Facile Synthesis of AM-Toxin II

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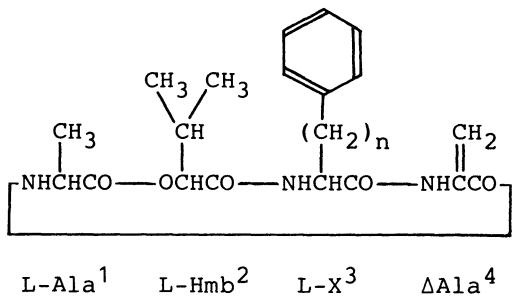
In a search for a more effective synthetic route for AM-toxins (cyclic tetradepsipeptides), the syntheses of [L-Phe³]AM-toxin II as a preliminary peptide and AM-toxin II as a target were attempted. The cyclization of H-L-Ser(Bzl)-L-Ala-L-Hmb-L-Phe-ONSu (Hmb, 2-hydroxy-3-methylbutanoic acid) in pyridine gave a cyclic monomer in high yield (59%). The removal of *O*-Bzl in the cyclopeptide and a subsequent dehydration of Ser with carbodiimide-CuCl afforded [L-Phe³]AM-toxin II in 24% yield. AM-Toxin II was synthesized in similar yield as in the case of the analog. The characteristic features of synthetic AM-toxin II were identical to those of the natural one.

AM-Toxins are host-specific phytotoxins and cause a spot disease on apple leaves. The structures of three congeners (I, II, and III) of AM-toxins were determined to be cyclotetradepsipeptides, the structure of the II being shown in Fig. 1. AM-Toxin I and III contain L-Amp and L-Ahp, respectively, instead of L-App residue at position 3.[1] We have already synthesized three congeners in order to confirm their structures, and also various analogs to clarify the structure-activity relationship. However, the synthetic yield has still been unsatisfactory from the standpoints of two crucial steps involving preferential monomeric cyclization from linear tetrapeptides and effective ΔAla formation in cyclotetrapeptide. Therefore, it has been our continuing interest to search for more facile synthetic routes for AM-toxins.

We previously found that the cyclization of a linear tetrapepetide containing an L-Ser residue at N-terminus gave a considerable amount of cyclic polymers in addition to the desired cyclic monomer (see, Table 1),[2,3] while a linear peptide with a D-Ser residue gave a cyclic monomer exclusively.[2] However, the subsequent conversion of the D-Ser residue in cyclotetrapeptide into ΔAla with Tos-Cl, followed by Et_2NH (Photaki method), was impossible because of a steric hindrance of the D-Ser (see, Table 2).[2] On the contrary, the L-Ser in cyclopeptide was converted into ΔAla, even though yields were poor (15% and 2% in Table 2).[3,4]

In the present study, we first attempted an easier



	n	X
AM-Toxin II	3	App
[L-Phe³]AM-Toxin II	1	Phe

Fig. 1. Structure of AM-toxin II and [L-Phe³]AM-toxin II.

Table 1. Formation of Cyclic Monomer and Cyclic Polymers after Cyclization of Linear Tetrapeptide Active Ester

Linear tetrapeptide active ester[a]	Formation of cyclic peptides		Ref.
	Yield(%)[b] of cyclic monomer	Formation of cyclic polymers	
H-Ser-Ala-Hmb-Phe-ONSu	11	+++	2
H-Ser-Ala-Hmb-Amp-ONSu	18	+++	3
H-D-Ser-Ala-Hmb-Phe-ONSu	52	−	2
H-D-Ser-Ala-Hmb-App-ONSu	51	−	2
H-Ser(Bzl)-Ala-Hmb-Phe-ONSu	59	±	This study
H-Ser(Bzl)-Ala-Hmb-App-ONSu	57	±	This study
H-D-Dpr(Z)-Ala-Hmb-Amp-ONSu	57	−	9

a) Initial concentration, 3 mM in pyridine; reaction time, 2 d at 20 °C. b) Isolated yield based on Boc-tetradepsipeptide-ONSu.

