# LEUALACIN, A NOVEL CALCIUM BLOCKER FROM *Hapsidospora irregularis*

## II. STRUCTURE DETERMINATION

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The structure of leualacin was determined by MS and NMR analysis to be a cyclic depsipeptide consisting of L-leucine, L-*N*-methylphenylalanine, β-alanine, and *R*- and *S*-leucic acids.

Leualacin is a new calcium channel blocker with a unique cyclic depsipeptide structure; it was isolated from a fungi *Hapsidospora irregularis*.

The fermentation, isolation and characterization of leualacin have been reported in a previous paper[1]. In this report, we describe the structure determination of leualacin (11-benzyl-2,5,8-triisobutyl-10-methyl-1,7-dioxa-4,10,13-triazacyclohexadecane-3,6,9,12,16-pentone) in detail based on the results of chemical degradation and spectroscopic studies.

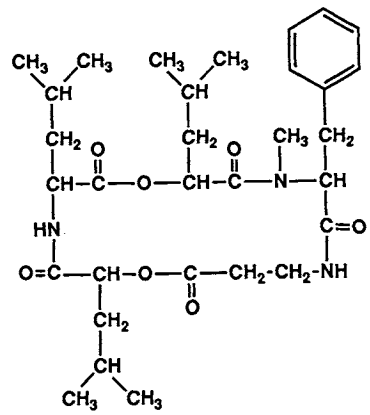
## Results and Discussion

The molecular formula of leualacin **1** (Fig. 1) was established as $C_{31}H_{47}N_3O_7$ from FAB-MS ($(M+H)^+$ *m/z* 574, $(M-H)^-$ *m/z* 572) and EI-MS ($M^{+\cdot}$, *m/z* 573), together with the elemental analysis (*Anal* Calcd for $C_{31}H_{47}N_3O_7$: C 64.65, H 8.33, N 7.21, Found: C 64.90, H 8.26, N 7.32).

This is also supported by the $^{13}C$ NMR spectrum of **1**, which showed signals due to five carbonyl carbons, six aromatic carbons, seven aliphatic methines, six aliphatic methylenes and seven methyls. Since **1** was deduced to be a peptide-related compound from $^{13}C$ NMR data, complete acid hydrolysis (6N HCl, 110°C, 24 hours) was performed and leucine, *N*-methylphenylalanine and β-alanine were detected by amino acid analysis. From the ether extract of the acid hydrolysate, leucic acid was isolated.

On the other hand, mild hydrolysis of **1** in 0.1 N ammonium hydroxide in 50% methanol at room temperature for 1 hour yielded a methanolysis

Fig. 1. Structure of leualacin **1**.



product **2**, $C_{32}H_{51}N_3O_8$, FAB-MS $(M+H)^+$ *m/z* at 606. EI-MS of the TMS derivative of **2** gave the molecular ion at *m/z* 677 as well as a series of fragment ions, such as at *m/z* 575, 547, 414, 300, 272 and 159. High-resolution measurements of these ions revealed that they are ascribed to the fragments as shown in Fig. 2, suggesting the structure of **2** to be a depsipeptide with the sequence of leucic acid-leucine-leucic acid-*N*-methyl phenylalanine-*β*-alanine-OMe. This sequence was also supported by the diagnostic fragment

Fig. 2. The structure of **2** and assignments of the fragment ions.

—; Positive ion FAB-MS fragment ions (R=H). =; Negative ion FAB-MS fragment ions (R=H).
( ); Positive ion EI-MS fragment ions (R=TMS).

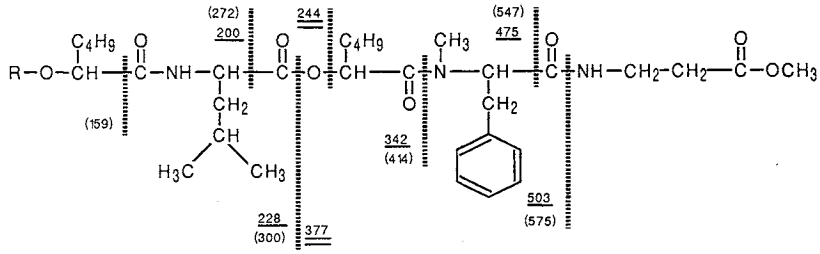



Fig. 3. The MS/MS spectra of *m/z* 574 (A) and *m/z* 572 (B) derived from positive and negative ion FAB-MS of **1**, respectively.

