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Synthesis and Activity of Cereulide, a Cyclic Dodecadepsipeptide Ionophore as Emetic Toxin from *Bacillus cereus*

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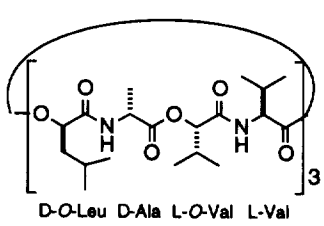
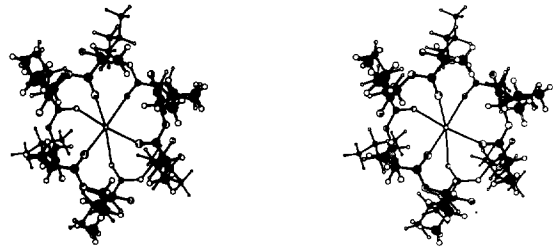
Abstract : Cereulide is the emetic toxin caused by Bacillus cereus as food contaminant. This cyclic depsipeptide was synthesized from L-O-Val, L-Val, L-O-Leu and D-Ala by coupling the former 2 and the latter 2, with inversion of the configuration of L-O-Leu into D-O-Leu, to form a tetrapeptide having TBDMS and benzyl protection. After selective deprotection of this tetrapeptide into the corresponding alcohol and acid, they were coupled twice and then transformed into a dodecapeptide (seco-acid) that was finally cyclized under high-dilution condition to afford cereulide. The final preparation showed, in fact, emetic toxicity as well as vacuole formation to HEp2 cells at the same level as the natural cereulide.

Cereulide (**1**) was first isolated by Agata *et al.* from *Bacillus cereus* as the producer of emetic toxin.[1] It is assigned as a heat resistant principle in the contamination of food poisonings. We have collaborated with Agata, Ohta *et al.*, and purified the toxic principle based on assay using vacuole formation in the HEp2 cell due to destruction of mitochondria.[2] One hundred microgram of cereulide (**1**) was isolated from 2L of culture broth, and the sample was analyzed by NMR and FAB-MS to elucidate a 36 membered dodecadepsipeptide.[3] The absolute stereochemistry of the 4 asymmetric centers in the 3-times repeated tetrapeptide was established as [L-*O*-Val-L-Val-D-*O*-Leu-D-Ala]$_3$ based on chemical degradation in *ultra*-microscale. Since we found **1** to be an ion as ion-selective ionophore, we have further established the higher structure of cereulide **1** as its complexation with K^+ ion. The main chain of this complex is a hexagonal cylinder-like framework, and all the side chains stick outside of this frame. The whole structure was assigned by NMR and molecular mechanic calculations and the result was similar to valinomycin, for which X-ray crystallographic analysis has established the higher structure.[4] Very recently, Uemura *et al.* reported cereulide and homo-cereulide from *Bacillus cereus*.[5] In the present paper we describe the chemical synthesis of cereulide (**1**), which includes alternative peptide and ester bonds, and the biological evaluation with the synthetic **1**.