Table 2. ΔAla Formation from Ser or Dpr Residue in Cyclic Tetrapeptide

Cyclic tetrapeptide	Method[a]	Product	Yield(%)[b] of the product	Ref.
Cyclo(-Ser-Ala-Hmb-Tyr(Me)-)	Photaki	[L-Tyr(Me)^3]AM-toxin I	15	4
Cyclo(-Ser-Ala-Hmb-Amp-)	Photaki	AM-toxin I	2	3
Cyclo(-D-Ser-Ala-Hmb-Phe-)	Photaki	[L-Phe^3]AM-toxin II	trace	2
ditto	Modified Photaki	ditto	4	
Cyclo(-D-Ser-Ala-Hmb-App-)	Photaki	AM-toxin II	trace	2
ditto	Modified Photaki	ditto	6	
Cyclo(-Ser-Ala-Hmb-Phe-)	Photaki	[L-Phe^3]AM-toxin II	6.5	This study
ditto	Miller	ditto	24	This study
Cyclo(-Ser-Ala-Hmb-App-)	Miller	AM-toxin II	20	This study
Cyclo(-D-Dpr-Ala-Hmb-Amp-)	Shiba	AM-toxin I	26	9

a) Photaki method, Tos-Cl and Et_2NH; Shiba method, CH_3I and $KHCO_3$; Miller method, EDC·HCl and CuCl; modified Photaki method, Ms-Cl and Et_2NH.

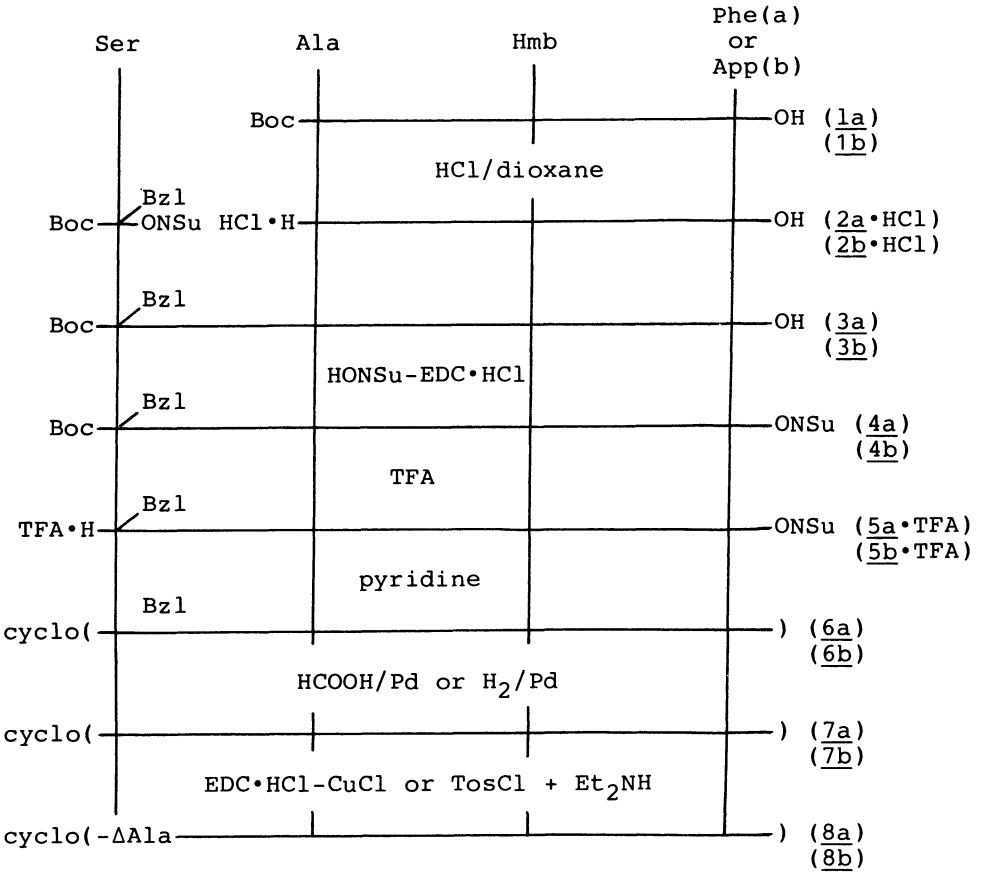


Fig. 2. Synthesis of [L-Phe^3]AM-toxin II (**8a**) and AM-toxin II (**8b**).

