ISOLATION OF (4*R*)-4-[(*E*)-2-BUTENYL]-4-METHYL-L-THREONINE, THE CHARACTERISTIC STRUCTURAL ELEMENT OF CYCLOSPORINS, FROM A BLOCKED MUTANT OF *TOLYPOCLADIUM INFLATUM*†

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By mutagenic treatment of a strain of *Tolypocladium inflatum*, a cyclosporin non-producing mutant was obtained which accumulated the characteristic building unit of cyclosporins, (4*R*)-4-[(*E*)-2-butenyl]-4-methyl-L-threonine (abbreviation Bmt; systematic name: (2*S*,3*R*,4*R*,6*E*)-2-amino-3-hydroxy-4-methyl-6-octenoic acid) in free form. The isolation from a culture filtrate was performed by extraction, chromatographic separation and final crystallization from methanol - water. The structure and stereochemistry of this amino acid was determined by chemical transformation and correlation to dihydro-MeBmt, with known chirality [(2*S*,3*R*,4*R*)-3-hydroxy-4-methyl-2-methylamino-octanoic acid], obtained by hydrolysis of dihydrocyclosporin A.

The fungus *Tolypocladium inflatum* W. Gams produces the cyclosporins, a group of neutral cyclic oligopeptides composed of 11 amino acids, which exhibit remarkable biological effects[1]. Cyclosporin A (Fig. 1), the main metabolite, represents a potent, specific immunosuppressant (Sandimmun)[2,3]. Cyclosporins show also activity against filamentous fungi. Characteristic structural features of natural cyclosporins consist in the presence of a unique unsaturated β-hydroxy-α-amino acid with a C_9-skeleton, the *N*-methylation of several amino acids and the occurrence of a D-alanine unit in the molecule[4]. The biosynthesis of cyclosporins proceeds on a multienzyme thiotemplate complex[5].

The "C_9-amino acid" has the structure (2*S*,3*R*,4*R*,6*E*)-3-hydroxy-4-methyl-2-methylamino-6-octenoic acid, designated as (4*R*)-4-[(*E*)-2-butenyl]-4,*N*-dimethyl-L-threonine (abbreviation MeBmt) according to IUPAC/IUB rules. Feeding experiments with [^{13}C]acetate, labeled either at the methyl or the carboxyl group, demonstrated that the carbon skeleton of the amino acid MeBmt is built up by head-to-tail coupling of four acetate units whereas the *C*-methyl in position 4 and the *N*-methyl group originate from methionine[6].

The presence of MeBmt in the cyclosporin molecule is essential for high immunosuppressive activity; *per se* MeBmt and its *N*-demethylated derivative, Bmt, possess neither an antifungal nor an immunological effect. Several laborious procedures for the total synthesis of MeBmt[7~11] and Bmt (P. BOLLINGER (Sandoz Ltd.), unpublished results) have been elaborated. In culture broths of a wild-type strain *T. inflatum* (NRRL 8044) and of subsequent high producing mutants, free Bmt could not be detected. Recently, the isolation of *N*-acetyl-Bmt, beside cyclosporin A, from *Neocosmospora vasinfecta* has been described[12].

By hydrolytic cleavage of cyclosporin A with 6 N hydrochloric acid, MeBmt was not obtained in genuine form but as a cyclic artifact[2]. It is however possible to isolate dihydro-MeBmt from the hydrolysis

