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Efficient, Optimized Applications of *p*-Nitrophenyl Active Ester Temporarily Protecting Groups Together with Simultaneous Activation in Synthesis of Special Glu and Lys Peptides

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Benzyloxycarbonyl-glutamylpeptide *p*-nitrophenylesters were prepared from protected amino acids, e.g.: *p*-nitrophenyl-glutamate as carboxyl component and aminoacid or peptide *p*-nitrophenylesters as amino components by different kinds of peptide coupling methods. Mixed carbonic anhydride and azide methods gave good results. The *p*-nitrophenylesters existed as temporary protecting groups, so the peptide couplings proceeded together with simultaneous activation. The conditions and applications of the procedure are discussed. The peptides having one or more active ester groups were used to form amides by their aminolysis with derivatives of ethylamine or were polycondensated (after deprotection) to obtain polypeptides.

INTRODUCTION

Certain conditions permit the use of activated esters as carboxyl protecting groups.[1,3] In these cases they represent dual function groups.[3a] Esters with a higher degree of activation (e.g. phenylesters, ethyl-tiolesters)[4-6] than methyl or ethyl esters are at an intermediate level between activated esters and carboxyl protecting groups. They are not sufficiently activated to give rise to side reactions during peptide synthesis. Their main advantage is the ready amide (or hydrazide) formation. If we use activated ester (for example *p*-nitrophenylester) as temporary carboxyl protecting group, the coupling method employed must provide a higher degree of activation than the activated ester or it will not be possible to prevent side reactions: the aminolysis of *p*-nitrophenylesters, cyclization and so on.[7]

On the one hand, the esters can be arranged with respect to their activating properties in approximately the order:[3,8-13]

$$OEt < OBzl < OMe < OPh < SPh < OCH_2CN < ONp < OSu < OPCP < OPFP$$

The level of activity is demonstrated by the values of infrared stretching frequencies for the carbonyl groups in carboxyl derivatives (see Table 1). The activity is increasing downward.

On the other hand, in order to utilize amino acid or peptide component containing active ester as a temporary carboxyl protecting group, it is necessary, that the coupling reaction employed proceeds rapidly compared with the aminolysis of the active *p*-nitrophenylester group. Normally the complete acylation of a peptide amino group by *p*-nitrophenylester in general requires 12-48 hours at room temperature. With the mixed carbonic anhydride procedure, the reaction is usually over in 1-2 hours. It is unlikely, therefore, that a competitive acylation involving the *p*-nitrophenyl group will interfere with the coupling reaction. This is further indicated by the fact that dicyclohexylcarbodiimide, which requires some 4 hours for complete reaction, has been used successfully in some cases for coupling with amino acid *p*-nitrophenylesters.[14] An alternative ap-

Table 1. Characteristic Infrared Absorption Frequencies for Carbonyl Groups in Activated Glutamyl Carboxyl Groups Compared with Other Derivatives (for IR spectra Zeiss UR 10 Spectrometer was used; the compounds were benzyloxycarbonyl-α- and γ-glutamates prepared by our laboratory)

O ‖ -C-OR	R	Frequency $\nu_{C=O}$ (cm^{-1})
Acid	H	1715-20
protecting	CH_3	1725-30
groups	$CH_2C_6H_5$	1735-45
	CH_2CH_2Cl	1740
	Ph	1750-60
Active	Np	1755-65
esters	Su	1765-70
	PCP	1780-85
Mixed anhydride	$COCH_2CH(CH_3)_2$	1780 and 1820
in Leuchs-anhydride		1805 and 1870
in -CONH (peptide bond)		1650-65
in -CONH (Z)		1695-1705

proach would be the use of active esters which are substantially more reactive than the *p*-nitrophenyl derivatives for the coupling stages. Examples of such compounds are the 2,4-dinitrophenyl-, tri and pentachlorophenyl-, *N*-hydroxysuccinimideesters, which can couple generally within 1-2 hours. The time values depend on the quality of amino acid residues, too. The acylazides, acylchlorides acylate also very rapidly.[8]

Using the given possibilities Goodman and Stueben[14] and Goodman, Schmitt and Yphantis[15] found at first, that a benzyloxycarbonyl-amino acid (Ia) could be coupled directly with an amino acid-*p*-nitrophenylester (III) by the mixed carbonic anhydride procedure and sometimes by dicyclohexylcarbodiimide, too. This reaction, in which the *p*-nitrophenyl acts as a protecting group, depends on the fact, that the coupling proceeds at a considerably greater rate than the aminolysis of *p*-nitrophenylester groups.

