o-Nitrophenyl Esters in Solid Phase Peptide Synthesis

MIKLOS BODANSZKY* AND KENNETH W. FUNK

Department of Chemistry, Case Western Reserve University, Cleveland, Ohio 44106

Received November 10, 1972

Stepwise synthesis of the heptapeptide amide, L-Leu-L-Gln-L-Asn-L-Cys(Bzl)-L-Pro-L-Leu-Gly-NH_2 has been carried out by the solid phase method (a) with *o*-nitrophenyl esters as acylating agents and (b) with DCC as coupling reagent at each chain-lengthening step. Countercurrent distribution revealed only minor impurities in the product obtained with active esters, but not insignificant amounts of by-products in the material prepared by the DCC method.

Nitrophenyl esters,[1] in spite of their pronounced reactivity in aminolysis, are not the best acylating agents in reactions involving derivatives of single amino acids or dipeptides as amino components. Diketopiperazine formation competes with the desired acylation, particularly in the case of dipeptides where the ring closure, an unimolecular reaction, competes with the desired bimolecular acylation. However, many advantages of active esters, such as unequivocal reactions or the ease of removal of excess reagent, fully emerge when an already existing shorter or longer peptide chain needs to be lengthened by the addition of a single amino acid residue. These observations and the general concern about racemization during coupling led to the stepwise strategy first demonstrated on a synthesis of oxytocin.[2] The then new approach, stepwise synthesis of peptides with active esters,[3] was considered by Merrifield[4] to be ideal for solid phase peptide synthesis (SPPS), but was immediately abandoned for technical reasons. After Bodanszky and Sheehan[5] proved that active esters are indeed applicable in SPPS, the *p*-nitrophenyl esters of protected asparagine and glutamine became accepted tools of solid phase peptide chemists. A more general use of active esters in SPPS was reported only in a few cases.[6] Probably because of the moderate rates of acylation of resin-bound amino components,[7] the pronounced solvent dependence of these rates, and incomplete reactions with hindered amino acids,[8] coupling with dicyclohexylcarbodiimide (DCC)[9] remained the most widely used method in SPPS. Carbodiimides and several other coupling reagents[10] are highly efficient and can provide the desired amides even in minutes,[11] but they suffer from the disadvantage of overactivation[12] and can lead to undesired side reactions. One of these, the formation of ninhydrin-positive impurities in the reaction of DCC with *tert*-butyloxycarbonyl (Boc) amino acids, was recently reported.[13] More importantly, the high reactivity of intermediates such as *o*-acylisoureas or symmetrical anhydrides[14] necessitates global protection. Side chain hydroxyl and carboxyl groups must be protected. Selective acylation of amino groups can be achieved only at the expense of reactivity and, hence, of speed. Yet, with unprotected hydroxyl and carboxyl functions, there is a considerable gain in the freedom of planning of syntheses (*e.g.*, with free side chain carboxyl groups, the absence of esters permits the removal of completed chains from the resin by aminolysis,[5] hydrazinolysis,[15] or ester exchange[16]).

The advantages of selective acylation prompted a reexamination[17] of different active esters with respect to their usefulness in SPPS. It soon became clear that rate measurements carried out in solution are not necessarily valid when the amino component is attached to an insoluble support. The matrix of the resin itself is the cause of serious steric hindrance, which is subsequently compounded by the hindrance from the growing peptide chain.[13,18] It is understandable, therefore, that bulky activating groups, such as the one in pentachlorophenyl esters,[19] render the corresponding derivative, that was highly active in solution, quite inefficient in SPPS. The initially applied[5] *p*-nitrophenyl esters are better in this respect, but not particularly fast. On the other hand, *o*-nitrophenyl esters, while only somewhat more reactive in solution than their para isomers, were found quite promising in SPPS. An additional advantage of *o*-nitrophenyl esters is that, in contrast to the para isomers, their reaction rates are only slightly solvent dependent.[13]

To test the applicability of *o*-nitrophenyl esters in actual SPPS, the heptapeptide amide L-leucyl-L-glutaminyl-L-asparaginyl-*S*-benzyl-L-cysteinyl-L-prolyl-

(1) M. Bodanszky, *Nature (London)*, **175**, 685 (1955).

(2) M. Bodanszky and V. du Vigneaud, *Nature (London)*, **183**, 1324 (1959); M. Bodanszky and V. du Vigneaud, *J. Amer. Chem. Soc.*, **81**, 5688 (1959).

(3) M. Bodanszky, *Ann. N. Y. Acad. Sci.*, **88**, 655 (1960).

(4) R. B. Merrifield, *J. Amer. Chem. Soc.*, **75**, 2149 (1963).

(5) M. Bodanszky and J. T. Sheehan, *Chem. Ind. (London)*, 1423 (1964).

(6) S. Hörnle, *Z. Physiol. Chem.*, **348**, 1355 (1967); V. Weber, S. Hörnle, G. Grieser, K. H. Herzog, and G. Weitzel, *ibid.*, **348**, 1715 (1967).

