Oligomeric Peptides derived from γ-*p*-Nitrobenzyl-L-glutamate. Synthesis and Optical Activity of the Dimer and the Trimer

By **Nicole Helbecque** and **Marie-H. Loucheux-Lefebvre,*** Laboratoire de chimie macromoléculaire, Université des Sciences et Techniques de Lille, B.P. 36, 59650 Villeneuve d'Ascq, France

Both the dimer and the trimer of γ-*p*-nitrobenzyl-L-glutamate have been prepared using either solid phase peptide synthesis or a step-by-step method. From a c.d. study, it has been shown that the nitrobenzylic chromophore is optically active for both oligomers. This result may be explained by folding of the side chain towards the asymmetric carbon atom.

O.R.D. and c.d. are two widely used methods of determining the detailed structure of proteins. However, it is often difficult to interpret the results. This is due in part to the Cotton effects associated with the absorption bands of aromatic side chains, the precise origin of which is not known. Therefore, we have studied nitrobenzyl-polyglutamates and -glutamates in order to explain the origin of those effects.[1] The nitrobenzyl group is a good one for our purpose because it is an intrinsically symmetric chromophore, with absorption bands at *ca.* 270, 290, and 350 nm,[2] which are easily distinguished from those due to the peptide chromophore.

[1] P. le Barny and M. H. Loucheux, *Biopolymers*, in the press.

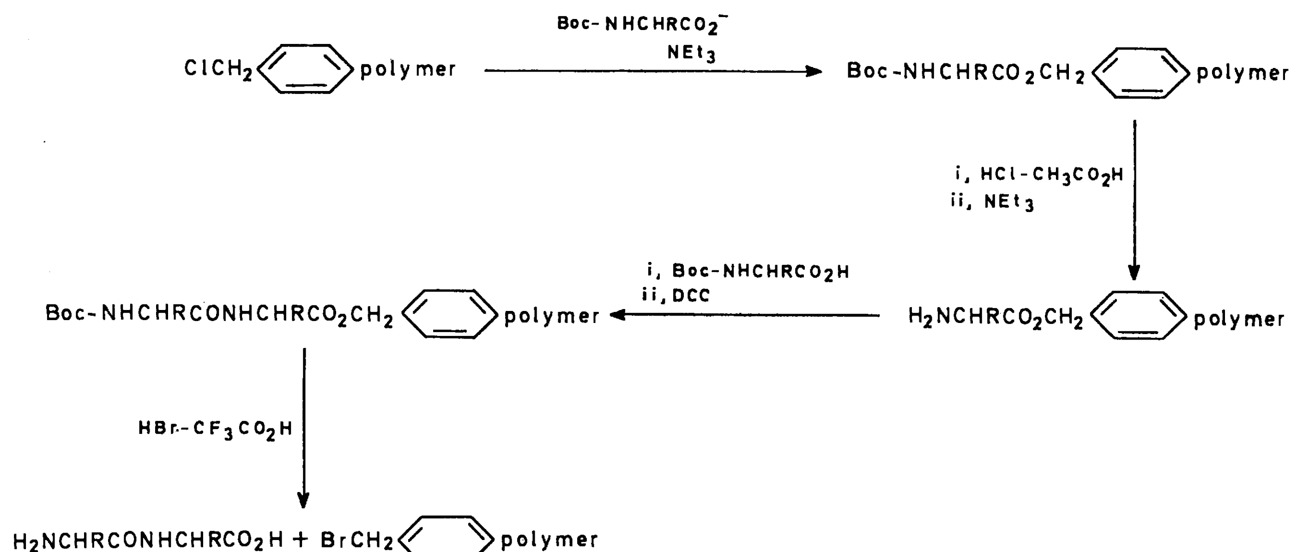
[2] M. Goodman, A. M. Felix, C. M. Deber, A. R. Brause, and G. Schwartz, *Biopolymers*, 1963, **1**, 371.

