solution and with water until neutral and concentrated in vacuo. The product was precipitated by the addition of ether (100 ml), collected on a filter, and washed with ether. The air-dried material weighed 8.15 g. The crude product showed two major spots on TLC, R_f (A) 0.65 and 0.74, R_f (B) 0.45 and 0.71. For the separation of the components and isolation of XI in pure form, cf. the following paper.

B. The hexapeptide ditrifluoroacetate X (100 mg, 0.1 mmol) was dissolved in DMF (0.6 ml) and treated with diisopropylethylamine (0.017 ml, 0.11 mmol), *tert*-butyloxycarbonyl-β-benzyl-L-aspartic acid *p*-nitrophenyl ester (Bachem, 42 mg, 0.1 mmol), and 1-hydroxybenzotriazole (13 mg, 0.1 mmol). Next day, ether (40 ml) was added and the mixture was kept overnight in the refrigerator. The resulting solid was disintegrated, filtered, washed with ether (25 ml), and dried over P_2O_5 in vacuo. The product weighed 115 mg (98%), mp 109–110 °C, $[\alpha]^{24}D$ −35° (*c* 1, DMF). On TLC, in addition to the main product [R_f (A) 0.65, R_f (B) 0.45], only a trace of the *O*-acyl derivative [R_f (A) 0.74, R_f (B) 0.71] could be detected. Amino acid analysis: Asp 1.0, Ser 1.7, Pro 1.0, Ile 1.0, His 1.1, Arg + Orn 1.0.

Anal. Calcd for $C_{53}H_{75}N_{13}O_{16}\cdot H_2O$: C, 54.4; H, 6.6; N, 15.6. Found: C, 54.5; H, 6.7; N, 15.6.

***tert*-Butyloxycarbonyl-L-aspartyl-L-prolyl-L-seryl-L-histidyl-L-arginyl-L-isoleucyl-L-serine Hydrazide (XIII).** A sample (1.12 g) of the protected heptapeptide ester XI was dissolved in a mixture of 95% ethanol (125 ml), H_2O (20 ml), and AcOH (2.5 ml) and hydrogenated in the presence of a 10% Pd on charcoal catalyst (0.3 g) for 3 days. The catalyst was removed by filtration and the solvent by evaporation in vacuo, and the residue was treated with dry ether (50 ml). The solid product was collected on a filter, washed with dry ether, and dried in vacuo over P_2O_5 and KOH for 24 h. The product (XII) weighed 0.80 g: mp 77–78 °C; $[\alpha]^{24}D$ −57° (*c* 1, MeOH); TLC R_f (A) 0.33. No significant uv absorption was found at 270 nm. Elemental analysis suggests the retention of both acetic acid and water.

Anal. Calcd for $C_{39}H_{64}N_{12}O_{14}\cdot 2.5CH_3COOH\cdot 2H_2O$: C, 47.7; H, 7.2; N, 15.5. Found: C 47.5; H, 7.1; N, 15.6.

A sample (0.88 g) of the partially protected heptapeptide methyl ester XII was dissolved in CH_3OH (18 ml) and treated with hydrazine hydrate (2 ml). After 12 h, the solvent was removed in vacuo, and the residue dried in vacuo over P_2O_5 and concentrated H_2SO_4 for 24 h. The product was dissolved in H_2O and freeze-dried. This was repeated four times. The hydrazide XIII (0.88 g) melted at 99–100 °C; $[\alpha]^{24}D$ −53° (*c* 1, CH_3OH); TLC R_f (A) 0.18, R_f (C) 0.33. Amino acid analysis: Asp 1.0, Ser 1.8, Pro 0.95, Ile 1.0, His 1.0, Arg 1.0.

Anal. Calcd for $C_{38}H_{64}N_{14}O_{13}\cdot 6H_2O$: C, 44.2; H, 7.6; N, 19.5. Found: C, 43.9; H, 7.5; N, 19.3.