(7) H. C. Beyerman, C. A. M. Boers-Boonekamp, and H. Maassen Van Den Brink-Zimmermannova, *Recl. Trav. Chim. Pays-Bas*, **87**, 257 (1968).

(8) K. Lübke, personal communication.

(9) J. C. Sheehan and G. P. Hess, *J. Amer. Chem. Soc.*, **77**, 1067 (1955).

(10) F. M. Bumpus, M. C. Khosla, and R. R. Smeby, 153rd National Meeting of the American Chemical Society, Miami Beach, Fla., April 9–14, 1967, Abstract M40.

(11) L. Corley, D. H. Sachs, and C. B. Anfinsen, *Biochem. Biophys. Res. Commun.*, **47**, 1353 (1972).

(12) M. Brenner in "Proceedings of the Eighth European Peptide Symposium, 1966," H. C. Beyerman, A. Van de Linde, and W. Maassen-van den Brink, Ed., North-Holland Publishing Co., Amsterdam, 1967, p 1.

(13) M. Bodanszky, R. J. Bath, A. Chang, M. L. Fink, K. W. Funk, S. M. Greenwald, and Y. S. Klausner in "Proceedings of the Third American Peptide Symposium," in press.

(14) H. G. Khorana, *Chem. Ind. (London)*, 1087 (1955).

(15) W. Kessler and B. Iselin, *Helv. Chim. Acta*, **49**, 1330 (1966); M. Ohno and C. B. Anfinsen, *J. Amer. Chem. Soc.*, **89**, 5994 (1967); *cf.* also ref 5.

(16) M. Bodanszky and J. T. Sheehan, *Chem. Ind. (London)*, 1597 (1966); *cf.* also H. C. Beyerman, H. Hendriks, and E. W. B. de Leer, *Chem. Commun.*, 1668 (1968).

(17) M. Bodanszky and R. J. Bath, *Chem. Commun.*, 1259 (1969).

(18) M. Bodanszky in "Peptides: Chemistry and Biochemistry, Proceedings of the First American Peptide Symposium at Yale University, 1968," B. Weinstein and S. Lande, Ed., Marcel Dekker, New York, N. Y., 1970, p 1; *cf.* also H. Hagenmaier, *Tetrahedron Lett.*, 283 (1970).

(19) G. Kupryszewski and M. Kaczmarek, *Rocz. Chem.*, **35**, 935 (1961).

L-leucylglycinamide[20] was synthesized first by the exclusive use of *o*-nitrophenyl esters, and then, for comparison, also by DCC couplings in each step. The aminobenzhydryl resin described by Rivaille and his associates[21] was acylated by *tert*-butyloxycarbonylglycine *o*-nitrophenyl ester (Boc-Gly-ONO). The protecting group was removed with trifluoroacetic acid (TFA) and the resulting amine was treated with Boc-L-Leu-ONO. This procedure was followed until the complete chain of the heptapeptide was assembled. The acylation steps were monitored by measurements (uv absorption) of the released *o*-nitrophenol. The completeness of these reactions was checked by the ninhydrin method of Kaiser and his coworkers.[22] In the alternative procedure, the rapid method proposed by Corley, Sachs, and Anfinsen[11] was followed. The weight increase of the resin was about the same in the two syntheses. The crude heptapeptide amide, removed by the prolonged action of TFA,[23] was secured in both cases as the trifluoroacetate in amounts that correspond to the capacity of the resin. In view of the use of all reagents in considerable excess, the calculation of yields in the manner conventional in organic syntheses may not be justified.[24]

Countercurrent distribution[25] of the crude heptapeptide amide trifluoroacetate, prepared *via* active esters in the solvent system 1-butanol–ethanol–0.1% acetic acid (4:1:5) through 60 transfers, resulted in the distribution curve shown in Figure 1. A detailed study of the main distribution band revealed only slight amounts of impurities, apparently formed during the cleavage of the peptide from the resin by acidolysis.

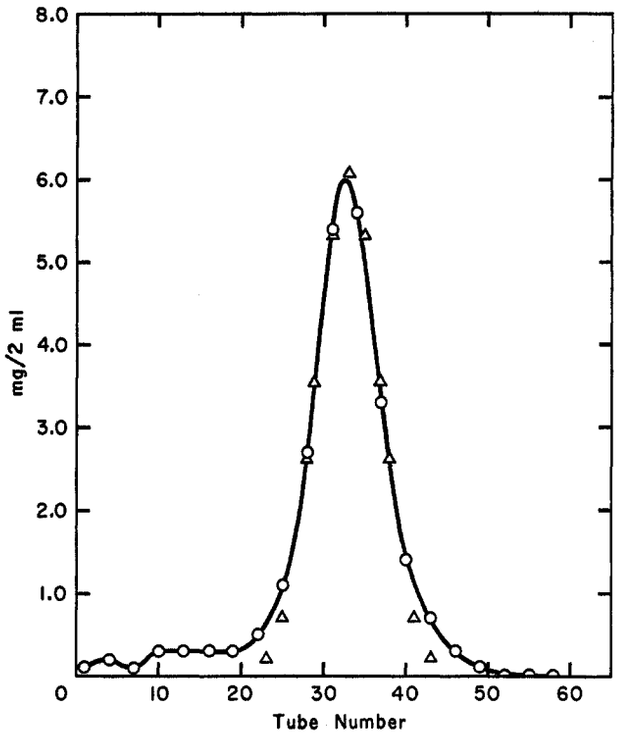

Figure 1.—Countercurrent distribution of crude heptapeptide amide I, prepared *via* *o*-nitrophenyl esters (○, experimental values; △, calculated values).