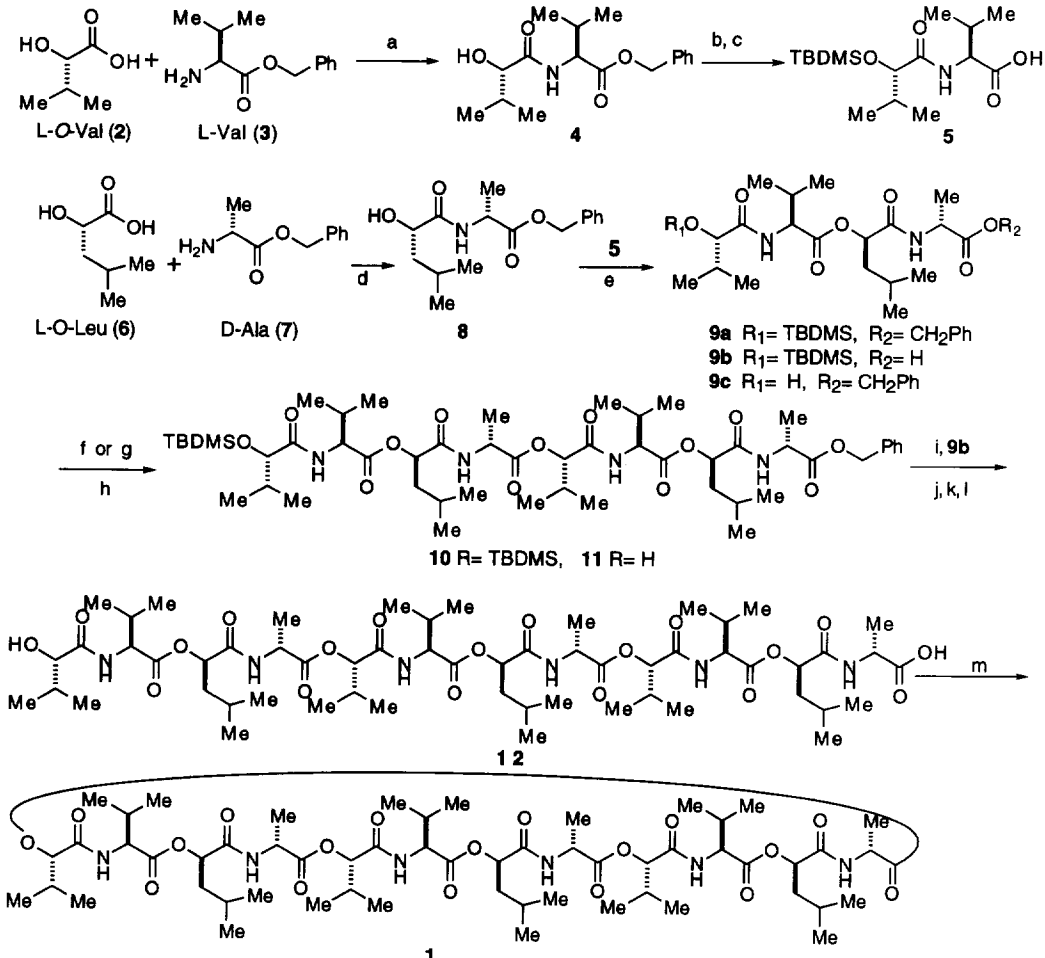
Cereulide 1



Stereo view of 1 with potassium ion[4]

Commercially available components for chemical synthesis of cereulide are limited to L-*O*-Val, L-Val and D-Ala, but D-*O*-Leu is not available. We employed a method to invert the configuration of *O*-Leu since L-*O*-Leu is commercially available.[6] Eventually we decided the route illustrated in **Scheme 1**; including this crucial inversion step in the tetrapeptide synthesis by Mitsunobu reaction[7] between the two segments TBDMS-[L-*O*-Val-L-Val]-OH (**5**) and H-[D-Ala-D-*O*-Leu]-OBn (**8**) having suitable protective groups as *tert*-butyldimethylsilyl and benzyl group, respectively. The tetrapeptide, L-*O*-Val-L-Val-D-*O*-Leu-D-Ala with two protective groups at the both ends (**9a**) was separated in 3 portions, and two portions were debenzylated by hydrogenolysis to give **9b** and one portion was desilylated with *tetra*-*n*-butylammonium fluoride to afford **9c**.

