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Total Synthesis of Ovine β-Lipotropin by the Solid-Phase Method

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Abstract: Ovine β-lipotropin with a linear structure of 91 amino acid residues has been synthesized by the solid-phase method. The synthetic material was purified by gel filtration, chromatography on carboxymethylcellulose, and partition chromatography on agarose. The final product has been found to be indistinguishable from the natural hormone in its R_f value on partition chromatography, mobility in paper electrophoresis, behavior on thin layer chromatography, amino acid composition of both acid and enzymic hydrolysates, NH_2-terminal residue, behavior on peptide mapping, isoelectric focusing, circular dichroism spectra, optical rotation, lipolytic activity, and immunoreactivity.

The lipotropic hormone, β-lipotropin (β-LPH),[1] was isolated from sheep pituitary glands[2,3] and its primary structure determined[4-6] (Figure 1). Its sequence from residues 41 to 58 was noted to be identical with that of β-melanotropin,[7] and residues 61–91 correspond to the sequence of the opioid peptide β-endorphin.[8] Solid-phase synthesis[9] of a peptide corresponding to residues 42–91 has been reported.[10] Subsequent syntheses of β-melanotropins,[11-13] β-endorphins,[14,15] and β_s-LPH-(41–91)[16] have been accomplished in relatively good yields. It was therefore decided to undertake total synthesis of β-lipotropin.

Recently, a second form of β_s-LPH has been isolated.[17] Partition chromatography of a hormone preparation on agarose gel cleanly separated this form from β_s-LPH and its structure was shown to be identical with that of the hormone with the sole exception that the glutamic acid residue in position 1 was replaced by a pyroglutamyl residue. This result afforded a new and effective means of purification while at the same time providing a highly purified preparation of natural β_s-LPH.

Since use of liquid HF[18,19] was planned to be the penultimate synthetic step, preliminary experiments were carried out with the natural hormone. The susceptibility of glumatic acid containing peptides to side reactions with anisole in HF[20,21] posed a potential hazard for this hormone since it contains 12 such residues. The best results were obtained by use of 2,6-dimethylanisole as scavenger. The hormone was recovered in 34% yield after chromatography on carboxymethylcellulose (CMC). Partition chromatography of this material on agarose gel gave practically the identical pattern as found with untreated hormone. These results suggested that a synthetic preparation would be able to survive a standard treatment with HF.

For solid-phase synthesis of β_s-LPH, the anchoring linkage of the COOH-terminal glutamine residue was to brominated styrene–1% divinylbenzene polymer. This linkage when used in conjunction with N^{α}-*tert*-butyloxycarbonyl protection has been shown to be cleaved only 0.03% per cycle and is approximately 15–50 times as stable as the standard linkage.[15] Coupling was performed by a symmetrical anhydride technique[22] which has been used to advantage for the β-LPH sequence.[10] Trifluoroethanol was employed in the second stage of anhydride couplings to enhance the efficiency of this reaction.[23] The side chain of tryptophan was protected with the formyl group[24-26] in view of its susceptibility to destruction under conditions of repeated acidolysis of Boc groups.[25-28] For histidine, the benzyloxycarbonyl group was used since it has been employed in synthesis of peptide analogues of the carboxyl terminal plasmin fragment of human somatotropin.[29] For threonine, serine, and glutamic acid, the very stable *p*-halobenzyl protecting groups[30,31] were used through residue 66 and benzyl protection thereafter. Selection of the other protecting groups has been discussed previously.[32-34] Since methionine was not protected, the last Boc group was removed with TFA to reduce *tert*-butylation that occurs in HF.[35]

The scheme for isolation is shown in Figure 2 with recoveries for various steps indicated. The first purification step on CMC gave a sharply defined peak close to the position previously reported for the natural hormone.[4] It is evident from the recovery of material that this procedure constituted the major purification step in the scheme. The slower moving materials which represent the major side products of synthesis apparently consist of shorter sequences as shown by both amino acid analysis and peptide mapping. The formyl group on tryptophan was then removed by brief treatment at pH 11.5 with NaOH.[12] Rechromatography on CMC gave a sharply defined peak as does natural β_s-LPH. The ultraviolet spectrum of this material was identical with that of the natural hormone in the region of 245–360 nm, indicating that deformylation was complete.[24]

Final purification was effected by partition chromatography in a biphasic solvent system on agarose. The R_f value of the major peak (0.275) which is a reflection of its distribution constant in the solvent system was, within experimental error,