The extrinsic Cotton effects induced in the side chain chromophores of a helical polypeptide may be due to many factors: the asymmetric field of the helix at the place where the chromophore is bound to it;[3] the interaction between two identical chromophores, bound to the same helix, the backbone of which determines their respective geometry;[4] and the interaction between the asymmetric C(α) atom and the chromophore.[1]

Our previous studies did not allow the measurement of the influence of each interaction, so we began to study the corresponding oligopeptides. In this paper we describe the synthesis and a c.d. study of the di- and tri-peptides derived from γ-*p*-nitrobenzyl-L-glutamate.

when this salt was synthesized from the *N*-t-butoxycarbonylamino-acid and crystallized from dimethyl sulphoxide was checked by the process of Allenmark.[6] Another method consisted of elimination of the *N*-protecting group followed by neutralization by triethylamine. The quantity of amino-acid bound to the resin was then determined by the potentiometric titration of triethylammonium chloride. The unchanged chloromethyl groups carried by the resin can be estimated by titration.

In the second step, the *N*-blocking group was removed by a solution of anhydrous hydrogen chloride in glacial acetic acid. The resin–amino-acid hydrochloride then



SCHEME 1 Boc = t-butoxycarbonyl; DCC = dicyclohexylcarbodi-imide; R = $[CH_2]_2CO_2CH_2C_6H_4NO_2$-*p*

The choice of a suitable method for the synthesis of our derivatives was difficult because the nitrobenzyl group is sensitive to alkaline hydrolysis; it is cleaved at pH 8. Therefore, the azide synthesis could not be used, despite the fact that it gives optically active products. Consequently we tried to synthesize our products in two different ways, the Merrifield method and the so-called ' step-by-step ' method.

Synthesis by Merrifield Method.—We used the method described by Stewart *et al.*[5] This process consists of many successive reactions and washings of the Merrifield resin by agitation in different solvents and reagents, each solvent eliminating the previous reagent or solvent and preparing the product for the next reagent. In this method the reaction time is very important. Comparatively long reaction times are needed to obtain maximum yields.

The synthesis is depicted in Scheme 1. The binding of the first amino-acid to the chloromethylated resin proceeded through the triethylammonium salt of the *N*-t-butoxycarbonylamino-acid. The extent of reaction

reacted with triethylamine in chloroform to neutralize hydrochloric acid and liberate the amine function. The efficiency of liberation of the amine group was checked by Kaiser's test[7] using Troll and Canan's ninhydrin reagent.[8] This test allowed rapid detection of incomplete coupling which could not be detected by amino-acid analysis.

The next coupling step proceeded through the action of a very large excess of *N*-t-butoxycarbonylamino-acid in the presence of dicyclohexylcarbodi-imide. We used Kaiser's test[7] to study the extent of coupling.

The *N*-terminal group was then liberated by a solution of anhydrous hydrogen chloride in glacial acetic acid as before. At each step the growing peptide was isolated by simple filtration. The derived oligomer was then separated from the resin by a solution of anhydrous hydrogen bromide in trifluoroacetic acid.

By this process we prepared the dimer and the trimer of γ-*p*-nitrobenzyl-L-glutamate: dimer, m.p. 146—148°, R_F 0·75 [n-butanol-acetic acid–water 4 : 1 : 1 (v/v)], $\nu_{max.}$ 1610 (amide I), 1535 (amide II), and 1350 (C–O)

[3] M. H. Loucheux and C. Duflot, *Biopolymers*, 1973, **12**, 121.
[4] M. H. Loucheux and S. Graff, *Compt. rend.*, 1969, **269**, 1509.
[5] J. M. Stewart, J. D. Young, E. Benjamini, M. Shimizu, and C. Y. Leung, *Biochemistry*, 1966, **5**, 3396.
[6] S. Allenmark, *Acta Chem. Scand.*, 1966, **20**, 910.
[7] E. Kaiser, R. L. Colescott, C. D. Bossinger, and P. I. Cook, *Analyt. Biochem.*, 1970, **34**, 595.
[8] W. Troll and R. K. Cannan, *J. Biol. Chem.*, 1953, **200**, 803.

cm^{-1}, $[\alpha]_{546}^{23}$ $-4{\cdot}8°$ (*c* 0·42, trimethyl phosphate), $\lambda_{max.}$ 272 nm (ε 18,600); trimer, m.p. 125—126°, R_F 0·96 [ethyl acetate–pyridine–acetic acid–water 5 : 5 : 1 : 3 (v/v)], $\nu_{max.}$ 1610, 1535, and 1350 cm^{-1}, $[\alpha]_{546}^{23}$ $-3{\cdot}8°$ (*c* 0·79, trimethyl phosphate), $\lambda_{max.}$ 272 nm (ε 30,800).

