Acknowledgment.—We thank Mr. W. F. Hain and Mr. W. T. Yeung for skilled technical assistance. This work was supported in part by the American Cancer Society, The Geffen Foundation, and The Allen Foundation.

Registry No.—I, 41174-26-9; II, 41117-58-2; N^{α}-Boc-*S*-carbamidomethylcysteine, 41117-59-3; *S*-carbamidomethylcysteine, 17528-66-4; *p*-nitrophenyl N^{α}-Boc-*S*-carbamidomethylcysteinate, 41117-61-7; γ-*p*-bromobenzyl glutamate, 20806-21-7; *p*-bromobenzyl bromide, 589-15-1; N^{α}-Boc-γ-(*p*-bromobenzyl)-glutamic acid, 41117-62-8; triethylamine, 121-44-8; Boc-azide, 1070-19-5; 3,4-dimethylbenzylcysteine, 41594-21-2; cysteine, 52-90-4; ammonium chloride, 12125-02-9; 3,4-dimethylbenzyl chloride, 102-46-5; N^{α}-Boc-*S*-(3,4-dimethylbenzyl)cysteinedicyclohexylamine salt, 41117-64-0; N^{α}-Boc-*S*-(*p*-methoxybenzyl)-cysteine, 18942-46-6; N^{α}-Boc-*S*-(3,4-dimethylbenzyl)cysteine, 41117-66-2; human pituitary growth hormone, 9002-72-6.

o-Nitrophenyl Esters of *tert*-Butyloxycarbonylamino Acids and Their Application in the Stepwise Synthesis of Peptide Chains by a New Technique[1]

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Synthesis of the protected nonapeptide *tert*-butyloxycarbonyl-L-leucyl-L-glutaminyl-N^{ϵ}-2,6-dichlorobenzyloxycarbonyl-L-lysyl-L-leucyl-L-leucyl-L-glutaminylglycyl-L-leucyl-L-valinamide, corresponding to the C-terminal sequence of a secretin analog, is described. Acylation of L-valinamide with the *o*-nitrophenyl ester of *tert*-butyloxycarbonyl-L-leucine yielded a dipeptide derivative which, after deprotection with trifluoroacetic acid, was converted to a protected tripeptide amide by reaction with *tert*-butyloxycarbonylglycine *o*-nitrophenyl ester. The chain was lengthened in the same manner; *o*-nitrophenyl esters of Boc-amino acids were used as acylating agents. All the operations were carried out in the same vessel, from which the intermediates were not removed throughout the synthesis. The preparation and properties of *o*-nitrophenyl esters of Boc-amino acids are also reported.

Stepwise synthesis of peptide chains was proposed, and demonstrated on the example of oxytocin, by Bodanszky and du Vigneaud.[2] This approach proceeds through isolated intermediates. If unequivocal methods are used in the acylation and deprotection reactions, it permits rapid chain lengthening, since an extensive purification of the intermediates is often unnecessary. Removal of excess reagents and of by-products was accomplished, in the majority of steps, simply by washing with appropriately chosen solvents. Active esters, such as *p*-nitrophenyl esters, were found as the acylating agents of choice in this method. The repetitiveness of the operation led to the suggestion[3] that mechanization and automation of the procedure would eventually be possible. In the following years, the stepwise strategy indeed lent itself to the development of mechanized and automated syntheses of long chains, but only through the introduction of the solid-phase method by Merrifield.[4] Without a solid support, peptides of only moderate size[5-7] were built by the stepwise approach. In this paper we propose a new technique by which the stepwise synthesis of peptides, *not attached to an insoluble polymer*, can be considerably facilitated.

tert-Butyloxycarbonyl-L-valinamide was deprotected with trifluoroacetic acid, the reagent was removed *in vacuo*, and the residue was triturated with dry ether. These operations were carried out in a centrifuge tube provided with a standard tapered joint through which the tube could be attached to a rotary evaporator. After separation of the trifluoroacetate salt of L-valinamide from the ethereal solution by centrifugation, the supernatant was removed, and the precipitate was washed with ether and dried *in vacuo*. After dissolution of the trifluoroacetate salt in dimethylformamide and the addition of a tertiary amine,[8] the amino component was acylated with an active ester. *o*-Nitrophenyl esters of protected amino acids, reagents that remain efficient even under hindered conditions,[9,10] *e.g.*, in solid-phase synthesis,[11] performed quite satisfactorily in this procedure. High concentration of the reactants is desirable for practical rates and for the suppression of intramolecular side reactions.[12] Therefore, with the growing chain length, the *o*-nitrophenyl esters were applied in gradually increasing excess, their concentration at the start of each acylation being not less than 0.1 *M*. Completion of the acylation was ascertained by a spot test with ninhydrin. The amount of the released *o*-nitrophenol also is a good measure of the extent of acylation. Evaporation of the solvent was followed by the addition of a "nonsolvent." Ethyl acetate is particularly useful in this role[13] because it precipitates many of the common protected peptide intermediates while keeping in solution the excess active esters, the released *o*-nitrophenol and—importantly—also the trifluoroacetate salts of triethyl-

(1) This study was supported by a grant from the U. S. Public Health Service (NIH 5-R01-AM12473).

(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.*, **85**, 2149 (1963); *cf.* also R. L. Retsinger and M. J. Kornet, *ibid.*, **85**, 3045 (1963).

(5) *E.g.*, M. Bodanszky, J. Meienhofer, and V. du Vigneaud, *J. Amer. Chem. Soc.*, **82**, 3195 (1960).

(6) R. Schwyzer and P. Sieber, *Helv. Chim. Acta*, **49**, 134 (1966).

(7) M. Bodanszky and N. J. Williams, *J. Amer. Chem. Soc.*, **89**, 685 (1967); M. Bodanszky, M. A. Ondetti, S. D. Levine, and N. J. Williams, *ibid.*, **89**, 6753 (1967).

(8) For the incorporation of N-protected derivatives of *S*-benzyl-L-cysteine and *O*-benzyl-L-serine *via* their active esters, diisopropylethylamine is preferred [*cf.* M. Bodanszky and A. Bodanszky, *Chem. Commun.*, 591 (1967)].

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

(10) M. Bodanszky, R. J. Bath, A. Chang, M. L. Fink, K. W. Funk, S. M. Greenwald, and Y. S. Klausner in "Chemistry and Biology of Peptides," J. Meienhofer, Ed., Ann Arbor Science Publishers, Ann Arbor, Mich., 1972, p 203.