From tubes no. 30–40, on evaporation of the solvent, the trifluoroacetate of I was obtained in crystalline form. This material was shown to be chromatographically and analytically pure. A second sample of the same crude peptide trifluoroacetate was converted to the free base and was crystallized from water.

Distribution of the crude heptapeptide amide obtained by the rapid method[11] with DCC revealed (Figure 2) the presence of a considerable amount of impurities. This comparison clearly demonstrates that the active ester approach, although not particularly fast, can lead to products of good quality that can be easily purified. Further information is expected from our continued use of *o*-nitrophenyl esters in the SPPS of peptides containing residues with functional (OH, COOH) side chains.

Experimental Section

Capillary melting points are reported uncorrected. On thin layer chromatograms, the protected peptides were revealed by *tert*-butyl hypochlorite–KI–starch reagents. The following solvent systems were applied for development: A, *n*-BuOH–AcOH–H_2O (4:1:1); B, $CHCl_3$–MeOH (9:1). For paper chromatography, *n*-BuOH–pyridine–AcOH–H_2O (30:24:6:20)[26] was used.

For amino acid analyses, polymer-bound samples were hydrolyzed with propionic acid–6 *N* HCl (1:1 v/v)[27] in evacuated, sealed ampoules at 130° for 24 hr, and analyzed by the Spackman–Stein–Moore method[28] on a Beckman Spinco 120C amino acid analyzer. All other samples were hydrolyzed with constant-boiling HCl in evacuated, sealed ampoules at 110° for 16 hr.

***tert*-Butyloxycarbonylglycine *o*-Nitrophenyl Ester.**—*t*-Boc-Gly (4.81 g, 27.5 mmol) and *o*-nitrophenol (6.95 g, 50 mmol) were dissolved in pyridine (75 ml) and cooled in an ice–water bath.

(20) Initially the synthesis of oxytocin was planned. After the incorporation of the seventh residue, amino acid analysis revealed that, instead of L-isoleucine, L-leucine was added to the hexapeptide chain. The error was traced to a preparation of Boc-L-Leu mislabeled by the supplier as Boc-L-Ile. The material was checked by us only for homogeneity but not for identity and therefore the error was detected too late. A possible continuation of the synthesis, that would have resulted in the hormone analog 3-L-leucine oxytocin, was briefly considered. However, instead of persisting in the preparation of oxytocin or one of its analogs, it seemed more attractive to terminate the chain building at this point. From the point of view of exploration of advantages and disadvantages of *o*-nitrophenyl esters in SPPS, the heptapeptide amide I is not inferior, as a model, to oxytocin, in which complicating factors arise from ring closure, dimerization, etc. A question about the incorporation of the isoleucine residue was settled in separate experiments (with the participation of Mr. A. Chang): complete acylation of resin-bound valine was achieved with (*Z*)-L-Ile-ONO.

(21) P. Rivaille, A. Robinson, M. Kamen, and G. Milhaud, *Helv. Chim. Acta*, **54**, 2772 (1971).

(22) E. Kaiser, R. L. Colescott, C. D. Bossinger, and P. I. Cook, *Anal. Biochem.*, **34**, 595 (1970).

(23) Removal of the completed peptide amide from the resin with TFA is probably too slow for many practical purposes. Furthermore, short-column analysis of unhydrolyzed compound I in its crude form revealed two by-products, with maxima at 19 and 27 min, and therefore less basic than compound I, which is eluted at 46 min. During the slow process of cleavage, some hydrolysis of asparagine and/or glutamine residues seems to have occurred, probably owing to the presence of traces of water. Indeed, when samples of the protected heptapeptidyl resin (10 mg) were treated with TFA (0.5 ml) for periods from 1 to 16 days, not only a gradual increase in the amount of the by-product(s) that give a peak at 27 min and in the area of the ammonia peak was observed, but also a corresponding decrease in the size of the principal product peak at 46 min. An increase with time in the least basic peak at 19 min was also noted. These less basic by-products result therefore from hydrolysis during cleavage and are not the consequence of side reactions in the chain-building procedures.

(24) In the active ester approach, the amount of the Boc-amino acids used for the preparation of 1 mmol of crude heptapeptide amide I (trifluoroacetate) totalled 21 mmol. The rapid acylation procedure (with DCC) required 110 mmol of Boc-amino acid for the same amount of crude material. Because of the considerable difference in the quality of the crude peptides obtained by the two methods, the economy of the active ester method would become even more pronounced if the calculations would be based on purified products. For a detailed discussion of this question, *cf.* ref 18.

