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Tritylation of a Partially Protected Pentapeptide Synthesized by the Merrifield Solid Phase Method*

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The solid phase synthesis according to Merrifield of the partially protected pentapeptide, Asp(OBzlNO_2)-Glu(OBzlNO_2)-Ala-Asp-(OBzlNO_2)-Pro, in 55 % yield, is described. Tritylation of the *C*-terminal carboxyl group as well as the *N*-terminal amino group was observed upon treatment with trityl chloride and triethylamine in pyridine solution. The N^{α}-trityl pentapeptide trityl ester was isolated in 32 % over-all yield, and characterized by its PMR-spectrum. Selective detritylation of the carboxyl group to give the N^{α}-trityl pentapeptide in 25 % over-all yield was effected by methanolysis at room temperature for 24 h. The methanolysis product was identical with the monotritylated byproduct from the tritylation. The total over-all yield of N^{α}-trityl pentapeptide was thus 29 %.

The increasing need for easily cleaved amino-protecting groups to be used in the synthesis of, *e.g.*, tryptophan containing sequences [1] has led to development of among others the Bpoc[2] [2-(*p*-biphenylyl)-isopropyloxy-carbonyl-] and Nps [3] (*o*-nitrophenyl-sulfenyl-) groups. The trityl group,[4,5] which can also be cleaved from amino groups under very mild conditions, is not suitable for stepwise chain elongation because of sterical hindrance.[6] As this hindrance is not apparent when the N^{α}-trityl group is further removed from the *C*-terminus,[6] the trityl group has found application in several instances,[7–9] where it was introduced into peptide esters, synthesized with a different N^{α}-protection. While N^{α}-protected peptide esters are applicable to fragment condensation by the azide method, the use of other coupling methods, such as the carbodiimide method,[10] which is to date the most frequently applied,[11] requires the liberation of the *C*-terminal carboxyl group. As the selective saponification of protected polypeptide esters is sometimes difficult,[12] a way of introducing the trityl group into the α-amino group of free peptides under conditions, which will not affect the side-chain protection, would perhaps lead to a more

* Abbreviations follow the rules of the IUPAC-IUB commission on biochemical nomenclature *J. Biol. Chem.* **241** (1966) 2491.

extensive use of this valuable protecting group. Combined with the fully automatized solid phase synthesis,[13] such a method would permit the rational synthesis of N^{α}-tritylated peptide fragments for use in the controlled assembly of proteins.

The tritylation procedure was studied on a pentapeptide, which corresponds to the sequence 98 – 102 of tobacco mosaic virus coat protein.[14] The Asn and Gln residues were introduced as the β- and γ-*p*-nitrobenzyl esters of Asp and Glu. Since the formation of the amides by ammonolysis [15] at the end of a synthesis depends on the stability of the esters during the synthesis, it was of interest to see, if the base-labile *p*-nitrobenzyl esters would remain intact during the introduction of the trityl group.

The protected peptide resin was synthesized in a stepwise manner according to the procedure of Merrifield,[16] using methylene chloride, ethanol, and acetic acid as solvents, and deblocking twice with N HCl in acetic acid in order to ensure a complete cleavage of the Boc-group. The excess of Boc-amino acids and carbodiimide employed in the coupling step was for Asp and Ala threefold, and for Glu four-fold, calculated from the chloride content of the previous neutralization filtrate, determined by Volhard titration. Cleavage of the partially protected peptide from the resin was effected by treatment with HBr in trifluoroacetic acid. The yield was 55 % of theory, based on the quantity of Boc-Pro resin employed. Elemental analysis showed that the *p*-nitrobenzyl ester groups were still intact.

