THE STRUCTURES OF TRIDECAPTINS B AND C

(STUDIES ON ANTIBIOTICS FROM THE GENUS *BACILLUS*. XXV)[1]

TOSHIYUKI KATO, RYUJI SAKAZAKI, HIROSHI HINOO and JUN'ICHI SHOJI

Shionogi Research Laboratory, Shionogi & Co., Ltd.,
Fukushima-ku, Osaka 553, Japan

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On examining the structures of the antibiotics tridecaptins B and C, the constituent amino acids were separated and their chiralities were determined. The constituent fatty acids were identified by gas chromatography and mass spectrometry. Cleavage with N-bromosuccinimide and sequential analysis by EDMAN degradation demonstrates the sequence of the C-terminal side of tridecaptins B and C. Deacylation with polymyxin acylase of tridecaptin B and successive EDMAN degradation revealed the sequence of the N-terminal side of tridecaptin B. Finally partial acid hydrolysis on tridecaptin C clarified the sequence of the N-terminal side of tridecaptin C.

Tridecaptins B and C, members of the tridecaptin group of antibiotics produced by strains of *Bacillus polymyxa*, are active against Gram-negative and Gram-positive bacteria *in vitro* and *in vivo*. They are acyl tridecapeptides whose constituents have been already reported[2] (summarized as in Table 1). Degradative studies described here elucidated the structure of tridecaptins B and C as in Fig. 1.

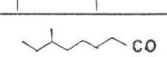
From the acid hydrolyzates of tridecaptins B and C, each amino acid was isolated by preparative paper chromatography, column chromatography on a porous polymer Amberlite XAD-2 and ion exchange column chromatography on Dowex 50. Comparison of their $[M]_D$ values with those of the pure isomers and the COTTON effects in their CD curves (Table 2) clarified the chirality of each amino acid as follows: Ser (2L, 1D), Glu (L), Val (D,L mixture, more abundunt in L-form), Ile (L), aIle (D), Dab (2D, 1L) and Trp (D) in tridecaptin B, and Ser (2L, 1D), Glu (L), Val (D,L mixture, more abundunt in D-form), aIle (D), Phe (L), Dab (2D, 1L) and Trp (D) in tridecaptin C.

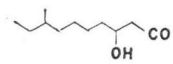
The acid hydrolyzates of tridecaptins B and C were extracted with ethyl ether and the ethereal extracts were methylated and examined by gas chromatography (Fig. 2). From the chromatogram, methyl anteisononanoate (abbreviated hereafter as *a*-C_9) from tridecaptin B, and methyl β-hydroxy an-

Table 1. Constituent amino acids and fatty acids of tridecaptins B and C[2].

Antibiotic	Amino acids										Fatty acids
	Dab	Trp	Ser	Glu	Gly	Ala	Val	aIle	Ile	Phe	
Tridecaptin B	3	1	3	1	2		1.6	0.7	0.7		*a*-C_9
Tridecaptin C	3	1	3	1	1		2.8	0.2		1	*a*-$C_{11}h^3$, *i*-$C_{10}h^3$

Dab : 2,4-diaminobutyric acid

a-C_9 : 

a-$C_{11}h^3$: 

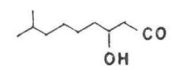
i-$C_{10}h^3$: 

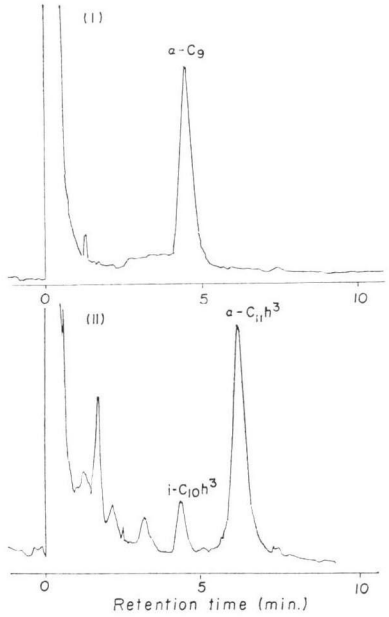
Fig. 1. Structures of tridecaptins B and C.