synthesis of cyclopeptide with the L-Ser residue from a linear peptide precursor. In a peptide synthesis, there is a tendency that a peptide with an L-Ser(Bzl) residue is obtained as a better crystalline product than a peptide with L-Ser. We therefore chose H-Ser(Bzl)-Ala-Hmb-Phe-ONSu (**5a**) as a preliminary precursor, the synthetic route of **5a** being shown in Fig. 2. Then, the cyclization of **5a** was carried out in pyridine at a concentration of 3 mM (1 M=1 mol dm^{-3}) at 20 °C for 2 d. Unexpectedly, the TLC of the reaction mixture showed the presence of a major cyclic monomer (**6a**) and a trace cyclic polymer. Indeed, an isolated solid was almost **6a**; and pure **6a** was obtained in good yield (59%) after simple recrystallization. The results indicate that the cyclization-mode of a linear tetrapeptide precursor is influenced by not only the configuration, but also the side-chain nature of the N-terminal amino acid residue in the precursor. For the synthesis of AM-toxin II, the cyclization of **5b** with L-App was carried out similarly in pyridine, and pure

6b was obtained in 57% yield.

The removal of the *O*-Bzl group in **6a** and **6b** was achieved by catalytic transfer hydrogenation[5] using Pd black and formic acid in almost quantitative yield after 2 h to produce the desired **7a** and **7b**. The **6a** was subjected to conventional hydrogenation in a solvent of DMF with Pd black; **7a** was obtained after 7d in 76% yield.

Secondly we attempted an easier formation of the ΔAla residue from L-Ser in **7a**. Recently, Miller reported an efficient one-pot dehydration of the Ser derivative to form the ΔAla derivative using carbodiimide and CuCl.[6] Thus, we examined the applicability of the Miller method for the synthesis of [L-Phe^3]AM-toxin II (**8a**) from cyclotetrapeptide (**7a**) containing the L-Ser residue. The **7a** was treated with hydrochloride of water-soluble carbodiimide (EDC·HCl) in the presence of CuCl. In this case, more reagents and a longer time than reported in the literature[6] were needed for the completion of the reaction; however, we could obtained pure **8a** in 24% yield. When the Photaki method[7] was applied to **7a**, the yield of pure **8a** was 6.5%. We applied the procedure of the Miller method for **7b**, and could isolate pure AM-toxin II (**8b**) in 20% yield. The physicochemical properties of **8b** were identical to those reported for natural AM-toxin II, and the minimum toxic activity of the **8b** and natural II was the same for the induction of necrosis on apple leaves.

For the syntheses of natural AM-toxins and their analogs, a route using the D-Dpr residue[8] has been giving the best yield; the yields for the cyclization of the linear peptide and dehydration in cyclotetrapeptide are approximately 57%[9] (Table 1) and 26%[9] (Table 2), respectively. Yields in the present study are comparable with those in the D-Dpr route; it should be further noted that the preparation of an amino acid, L-Ser(Bzl), is easier than that of D-Dpr(Z). Thus, we believe that the present study gives a facile method for synthesizing AM-toxin II and other related cyclotetrapeptides.

Experimental

Optical rotations were measured with a JASCO polarimeter Model DIP-140. Mass spectra were taken on a Jeol mass spectrometer Model JMS-01SG operating at 75 eV. IR spectra were recorded with KBr disks on a JASCO IRA-2 spectrophotometer. UV spectra were taken on a Hitachi 124 spectrophotometer. TLC was performed on silica gel 60 F_{254} pre-coated plates (Merck) with the following solvent systems: R_f^1, $CHCl_3$–MeOH (5:1); R_f^2, $CHCl_3$–MeOH–AcOH (95:5:1); R_f^3, *n*-BuOH–AcOH–H_2O (4:1:1); R_f^4, *n*-BuOH–AcOH–pyridine–H_2O (15:3:10:12); R_f^5, EtOAc–$CHCl_3$ (1:1).

Boc-Ala-Hmb-Phe-OH (1a). Boc-Ala-Hmb-Phe-OBzl (5.27 g, 10 mmol)[2] dissolved in THF (20 ml) was hydrogenated in the presence of Pd black at 20 °C for 5 h. After removal of the catalyst, the filtrate was evaporated in vacuo and the residual oil was solidified by the addition of hexane. The resulting solid was recrystallized from EtOAc-ether-hexane: yield, 3.99 g (91%); mp 145 °C; $[\alpha]_D^{25}$ −6.9° (*c* 1, $CHCl_3$); R_f^1 0.49, R_f^2 0.33.