(11) M. Bodanszky and K. W. Funk, *J. Org. Chem.*, **38**, 1296 (1973).

(12) M. Bodanszky in "Prebiotic and Biochemical Evolution," A. P. Kimball and J. Oró, Ed., North-Holland Publishing Co., Amsterdam, 1971, p 217.

(13) In an unpublished synthesis of oxytocin, 95% ethanol was used as the "nonsolvent."

TABLE I

o-NITROPHENYL ESTERS OF *tert*-BUTYLOXYCARBONYLAMINO ACIDS

Registry no.	*tert*-Butyloxycarbonylamino acid *o*-nitrophenyl ester	Mp, °C	$[\alpha]^{25}D$ (*c* 1–2), DMF containing 1% AcOH	Tlc R_fA	Tlc R_fB	Formula	Mol wt	Calcd, % C	Calcd, % H	Calcd, % N	Found, % C	Found, % H	Found, % N
41120-55-2	Ala	86–87	−79	0.70	0.60	$C_{14}H_{18}N_2O_6$	310.3	54.2	5.8	9.0	54.3	5.9	9.0
41120-56-3	Asp(Bzl)	94–95	−42	0.87	0.77	$C_{22}H_{24}N_2O_8$	444.4	59.5	5.4	6.3	59.7	5.6	6.3
38605-58-2	Asn	144.5–146.5	−55	0.77	0.41	$C_{15}H_{19}N_3O_7$	353.3	51.0	5.4	11.9	50.8	5.6	11.6
38605-57-1	Cys(Bzl)	103–105	−75	0.82	0.67	$C_{21}H_{24}N_2O_6S$	432.5	58.3	5.6	6.5	58.1	5.5	6.8
41120-59-6	Glu(Bzl)	123–125	−54	0.87	0.77	$C_{23}H_{26}N_2O_8$	458.5	60.3	5.7	6.1	60.5	5.8	6.2
38605-59-3	Gln	149.5–151	−55	0.79	0.40	$C_{16}H_{21}N_3O_7 \cdot {}^1/_2H_2O$	367.4	51.1	5.9	11.2	51.3	5.9	11.3
38605-09-6	Gly	96.5–98		0.85	0.63	$C_{13}H_{16}N_2O_6$	296.3	52.7	5.4	9.5	52.8	5.4	9.4
41120-62-1	Ile	Oil	−37	0.84	0.74	$C_{17}H_{24}N_2O_6$	352.4	57.9	6.9	8.0	58.0	6.7	7.9
24868-52-8	Leu	56–57	−68	0.87	0.65	$C_{17}H_{24}N_2O_6$	352.4	57.9	6.9	8.0	58.0	6.7	8.1
41120-64-3	Lys(DCZ)[a]	135–137	−37	0.87	0.74	$C_{25}H_{29}N_3O_8Cl_2$	570.4	52.6	5.1	7.4	52.6	5.1	7.3
41120-65-4	Lys(INOC)[a]	94–98	−41	0.64	0.47	$C_{24}H_{30}N_4O_8$	502.5	57.4	6.0	11.1	57.4	5.8	10.9
41120-66-5	Met	104–105	−73	0.80	0.73	$C_{16}H_{22}N_2O_6S$	370.4	51.9	6.0	7.6	51.8	6.0	7.5
41120-67-6	Phe	146–146.5	−65	0.80	0.66	$C_{20}H_{22}N_2O_6$	386.4	62.2	5.7	7.3	62.1	5.7	7.2
38605-56-0	Pro	63–70	−84	0.84	0.66	$C_{16}H_{20}N_2O_6$	337.4	57.1	6.0	8.3	57.0	6.2	8.1
41120-69-8	Ser(Bzl)	49–52	−30	0.88	0.77	$C_{21}H_{24}N_2O_7$	416.4	60.6	5.8	6.7	60.6	5.9	6.7
41120-70-1	Trp	155–156	−62	0.80	0.64	$C_{22}H_{23}N_3O_6$	425.4	62.1	5.5	9.9	62.3	5.7	9.8
41120-71-2	Tyr(Bzl)	139–140	−51	0.82	0.78	$C_{27}H_{28}N_2O_7$	492.5	65.8	5.7	5.7	66.1	5.7	5.7
41120-72-3	Val	53–56	−44	0.84	0.64	$C_{16}H_{22}N_2O_6$	338.4	56.8	6.6	8.3	57.0	6.3	8.3

[a] DCZ, 2,6-dichlorobenzyloxycarbonyl; INOC, isonicotinyloxycarbonyl.

amine or diisopropylethylamine, etc. The protected peptide, lengthened by one amino acid, was separated by centrifugation and washed with the "nonsolvent" in the same way. Drying *in vacuo* completed the cycle. The next residue was incorporated in the same manner. An essential feature of this technique is that the *intermediates of the synthesis remain in the centrifuge tube throughout the operations and throughout the series of chain-lengthening steps.*[14] The use of a reaction vessel that permits mixing, evaporation, centrifugation, and drying simplifies the necessary manipulations and may lend itself to further developments, including mechanization and automation of the procedure. In the Experimental Section, further details of the synthesis of the protected nonapeptide, *tert*-butyloxycarbonyl-L-leucyl-L-glutaminyl-N^{ϵ}-2,6-dichlorobenzyloxycarbonyl-L-lysyl-L-leucyl-L-leucyl-L-glutaminylglycyl-L-leucyl-L-valinamide, corresponding to the C-terminal sequence of a secretin analog, are described (*cf.* also Chart I). A nonapeptide with an analogous sequence had been prepared earlier,[7] but with different protecting groups. In the present study the N^{ϵ}-amino group of the lysine residue, which replaced arginine in position 21 of secretin, was protected with the 2,6-dichlorobenzyloxycarbonyl group. This protection is not affected during the removal of *tert*-butyloxycarbonyl groups with trifluoroacetic acid. The synthesis of the C-terminal hexapeptide portion was carried out both by conventional operations and by the new technique.

It seems to be possible to apply acylation methods other than the active ester procedure with the same technique; *e.g.*, dicyclohexylcarbodiimide[15] or carbonyldiimidazole[16] (but not mixed anhydrides[17]) could be used for activation in the coupling steps. However, in order to avoid overactivation[18] and the concomitant formation of by-products that in turn might necessitate extensive purification between the chain-lengthening steps, we feel that it is imperative to use moderately active and therefore sufficiently selective acylating agents.