The procedure was introduced generally as so called "backing-off" method for preparation of protected peptide active-esters. It was then extended to the stepwise synthesis of a complete series of sequential polypeptides.[16-18] Then trichlorophenylesters,[19] pentachlorophenylesters,[20-23] *N*-hydroxysuccinimidesters,[22-24] 8-hydroxyquinolyester[24] and pentafluorophenylester,[25,39,40] have been used as an active temporary protecting group.

For the coupling reactions with these compounds mixed carbonic anhydride,[14,15,17-21] azide,[26,27] dicyclohexylcarbodiimide[16] and more active ester[9,28] methods were used.

Recently pentafluorophenylesters have been used for temporary carboxyl group protection in solid phase synthesis of *N*- and *O*-linked glycopeptides.[39,40]

RESULTS AND DISCUSSION

In our investigations the use of active ester temporary protecting groups has been extended to the stepwise synthesis of a complete series of oligoglutamyl peptides with 1, 2, 3, ... 6 *p*-nitrophenylester groups at α and γ position (VIII, IX) and not only at the C, but at the *N*-terminal residue too [VIII, IX]. Dipeptides containing glycine [434] and tyrosine residues[38] were also synthesized by this procedure.

In order to test this possibility by means of a simple model synthesis, at first glycinesters and alkylglutamates, then *p*-nitrophenylglutamates were subjected to couplings with benzyloxycarbonyl-γ-*p*-nitrophenyl-L-glutamate (VI) (Fig. 3, 5).

Dicyclohexylcarbodiimide, mixed anhydride, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline,[36] azide and *N*-hydroxysuccinimide ester methods were applied successfully for the couplings and compared at the same time. Table 2 contains a list of dipeptide active esters, which were synthesized. From the comparison the mixed carbonic anhydride (MCA) seems to be the most satisfactory method. During the synthesis isobutylchloroformate and *N*-methylmorpholine or triethylamine were used for the activation. The amino components were liberated during the coupling gradually by the same tertiary bases from their hydrochloride or hydrobromide salts. The reactions have been carried out in a variety of solvents: anhydrous acetonitrile was found to be particularly satisfactory because the endproducts were precipitated from the reaction mixtures. The high crystallizability of the *p*-nitrophenyl derivatives facilitates their purification. *p*-Nitrophenyl-esters show a characteristic UV absorption at 268-270 nm different from that of *p*-nitrophenol (400 nm). This facilitates determination of the rate of aminolysis or hydrolysis and makes it possible to detect the purity of endproducts according to De Tar.[16] Racemization does not occur to any significant extent because the acidic component is a benzyloxycarbonyl-aminoacid.[17,29]

The summarized advantages of applications of *p*-nitrophenylesters are the following:

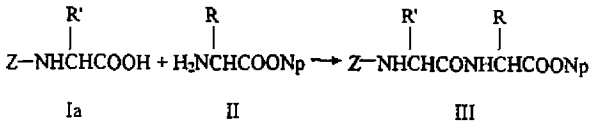


Fig. 1. "Backing off" procedure for dipeptide.

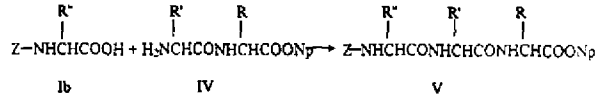


Fig. 2. "Backing off" procedure for tripeptide.

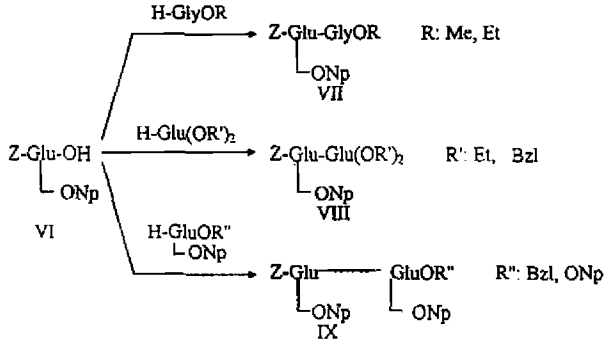


Fig. 3. Dipeptide derivatives from benzyloxycarbonyl-γ-*p*-nitrophenyl-glutamate VI.