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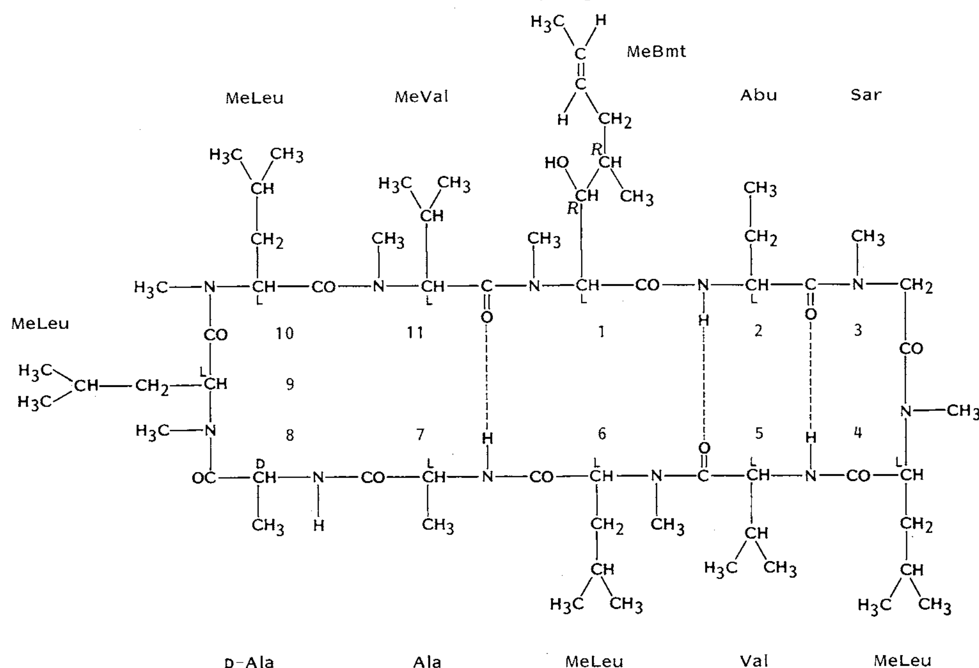
Fig. 1. Structure of cyclosporin A.



products of dihydrocyclosporin A. Only recently, intact MeBmt has become available by mild cleavage reaction of cyclosporin A[13].

The goal of this project was to obtain mutants able to produce Bmt and to characterize the chemical features of this key amino acid. As strategy, mutants devoid of antifungal activity, and therefore blocked in cyclosporin biosynthesis, were selected and hereafter tested for Bmt production.

Materials and Methods

Strains

Cyclosporin-producer: The *T. inflatum* strain Cyb 156, an improved high producing mutant originated from a wild strain (NRRL 8044) was used as parental strain. The propagation was performed on agar medium SA-1 at 27°C for 14 days.

Test Organism: *Aspergillus niger* strain S7281, cultivated on agar medium SA-1 at 27°C, was employed for detection of the antifungal activity of cyclosporins. The sensitivity to cyclosporin A was 10 μg/ml.

Media

Agar Medium SA-1: Malt extract (liquid, Wander)20 g, yeast extract (Gistex) 4 g, Bacto agar (Difco) 20 g, demineralized water up to 1 liter, pH 5.6~5.8; sterilization: 20 minutes at 120°C.

Agar Medium SA-2: Sucrose 20 g, ammonium malate 3.5 g, KH_2PO_4 0.35 g, $MgSO_4 \cdot 7H_2O$ 0.20 g, $CaCl_2 \cdot 6H_2O$ 0.10 g, $FeSO_4 \cdot 7H_2O$ 5.5 mg, $ZnSO_4 \cdot 7H_2O$ 4 mg, $MnCl_2 \cdot 4H_2O$ 0.2 mg, $CuSO_4 \cdot 7H_2O$ 0.08 mg, $(NH_4)_6\ Mo_7O_{24} \cdot 4H_2O$ 0.02 mg, Bacto agar 20 g, demineralized water up to 1 liter, pH 5.0; sterilization: 20 minutes at 120°C.

Liquid Medium L-1: Malt extract (liquid, Wander) 20 g, yeast extract (Gistex) 4 g, demineralized water up to 1 liter, pH 5.6; sterilization: 20 minutes at 120°C.

Liquid Medium L-2: Fructose 75 g, casein (Amber EHC) 25 g, KH_2PO_4 5 g, KCl 2.5 g, demineralized water up to 1 liter, pH 5.5; sterilization: 20 minutes at 120°C.

