Oligomerization during the Acylation of *O*-(Benzyloxycarbonylsarcosyl-L-*N*-methylvalyl)-L-threonyl-D-valyl-L-proline with 2-Nitro-3-benzyloxy-4-methylbenzoic Acid[1,2]

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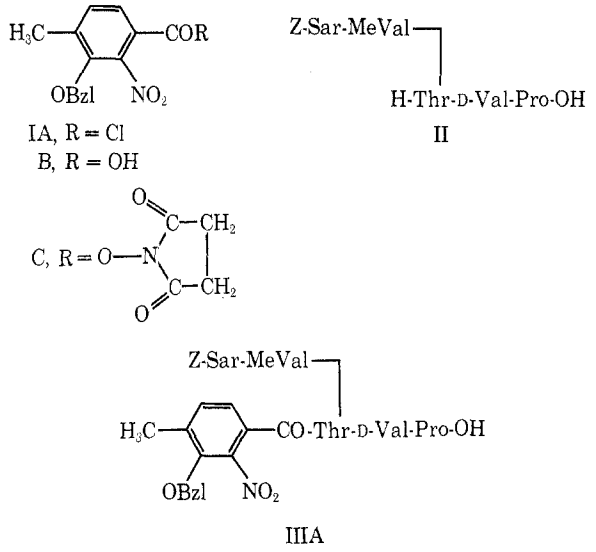
Reaction of the β-pentadepsipeptide *O*-(benzyloxycarbonylsarcosyl-L-*N*-methylvalyl)-L-threonyl-D-valyl-L-proline, with 2-nitro-3-benzyloxy-4-methylbenzoyl chloride resulted in considerable oligomerization. Oligomers up to the pentamer were isolated by means of LH-20 chromatography and characterized. Other methods of activation of the benzoyl moiety were investigated including mixed carbonic carboxylic anhydride, *N,N'*-carbonyldiimidazole, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline, *N*-hydroxysuccinimide ester, symmetrical anhydride, and azide. The best yields of the desired monomer were obtained from the symmetrical anhydride generated by the reaction of dicyclohexylcarbodiimide with 2-nitro-3-benzyloxy-4-methylbenzoic acid, although dimer and trace amounts of trimer were also formed. Monomer only was obtained from (a) the acid chloride by controlling the addition of base and (b) the azide, but the total yields were low in these procedures.

The reaction of 2-nitro-3-benzyloxy-4-methylbenzoyl chloride (IA)[4] with *O*-(benzyloxycarbonylsarcosyl-L-*N*-methylvalyl)-L-threonyl-D-valyl-L-proline (II) was described in an earlier communication on the synthesis of actinomycin D.[5] Purification of the crude product by column chromatography on Sephadex LH-20 in methanol or ethanol gave five discrete peaks of peptide derivatives (Figure 1).[6]

The desired intermediate for the synthesis of actinomycin D, *O*-(benzyloxycarbonylsarcosyl-L-*N*-methylvalyl)-*N*-(2-nitro-3-benzyloxy-4-methylbenzoyl)-L-threonyl-D-valyl-L-proline (IIIA) was contained in the largest and slowest moving peak, A. Isolation and examination of the four faster moving peaks by carboxyl group titration revealed that they were due to dimer IIIB, trimer IIIC, tetramer IIID, and pentamer IIIE (Figure 2). Authentic dimer was subsequently synthesized from *O*-(benzyloxycarbonylsarcosyl-L-*N*-methylvalyl)-*N*-(2-nitro-3-benzyloxy-4-methylbenzoyl)-L-threonyl-D-valyl-L-proline (IIIA) and *O*-(benzyloxycarbonylsarcosyl-L-*N*-methylvalyl)-L-threonyl-D-valyl-L-proline (II), and its physical characteristics agreed with the dimer IIIB obtained from chromatographic fractionation of the crude reaction mixture.