Found: C, 60.58; H, 7.37; N, 6.40%. Calcd for $C_{22}H_{32}O_7N_2$: C, 60.53; H, 7.39; N, 6.42%.

Boc-Ala-Hmb-App-OH (1b). Boc-Ala-Hmb-ONSu (2.07 g, 5.4 mmol)[10] was added to a suspension of H-L-App-OH (1.14 g, 5.9 mmol)[11] and Et_3N (0.83 ml, 5.9 mmol) in DMF (40 ml) at 0 °C. The mixture was stirred at 4 °C for 24 h, and the filtrate was evaporated. The residue was dissolved in EtOAc (80 ml) and washed with 10% citric acid and water, and dried over $MgSO_4$. After evaporation, the residual oil was solidified by the addition of petroleum ether, and the resulting solid was recrystallized from EtOAc–ether–petroleum ether: yield, 2.10 g (84%); mp 113 °C; $[\alpha]_D^{20}$ −10.2° (*c* 1, $CHCl_3$); R_f^1 0.56.

Found: C, 61.84; H, 7.81; N, 6.00%. Calcd for $C_{24}H_{36}O_7N_2$: C, 62.05; H, 7.81; N, 6.03%.

H-Ala-Hmb-Phe-OH·HCl (2a·HCl). Compound **1a** (3.49 g, 8 mmol) was dissolved in dioxane (13 ml) and treated with 6 M HCl in dioxane (13.3 ml, 80 mmol) at 20 °C for 3 h. The solvent was evaporated, and the residual solid was triturated with ether and collected: yield, 2.96 g (99%); mp 206—208 °C; $[\alpha]_D^{25}$ −18.5° (*c* 1, DMF); R_f^3 0.33.

H-Ala-Hmb-App-OH·HCl (2b·HCl). This compound was prepared from **1b** (1.86 g, 4 mmol) as described for **2a**·HCl: yield, 1.60 g (100%); mp 198—199 °C; $[\alpha]_D^{25}$ −17.3° (*c* 1, DMF); R_f^3 0.38.

Boc-Ser(Bzl)-Ala-Hmb-Phe-OH (3a). Boc-L-Ser(Bzl)-ONSu (2.88 g, 7.4 mmol)[12] was added to a solution of **2a**·HCl (2.61 g, 7 mmol) and Et_3N (1.96 ml, 14 mmol) in DMF (30 ml) at 0 °C. The mixture was stirred at 0 °C for 1 h and at 20 °C overnight. The mixture was treated as described for **1a**: yield, 4.20 g (98%); mp 103 °C; $[\alpha]_D^{25}$ −4.3° (*c* 1, $CHCl_3$); R_f^1 0.43.

Found: C, 62.40; H, 6.99; N, 6.79%. Calcd for $C_{32}H_{43}O_9N_3$: C, 62.62; H, 7.06; N, 6.85%.

Boc-Ser(Bzl)-Ala-Hmb-App-OH (3b). This compound was prepared from Boc-L-Ser(Bzl)-ONSu (1.57 g, 4 mmol) and **2b**·HCl (1.73 g, 4.3 mmol) as described for **3a**: yield, 2.07 g (81%); mp 114 °C; $[\alpha]_D^{20}$ −0.4° (*c* 1, $CHCl_3$); R_f^1 0.48.

Found: C, 62.79; H, 7.37; N, 6.64%. Calcd for $C_{34}H_{47}O_9N_3 \cdot 1/2H_2O$: C, 62.75; H, 7.44; N, 6.46%.

Boc-Ser(Bzl)-Ala-Hmb-Phe-ONSu (4a). To a stirred solution of **3a** (3.68 g, 6 mmol) and HONSu (1.04 g, 9 mmol) in DMF was added EDC·HCl (1.50 g, 7.8 mmol) at 0 °C. The mixture was stirred at 4 °C for 24 h. After evaporation, the residue was solidified by the addition of cold water and dried in vacuo over P_2O_5: yield, 4.30 g (100%); R_f^1 0.83, R_f^2 0.37. This was used to the next step without further purification.