FA→W[1]→Dab[2]→Gly[3]→Ser[4]→Trp[5]→Ser[6]→Dab[7]—
(D) (D) (D) (L) (L)
→Dab[8]→X[9]→Glu[10]→Val[11]→Y[12]→Ser[13]
(D) (L) (L) (L) (D) (L)

Antibiotics	FA	W	X	Y
Tridecaptin B	*a*-C_9	Gly	{Ile {Val	{aIle {Val
Tridecaptin C	{*a*-$C_{11}h^3$ {*i*-$C_{10}h^3$	D-Val	Phe	{Val {aIle

Fig. 2. Gas chromatogram of fatty acid methyl esters from tridecaptins B and C.

i) Tridecaptin B: analyzed at 105°C
ii) Tridecaptin C: analyzed at 172°C



teisoundecanoate (*a*-$C_{11}h^3$) and methyl β-hydroxy isodecanoate (*i*-$C_{10}h^3$) from tridecaptin C were tentatively identified by comparison of retention times with those of authentic specimens (*a*-C_9 from colistin, and *a*-$C_{11}h^3$ and *i*-$C_{10}h^3$ from cerexin B)[3]. The identity of the fatty acids was further confirmed by gas chromatography-mass spectrometry.

Oxidation of tridecaptin B with N-bromosuccinimide cleaved the antibiotic into two peptide fragments at the Trp residue. An octapeptide, which was presumed to be a C-terminal fragment because it has no UV absorption depending on Trp residue, was isolated. EDMAN degradation of this fragment showed the sequence of this fragment to be Ser→Dab→Dab→(Ile, Val)→Glu→Val→(aIle, Val)→Ser. Thus the amino acid sequence of the C-terminal side from the Trp residue was determined.

Polymyxin acylase from the cells of *Pseudomonas* sp. M-6-3 has previously been used to deacylate cerexin A[4], brevistin[5], and polymyxins S[6] and T[7]. Tridecaptin B was easily deacylated by the

Table 2. Comparisons of molecular rotations and CD curves.

Amino acids	Pure isomer (COTTON effect)		Isolated from tridecaptin B (COTTON effect)	Isolated from tridecaptin C (COTTON effect)
Dab	L-form	+39.0°	−14.8±0.8°	−6.9±0.5°
	Calcd. for a mixture of D-form(2) and L-form(1)	−13.0°		
Ser	L-form	+15.9°	+3.4±1.3°	+3.3±0.8°
	Calcd. for a mixture of L-form(2) and D-form(1)	+5.3°		
Glu	L-form	+47.3°	+33.3±3.6° (positive)	+46.7±3.6° (positive)
Val	L-form	+33.7°	+8.9±1.6°	−7.4±1.5°
Ile	L-form	+53.1°	+47.4±4.3° (positive)	
aIle	D-form	−45.1°	−45.9±6.3° (negative)	−37.4±6.3° (negative)
Phe	L-form	−7.4°		−8.9±2.3° (positive)
Trp	L-form	(positive)	(negative)	(negative)

action of the enzyme to give deacyl tridecaptin B. EDMAN degradation of the deacyl tridecaptin B showed the sequence of the N-terminal side to be Gly→Dab→Gly→Ser→Trp. Thus the entire amino acid sequence of tridecaptin B was determined as in Fig. 1.

The chirality of individual Ser and Dab residues were determined as follows: From the N-terminal fragment obtained by NBS cleavage, Dab^2 was isolated. Similarly from the C-terminal fragment $Ser^{6,13}$ and $Dab^{7,8}$ were isolated. Their CD curves were measured and $Ser^{6,13}$ were deduced to be L-forms and Dab^2 to be D-form and $Dab^{7,8}$ to be a mixture of one D-form and one L-form. Thus the remaining Ser^4 was deduced to be D-form. Although the chiralities of Dab^7 and Dab^8 were not determined, they were assumed to be identical with those of the Dab units in tridecaptin A[6], namely Dab^7 to be L-form and Dab^8 to be D-form.

From these results we concluded the total structure of tridecaptin B, except for the configuration of the fatty acyl residue, to be as shown in Fig. 1.

Tridecaptin C was similarly oxidized with N-bromosuccinimide, and the C-terminal octapeptide isolated. EDMAN degradation of this fragment showed the sequence to be Ser→Dab→Dab→Phe→Glu Val→(Val, aIle)→Ser, thereby clarifying the amino acid sequence of the C-terminal side from the Trp residue.