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Table I. Variation of ΔR_M as a Function of Chain Length

structural change	chain length	peptide pairs separated	support	solvent system[a]	R_f	ΔR_{MX}
-Met- ↓ -Met(O)-	7	H-Leu-Gly-Arg-Leu-Gly-Met-Phe-OH H-Leu-Gly-Arg-Leu-Gly-Met(d-O)-Phe-OH	Sephadex G-50	A	0.59 0.35	−0.99
	31	β_h-endorphin [Met(O)^5]-β_h-endorphin	Sephadex G-50	B	0.53 0.35	−0.74
	39[b]	[Trp(NPS)^9]-α_s-ACTH [Met(O)^4,Trp(NPS)^9]-α_s-ACTH	Sephadex G-50	A	0.48 0.36	−0.50
		[Trp(DNPS)^9]-α_s-ACTH [Met(O)^4,Trp(DNPS)^9]-α_s-ACTH			0.48 0.36	−0.50
		[Trp(NCPS)^9]-α_s-ACTH [Met(O)^4,Trp(NCPS)^9]-α_s-ACTH			0.33 0.24	−0.46
		[Trp(NCMPS)^9]-α_s-ACTH [Met(O)^4,Trp(NCMPS)^9]-α_s-ACTH			0.40 0.27	−0.59
	91	β_s-LPH [Met(O)]-β_s-LPH	Agarose A-1.5m	C	0.285 0.244	−0.21
H_2N-Glu ↓ <Glu-	6	H-Glu-Leu-Thr-Gly-Glu-Arg-OH <Glu-Leu-Thr-Gly-Glu-Arg-OH	Agarose A-1.5 M	C	0.22 0.49	+1.23
	91	β_s-LPH [<Glu^1]-β_s-LPH	Agarose A-1.5m	C	0.285 0.372	+0.40

[a] Solvent systems: A, 1-butanol–pyridine–0.1% acetic acid (5:3:11); B, 1-butanol–pyridine–0.1 N NH_4OH containing 0.1% acetic acid (2:1:3); C, as described in Experimental Section. [b] Data taken from E. Canova-Davis and J. Ramachandran, *Biochemistry*, **15**, 921 (1976). NPS, 2-nitrophenylsulfenyl; DNPS, 2.4-dinitrophenylsulfenyl; NCPS, 2-nitro-4-carboxyphenylsulfenyl; NCMPS, 2-nitro-4-carbamidophenylsulfenyl.

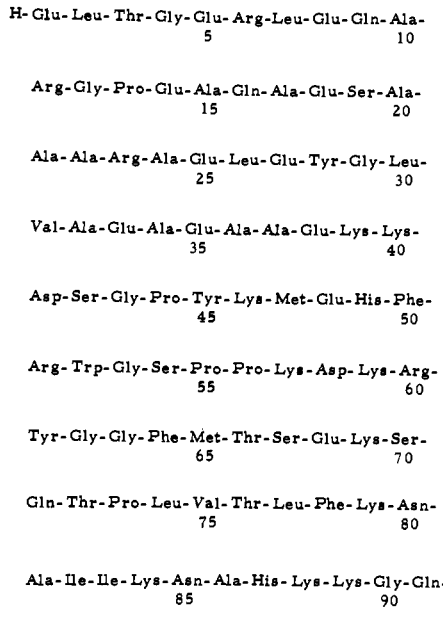

Figure 1. Amino acid sequence of ovine β-LPH.

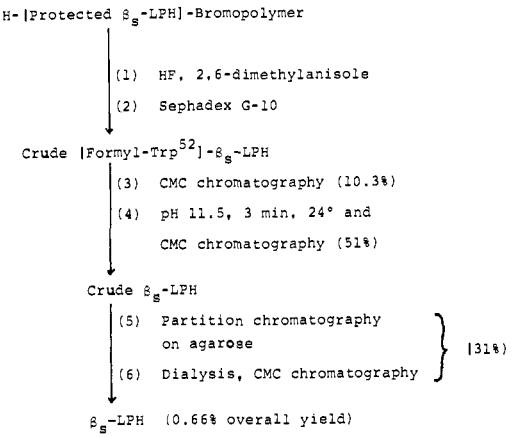

Figure 2. Scheme for isolation of synthetic β_s-LPH.

indistinguishable from that of natural β_s-LPH. Under these conditions, natural β_s-LPH gave R_f 0.285 and <Glu^1-β_s-LPH gave R_f 0.37.