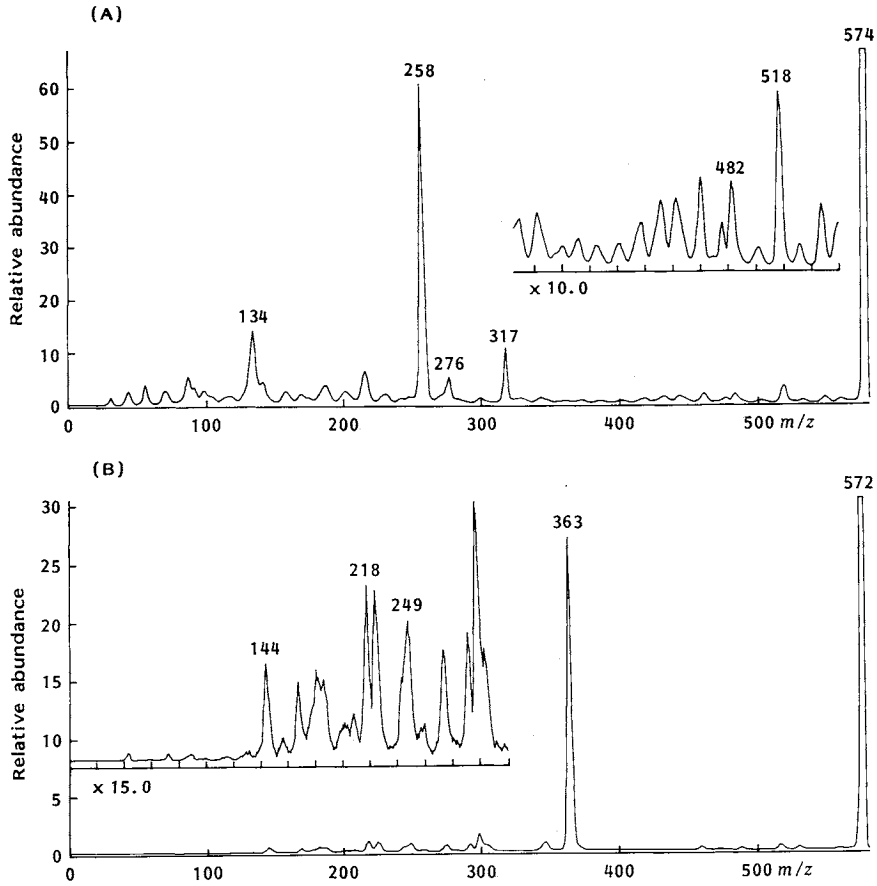


Fig. 4. Fragmentations of **1** by FAB-MS.

⊂⊃; Positive ion FAB-MS fragmentations. ——; Negative ion FAB-MS fragment ion.