Tritylation was carried out by dissolving the partially protected peptide in anhydrous pyridine, removing traces of water by repeated evaporations with fresh pyridine, followed by addition of trityl chloride and triethylamine at 0°C. After standing for 16 h at room temperature in the dark and evaporation to dryness, the solid residue was dissolved in a mixture of ethyl acetate

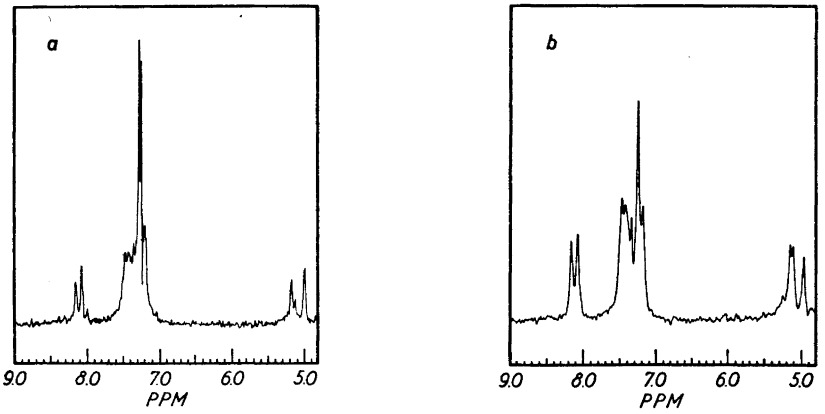

Fig. 1a: N^{α}-Trityl pentapeptide trityl ester. *Fig. 1b:* N^{α}-Trityl pentapeptide acid. Resonance maxima deriving from the protons in ortho position to the nitro groups appear at 8.17 and 8.08 ppm and from the protons in meta position in the region of the two trityl groups at higher field. The maximum for the tritylester group and the N^{α}-trityl group appear respectively at 7.30 and 7.25 ppm. The resonances at higher field are due to CH_2 of the three different *p*-nitrobenzyl protecting groups.

and water, and the resulting alkaline mixture immediately neutralized at 10°C by addition of ice and acetic acid. On working up the organic phase, a product was obtained, from which the only impurity, *viz.* the N^{α}-tritylated peptide acid, could be completely removed by sodium bicarbonate extraction to give a chromatographically homogeneous product in 32 % over-all yield, based on the quantity of Boc-Pro resin employed. The elemental analysis values corresponded closely to those calculated for the N^{α}-trityl pentapeptide trityl ester, and further evidence was supplied by PMR-spectra, showing the presence of three nitrobenzyl groups and two trityl groups (Fig. 1a).

Detritylation of the *C*-terminal carboxyl group was effected selectively under very mild conditions by letting a methanolic solution of the fully protected peptide stand for 24 h in the dark at room temperature. The methanolysis [17] was followed chromatographically and was shown to be quantitative, producing one tolidine-positive substance only, which could be isolated by concentration and crystallization from benzene/petroleum spirit in 25 % over-all yield. The product was chromatographically identical with the by-product from the tritylation (over-all yield 4 %), and the values found by elemental analysis corresponded to those calculated for the N^{α}-tritylated peptide acid. Treatment of a sample with 80 % acetic acid at room temperature for 1 h was checked chromatographically, and was found by thin layer chromatography to produce quantitative conversion to the original peptide. The optical rotation of the solution of the partially protected peptide obtained in this way was identical within the experimental error to that of a corresponding solution of the original peptide. The PMR-spectrum of the N^{α}-trityl pentapeptide confirmed the presence of one trityl group. Comparison of the integrated signals from the protons in *ortho* position to the nitro group with the integrated signals from the protons in *meta* position and from the trityl group protons shows that detritylation of the carboxyl group does not affect the *p*-nitrobenzyl protecting groups (Fig. 1b).

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EXPERIMENTAL

Melting points are uncorrected. Ascending thin-layer chromatography was performed on commercial plates (DC-Fertigplatten, Kieselgel F 254, E. Merck AG., Darmstadt). Solvent systems: S1 (chloroform/acetic acid/methanol by volume 90/5/5), S2 (2-butanol/formic acid/water by volume 75/15/10), S4 (1-butanol/pyridine/acetic acid/water by volume 30/20/6/24) and S5 (*tert.*butanol/pyridine/heptane by volume 33/13/54). Chromatograms were visualized by spraying with *tert.* butyl hypochlorite, followed by *p*-tolidine/potassium iodide. Optical rotations were measured with the Perkin-Elmer model 141 photoelectric polarimeter. The apparatus used for the manual solid phase synthesis was similar to the one described by Kusch.[18] The resin (Bio-beads S-X2, 200–400 mesh) was obtained from Bio-Rad Laboratories, Richmond, California, and was chloromethylated and esterified with the first amino acid, proline, according to the general procedure of Merrifield.[19] Methylene chloride (May and Baker Ltd., Dagenham, Essex) was stored over potassium carbonate and distilled before use. Acetic acid ("Pronalys" glacial acetic acid, May and Baker Ltd.) was used as such. Ethanol was commercial absolute ethanol. Boc-Ala and Boc-Pro were prepared according to Schnabel.[20]