After preliminary comparisons of different active esters,[9,10] *o*-nitrophenyl esters were found to be practical tools in the execution of the technique proposed here and therefore we include methods for their preparation and list in Table I the physical properties of *o*-nitrophenyl esters of the more commonly used *tert*-butyloxycarbonylamino acids.[19]

In the preparation of some *o*-nitrophenyl esters, difficulties were encountered when the reactions were carried out in ethyl acetate, the solvent commonly applied in the synthesis of *p*-nitrophenyl esters.[20] The progress of active ester formation was monitored with ir spectra and its completion was indicated by the disappearance of the band at 4.8 μ, which is characteristic for the condensing agent, dicyclohexylcarbodiimide. Slow rates, especially in the case of protected amino acids with bulky side chains, led to the formation of *N*-acyldicyclohexylureas in not insignificant amounts. This undesirable side reaction was avoided by applying *o*-nitrophenol in excess and particularly by the use of pyridine as solvent. The nucleophilic character of the hydroxyl of *o*-nitrophenol is enhanced

(14) On the other hand, if necessary, intermediates can be removed from the apparatus, purified, and returned into the centrifuge tube for subsequent cycles.

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

(16) H. A. Staab, *Justus Liebigs Ann. Chem.*, **609**, 75 (1957). G. W. Anderson and R. Paul, *J. Amer. Chem. Soc.*, **80**, 4423 (1958); **82**, 4596 (1960).

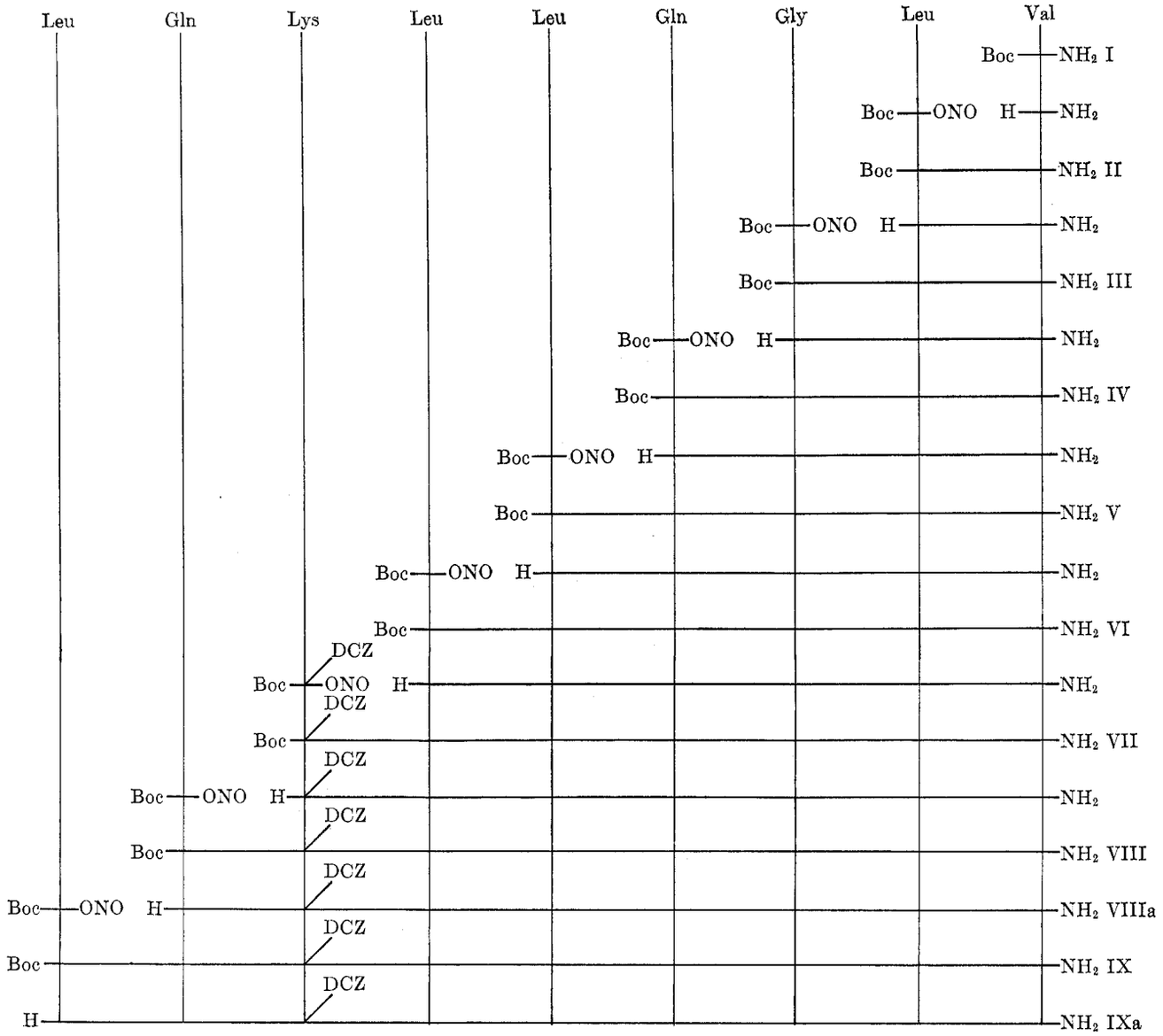
(17) Concerns about the use of mixed anhydrides in stepwise syntheses of larger chains were mentioned earlier (ref 2). Nevertheless, such syntheses were carried out with the exclusive application of mixed anhydrides by R. Sarges and B. Witkop [*J. Amer. Chem. Soc.*, **86**, 1862 (1964)] and also by M. A. Tilak [*Tetrahedron Lett.*, 849 (1970)]. The absence of the expected urethanes (*cf.* also H. C. Beyerman in "Chemistry and Biology of Peptides," J. Meienhofer, Ed., Ann Arbor Science Publishers, Ann Arbor, Mich., 1972, p 351) was not demonstrated in these studies. The coupling reagent EEDQ [B. Belleau and G. Malek, *J. Amer. Chem. Soc.*, **90**, 1651 (1968)] that produces ethoxycarbonyl mixed anhydrides as reactive intermediates indeed gives rise to the formation of urethanes (A. Bodanszky and M. Bodanszky, unpublished; *cf.* also Y. S. Klausner, C. Yang Lin, V. Mutt, and M. Bodanszky, *Bioorg. Chem.*, in press).