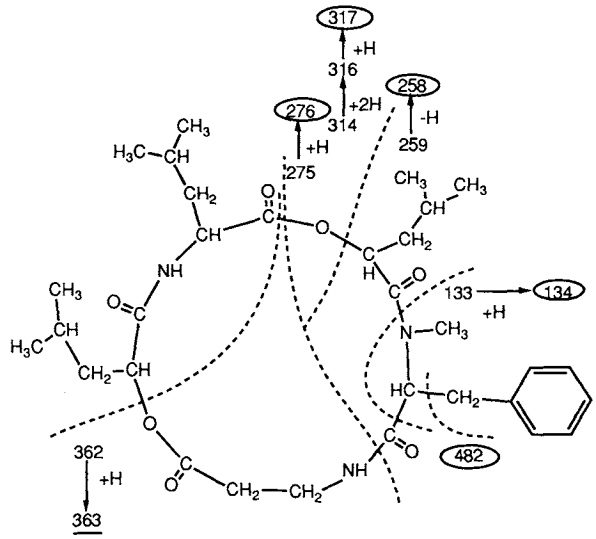
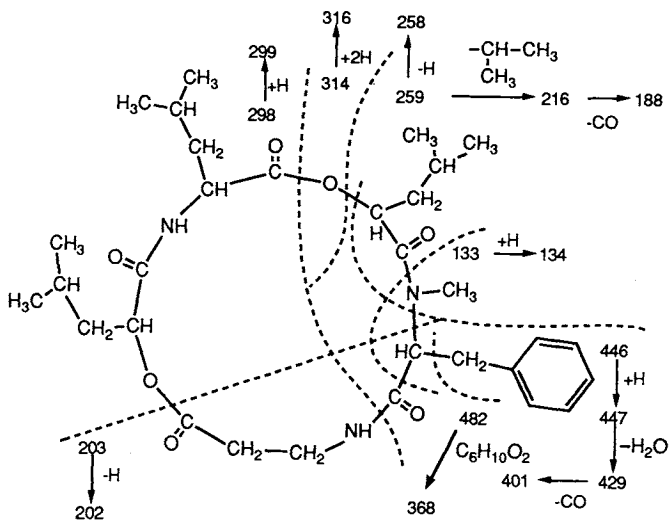



Table 1. High-resolution EI-MS measurements of **1**.

| *m/z* | Obsd | Calcd | Composition | *m/z* | Obsd | Calcd | Composition |
|---|---|---|---|---|---|---|---|
| 573 | 573.3391 | 573.3419 | $C_{31}H_{47}N_3O_7$ | 299 | 299.1995 | 299.1971 | $C_{15}H_{27}N_2O_4$ |
| 482 | 482.2879 | 482.2866 | $C_{24}H_{40}N_3O_7$ | 258 | 258.1473 | 258.1493 | $C_{16}H_{20}N_1O_2$ |
| 447 | 447.2502 | 447.2496 | $C_{24}H_{35}N_2O_6$ | 216 | 216.1045 | 216.1025 | $C_{13}H_{14}N_1O_2$ |
| 429 | 429.2382 | 429.2389 | $C_{24}H_{33}N_2O_5$ | 202 | 202.0842 | 202.0838 | $C_{12}H_{12}N_1O_2$ |
| 401 | 401.2384 | 401.2439 | $C_{23}H_{33}N_2O_4$ | 188 | 188.1103 | 188.1076 | $C_{12}H_{14}N_1O_1$ |
| 368 | 368.2155 | 368.2185 | $C_{18}H_{30}N_3O_5$ | 134 | 134.0958 | 134.0969 | $C_9H_{12}N_1$ |