Loss of weight on drying at room temperature for 24 h and then at 60 °C for 16 h was 8.3%. Calcd (for $6H_2O$): 10.7. After this drying, the melting point changed to 124–133 °C dec, with sintering at 118 °C.

Acknowledgment. This study was supported by a grant from the U.S. Public Health Service (NIH AM-12473).

Registry No.—I, 60526-22-9; II, 60562-29-0; III, 60526-23-0; IV, 60562-30-3; V, 60526-24-1; VI, 60526-26-3; VII, 60526-27-4; VIII, 60526-29-6; IX, 60526-30-9; X, 60526-32-1; XI, 60512-76-7; XII, 60526-33-2; XIII, 60526-34-3; serine methyl ester hydrochloride, 5680-80-8; benzyloxycarbonyl-L-isoleucine nitrophenyl ester, 2130-99-6; z-L-Ile, 3160-59-6; Ser-OCH_3, 2788-84-3; *tert*-butyloxycarbonylnitro-L-arginine, 2188-18-3; *tert*-butyloxycarbonylnitro-L-argine *N*-hydroxysuccinimide ester, 60526-35-4; N^{α}-N^{im}-bis(*tert*-butyloxycarbonyl)-L-histidine *p*-nitrophenyl ester, 20866-47-1; *tert*-butyloxycarbonyl-*O*-benzyl-L-serine *p*-nitrophenyl ester, 16948-39-3; *tert*-butyloxycarbonyl-L-proline *p*-nitrophenyl ester, 28310-65-8; *tert*-butyloxycarbonyl-β-benzyl-L-aspartic acid *N*-hydroxysuccinimide ester, 13798-75-9; *tert*-butyloxycarbonyl-β-benzyl-L-aspartic acid *p*-nitrophenyl ester, 26048-69-1.

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Side Reactions in Peptide Synthesis. 4. Extensive O-Acylation by Active Esters in Histidine Containing Peptides[1]

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Received June 11, 1976

Acylation of the partially protected hexapeptide Pro-Ser(Bzl)-His-Arg(NO_2)-Ile-Ser-OCH_3 with the *N*-hydroxysuccinimide ester of *tert*-butyloxycarbonyl-β-benzyl-L-aspartic acid in the presence of 1-hydroxybenzotriazole produced, in addition to the desired heptapeptide derivative, a by-product, often in significant amounts. Examination of this material revealed that acylation of the free amino group of the partially protected hexapeptide was accompanied by acylation of the unprotected hydroxyl group of the C-terminal serine residue. Model experiments demonstrated that the extensive O-acylation was due to the presence f a histidine residue in the amino component. The imidazole in the side chain of this amino acid acts as catalyst in the alcoholysis of the active esters used for chain lengthening. Conditions that can reduce the extent of O-acylation were explored. The implications of this side reaction on the problems of minimal vs. global protection and of the application of excess acylating agents ("the principle of excess") are also discussed.

In the course of our continued effort[2] toward the synthesis of the gastrointestinal hormone cholecystokinin,[3] a protected heptapeptide corresponding to the partial sequence 17–23 of the 33-membered chain was prepared. Building of the protected derivative *tert*-butyloxycarbonyl-β-benzyl-L-aspartyl-L-prolyl-*O*-benzyl-L-seryl-L-histidylnitro-L-ar-