(18) 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.

(19) An extension of the same technique to benzyloxycarbonylamino acid *o*-nitrophenyl esters is being carried out in this laboratory.

(20) M. Bodanszky and V. du Vigneaud, *Biochem. Prep.*, **9**, 110 (1962).

CHART I



in this solvent, perhaps because of interference with intramolecular hydrogen bonding between the hydroxyl and nitro groups.

The higher reactivity of *o*-nitrophenyl esters as compared with *p*-nitrophenyl esters[9,10] is reflected also in their ir spectra: the active ester carbonyl appears at 5.62–5.63 μ in *o*-nitrophenyl esters and at 5.65 μ in their para isomers. The rates of aminolysis of *o*-nitrophenyl esters are less solvent dependent than the rates observed with *p*-nitrophenyl esters.[9] This difference points to some intramolecular engagement of the nitro group. The rotations of *o*-nitrophenyl esters are consistently and significantly higher than those of the corresponding para isomers, and this suggests that the asymmetric carbon atom is part of a rigid system in the ortho esters.[21] A diminished conformational freedom in these molecules might explain why they are not overly sensitive to steric hindrance such as that encountered in solid-phase syntheses.[9-11]

Since stepwise synthesis with *o*-nitrophenyl esters by the here-outlined technique permits facile chain lengthening, and also the purification of any intermediate should this be necessary, we continue our studies of this new approach for which the expression "peptide synthesis *in situ*" is proposed.

(21) W. Kauzmann and H. Eyring, *J. Chem. Phys.*, **9**, 41 (1941).

Experimental Section

Uncorrected capillary melting points are reported. On thin layer chromatograms, the protected peptides were revealed by *tert*-butyl hypochlorite–KI–starch reagents.[22,23] Active esters were detected by their uv absorption and through exposure to ammonia. For development, the following systems were applied: A, *n*-BuOH–AcOH–H_2O (4:1:1); B, $CHCl_3$–MeOH (9:1); C, EtOAc–pyridine–AcOH–H_2O (60:20:6:11). Reagent-grade commercial solvents were used without purification, but DMF was dried over a molecular sieve (Linde 4A or 4AXH). For amino acid analyses, samples were hydrolyzed with constant-boiling 6 *N* HCl in evacuated, sealed ampoules at 110° for 16 hr and analyzed by the Spackman–Stein–Moore method.[24]

The abbreviations for amino acids and protecting groups are those of the IUPAC-IUB Commission on Biochemical Nomen-

(22) R. H. Mazur, B. W. Ellis, and P. Cammarata, *J. Biol. Chem.*, **237**, 1619 (1962).

(23) D. P. Schwartz and M. J. Pallansch, *Anal. Chem.*, **30**, 219 (1958).

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

clature [*Biochemistry*, **5**, 1445, 2485 (1966); **6**, 362 (1967); *J. Biol. Chem.* **241**, 2491 (1966); 247, 977 (1972)]. Furthermore, DCC was used for dicyclohexylcarbodiimide, DCU for *N*,*N*′-dicyclohexylurea, TFA for trifluoroacetic acid, DMF for dimethylformamide, Z for benzyloxycarbonyl, INOC for isonicotinyloxycarbonyl, DCZ for 2,6-dichlorobenzyloxycarbonyl, Boc for *tert*-butyloxycarbonyl, ONP for *p*-nitrophenyloxy, and ONO for *o*-nitrophenyloxy groups.

***tert*-Butyloxycarbonyl-*O*-benzyl-L-serine *o*-Nitrophenyl Ester.**[25]—Boc-L-Ser(Bzl) (1.62 g, 5.5 mmol) and *o*-nitrophenol (1.39 g, 10 mmol) were dissolved in pyridine (5 ml) and cooled in an ice–water bath. DCC (1.03 g, 5 mmol) was added to the stirred solution and rinsed in with more pyridine (5 ml). After 30 min, the ice bath was replaced with a bath at room temperature. After 5 hr the DCU was removed by filtration and the pyridine was removed by evaporation *in vacuo*. The oil was taken up in ether and filtered to remove more DCU. The solvent was evaporated and the residue was dissolved in $CHCl_3$ (100 ml). The solution was extracted with 5% citric acid (50 ml in two portions) and 0.1 *N* NaOH (300 ml in four portions). The organic layer was washed with water (100 ml), dried over $MgSO_4$, and filtered, and the solvent was removed *in vacuo*. The residue was taken up in $CHCl_3$ (1.5 ml) and chromatographed on a column of silica gel (25 g) with $CHCl_3$. The fractions shown to be homogeneous by tlc were pooled and evaporated to give 1.95 g of oil. The product was crystallized from hot petroleum ether (150 ml, bp 38–47°), filtered, washed with cold petroleum ether (50 ml), and dried *in vacuo* over P_2O_5 to give 1.32 g (63%), mp 49–52°. A second crop (223 mg, mp 49–52°) and third crop (88 mg, mp 49–52°) were recovered from the mother liquor, total yield 78%. For specific rotation, R_f values on tlc, and analytical data, *cf.* Table I.

***tert*-Butyloxycarbonyl-L-alanine *o*-nitrophenyl ester** was prepared according to the method described above, with a reaction time of 6 hr. The crude, crystalline product (5.2 g, 61%) was recrystallized from warm EtOH to give a total of 3.8 g (49%) in successive crops, mp 86–87°.

***tert*-Butyloxycarbonyl-L-isoleucine *o*-nitrophenyl ester** was prepared according to the procedure described for Boc-L-Ser(Bzl)-ONO. The crude product from a 5-mmol preparation was taken up in $CHCl_3$ (1 ml) and chromatographed on a column of silica gel (20 g) with $CHCl_3$. The material running with the front was collected (20 ml) and shown to be homogeneous by tlc. The solvent was removed *in vacuo* to give 1.76 g (99%) of an oil which has thus far resisted attempts at crystallization.

***tert*-Butyloxycarbonyl-L-valine *o*-nitrophenyl ester** was prepared according to the procedure described for Boc-L-Ser(Bzl)-ONO with a reaction time of 3 hr. The crude product from a 50-mmol preparation was taken up in ether and filtered to remove more DCU. The solvent was removed by evaporation and the oily residue was dried *in vacuo* over P_2O_5 to give the desired product in quantitative yield. The purified material crystallized in the refrigerator after many weeks, mp 53–56°.