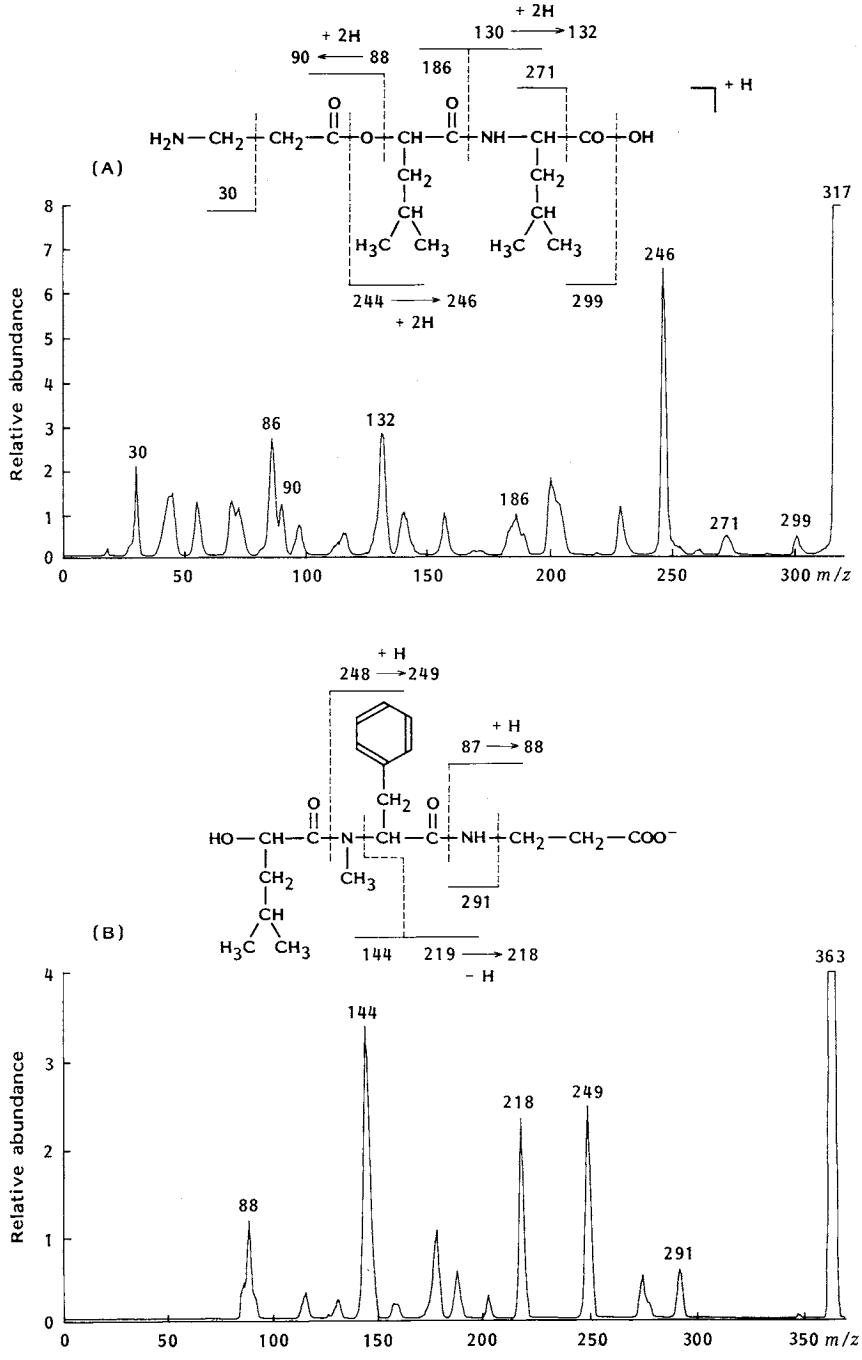
Fig. 5. Interpretation of fragment ions of **1** by positive EI high resolution MS.



ions at *m/z* 503, 475, 342, 228 and 200 in the positive FAB-MS of **2**, and ions at *m/z* 377 and 244 in the negative FAB-MS of **2**, as shown in Fig. 2.

The methyl ester at *C*-terminal should originate from methanol used in the solvent of the reaction, since no methoxy signal was observed in the $^1$H NMR spectrum of **1**. Based on the structure of **2** together

Fig. 6. The MS/MS spectra of ions at *m/z* 317 (A) and 363 (B) from **1** by positive and negative ion FAB-MS, respectively.



with the molecular formula of **1**, the structure of **1** was deduced to be a cyclic depsipeptide in which an ester linkage is formed from the carboxylic group of β-alanine and the hydroxy group of leucic acid.

The cyclic structure of **1** was further supported by MS analysis. The positive ion FAB-MS/MS of the *quasi*-molecular ion ($(M+H)^+$) at *m/z* 574 as a precursor ion gave main peaks at *m/z* 482, 317, 276, 258

Table 2. Proton chemical shifts and coupling constants of **1**.