To determine the amino acid sequence of the N-terminal side, we first attempted deacylation of tridecaptin C with polymyxin acylase. Since enzymic deacylation of tridecaptin C was unsuccessful, tridecaptin C was partially hydrolyzed with a mixture of formic acid and conc.hydrochloric acid[1]. From the hydrolyzate four significant fragments were isolated. They were deduced to be FA→Val, FA→Val→Dab, FA→Val→Dab→Gly, and Ser→Trp* (*Trp residue was degraded during the acid hydrolysis) from their amino acid compositions and by direct comparison with fragments obtained from tridecaptin A[1]. Thus the amino acid sequence of the N-terminal side was determined to be FA→Val→Dab→Gly→Ser→Trp.

The chiralities of individual Ser, Val and Dab residues were not determined, but they were assumed to be identical with the corresponding amino acids of tridecaptins A and B.

From these considerations, we concluded the structure of tridecaptin C, except for the configuration of the fatty acyl residue, to be as shown in Fig. 1.

Tridecaptins B and C are assumed to be complexes of components which differ at the fatty acyl residue or amino acid residues (FA, X and Y in Fig. 1). In this paper the structures of tridecaptins B and C were investigated on the complexes. Separation of the complexes into their components by high performance liquid chromatography and elucidation of the structure of each component is reported in the next paper[8].

Experimental

The experimental procedures are the same as those described in the preceding paper[1].

The constituent fatty acids

Some 10 mg each of tridecaptins B and C were hydrolyzed with constant boiling hydrochloric acid at 110°C for 1.0 hour. The ethereal extracts of the acid-hydrolyzate were methylated with diazomethane and analyzed with gas chromatography. From tridecaptin B, a main peak of identical retention time with *a*-C_9, prepared from polymyxin E, was found. Similarly from tridecaptin C, two main peaks of identical retention times with *a*-$C_{11}h^3$ and *i*-$C_{10}h^3$, prepared from cerexin B, were obtained. Their identities were further confirmed with GC-MS. The ion peaks contained in the GC-MS were the same as those described in the preceding paper[3].

Isolation of constituent amino acids of tridecaptin B

Tridecaptin B trihydrochloride (150 mg) was hydrolyzed with constant boiling hydrochloric acid containing 4% thioglycollic acid in a vacuum sealed tube. The hydrolyzate was concentrated to dryness. The residue was dissolved in water and extracted with ethyl acetate to remove thioglycollic acid. The aqueous layer was concentrated to dryness, and the residue was dissolved in 2 ml of water, neutralized to pH 7.0 and placed on the top of an Amberlite XAD-2 column (0.8×27 cm) which was eluted with water, slowly. The amino acid mixtures, except for Trp, were passed through the column and appeared in the fraction of 4~25 ml, whereas Trp was retarded by the resin and appeared in the fraction of 94~215 ml. Each fraction was treated as below.

(1) The eluate containing Trp was adsorbed on a small column of Dowex 50×8 (NH_4), which was washed with water and then eluted with 0.3 N NH_4OH. Lyophilization of the eluate gave Trp (5.5 mg) as a colorless powder.

(2) The eluate of the mixture of the amino acid was concentrated to dryness. When this residue was treated in the same manner as that from tridecaptin A, Val (9.2 mg), Glu (6.4 mg), Ser (9.3 mg), Gly (7.8 mg), Dab·HCl (38.1 mg) and a mixture of Ile and aIle (6 mg) were obtained as colorless crystalline powders.

The mixture of Ile and aIle was dissolved in a small amount of 0.2 M pyridine-acetate buffer (pH 3.03) and placed on a column of Dowex 50×8 (0.9×85 cm) and then eluted with the same buffer. aIle was eluted in the fraction of 101~110 ml and Ile in the fraction of 112~124 ml. Each fraction was evaporated to dryness, and aIle (1.7 mg) and Ile (3.0 mg) were obtained as colorless powders.

When CD curves and $[\alpha]_D$ values were measured for the separated amino acids, the following values were obtained.