Protective Medium GG: Glycerol 20 g, gelatine 10 g, peptone 5 g, demineralized water up to 1 liter;

sterilization: 20 minutes at 120°C.

Mutagenic Treatments

UV-Light: A 10^6/ml-suspension of spores in a 0.05-M Tris-maleate buffer (pH 6.0) containing 100 mg/liter of sodium lauryl sulfate was dispersed into a glass Petri dish and irradiated at 254 nm with a UV lamp placed 30 cm above the surface of the liquid for 30 seconds to 4 minutes.

N-Methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG): 10^7 spores/ml were suspended in a 0.05-M Tris-maleate buffer (pH 6.0) containing 100 mg/liter of sodium lauryl sulfate and 150 mg/liter of MNNG. The suspension was incubated at 27°C on a rotary shaker for 1 to 4 hours. After this mutagenic treatment, the suspension of spores was diluted in a 0.05-M Tris-maleate buffer (pH 6.0), spread on agar medium SA-2 and incubated at 27°C for 3 weeks.

Selection and Cultivation of Strains

Preselection on Agar: Colonies were suspended in 2.5 ml of a protective medium (GG). Agar medium SA-2 was poured into mini-wells (2.5 ml, Linbro Flow Lab.) and each well was inoculated with 100 µl of a colony suspension. The cultures were incubated for 3 weeks at 27°C. The agar pieces were extracted with methanol, and the extracts tested for activity against *A. niger*.

Selection in Liquid Culture: Slants of agar medium SA-1 were prepared with the suspension of the colonies devoid of antifungal activity and incubated for 2 weeks at 27°C. The conidia were suspended in 10 ml of saline. Seed medium L-1 (200 ml in 500-ml Erlenmeyer flasks) was inoculated with 3 ml of the suspension and incubated at 27°C on a rotary shaker (200 rpm) for 120 hours. A 100-ml of production medium L-2 in 500-ml Erlenmeyer flasks was inoculated with 10 ml of 5-day old seed culture. This main culture was incubated at 27°C on a rotary shaker (200 rpm) for 8~12 days. The contents of cyclosporins and of Bmt in the broths were determined by HPLC and TLC.

Analytical Methods

Determination of Cyclosporin Content: A 10-ml portion of culture broth was mixed with 20 ml methanol under agitation for 1 hour. After centrifugation, an aliquot of the supernatant was analyzed by HPLC (stationary phase: LiChrosorb RP8, mobile phase: acetonitrile - water - *ortho*-phosphoric acid 630 : 370 : 0.1; flow-rate: 2.4 ml/minute, column temperature: 75°C, detection at 210 nm).

Determination of Bmt: 20 ml of culture filtrate were extracted twice with 20 ml butanol and evaporated under vaccuum.

TLC: The extract was suspended in 2 ml of the TLC solvent system chloroform - methanol - water - acetic acid (60 : 30 : 5 : 5). 10 µl were spotted onto a Silica gel plate 60 F_{254} (Merck) and developed with the same solvent for 1 hour. The amino acid spots were visualized with ninhydrin reagent. Chemically synthesized Bmt was used as standard.

HPLC: 100 µl of a sample (with a Bmt content between 5 and 50 µg/ml) were mixed with 400 µl of a *ortho*-phthaldialdehyde/mercaptoethanol solution for derivatization (dissolve 25 mg of *ortho*-phthaldialdehyde in 0.8 ml methanol, add 20 ml of 0.4 M sodium borate buffer (pH 10) and 50 µl of mercaptoethanol). After 10 minutes, 50 to 100 µl of the solution were subjected to HPLC (column: Knauer, Spherisorb ODS II 3 µm, 4 × 120 mm; phase A: 0.05 M Na_2HPO_4, pH 6.5; phase B: phase A - methanol (35 : 65); gradient: 75% B to 100% B in 10 minutes, flow 1.4 ml/minute; detection by fluorescence: excitation 344 nm, emission 443 nm; internal control: 20 µg/ml Bmt in methanol).