(25) L. C. Craig and T. P. King in "Methods of Biochemical Analysis," Vol. 10, D. Glick, Ed., Interscience, New York, N. Y., 1962, p 201.

(26) S. G. Waley and J. Watson, *Biochem. J.*, **57**, 529 (1954).

(27) J. Schotchler, R. Lozier, and A. Robinson, *J. Org. Chem.*, **35**, 3151 (1970).

(28) D. H. Spackman, W. H. Stein, and S. Moore, *Anal. Chem.*, **30**, 1190 (1958).

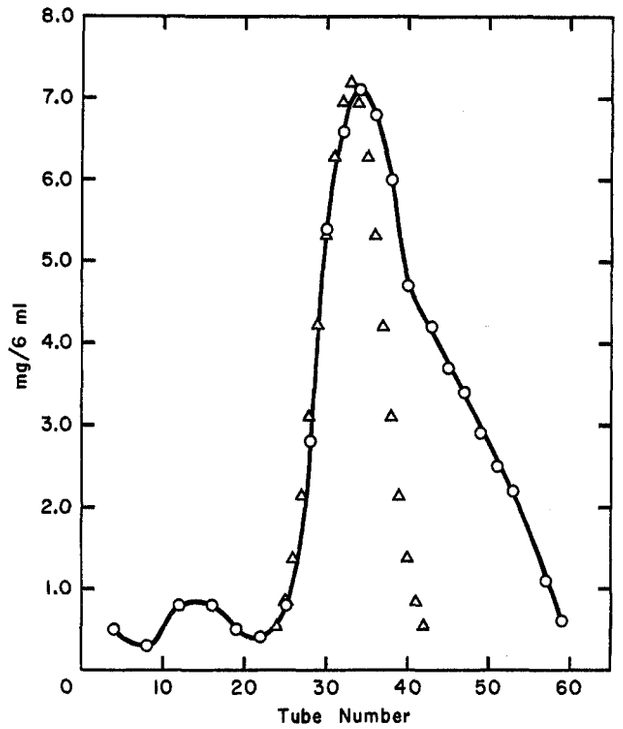

Figure 2.—Countercurrent distribution of crude heptapeptide amide I with DCC applied in all coupling steps (O, experimental values; Δ, calculated values).

DCC (5.15 g, 25 mmol) was added to the stirred solution and rinsed in with more pyridine (25 ml). After 30 min of stirring, the ice bath was replaced with a bath at room temperature. The reaction was followed by the disappearance of the diimide band at 4.8 μ in the ir spectrum. After a total of 4 hr, the *N,N'*-dicyclohexylurea (DCU) was removed by filtration and the pyridine by evaporation *in vacuo* below room temperature. The resulting oil was dissolved in ether and filtered to remove some more DCU. The solvent was evaporated and the crystalline residue was dissolved in $CHCl_3$ (100 ml). The solution was extracted with 5% citric acid (75 ml in three portions), and then with small volumes of 0.1 *N* NaOH. The first few extracts were yellow, the subsequent ones red. When the extracts were again light in color (orange), the washing with alkali was discontinued and the organic layer was washed with water (100 ml). The solution was dried over $MgSO_4$ and filtered, and the solvent was evaporated *in vacuo*. The crystalline solid was dissolved in warm 95% EtOH (75 ml). On cooling, long, white needles formed. The crystals were collected in the cold room, washed with cold 95% EtOH, and dried in air and finally in a desiccator *in vacuo* to give 5.73 g (78%): mp 96.5–98°; tlc R_fA 0.85, R_fB 0.63; active ester carbonyl, 5.62 μ.[29]

Anal. Calcd for $C_{18}H_{16}N_2O_6$ (296.4): C, 52.7; H, 5.4; N, 9.5. Found: C, 52.8; H, 5.4; N, 9.4.

***tert*-Butyloxycarbonyl-L-Leucine *o*-nitrophenyl ester** was prepared according to the procedure described above. The crude oil from a 65-mmol preparation was taken up in warm (35°) petroleum ether (bp 37–50°). Cooling to room temperature produced long, fine, white needles. After a night in a cold room, the crystals were collected, washed with cold petroleum ether, and dried. The active ester, 20.0 g (87%), melts at 56–57°: $[\alpha]^{25}D$ −68° (*c* 1, DMF); tlc R_fA 0.87, R_fB 0.65; active ester carbonyl, 5.62 μ.

Anal. Calcd for $C_{17}H_{24}N_2O_6$ (352.5): C, 57.9; H, 6.9; N, 8.0. Found: C, 58.0; H, 6.7; N, 8.1.

