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Enniatin Production by the Entomopathogenic Fungus *Verticillium hemipterigenum* BCC 1449

SUMALEE SUPOTHINA, MASAHIKO ISAKA,* KANYAWIM KIRTIKARA, MORAKOT TANTICHAROEN and YODHATHAI THEBTARANONTH

National Center for Genetic Engineering and Biotechnology (BIOTEC),
113 Phaholyothin Road, Klong 1, Klong Luang, Pathumthani 12120, Thailand

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Optimal fermentation conditions for enniatin production using the entomopathogenic fungus *Verticillium hemipterigenum* BCC 1449 have been investigated. Among various liquid media tested, highest efficiency of enniatin production was achieved by fermentation in yeast extract sucrose. Application of this condition to large-scale fermentation resulted in the isolation of three new analogs, O_1, O_2 and O_3, which are closely related isomers that were characterized as an inseparable mixture, along with seven known enniatins.

Recently, we reported the isolation and structure elucidation of two new enniatins[1] and two new diketopiperazines[2] from the entomopathogenic fungus *Verticillium hemipterigenum* BCC 1449. This fungus has also been shown to be useful for the studies on precursor-directed biosynthesis of enniatin analogs. Feeding L-leucine resulted in the selective uptake of this precursor in the *N*-methylamino acid residue of the enniatin molecule, while L-isoleucine was used as precursor for the 2-hydroxycarboxylic acid residue. In the above mentioned preliminary studies,[1] however, the low efficiency of enniatin production by BCC 1449 was a problem. Some minor enniatin analogs eluded the isolation/characterization process due to their low relative and absolute amounts in the crude extract from large-scale fermentation. This technical limitation in the precursor-feeding experiments has also been responsible for the problems in characterizing minor enniatin analogs. Considering the significant biological activities of this class of cyclodepsipeptide antibiotics as well as the aim to create a mini-library of enniatin analogs by precursor-directed biosynthesis[1,3,4] we decided to optimize the fermentation conditions for enniatin production using strain BCC 1449. In this paper, we report the results of the studies on fermentation conditions for BCC 1449 and the isolation of enniatins from a large-scale fermentation broth.

Results and Discussion

Fungal Material

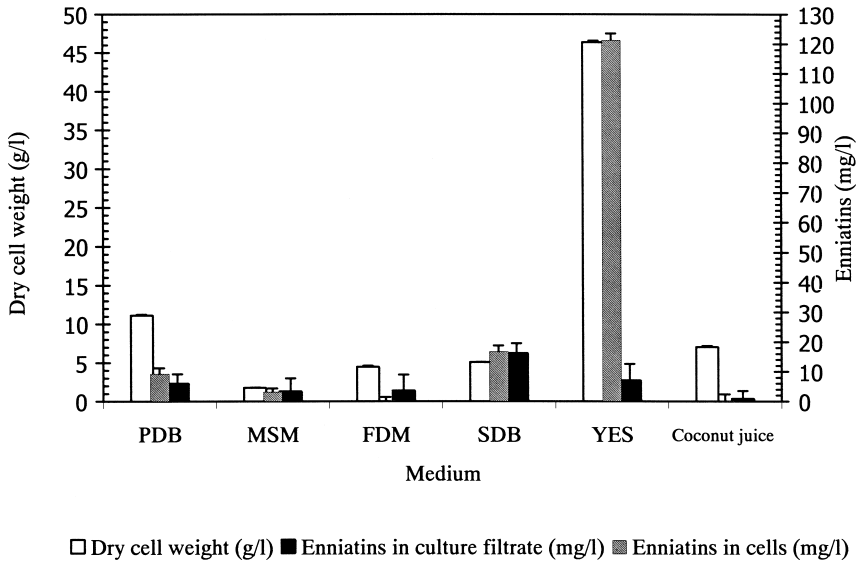
V. hemipterigenum was collected from Khlong Nakha Wildlife Sanctuary, Phetchaboon province, northern Thailand, on Homoptera-adult leafhopper, and identified by Dr. NIGEL L. HYWEL-JONES of the Mycology Research Unit, BIOTEC.[5] The fungus was deposited at the Thailand BIOTEC Culture Collection as BCC 1449.

Fermentation, Extraction, and Metabolites Analysis for Optimization

For the optimization of enniatin production, small scale cultivations were conducted in six liquid media (25°C, 21 days); 1) PDB (potato dextrose broth: potato infusion 200 g, Bacto dextrose 20 g, per liter distilled water), 2) SDB (Sabouraud's dextrose broth: peptone 15 g, glucose 20 g, per liter distilled water), 3) MSM (minimum salt medium: glucose 20 g, yeast extract 1.0 g, NH_4NO_3 3.0 g, KH_2PO_4 0.5 g, NaH_2PO_4 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $CaCl_2$ 0.5 g, per liter distilled water), 4) FDM (sucrose 25 g, $NaNO_3$ 4.25 g, NaCl 5.0 g, $MgSO_4 \cdot 7H_2O$ 2.5 g, KH_2PO_4 1.36 g, $FeSO_4 \cdot 7H_2O$ 0.1 g, $ZnSO_4 \cdot 7H_2O$ 0.0029 g, per liter distilled water), 5) YES (yeast extract sucrose: yeast extract 20 g, sucrose 150 g, per liter distilled water), and 6) coconut

* Corresponding author: isaka@biotec.or.th

Fig. 1. Comparison of liquid medium type for enniatins production by BCC 1449.



juice.