Table 2. Benzyloxycarbonyl-dipeplide-*p*-nitrophenylesters (the preparation methods are given in Experimental. Optical rotations were measured at concentration 1.0 g/100 mL in ethylacetate and *dimethylformamide)

Compounds	Methods	Yields (%)	M.P. (°C)	$[\alpha]_D^{20}$	R_F[a]	IR $\nu_{C=O}$ cm^{-1}	^{1}H NMR[b] δ ppm -ONp+NH
Z-Glu(ONp)GlyOEt	DCCI	35	131	-15.1°	0.85	1730 (COOEt)	7.25 (2H)
	MCA	65	132	-15.3	0.85	1762 (COONp)	7.64 (4H)
Z-Glu(ONp)GlyOMe	DCCI	25	152	-14.1	0.75	1695 (Z)	7.25 (2H)
	MCA	60	153-154	-14.4	0.75	1660 (Amide I)	7.64 (4H)
	EEDQ	55	151-153	-14.4	0.75	1735 (COOMe)	-
	AZIDE	40	153	-14.4	0.75		-
Z-Glu(ONp)Glu(OBzl)$_2$	DCCI	42	129	-12.8	0.31	1740 (COOBzl)	-
	MCA	68	130	-13.0	0.31	1770 (COONp)	7.25 (2H)
Z-Glu(ONp)Glu(OBzl) └ONp	MCA	72	143-144	-20.9	0.72	1645 (Amide I) 1770 (COONp)	7.64 (4H) 7.25 (4H)
Z-Glu(ONp)Glu(ONp)$_2$	MCA	88	179-180	-30.8*	0.56	1700 (Z)	7.64 (6H)
	EEDQ	70	179-181	-30.6*	0.56	1765 (COONp)	7.25 (6H)
	AZIDE	45	178-179	-30.2	0.56		7.64 (8H)
Z-Glu(ONp)Tyr(OEt)	MCA	41	168-170	-19.9*	0.95	1725 (COOEt)	-
Z-Glu(ONp)Tyr(OBzl) └Bzl	MCA	65	133-135	-12.9	0.98	1735 (COOBzl)	-
	EEDQ	81	133-134	-13.0*	0.98	1770 (COONp)	-
Z(Glu)$_3$(ONp)$_4$	MCA	79	185-186	-25.2*	0.51	1770 (COONp)	7.25 (8H)
Z(Glu)$_4$(ONp)$_5$	MCA	68	170-172	-19.7*	0.48	1660 (Amide I)	7.64 (14H)
Z(Glu)$_5$(ONp)$_6$	MCA	64	166-167	-16.3*	0.42	1700 (Z)	7.64 (17H)
Z-Lys(BOC)-(Z-Lys-ONp)	MCA	89	128-131	-15.4*	2.2[c]	1765 (COONp)	7.64 (4H)
Z-Lys(BOC)-(Z-Lys)$_2$-ONp	MCA	95	119-125	-8.7*	8.1[c]	1695 (Z)	7.25 (2H)

[a] TLC Merck-silica, *n*-BuOH:AcOH:H_2O 4:1:1.
[b] see Experimental; signals due to Z groups: ~7.20 (s) and 4.95 (s) in all compounds.
[c] k' (HPLC) see Experimental.

- The peptide *p*-nitrophenylesters are very well and easily crystallizable compounds.

- *p*-nitrophenol is a very useful indicator because of its yellow color for any side reaction forming it and *p*-nitrophenylester group means a very well detectable chromophore.

- the active-ester content can be determined very simply.

- during HPLC measurements there is no decomposition, but the couplings can be very well monitored because of their detectability (see Table 2).

- aminolysis of *p*-nitrophenylesters can be performed very easily (see Table 3).

- *p*-nitrophenylesters are easily characterized by IR, UV, and ^{1}H NMR (see Exp. data).

