in every instance where the tosyl group is alpha to the carboxyl group being reacted with, except in the case of N-tosyl-L-pyroglutamic acid, II, and N-tosyl-L-proline, III. The significance of the structure of these two tosylamino acids

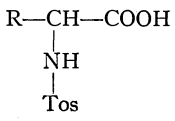


II III

(Tos = tosyl = CH_3—  —SO_2—)

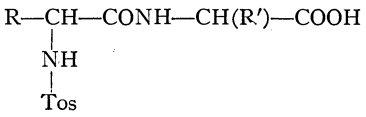
will be discussed later. In order to rule out the possibility of steric hindrance being a factor in the failure of the mixed anhydride method with the N^{α}-tosylamino acids, we prepared (or report numerous instances from the literature) sequences in which the amino acid residues are C-terminal in one instance and N-terminal in another. It is clear, from a study of Table I, that the failure of the desired reactions, in the case of tosylamino acids is ascribable to the tosyl group.

If one examines the structure of the tosylamino derivatives in which mixed anhydride or *p*-nitrophenyl ester formation *fails*, one will see that, in general, the derivatives have the structure IV. On the other hand, those instances where these reactions are successful,



IV

have the structure II, III, or V. It is evident, therefore, *that if the tosyl group is alpha*



V

to the carboxyl group undergoing activation, and if there is a hydrogen atom on the nitrogen atom to which the tosyl group is attached, there will be interference with *both* of the aforementioned reactions; otherwise the tosyl group does *not* cause interference with *either* of the reactions. This represents, in our view, an important extension of the conclusion

TABLE I

No.	Sequence attempted	Protecting group: Amino	Protecting group: Carboxyl	Method	Result	Reference
IX	Cy(SBz)*Asp(NH_2)	Cbz	Na salt	$ClCO_2Et$	+ve	This paper, 15
		Tos	Na salt	$ClCO_2Et$	−ve	T.M.
		Tos	Mg salt	Acid chloride	−ve	T.M.
X	Glu(NH_2)*Asp(NH_2)	Tos	Mg salt	Pyroglutamyl chloride	+ve	16
XI	Cy(SBz)-Tyr*Phe-Glu(NH_2)-Asp(NH_2)	Tos	Et_3N salt	$ClCO_2Et$	+ve	17
XII	Cy(SBz)*Gly	Tos	Et	$ClCO_2Et$	−ve	11
		Cbz	Et	$ClCO_2Et$	+ve	T.M., 18
		Tos	Et	DCC	+ve	11
		Tos	Et	Acid chloride	+ve	11
		Tos	PNB	DCC	+ve	This paper
XIII	Phe*Cy(SBz)	Phth	Et	$ClCO_2Et$	+ve	11
XIV	Tyr*Cy(SBz)	Tos	Et	$ClCO_2Et$	−ve	11
		Tos	Et	DCC	+ve	11
XV	π-Glu*Gly	Tos	Et	$ClCO_2Et$	+ve	This paper
		Tos	Et	Pyroglutamyl chloride	+ve	5
XVI	π-Glu*Cy(SBz)	Tos	Et	$ClCO_2Et$	+ve	This paper
		Tos	Et	Pyroglutamyl chloride	+ve	19
XVII	Cy(SBz)-Gly*Glu(OMe)	Tos	Na salt	$ClCO_2Et$	+ve	T.M.
XVIII	Cy(SBz)-Gly*Ser	Tos	Et	$ClCO_2Et$	+ve	This paper
XIX	Gly*PNP	Tos	Free	DCC	−ve	This paper
		Phth	Free	DCC	+ve	T.M.
XX	Cy(SBz)*PNP	Tos	Free	DCC	+ve	This paper
		Tos	Free	$ClCO_2Et$	−ve	T.M.
		Cbz	Free	DCC	+ve	7
XXI	π-Glu*PNP	Tos	Free	DCC	+ve	This paper
XXII	Pro*PNP	Tos	Free	DCC	+ve	This paper
XXIII	Cy(SBz)-Gly*PNP	Tos	Free	DCC	+ve	This paper

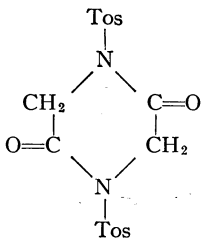
NOTE: Asterisk (*) denotes formation of a new bond; T.M. = unpublished results of one of the authors; Bz = benzyl; Cbz = carbobenzoxy; DCC = N,N′-dicyclohexylcarbodiimide; Et = ethyl; Me = methyl; PNB = *p*-nitrobenzyl; Phth = phthaloyl; PNP = *p*-nitrophenyl; Tos = tosyl = *p*-toluenesulfonyl; +ve denotes a successful reaction; −ve denotes an unsuccessful reaction.