Results

Selection of Bmt Producers

In medium L-2, strain Cyb 156 produced 500~700 mg per liter of cyclosporins (main component; cyclosporin A).

After 2 minutes of irradiation with UV the survival rate of conidia of strain Cyb 156 was *ca.* 4%; 615 colonies were isolated. By a 4-hour treatment with MNNG, 815 colonies were isolated, corresponding

to a survival rate of about 2%. Among these 1,430 strains cultivated on agar medium SA-2, 49 strains (4 from the UV light treatment and 45 from the MNNG treatment) did not show any activity against *A. niger*, thus seemingly not producing cyclosporins. 15 strains were eliminated due to very little growth in liquid medium L-2. The rest 34 strains were again tested for the absence of antifungal activity; 7 strains were found to synthesize Bmt in small amount (< 10 mg/liter) and only one mutant, YP 582 (from the MNNG tratment), accumulated Bmt at 25 to 50 mg/liter after 10~12 days of cultivation.

Properties of Cyclosporin Non-producing Mutants

The loss of the capacity to synthesize cyclosporins by the mutant YP 582 was accompanied by a reduction of conidia formation. Whereas the parental strain Cyb 156 formed over 3×10^8 conidia/cm^2 of agar medium, mutant YP 582 produced only 8×10^6 after the first transfer. The parental strain produced white mycelium and no pigment; mutant YP 582 showed a greyish mycelium and synthesized a grey-purple pigment.

In mutant YP 582, the capacity to produce Bmt as free amino acid was gradually lost by transfer of spores on agar media while cyclosporin biosynthesis was partially restored (Table 1). After transfer of spores, two main morphological types of colonies appeared: (i) Grey colonies, diffusible grey-purple pigment, poor sporulation and (ii) white colonies, no pigment, rich sporulation. Only the strains derived from the first type were Bmt producers. Strains of the second type were cyclosporin producers.

This degeneration occured also rapidly in liquid culture. Therefore, the production medium had to be inoculated directly with the first seed culture, without any additional propagation step. Due to this phenomenon, it was necessary to re-isolate continuously selectants of strain YP 582.

Isolation and Characterization of Bmt [(2*S*,3*R*,4*R*,6*E*)-2-amino-3-hydroxy-4-methyl-6-octenoic acid]

For the isolation of Bmt (**1**), the culture broth was extracted with butanol and the crude extract separated by repeated column chromatography on silica gel and cellulose. Final crystallization from methanol - water yielded pure Bmt as white needles.

The spectroscopic data of IR (Fig. 2), ^1H NMR (Fig. 3), and FAB-MS (*m/z* 188 (M+H) $C_9H_{17}NO_3$)

Table 1. Loss of capacity of producing free Bmt by mutant YP 582 of *Tolypocladium inflatum*.

Transfer	Production (mg/liter)		Conidia formation (No. of conidia/ cm^2 agar)	White colonies (%)
	Bmt	Cyclosporins		
1	25~50	<5	8×10^6	<1
2	10~25	20~ 80	1×10^8	60
3	<5	120~150	4×10^8	>99

Fig. 2. IR spectrum of Bmt (**1**) in KBr.