- there is no racemization at all of α-*p*-nitrophenylester at *N* and *C* terminal (it was controlled by Marfey's-method).

The traditional mixed carbonic anhydride method could be substituted by the use of *N*-ethoxy-carbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ).[31] The azide coupling technique proceeded with the use of the *t*-butyloxycarbonylhydrazyl (X) blocking group for the protection of the terminal carboxylic acid group.[39] We employ this blocking group in conjunction with the benzyloxycarbonyl group. Treating the derivative (XI) with trifluoroacetic acid or hydrogen chloride in acetic acid, removes the *t*-butyloxy carbonyl group without affecting the benzyloxycarbonyl group yielding a hydrazide (XII), which can subsequently be used in an azide reaction. The azide reaction involves a homogeneous non-aqueous medium and azide forming agents such as isoamyl or *t*-butyl-nitrite and the coupling proceeds without the isolation of azide (XIII). Details of the synthesis can be seen from Fig. 4.

In all cases, active esters were at the *C*-terminal end and the peptide chains were lengthened from *C*-terminal residues of amino acids. The method was optimized by our laboratory in the case of α-glutamyl-peptides [Table 6]. The peptide couplings with simultaneous activation were systematically investigated with the help of multi-*p*-nitrophenylesters of oligoglutamyl peptides, where *p*-nitrophenylesters were used as temporary protecting groups. The first applications took place in the case of sequential polypeptides, where the tripeptide active esters for polycondensation were prepared in this way without basic treatment, racemization free.[16]

On the other hand homoisopeptides could be synthe-

sized by this procedure without basic treatment, racemization free. According to our method the monomer protected lysine-*p*-nitrophenylester serves as starting material. The *t*-butyloxycarbonyl group was removed by acidolysis, and the protected lysine unit was coupled to it by the mixed anhydride method. The conditions most favorable to the mixed-anhydride coupling method were the following: solvent: acetonitrile; reagent forming anhydride: isobutyl-chloroformate; tertiary base: triethylamine; time of activation: 15-20 min.; temperature: -10 °C; neutralization of amino component salt: with triethylamine. After removal of the acid sensitive protecting group, the dipeptide could be polycondensated or deprotected and coupled again with the next protected lysine. The best results were obtained by repeating the couplings to tripeptide. After polycondensation, isopolylysines were formed with the desired number of residues. Removal of the benzyloxycarbonyl protecting groups was performed by HBr in acetic acid (See Fig. 6).

The poly-isopeptides preparations were analyzed using modern separation techniques such as HPLC, IEC, GC and by structure determination methods as UV, IR, CD (See Table 4; detailed experimental data will be published elsewhere).

The polymers (see Table 4) were characterized by viscosity, molecular weight, dispersity, optical rotation, and purity. It was concluded, that poly-isolysines of a given Mw, with low polydispersity and in a wide Mw range can be reproducibly synthesized by our method. The intermediates are well crystallizable products, and the endproduct polymers are substances of high purity. Cyclolysines were synthesized by Iwai with a similar method.[34,35]

Summarizing the results, according to the experimental data *p*-nitrophenyl groups could function as temporary protecting groups in all positions. The couplings proceeded in the right direction using the mixed anhydride, EEDQ, and azide technique. The dicyclohexylcarbodiimide (DCCI) method was suitable only with *p*-nitrophenylester as a carboxylic component, but not in other case. The activities of dicyclohexylcarbodiimide complex and *N*-hydroxysuccinimidester of benzyloxycarbonyl-*p*-nitrophenyl-glutamate were not enough for total right coupling side reactions to proceed too, liberating *p*-nitrophenol. The side reactions were attributed to the possible formation of pyroglutamyl-ring, diketopiperazine derivatives or cyclic and linear polypeptides because of the aminolysis of active ester groups.[7] It is well known that γ-glutamate and dipeptide esters can easily be cyclized to form pyroglutamyl ring and diketopiperazine, respectively. A side reaction indicates itself because *p*-nitrophenol, liberated, exhibits a yellow colour that can be seen in the reaction mixture and on the chromatogram too. In some cases byproducts were isolated;[30] the free *p*-nitrophenol reacted with the activated *N*-terminal component producing the suitable acylated amino acid *p*-nitrophenylesters.