Trp $[\Theta]_{305}$ 0 $[\Theta]_{260}$ −820 $[\Theta]_{224}$ −8700 $[\Theta]_{215}$ 0 (*c* 0.104, 0.5 N HCl)
Ile $[\alpha]_D^{25.0}$ +36.1±3.3° (*c* 0.230, 5 N HCl)
$[\Theta]_{245}$ 0 $[\Theta]_{209}$ +4800 $[\Theta]_{200}$ +4110 (*c* 0.220, 0.5 N HCl)
aIle $[\alpha]_D^{28.0}$ −35.0±4.8° (*c* 0.157, 5 N HCl)
$[\Theta]_{250}$ 0 $[\Theta]_{210}$ −5390 $[\Theta]_{200}$ −4580 (*c* 0.146, 0.5 N HCl)
Val $[\alpha]_D^{24.0}$ +7.6±1.4° (*c* 0.330, 5 N HCl)
Ser $[\alpha]_D^{25.0}$ +3.2±1.2° (*c* 0.378, 5 N HCl)
Glu $[\alpha]_D^{25.0}$ +21.3±2.3° (*c* 0.268, 5 N HCl)
$[\Theta]_{250}$ 0 $[\Theta]_{206}$ +4010 $[\Theta]_{200}$ +3070 (*c* 0.2149, 0.5 N HCl)
Dab·HCl $[\alpha]_D^{24.0}$ −9.6±0.5° (*c* 1.015, 5 N HCl)

Isolation of the constituent amino acids of tridecaptin C

Tridecaptin C (150 mg) was hydrolyzed in the same manner as tridecaptin B. Thioglycollic acid was removed by ethyl acetate extraction. The aqueous layer was evaporated to dryness and the residue was placed on a column of porous polymer Amberlite XAD-2 (1.6×30 cm), which was eluted with 470 ml of water and then 5% aqueous methanol. The amino acids, except for Trp and Phe, passed through the column and appeared in the fraction of 9~70 ml, whereas Phe was slightly retarded by the resin and appeared in the fraction of 128~238 ml. Trp was strongly retarded, appearing in the fraction of 345~729 ml.

The fractions containing Phe and Trp were treated with a small column of Dowex 50×8 (NH_4). Lyophillization of the eluate containing Phe and crystallization of the residue from water and ethanol gave Phe (8 mg) as colorless crystals. Lyophilization of the eluate containing Trp and crystallization of the residue from water and acetone gave Trp (6 mg) as colorless crystals.

When the fraction containing other amino acids was treated in the same manner as that of tridecaptin B, aIle (1.4 mg), Val (24.2 mg), Ser (12 mg), Glu (6 mg), and Dab·HCl (21.5 mg) were obtained as colorless crystalline powders.

When CD curves and $[\alpha]_D$ values were measured for the separated amino acids, the following values were obtained.

Trp $[\Theta]_{310}$ 0 $[\Theta]_{262}$ −1700 $[\Theta]_{225}$ −16500 $[\Theta]_{214}$ 0 (*c* 0.0815, 0.5 N HCl)
Phe $[\alpha]_D^{28.0}$ −5.4±1.4° (*c* 0.314, 5 N HCl)
$[\Theta]_{270}$ 0 $[\Theta]_{217}$ +14300 $[\Theta]_{10}$ +7250 (*c* 0.1029, 0.5 N HCl)

aIle $[\alpha]_D^{26.0}$ $-28.5\pm4.8°$ (*c* 0.144, 5 N HCl)
$[\Theta]_{250}$ 0 $[\Theta]_{207}$ -5900 $[\Theta]_{200}$ -4600 (*c* 0.218, 0.5 N HCl)
Val $[\alpha]_D^{26.0}$ $-6.3\pm1.3°$ (*c* 0.352, 5 N HCl)
Ser $[\alpha]_D^{26.0}$ $+3.1\pm0.8°$ (*c* 0.514, 5 N HCl)
Glu $[\alpha]_D^{26.0}$ $+18.6\pm1.4°$ (*c* 0.353, 5 N HCl)
$[\Theta]_{250}$ 0 $[\Theta]_{210}$ $+4310$ $[\Theta]_{200}$ $+3460$
Dab·HCl $[\alpha]_D^{26.0}$ $-6.9\pm0.6°$ (*c* 1.021, 5 N HCl)

Cleavage reaction with N-bromosuccinimide

Tridecaptin B or C (5 mg) was dissolved in 70% aqueous acetic acid (1.0 ml). N-Bromosuccinimide (5 mg) was added to the solution, which was allowed to stand for 10 minutes. To decompose excess N-bromosuccinimide, a few drops of formic acid were added to the solution, which was allowed to stand for 5 minutes and then lyophilized. Both the N-terminal fragment and C-terminal one were separated by thin-layer chromatography on a silica gel plate and extracted with slightly acidified aqueous methanol. The extracts were concentrated to dryness. The C-terminal fragment was further purified by preparative paper chromatography on Toyo Roshi No. 51, developed with *n*-butanol - acetic acid - water (4: 1: 2) in descending manner for 20 hours, to remove inorganic material. The zone detected by ninhydrin coloration was extracted with slightly acidified aqueous methanol. The extracts were concentrated to dryness. The C-terminal fragment was obtained in 10~20% yield in several runs. The amino acid compositions and chromatographic behavior of these fragments are as follows:

Fragments obtained from tridecaptin B

	Rf value*	Amino acids found (in ratio)							
		Ser	Glu	Gly	Val	aIle	Ile	Dab	Trp**
N-terminal one	0.47	0.94	—	2.00	—	—	—	1.05	+
C-terminal one	0.22	1.60	1.00	—	1.62	0.70	0.73	1.80	—

Fragments obtained from tridecaptin C

	Rf value*	Amino acids found (in ratio)							
		Ser	Glu	Gly	Val	aIle	Phe	Dab	Trp**
N-terminal one	0.47	0.88	—	1.00	1.05	—	—	1.05	+
C-terminal one	0.23	1.60	1.00	—	1.66	0.28	0.95	1.99	—

* Precoated silica gel F_{254} plate (Merck) developed with *n*-butanol - acetic acid - water (4: 1: 2).
** Present in the oxidized form.

EDMAN degradation of the C-terminal fragment obtained by NBS-cleavage

Successive EDMAN degradation on the C-terminal octapeptide, described in the former section, was carried out by the same procedure used for tridecaptin A.[1] The results of EDMAN degradation (Table 3) showed the sequence to be Ser→Dab→Dab→(Ile, Val)→Glu→Val→(aIle, Val)→Ser.

Similarly the results of EDMAN degradation on the C-terminal fragment of tridecaptin C (Table 4) showed the sequence to be Ser→Dab→Dab→Phe→Glu→Val→(Val, aIle)→Ser.

Deacyl tridecaptin B

Tridecaptin B trihydrochloride (30 mg) was suspended in 0.05 M phosphate buffer (pH 7.5). Polymyxin acylase (6 mg) was added to the solution, which was stirred for 20 hours at 37°C. The reaction mixture was acidified to pH 3.0 with hydrochloric acid and centrifuged. The supernatant was lyophilized. The residue was subjected to paper chromatography on Toyo Roshi No. 51, developed with *n*-butanol - acetic acid - water (4: 1: 2). A ninhydrin positive zone with Rf 0.33 was extracted with water. Lyophilization of the extract gave colorless powder of deacyl tridecaptin B (8.1 mg).

Table 3. Results of EDMAN degradation of the C-terminal octapeptide from tridecaptin B.

	PTH-Amino acid	Amino acids found (in ratio)					
		Ser	Dab	Ile	Glu	Val	aIle
Original peptide		1.60	1.75	0.80	1.00	1.55	0.76
1st step	not detected	**1.12**	1.52	0.67	1.00	1.24	0.65
2nd step	γ-PTC-Dab*	0.96	**0.89**	0.63	1.00	1.20	0.63
3rd step	γ-PTC-Dab	0.93	**0.0**	0.71	1.00	1.33	0.66
4th step	Ile, Val	1.05	0.0	**0.15**	1.00	**1.21**	0.77
5th step	Glu	1.00	0.0	0.14	**0.34**	1.03	0.67
6th step	Val	1.00	0.0	0.12	0.34	**0.54**	0.63
7th step	aIle, Val	+**	—	—	—	—	—

* Phenylthiohydantoin of γ-phenylthiocarbamyl-2,4-diaminobutyric acid.
** Analyzed before hydrolysis.

Table 4. Results of EDMAN degradation on the C-terminal octapeptide from tridecaptin C.

	PTH-Amino acid	Amino acids found (in ratio)					
		Ser	Dab	Phe	Glu	Val	aIle
Original peptide		1.60	1.96	0.85	1.00	1.85	0.11
1st step	not detected	**0.90**	1.31	0.81	1.00	1.77	0.11
2nd step	γ-PTC-Dab	0.92	**0.76**	0.85	1.00	1.81	0.13
3rd step	γ-PTC-Dab	0.90	**0.13**	0.77	1.00	1.77	0.11
4th step	Phe	0.90	0.16	**0.14**	1.00	1.95	0.18
5th step	Glu	0.97	0.15	0.14	**0.52**	1.80	0.12
6th step	Val	1.00	0.13	0.14	0.49	**1.41**	0.11
7th step	Val, aIle	+*	—	—	—	—	—

* Analyzed before hydrolysis.