***tert*-Butyloxycarbonyl-L-aspartic acid α-*o*-nitrophenyl ester β-benzyl ester** was prepared according to the procedure described for Boc-L-Ser(Bzl)-ONO. The crude crystalline product from a 5-mmol preparation was dried *in vacuo* over P_2O_5 to give 2.12 g. It was recrystallized from hot 95% EtOH (50 ml), filtered, washed with cold 95% EtOH (30 ml in three portions), and dried *in vacuo* to give 1.35 g (51%), mp 90–93°. A second crop (0.51 g, mp 90–93°) was recovered from the mother liquor, total yield 84%. A sample (1.25 g) was dissolved in hot 95% EtOH (15 ml) and allowed to crystallize at room temperature. The crystals were filtered, washed with cold 95% EtOH (15 ml), and dried. The recovered material (1.09 g) melts at 94–95°.

***tert*-Butyloxycarbonyl-L-glutamic acid α-*o*-nitrophenyl ester γ-benzyl ester** was prepared according to the procedure described for Boc-L-Ser(Bzl)-ONO. The crude crystalline product from a 5-mmol preparation was dried *in vacuo* over P_2O_5 to give 2.18 g. The product was crystallized from hot 95% EtOH (35 ml), filtered, washed with cold 95% EtOH (30 ml in three portions), and dried *in vacuo* to give 1.96 g (86%), mp 124–125°. A sample (1.25 g) was dissolved in hot 95% EtOH (20 ml) and allowed to crystallize at room temperature. The crystals were filtered, washed with cold 95% EtOH (15 ml), and dried. The purified material (1.19 g) melts at 123–125°.

***tert*-Butyloxycarbonyl-L-methionine *o*-nitrophenyl ester** was prepared on a 4-mmol scale, with a reaction time of 3 hr, according to the procedure described for Boc-L-Ser(Bzl)-ONO. The crude product was recrystallized from warm 95% EtOH containing 0.1% AcOH, yield 1.15 g (78%), mp 104–105°.

***tert*-Butyloxycarbonyl-*O*-benzyl-L-tyrosine *o*-nitrophenyl ester** was prepared according to the procedure described for Boc-L-Ser(Bzl)-ONO. The reaction was carried out on a 6-mmol scale and was complete after 3 hr. The crude oil was dissolved in 95% EtOH containing 0.1% AcOH. The crystals which separated on cooling were collected, washed with cold 95% EtOH, and dried *in vacuo* to give 2.3 g (78%) of product, mp 139–140°.

***tert*-Butyloxycarbonyl-L-tryptophan *o*-nitrophenyl ester** was prepared according to the procedure described for *p*-nitrophenyl esters.[20] The reaction was carried out in EtOAc as solvent on a 1-mmol scale. The crude product was recrystallized from warm 95% EtOH containing 0.1% AcOH to give 0.23 g (53%), mp 155–156°. A second crop (0.12 g, 28%, mp 155–156°) was recovered from the mother liquor.

***tert*-Butyloxycarbonyl-L-phenylalanine *o*-nitrophenyl ester** was prepared according to the method used for the tryptophan derivative described above. The crude product from a 51.5-mmol preparation was recrystallized from warm 95% EtOH containing 0.1% AcOH (60 ml) to give 6.8 g (34%), mp 146–146.5°. A second crop from the mother liquor (4.65 g, mp 145–146°) raised the total yield to 57%.

***N*$^{\alpha}$-*tert*-Butyloxycarbonyl-*N*$^{\epsilon}$-2,6-dichlorobenzyloxycarbonyl-L-lysine *o*-Nitrophenyl Ester.**—Phosgene (12.5% in benzene, 130 ml) was cooled to 0° in an ice–water bath. 2,6-Dichlorobenzyl alcohol (24 g, 136 mmol) was added to the cooled solution with vigorous stirring. After 30 min, the ice–water bath was replaced with a bath at room temperature. The 2,6-dichlorobenzyl alcohol dissolved when the solution warmed to room temperature. After 4 hr, the benzene was removed by evaporation *in vacuo* at 40° bath temperature, giving an oil that in part crystallized in the cold room overnight. To complete the conversion, the product was again treated with phosgene (12.5% in benzene, 130 ml) and stirred overnight. The benzene was removed by evaporation at 40° *in vacuo* to give 34 g of 2,6-dichlorobenzyl chloroformate, an oil.

Lysine hydrochloride (12.2 g, 67 mmol) was dissolved in water (100 ml). Sodium hydroxide (4 *N*, 33.5 ml) was added, followed by a solution of $CuSO_4 \cdot 5H_2O$ (8.4 g, 33.5 mmol) in water (100 ml). Sodium carbonate (14.2 g, 134 mmol) was added to the deep blue solution, followed immediately by 2,6-dichlorobenzyl chloroformate (19.4 g, 81 mmol). The reaction mixture was stirred vigorously for 4 hr. The blue precipitate was filtered and washed with water (500 ml), 95% ethanol (250 ml), acetone (200 ml), and ether (200 ml). The copper complex was air dried to give 20.2 g. It was suspended in water (300 ml) and H_2S was bubbled through the vigorously stirred suspension for 1 hr. Cupric sulfide and the desired product precipitated out rapidly, causing thickening and foaming. Water (300 ml) was added to aid stirring. The flask was stoppered and the mixture was allowed to stand overnight. After brief boiling, charcoal (3 g) and Celite (6 g) were added. The mixture was diluted with water (700 ml), 95% ethanol (800 ml), and acetic acid (60 ml), and heated to boiling to dissolve the white, crystalline product. The solution was filtered through a steam-heated funnel and the cake was washed with a mixture of hot water (300 ml), 95% ethanol (300 ml), and acetic acid (25 ml). The N$^{\epsilon}$-protected lysine crystallized in the cold room overnight. The crystals were filtered, washed with water (100 ml) and acetone (100 ml), and dried *in vacuo* over P_2O_5 to give 14.9 g, mp 245–248°. The filtrate was concentrated *in vacuo* to a volume of 150 ml. The product that separated was filtered, washed with water (30 ml), and dried to give 2.1 g, mp 245–246.5°. A third crop (0.8 g) was recovered in a similar manner.