IIIA

The occurrence of oligomerization can be explained by intermediate mixed anhydride formation due to nucleophilic attack by the carboxylate of the C-terminal proline residue on 2-nitro-3-benzyloxy-4-methylbenzoyl chloride (IA). Kopple, *et al.*, previously observed and discussed the rationale of oligomer formation during reactions of benzyl chloroformate with tripeptides.[7] Indeed, the first recorded peptide synthesis occurred during benzoylation of silver glycinate.[8]

To obtain optimal yields of monomer required for the synthesis of actinomycin D and analogs, several other methods of activation of 2-nitro-3-benzyloxy-4-methylbenzoic acid were investigated. In these experiments the overall yield of monomer in relation to the degree of oligomer formation was an important factor in choosing a suitable method of activation. The results are summarized in Table I.

The mixed carbonic carboxylic anhydride method utilizing isobutyl chloroformate[9] gave a low yield of the monomer IIIA and substantially more oligomerization than the acid chloride reaction. Likewise, the *N,N'*-carbonyldiimidazole[10] and the *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ)[11] methods gave similar results.

The *N*-hydroxysuccinimide[12] ester (IC) of 2-nitro-3-benzyloxy-4-methylbenzoic acid gave only monomer

(1) Contribution VII in the series, Syntheses of Actinomycin and Analogs. Part VI: J. Meienhofer, R. Cotton, and E. Atherton, *Peptides, Proc. Eur. Symp., 11th*, in press.

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(6) Additional peaks of by-products and side products of lower molecular weight were observed eluting after the peptide monomer. One of these peaks was identified as 2-nitro-3-benzyloxy-4-methylbenzoic acid.

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TABLE I

PROPERTIES OF THE OLIGOMERS AND YIELDS FOR VARIOUS METHODS OF ACTIVATION

	Mp, °C	$[\alpha]^{20}D$, deg	Formula, (mol wt)	Calcd (found) C, %	Calcd (found) H, %	Calcd (found) N, %	Mol wt[a] by titration	% yields Acid chloride	Mixed anhydride	N,N'-Carbonyldiimidazole	EEDQ	N-Hydroxysuccinimide ester	Symmetrical anhydride	Acid chloride base control	Azide
Monomer IIIA	115–120	−2.3 (c 1, MeOH)	$C_{46}H_{58}N_6O_{13}$ (903.0)	61.19 (61.18)	6.47 (6.84)	9.31 (9.02)	985	55.0	7.7	15.0	10.0	48.2	80.0	33.0	28.0
Dimer IIIB	116–120	−19.9 (c 1, MeOH)	$C_{77}H_{102}N_{11}O_{21}$ (1518.73)	60.90 (60.82)	6.84 (7.05)	10.15 (9.98)	1532	21.0	11.0	20.0	19.3	Trace	5.5	0	0
Trimer IIIC	119–124	−25.6 (c 1, MeOH)	$C_{108}H_{148}N_{16}O_{29}$ (2134.45)	60.77 (60.94)	6.99 (7.38)	10.50 (10.06)	2000	1.5	24.5	5.0	11.0	0	Trace	0	0
Tetramer IIID	123–126	−54.5 (c 0.29, MeOH)	$C_{139}H_{192}N_{21}O_{37}$ (2750.18)	60.69 (60.26)	7.08 (7.19)	10.69 (10.58)	2682	Trace	Trace	0	4.7	0	0	0	0
Pentamer IIIE	127–131	−41.0 (c 0.29, MeOH)	$C_{170}H_{238}N_{26}O_{45}$ (3365.91)	60.65 (60.48)	7.14 (7.12)	10.82 (10.71)		Trace	Trace	0	1.5	0	0	0	0

[a] Molecular weights were determined by titration of an aqueous ethanolic solution of each oligomer with a standardized solution of sodium hydroxide.