To the synthesis of a complete series of oligoglutamylpeptides (IXc, n = 2, 3, 4, 5) the peptide chain was lengthened from the *C*-terminal residue. The stepwise synthesis started with the preparation of di-*p*-nitrophenyl-glutamate hydrobromide (XIII, n = 2). The benzyloxycarbonyl protecting group was removed in every step with 2N hydrogen bromide in acetic acid in the usual way. Where possible the resultant peptide *p*-nitrophenylester hydrobromides were recrystallized, but in a number of cases the products were hygroscopic and were used after washing and drying directly for the following coupling stages with the mixed carbonic anhydride method. The peptide chain was built up

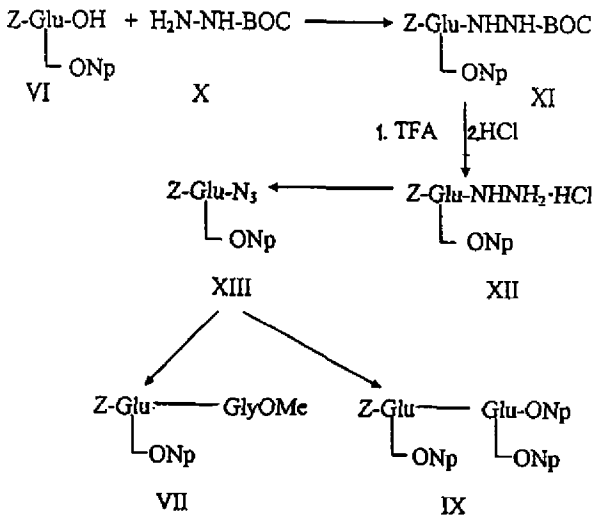


Fig. 4. Acylazide method.

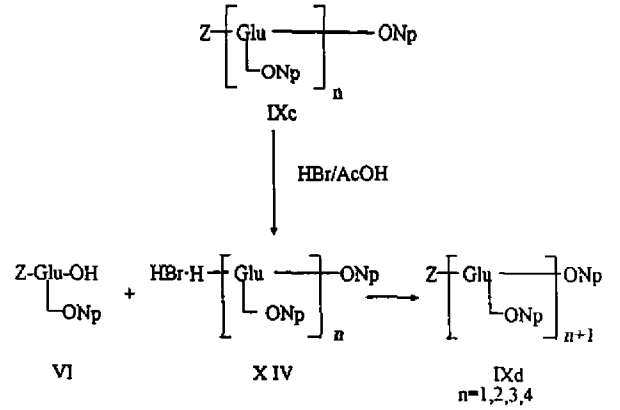


Fig. 5. Stepwise synthesis of benzyloxycarbonyl-oligopeptide-*p*-nitrophenylesters.

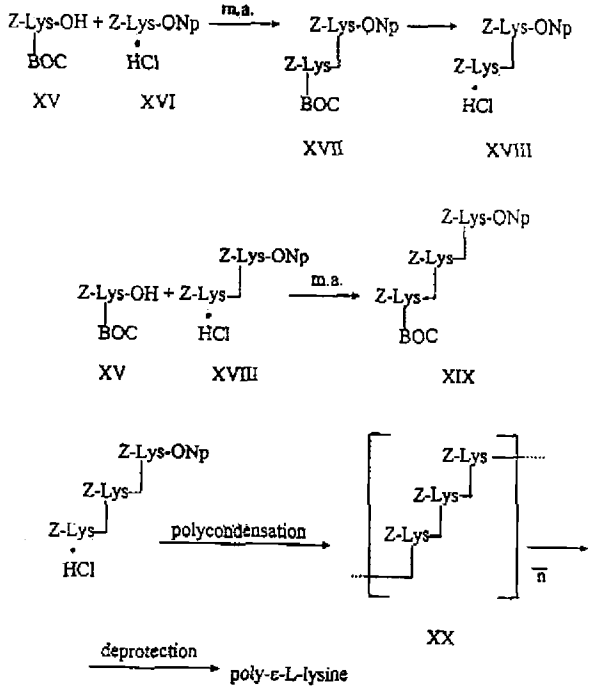
Table 3. Amides Prepared by Aminolysis of the Suitable *p*-Nitrophenylester. Optical Rotations Were Measured at c = 1.0 in EtOH and *TFE