N$^{\epsilon}$-2,6-Dichlorobenzyloxycarbonyl-L-lysine[26] (1.7 g, 5 mmol) was dissolved in a mixture of dioxane (4 ml), water (1 ml), and 2 *N* sodium hydroxide (3 ml). *tert*-Butyl azidoformate (0.9 g, 5.5 mmol) was added with vigorous stirring. The pH was maintained at 10.2 for 24 hr by the addition of 2 *N* sodium hydroxide

(25) The esterification of Boc-L-Thr, unprotected on the OH group, with *o*-nitrophenyl was attempted both in EtOAc and in pyridine solution. In pyridine, the formation of the desired active ester was observed by ir and by tlc, but the material was impure even after chromatography on silica gel [*cf.* M. Bodanszky and M. A. Ondetti, *Chem. Ind. (London)*, 26 (1966)].

(26) B. W. Erickson and R. B. Merrifield "in Chemistry and Biology of Peptides," J. Meienhofer, Ed., Ann Arbor Science Publishers, Ann Arbor, Mich., 1972, p 191.

in small portions.[27] A viscous white gum separated, making stirring difficult. Dioxane–water (50%, 25 ml) was added to dissolve it. After 2 more days of stirring, no more base was consumed. The reaction mixture was diluted with 50% dioxane–water (50 ml) and extracted with ether (100 ml in two portions). The aqueous layer was filtered and acidified to pH 3.1 with 20% citric acid. The product deposited as a viscous gum on the walls of the flask. The supernatant liquid was decanted, and the product was rinsed with water (100 ml), dissolved in ether (25 ml), and filtered from a small amount of an impurity. The solvent was removed *in vacuo* to give 1.6 g (69%) of a fluffy material: mp 55–60°; $[\alpha]^{25}D$ −8° (*c* 2, AcOH); tlc R_fA 0.74.

N^{α}-*tert*-Butyloxycarbonyl-N^{ϵ}-2,6-dichlorobenzyloxycarbonyl-L-lysine (989 mg, 2.2 mmol) and *o*-nitrophenol (556 mg, 4 mmol) were dissolved in pyridine (5 ml) and stirred in an ice–water bath. Dicyclohexylcarbodiimide (412 mg, 2 mmol) was added. After 30 min, the ice bath was replaced with a bath at room temperature. After a total of 7.5 hr, DCU was removed by filtration and washed with pyridine (5 ml) and ether (5 ml). The solvent was removed by evaporation *in vacuo* and the crude oily product was triturated with ether (25 ml). The crystals were filtered, washed with ether (20 ml), and dried to give 888 mg (78%), mp 131–135°. More crystals separated from the mother liquor on standing. These were collected, washed with ether (15 ml), and dried to give 118 mg, mp 131–135°, a total yield of 88%. For analysis, a sample (100 mg) was dissolved in $CHCl_3$ (1 ml) and chromatographed on a column of silica gel (2 g). The purified active ester had a melting point of 135–137°.

N^{α}-*tert*-Butyloxycarbonyl-N^{ϵ}-isonicotinyloxycarbonyl-L-lysine *o*-nitrophenyl ester was prepared according to the method described for Boc-L-Ser(Bzl)-ONO with a reaction time of 7 hr. The crude oil from a 0.9-mmol preparation crystallized on standing at room temperature to give 447 mg. A sample (60 mg) was dissolved in warm 95% EtOH containing 0.1% AcOH (0.75 ml) and centrifuged to remove a small amount of an impurity. The product crystallized in the freezer. It was filtered, washed with cold 95% EtOH, and dried *in vacuo* to give 23 mg (38%). The remaining crude product was dissolved in $CHCl_3$ (0.5 ml) and chromatographed on a column of silica gel (10 g) with $CHCl_3$, followed by 1, 3, and 5% MeOH in $CHCl_3$. The fractions containing the purified product were pooled and the solvent was removed by evaporation to give 339 mg of oil which was crystallized from 95% EtOH containing 0.1% AcOH in two crops of equal purity to give 204 mg (60%), mp 94–98°.

***tert*-Butyloxycarbonyl-L-valinamide (I).**—A stream of dry NH_3 was led over a solution of Boc-L-Val-ONO (16.77 g, 49.6 mmol) in THF (100 ml) for 3 hr. The flask was stoppered and allowed to stand for an additional 1 hr. The ammonium salt of *o*-nitrophenol separated on standing and was removed by filtration. The THF was removed *in vacuo*, and the yellowish-white crystalline residue was triturated with ether (100 ml), filtered, washed with cold ether (100 ml), and dried *in vacuo* over P_2O_5 to give 8.93 g (83%), mp 157–158° (lit.[28] mp 156–157°). Additional amounts of the same product were isolated from the mother liquor, increasing the total yield to 86%. Thin layer chromatography revealed a few per cent of DCU contamination. This impurity was removed by countercurrent distribution (60 transfers) in a $CHCl_3$–toluene–MeOH–H_2O (5:5:8:2) system.

***tert*-Butyloxycarbonyl-L-leucyl-L-valinamide (II).**—A sample of compound I (6.9 g, 32 mmol) was dissolved in TFA (32 ml). After 15 min at room temperature, the excess TFA was removed *in vacuo* and the trifluoroacetate salt was triturated with ether (100 ml), filtered, washed with ether (50 ml), and dried *in vacuo* over P_2O_5: 7.3 g (99%); mp 123.5–125.5°; $[\alpha]^{25}D$ +55° (*c* 1, DMF). The salt (5.8 g, 25 mmol) was dissolved in DMF (40 ml). Triethylamine (3 ml) was added to the solution, followed by Boc-L-Leu-ONO (10.6 g, 30 mmol), and TEA (0.6 ml) was added in small portions to maintain alkalinity. The next day, the semisolid mass of crystals was filtered and washed with DMF (6 ml) and EtOAc (20 ml). The product, dried *in vacuo* over P_2O_5, weighed 3.2 g (39%): mp 187–188°; $[\alpha]^{25}D$ −47° (*c* 2, AcOH). The filtrate and washings were evaporated *in vacuo*, and the crystalline residue was triturated with EtOAc (50 ml), filtered, and washed with EtOAc (50 ml) to give an additional 3.65 g (44%), mp 187–189°. A third crop (0.91 g), mp 187–189°, was recovered from the mother liquor, raising the total yield to 94%. The crops are identical on tlc, R_fB 0.44. Amino acid analysis: Val, 1.00; Leu, 0.94.