PMR-Spectra. The samples were partially deuterated (NH, OH) by addition of 10 µl D_2O after dissolving in $CDCl_3$. A Varian HA 100 instrument was used for the measure-

ments and TMS was used as internal standard. The chemical shifts are given in δ-ppm values.

Boc-L-glutamic acid γ-p-nitrobenzylester dicyclohexylammonium salt. L-Glutamic acid γ-*p*-nitrobenzylester [21] (16.0 g, 56.7 mmol) was reacted with Boc-azide (12 ml, 84 mmol) and triethylamine (22 ml, 152 mmol) in dimethylsulfoxide (400 ml) according to Stewart [22] for 17 h at room temperature. The oil (15 g) resulting from the work-up was converted to the dicyclohexylammonium salt by treatment with dicyclohexylamine (10 ml, 50 mmol) in dry ether (150 ml). After filtration and recrystallization from water (1100 ml) the yield was 9.6 g (17.1 mmol, 30 %). M.p. 146–147.5°C. $[\alpha]_D^{25} = +10.7$, $[\alpha]_{578}^{25} = +11.2$ ($c=2$ in DMF). Chromatographically homogeneous (S1) and identical with a product synthesized by the general procedure of Schnabel [20] in a yield of 15 %; it gave the following analytical values: M.p. 144–146°C, $[\alpha]_D^{25} = +10.7$, $[\alpha]_{578}^{25} = +11.1$ ($c=2$ in DMF). (Found: C 61.7; H 8.3; N 7.5. Calc. for $C_{29}H_{45}N_3O_8$ (563.7): C 61.8; H 8.1; N 7.5).

Boc-L-aspartic acid β-p-nitrobenzylester. L-Aspartic acid β-*p*-nitrobenzylester [21] (25.0 g, 93.0 mmol) was reacted with Boc-azide (19 ml, 133 mmol) and triethylamine (34 ml, 242 mmol) in dimethylsulfoxide (600 ml) according to Stewart [22] for 20 h at room temperature. The resulting oil was recrystallized from ethyl acetate/petroleum spirit to give 20 g product (54.3 mmol, 58 %). M.p. 130–132°C, $[\alpha]_D^{25} = +2.4$, $[\alpha]_{578}^{25} = +1.7$ ($c=1$ in methanol); chromatographically homogeneous, R_FS1 = 0.5. (Found: C 52.4; H 5.6; N 7.7. Calc. for $C_{16}H_{20}N_2O_8$ (368.4): C 52.2; H 5.5; N 7.6.

L-Asp(OBzlNO$_2$)-L-Glu(OBzlNO$_2$)-L-Ala-L-Asp(OBzlNO$_2$)-L-Pro. Boc-L-Pro resin (5.0 g, 5.8 mmol proline) was transferred to a 100-ml Kusch-apparatus [18] and reacted in the usual manner [23] with the appropriate amino acid derivatives, as illustrated in Table 1.

Table 1. Synthesis of the pentapeptide on the resin.

Volhard chloride determination on N $(Et)_3$-filtrate (mmol of Cl^-)	Derivative added	Quantity g	Quantity mmol	Time allowed for salt formation (minutes)	Addition of dicyclohexyl-carbodiimide (ml 1 N)	Coupling time (hours)
5.8	Boc-L-Asp(OBzl(NO$_2$))	9.0	24.5	30	25	2
6.1	Boc-L-Ala	4.7	25.0	10	25	3
5.3	Boc-L-Glu(OBzl(NO$_2$))	10.1 [a]	26.0	15	25	16 [b]
5.6	Boc-L-Asp(OBzl(NO$_2$))	8.5	23.1	30	25	2

[a] Boc-L-Glu(OBzl(NO$_2$)) DCHA salt (15.1 g, 27.0 mmol) was mixed with citric acid (10.0 g, 47.6 mmol), ethyl acetate (150 ml), and water (100 ml). The mixture was stirred vigorously for 30 min (after the first 5 min, a clear solution was obtained). After extraction of the aqueous layer with more ethyl acetate, wash of the organic layer with six portions of water, drying over $MgSO_4$ and concentration to dryness at 40°C. and 1 mmHg, 10.1 g of a glassy residue was obtained (26.0 mmol, 96 %). [b] Left overnight.