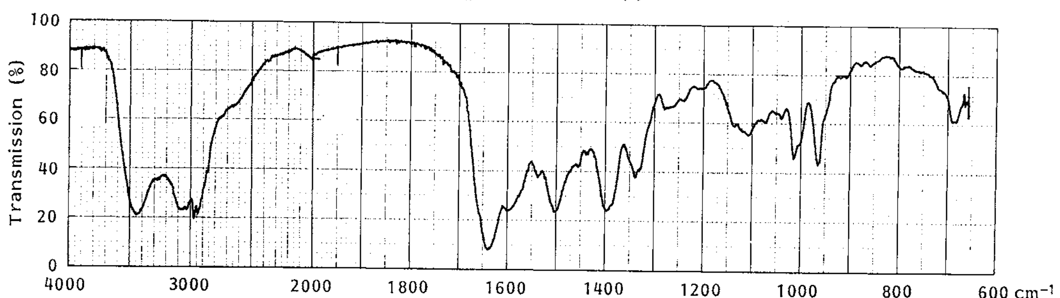
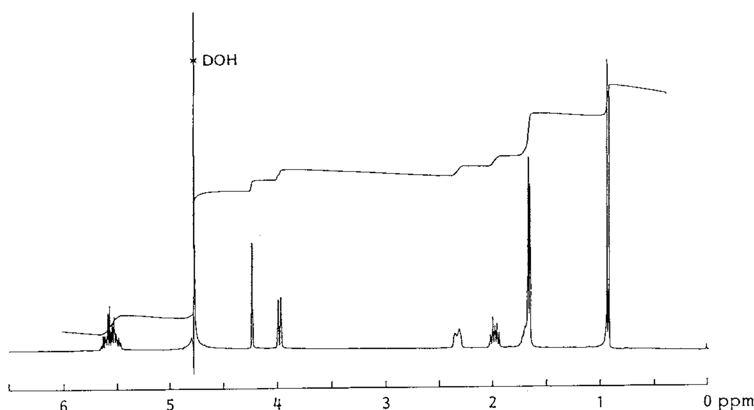
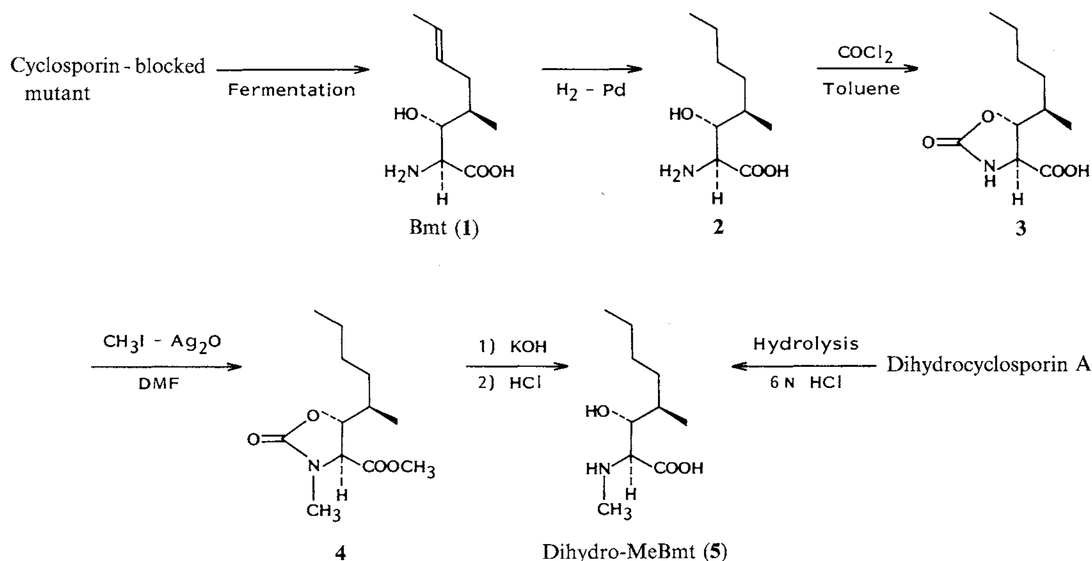


Fig. 3. ^1H NMR spectrum of Bmt (1) in D_2O+DCl.



Scheme 1. Chemical correlation of Bmt (1) with dihydro-MeBmt (5).



and the values of elemental analysis (see Experimental section) were in agreement with the assigned structure of **1**. The IR absorption at $970\,cm^{-1}$ is indicative of the *trans*-geometry of the double bond.