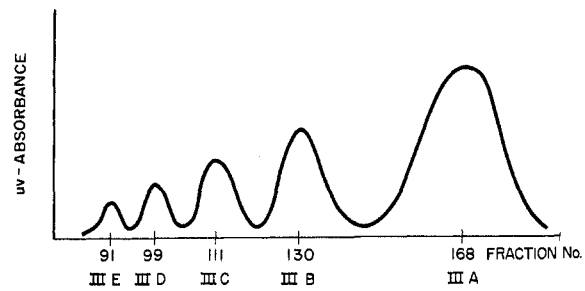

Figure 1.—Uvicord recording of a chromatographic fractionation of the product obtained from a reaction of 2-nitro-3-benzyloxy-4-methylbenzoyl chloride with *O*-(benzyloxycarbonylsarcosyl-L-*N*-methylvalyl)-L-threonyl-D-valyl-L-proline, Sephadex LH-20 column (190 × 5 cm) using ethanol at 5° as eluent and collecting 15-ml fractions.

IIIA and trace amounts of dimer IIIB. Formation of the dimer in this instance could occur through ester interchange involving transfer of the *N*-hydroxysuccinimide ester from the 2-nitro-3-benzyloxy-4-methylbenzoyl moiety to the peptide moiety[13] or through a mixed anhydride intermediate.[14] The low overall yield of monomer, however, rendered this method unsuitable.

Reaction of the symmetrical anhydride,[15,16] generated *in situ* by the action of dicyclohexylcarbodiimide on 2-nitro-3-benzyloxy-4-methylbenzoic acid, with the pentadepsipeptide II produced a high yield of monomer IIIA. A small amount of dimer was observed and trace amounts of trimer. In this reaction 1 equiv of base was initially used to neutralize the pentadepsipeptide hydrochloride and another 1.2 equiv was added over a period of 2 hr, thus optimizing conditions for obtaining the monomer and minimizing the chance of oligomer formation.

This method of controlled addition of base, utilized in the symmetrical anhydride reaction, was tried in a reexaminition of the acid chloride method. The experiment resulted in monomer formation only; however, the yield was quite low. Similarly, the azide[17] method of activation gave monomer only but in low yield.

In these series of experiments conditions were found under which oligomerization was suppressed, chiefly through controlled addition of base. Furthermore, weak activation minimized oligomer formation. However, under such conditions of comparatively slow reaction, the desired monomer was obtained in low yields, probably due to degradation of the pentadepsipeptide. It was concluded that the best method of activation of 2-nitro-3-benzyloxy-4-methyl benzoic acid, in the reaction under investigation, was the generating of its symmetrical anhydride. Although some dimer and trace amounts of trimer were formed, the best overall yields of monomer IIIA (70–80%) were obtained.

Experimental Section

Details on materials and methods have been described before.[5] *O*-(Benzyloxycarbonylsarcosyl-L-*N*-methylvalyl)-L-threonyl-D-

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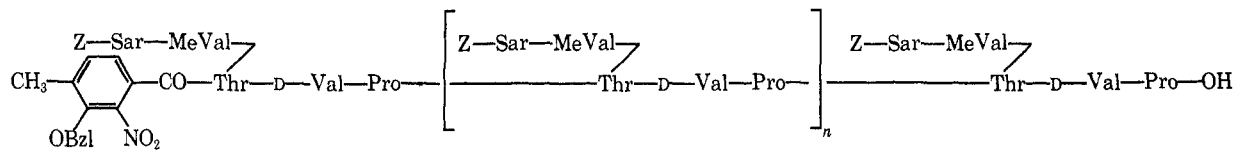

IIIB, $n = 0$; IIIC, $n = 1$; IIID, $n = 2$; IIIE, $n = 3$

Figure 2.—The structure of oligomers formed during N-acylation of *O*-(benzyloxycarbonylsarcosyl-L-*N*-methylvalyl)-L-threonyl-D-valyl-L-proline.

valyl-L-proline II. **A. Trifluoroborate.**—This was prepared in a similar manner to that described previously.[5]

B. Hydrochloride.[18]—The trifluoroborate salt of II was dissolved in dioxane and treated with a slight excess of 2.5 *N* HCl in ether. An oil was formed which on trituration with anhydrous ether solidified to a fine white solid (80–90% yields), mp 158–163°, $[\alpha]^{20}D$ −43° (*c* 1, methanol).