Racemization. A suspension of each peptide in 6N-hydrochloric acid (5·00 ml) was heated for 18 h at 110° in an oil-bath. A standard solution of γ-*p*-nitrobenzyl-L-glutamate was treated in a similar manner. The optical rotations of those solutions were measured at 366 nm. The results show that both peptides are partly racemized.

Synthesis by a 'Step-by-step' Method.—Choice of a protecting group. One of the main problems in peptide synthesis is the choice of protecting groups which allow crystallization of the products. Our choice was influenced by several factors, the most important being that the protecting groups had to be free of chromophores absorbing at the same wavelengths as the *p*-nitrobenzyl group in order to allow optical studies.

N-Protecting groups. Urethane-type protecting groups appeared to be useful in our case. Their steric hindrance is not too large, and they can be introduced and eliminated without affecting the *p*-nitrobenzyl group. The benzyloxycarbonyl group was not suitable for our purpose because it must be introduced in an alkaline medium. The t-butoxycarbonyl group was preferable because it can be introduced using dimethyl sulphoxide as solvent,[9,10] *i.e.* without any risk of alkaline hydrolysis.

C-Protecting groups. Many protecting groups have been used in this case, *e.g.* methyl and ethyl esters. However, the oligomers protected by these groups did not crystallize. We therefore used an active ester, well known for giving good crystalline products: *N*-hydroxysuccinimidyl ester which does not absorb at the same wavelengths as the *p*-nitrobenzyl group.

Choice of the coupling process. The process we chose is nearly the same as that used by Spach *et al.*[11,12] to synthesize the oligomers of γ-benzyl-L-glutamate (Scheme 2). By this method the completely blocked dimer and trimer Boc[Glu (O-*p*-NB)]$_n$OSu ($n = 2$ or 3) were prepared in good yields: completely blocked dimer, m.p. 78—80°, $\nu_{max.}$ 1610, 1535, and 1350 cm^{-1}, $[\alpha]_{546}^{23}$ $-5{\cdot}8°$ (*c* 1·196, trimethyl phosphate), $\lambda_{max.}$ 272 nm (ε 21,000); completely blocked trimer, m.p. 65—66°, $\nu_{max.}$ 1610, 1535, and 1350 cm^{-1}, $[\alpha]_{546}^{23}$ $-4{\cdot}2°$ (*c* 1·181, trimethyl phosphate), $\lambda_{max.}$ 272 nm (ε 31,000).

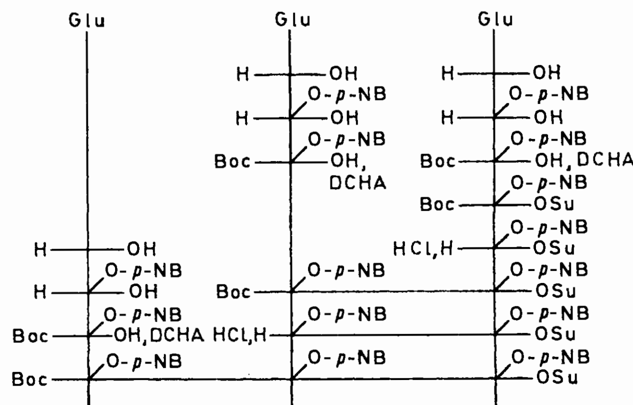
Racemization. Racemizations were carried out as before and showed that both peptides were partly racemized.

Comparison of the Two Processes.—Yields. Although the Merrifield method seems more promising because the intermediate products need not be separated, it gives very low yields. The final product must be recrystallized many times. It is a long and tedious process, although the problem here was not difficult, because we had only to purify a mixture containing essentially the monomer, the dimer, and the trimer. Moreover the longer oligomers are insoluble in methanol which facilitates their purification.