Chemical Correlation of Bmt (1) with Dihydro-MeBmt (5)

For structural and stereochemical proof, the amino acid Bmt (**1**) isolated from the fermentation broth, was correlated by a series of chemical transformations to dihydro-MeBmt of known absolute configuration [(2*S*,3*R*,4*R*)-3-hydroxy-4-methyl-2-methylamino-octanoic acid] (**5**), which was obtained by hydrolytic cleavage of dihydrocyclosporin A (Scheme 1). Thereto, the olefinic double bond in **1** was hydrogenated over palladium to the saturated compound **2**, which reacted with phosgene in toluene to the oxazolidinone derivative **3**. In the ^1H NMR spectrum of **3**, the observed coupling constant of 5 Hz between the α- and β-proton indicates the *threo* configuration for the amino and hydroxy groups fixed in the five-membered ring[14]. Subsequent treatment of **3** with methyl iodide in the presence of silver oxide yielded the *N*-methylated oxazolidinone methyl ester **4**. Removal of the protecting group and hydrolysis of the ester function led to the amino acid **5**. This material was found to be identical in chromatographic and spectroscopic data

as well as in optical rotation with an authentic sample of dihydro-MeBmt, thus confirming the chirality in Bmt as (2*S*,3*R*,4*R*).

Discussion

This is the first report on the isolation of the novel amino acid Bmt, the characteristic structural element of cyclosporins. Mutants accumulating a precursor of microbial peptides have been rarely described; the best example are mutants of *Cephalosporium acremonium* defective in the isopenicillin N synthetase, accumulating the intermediate tripeptide ACV as disulfide[15]. The loss of the capacity to produce cyclosporins by the *T. inflatum* mutant is correlated with a reduction in sporulation ability. Inversely, the restoration of cyclosporin biosynthesis paralleled an abundant sporulation. It may be that cyclosporin is involved in the induction of the sporulation of *T. inflatum* strain Cyb 156. In many cases with fungi, however, conidia formation and production of secondary metabolites are separate events[16]. Noteworthy is the rapid, but not complete restoration of cyclosporin biosynthesis. Therefore, it seems that the multienzyme complex was only slightly modified by the mutational treatment.

The amino acid Bmt, representing an essential building unit, has found use in the enzymatic total *in vitro* synthesis of cyclosporins[17]. By applying this methodology, several new cyclosporin analogues, *e.g.* [D-Abu^8]cyclosporin A, not available by precursor directed biosynthesis in the fungus, were preparted[18]. Furthermore, the cyclosporin-blocked mutant will allow to study the biosynthesis of the key intermediate Bmt.

Experimental

Isolation of Bmt [(2*S*,3*R*,4*R*,6*E*)-2-Amino-3-hydroxy-4-methyl-6-octenoic Acid] (**1**)

The lyophilized culture broth (14.5 liters) was mixed with ethanol - water (9 : 1) at 70°C for 1 hour. The extract was evaporated under vacuum to water (400 ml), diluted with the same volume of water and extracted four times with butanol (500 ml). The combined organic extracts were washed with water and evaporated to dryness *in vacuo*. The crude residue (45.7 g) was chromatographed on silica gel using chloroform - methanol - water - acetic acid (60 : 30 : 5 : 5). The fractions were analyzed by TLC on silica gel and cellulose plates (Polygram CEL 400) developed with the same solvent system; detection was performed by ninhydrin and iodine vapor. Fractions containing semi-pure Bmt (**1**) were further purified by chromatography on cellulose (Avicel) using the above eluent. Fractions which contained pure **1** as estimated by TLC were collected and decolorized with charcoal. After evaporation to dryness it was crystallized from methanol - water (4 : 1) yielding 204 mg of **1** as white needles: MP 209~211°C; $[\alpha]_D^{20}$ +22.5° (*c* 0.53, 0.1 N HCl); UV (MeOH): End absorption; IR: See Fig. 1; ^{1}H NMR (D_2O+DCl) δ 0.93 (3H, d, *J*=6 Hz, 4-CH_3), 1.68 (3H, d, *J*=6 Hz, 8-H_3), 1.7 (1H, m, 4-H), 2.0 and 2.35 (2H, 2m, 5-H_2), 4.00 (1H, dd, *J*=9 and 3 Hz, 3-H), 4.25 (1H, d, *J*=3 Hz, 2-H), 5.5~5.7 (2H, m, 6-H and 7-H) (Fig. 2); FAB-MS *m/z* 188 $(M+H)^+$.