Boc-Ser(Bzl)-Ala-Hmb-App-ONSu (4b). This compound was prepared from **3b** (1.60 g, 2.5 mmol) as described for **4a**: yield, 1.85 g (100%); R_f^1 0.73.

Cyclo(-Ala-Hmb-Phe-Ser(Bzl)-) (6a). A solution of **4a** (711 mg, 1 mmol) in TFA (4 ml) was allowed to stand at 0 °C for 30 min. After evaporation, the residual oil was solidified by the addition of ether to afford H-Ser(Bzl)-Ala-Hmb-Phe-ONSu·TFA (**5a**·TFA). This was dissolved in DMF (13 ml) and the solution was added dropwise into dry pyridine (320 ml) with stirring at 20 °C. The stirring was

continued at 20 °C for 2 d and the mixture was evaporated. The resulting solid was collected with the aid of cold water, washed with 10% citric acid and dried over P_2O_5. This was recrystallized from DMF-EtOAc: yield, 292 mg (59% from **4a**); mp 288—289 °C (decomp); $[\alpha]_D^{25}$ −140° (*c* 0.5, DMF); MS, *m/z* 495 (M^+); R_f^1 0.79, R_f^2 0.24.

Found: C, 64.43; H, 6.73; N, 8.49%. Calcd for $C_{27}H_{33}O_6N_3 \cdot 1/2H_2O$: C, 64.27; H, 6.79; N, 8.33%.

Cyclo(-Ala-Hmb-App-Ser(Bzl)-) (6b). This compound was prepared from **4b** (739 mg, 1 mmol) as described for **6a**: yield, 299 mg (57% from **4b**); mp 233—234 °C (decomp); $[\alpha]_D^{20}$ −187° (*c* 0.5, DMF); MS, *m/z* 523 (M^+); R_f^1 0.80, R_f^2 0.32.

Found: C, 65.49; H, 7.15; N, 7.97%. Calcd for $C_{29}H_{37}O_6N_3 \cdot 1/2H_2O$: C, 65.39; H, 7.19; N, 7.89%.

Cyclo(-Ala-Hmb-Phe-Ser-) (7a). This compound was prepared by catalytic transfer hydrogenation as follows. To a stirred solution of **6a** (130 mg, 0.26 mmol) in DMF (10 ml) were added Pd black and HCOOH (0.5 ml) at 20 °C. The stirring was continued at 20 °C for 2 h. The filtrate was evaporated and the residual solid was recrystallized from DMF-EtOAc: yield, 103 mg (97%); mp 219 °C; $[\alpha]_D^{25}$ −221° (*c* 0.5, DMF); MS, *m/z* 405 (M^+); R_f^1 0.31, R_f^2 0.10.

Found: C, 57.04; H, 6.58; N, 10.19%. Calcd for $C_{20}H_{27}O_6N_3 \cdot 4/5H_2O$: C, 57.21; H, 6.87; N, 10.01%.

The same compound was obtained by conventional hydrogenation. Compound **6a** (53 mg, 0.11 mmol) in DMF (4 ml) was hydrogenated in the presence of Pd black at 20 °C for 7 d. The filtrate was treated as above: yield, 34 mg (76%); mp 218 °C; $[\alpha]_D^{25}$ −220° (*c* 0.5, DMF); R_f^1 0.31, R_f^2 0.10.

Cyclo(-Ala-Hmb-App-Ser-) (7b). This compound was prepared from **6b** (236 mg, 0.45 mmol) by catalytic transfer hydrogenation as described for **7a**: yield, 182 mg (93%); mp 199—200 °C; $[\alpha]_D^{20}$ −248° (*c* 0.5, DMF); MS, *m/z* 433 (M^+); R_f^1 0.44.

Found: C, 58.99; H, 7.13; N, 9.48%. Calcd for $C_{22}H_{31}O_6N_3 \cdot 4/5H_2O$: C, 58.99; H, 7.34; N, 9.38%.