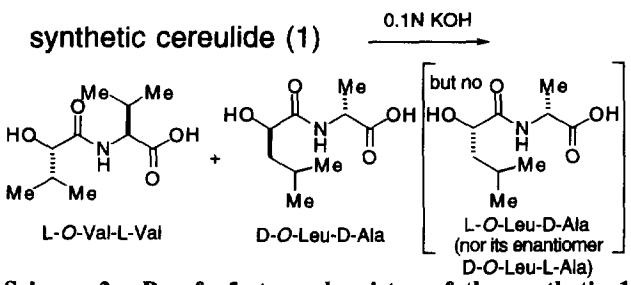
Scheme 1



(a) DCC (2 equiv.), HOBT (1.5 equiv.), THF, rt, 1 day; (b) TBDMSCl, imidazole; (c) H_2, Pd-C (98%, 3 steps); (d) same condition as (a) (81%); (e) DEAD, Ph_3P, THF, 0°C ~ rt, 6 hr (94%); (f) H_2, Pd-C, (99%); (g) TBAF, AcOH, CH_3CN (91%); (h) Et_3N, TCBC, DMAP, toluene (44%, corrected yield 73% based on recovered mass); (i) TBAF, AcOH, CH_3CN (87%); (j) Et_3N, TCBC, DMAP, toluene, 3 days (67%); (k) TBAF, AcOH, MeCN, rt, 5 days; (l) Pd-C H_2, EtOH, rt, 1 day (99%); (m) 3%, Et_3N, TCBC, KNO_3; then 0.05% DMAP, toluene, rt 1 day (40%)

Each one portion of these two peptides (**9b** and **9c**) with free alcohol and free carboxylic acid, respectively, were subjected to couple under Yamaguchi condition using trichlorobenzoic anhydride for activation to provide **10**. This octapeptide, after removing its TBDMS group, was coupled again with **9b** under the similar condition after Yamaguchi method to yield the dodecapeptide, from which two protective groups were removed to give the seco-acid **12**. The final cyclization was successfully achieved under high dilution condition at a concentration of 0.05% (w/v) to yield **1** in 40%.

The synthetic cereulide showed identical ^1H NMR (in $CDCl_3$ in the presence of K^+),[8] FAB-MS and HPLC with the natural sample. ^{13}C NMR data were also satisfactory.[9] Optical rotation of synthetic sample of **1** showed $[\alpha]_D$ -1.76°, while natural sample showed $[\alpha]_D$ -1.3°, that is considered to be identical.



Scheme 2. Proof of stereochemistry of the synthetic 1.
No racemized diastereoisomer greater than 3% of the dipeptides was detectable in above hydrolysate by HPLC as shown in Fig 1.

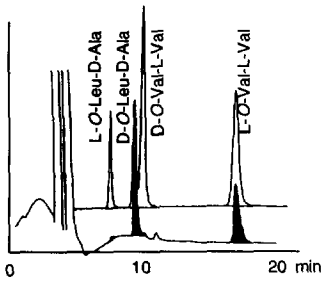


Fig. 1 HPLC of hydrolysate.
An ODS column (4 x 250 mm) 1 ml/min 21% CH_3CN-H_2O with 0.1% TFA

Racemization with the stereogenic centers was examined by chemical degradation of the final synthetic cereulide **1** under the same method that was employed for establishing the stereochemistry of natural sample. Namely, a solution of **1** (10 μg/ml) was hydrolyzed by addition of 20 μl of 0.1*N* KOH, while no starting **1** was detected by HPLC, and dipeptides were analyzed with HPLC as **Fig. 1**. Conclusion was that no racemization higher than 3% did not occur during these synthetic process.

Table 1. Emetic Effects of Cereulide in *Suncus murinus*

Emetics (Route)	Dose (μg/kg)	No. of suncus vomited	No. of vomiting episodes	Latency (min)
Natural	4	0 / 5	-	-
Cereulide	8	1 / 5	3	6
(p.o.)	**16**	3 / 5	5.3 ± 2.5	6.3 ± 1.5
	32	5 / 5	7.0 ± 1.9	4.4 ± 1.5
(i.p.)	2	0 / 3	-	-
	4	1 / 5	4	15
	8	2 / 5	6.8	5.7
	16	3 / 5	7.3 ± 1.5	5.3 ± 1.6
	32	5 / 5	8.4 ± 2.7	5.9 ± 4.0