Acylation of *O*-(Benzyloxycarbonylsarcosyl-L-*N*-methylvalyl)-L-threonyl-D-valyl-L-proline with 2-Nitro-3-benzyloxy-4-methylbenzoic Acid.—All procedures described were carried out in reduced light. The general work-up procedure was the same in every reaction. After completion, the reaction mixture was poured into water and the oily suspension which formed was extracted into ethyl acetate. The ethyl acetate solution was washed with dilute acid two times, followed by saturated NaCl, dried over $MgSO_4$, and concentrated under reduced pressure. The oil obtained was dissolved in 95% ethanol and chromatographed at 5° on a 190 × 5 cm column of Sephadex LH-20 using ethanol as the eluent. Fractions obtained were concentrated under reduced pressure and precipitated from ethyl acetate by the addition of hexane. The results are summarized in Table I.

Methods of Activation. A. Acid Chloride.[5]—The trifluoroborate salt of II (6.00 g, 9.45 mmol) was dissolved in 25 ml of dioxane and cooled to 0°. *N*-Methylmorpholine (3.16 ml, 28.26 mmol) was added, followed by Ia[4] (2.88 g, 9.42 mmol). The reaction mixture was allowed to warm to room temperature and after stirring for 1 hr was worked up as described above.

B. Mixed Carbonic Carboxylic Anhydride.—To a stirred solution of IB (102.1 mg, 0.36 mmol) in dioxane (2 ml) at room temperature was added *N*-methylmorpholine (0.04 ml, 0.36 mmol) and isobutyl chloroformate (0.047 ml, 0.36 mmol). After 1 min a solution of the trifluoroborate salt of II (250 mg, 0.36 mmol) and *N*-methylmorpholine (0.12 ml, 1.07 mmol) in dioxane (2 ml) was added. After stirring for 1 hr, the reaction mixture was worked up as described.

C. *N,N'*-Carbonyldiimidazole.—IB (86 mg, 0.30 mmol) was dissolved in dioxane (5 ml). *N,N'*-Carbonyldiimidazole (47 mg, 0.30 mmol) was added and the mixture was stirred for 10 min at room temperature. The hydrochloride of II (200 mg, 0.30 mmol) was dissolved in 5 ml of dioxane and *N*-methylmorpholine (0.1 ml, 0.90 mmol) was added. The two solutions were mixed and stirred at room temperature for 16 hr and then worked up as described.

D. *N*-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline.—The hydrochloride of II (1 g, 1.49 mmol) was dissolved in dimethylformamide (5 ml) cooled to 0° and *N*-methylmorpholine (0.33 ml, 2.98 mmol) was added. IB (0.43 g, 1.49 mmol) and *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline were dissolved in dimethylformamide at 0° and stirred for approximately 30 sec before the addition of the peptide solution. The mixture was stirred for 19 hr at room temperature and worked up as described after the addition of two drops of concentrated HCl.

E. *N*-Hydroxysuccinimide Ester.—IC[19] (157 mg, 0.41 mmol) was added to a dimethylformamide (5 ml) solution of the hydrochloride of II (0.25 g, 0.37 mmol) preneutralized with triethylamine (0.064 ml, 0.45 mmol). The mixture was stirred for 24 hr and worked up as previously described. Unreacted IC was filtered off before column chromatography.

F. Symmetrical Anhydride.—IB (0.944 g, 3.27 mmol) was dissolved in ethyl acetate (15 ml) and dicyclohexylcarbodiimide (0.323 g, 1.56 mmol) in ethyl acetate (7 ml) was added. The mixture was warmed slightly and allowed to stir at room temperature for approximately 5 min, before the addition of the hydrochloride of II (1 g, 1.49 mmol) dissolved in dimethylformamide (5 ml), and neutralized with *N*-methylmorpholine (0.166 ml, 1.49 mmol). After intervals of 1 and 2 hr, 0.083 ml and 0.12 ml of *N*-methylmorpholine were added respectively. After 3.5 hr, *N,N'*-dicyclohexylurea was filtered off and the mixture was worked up as described.