Cyclo(-Ala-Hmb-Phe-ΔAla-) ([L-Phe³]AM-toxin II, 8a). This compound was prepared by Miller method as follows. To a suspension of **7a** (41 mg, 0.1 mmol) in CH_2Cl_2 (10 ml) were added CuCl (5 mg, 0.05 mmol) and EDC·HCl (38 mg, 0.2 mmol). The mixture was refluxed with stirring in the dark. After 2 d, the same amounts of CuCl and EDC·HCl were added, the stirring being continued for further 4 d. Then, the solution was evaporated, and the residue was dissolved in $CHCl_3$ (3 ml). The solution was applied to a silica-gel column (0.9×9 cm, Wako silica gel C-200, 100—200 mesh) and eluted with a solvent of $CHCl_3$–acetone (4:1). The fractions (9—42 ml) were evaporated and the residual solid was recrystallized from EtOAc–petroleum ether: yield, 9.3 mg (24%); mp 230—232 °C; MS, *m/z* 387 (M^+); IR (KBr) 1740, 1655, 1630, 1520, 1040 cm^{-1}; R_f^2 0.24, R_f^3 0.81, R_f^4 0.86, R_f^5 0.24. Reported value,[2] mp 231—232 °C.

Found: C, 61.12; H, 6.65; N, 10.65%. Calcd for $C_{20}H_{25}O_5N_3 \cdot 1/3H_2O$: C, 61.05; H, 6.58; N, 10.68%.

Same **8a** was prepared by the Photaki method as follows. To a chilled solution of **7a** (80 mg, 0.2 mmol) in pyridine (2 ml) was added Tos-Cl (76 mg, 0.4 mmol) at 0 °C. After 24 h, the solution was evaporated and the solid was collected by the aid of cold water. The solid was washed with a mixture of ether–petroleum ether to remove excess Tos-Cl: yield of cyclo(-Ala-Hmb-Phe-Ser(Tos)-), 105 mg (94%); R_f^5 0.64. This tosylated cyclopeptide (105 mg, 0.19 mmol) in DMF (1 ml) was treated with 1 M Et_2NH in dioxane (0.38 ml, 0.38 mmol) at 20 °C. After 6 h the solution was evaporated and the solid was collected with cold water. The solid was suspended in EtOAc (4 ml), and the filtrate was subjected to purification by preparative TLC on silica gel 60 F_{254} precoated plate (layer thickness: 2 mm, Merck) using a solvent EtOAc–$CHCl_3$ (1:1). A portion of R_f^5 0.16 on the plate was extracted with EtOAc, evaporated and the solid was recrystallized from EtOAc–ether–petroleum ether: yield, 4.7 mg (6.5% from **7a**); mp 228—230 °C; R_f^2 0.24, R_f^4 0.86.

Cyclo(-Ala-Hmb-App-ΔAla-) (AM-toxin II, 8b). This compound was prepared from **7b** (43 mg, 0.1 mmol) by the procedure of Miller as described for **8a**: yield, 8.2 mg (20%); mp 212—213 °C; MS, *m/z* 415 (M^+); IR (KBr) 1656, 1630, 1519, 1040 cm^{-1}; UV λ_{max} (MeOH) 202 nm (ε 5500), 208 (11300), 224 (6000), 267 (800); R_f^2 0.30, R_f^3 0.83, R_f^4 0.87, R_f^5 0.30. Reported values; mp 213—214 °C,[13] 212—213 °C.[2]

Bioassays. Bioassays on apple leaves (susceptible cultivar, Indo) was carried out as described in the literature.[13] Compound **8b** and natural AM-toxin II possessed the same minimum toxic activity for the induction of necrosis at 0.02 μg ml^{-1}, where [L-Phe³]AM-toxin II (**8a**) showed the activity at 20 μg ml^{-1} level.

References

1) Abbreviations: Ahp, 2-amino-5-(*p*-hydroxyphenyl)-pentanoic acid; Amp, 2-amino-5-(*p*-methoxyphenyl)pentanoic acid; App, 2-amino-5-phenylpentanoic acid; Bzl, benzyl; ΔAla, 2,3-dehydroalanine; DMF, *N,N*-dimethylformamide; Dpr(Z), 3-benzyloxycarbonyl-2,3-diaminopropanoic acid; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; Hmb, 2-hydroxy-3-methylbutanoic acid; HONSu, *N*-hydroxysuccinimide; Ms-Cl, methanesulfonyl chloride; TFA, trifluoroacetic acid; THF, tetrahydrofuran; Tos-Cl, *p*-toluenesulfonyl chloride. Amino acid symbols except D-amino acids denote the L-configuration unless otherwise indicated.

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