| Assignment | δ (ppm) | Multiplicity (*J*=Hz) | No. of proton |
|---|---|---|---|
| Aα | 2.57 | d, d (6.7, 4.0) | 2 |
| Aβ | 4.05 | d, d, t (13.8, 9.8, 4.0) | 1 |
| | 3.37 | d, t, d (13.8, 6.7, 2.4) | 1 |
| Aβ NH | 7.48 | d, d (9.8, 2.4) | 1 |
| Fα | 4.67 | d, d (11.5, 3.4) | 1 |
| Fβ (a) | 3.40 | d, d (14.5, 3.4) | 1 |
| (b) | 2.95 | d, d (14.5, 11.5) | 1 |
| Fo | 7.10 | d (6.9) | 2 |
| Fm | 7.30 | d, d (7.4, 6.9) | 2 |
| Fp | 7.24 | t (7.4) | 1 |
| Fα –NMe | 2.88 | s | 3 |
| Vα | 4.68 | d, d (11.4, 2.4) | 1 |
| Vβ (a) | 1.42[a] | m | 1 |
| (b) | −0.41 | d, d, d (14.0, 9.0, 3.9) | 1 |
| Vγ | 1.46[a] | m | 1 |
| Vδ | 0.68 | d (6.6) | 3 |
| | 0.57 | d (6.6) | 3 |
| V1α | 5.17 | d, d (7.9, 6.4) | 1 |
| V1β (a) | 1.79[a] | m | |
| (b) | 1.75[a] | m | 1 |
| V1γ or Lγ | 1.75[a] | m | 1 |
| | 1.65 | m | 1 |
| V1δ or Lδ | 0.95 | d (6.6) | 6 |
| | 0.91 | d (6.6) | 6 |
| Lα | 4.53 | d, d, d (10.7, 10.1, 3.9) | 1 |
| Lβ (a) | 1.79 | m | 1 |
| (b) | 1.54 | d, d, d (11.0, 10.7. 1.5) | 1 |
| Lα –NH | 6.18 | d (10.1) | 1 |

[a] Estimated value based on $^1H$-$^1H$ and $^{13}C$-$^1H$ COSY spectra.

Table 3. $^{13}C$ NMR spectral data of **1**.

| Assignments | δ (ppm) | Multiplicity | Assignments | δ (ppm) | Multiplicity |
|---|---|---|---|---|---|
| Aα | 34.58 | t | Vδ | 20.68 | q |
| Aβ | 34.45 | t | Vα –C=O | 171.30 | s |
| Aα –C=O | 173.18 | s | V1α | 72.66 | d |
| Fα | 63.62 | d | V1β | 37.33 | t |
| Fβ | 34.12 | t | V1α –C=O | 170.33 | s |
| Fγ | 137.82 | s | V1γ or Lγ | 25.16 | d |
| Fo | 129.51 | d | | 24.17 | d |
| Fm | 129.20 | d | V1δ or Lδ | 22.86 | q |
| Fp | 127.08 | d | | 22.75 | q |
| Fα –NMe | 29.99 | q | | 22.24 | q |
| Fβ –C=O | 168.30 | s | | 21.03 | q |
| Vα | 70.64 | d | Lα | 51.19 | d |
| Vβ | 37.71 | t | Lβ | 38.70 | t |
| Vγ | 24.14 | d | Lα –C=O | 174.50 | s |
| Vδ | 22.78 | q | | | |

and 134 (Fig. 3A). On the other hand, the negative ion FAB-MS/MS of the *quasi*-molecular ion ($(M-H)^-$) at *m/z* 572 as a precursor ion gave the major fragment ion at *m/z* 363 (Fig. 3B). These fragmentation patterns were interpreted as shown in Fig. 4. The results of high resolution measurements of EI-MS of **1** (Table 1) were also satisfactorily explained as shown in Fig. 5. Moreover, the proposed structure of **1** was consistent with the result of MS/MS analysis of the fragment ions at *m/z* 317 and 363 from leualacin by positive and negative ion FAB-MS, respectively (Fig. 6).

The deduced structure of leualacin was also in good agreement with the result of the NMR analysis. Detailed analysis of the $^1H$-$^1H$ COSY spectrum of **1** as well as the heteronuclear multiple bond correlation

Fig. 7. Long-range coupling correlations between the protons and the carbonyl carbons.