Some comment on the present capabilities of partition chromatography as it relates to the purification of large synthetic peptides is required since this has a bearing on assessments of homogeneity and identity. If we assume that the two peptides A and B differ in a single amino acid residue, the difference in chromatographic behavior can be measured with use of the Bate-Smith and Westall[36] concept of R_M. We can define R_M as

$$R_M = \ln\left(\frac{1 - R_f}{R_f}\right)$$

and the difference in R_M values for peptides A and B can be recorded as

$$\Delta R_{MX} = R_{MB} - R_{MA}$$

where X refers to the structural changes in going from A to B. Effective separations of peptides can be easily achieved for ΔR_M values as small as 0.20 as is evident in recent work on diastereoisomeric analogues of β-endorphin.[37,38] Some data on the variation of ΔR_{MX} as a function of chain length are presented in Table I for two structural changes. It may be noted that the hexapeptides correspond to the H_2N-terminal sequence of β_s-LPH and [<Glu^1]-β_s-LPH. Although the value of ΔR_{MX} for either change decreases with molecular size, it remains large enough to allow separations for peptides having the size of β-LPH. In view of this it can be concluded that careful measurement of R_f values of synthetic and natural β_s-LPH is a meaningful comparison of a physical property that is sensitive to small structural changes.

The final highly purified material was obtained after dialysis and CMC chromatography in which a single sharp peak was obtained. The synthetic preparation was homogeneous and

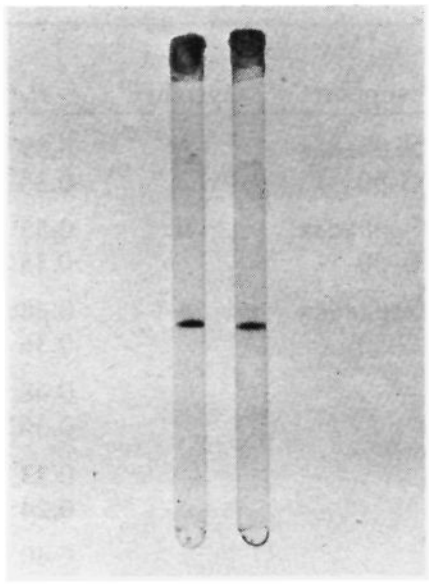

Figure 3. Isoelectric focusing on polyacrylamide gel (0.6 × 10 cm) of natural β_s-LPH (left in figure, 0.10 mg) and synthetic β_s-LPH (right in figure, 0.10 mg) in 8 M urea, 2% Ampholine pI 5–8, 100 V for 8 h and stained with Coomassie Brilliant Blue G-250 in perchloric acid solution; anode, top; and cathode, bottom.

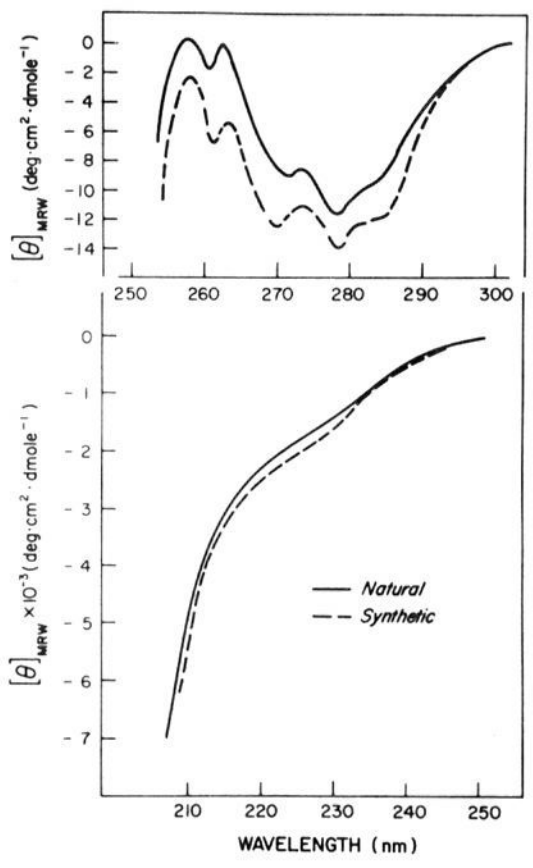

Figure 4. Circular dichroism spectra of natural and synthetic β_s-LPH in 0.1 M Tris buffer of pH 8.2.

identical with natural β_s-LPH on paper electrophoresis at two pH values and on thin layer chromatography. Amino acid analysis of an HCl hydrolysate was in agreement with expected values (Table II). Amino acid analysis of a total enzyme digest was in close agreement with that previously reported for natural β_s-LPH.[17] The synthetic peptide showed only glutamic acid as H_2N-terminal as does natural β_s-LPH. A peptide map of a tryptic digest gave a pattern which was close to that given by the natural hormone. Since the behavior of peptides on a map can vary from one map to another, a map of a mixture of synthetic and natural materials was obtained. No difference between the two materials could be discerned including colorations of various peptides to ninhydrin. The synthetic material showed behavior identical with that of natural β_s-LPH upon isoelectric focusing (Figure 3). It may be noted that natural β_s-LPH and <Glu^1-β_s-LPH are not separable by isoelectric focusing, presumably because no net difference in charge exists.