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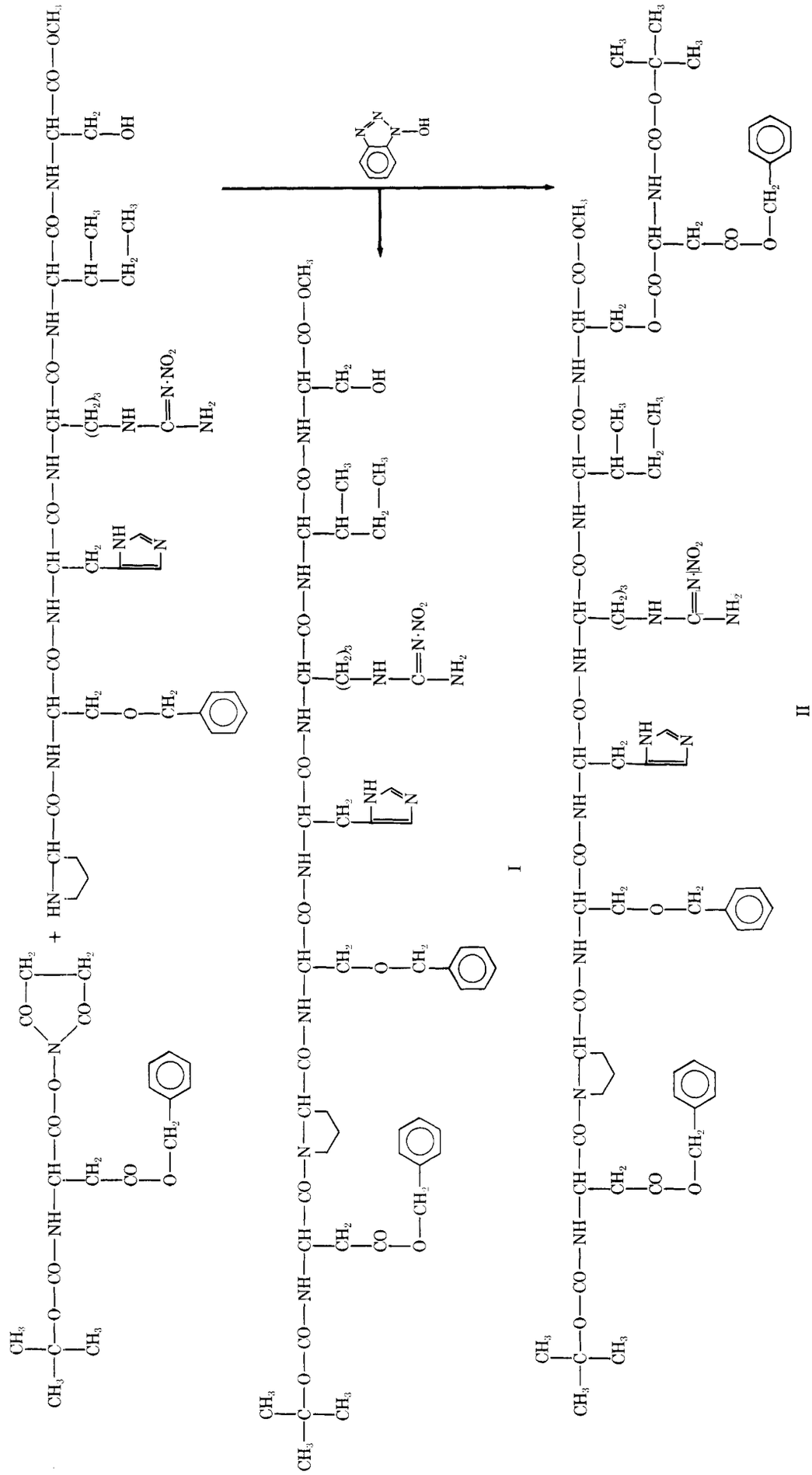
Scheme I. Formation of an *O*-acyl Derivative (II) in the Preparation of Compound I



ginyl-L-isoleucyl-L-serine methyl ester (I)[4] by stepwise chain lengthening[5] with active esters[6] was carried out without major difficulties until the hexapeptide stage was reached. For the incorporation of the N-terminal residue, aspartic acid, *tert*-butyloxycarbonyl-β-benzyl-L-aspartic acid *N*-hydroxysuccinimide ester[7] was applied, and the reaction was catalyzed by the addition of 1-hydroxybenzotriazole[8] (Scheme I). Care was taken to use the acylating agent in only moderate excess, and to keep the reaction mixture only slightly alkaline by the controlled addition of tertiary base. Nevertheless, an examination of the crude product by thin layer chromatography (TLC) revealed the presence of impurities. One of these, a material that moved faster on the silica gel plates than the desired product, was present in varying but always significant amounts, in some cases exceeding that of the desired heptapeptide derivative (I). The by-product (II) was isolated by chromatography on a silica gel column, and was secured in crystalline form. Both elemental analysis and amino acid analysis indicated the incorporation of *two* protected aspartyl residues. Firm evidence for the assignment of structure II (Scheme I) was found in the chromic acid oxidation of II, followed by hydrolysis and amino acid analysis. Two moles of serine was present in the hydrolysate, while in a parallel experiment, in which compound I was similarly oxidized and hydrolyzed, the serine content was reduced almost to 1 mol. Thus, the second aspartyl residue was attached, via an ester bond, to the hydroxyl group of the C-terminal serine.[9]