Arrows indicate the coupling correlations proved by HMBC and COLOC spectra and dotted arrows show those by LSPD spectrum.

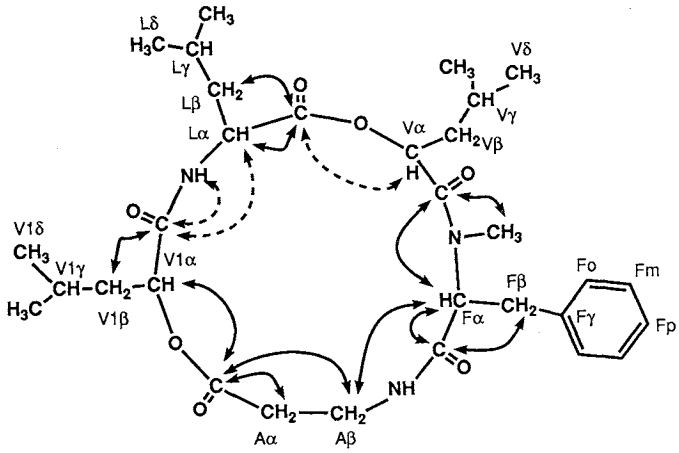
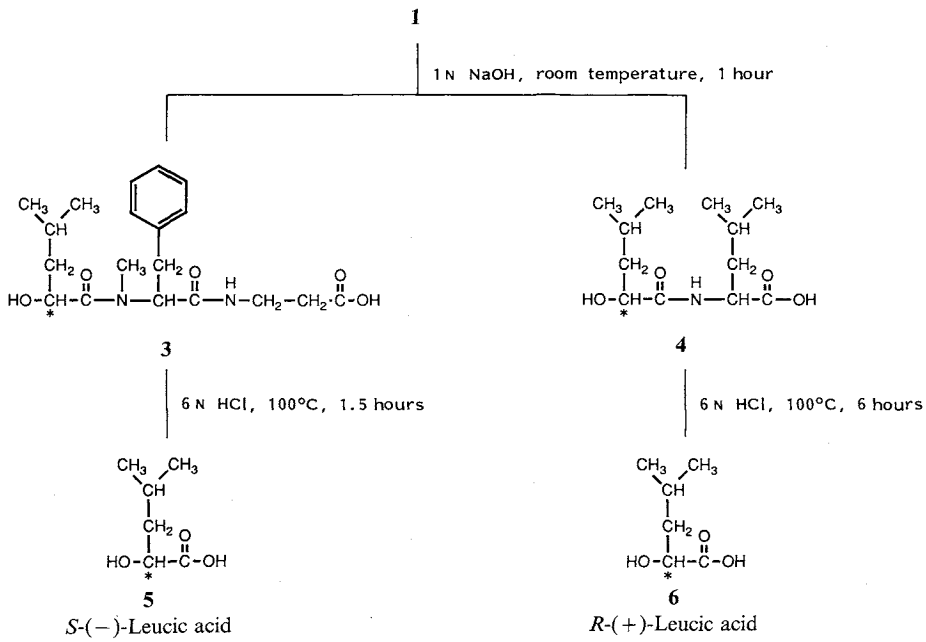
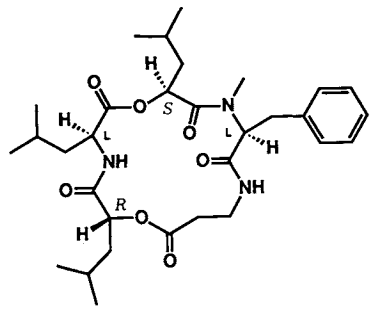



Fig. 8. Isolation of leucic acids.



(HMBC) spectrum and correlation spectroscopy *via* long-range couplings (COLOC) measurement revealed spin systems attributable to the following structural unit of **1**: 1×–NH–$CH_2$–$CH_2$–C=O for β-alanine, 1×–NH–CH($CH_2$($CH_3$)$_2$)–CO– for leucine, 1×NH–CH–$CH_2$–$C_6H_5$ for phenylalanine, and 2×O–CH($CH_2$($CH_3$)$_2$)–CO– for leucic acids (Tables 2 and 3). The sequence of the amino acids was elucidated by $^{13}C$-$^{1}H$ long-range coupling (Fig. 7) between the protons and carbonyl carbons detected by HMBC, COLOC and long-range spin-spin proton decoupling (LSPD) spectra (data not shown).†

Fig. 9. Stereochemistry of **1**.