See reviews by Fruton (20), Shapiro (21), Wieland (22), du Vigneaud (23), Springall and Law (24), Goodman and Kenner (25).

which Zaoral and Rudinger (12) had made regarding the *mixed carbonic anhydride method*. Furthermore, to the best of our awareness, the case of tosylated *p*-nitrophenyl ester formation does not seem to have been dealt with in the published literature.

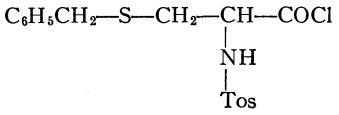
From the present work, and that of others (12, 13), we know, now, that we can form mixed carbonic anhydrides, and thence peptides, of N-tosylamino acids and peptides such as II, III, or V in the conventional manner (8), while mixed anhydrides of tosylamino acids such as IV may be prepared using pivaloyl chloride (13).

Each of the N^{α}-tosylamino acids investigated reacted with an ester of another amino acid to form dipeptides, in the presence of N,N′-dicyclohexylcarbodiimide (10). In contrast to this, only *certain* N^{α}-tosylamino acids formed *p*-nitrophenyl esters, even though the expected coproduct, N,N′-dicyclohexylurea (10), was observed to have formed in *every* case. The reaction of N-tosylglycine, IV (R = H), and N,N′-dicyclohexylcarbodiimide, alone and in the presence of an equimolar amount of each of these ingredients and *p*-nitrophenol, was investigated. In the former instance, N,N′-dicyclohexylurea and the diketopiperazine, VI, arising from N-tosylglycine, IV (R = H), were isolated in essentially quantitative yield. In the latter instance, the same two products were isolated, and 90% of the initial amount of *p*-nitrophenyl was recovered, unreacted. Since diketopiperazines were never detected among the reaction products, in the case of dipeptide formation, it would seem that they are *not* precursors of the dipeptides. Diketopiperazine formation appears to compete successfully with tosylated *p*-nitrophenyl ester formation, however, in those cases where one would predict interference with the desired reaction on the basis of the hypothesis enunciated previously.

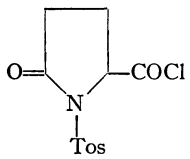


VI

The divergent behavior of N-tosyl-S-benzyl-L-cysteinyl chloride, VII, and N-tosyl-L-pyroglutamyl chloride, VIII (see Table I), towards the magnesium salt of L-asparagine



VII



VIII

is unexplained, but it may represent another aspect of the peculiar role of the amino hydrogen atom (12) under the influence of the tosyl group. Since the mechanism of Beecham (14) does not appear to be applicable to the foregoing case, it seems likely that the probable product from VII was the diketopiperazine analogous to VI. No attempt was made to identify the reaction product, however, once it was shown to be a product other than the one expected.

EXPERIMENTAL[3, 4]

N-Carbobenzoxy-S-benzyl-L-cysteinyl-L-asparagine, IX

This compound was prepared from N-carbobenzoxy-S-benzyl-L-cysteine (26) (10.35 g; 0.03 mole) and L-asparagine (3.96 g; 0.03 mole) by the mixed carbonic anhydride method, as reported by Leach and Lindley (15). The yield of the product was 6.5 g (47%), after recrystallization from boiling methanol (150 ml); m.p. 157–158° C (lit. (15): 180° C), $[\alpha]_D^{22}$ −32.7 (*c*, 2.43; *N,N*-dimethylformamide). Anal. Calc. for $C_{22}H_{25}O_6N_3S$ (459.5): C, 57.50; H, 5.48; N, 9.15%. Found: C, 57.87; H, 5.47; N, 9.21%.

N-Tosyl-S-benzyl-L-cysteinyl-glycine p-Nitrobenzyl Ester, XII

Glycine *p*-nitrobenzyl ester hydrobromide (27) (5.39 g; 18.5 mmoles) was dissolved in chloroform (75 ml) containing triethylamine (2.59 ml; 18.5 mmoles), and the solution was stirred for 10 minutes. N-Tosyl-S-benzyl-L-cysteine (17) (6.76 g; 18.5 mmoles) was added, the reaction mixture was cooled to 0° C, treated with a solution of *N,N*-'dicyclohexylcarbodiimide (3.82 g; 18.5 mmoles) in dry tetrahydrofuran (25 ml) and stirred for 30 minutes in the cooling bath. The viscous suspension was then transferred to a Burrell wrist action shaker, and agitated for 16 hours at room temperature. The precipitated N,N'-dicyclohexylurea (2.24 g; 57% of the theoretical) was filtered, and the filtrate was concentrated to a semisolid oil *in vacuo*. This oil was dissolved in chloroform (75 ml), and the resulting solution was washed successively with water, 1 *N* sodium bicarbonate, 1 *N* hydrochloric acid, water, and then it was dried over sodium sulphate. Petroleum ether (b.p. 65–110° C) was added to opalescence, and the product was allowed to crystallize at room temperature. The product, thus obtained, weighed 5.57 g (54%), m.p. 116–117° C. Further recrystallizations raised the melting point to a constant value of 118–119° C. $[\alpha]_D^{25}$ −3.12 (*c*, 1.41 in chloroform). Anal. Calc. for $C_{26}H_{27}O_7N_3S_2$ (557.6): C, 56.00; H, 4.88; N, 7.54%. Found: C, 55.62; H, 4.64; N, 7.16%.