Emetics (Route)	Dose (μg/kg)	No. of suncus vomited	No. of vomiting episodes	Latency (min)
Synthetic	-	-	-	-
Cereulide	8	0 / 5	-	6
(p.o.)	**16**	3 / 5	6.3 ± 3.5	8.3 ± 2.1
	32	5 / 5	7.6 ± 2.2	8.2 ± 1.1
(i.p.)	-	-	-	-
	4	0 / 5	-	-
	8	1 / 5	5	7
	16	5 / 5	10.3 ± 2.6	6.0 ± 2.2

The Vacuole Response in the HEp-2 Cells

Natural Cereulide (**1**)	5 ng/ml
Synthetic Cereulide (**1**)	3 ng/ml
C. Seco-Acid (**12**)	12000 ng/ml
SA Both ends protected	>20000 ng/ml

Bold numbers : $ED_{1/2}$
Values for the number of vomiting episodes and the latency per vomited animal are mean S.D., but actual values are indicated when the number of vomiting animals was less than three; -, No. suncus vomited at these doses.
The effect of ondansetron hydrochloride and surgical vagotomy against cereulide-induced emessis in *Suncus murinus*. Cereulide (50 mg/kg), 5 / 5; Ondansetron+Cereulide, 0 / 5.
For detail of assay system, see ref. # 2.

The toxicity of the synthetic cereulide is compared with the sample from natural origin, *B. cereus* in Table 1. The data of natural cereulide was taken from our previous paper[3] for comparison. Both of synthetic and natural cereulide (**1**) showed identical toxicity in the emetic effects in *Suncus murinus*. In the vacuole response in the HEp-2 cells both of the cereulide were as active to each other. Valinomycin shows almost identical nature as potassium ionophore, but it did not show emetic toxicity as compared cereulide as reported previously. Vacuole response of the seco-acid (**12**), the sample before the final cyclization, and its protected derivative were not active either. The selective toxicity of cereulide is of quite interesting to the low toxicity (1/60) of valinomycin, although they have similar potassium ionophore nature that might disturb the ion potential of the organelle.[2]

The current achievements of the total synthesis of cereulide demonstrated that (1) cereulide is in fact the emetic toxin, (2) synthesis provides enough material for mechanistic studies of the toxicity and ionophoric nature and (3) the synthetic methods could allow other synthesis of analogous compounds useful for biological studies.

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6. L-*O*-Leu = (-)-2-Hydroxyisocaproic acid (98%) [namely L-leucic acid or 2-hydroxy-4-methylvaleric acid from Aldrich # 21,982-7]; L-*O*-Val = (-)-2-Hydroxyisovaleric acid (99%) [namely L-varic acid or 2-hydroxy-3-methylburyric acid from Aldrich # 37,909-3] were used as the starting material for the synthesis of cereulide.
7. Mitsunobu, O. *Synthesis*, **1981**, 1-28.
8. Data of Synthetic Cereulide (**1**): ^{1}H NMR ($CDCl_3$) δ 0.89 (d, *J*= 6.3 Hz), 0.95 (d, *J*= 6.3 Hz), 0.97 (d, *J*= 5.8 Hz), 1.01 (d, *J*= 7.0 Hz), 1.03 (d, *J*= 7.0 Hz), 1.14 (d, *J*= 6.3 Hz), 1.49 (d, *J*= 7.1 Hz), 1.66-1.72 (m, 9H), 2.18-2.38 (m, 6H), 3.83 (dd, *J*= 11.0, 5.1 Hz, 3H), 4.29 (qd, *J*= 7.1, 4.1 Hz), 4.63 (d,*J*= 3.0 Hz), 4.78 (dd,*J*= 10.0, 3.5 Hz), 8.29 (d,*J*= 5.1 Hz), 8.39 (d, *J*= 4.1 Hz). ^{13}C NMR ($CDCl_3$) δ 15.2, 16.6, 18.6, 19.2, 20.2, 21.1, 23.1, 24.3, 28.5, 30.2, 40.8, 50.3, 61.8, 73.7, 79.8, 171.5, 172,3, 175.8, 176.3. IR (KBr) ν 1743, 1653, 1508 /cm. HRMS found 1192.6420; calcd 1191.6418 as K^+ form.

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