The stereochemistry of **1** was established as follows. The leucine residue was determined to be L by chiral HPLC analysis of the sample purified from acid hydrolysate of **1**. By the same manner, *N*-methylphenylalanine was determined to be L form from its optical rotation. Leucic acid obtained from acid hydrolysate showed no optical rotation, suggesting that it is either racemic or 1 : 1 mixtures of *R* and *S* forms. In order to discriminate between these two possibilities, **1** was hydrolyzed in the presence of 0.1 N NaOH at room temperature for 1 hour, and the resultant fragments, **3** and **4**, were purified by HPLC. Each fragment was further hydrolyzed in the presence of HCl to obtain the leucic acids (Fig. 8).

The specific optical rotation of leucic acid from **3** ($[\alpha]_D^{25}$ −25.13° (*c* 1.55, 1 N NaOH)) and that from **4** ($[\alpha]_D^{25}$ +26.58° (*c* 1.61, 1 N NaOH)) suggest that their absolute configurations are *S* and *R*, respectively[2]. Based on these results, the stereochemistry of **1** was concluded to be as shown in Fig. 9.

## Experimental

### MS and NMR

EI-MS, FAB-MS and FAB-MS/MS spectra were obtained with a Jeol-HX 100 tandem mass spectrometer, which consisted of a conventional geometry double-focusing mass spectrometer (MS-I) followed by an electrostatic analyzer used as MS-II. Xenon was used to provide the primary beam of atoms (6 kV). The liquid matrix used for FAB ionization was 3-nitrobenzylalcohol. MS/MS spectra were obtained activating the ions in the third field free region by collision with argon gas (sufficient to suppress the precursor ion beam by 20%) and by scanning MS-II. All NMR spectra were recorded on a Jeol JNM-GX-500 spectrometer operating at 500 MHz for $^{1}H$ and 125 MHz for $^{13}C$. Tetramethylsilane was used as an internal reference for $^{1}H$ NMR in the $CDCl_3$ solution. For the $^{13}C$ chemical shift reference, the $^{13}C$ peak at $\delta$ 77.0 ppm of $CDCl_3$ was used.

### Compound 2

A solution of 24.9 mg of **1** in 1 ml of methanol and 1 ml of 0.2 N ammonium hydroxide was stirred at room temperature and the reaction was monitored by HPLC on an ODS column using 60% methanol as the eluent. After 2 hours starting material was not detected, and a peak due to **2** was detected at a retention time of 5 minutes. Methanol was evaporated and the residual mixture was extracted twice with 2 ml of ethyl acetate. The combined extract was chromatographed on an ODS column (Senshu Pak ODS-H-5251, 20 mm × 25 cm) eluted with 60% methanol, at a flow rate of 6 ml/minute. The effluent corresponding to a major peak eluted at 40 minutes was collected and evaporated, to yield 6.4 mg of pure **2**.

† A detailed discussion of the NMR analysis will be reported in a separate paper.

Leucine and *N*-Methylphenylalanine

**1** (13 mg) was hydrolyzed in 20 ml of 6 N HCl at 100°C for 15 hours. After HCl was evaporated under reduced pressure, water was added and the aqueous solution was extracted twice with ethyl acetate. The organic layer was evaporated to yield leucic acid. The optical rotation of the leucic acid thus obtained was 0 degrees. Leucine and *N*-methylphenylalanine was purified from the residual water phase of the hydrolysate. After the evaporation of water, the residue was chromatographed on a semi-preparative C18 reverse phase HPLC column ($\mu$Bondapak C18, 10 × 300 mm, Waters) eluted with 0.1% trifluoroacetic acid at 5 ml/minute, to yield leucine and *N*-methylphenylalanine, which were eluted at 4.5 minutes and 7.7 minutes, respectively.