Products. Despite the low yields the Merrifield method makes the unprotected oligopeptides easily obtainable. This is of interest only because the corresponding oligomers were obtained as crystalline products.

The low yields can be explained by a side reaction recently described by Merrifield *et al.*,[13] *i.e.* a carboxylic acid catalysed intramolecular aminolysis of the ester bond to the resin at the dipeptide stage.

Racemization. It is now well known that some degree of racemization of amino-acids takes place when proteins or peptides are hydrolysed with acid.[14] In the case of glutamic acid racemization would occur through the formation of a glutarimide intermediate which rearranges, giving a mixture of L- and D-derivatives.[15] On the other hand many authors have studied the influence of different coupling methods and protecting groups on racemization. Bayer *et al.*[16] did not observe any racemization when using the solid phase synthesis under the conditions employed by Merrifield. The t-butoxycarbonyl protecting group is also known to prevent racemization.[17] Therefore, we think that the racemization observed occurred through a rearrangement during the hydrolysis.



SCHEME 2 Boc = t-Butoxycarbonyl, Su = *N*-hydroxysuccinimidyl, DCHA = dicyclohexylammonium, *p*-NB = 4-nitrobenzyl

C.d. Study of Oligomers.—Both the dimer and trimer of *p*-nitrobenzyl-L-glutamate have been studied by c.d. over two wavelength ranges, the first corresponding to the peptide chromophore which has absorption bands at

[9] J. M. Stewart and J. D. Young, 'Solid Phase Peptide Synthesis,' Freeman, San Francisco, 1969.
[10] J. Halstrøm, O. Schou, K. Kovacs, and K. Brunfeldt, *Hoppe-Seyler's Z. Physiol. Chem.*, 1970, **351**, 1576.
[11] Y. Trudelle and G. Spach, *Bull. Soc. chim. France*, 1971, 4495.
[12] A. Brack and G. Spach, *Bull. Soc. chim. France*, 1971, 4484.
[13] B. F. Gisin and R. B. Merrifield, *J. Amer. Chem. Soc.*, 1972, **94**, 3102.
[14] J. Manning and S. Moore, *J. Biol. Chem.*, 1968, **243**, 5591.
[15] H. D. Law, 'The Organic Chemistry of Peptides,' Wiley-Interscience, London, 1970, p. 14.
[16] E. Bayer, E. Gil-Av, W. A. Konig, S. Nakaparksin, T. Oro, and W. Parr, *J. Amer. Chem. Soc.*, 1970, **92**, 1738.
[17] H. Determann, J. Heuer, P. Pfander, and M. L. Reinartz, *Annalen*, 1966, **694**, 190.

ca. 222, 207, and 197 nm, and the second corresponding to the nitrobenzyl chromophore which shows three absorption bands at *ca.* 350, 290, and 270 nm.

Peptide chromophore –CO–NH–. The c.d. band due to the peptide $n \longrightarrow \pi^*$ transition has been recorded for all oligomers, protected or not, for trimethyl phosphate solutions, and for the monomer in the form Boc–Glu(O-*p*-NB)–OSu in order to make it soluble in trimethyl phosphate. Table 1 gives the values for λ_j, the wavelength of the extremum in the c.d. curve,

TABLE 1

C.d. data for peptide $n \longrightarrow \pi^*$ transitions in trimethyl phosphate solution

	λ_j/nm [a]	$[\theta]_j^0$/degree cm^2 $dmol^{-1}$ [b]	R_j/erg cm^{-2} radian [c]
Protected monomer [d,e]	213	2980	0·0334
Protected dimer	214	29,320	0·2006
Unprotected dimer	211	9080	0·0573
Protected trimer	217·5	11,000	0·0706
Unprotected trimer	220	15,900	0·1715

[a] Wavelength of extremum. [b] Value of ellipticity $[\theta]_j$ at extremum. [c] Rotational strength. [d] Protected peptides *H*–[Glu(O-*p*-NB)]$_n$–OH; n = 1—3. [e] Unprotected peptides Boc–[Glu(O-*p*-NB)]$_n$–OSu; n = 1—3.