Anal Calcd for $C_9H_{17}NO_3$: C 57.7, H 9.2, N 7.5, O 25.6.
Found: C 57.8, H 9.1, N 7.7, O 25.6.

Chemical Correlation of Bmt (**1**) with Dihydro-MeBmt (**5**)

Dihydro-Bmt (**2**): 748 mg (4 mmol) of compound **1** dissolved in a mixture of 20 ml of ethanol and 6 ml of 0.5 N NaOH were hydrogenated over 10% palladium-carbon (600 mg) at room temperature and atmospheric pressure. After filtering off the catalyst, the reaction mixture was diluted with water and the ethanol removed by evaporation *in vacuo*. On addition of 2 N HCl to neutralize the solution, 654 mg of **2** (87%) were obtained as white precipitate: MP 203~205°C; $[\alpha]_D^{20}$ +3.4° (*c* 0.75, methanol - 0.1 N HCl, 1 : 1); IR (KBr) cm^{-1} as **1** but absorption at 970 (CH=CH trans) missing; ^{1}H NMR (D_2O+DCl) δ 0.90 (3H, t, *J*=6 Hz, 8-H_3), 0.97 (3H, d, *J*=6 Hz, 4-CH_3), 1.2~1.7 (7H, m, 4-H, 5-H_2, 6-H_2 and 7-H_2), 3.98 (1H, dd, *J*=9 and 3 Hz, 3-H), 4.30 (1H, d, *J*=3 Hz, 2-H); FAB-MS *m/z* 190 $(M+H)^+$.

Anal Calcd for $C_9H_{19}NO_3$: C 57.1, H 10.1, N 7.4, O 25.4.
Found: C 57.1, H 10.0, N 7.6, O 25.3.

Oxazolidinone Derivative (**3**) of Dihydro-Bmt (**2**): To 564 mg (3 mmol) of compound **2** in 60 ml of 1 N NaOH, phosgene (30 ml of a 20% w/w solution in toluene) was added all at once at 0°C. After stirring for 5.5 hours, the aqueous layer was separated, acidified with conc HCl and extracted with chloroform. The extract was purified by silica gel column chromatography using ethyl acetate (saturated with water)-methanol (9:1) as eluent, to give 348 mg (54%) of **3** as white powder: MP 156~158°C; $[\alpha]_D^{20}$ +89.0° (*c* 0.87, methanol); IR (KBr) cm^{-1} 1730, 1610, 1420, 1230, 1040, 770; ^1H NMR (DMSO-d_6) δ 0.87 (6H, t and d, 8-H_3 and 4-CH_3), 1.0~1.5 (6H, m, 5-H_2, 6-H_2 and 7-H_2), 1.7 (1H, m, 4-H), 3.65 (1H, d, J=5 Hz, 2-H), 4.3 (1H, t, J=5 Hz, 3-H), 7.87 (1H, s, NH), 3.3~3.7 (1H, br s, exchangeable with D_2O, COOH); FAB-MS *m/z* 216 $(M+H)^+$.