The circular dichroism spectra of the synthetic and natural hormones are shown in Figure 4. The differences between the

Table II. Amino Acid Analyses of Synthetic β_s-LPH

acid hydrolysate			enzyme hydrolysate		
amino acid	expected	found	amino acid	expected	found[a]
Trp	1		Trp	1	0.96
Lys	10	9.85	Lys	10	9.20
His	2	1.72	His	2	1.66
Arg	5	4.68	Arg	5	4.68
Asp	4	4.11	Asp	2	2.12
Thr	4	3.62	Thr + Ser + Asn + Gln	15	13.9
Ser	5	4.50			
Glu	16	16.5	Glu	12	12.4
Pro	5	4.82	Pro	5	4.63
Gly	8	8.27	Gly	8	7.64
Ala	13	13.5	Ala	13	13.9
Val	2	2.22	Val	2	2.55
Met	2	1.89	Met	2	1.81
Ileu	2	1.34[b]	Ile	2	2.11
Leu	6	6.00	Leu	6	6.30
Tyr	3	3.00	Tyr	3	3.00
Phe	3	3.03	Phe	3	3.14

[a] Degree of hydrolysis was 92% relative to acid hydrolysis. [b] Low Ile value accounted for by acid-resistant Ile-Ile sequence.

Table III. Lipolytic Activity of Synthetic β_s-LPH

β_s-LPH	dose, μg/mL	glycerol production[a]
natural	0.37	2.98 ± 0.07
	1.10	3.70 ± 0.13
synthetic[b]	0.37	2.85 ± 0.05
	1.10	3.60 ± 0.11

[a] μmol/g of cells per hr; mean ± SE: determination in triplicate. [b] Relative potency to the natural hormone, 0.84 times, a confidence limit of 0.58–1.18 and λ = 0.11.

two spectra are probably within the expected error as determined by multiple runs of different preparations of a single protein.[39] The optical rotations of synthetic hormone taken in a wavelength region of high sensitivity were $[\alpha]^{27}_{300nm}$ −604° and $[\alpha]^{27}_{250nm}$ −1820° at concentration 0.1% in 0.1 M Tris buffer of pH 8.2. The corresponding values for natural hormone were $[\alpha]^{27}_{300nm}$ −590° and $[\alpha]^{27}_{250nm}$ −1840°.

The biological activities of synthetic and natural β_s-LPH were compared in isolated rabbit fat cells as summarized in Table III. The lipolytic activity of the synthetic product is nearly identical with that for the natural hormone. As shown in Figures 5 and 6, the immunoreactivity of the synthetic hormone is the same as that of the natural β_s-LPH as revealed by both radioimmunoassay and complement fixation using rabbit antiserum to the natural hormone.

Thus, the synthetic material has been found to be indistinguishable from the natural hormone in its R_f value on partition chromatography, mobility in paper electrophoresis at two pH values, behavior on thin layer chromatography, amino acid composition of both acid and enzymatic hydrolysates, H_2N-terminal residue, behavior on peptide mapping, pI value in isoelectric focusing, circular dichroism spectra, optical rotation, lipolytic activity, and immunoreactivity. The presence of some deletion sequences involving neutral residues cannot be excluded. However, the closeness in properties of synthetic material to those of the natural hormone supports the conclusion that the synthetic product is β_s-lipotropin.

Experimental Section

Dichloromethane was distilled from P_2O_5. Dioxane was distilled from sodium and stored at 4 °C. DIEA was washed with water, dried

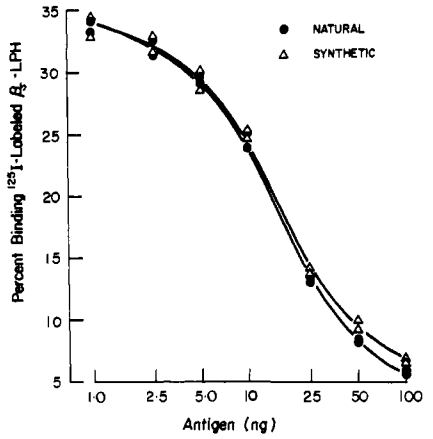

Figure 5. Competition of the natural and synthetic β_s-LPH in the β_s-LPH radioimmunoassay system. Final dilution of rabbit antiserum was 1/4000.