Anal. Calcd for $C_{16}H_{31}N_3O_4$ (329.4): C, 58.3; H, 9.5; N, 12.8. Found: C, 58.6; H, 9.2; N, 12.8.

***tert*-Butyloxycarbonylglycyl-L-leucyl-L-valinamide (III).**—The *tert*-butyloxycarbonyl group was removed from compound II (7.4 g, 22.5 mmol) with TFA (30 ml) as described in the previous paragraph. The trifluoroacetate salt was dissolved in DMF (40 ml), and TEA (11 ml) was added, followed by Boc-Gly-ONO (8.3 g, 28 mmol). After the acylation was complete, the DMF was removed *in vacuo* at 35°, and the yellow oily residue was triturated with EtOAc (100 ml), filtered, and washed with EtOAc (200 ml). The crystalline product was dried *in vacuo* over P_2O_5 to give 7.65 g (88%): mp 205–206.5°; $[\alpha]^{25}D$ −32.5° (*c* 2, AcOH); tlc R_fB 0.32. Amino acid analysis: Gly, 1.00; Val, 0.95; Leu, 0.95. Additional material (0.75 g, 9%) of the same purity was obtained from the mother liquors.

Anal. Calcd for $C_{18}H_{34}N_4O_5$ (386.5): C, 55.9; H, 8.9; N, 14.5. Found: C, 55.8; H, 8.7; N, 14.4.

***tert*-Butyloxycarbonyl-L-glutaminylglycyl-L-leucyl-L-valinamide (IV).**—The *tert*-butyloxycarbonyl group was removed from compound III (7.7 g, 20 mmol) with TFA (20 ml) as described. The trifluoroacetate salt was dissolved in DMF (35 ml) and the solution was made basic with TEA (6.5 ml). Boc-L-Gln-ONO (8.8 g, 24 mmol) was added and rinsed in with DMF (15 ml). After 3 hr, DMF (35 ml) was added to the semisolid mass of crystals to aid homogenization. Acylation appeared complete in 24 hr. The DMF was removed *in vacuo* at 35° and the crude product was triturated with EtOAc (75 ml), filtered, washed with EtOAc (175 ml in small portions), and dried *in vacuo* at 46° for 4 hr to give 10.0 g (97%): mp 219–220°; $[\alpha]^{25}D$ −31° (*c* 2, AcOH); tlc R_fA 0.62. Amino acid analysis: Glu, 1.00; Gly, 1.00; Val, 1.02; Leu, 1.13.

Anal. Calcd for $C_{23}H_{42}N_6O_7$ (514.6): C, 53.7; H, 8.2; N, 16.3. Found: C, 53.8; H, 8.3; N, 16.1.

***tert*-Butyloxycarbonyl-L-leucyl-L-glutaminylglycyl-L-leucyl-L-valinamide (V).**—The *tert*-butyloxycarbonyl group was removed from a sample (5.1 g, 10 mmol) of compound IV with TFA (15 ml) as described above. The trifluoroacetate salt was suspended in DMF (25 ml); a thick gel formed. Triethylamine (4.5 ml) was added, followed immediately by Boc-L-Leu-ONO (7.0 g, 20 mmol). Subsequently the mixture was diluted with DMF (25 ml). Acylation was complete in 20 hr. The DMF was removed *in vacuo* at 35° bath temperature, and the crude product was triturated with EtOAc (100 ml), filtered, washed with EtOAc (150 ml), and dried *in vacuo* over P_2O_5 to give 6.1 g (96%): mp 240–242°; $[\alpha]^{25}D$ −40° (*c* 2, AcOH); tlc R_fA 0.67. Amino acid analysis: Glu, 0.97; Gly, 1.00; Val, 0.97; Leu, 1.98.

Anal. Calcd for $C_{29}H_{53}N_7O_8$ (627.8): C, 55.5; H, 8.5; N, 15.6. Found: C, 55.7; H, 8.6; N, 15.4.

***tert*-Butyloxycarbonyl-L-leucyl-L-leucyl-L-glutaminylglycyl-L-leucyl-L-valinamide (VI).**—The *tert*-butyloxycarbonyl group was removed from compound V (5.8 g, 9.3 mmol) with TFA (15 ml) as described above. The trifluoroacetate salt was dissolved in DMF (40 ml). Triethylamine (2.8 ml) and Boc-L-Leu-ONO (4.9 g, 13.9 mmol) were added with vigorous mixing. Since the reaction mixture thickened to a semisolid mass of crystals in 30 min, DMF (20 ml) was added to aid homogenization. After 3 days the solvent was removed *in vacuo* at 35°, and the crude product was triturated with EtOAc (50 ml), filtered, and washed with EtOAc (50 ml). The product was dried in air, powdered, and washed again with EtOAc (150 ml). The protected hexapeptide amide was dried *in vacuo* to give 6.75 g (98%). The crystals sinter at 255° and melt with decomposition at 263–265°; $[\alpha]^{25}D$ −48° (*c* 2, AcOH); tlc R_fA 0.75. Amino acid analysis: Glu, 1.00; Gly, 1.00; Val, 0.99; Leu, 3.10.

Anal. Calcd for $C_{35}H_{64}N_8O_9$ (740.95): C, 56.7; H, 8.7; N, 15.1. Found: C, 56.6; H, 8.6; N, 14.9.

L-Leucyl-L-glutaminyl-N^{ϵ}-2,6-dichlorobenzyloxycarbonyl-L-lysyl-L-leucyl-L-leucyl-L-glutaminylglycyl-L-leucyl-L-valinamide Trifluoroacetate (IXa).—All the following operations were carried out in a 40-ml centrifuge tube fitted with a 24/40 ground glass joint to allow its attachment to a rotary evaporator. The intermediates were not removed from the vessel at any time during the chain-lengthening steps. Trifluoroacetic acid was used for the (partial) deprotection of the peptides and TEA for the liberation of the amines from their trifluoroacetate salts. Stepwise acylation was accomplished with a gradual increase from 20 to 50% excess of the respective protected amino acid *o*-nitrophenyl esters, except in the preparation of the tetrapeptide,

(27) E. Schnabel, *Justus Liebigs Ann. Chem.*, **702**, 188 (1967).