N-Tosyl-L-pyroglutamyl-glycine Ethyl Ester, XV

To a stirred solution of N-tosyl-L-pyroglutamic acid (28) (2.83 g; 0.01 mole) in chloroform (30 ml), cooled to 0° C, was added triethylamine (1.4 ml; 0.01 mole) and ethyl chloroformate (0.95 ml; 0.01 mole). After 10 minutes a precooled solution of glycine ethyl ester (prepared from 1.39 g, 0.01 mole, of the hydrochloride and 1.4 ml; 0.01 mole, of triethylamine) in methylene chloride (40 ml) – chloroform (10 ml) was added. The solution was stirred for 30 minutes at 0° C, and 1.5 hours at room temperature, then it was washed to neutrality in the usual way, and dried over magnesium sulphate. The washed and dried solution was evaporated to an oil *in vacuo*, and the oily residue was taken up in 95% ethanol (5 ml) with slight warming. The yield of recrystallized product was 2 g (54%), m.p. 131–132° C (lit. (5): 132–133° C); $[\alpha]_D^{28}$ −21.8 (*c*, 1.19 in dioxane).

N-Tosyl-L-pyroglutamyl-S-benzyl-L-cysteine Ethyl Ester, XVI

This compound was prepared exactly as in the preceding case, from N-tosyl-L-pyroglutamic acid (28) (2.83 g; 0.01 mole) and S-benzyl-L-cysteine ethyl ester (20) (from 2.77 g; 0.01 mole of the hydrochloride).

The crude product was recrystallized from boiling absolute ethanol (2.0 ml) to give 2.21 g (43.8%) of the pure product, m.p. 145–146° C (lit. (19): 142–145° C), $[\alpha]_D^{28}$ −16.7 (*c*, 1.19; dioxane) (lit. (19): $[\alpha]_D^{21.5}$ −17 (*c*, 1.8; dioxane)).

N-Tosyl-S-benzyl-L-cysteinyl-glycyl-L-serine Ethyl Ester, XVIII

A solution of the mixed anhydride of N-tosyl-S-benzyl-L-cysteinyl-glycine (11) (2.12 g; 5 mmoles) in dry tetrahydrofuran (20 ml) was prepared as in XV. To this solution was added a precooled suspension of L-serine ethyl ester (prepared from 0.857 g; 5 mmoles of the hydrochloride (29), on treatment with triethylamine). After being stirred for 30 minutes in the cooling bath and overnight at room temperature, the reaction mixture was evaporated to dryness in a rotary evaporator. The residue was dissolved in boiling ethyl acetate, the resulting solution was filtered, and the filtrate was evaporated to dryness. The semicrystalline oil was dissolved in 10 ml each of warm absolute ethanol and ether. Petroleum ether (b.p. 30–52° C) was added to the solution until it became opalescent. Crystallization of the product was induced by scratching. The yield of crude product was 1.76 g (65%).

For analysis, the product was recrystallized from ethanol–water; m.p. 147.5–148.5° C, $[\alpha]_D^{23.5}$ −30.6 (*c*, 1.37 in ethanol). Anal. Calc. for $C_{24}H_{31}O_7N_3S_2$ (537.6): C, 53.61; H, 5.81; N, 7.82%. Found: C, 53.12; H, 5.78; N, 7.97%.

Products from the Attempted Preparation of p-Nitrophenyl-N-tosylglycinate, XIX

N-Tosylglycine (30) (2.29 g; 0.01 mole) and *p*-nitrophenol (1.39 g; 0.01 mole) were dissolved in a mixture of methylene chloride (100 ml) – dimethylformamide (10 ml), and the solution was cooled to 0° C. N,N'-Dicyclohexylcarbodiimide (2.06 g; 0.01 mole) was then added. The reaction mixture was stirred for 30 minutes at 0° C, and overnight at room temperature, prior to being filtered. The residue (3.6 g) "r_1" was

[3]*Melting points were determined in capillary tubes and are uncorrected.*

[4]*Microanalyses were performed by Dr. C. Daesslé, Organic Microanalyses, 5757 Decelles St., Montreal, Que.*