***tert*-Butyloxycarbonyl-L-proline *o*-nitrophenyl ester** was prepared as described for the above active esters. The crude oil from a 10-mmol preparation was taken up in ether; the solution was filtered and evaporated *in vacuo*. The residual oil slowly solidified on standing at room temperature. One-half of the crude solid was dissolved in warm (35°) 95% EtOH (12 ml), cooled, and seeded with the crude crystals. After the addition of water (3 ml), large, yellowish crystals grew over a 2-day period. The crystals were filtered, washed with cold 95% EtOH, and dried *in vacuo* to give 0.30 g, mp 61–70°. The filtrate was concentrated to an oil that, when combined with the rest of the crude solid, was taken up in warm (35°) 95% EtOH. Water was added to turbidity, and the solution was seeded and placed in the cold room. The crystals were collected, washed with cold 90% EtOH, and dried in air to give 1.7 g: mp 63–70° (a third crop weighed 0.73 g, mp 58–62.5°); $[\alpha]^{25}D$ −83° (*c* 1, DMF, 1% AcOH); tlc R_fA 0.84, R_fB 0.66; active ester carbonyl, 5.67 μ. A sample of the active ester was dissolved in hexane and filtered, and the filtrate was concentrated to an oil. The oil was triturated with a small volume of hexane until it solidified, and dried for analysis *in vacuo* over P_2O_5.

Anal. Calcd for $C_{16}H_{20}N_2O_6$ (336.4): C, 57.1; H, 6.0; N, 8.3. Found: C, 57.0; H, 6.2; N, 8.1.

***tert*-Butyloxycarbonyl-*S*-benzoyl-L-cysteine *o*-nitrophenyl ester** was prepared according to the procedure described for the active ester of glycine. The crude oil from a 10-mmol preparation was dissolved in warm 95% EtOH. On cooling, long white needles formed, which were then collected, washed with cold 95% EtOH, and dried to give 3.3 g (77%): mp 103–105°; $[\alpha]^{25}D$ −74° (*c* 1, DMF, 1% AcOH); tlc R_fA 0.82, R_fB 0.67; active ester carbonyl, 5.62 μ.

Anal. Calcd for $C_{21}H_{24}N_2O_6S$ (432.6): C, 58.3; H, 5.6; N, 6.5; S, 7.4. Found: C, 58.1; H, 5.5; N, 6.8; S, 7.6.

***tert*-Butyloxycarbonyl-L-asparagine *o*-nitrophenyl ester** was prepared according to the procedure described for Z-Asn-ONP.[30] From a 38-mmol preparation, 8.5 g of crude product was obtained, mp 137–141°, $[\alpha]^{25}D$ −48.0° (*c* 2, DMF). Trituration with EtOAc gave 6.7 g, mp 146–149°. Dissolution in DMF and precipitation by the addition of water yielded 5.6 g, mp 147.5–150°, $[\alpha]^{25}D$ −49.8° (*c* 2, DMF). Recrystallization from hot EtOAc produced 4.2 g (36%): mp 144.5–146.5; $[\alpha]^{25}D$ −52.0°; tlc R_fA 0.77, R_fB 0.41.

Anal. Calcd for $C_{15}H_{19}N_3O_7$ (353.3): C, 51.0; H, 5.4; N, 11.9. Found: C, 50.8; H, 5.6; N, 11.6.

***tert*-Butyloxycarbonyl-L-glutamine *o*-nitrophenyl ester** was prepared according to the procedure described in ref 30. From a 68-mmol preparation, the yield of crude product was 15.3 g, mp 132–137°. Trituration with EtOAc gave 14.9 g, mp 149–151°, $[\alpha]^{25}D$ −53.0° (*c* 2, DMF). Precipitation with water from DMF yielded 14.0 g, mp 149.5–151°, $[\alpha]^{25}D$ −52.3° (*c* 2, DMF). Recrystallization from hot EtOAc gave 8.7 g (without additional purification): mp 148.5–151°; $[\alpha]^{25}D$ −52.3° (*c* 2, DMF); tlc R_fA 0.79, R_fB 0.40.

Anal. Calcd for $C_{16}H_{21}N_3O_7$ (367.4): C, 52.3; H, 5.8; N, 11.4. Calcd for $C_{16}H_{21}N_3O_7 \cdot {}^1/_2H_2O$ (376.4): C, 51.1; H, 5.9; N, 11.2. Found: C, 51.3; H, 5.9; N, 11.3.[31]

Benzhydrylamine Resin.—The hydrochloride (6.0 g)[21] was treated with a mixture of triethylamine (4 ml) and dichloromethane (26 ml) in a sintered glass funnel. After about 1–2 min, the solvent was removed and the same treatment was repeated, this time for 5 min. The resin was washed with dichloromethane (160 ml in eight portions) and with methanol (60 ml in three portions) and dried *in vacuo* at room temperature. The incorporation of Boc-Gly, as determined by amino acid analysis, varied between 0.7 and 1.0 mmol per gram of resin. Amino acid analysis, after acylation of the resin with Boc-Gly-ONO as described below, gave similar results. However, in both procedures the increase in weight corresponds to an uptake of about 1.4 mmol of Boc-glycine by 1 g of aminobenzhydryl resin.

Reaction Vessel.—The stem of a sintered glass filter funnel (60 ml, F) was equipped with a two-way stopcock. This allowed the rapid interchange of N_2 (dried over KOH and $CaCl_2$) used for gentle agitation of the resin and aspiration used for filtration. A mercury regulator ensured constant N_2 pressure. The filter was kept loosely covered with a polyethylene stopper. The reagents were added manually.