The strain BCC 1449 was maintained on potato dextrose agar at 25°C for 10 days. The agar plate culture was cut into pieces (1×1 cm) with a sterilized surgical knife and transferred into 250 ml Erlenmeyer flasks (2 pieces per flask) each containing 50 ml of PDB medium. The seed cultures were incubated on a rotary shaker (200 rpm) at 25°C for 4 days. A 5 ml portion of the seed culture was inoculated into twenty-five 250 ml Erlenmeyer flasks containing 45 ml of the production medium (PDB) and incubated without shaking at 25°C. After 7, 14, 21, 28 and 35 days, 5 flasks (total 250 ml broth) were harvested and subjected to extraction and analysis of the metabolites. This set of experiment was performed in triplicate. Cultivations in five other media were also carried out using the PDB seed culture.

The cultures in five Erlenmeyer flasks were filtered to separate filtrate and wet mycelia. The combined filtrate (250 ml) was extracted twice with EtOAc (250 ml each). The EtOAc solution was dried over $MgSO_4$, filtered and concentrated under reduced pressure to obtain a crude extract. The wet mycelia were freeze-dried to measure dry cell-weight, and were extracted with MeOH (50 ml, room temp, 2 days). The methanol extract of mycelia was filtered and 5 ml of H_2O was added to the filtrate and washed with 30 ml of hexane. The aqueous MeOH layer was separated from the hexane layer, and partially concentrated under reduced pressure. The residue was dissolved in EtOAc (50 ml) and washed with H_2O (30 ml), and concentrated to yield a crude mycelial extract.

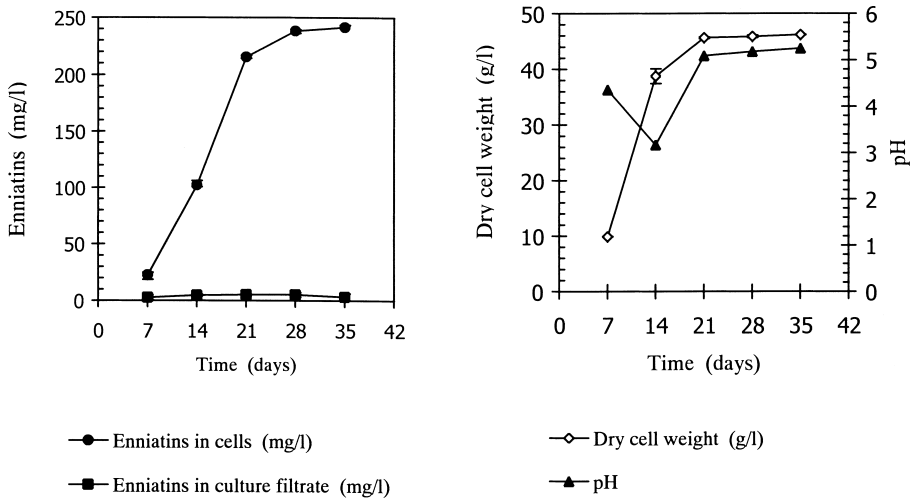
Quantitative analysis of enniatins in the extracts both from culture filtrate and mycelia was carried out by HPLC/UV (detection at 210 nm) using a reverse-phase column (NovaPak C_{18}, 4 μm, 3.9×150 mm; MeCN/H_2O=65 : 35; flow rate, 1 ml/minute). Ethyl 4-phenyl-benzoate was used as an internal standard.

Amount of enniatins from a total 250 ml culture (5×50 ml; calculated in mg/liter), and cell dry-weights (calculated in g/liter) are shown in Fig. 1. Enniatins were produced best in YES medium, which corresponded to the efficiency of cell growth. Incubation in SDB medium also gave better production of enniatins compared to PDB, while other liquid media (MSM, FDM, coconut juice) were not sufficient for enniatin production. Compositions of enniatin isomers in the crude extracts were similar in all cases. In YES cultures, enniatins were present mainly in mycelia (Fig. 1). Examination of the time-course in YES medium revealed that enniatin production reached the maximum level within 4 weeks incubation (Fig. 2). Therefore, we set up the optimal liquid-medium fermentation conditions in laboratory scale: static fermentation in YES, at 25°C, for 28 days.