(28) R. Schwyzer, A. Costopanagiotis, and P. Sieber, *Helv. Chim. Acta*, **46**, 870 (1963).

where, to suppress pyroglutamyl peptide formation, a 100% excess was applied. The acylation of the N-terminal leucyl residue of the pentapeptide amide with Boc-L-Leu-ONO was observed to proceed with a slower than desirable rate; this probably could be increased by the application of a larger excess of the active ester.[29]

A sample of Boc-L-Val-NH_2 (I, 400 mg, 1.85 mmol) was placed in the centrifuge tube and dissolved in TFA (1 ml). After 15 min at room temperature, the excess TFA was removed *in vacuo*. The trifluoroacetate salt was triturated with ether, the solution was removed after centrifugation, and the solid was washed with ether (45 ml in three portions) and dried *in vacuo* to give compound Ia (416 mg, 98%). The amide trifluoroacetate was dissolved in DMF (2 ml), and TEA (270 μl) was added followed by Boc-L-Leu-ONO (763 mg, 2.2 mmol). After 24 hr the DMF was removed *in vacuo* at 35°. The residue was triturated and washed with EtOAc (15 ml in five portions). The dry product II weighed 534 mg (90%). Melting points and R_f values were similar to those of the respective compounds reported above. No attempt was made to secure additional material from the mother liquors and washings.

Following this procedure, the protected tripeptide amide III was secured in 82% yield (514 mg). The tetrapeptide IV and all subsequent peptides were triturated and washed with EtOAc (90 ml in six portions). Compound IV was obtained in quantitative yield, 550 mg. The pentapeptide V (610 mg, 97%) showed a trace of a slower moving ninhydrin-negative material on tlc which is probably pyroglutamyltetrapeptide amide. The hexapeptide VI (701 mg, 97%), with the trace impurity still noticeable, gave a satisfactory amino acid analysis: Glu, 1.01; Gly, 1.00; Val, 1.00; Leu, 2.96.

tert-Butyloxycarbonyl-N^{ϵ}-2,6-dichlorobenzyloxycarbonyl-L-lysyl-L-leucyl-L-leucyl-L-glutaminylglycyl-L-leucyl-L-valinamide (VII) was isolated in a yield of 948 mg (98%): sintering at 263°, mp 270–273° dec; $[\alpha]^{25}D$ −42° (*c* 1, AcOH); tlc R_fA 0.76, R_fC 0.83. Amino acid analysis: Lys, 0.97; Glu, 1.02; Gly, 1.00; Val, 1.00; Leu, 2.98; NH_3, 2.10.

Anal. Calcd for $C_{49}H_{80}N_{10}O_{12}Cl_2$ (1072.1): C, 54.9; H, 7.5; N, 13.1. Found: C, 54.7; H, 7.4; N, 12.8.

tert-Butyloxycarbonyl-L-glutaminyl-N^{ϵ}-2,6-dichlorobenzyloxycarbonyl-L-lysyl-L-leucyl-L-leucyl-L-glutaminylglycyl-L-leucyl-L-valinamide (VIII) was secured in a yield of 919 mg (96%): sinters at 266°, mp 267–269° dec; tlc R_fA 0.69, R_fC 0.64. A sample (20 mg) was removed from the reaction vessel, deprotected with TFA (0.5 ml) and filtered to remove mechanical impurities, precipitated, washed with ether, and dried for analysis; VIIIa sinters at 220°, darkens at 260°, mp 270–273° dec, tlc R_fA 0.49. Amino acid analysis: Lys, 1.05; Glu, 1.96; Gly, 1.00; Val, 1.01; Leu, 3.10; NH_3, 3.40.

Anal. Calcd for $C_{56}H_{89}N_{12}O_{16}Cl_2F_3$ (1214.2): C, 50.4; H, 6.7; N, 13.8. Found: C, 50.5; H, 6.8; N, 13.6.

tert-Butyloxycarbonyl-L-leucyl-L-glutaminyl-N^{ϵ}-2,6-dichlorobenzyloxycarbonyl-L-lysyl-L-leucyl-L-leucyl-L-glutaminylglycyl-L-leucyl-L-valinamide (IX) was prepared as described above. A 100% excess of Boc-L-Leu-ONO was applied, followed later by an additional 65%.[29] The yield of compound IX was 955 mg (98%): sinters at 275°, mp 283–286° dec; tlc R_fA 0.70, R_fC 0.63. A sample (10 mg) was deprotected and prepared for analysis as described for VIIIa. The trifluoroacetate salt IXa sinters at 200° and darkens at 260°, mp 278–279° dec, tlc R_fA 0.51. Amino acid analysis: Lys, 1.04; Glu, 1.94; Gly, 1.00; Val, 1.00; Leu, 4.06; NH_3, 3.2.

Anal. Calcd for $C_{62}H_{100}N_{13}O_{17}Cl_2F_3$ (1327.3): C, 51.6; H, 7.0; N, 13.7. Found: C, 50.0; H, 6.6; N, 13.0.

These values suggest that the sample contains more than 1 mol of TFA, even though it was dried at 40° *in vacuo* for 2.5 hr. Attempts to dry the sample at higher temperatures resulted in decomposition.

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Registry No.—I, 35150-08-4; II, 33900-16-2; II TFA salt, 41641-83-2; III, 41120-77-8; IV, 41120-78-9; V, 33529-91-8; VI, 7802-00-8; VII, 41120-80-3; VIII, 41594-22-3; VIII TFA salt, 41120-82-5; IX, 41120-83-6; IX TFA salt, 41594-23-4; Boc-L-Ser(Bzl), 23680-31-1; *o*-nitrophenol, 88-75-5; 2,6-dichlorobenzyl alcohol, 15258-73-8; *tert*-butyl azidoformate, 1070-19-5; dicyclohexylcarbodiimide, 538-75-0; triethylamine, 121-44-8.

(29) The completion of the coupling reaction required 1 week. Similar difficulties encountered in the preparation of the protected nonapeptide IX were overcome by the application of the active ester in 100% excess and by catalysis with 1-hydroxybenzotriazole used in an amount equimolar to the active ester (W. König and R. Geiger in "Chemistry and Biology of Peptides," J. Meienhofer, Ed., Ann Arbor Science Publishers, Ann Arbor, Mich., 1972, p 343). Under these conditions, acylation was complete in less than 1 day.