G. Acid Chloride with Controlled Addition of Base.—The hydrochloride of II (1 g, 1.49 mmol) was dissolved in dimethylformamide (10 ml) and *N*-methylmorpholine (0.167 ml, 1.49 mmol) was added, followed by IA (0.455 g, 1.49 mmol). Over the next 30 min at intervals of 5, 10, 20, and 30 min, *N*-methylmorpholine (0.04 ml each) was added. After 3 hr of stirring at room temperature, the mixture was worked up as described previously.

H. Azide. 2-Nitro-3-benzyloxy-4-methylbenzoylhydrazide.—IC (1.36 g, 3.5 mmol) was dissolved in dimethylformamide (5 ml) and hydrazine hydrate (3.5 ml, 70 mmol) was added. A dark yellow solution resulted which after standing 18 hr at room temperature turned to a solid green mass. The mixture was diluted with water (50 ml) and filtered, and the filtrate was washed with water and then dried to give 0.9 g of hydrazide. Crystallization from ethyl acetate–hexane gave colorless crystals, mp 162–163°.

Anal. Calcd for $C_{15}H_{15}N_3O_4$ (301.31): C, 59.80; H, 5.02; N, 13.95. Found: C, 60.27; H, 5.03; N, 13.92.

2-Nitro-3-benzyloxy-4-methylbenzoyl Azide.—The hydrazide (0.5 g, 1.66 mmol) was dissolved in acetic acid (1 ml) and water (4 ml), cooled to 0°, and sodium nitrite (0.114 g) dissolved in water (2 ml) was added. The mixture was stirred for 20 min at 0° and allowed to warm to room temperature. The solid formed was extracted into ethyl acetate and washed with 1 *M* $NaHCO_3$ and water and dried ($MgSO_4$). Evaporation under reduced pressure gave an oil. Unchanged hydrazide (90 mg) was removed by filtration after treatment with ethyl acetate–hexane. On evaporation of the mother liquor pure solid azide was obtained: 200 mg (38.5%); mp 72–73°; ir (KCl) 2075 cm^{-1} (strong singlet).

Anal. Calcd for $C_{15}H_{12}N_4O_4$ (312.28): C, 57.68; H, 3.87; N, 17.94. Found: C, 57.52; H, 3.98; N, 17.03.

Coupling Reaction.—2-Nitro-3-benzyloxy-4-methylbenzoyl azide (124 mg, 0.39 mmol) was dissolved in dimethylformamide and cooled to 0°. The hydrochloride of II (250 mg, 0.37 mmol) was dissolved in dimethylformamide, cooled to 0°, and *N*-methylmorpholine (0.041 ml, 0.37 mmol) was added. After 30 sec the two solutions were mixed and stirred for 24 hr at room temperature and worked up as described.

Synthesis of Authentic Dimer IIIB.—To a stirred solution of monomer IIIA (200 mg, 0.22 mmol) in tetrahydrofuran at −10° was added *N*-methylmorpholine (0.025 ml, 0.22 mmol) followed by isobutyl chloroformate (0.029 ml, 0.22 mmol). After 2 min a solution of the trifluoroborate salt of II (156 mg) and *N*-methylmorpholine (0.075 ml, 0.66 mmol) in dioxane (2 ml) was added and the mixture was stirred for 30 min at −10° and 17 hr at room temperature. It was worked up as described above to yield dimer 65%, trimer 12%, and trace amounts of tetramer and pentamer.

Registry No.—IB, 6623-31-0; II, 31729-72-3; II HCl, 21148-62-9; IIIA, 21148-63-0; IIIB, 31729-75-6; IIIC, 31729-76-7; IIID, 31729-77-8; IIIE, 31729-78-9; 2-nitro-3-benzyloxy-4-methylbenzoylhydrazide, 31729-79-0; 2-nitro-3-benzyloxy-4-methylbenzoyl azide, 31729-80-3.

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