After repeated washing of the peptide resin with methylene chloride and ethanol (six portions of each), it was dried under vacuum in the apparatus at 40°C. Detachment from the resin was effected by two 60-min treatments with HBr in trifluoroacetic acid. Evaporation of the filtrates left an oil (4.5 g), which was redissolved in DMF (5 ml) and precipitated by the addition of dry ether (150 ml) to remove remaining trifluoroacetic acid. After

decantation and washing with dry ether the oil was dissolved in DMF (15 ml) and triethylamine (1.0 ml) added in excess. Glacial acetic acid (*ca.* 1 ml) was immediately added in excess. The presence of an excess of volatile acid or base was detected by positioning a strip of moist indicator paper (E. Merck A.G., pH 1—10) above the liquid surface. The peptide acetate was finally precipitated from the light yellow solution by dropwise addition of ether (450 ml Pharm.Dan.), freed from peroxides by treatment with aluminium oxide (M. Woelm, W 200 basic). The light yellow oil was crystallized by trituration with hot ethyl acetate (450 ml). Yield: 2.7 g white, compact powder, chromatographically homogeneous (S2 and S4). R_FS2=0.60; R_FS4=0.84; ninhydrin colour: yellow. $[\alpha]_D^{25}=-27.6$, $[\alpha]_{578}^{25}=-29.0$ ($c=1$ in DMF). (Found: C 50.4; H 5.2; N 10.5; CH_3CO 8.5. Calc. for $C_{42}H_{46}N_8O_{16}.2.2\ CH_3COOH,1\ H_2O$ (1101.0): C 50.6; H 5.2; N 10.2; CH_3CO 8.5). Amino acid analysis (mol per 1101 g): Ala 1.01; Asp 2.04; Glu 1.02; Pro 1.02.

By working up the light yellow ethyl acetate mother liquor a further quantity of the product could be isolated. On concentration, a crystalline precipitate (0.7 g), slightly yellow, but almost chromatographically pure (S2 and S4), was recovered. The filtrate, upon addition of an equal volume of dry ether, became turbid, and on standing in the refrigerator left a fine deposit (0.3 g), which, however, contained other products (S2 and S4) as well. The total yield, based on L-Pro resin (5.8 mmol) was thus *ca.* 3.5 g (55 %), of which 2.7 g (42 %) was analytically pure.