[5]*We were unable to obtain the melting point reported in reference 15, despite repeated recrystallization of the product or repetition of the preparation.*

retained. On concentrating the filtrate, *in vacuo*, there was obtained a semicrystalline residue "r_2", which was treated with anhydrous ether (50 ml) and filtered. The ether-insoluble residue "r_3" was combined with "r_1". These combined residues ("r_1"+"r_3") were treated with hot absolute ethanol (50 ml), filtered, and the alcohol-insoluble portion "r_4" was weighed (1.7 g; 80%), m.p. 260° C. For analysis, "r_4" was recrystallized from either pyridine or dimethylformamide–ether, m.p. 262° C (lit. (12, 31): 295–296, 275° C), then dried *in vacuo* at 100° C for at least 4 hours. The analysis of the fraction "r_4" corresponded to that for 1,4-ditosylpiperazine-2,5-dione, VI. Anal. Calc. for $C_{18}H_{18}N_2O_6S_2$: N, 6.63%. Found: N, 6.83%.

From the hot alcoholic solution, there separated in an essentially quantitative yield (2.1 g), a product, m.p. 213–215° C, raised to 230–232° C (undepressed by admixture with authentic N,N-'dicyclohexylurea), on recrystallization from hot dimethylformamide. The product gave the correct analysis for N,N'-dicyclohexylurea. Anal. Calc. for $C_{13}H_{24}ON_2$: N, 12.49%. Found: N, 12.61%.

p-Nitrophenol was recovered on evaporation of the ether solution, followed by recrystallization of the residue from toluene. Alternatively, the isolation was achieved by extracting the *initial* filtrate with three portions of 1 *N* sodium hydroxide, acidifying the aqueous alkaline extract with 1 *N* hydrochloric acid, and extracting the acidified solution with ether. Evaporation of the ether, followed by recrystallization of the residue from toluene, gave 1.2 g (86%) of *p*-nitrophenol, m.p. and mixed m.p. 114° C.

N-Tosyl-L-pyroglutamic Acid p-Nitrophenyl Ester, XXI

N-Tosyl-L-pyroglutamic acid (28) (2.83 g; 0.01 mole) and *p*-nitrophenol (1.39 g; 0.01 mole) were dissolved in dry tetrahydrofuran (50 ml). The solution was cooled to 0° C. N,N'-Dicyclohexylcarbodiimide (2.06 g; 0.01 mole) was added, and the reaction mixture was stirred for 30 minutes at 0° C, and for 16 hours at room temperature. The precipitated N,N'-dicyclohexylurea was filtered, and the filtrate was concentrated to a semicrystalline oily residue, *in vacuo*, at about 40° C. The residue was dissolved in a minimum of absolute ethanol, and the resulting solution was filtered. Crystallization of the product was allowed to proceed at room temperature. The yield of recrystallized product was 1.82 g (45%), m.p. 126–128° C (colorless needles), $[\alpha]_D^{24}$ −19.8 (*c*, 1.21 in dimethylformamide). Anal. Calc. for $C_{18}H_{16}O_7N_2S$ (404.4): N, 6.93%. Found: N, 7.16%.

N-Tosyl-L-proline p-Nitrophenyl Ester, XXII

This compound was prepared exactly as in the above case, from N-tosyl-L-proline hemibenzenate (32) (3.06 g; 0.01 mole) and *p*-nitrophenol (1.39 g; 0.01 mole). The yield of the product was 2.90 g (74.8%), m.p. 105–106° C (from ethanol), $[\alpha]_D^{27}$ −106 (*c*, 1.43 in dioxane). Anal. Calc. for $C_{18}H_{18}O_6N_2S$ (390.4): N, 7.18%. Found: N, 7.24%.

N-Tosyl-S-benzyl-L-cysteinyl-glycine p-Nitrophenyl Ester, XXIII

To a stirred, ice-cooled solution of N-tosyl-S-benzyl-L-cysteinyl-glycine (11) (2.12 g; 5 mmoles) and *p*-nitrophenol (0.84 g; 6 mmoles) in dry tetrahydrofuran (20 ml) was added N,N'-dicyclohexylurea (1.24 g; 6 mmoles). The reaction mixture was stirred for 30 minutes in the cooling bath, and 3.5 hours at room temperature. The precipitated dicyclohexylurea was filtered, and the filtrate was evaporated to dryness *in vacuo*. The residue was triturated under ether, then it was dissolved in a minimum of boiling absolute alcohol (5 ml). Almost immediately, the product crystallized out. It was washed with ether then dried at the water pump. The yield of the product was 1.40 g (52%), m.p. 144–145° C, $[\alpha]_D^{26}$ +17.5 (*c*, 1.66; dimethylformamide). Anal. Calc. for $C_{25}H_{25}O_7N_3S_2$ (543.6): C, 55.23; H, 4.64; N, 7.73%. Found: C, 54.91; H, 4.86; N, 8.00%.

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