$[\theta]_j^0$, the value of the ellipticity at the extremum, and R_j, the rotational strength associated with a single c.d. band. Moscowitz [18] showed that, if the c.d. band is approximately Gaussian, R_j is given by equation (1)

$$R_j = 0{\cdot}696\pi^{\frac{1}{2}}10^{-42}[\theta]_j^0\Delta_j\lambda_j \qquad (1)$$

where Δ_j is the half-width of the c.d. band (half the separation in nm between the wavelengths at which $[\theta]_j$ falls to $1/e[\theta]_j^0$).

strength for molecules containing two peptide units as a function of the conformational angles ϕ and ψ for a number of the structural situations which arise in polypeptides. Comparison of the sign of the rotatory strength obtained from the $n \longrightarrow \pi^*$ transition of our unprotected trimer with these calculated rotational strength maps permits the determination of a set of values, corresponding to the positive rotational strengths for a *trans,trans*-peptide.

Nitrobenzyl chromophore. In the wavelength range measured, the nitrobenzyl chromophore shows three absorption bands at *ca.* 270 (intramolecular charge transfer band), 290 (α band of benzene), and 350 nm ($n \longrightarrow \pi^*$ transition).[1] C.d. spectra were recorded from 400 to 250 nm for monomer and oligomers. The values of the wavelengths of the extrema of the c.d. bands and the corresponding ellipticities are given in Table 2. The $[\theta]_j$ values are based on that for one nitrobenzyl chromophore.

Generally, the Cotton effects are weak, too weak in a few cases to allow a precise value of the ellipticity to be given. But it has been shown that each product studied gives three c.d. bands corresponding to absorption bands. As in dichloroacetic acid solution,[1] γ-*p*-nitrobenzyl-L-glutamate shows three Cotton effects in aqueous solution. Since the solutions used in the spectroscopic studies are very dilute, side chain–side chain interactions do not exist; therefore the optical activity of the nitrobenzyl group is necessarily induced by an interaction between the chiral carbon atom and the extrinsic chromophore which would be due to a folding of the side chain. Three Cotton effects were also

TABLE 2

C.d. data for the nitrobenzyl chromophore

Product	Solvent	λ_j/nm	$[\theta]_j^0$/ degree cm^2 $dmol^{-1}$	λ_j/nm	$[\theta]_j^0$/ degree cm^2 $dmol^{-1}$	λ_j/nm	$[\theta]_j^0$/ degree cm^2 $dmol^{-1}$
Unprotected monomer	0·1N-NaOH	343	28	287	26	261	52
Protected monomer	Me_3PO_4	341	125	288	1219	263	
Unprotected dimer	Me_3PO_4	339	79	293		272	739
Protected dimer	Me_3PO_4	342		290		258	3141
Unprotected trimer	Me_3PO_4	345		302		266	3860
Protected trimer	Me_3PO_4	364	432	288		265	1530

In all cases, the rotational strength is positive. The maximum of the $n \longrightarrow \pi^*$ transition band is at *ca.* 212 nm for the monomer and the dimers, but at *ca.* 218 nm for the trimers. For polypeptides the maximum of this band is observed at *ca.* 222 nm when the polymer adopts an α helical structure and at *ca.* 217 nm when the polymer adopts a random coil conformation. Therefore, it is obvious that the position of the trimer $n \longrightarrow \pi^*$ transition band should be centred at 217 nm, since the chain is not long enough to adopt a helical structure. For the monomers and dimers the $n \longrightarrow \pi^*$ transition band exhibits a blue shift in good agreement with the calculations and observations of Schellman and his co-workers who localized this band at *ca.* 212 ± 2 nm.[19] Furthermore, they have calculated the rotational

[18] A. Moscowitz in 'Optical Rotatory Dispersion,' ed. C. Djerassi, McGraw Hill, New York, 1960, p. 150.

recorded for the protected monomer in organic solutions, but the maximum wavelengths of the corresponding c.d. bands were shifted when compared with those for aqueous solution.