Starting active esters	Amines	M.P. of amides (°C)	Yields (%)	$[\alpha]_D^{20}$	R_F	IR $\nu_{C=O}$ cm^{-1}
Z-Glu(ONp)GlyOEt	$H_2NCH_2CH_2N(CH_3)_2$	99-101	80	-23.0°	0.64[a]	1690 (Z), 1645 (Amide I)
Z-Glu(ONp)Glu(OBzl)$_2$	$H_2NCH_2CH_2N(CH_3)_2$	111-112	83	-14.0	0.73[a]	1730 (COOBzl), 1690 (Z)
Z-Glu(ONp)Glu(OBzl) ⌊ONp	$H_2NCH_2CH_2N(CH_3)_2$	168-169	98	-21.0	0.37[a]	1645 (Amide I), 1730 (COOBzl)
Z-Glu(ONp)Glu(ONp)$_2$	$H_2NCH_2CH_2N(CH_3)_2$	192-193	89	-10.2	0.38[a]	1690 (Z), 1645 (Amide I)
Z-Glu(ONp)-Tyr-(OBzl) ⌊Bzl	$H_2NCH_2CH_2SH$	188-189	73	-32.4	0.74[a]	1690 (Z), 1730 (COOBzl)
Z-Glu(ONp)GlyOEt	$H_2NCH_2CH_2SH$	172-174	81	-28.5	0.57[a]	1748 (COOEt)
Z(Glu)$_3$(ONp)$_4$	$H_2NCH_2CH_2N(CH_3)_2$	200-201	91	-18.1*	0.26[a]	1645 (Amide I)
Z(Glu)$_4$(ONp)$_5$	$H_2NCH_2CH_2N(CH_3)_2$	208-209	85	-13.6*	0.13[b]	1695 (Z)
Z(Glu)$_5$(ONp)$_6$	$H_2NCH_2CH_2N(CH_3)_2$	218-220	82	-12.7*	0.35[c]	1645 (Amide I), 1695 (Z)

R_F: TLC Merck-silica
[a] *n*-BuOH:Pyr:AcOH:H_2O 30:60:20:24
[b] the same, but 30:6:20:24
[c] *n*-BuOH:Pyr:AcOH:H_2O:DMF 30:60:20:24:15 (v/v)

as shown on Fig. 4.

The good reactivity of peptide and amino acid *p*-nitrophenylesters in aminolysis resulted in a lot of γ-amides in reasonable yields. The reaction proceeded with equivalent amines calculated for the number of *p*-nitrophenylester groups.[2,29,30] Table 3 contains a list of compounds synthesized by this method.



(m.a. = mixed anhydride method)

Fig. 6. Synthesis of poly-ε-L-lysine.

The summarized applications of our method are the following:

1. Sequential polypeptides[1,16,17]
2. Poly-active esters of oligoglutamyl peptides
3. Glutaminyl peptides e.g. basic derivatives of glutamyl peptides, glutamylamides[29,30,33]
4. γ-L-glutamyl-taurine peptides and their intermediates
5. ε-poly-lysines, lysine isopeptides, cyclolysines[34-36]
6. Branched peptides (e.g. bacitracin), cyclic peptide antibiotics[37]
7. *N*-glycopeptides (Asn-sugar)[39,40]

EXPERIMENTAL

All reagents and solvent were reagent grade from REANAL Ltd., Hungary. Protected amino acids were obtained from the same firm. All melting points were uncorrected. Optical rotations were measured with a Zeiss Polamat A polarimeter at 20 °C. TLC was performed on Merck's silicagel. Visualization of spots on TLC plates took place by use of TMD reagent (*N,N,N',N''*-tetramethyl-4,4'-diaminodiphenyl methane) after chlorination. ^{1}H NMR spectra were obtained using a ZKR60 spectrometer at 60 MHz in $(CD_3)_2SO$/TMS.

For infrared spectra a Zeiss UR10 spectrometer was used.