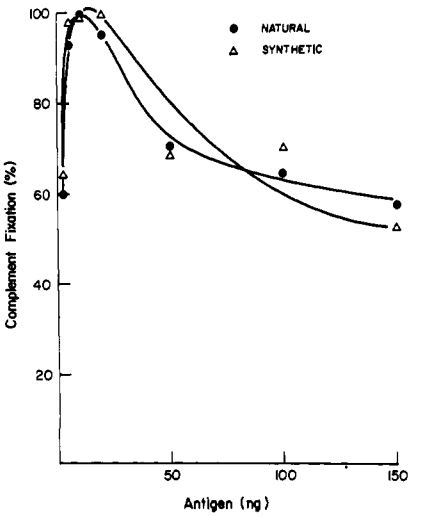

Figure 6. Microcomplement fixation curves obtained with natural β_s-LPH or the synthetic product and rabbit antiserum diluted 1/1000.

over NaOH, and distilled. *N*-Methylmorpholine was dried over NaOH and distilled. TFA, ethanol, and dicyclohexylcarbodiimide were distilled.

Chromatography on CMC[40] was performed at 24 °C in a 1.0 × 55 cm column with an initial buffer of 0.01 M NH_4OAc of pH 4.5 and collection of 10-mL fractions at a flow rate of ca. 200 mL/h. In procedure A, a gradient with respect to pH and salt concentration was effected through a 500-mL constant volume mixing chamber containing the starting buffer with introduction of 0.1 M NH_4OAc after fraction no. 10 and 0.2 M NH_4OAc after fraction no. 50. In procedure B, the gradient with 0.1 M buffer was started immediately and with 0.2 M buffer after fraction no. 40. Peptides were detected spectrophotometrically at 280 nm.

Partition chromatography[41,17] on agarose was performed in a 1.06 × 20.0 cm column of Bio-Gel A-5 m or A-1.5 m (200–400 mesh, BioRad Labs) in a biphasic solvent system composed of 2-butanol-water azeotrope (150 mL), water (97 mL), glacial acetic acid (4.3 mL), 10% (w/w) aqueous trichloroacetic acid (7.5 mL), and sodium chloride (4.4 g). Fraction volume was 0.402 mL; flow rate, 2 mL/h; hold-up volume, 5.45 mL; detection, Folin–Lowry.

Isoelectric focusing on polyacrylamide gel[42] was carried out at 20 °C as described previously[43] and staining performed by reported procedures.[44]

Peptides for Chromatographic Studies. Syntheses of H-Leu-Gly-Arg-Leu-Gly-Met-Phe-OH and its *d*-sulfoxide derivative have been reported[35,45] as well as H-Glu-Leu-Thr-Gly-Glu-Arg-OH and <Glu-Leu-Thr-Gly-Glu-Arg-OH.[17] The β_h-endorphin is a synthetic preparation.[15] The β_s-LPH and [<Glu^1]-β_s-LPH were isolated from natural sources.[4,17] The preparation and chromatographic properties of all ACTH derivatives have been reported.[46] The [Met(O)^5]-β_h-endorphin was isolated as described in Table I from a preparation of β_h-endorphin that had been exposed to air at room temperature for 2 weeks: yield, 17% based on weight of starting hormone. Amino acid analyses of a total enzyme digest showed (theoretical values in parentheses): Lys, 4.7 (5); Met(O), 1.1 (1); Thr + Ser + Asn + Gln, 8.1 (8); Glu, 2.0 (2); Pro, 1.0 (1); Gly, 2.9 (3); Ala, 2.0 (2); Val, 1.2 (1); Ile, 1.7 (2); Leu, 2.1 (2); Tyr, 2.1 (2); Phe, 2.1 (2). The [Met(O)]-β_s-LPH was isolated by partition chromatography on agarose A-1.5 m from a preparation of natural β_s-LPH (21.5 mg), yield 1.9 mg. Amino acid analysis of a total enzyme digest showed: Trp, 0.9 (1); Lys, 9.0 (10); His, 1.8 (2); Arg, 4.4 (5); Met(O), 0.8 (1); Asp, 2.4 (2); Thr + Ser + Asn + Gln, 15.9 (15); Glu, 12.0 (12); Pro, 4.5 (5), Gly, 8.6 (8); Ala, 13.0 (13); Val 2.3 (2); Met, 1.2 (1); Ile, 2.1 (2); Leu, 6.1 (6); Tyr, 2.9 (3); Phe, 2.9 (3). A tryptic map gave the same pattern as given by β_s-LPH. The positions of the Met(O) and Met residues were not determined.