N-Methylation of Compound **3** and Preparation of Methyl Ester **4**: A solution containing 324 mg (1.5 mmol) of compound **3**, 4.26 g of CH_3I and 3.48 g of Ag_2O in 15 ml of dry DMF was stirred for 24 hours in the dark. The precipitate was filtered off. The solution was evaporated and the crude residue was chromatographed on silica gel using ethyl acetate saturated with water, to yield 274 mg (75%) of **4** as a viscous oil: $[\alpha]_D^{20}$ +44.8° (*c* 1.43, methanol); IR (CCl_4) cm^{-1} 1775 (oxazolidinone C=O), 1740 (ester C=O); ^1H NMR ($CDCl_3$) δ 0.90 (3H, t, J=6 Hz, 8-H_3), 0.97 (3H, d, J=6 Hz, 4-CH_3), 1.2~1.6 (6H, m, 5-H_2, 6-H_2 and 7-H_2), 1.80 (1H, m, 4-H), 2.92 (3H, s, NCH_3), 3.82 (3H, s, $COOCH_3$), 3.96 (1H, d, J=5 Hz, 2-H), 4.28 (1H, dd, J=7 and 5 Hz, 3-H); FAB-MS *m/z* 244 $(M+H)^+$.

Anal Calcd for $C_{12}H_{21}NO_4$: C 59.2, H 8.7, N 5.8, O 26.3.
Found: C 59.3, H 8.8, N 5.8, O 26.4.

Removal of Oxazolidinone Protecting Group and Hydrolysis of Methyl Ester Function in **4**→Compound **5**: 195 mg (0.8 mmol) of **4** were heated in 4 ml of 2 N KOH at 80°C for 3 hours. The solution was cooled to room temperature, adjusted to pH 5 by the addition of 1 N HCl and evaporated. The residue was partitioned between water and butanol. The organic layer was evaporated yielding 182 mg of crude product which was crystallized from methanol-water to give prismatic needles: MP 260~261°C; $[\alpha]_D^{20}$ +25.8° (*c* 0.66, methanol-0.1 N HCl, 1:1).

This material was found to be identical by TLC as well as in physico-chemical and spectroscopic data with an authentic sample of (2*S*,3*R*,4*R*)-3-hydroxy-4-methyl-2-methylamino-octanoic acid (**5**).

Isolation of Dihydro-MeBmt [(2*S*,3*R*,4*R*)-3-Hydroxy-4-methyl-2-methylamino-octanoic Acid] (**5**) from Hydrolysis of Dihydrocyclosporin A

Dihydrocyclosporin A (24 g, 20 mmol) was heated for 20 hours under reflux in 700 ml of 6 N HCl. The reaction solution was evaporated, and the residue was dissolved in a small amount of water, filtrated through a weak basic anion exchange residue (Amberlite IRA-93, acetate form) and washed with water until the eluate was ninhydrin-negative. After evaporating to dryness, the crude mixture of amino acids was separated by column chromatography on cellulose (Avicel PH 101) using *sec*-butanol saturated with water. Dihydro-MeBmt (**5**) was eluted (2.70 g, 67%), followed by the other amino acid constituents (*N*-methylleucine, *N*-methyl valine, valine, α-aminobutyric acid, DL-alanine, sarcosine[3]), and crystallized from ethanol-water to yield prismatic needles: MP 262~264°C; $[\alpha]_D^{20}$ +25.6° (*c* 0.79, methanol-0.1 N HCl, 1:1); IR (KBr) cm^{-1} 1630, 1570, 1465, 1380; ^1H NMR (D_2O+DCl) δ 0.88 (3H, t, J=6 Hz, 8-H_3), 0.98 (3H, d, J=6 Hz, 4-CH_3), 1.2~1.75 (7H, m, 4-H, 5-H_2, 6-H_2 and 7-H_2), 2.82 (3H, s, NCH_3), 3.90 (1H, t, 3-H), 4.13 (1H, d, J=4 Hz, 2-H); FAB-MS *m/z* 204 $(M+H)^+$.

Anal Calcd for $C_{10}H_{21}NO_3$: C 59.1, H 10.4, N 6.9, O 23.6.
Found: C 59.2, H 10.4, N 6.9, O 23.6.

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