Reaction of active esters with alcohols is not obvious: most of these compounds can be recrystallized from boiling ethanol[10] if care is taken that basic materials, that could catalyze their alcoholysis, are absent. Still, unexpected O-acylation of a tyrosine residue was reported by Ramachandran[11] and that of a serine side chain by Zahn and his associates.[12] In our laboratories, however, by avoiding an excess of the tertiary base used for the liberation of a free amine from a salt of the amino component, we were able to build peptides in excellent yield and high purity by stepwise cain lengthening with nitrophenyl esters without protection of the hydroxyl group of tyrosine residue. E.g., a hendecapeptide corresponding to the C-terminal sequence of the vasoactive intestinal peptide (VIP) from chicken[13] Ala-Val-Lys-Lys-Tyr-Leu-Asn-Ser-Val-Leu-Thr-NH_2[14] was prepared in this manner. Similarly, no O-acylation was observed when this sequence was assembled again, with both the serine and tyrosine side chains unprotected. Therefore, the extensive O-acylation which produced *significant* amounts of II was unexpected, and prompted an examination of the conditions that are conducive to this side reaction.

Since the participation of the imidazole[15] in the side chain of the histidine residue in the alcoholysis of the active ester was suspected, Boc-Arg(NO_2)-Ile-Ser-OCH_3 (III), the C-terminal (protected) tripeptide intermediate of the synthesis of the heptapeptide, was chosen as a model in which no histidine is present. Samples of III were treated with trifluoroacetic acid, and the partially deprotected tripeptide ester trifluoroacetate salt was acylated, in the presence of tertiary base, with active esters of *tert*-butyloxycarbonyl-β-benzyl-L-aspartic acid. The reactions were carried out in the presence and in the absence of catalyst (1-hydroxybenzotriazole, HOBt), and also with and without the addition of imidazole that was used to play the role of the histidine side chain. The crude products were examined by TLC; the results are summarized in Table I.

With hydroxysuccinimide esters, the presence of HOBt was relatively harmless: the expected protected tetrapeptide Boc-Asp(Bzl)-Arg(NO_2)-Ile-Ser-OCH_3 (IV) was the main product, while only a small amount of *N,O*-diacyl derivative (V) could be detected. The addition of imidazole, however, led to a substantial increase in the formation of V, as did the presence of both imidazole and HOBt. With the *p*-nitrophenyl ester as acylating agent, the effects of HOBt and imidazole were similar in causing the formation of V in substantial quantity, but *very little diacyl derivative was found in the crude product of the experiment in which both HOBt and imidazole were added* to the mixture of the reactants. Since in histidine-containing peptides with unprotected imidazole the choice is reduced to the addition or omission of HOBt, the *p*-nitrophenyl rather than the *N*-hydroxysuccinimide ester of (protected) aspartic acid was applied for the preparation of the desired heptapeptide derivative, and HOBt was used to counteract the influence of the imidazole of histidine. The results were encouraging: only a very small amount of V could be detected by TLC in the crude protected heptapeptide.[4]

Table I.[a] Products Formed in the Acylation of Arg(NO_2)-Ile-Ser-OCH_3[b] with Active Esters of Boc-Asp(Bzl)