HPLC measurements were performed on a Knauer instrument (Berlin, Germany) consisting of two pumps (Model 64), a programmer (Model 50), a variable-wave-

Table 4. Characterisation of Isopolylysines (ε-poly-lysines-HBr) Prepared by Polycondensation of Tripeptide Active Esters Synthesized with "Backing Off" Procedure

Starting material (for polycondensation)	Charge No of polymers	MW (±300)	Polym.* degree (±2)	Viscosity η spec/c (protected polymer)	$[\alpha]_D^{20}$	Br%
BOC(Z-Lys)$_3$-ONp	SZTP3	8700	68	28.8	+30.5	32.4
	SZTP4	10400	81	30.4	+31.8	36.7
	SZTP7	10600	82	30.9	+31.9	36.6
	SZTP14	12700	99	31.9	+32.4	36.8

*They were determined with UC measurement by P. Kovács (Dept. Colloid Chemistry, Eötvös University, Budapest).

length UV monitor, and a sample injector (Rheodyne 7010, Berkeley, CA, USA). The column (125 × 4 mm) packing material was Hypersil-ODS6 (Shandon Southern Products, Runcorn, UK). Peaks were recorded on a Model OH-314-1 chart recorder (Radelkis, Hungary). The chromatograph was operated isocratically at flow rate 1 cm^3/min. MeOH/ 0.02 M NaOAc buffer (pH 4.0)-CH_3CN 40:30:30 (v/v/v) mixture was used as eluent, detection at 254 nm.

Determination of *p*-Nitrophenylester-groups

1.0 mg sample of compound containing one or more *p*-nitrophenylester groups was dissolved in 25 mL of 0.1 N sodium hydroxide at room temperature during some hours. The absorbance of this solution was measured at 400 nm by UNICAM SP 700 spectrophotometer. Recrystallized *p*-nitrophenol was the suitable reference standard with molar extinction coefficient: 18400.

General Procedures for Preparation of Benzyloxycarbonyl-peptide *p*-Nitrophenylesters (Table 2)

(A) by mixed carbonic anhydride method (MCA)

A solution of the benzyloxycarbonyl-γ-*p*-nitrophenyl-glutamate (VI 2.01 g, 5 mmole) or Z-Lys(Boc)-OH (XV) and triethylamine (0.70 mL; 5 mmole) in anhydrous acetonitrile (35 mL) was cooled to -20 °C in salt-ice bath, and isobutylchloroformate (0.70 mL; 5 mmole) was added dropwise with vigorous stirring. After a further 20 min at this temperature, the amino acid or peptide *p*-nitrophenylester salt (hydrobromide or hydrochloride; 5 mmole) was added, followed by the dropwise addition of a cold solution of triethylamine (0.70 mL; 5 mmole) in acetonitrile (5 mL). Stirring was continued for 1 hr at -20 °C and the mixture containing precipitated end-product was allowed to reach 0 °C gradually during the next hour. The mixture was diluted with 300 mL 0.5 N HCl. The solid product was collected, washed with dilute HCl, water, dried over P_2O_5 in vacuum and finally recrystallized. If oil product was obtained, it was extracted with ethylacetate, washed with N HCl, water, 4% sodium bicarbonate, water and finally dried over sodium sulphate. The solution was evaporated and the solid residue was recrystallized from acetonitrile or ethanol.

(B) by *N*-ethoxy-carbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ)

To a solution of benzyloxycarbonyl-γ-*p*-nitrophenyl-glutamate (VI; 0.402 g; 1 mmole) and aminoacid-ester (1 mmole, freshly prepared from its hydrochloride or hydrobromide salts with an equivalent amount of *N*-methylmorpholine) EEDQ (1 mmole) was added at 0 °C. When the amino component contains a *p*-nitrophenyl group too, the reaction time is 4 hr at -5 °C. The mixture was poured into dilute HCl. The precipitated product was collected, washed, dried, and finally recrystallized from acetonitrile or ethanol.

(C) by dicyclohexylcarbodiimide (DCCI)

To s solution of benzyloxycarbonyl-γ-*p*-nitrophenyl-glutamate (VI, 2.01 g; 5 mmole) and aminoacid-ester (5 mmole) in anhydrous acetonitrile (30 mL) DCCI (1.05 g; 5 mmole) was added at 0 °C with stirring. After one hour the reaction was allowed to warm to room temperature and sit for an additional 3 hr. The white precipitate (dicyclohexylurea) was filtered and washed. The combined filtrate was evaporated under reduced pressure at 20 °C. The residue was taken up in 50 mL of ethylacetate, extracted with 1 M HCl, water, 4% sodium bicarbonate (until the organic layer was colorless), water, and finally dried over sodium sulphate. The dried solution was evaporated and recrystallized from ethanol-diethylether.