We are not able to compare the c.d. spectra of the protected and unprotected oligomers. Especially for the dimer, the influence of the protecting groups cannot be neglected. Therefore we will discuss only the results obtained with unprotected products. The c.d. band at *ca.* 350 nm always exhibits weak ellipticity for each compound studied. Since this band corresponds to the $n \longrightarrow \pi^*$ transition its optical activity would be essentially due to side chain–side chain interactions; therefore, these interactions are very weak. The

[19] D. W. Urry in 'Spectroscopic Approaches to Biomolecular Conformation,' ed. D. W. Urry, American Medical Association, Chicago, 1970, p. 33.

random coil configurations of the oligomers which do not allow the side chains to be near enough to interact are compatible with this result. The absorption band at *ca.* 290 nm is always optically active, for monomer, dimer, and trimer. The charge transfer band is also always optically active. Its maximum ellipticity increases from monomer to dimer to trimer. Moreover, this band is characterized by a molar extinction coefficient $>10^3$. In this case, the optical activity is essentially due to interactions with vicinal chromophores exhibiting large absorption bands, *i.e.*, in this case, with the peptide chromophore.[20]

In conclusion, we can say, both for the monomer and for the oligomers, that the experimental results are in good agreement with folding of the side chain towards the peptide chromophore.

EXPERIMENTAL

Solid-phase Peptide Synthesis.—N-*t-Butoxycarbonyl-γ-*p-*nitrobenzyl-*L-*glutamate, dicyclohexylammonium salt*, Boc–Glu-(O-*p*-NB)–OH,DCHA. (a) Boc–Glu(O-*p*-NB)–OH.[9,10] This was purified by two recrystallizations from ethyl acetate to give a product with m.p. 104—105° (lit.,[10] 104—105°), $\lambda_{max.}$ 270 nm (ε 10,700), $[\alpha]_D^{20}$ −7·4° (*c* 10·42 g l^{-1}; methanol) (lit.,[10] $[\alpha]_D^{22}$ −8°), R_F 0·96 [42·5 : 5 : 2·5 (v/v) $CHCl_3$–CH_3OH–AcOH) (Found: C, 49·6; H, 6·2; O, 31·5; N, 6·1; S, 6·7. Calc. for $C_{19}H_{28}N_2O_9S$: C, 49·6; H, 6·1; O, 31·3; N, 6·1; S, 7·0%). The product contained one DMSO molecule per molecule determined by Allenmark's method.[6]

(b) Boc–Glu(O-*p*-NB)–OH,DCHA.[19,21] The salt (74·5%) was recrystallized from water, m.p. 146—150° (lit., 144—146°,[21] 146—148° [22]), $\lambda_{max.}$ 270 nm (ε 10,000).

Oligomer syntheses. The resin used (Fluka) had a degree of chloromethylation of 0·7 Cl mequiv. per g of resin, determined by Volhard's method.[23] Before use, the resin was swollen by stirring in methylene chloride at room temperature for 48 h.

(*a*) Attachment of the first amino-acid to the resin. Brunfeldt and Halstrøm's method [22] was used for the neutralization of Boc–Glu(O-*p*-NB)–OH,DCHA, giving the product as an oil.

N-t-Butoxycarbonyl-*γ*-*p*-nitrobenzyl-L-glutamate, triethylammonium salt, Boc–Glu(O-*p*-NB)–OH,TEA was obtained by suspending the oil from the previous step (3·824 g, 0·01 mol) in ethyl acetate (25 ml) and adding freshly distilled triethylamine (1·40 ml, 0·01 mol). The solvent was then evaporated *in vacuo* giving an oily residue which crystallized in a few hours at room temperature. A mixture of chloromethylated resin (1 g) and Boc–Glu-(O-*p*-NB)–OH,TEA (0·34 g, 0·7 mol) was gently refluxed in absolute ethanol for 48 h in an oil-bath heated to *ca.* 90°. The resin was filtered and washed successively three times with ethanol, water, methanol, and methylene chloride, allowing adequate contact time for the solvent to penetrate the resin beads and for solutes to diffuse out of the beads. The resin was dried overnight in a desiccator *in vacuo*.