Treatment of Natural β_s-LPH with HF. Natural β_s-LPH (8.95 mg) obtained as described previously[4] was stirred in 5 mL of liquid HF in the presence of 0.2 mL of 2,6-dimethylanisole for 1 h at 0 °C. The HF was removed with a rapid stream of nitrogen below 0 °C, and the residue was dried in vacuo over NaOH. The material was directly submitted to CMC chromatography (procedure A) to give a sharply defined major peak centered in fraction no. 63, followed by a smaller trailing peak. The material isolated from fractions 61–65 (3.0 mg, 34% recovery) was submitted to partition chromatography on Bio-Gel A-5 m and gave a pattern practically identical with that previously described for untreated natural hormone.[17]

Table IV. Schedule for Solid-Phase Synthesis of β_s-LPH

step	reagent (applications)	mix time each application, min
1	CH_2Cl_2 (4 times)	1
2	55% TFA/CH_2Cl_2 (1 time)	1
3	55% TFA/CH_2Cl_2 (1 time)	15
4	CH_2Cl_2 (2 times)	1
5	25% dioxane/CH_2Cl_2 (3 times)	1
6	CH_2Cl_2 (2 times)	1
7	5% NMM/CH_2Cl_2 (1 time)	0.5
8	CH_2Cl_2 (2 times)	1
9	repeat step 7	
10	repeat step 8	
11	repeat step 7	
12	repeat step 8	
13	1% DIEA/CH_2Cl_2 (1 time)	0.5
14	CH_2Cl_2 (6 times)	1
15	symmetrical anhydride (1 time) in CH_2Cl_2	20 and hold
16	NMM/TFE (1 time)	10
17	CH_2Cl_2 (3 times)	1
18	33% EtOH/CH_2Cl_2 (3 times)	1

Solid-Phase Synthesis Procedures. Boc-Gln bromopolymer (0.69 g, 0.34 mmol)[15,37] in a Beckman 990 peptide synthesizer was carried through the schedules shown in Table IV. All couplings except that of Boc-Asn-OH were by a symmetrical anhydride technique using 6.5 equiv of Boc-amino acid and 3.0 equiv of DCC in CH_2Cl_2.[10,22] In step 16 an amount of TFE was added sufficient to give an overall concentration of 20% (v/v) and the amount of NMM used gave 0.1%.[23] Boc-Asn-OH was coupled by the DCC–1-hydroxybenzotriazole method.[47,48] Side chain protecting groups were Trp, formyl;[26] Lys, 2-BrZ;[49] His, Z;[29] Arg, tosyl;[33] Asp, Bzl; Thr, 4-ClBzl[31] for residues 76, 72, and 66 and Bzl for residue 3; Ser, 4-BrBzl[31] for residues 70 and 67, and Bzl for 54, 42, and 19; Glu, 4-BrBzl[30] for residue 68 and Bzl thereafter; Tyr, 2-BrZ.[34] Steps 11 and 12 in the schedule were omitted for residues 90–61. Boc (Tos) Arg-OH and Boc-Gln-OH were dissolved in the minimum amount of DMF before proceeding to the anhydride preparation; in both cases the TFE in step 16 was omitted. The preparation of the anhydride of Boc-Leu-OH was started at room temperature since the acid had limited solubility in CH_2Cl_2 at 0 °C while the symmetrical anhydride was soluble. For Boc(4-BrBzl)Glu-OH and Boc(formyl)Trp-OH the symmetrical anhydrides

had low solubility at 0 °C but could be dissolved upon warming to room temperature and dilution with CH_2Cl_2. The Boc group of the last amino acid residue was removed by running steps 1-10 of the schedule. The yield of protected peptide resin was 4.29 g.

Isolation of Synthetic Material. A sample (0.56 g) of protected peptide resin was treated in 10 mL of liquid HF in the presence of 2,6-dimethylanisole (1.0 mL) for 30 min at −30 to −20 °C and 45 min at 0 °C. The HF was removed with a rapid stream of nitrogen at less than 0 °C over 15 min. Cold ethyl acetate (10 mL) was added at −60 °C, the mixture swirled briefly, and the supernatant decanted from the gummy material. The material was solidified under ethyl acetate (10 mL) at 24 °C and filtered off. The air-dried solid was extracted with 0.5 N HOAc (3 mL) and the resin (109 mg) removed by filtration. The filtrate was passed through a 2.16 × 25 cm column of Sephadex G-10 in 0.5 N HOAc. A single peak was detected (280 nm) and isolation by lyophilization gave 282 mg. The material was submitted to CMC chromatography (procedure A) to give a sharply defined peak with maximum in fraction no. 65 close to the position for natural β_s-LPH. Isolation of material corresponding to fractions 61-67 gave 29.1 mg. The remainder of the protected peptide resin was processed in 0.5-g batches in the same way.