Active ester	Additives	Relative amounts[c] of *N*-Acyl deriv IV, R_f 0.28	*N,O*-Diacyl deriv V, R_f 0.55
OSu		90	10
OSu	HOBt	90	5–10
OSu	Im	50	50
OSu	HOBt + Im	70	30
OTC		95	5
ONP		95	5
ONP	HOBt	70	30
ONP	Im	40	60
ONP[d]	Im	Trace	>90
ONP	AcOH + Im	40	60
ONP	HOBt + Im	90	5–10

[a] HOBt, 1-hydroxybenzotriazole; Im, imidazole; OSu, *N*-hydroxysuccinimide ester; ONP, *p*-nitrophenyl ester; OTC, 2,4,5-trichlorophenyl ester. [b] Applied as trifluoroacetate salt in the presence of triethylamine. [c] Estimated by inspection of TLC plates under uv and after charring. [d] In this experiment, 2.5 mmol of active ester was used for 1 mmol of tripeptide derivative, while in all other experiments, 1.25 mmol of active ester was applied.

Discussion

The model experiments revealed the role of the imidazole moiety in the histidine in the observed O-acylation of serine side chains. No clear rationale can be offered at this time for the different effects with the two kinds of active esters. The role of HOBt also requires further clarification.Its weak acidic character does not provide a satisfactory explanation: the addition of acetic acid instead of HOBt produced no beneficial effect. The need for a study in depth is indicated, including comparisons of various active esters, imidazole protecting groups, and additives. There are, however, several reasons for reporting these findings prior to such extensive investigations. A similar case of O-acylation, in the HOBt-catalyzed[8] reaction of a 2,4,5-trichlorophenyl ester[16] with a *histidine-containing* peptide, was reported—without discussion—by Geiger and his associates.[17] It seems to be necessary, therefore, to call attention to this side reaction before it is encountered by other investigators as well, and to point out not only the risk of the formation of O-acylated products, but also possible means for decreasing the extent of this side reaction. This report was motivated also by the desire to express certain reservations about approaches in peptide synthesis originating from this laboratory. The inertness of *p*-nitrophenyl esters toward alcoholysis[10] suggested that these mild acylating agents can be applied to amino components carrying minimal protection, possibly only on lysine and cysteine side chains. The observations here presented, and others reported by us recently,[1,18] show certain limitations in the application of minimal pro-

tection, even with mild acylating agents. It should also be pointed out that the "principle of excess",[19] that is, the use of the acylating agents in high enough concentration to secure practical reaction rates with amino components that cannot be present in high molar concentration, might be incompatible—at least in certain cases—with minimal protection.[20]

Conclusions

The presence of histidine in peptides is conducive to O-acylation of (unprotected) side chain hydroxyl groups by active esters. It was possible to keep this side reaction at a minimum when the acylation was carried out with a *p*-nitrophenyl ester in the presence of 1-hydroxybenzotriazole. The "principle of excess" in the use of acylating agent and minimal protection are probably mutually exclusive.

Experimental Section

Capillary melting points are reported uncorrected. Thin layer chromatograms were run on silica gel plates (Merck) with the solvent system $CHCl_3$–CH_3OH (9:1). Spots were revealed by charring. For amino acid analysis, samples were hydrolyzed in evacuated, sealed ampules with constant boiling hydrochloric acid at 110 °C for 16 h, and analyzed on a Beckman Spinco 120C instrument.

Boc-Asp(Bzl)-Pro-Ser(Bzl)-His-Arg(NO_2)-Ile-Ser-OCH_3 (I) and Compound II. A sample (1.0 g) of the crude mixture of I and II[4] was dissolved in $CHCl_3$ (3.5 ml) and applied to a column of silica gel (Baker, 2 × 58 cm). Fractions of 25 ml were collected. $CHCl_3$ was first used for elution; this was changed to $CHCl_3$ containing 5% (v/v) CH_3OH after four fractions were eluted. From fraction 15 on, the methanol ontent was raised to 8%, and from fraction 24 on, to 10%. The individual fractions were examined by TLC and evaporated to dryness with a stream of N_2. The weight of the residues were plotted. Two major peaks emerged: the one containing II (fractions 15–21), the second containing the originally expected heptapeptide derivative I (fractions 30–38). By pooling fractions 16–20, compound II was obtained: 0.35 g; mp 104 °C, sintering at 96 °C; $[\alpha]^{25}D$ −37° (*c* 1, CH_3OH); TLC R_f 0.43. Amino acid analysis: Asp 2.2, Ser 1.95, Pro 0.95, Ile 1.0, His 1.0, Arg (+ Orn) 0.85.