(*b*) Stepwise addition of peptides to the resin. Boc–Glu-(O-*p*-NB)–resin (1 g) was subjected to the routine operations used for solid phase peptide synthesis for the addition of each amino-acid.[9]

Cleavage of the finished peptide from the resin.[9] Since the higher oligomers were insoluble in methanol, purification by crystallization from this solvent is possible. The yields obtained for dimer and trimer preparations were very low.

Classical Method.—N-*t-Butoxycarbonyl-γ-*p-*nitrobenzyl-*L-*glutamate, succinimidyl ester*, Boc–Glu(O-*p*-NB)–OSu.[11] This was obtained as an oil; attempts to recrystallize it were unsuccessful.

*γ-*p-*Nitrobenzyl-*L-*glutamate, succinimidyl ester, chloride*, HCl, H–Glu(O-*p*-NB)–OSu.[12] This was obtained in 56% yield from Boc–Glu(O-*p*-NB)–OH,DCHA and was kept *in vacuo* as it was extremely hygroscopic. It was characterized by the u.v. spectra $\lambda_{max.}$ 267 nm (ε 9440).

The protected dimer, Boc–Glu(O-*p*-NB)–Glu O-*p*-NB)–OSu. Boc–Glu(O-*p*-NB)–OH,DCHA (2·82 g, 5 mmol) was dissolved in chloroform (55 ml), the solution cooled to −10°, and then DCC (1·03 g, 5 mmol) and HCl,H–Glu(O-*p*-NB)–OSu (2·08 g, 5 mmol) added. After 2 h at −10° and 2 h at room temperature urea was filtered off, chloroform evaporated *in vacuo*, and the oily residue dissolved in ethyl acetate. This operation was repeated twice, finally yielding an oil which crystallized after three days *in vacuo* (3·34 g, 90%). The dimer was purified by gel chromatography,[24] rather than by recrystallization from methanol [12] (LH 20 Sephadex; 16·5 × 2·0 cm column; flow rate 0·3 ml min^{-1}; detection by u.v.).

Chloride of protected dimer, HCl,H–Glu(O-*p*-NB)–Glu-(O-*p*-NB)–OSu. The protected dimer (2·60 g, 3·5 mmol) was dissolved in acetone (20 ml) and ether (20 ml) added. Anhydrous HCl was bubbled through for 10 min and the mixture stirred for 2 h at room temperature. Upon addition of an excess of ether the product separated as an oil and was used in this form after removal of the solvent.

Protected trimer, Boc–[Glu(O-*p*-NB)]$_3$–OSu. Boc–Glu-(O-*p*-NB)–OH,DCHA (0·829 g, 1·47 mmol) was dissolved in chloroform (10 ml) at −10° and DCC (0·304 g, 1·47 mmol) and HCl,H–[Glu(O-*p*-NB)]$_2$–OSu (1 g, 1·47 mmol) were added successively. The mixture was stirred for 1 h at −20° and then 4 h at room temperature. Urea was filtered off and the solvent evaporated. The oily residue was dissolved in warm ethyl acetate, the solution filtered, and the solvent evaporated. The trimer was then dissolved in chloroform (10 ml), ethanol (100 ml) added, and the solution concentrated *in vacuo*. The trimer crystallized (0·5 g, 33·7%). The crude product was purified by gel chromatography as above.

C.d. Spectra.—C.d. spectra were recorded with a Roussel–Jouan II dichrograph, using 1, 0·5, 0·1, and 0·01 cm pathlength cells.

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[20] P. M. Bayley, E. B. Nielsen, and J. A. Schellman, *J. Phys. Chem.*, 1969, **73**, 228.
[21] E. Schnabel, *Annalen*, 1967, **702**, 188.
[22] K. Brunfeldt and J. Halstrøm, *Acta Chem. Scand.*, 1970, **24**, 3013.
[23] P. B. Hawk, B. L. Oser, and W. H. Summersom, 'Practical Physiological Chemistry,' Blakiston, Philadelphia, 1954, 13th edn., p. 955.
[24] H. Determann, 'Chromatographic sur gel,' Masson, Paris, 1969.