A sample (74.5 mg) of the above material was dissolved in water (14.5 mL) and rapidly mixed with 0.50 mL of 1 N NaOH to a pH of 11.5. After 3 min at 24 °C the pH was quickly dropped to 4.8 with glacial acetic acid (50 μL). The solution was submitted to CMC chromatography (procedure A) to give essentially one major peak with maximum in fraction no. 68, and isolation of material from fractions 63-73 gave 38.0 mg of product. For partition chromatography a sample (ca. 15 mg) was dissolved in 50 μL of 0.5 N HOAc, diluted with 0.2 mL of the organic phase of the solvent system described above, and applied to a Bio-Gel A-1.5 m partition column. The elution profile showed a well-defined major peak with R_f 0.275. Fractions 44-60 corresponding to this peak were pooled with those from two other runs, diluted with an equal volume of water, and evaporated in vacuo below 24 °C till a single aqueous phase was obtained. The solution was dialyzed at 4 °C against 1 L of 0.1 N NH_4HCO_3-0.03 N NH_4OH (pH 9) for 2.5 h and then against fresh buffer for 18 h. After additional dialysis at 4 °C against 1 L of water for 5.5 h, the solution was lyophilized to give 14.4 mg from a total of 47 mg which had been submitted to partitioning. This material was chromatographed on CMC (procedure B) to give one sharply defined peak with maximum in fraction no. 55, and isolation of material from fractions 52-58 gave 10.8 mg of highly purified synthetic product (0.66% yield based on starting resin; 86% peptide content based on ϵ_{276} 9570).

Comparison of Synthetic Material with Natural β_s-LPH. Natural β_s-LPH that had been purified as described previously[17] was used in the following comparisons.

Paper electrophoresis of synthetic material (100-μg samples) on Whatman 3 MM at pH 3.7 (pyridine acetate buffer) and 6.7 (collidine acetate buffer) for 6 h at 400 V each gave a single spot (ninhydrin detection) with R_f^{Lys} values of 0.56 and 0.18, respectively, and identical in behavior with natural β_s-LPH. Thin layer chromatography of synthetic material (30 μg) on silica gel in 1-butanol/pyridine/acetic acid/water (5:5:1:4) gave a spot with R_f 0.25 and a trace at the origin (ninhydrin and Cl_2-tolidine), identical in behavior with natural β_s-LPH. Amino acid analysis[50] of synthetic material (0.60 mg) after 24-h hydrolysis in constant boiling HCl gave values shown in Table II. For total enzyme digestion synthetic material (0.68 mg) was treated in 0.28 mL of 0.05 M Tris buffer of pH 8 (0.01 M Mg^{2+}) with 14 μg each of trypsin (Worthington) and chymotrypsin (Worthington) for 24 h at 37 °C. The solution was heated at 100 °C for 15 min, cooled, and treated with 28 μg of leucine aminopeptidase (Worthington) at 37 °C for 48 h. After treatment with 1 N NaOH (35 μL) and lyophilization, amino acid analysis gave the results shown in Table II. Synthetic and natural materials were submitted to amino end group determination by the dansyl method.[51,52] For peptide mapping a sample (0.65 mg) was treated with 14 μg of trypsin in 35 μL of the above Tris buffer for 24 h at 37 °C. The entire sample was used for mapping on Whatman 3 MM by descending chromatography with upper phase of 1-butanol/acetic acid/water (4:1:5) followed by electrophoresis at pH 6.7 (collidine acetate buffer) and 400 V for 6 h. Detection was with ninhydrin.

Optical rotations and circular dichroism spectra were measured in 0.1 M Tris buffer (pH 8.2) in a Cary 60 recording spectropolarimeter equipped with a Model 6002 circular dichroism attachment. The procedures used for these measurements have been described.[53,54] Circular dichroism spectra were obtained on solutions of approximately 1 mg/mL at 27 °C in a 1.0-cm path length cell for wavelengths above 250 nm and in a 1.0 cell below 250 nm. Optical rotations were obtained on the same solutions, under the aforementioned conditions. In all measurements, dynode voltages did not exceed 500 V. Peptide concentrations were then determined on a Beckman DK-2A recording spectrophotometer after correction for light scattering[55] and with the assumption that $E^{0.1\%}_{1cm,276nm} = 9.6$.