Anal. Calcd for $C_{69}H_{94}N_{14}O_{21}$: C, 56.9; H, 6.5; N, 13.5. Found: C, 57.0; H, 6.8; N, 13.4.

The protected heptapeptide ester I was secured from fractions 31–37: 0.34 g; mp 132 °C, sintering from 120 °C; TLC R_f 0.18. Amino acid analysis: Asp 1.0, Ser 1.8, Pro 0.85, Ile 1.0, His 1.0, Arg (+ Orn) 0.85.

Anal. Calcd for $C_{53}H_{75}N_{13}O_{16}\cdot H_2O$: C, 54.5; H, 6.5; N, 15.6. Found: C, 54.7; H, 6.7; N, 15.7.

Oxidation with Chromic Acid. A sample of compound II (1.2 mg) was dissolved in acetic acid (0.25 ml) containing CrO_3 (8.3 mg) and pyridine (8.3 μl). The mixture was left to stand for 18 h at room temperature, 95% EtOH (5 ml) was added, the solvents were removed with a stream of N_2, and the residue was hydrolyzed for amino acid analysis. The following ratios were determined: Asp 2.2, Ser 1.9, Pro 0.8, Ile 1.0, His 1.0, Arg 0.6. A similarly treated sample of the protected heptapeptide derivative I gave Asp 1.2, Ser 1.1, Pro 0.9, Ile 1.0, His 1.0, Arg 0.6.

Model Experiments. The protected tripeptide ester Boc-Arg(NO_2)-Ile-Ser-OCH_3[4] (0.23 g, 0.44 mmol) was treated with 98% trifluoroacetic acid at room temperature for 15 min. The acid was removed by evaporation, and the residue was washed with ether and dried in vacuo. The trifluoroacetate salt was dissolved in DMF (0.5 ml); triethylamine (0.10 ml, 0.7 mmol) and *tert*-butyloxycarbonyl-β-benzyl-L-aspartic acid *p*-nitrophenyl ester, 2,4,5-trichlorophenyl ester, or *N*-hydroxysuccinimide ester (0.55 mmol) were added. In certain experiments, as indicated in Table I, imidazole (30 mg, 0.44 mmol) and/or 1-hydroxybenzotriazole (54 mg, 0.35 mmol) was also added. The reaction mixtures were stirred overnight. The solvent was removed in vacuo; the residues were taken up in EtOAc (30 ml) and extracted, several times, with saturated $NaHCO_3$ solution and water. The solvent was removed in vacuo, and the product precipitated with ether (10 ml), collected on a filter, washed with ether, and dried. Amino acid analysis of the two main products, separated by preparative TLC, showed that the faster moving material corresponds to the *N,O*-diacyl derivative V, while the slower moving band is that of the expected protected tetrapeptide ester IV. For the relative amounts of these materials in the crude products, cf Table I.

Acknowledgments. Amino acid analyses were carried out by Mrs. Delores J. Gaut, elemental analyses by the Baron Consulting Co., Orange, Conn. This study was supported by grants from the U.S. Public Health Service (NIH AM-12473 and HL-14187).

Registry No.—I, 60512-76-7; II, 60512-77-8; Boc-Asp(Bzl)-OSu, 13798-75-9; Boc-Asp(Bzl)-OTC, 43189-58-8; Boc-Asp(Bzl)-ONP, 26048-69-1; Arg(NO_2)-Ile-Ser-OCH_3, 60512-78-9.

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