(D) by acylazide-method

Benzyloxycarbonyl-(α-hydrazidyl-γ-*p*-nitrophenyl-glutamate hydrochloride (XII; 0.45 g, 1 mmole) was suspended in 5 mL of acetonitrile and cooled to -15 °C in an acetone-dry ice bath. 2 mL of 2 M hydrogen chloride in tetrahydrofurane (4 mmole) and isoamylnitrite (0.14 mL; 1 mmole) was added to the reaction mixture which was vigorously stirred until a clear solution was obtained with the suitable azide at -5 °C. The resulting solution was cooled to -40 °C and pre-cooled triethylamine in acetonitrile (0.7 mL;

5 mmole) was added to it. Then the aminoacid-ester or salt (1 mmole) was added at -20 °C, followed by the dropwise addition of a cold solution of triethylamine (5 mmole). Stirring was continued for 2 hr between -10 and -20 °C and the mixture containing the precipitated product was dropped into dilute HCl. The solid was collected, washed, dried and recrystallized from ethanol-diethylether.

(E) by *N*-hydroxy-succinimide-ester (OSu)

To a solution of benzyloxycarbonyl-α-*N*-hydroxysuccinimide-γ-*p*-nitrophenyl-glutamate (0.5 g; 1 mmole) in acetonitrile di-*p*-nitrophenyl-glutamate hydrobromide (XIV, $n = 1$) was added, followed by the dropwise addition of a cold solution of triethylamine (2 mmole) at -10 °C. After 4 hr, the reaction mixture was poured into dilute HCl, extracted with ethylacetate. The solution in ethylacetate was washed with *n* HCl, water, 4% sodium bicarbonate, water, and then finally dried and evaporated. The residue was crystallized from ethanol: 0.9 g, mp 112-114 °C identified as by-product: benzyloxycarbonyl-di-*p*-nitrophenyl-glutamate (IXc; $n = 1$) with IR and ONp-measurements.

For Peptide-*p*-nitrophenyl-ester-hydrobromides (XV; *n* = 1-4)

The benzyloxycarbonyl-peptide-*p*-nitrophenyl ester (1 g) was dissolved in 3 mL acetic acid and treated with 4 N hydrogen bromide in acetic acid (3 mL) for about 1 hr at room temperature. The pure reaction mixture was diluted with 100 mL ether. The precipitated hydrobromide salt was collected, washed with ether, and dried for at least 4 hr, over KOH and H_2SO_4 *in vacuo*.

For Aminolysis of Peptide *p*-Nitrophenylesters

The benzyloxycarbonyl-amino acid or peptide-*p*-nitrophenyl-ester (1 mmol) was dissolved in ethyl acetate (10-20 mL) or in a mixture of ethylacetate and dimethyl-formamide. The suitable amine (mmole equivalent to the number of *p*-nitrophenyl groups) was added dropwise at 0 °C. After 2 hrs the mixture was allowed to stand at room temperature. After cooling in a refrigerator the crystallized product was collected, washed with ether, and recrystallized. When no crystallization proceeded, after dilution with ethylacetate, the solution was purified with 5% $NaHCO_3$ and water, dried, and then evaporated. See the products in Table 3.

For Polycondensations

23 g (24 mmole) of tri-(N^{α}-benzyloxycarbonyl-L-lysine)-*p*-nitrophenylester-hydrochloride were condensed in 60 mL of dimethylsulfoxide, in the presence of 4.5 mL of triethylamine, up to gel formation. The polymer was isolated by pouring into an aqueous 5% Na_2CO_3 solution. 16 g of protected polymer of the previous step were dissolved in 100 mL of trifluoroacetic acid and 10 mL of 4N HBr/AcOH were added. The end-product was precipitated by ether.

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Key Words

Temporarily protecting groups; *p*-Nitrophenyl active esters; Simultaneous activation; Peptide synthesis; Poly-active esters.

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