Chain Building with *o*-Nitrophenyl Esters.—Aminobenzhydryl

(29) In the ir spectrum, the active ester carbonyl band of *o*-nitrophenyl esters appears at a lower wavelength (5.62 μ) than the corresponding band of the para isomers (5.65 μ). This is in harmony with the higher reactivity of the ortho derivatives.

(30) M. Bodanszky, G. S. Denning, Jr., and V. du Vigneaud, *Biochem. Prep.*, **10**, 122 (1963).

(31) The analytical sample was dried over P_2O_5 at 40° and *ca.* 0.1 mm for 2 hr. Drying at a higher temperature was not attempted because of the lack of stability of active esters of glutamine.

TABLE I

A STUDY OF POOLED FRACTIONS FROM COUNTERCURRENT DISTRIBUTION OF CRUDE COMPOUND I FROM ACTIVE ESTER SYNTHESIS

Pooled fraction (tube no.)	Tlc (R_fA)	Paper chromatogram	Area of peaks, cm² [a] 19 min	22 min	27 min	46[c] min	50 min	Amino acid analysis[b] Asp	Glu	Pro	Gly	Leu	Bzl-Cys
0–19	0.38 (w)[d]		6	0.5	0.5		1	0.8	0.9	0.7	1.0	1.4	
20–24	0.41[e]	0.75 (w)[d]						0.9	0.95	1.2	1.0	1.5	0.3
	0.32 (m)	0.71 (m)											
	0.21 (m)	0.61 (m)											
		0.50 (w)											
25–29	0.36[e] (s)	0.75 (s)			3	14		1.0	1.0	1.05	1.0	1.9	0.6
		0.61 (w)											
30–34	0.36 (s)	0.75 (s)			0.7	30		1.0	0.9	1.2	1.0	2.3	0.9
35–40	0.36 (s)	0.75 (s)				25		1.2	0.9	1.1	1.0	1.9	0.9
41–50	0.57 (w)	0.89 (t)				1		0.9	0.85	0.9	1.0	1.3	0.8
	0.39 (m)	0.77 (w)											
51–60	0.62 (w)							0.5	0.4	1.0	1.0	1.3	0.4
	0.41 (w)												

[a] 0.5 mg of unhydrolyzed sample was applied to the short column of the amino acid analyzer. 18 min, etc., indicates the elution time measured at the maxima. [b] Of hydrolysates. [c] Compound I. [d] Intensity: s, strong; m, medium; w, weak; t, trace. [e] Tailed.

resin (2.67 g) was placed into the reaction vessel and covered with a solution of *t*-Boc-Gly-ONO (1.18 g, 4 mmol) in DMF (20 ml, dried over Linde 4A Molecular Sieve) and gently agitated with a slow stream of N_2. Samples (5 μl) were removed at about hourly intervals and diluted to 10 ml with 95% EtOH containing 1% 1 *N* HCl. Absorption at 350 mμ (ϵ 2600) was used to measure the liberated *o*-nitrophenol. When no increase in the optical density was observed, the solution was removed by suction and the resin was washed with DMF (25 ml), followed by CH_2Cl_2 (60 ml in three portions). Small samples of the peptidyl resin were used for the ninhydrin test[22] (*ca.* 2 mg) and amino acid analysis (10 mg). Any remaining amino groups were acetylated with Ac_2O (0.5 ml) in CH_2Cl_2 (20 ml) for 45 min. The Boc group was removed by treatment with 25% TFA in CH_2Cl_2 (20 ml) for 1 min and by a second treatment for 25 min. The resin was then washed with CH_2Cl_2 (100 ml in four portions). Liberation of the amino groups was accomplished by treatment of the resin with triethylamine (TEA) in CH_2Cl_2 (2.5 ml/17.5 ml) for 1 min, removal of the solution by suction, and a second treatment, this time for 5 min, followed by washing with CH_2Cl_2 (240 ml in eight portions). The removal of Boc-protecting groups and the liberation of amino groups was carried out in the same manner throughout subsequent chain-lengthening steps. Acylation with the remaining protected active esters was carried out in DMF as follows. Boc-L-Leu-ONO (5 mmol) was added in three portions (two portions of 2 mmol each, followed by a 1-mmol portion), Boc-L-Pro-ONO (4.6 mmol) in three portions (3, 1, and 0.6 mmol), Boc-*S*-Bzl-L-Cys-ONO (4.5 mmol) in two portions (3.5 and 1 mmol), Boc-L-Asn-ONO (5.2 mmol) in three portions (3.2 mmol, and two portions of 1 mmol), Boc-L-Gln-ONO (5 mmol) in three portions (3 mmol, and two portions of 1 mmol), and Boc-L-Leu-ONO (8 mmol) in two portions (6 and 2 mmol). The final addition, with the exception of the asparagine active ester, led to no measureable reaction. The incorporation of the first two amino acids required only a few hours; the subsequent reactions were allowed to proceed overnight. At the completion of chain building, the protected amino acyl resin weighed 5.64 g, an increase of 2.97 g (without correction for samples removed). This corresponds to 3.25 mmol of protected heptapeptide or 1.2 mmol per gram of aminobenzhydryl resin. Amino acid analysis: Asp, 1.0; Glu, 0.95; Pro, 1.2; Gly, 1.0; Leu, 1.9, Bzl-Cys, 0.9.