Lipolytic activity was determined by the procedure previously described[10] using isolated fat cells from the perirenal fat pads of male New Zealand rabbits. For radioimmunoassay, the procedure of Rao and Li[56] was used employing a rabbit antiserum to native β_s-LPH. Microcomplement fixation was performed according to Wasserman and Levine.[57]

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References and Notes

(1) Symbols and abbreviations are in accordance with the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature, Collected Tentative Rules and Recommendations. Other abbreviations used: β_s-LPH, sheep β-lipotropin; TFA, trifluoroacetic acid; CMC, carboxymethylcellulose; <Glu, pyroglutamyl residue, DIEA, diisopropylethylamine; DCC, dicyclohexylcarbodiimide; NMM, *N*-methylmorpholine; DMF, dimethylformamide; TFE, trifluoroethanol.
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Synthetic Applications of *N*-Acylamino-1,3-dienes. An Efficient Stereospecific Total Synthesis of *dl*-Pumiliotoxin C, and a General Entry to *cis*-Decahydroquinoline Alkaloids[1]

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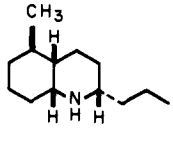
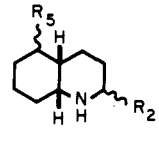
Abstract: Two efficient total syntheses of *dl*-pumiliotoxin C (**1**) are reported. A Diels–Alder strategy is employed which features the use of *trans*-1-*N*-acylamino-1,3-butadienes as synthetic equivalents for *trans*-1-amino-1,3-butadiene. In the best approach (Scheme II), **1** was prepared in a stereospecific fashion in three steps, and greater than 50% overall yield, from benzyl *trans*-1,3-butadiene-1-carbamate (**19**). The efficient preparation of other dialkyl *cis*-decahydroquinolines by this general synthetic strategy (Scheme III) is also reported.

Extensive investigations[3] of the toxic skin secretions of neotropical "poison-dart" frogs of *Dendrobates pumilio*,[4,5] *Dendrobates auratus*,[6] and *Dendrobates histrionicus*[7] have resulted in the isolation of more than 60[8] pumiliotoxins. The first member of this alkaloid group to be characterized was pumiliotoxin C, which was shown by x-ray analysis of the hydrobromide[4,7] to have the unusual *cis*-decahydroquinoline structure **1**.[9] Subsequently, a number of dendrobatid alkaloids (the pumiliotoxin C and hydroxypumiliotoxin C classes) have been tentatively assigned similar decahydroquinoline structures,[7,8] for example, alkaloids I (**2**), II (**3**), and III (**4**).[7,8]



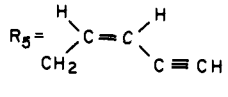
1



2, R_2 = n-C_4H_9, R_5 = H

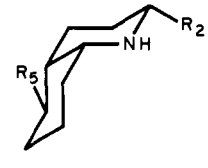
3, $R_2 = R_5$ = n-C_3H_7

4, $R_2 = (CH_2)_3CH{=}C{=}CH_2$



Alkaloids of the pumiliotoxin C class having a variety of saturated and unsaturated side chains, with lengths up to nine carbons, have been recognized.[8] The *cis*-decahydroquinoline ring is also a structural feature of the tricyclic dendrobatid alkaloids of the gephyrotoxin family.[7,8] The impossibility of isolation of more than milligram quantities of these toxins from natural sources, together with their significant neurological activities,[4,5,7,8,10] makes these alkaloids attractive targets for total synthesis.

A successful synthesis of alkaloids of the pumiliotoxin C class must deal with construction of the *cis*-decahydroquinoline ring,[11] and elaboration of the correctly oriented side chains at carbons 2 and 5. For alkaloids with the pumiliotoxin C stereochemistry, the two side chains are equatorially oriented in the most stable *cis*-decahydroquinoline conformation **5**.



5

Synthetic efforts to date have focused on pumiliotoxin C, culminating in a number of successful total syntheses.[11b,12,13] In this paper we present details of our total synthesis of racemic pumiliotoxin C,[14] which provides an excellent illustration of the application of *N*-acylamino-1,3-dienes[15,16] for solving formidable stereochemical problems in the alkaloid area. The generalization of this route to the synthesis of other pumiliotoxins and pumiliotoxin analogues is also detailed.

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