EDMAN degradation of deacyl tridecaptin B

Successive EDMAN degradation of deacyl tridecaptin B was carried out by the usual procedure[9]. The result showed the sequence before the Trp residue to be Gly→Dab→Gly→Ser→Trp.

Table 5. Results of EDMAN degradation on deacyl tridecaptin B.

	PTH-Amino acid	Amino acids found (in ratio)							
		Gly	Dab	Ser	Trp	Ile	Glu	Val	aIle
Original peptide		1.85	2.90	2.20	0.57	0.69	1.00	1.67	0.63
1st step	Gly	**1.05**	1.58	2.35	0.46	0.71	1.00	1.82	0.63
2nd step	γ-PTC-Dab	0.84	**1.16**	2.28	0.49	0.69	1.00	1.70	0.64
3rd step	Gly	**0.33**	1.13	2.27	0.57	0.73	1.00	1.69	0.68
4th step	trace Ser	0.27	1.20	**2.04**	0.43	0.72	1.00	1.68	0.65
5th step	Trp	0.28	1.11	1.82	**0.20**	0.62	1.00	1.64	0.61

Partial acid hydrolysis of tridecaptin C

Hydrochloric acid salt of tridecaptin C was dissolved in a mixture of formic acid and concentrated hydrochloric acid (1: 1). The solution was allowed to stand at 37°C for 20 hours and concentrated to dryness. When the residue was chromatographed on a silica gel plate with *n*-butanol -

Table 6. Peptide fragments obtained by partial acid hydrolysis of tridecaptin C.

Fragment	Rf value in TLC*			Amino acids found (in ratio)
	BAW	CEN	CEA	
a	0.70	—	—	Val
b	0.44	0.33	0.50	Val (1.31), Dab (1.00)
c	0.38	0.30	0.42	Val (3.36), Gly (1.00), Dab (1.00)
d	0.32	0.29	0.36	Ser

* Thin-layer chromatography on a precoated silica gel F_{254} plate (Merck).
BAW: *n*-butanol - acetic acid - water (4: 1: 2)
CEN: chloroform - ethanol - 14% ammoniacal water (4: 7: 2)
CEA: chloroform - ethanol - 10% aqueous acetic acid (4: 7: 2)

acetic acid - water (4: 1: 2) and sprayed with water, several spots were observed. Four lipophilic products were separated by thin-layer chromatography on a silica gel plate (Rf 0.70, 0.44, 0.38 and 0.32) and extracted with slightly acidified aqueous methanol. The extracts were concentrated to dryness and each residue was compared with those obtained from tridecaptin A for amino acid composition and chromatographic behavior.

The fragments obtained from tridecaptin C showed the same chromatographic behavior as those from tridecaptin A, in spite of the difference of fatty acyl residue. Fragment d has UV absorption ($\lambda_{max}^{0.01\,N\,HCl}$ 243 nm, 292 nm and 300 nm), presumably caused by partially decomposed Trp residue.

From these result fragment a was assumed to be FA→Val, fragment b to be FA→Val→Dab, fragment c to be FA→Val→Dab→Gly, and fragment d to be Ser→Trp* (*Trp was degraded during the hydrolysis).

Chirality of Dab and Ser residues of tridecaptin B

The N-terminal fragment of tridecaptin B obtained by NBS-cleavage was hydrolyzed and Dab^2 was isolated by paper chromatography. Approximately 540 mcg of $Dab^2 \cdot 2HCl$, which was estimated by the amino acid analyzer, was obtained.

Similarly from the C-terminal fragment, a mixture of Ser^6 and Ser^{13} (185 mcg) and a mixture of Dab^7 and Dab^8 (1.86 mg) were obtained. These isolated amino acids were subjected to CD measurement.

Dab^2 $[\Theta]_{260}$ 0 $[\Theta]_{210}$ −2600 $[\Theta]_{200}$ −2300 (*c* 0.108, 0.5 N HCl)
The mixture of Dab^7 and Dab^8 $[\Theta]_{260-200}$ 0 (*c* 0.186, 0.5 N HCl)
The mixture of Ser^6 and Ser^{13} $[\Theta]_{240}$ 0 $[\Theta]_{210}$ +3250 $[\Theta]_{205}$ +2840 (*c* 0.037, 0.5 N HCl)

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