Chain Building *via* Coupling with DCC.—Aminobenzhydryl resin (1.0 g), liberated from the hydrochloride as described above, was acylated with 2 mmol of Boc-Gly and 2 mmol of DCC, the latter applied in two equal portions. After the resin was washed alternately with CH_2Cl_2 and CH_3OH several times, the possibly remaining amino groups were acetylated with Ac_2O (0.5 ml) in CH_2Cl_2 (20 ml). The subsequent washings, deprotection and the incorporation of the next amino acid, etc., were carried out according to the procedure described in ref 11, except that a sintered glass filter (*cf.* above) was used as reaction vessel. The heat loss due to evaporation of CH_2Cl_2 was compensated by gentle warming of the filter with a warm air stream. The Boc derivatives of asparagine and glutamine were dissolved in CH_2Cl_2 with the addition of a small volume of DMF. All Boc-amino acids, with the exception of Boc-Gly, were applied in 20-mmol amounts. The weight increase after the completion of the synthesis of the protected heptapeptidyl resin, 1.06 g, corresponds to about 1.16 mmol of protected heptapeptide. Amino acid analysis: Asp, 1.0; Glu, 0.8; Pro, 0.9; Gly, 1.0; Leu, 1.9; Bzl-Cys, 0.8.

Acidolytic Removal of the Peptide Amide I from the Resin.—One-third (1.88 g) of the protected heptapeptidylaminobenzhydryl resin was treated with trifluoroacetic acid (TFA, 10 ml) overnight. The solution was separated from the resin by filtration and the latter was washed with TFA (10 ml) and CH_2Cl_2 (20 ml). Evaporation *in vacuo* left an oily residue that on trituration with ether (20 ml) afforded an off-white solid; this was dried *in vacuo* over KOH, 157 mg, mp 195–207°. A second treatment of the resin, for 24 hr, yielded 134 mg; a third and a fourth treatment, each for 24 hr, yielded 109 and 89 mg, respectively. Further exposure to TFA, for about 1 week each time, gave 239, 93, 27, and 18 mg, respectively. A total of 866 mg was collected. The first five fractions (a total of 728 mg) were found to be indistinguishable from each other on paper chromatograms (R_f 0.75 and a faint spot at R_f 0.61) and were pooled. On the short column of the amino acid analyzer, the unhydrolyzed material (0.5 mg) showed the peak corresponding to compound I at 46 min (20 cm²). Impurities emerged at 19 and 27 min with areas of 0.4 and 1 cm², respectively. An aliquot of this material was used for countercurrent distribution, as described below.

On completion of the second synthesis, in which DCC was used for coupling, a 67% aliquot of the protected heptapeptidylaminobenzhydryl resin (1.39 g) was cleaved similarly. The crude trifluoroacetate was collected in 513-, 128-, and 51-mg fractions after three exposures lasting for 5 days each. A total of 692 mg was collected. Paper chromatography of the largest fraction showed the major product at R_f 0.75, a slight impurity at R_f 0.62, and also a by-product at R_f 0.89. A sample (0.5 mg) applied unhydrolyzed to the short column revealed impurities at 27 and 66 min, with areas of 0.8 and 0.3 cm², respectively. The main product appeared with its maximum at 46 min (10 cm²).

Conversion of the Trifluoroacetate of Heptapeptide Amide I to the Free Amine.—This was carried out by following the procedure of Jost, Rudinger, and Šorm[32] described for the hydrobromide of a similar heptapeptide. From a sample of compound I (0.50 g) prepared *via* active esters, the free amine was obtained in crystalline form[33] (0.24 g): mp 179–182°; $[\alpha]^{25}D$ −54° (*c* 1, DMF); tlc R_fA 0.36. The unhydrolyzed material (0.5 mg) applied to the short column of the amino acid analyzer produced a single peak with its maximum emerging at 47 min. Amino acid analysis: Asp, 1.0; Glu, 0.95; Pro, 0.9; Gly, 1.05; Leu, 2.0; Bzl-Cys, 0.8; NH_3, 3.2.

Countercurrent Distribution of Compound I.—A sample (0.65 g) of I (trifluoroacetate) dissolved in the lower phase (10 ml) of the system 1-BuOH–EtOH–1% AcOH (4:1:5) was placed into the tube no. 0 of a 60-tube Craig apparatus and distributed with

(32) K. Jošt, J. Rudinger, and F. Šorm, *Collect. Czech. Chem. Commun.*, **26**, 2496 (1961).

(33) *Cf.* D. B. Hope and V. du Vigneaud, *J. Biochem.*, **237**, 3146 (1962).