Trt-L-*Asp(OBzlNO$_2$)*-L-*Glu(OBzlNO$_2$)*-L-*Ala*-L-*Asp(OBzlNO$_2$)*-L-*Pro-OTrt*. The pentapeptide (2.50 g, 2.27 mmole) was dissolved in pyridine (15 ml) and traces of water removed azeotropically by repeated (3) concentrations on the rotatory evaporator after addition of fresh pyridine (25 ml). To the resulting clear, yellow solution (12 ml) was added trityl chloride (1.9 g, 6.8 mmol) and, after cooling to 0°C, triethylamine (1.0 ml, 7.2 mmol). After standing for 16 h at room temperature in the dark the mixture was concentrated to dryness on the rotatory evaporator at 0.5 mmHg and a bath temperature of 30°C. The solid residue was stirred with a mixture of ethyl acetate (25 ml) and water (20 ml), in which it instantly dissolved. Under vigorous stirring, ice was added (5 g) and the pH adjusted to about 4 by the dropwise addition of glacial acetic acid. After extraction of the aqueous layer with ethyl acetate, the combined organic phases were washed six times with water, dried over $MgSO_4$, and concentrated to about 5 ml at 0.5 mmHg and 30°C. Addition of abs. ether/petroleum spirit (by volume 1/2, 100 ml) brought about the precipitation of an amorphous solid. After reprecipitation from ethyl acetate/petroleum spirit (150 ml, by volume 2/1), filtration and drying at 0.3 mmHg the yield of almost colourless material was 2.40 g (1.67 mmol, 74 %). M.p. interval 110—120°C, unchanged by repeated reprecipitation. Easily soluble in methylene chloride. (Found: C 66.0; H 5.3; N 8.5. Calc. for $C_{80}H_{74}N_8O_{18}$ (1435.5): C 66.9; H 5.2; N 7.8). Chromatographically homogeneous in solvent system S1 (R_FS1=0.55) without a trace of the free peptide (R_FS1=0). In solvent system S5 (R_FS5=0.35) an impurity with R_FS5=0 could be identified as the trityl-pentapeptide acid by a methanolysis study.[17] It could be removed quantitatively by sodium bicarbonate (0.1 N) extraction * of an ethyl acetate solution of the product (2.28 g). Recovery: 1.79 g. Melting point unchanged. Chromatographically homogeneous (S1 and S5). (Found: C 66.9; H 5.2; N 7.8; O 20.2. Calc. for $C_{80}H_{74}N_8O_{18}$ (1435.5): C 66.9; H 5.2; N 7.8; O 20.1). $[\alpha]_D^{22}=+60.4$ and $[\alpha]_{578}^{22}=-63.8$ ($c=1$ in ethyl acetate). PMR-spectrum: see Fig. 1a.

Trt-L-*Asp(OBzlNO$_2$)*-L-*Glu(OBzlNO$_2$)*-L-*Ala*-L-*Asp(OBzlNO$_2$)*-L-*Pro*. The fully protected pentapeptide (1.57 g, 1.09 mmol) was dissolved in ethyl acetate (10 ml). After addition of methanol (50 ml) the solution was left in the dark at room temperature for 24 h. The methanolysis was verified chromatographically (S5); no trace of the starting material remained. Moreover, the trityl-pentapeptide acid was the only tolidine-positive substance formed (S1 and S5). The solvents were removed on the rotatory evaporator at 30°C and 8 mmHg. The resulting oil was precipitated twice from ethyl acetate (25 ml) by addition of petroleum spirit (75 ml), and precipitated as an amorphous solid from benzene (80 ml) by addition of petroleum spirit (50—100 ml). Filtration and drying at 0.3 mm Hg afforded 1.01 g of a white material (0.85 mmol, yield 78 %). No well defined melting point (interval 90—110°C). (Found: C 61.0; H 4.9; N 9.3; O 24.6. Calc.

* From the bicarbonate extract the trityl-pentapeptide acid (0.20 g) was recovered by acidification at 0°C with citric acid, extraction with ethyl acetate, and working up as detailed below. It was chromatographically (S1 and S5) identical with the methanolysis product.

for $C_{81}H_{80}N_8O_{18}$ (1193.2): C 61.4; H 5.1; N 9.4; O 24.1). $[\alpha]_D^{22} = -39.4$ and $[\alpha]_{578}^{22} = -41.8$ ($c = 0.6$ in ethyl acetate). Neutralization equivalent: found 1370; calc. 1193. PMR-spectrum: see Fig. 1b.

In order to see if the original pentapeptide could be recovered in unchanged form, the N^{α}-trityl pentapeptide (27.3 mg, 22.9 μmol) was dissolved in 80 % aqueous acetic acid (5 ml), and left for an hour in the dark at room temperature. Chromatography in S1 showed no trace of the starting material (R_FS1 = 0.55); all of the applicated material remained on the application site and gave a yellow colour when sprayed with ninhydrin, in contrast to the starting material, which was ninhydrin-negative. In S4 the product was homogeneous with R_FS4 = 0.8. The optical rotation of the solution was measured, and the specific rotation calculated, based on an equivalent quantity of pentapeptide acetate (22.9 μmol, 25.2 mg). $[\alpha]_D^{22} = -35.3$ and $[\alpha]_{578}^{22} = -38.7$ ($c = 0.5$ in 80 % acetic acid). A reference solution of the original pentapeptide had $[\alpha]_D^{22} = -38.1$ and $[\alpha]_{578}^{22} = -42.5$ ($c = 0.5$ in 80 % acetic acid).

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