Stereochemistry of the leucine was analyzed by Crownpak CR(+) (Daicel Chemical Industries Ltd., 4 × 150 mm) eluted with $H_2O$-$HClO_4$, pH 2.0, at 0.8 ml/minutes. Elution of the amino acids was detected by UV absorbance at 200 nm. Under these conditions, the retention time of D-leucine was 4.2 minutes and that of L-leucine was 6.3 minutes, and the stereochemistry of the leucine obtained was found to be L form. The stereochemistry of the *N*-methylphenylanine obtained from **1** was determined by the specific optical rotation, $[\alpha]_D^{25}$ +20.3 (*c* 0.7, 6 N HCl), (*cf.* $[\alpha]_D^{25}$ +24.7 (*c* 1.06, 6 N HCl)) for an authentic sample.

Compound **3** and **4**

A solution of **1** (288 mg, 0.5 mmol) in $CH_3CN$ (20 ml) and $H_2O$ (20 ml) was mixed with 1.5 ml of 1 N NaOH. The mixture was stirred at room temperature for 1 hour, and then $CH_3CN$ was evaporated and the residual mixture was adjusted to pH 3.0 and extracted twice with 20 ml of ethyl acetate. The combined extracts were concentrated, to give an oily material that was subjected to a reverse phase HPLC column (ODS-H-5251, Senshu). The column was eluted with $CH_3CN$ - 0.2% TEAP in $H_2O$ (35 : 65) at 6 ml/minute. The chromatography was monitored with a refractive index detector. The fractions eluted at 22 minutes and at 30 minutes were collected. After evaporation of $CH_3CN$, each fraction was extracted with ethyl acetate. Evaporation of ethyl acetate yielded 140 mg of **3** from the former fraction and 86 mg of **4** from the latter.

**3**; MS *m/z* 365 $(M+H)^+$, $^1H$ NMR (500 MHz, $CDCl_3$) $\delta$ 0.91 (3H, s), 0.94 (3H, s), 1.2 (1H, m), 1.35 (1H, m), 1.90 (1H, m), 2.28 (2H, m), 2.94 (3H, s), 4.30 (1H, dd), 5.14 (1H, t), 6.98 (1H, t), 7.2 (5H, m).

**4**; MS *m/z* 246 $(M+H)^+$, $^1H$ NMR (500 MHz, $CDCl_3$) $\delta$ 1.5 (2H, m), 1.7 (2H, m), 1.9 (1H, m), 2.05 (1H, m), 4.1 (1H, dd), 4.5 (1H, m).

*S*-Leucic acid (**5**) and *R*-Leucic acid (**6**)

A solution of **3** (41 mg) in 6 N HCl (4 ml) was heated at 100°C for 90 minutes. The mixture was evaporated to dryness under reduced pressure. After resuspending the mixture in 20 ml of water, the residue was extracted twice with equal amounts of ethyl acetate. Evaporation of the organic layer yielded 18.2 mg of an oily material, which was identified to be leucic acid from the EI-MS and $^1H$ NMR analyses.

**5**; MS *m/z* 133 $(M+H)^+$, $^1H$ NMR (500 MHz, $CDCl_3$) $\delta$ 0.97 (6H, d, $J$=6.8 Hz), 1.64 (2H, m), 1.92 (1H, m), 4.28 (1H, dd, $J$=5.0 and 4.7 Hz).

$[\alpha]_D^{25}$ of this compound was −25.3° (*c* 1.55, 1 N NaOH), which coincided with that of *S* form of authentic leucic acid. Compound **4** was hydrolyzed in the similar manner, and the $[\alpha]_D^{25}$ of the resulting leucic acid (**6**) was +25.58° (*c* 1.61, 1 N NaOH), which was consistent with that of *R* form of authentic leucic acid.

## References

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2) MORI, K.: Synthesis of optical active forms of ispenol, the pheromone of IPS Bark beetles. Tetrahedron 32: 1101~1106, 1976