TABLE II

A STUDY OF POOLED FRACTIONS FROM COUNTERCURRENT DISTRIBUTION OF CRUDE COMPOUND I FROM SYNTHESIS USING DCC FOR COUPLING

Pooled fraction (tube no.)	Tlc (R_fA)	Paper chromatogram	Area of peaks, cm² [a] 19 min	22 min	27 min	46[c] min	52 min	62 min	66 min	Amino acid analysis[b] Asp	Glu	Pro	Gly	Leu	Bzl-Cys
0–7	0.33 (w)[d]									1.0	1.0		0.3	0.8	
	0.24 (w)			2											
	0.12 (w)														
8–20	0.56 (m)	0.53 (w)[d]	0.3	1.5	0.2		2		3	1.4	1.0	0.3	0.5	1.8	*f*
	0.46 (s)	0.33 (w)													
	0.30 (s)	0.26 (w)													
	0.12 (m)														
24–33	0.44 (m)	0.89 (m)			2	18				0.9	0.8	0.8	1.0	1.7	0.6
	0.36[e] (s)	0.75 (s)													
		0.61 (m)													
34–44	0.59 (m)	0.89 (m)				16				0.85	0.75	0.9	1.0	2.0	0.9
	0.36 (s)	0.75 (s)													
45–50	0.57 (w)	0.90 (s)						10		0.9	0.7	1.0	1.0	1.8	0.9
	0.51 (m)														

[a–e] For references *a–e*, *cf.* Table I. [f] This fraction contains sulfur. Thus the absence of Bzl-Cys in the amino acid analysis should be due to some side reaction involving this residue, and not to a lack of its incorporation.

10-ml phases through 60 transfers. A weight curve was determined by evaporation of samples from selected tubes, from the lower phase up to tube no. 30 and from the upper phase beyond this tube. The distribution curve is shown in Figure 1. A sample (0.40 g) of the crude trifluoroacetate from the DCC-mediated synthesis was distributed in the same system; the distribution curve is shown in Figure 2.

Samples from different areas of these distributions were examined on paper chromatograms, on tlc, and on the short column of the amino acid analyzer and also by quantitative amino acid analysis of their hydrolysates. The results of this study are summarized in Tables I and II.

A sample (8 mg) of fractions 45–50 from the distribution of the crude product of the second synthesis (with DCC, *cf.* Figure 2) was dissolved in liquid NH_3 (*ca.* 50 ml) and treated with CH_3OH (0.3 ml) and Na^{24} to produce a blue color which persisted for about 20 min. After evaporation of the ammonia, the residue was dissolved in H_2O (4 ml) and an aliquot (1 ml) was evaporated with a stream of N_2 with warming. The residue was stored *in vacuo* over H_2SO_4 overnight and then hydrolyzed with 6 *N* HCl for amino acid analysis in the usual way. No significant decrease of aspartic acid content was observed and only very small amounts of basic amino acids appeared. Thus, the presence of the fast-moving component cannot be explained by nitril formation from the asparagine or glutamine residues; the question about the nature of this by-product remained unresolved.

Purified material from the active ester synthesis was obtained by pooling the contents of tubes 30–40 (0.40 g): mp 195–207°; $[\alpha]^{25}D$ −78° (*c* 2, H_2O); tlc R_fA 0.36; paper chromatogram *R* 0.75. Amino acid analysis: Asp, 1.0; Glu, 0.85; Pro, 1.0; Gly, 1.0; Leu, 2.0; Bzl-Cys, 0.9.

Anal. Calcd for $C_{40}H_{61}N_{10}O_{11}SF_3$ (947.0): C, 50.7; H, 6.5; N, 14.8; S, 3.4; F, 6.0. Calcd for $C_{40}H_{61}N_{10}O_{11}SF_3 \cdot H_2O$ (965.1): C, 49.8; H, 6.6; N, 14.5; S, 3.3; F, 5.9. Found: C, 49.6; H, 6.6; N, 14.0; S, 3.5; F, 6.1.

Registry No.—I free amine, 38605-53-7; I trifluoroacetate salt, 38605-54-8; *t*-Boc-Gly *o*-nitrophenyl ester, 38606-09-6; *t*-Boc-Gly, 4530-20-5; *o*-nitrophenol, 88-75-5; *t*-Boc-L-Leu *o*-nitrophenyl ester, 24868-52-8; *t*-Boc-L-Pro *o*-nitrophenyl ester, 38605-56-0; *t*-Boc-*S*-benzyl-L-Cys *o*-nitrophenyl ester, 38605-57-1; *t*-Boc-L-Asp *o*-nitrophenyl ester, 38605-58-2; *t*-Boc-L-Gln *o*-nitrophenyl ester, 38605-59-3.

Acknowledgments.—The authors thank Mr. Alex Chang for the preparation of the *o*-nitrophenyl esters of Boc-Asn and Boc-Gln. Amino acid analyses were carried out by Mrs. Delores J. Gaut, and elemental analyses by Mr. J. Alicino (of The Squibb Institute for Medical Research). This study was supported by a grant from the U. S. Public Health Service (NIH AM-12473).

(34) M. Zaoral and J. Rudinger, *Collect. Czech. Chem. Commun.*, **24**, 1993 (1959).