Large-scale Fermentation and Isolation

Large-scale cultivation was carried out under the

Fig. 2. Time course study on enniatins production by BCC 1449 in YES medium.



conditions optimized for enniatin production. The strain BCC 1449 in 40×1 liter Erlenmeyer flasks each containing 250 ml of YES medium were incubated for 28 days. The flask cultures were filtered, and the residual wet mycelia were extracted with MeOH (1000 ml, 2 days), then filtered. To the filtrate was added 50 ml of H_2O and washed with 500 ml of hexane. The aqueous MeOH layer was separated from the hexane layer, and partially concentrated under reduced pressure. The residue was dissolved in EtOAc (500 ml), washed with H_2O (150 ml), and concentrated to yield a brown gum (2.0 g). This extract was passed through a Sephadex LH-20 column ($MeOH/CH_2Cl_2=1:2$) and the fractions containing enniatins were combined (1.29 g) and subjected to silica gel column chromatography ($EtOAc/CH_2Cl_2$). The enniatin-containing fractions were combined (673 mg), and subjected to preparative HPLC using a reversed phase column (NovaPak HR C_{18}, 40×100 mm, 6 μm) with $MeCN/H_2O=68:32$ as eluent at a flow rate of 20 ml/minute to separate into six fractions in the following elution order: **1**, **2**, (**3** and **5**), (**4**, **8a**, **8b** and **8c**), **6**, **7**. Each fraction was further separated by repeated preparative HPLC using an eluent of $MeOH/H_2O=80/20$ to afford pure compounds; **1** (286 mg), **2** (77 mg), **3** (13 mg), **5** (65 mg), **8a**/**8b**/**8c** mixture (19 mg), **4** (0.3 mg), **6** (4 mg), and **7** (0.3 mg).

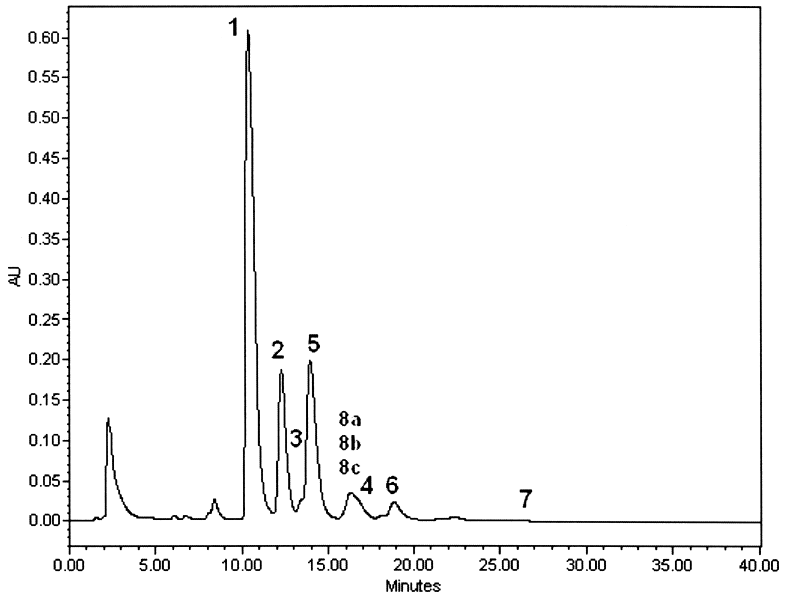
When the fermentation was repeated under the same conditions and scale, higher quantity of enniatin mixture (2.70 g) was obtained after partial purification by Sephadex LH20 and silica gel column (0.673 g, for the previous isolation). However, composition of enniatin analogs was similar in both of these fermentations. Presumably, the efficiency of the enniatin production may be affected by the seed culture preparation stage (on PDA) or other factors.

Structure Elucidation

Spectral data of known enniatins, **1**～**7**, were identical to those of our previous isolation from the same fungus[1] as well as those reported in the literature. It should be noted that enniatin C (**4**) was previously reported as a synthetic compound, and we recently obtained this compound by precursor-directed biosynthesis (feeding L-leucine) employing the same fungal strain.[1] Now it has been isolated, although in very low amount (0.3 mg), as a *bona fide* natural product.

A mixture obtained together with **1**～**7** composed of three new enniatins with an approximate ratio of 1 : 1 : 1, as indicated by its NMR spectra (^1H and ^{13}C). Attempts to separate each compound from this mixture using various chromatographic conditions met with failure, therefore, characterization was conducted for the mixture. The ESI-TOF mass spectrum indicated that the three inseparable compounds possess identical molecular formula of $C_{35}H_{61}N_3O_9$ (*m/z* 668.4502, $[M+H]^+$, $\Delta=1.6$ mmu), the same as that of enniatin G (**3**) and I (**6**). IR and UV spectral data and the specific rotation value were very similar to those of other enniatins. NMR analysis was supported by the full spectral data of related compounds **1**～**7** (all taken in $CDCl_3$), which revealed that the mixture of three compounds consists of six *N*-methylvaline, three *N*-

Fig. 3. HPLC chromatogram of enniatins mixture partially purified by silica gel column chromatography.



(NovaPak HR C_{18}, 3.9×150 mm; MeCN/H_2O=65 : 35; flow rate, 1 ml/minute).

Table 1. NMR data for enniatins O_1, O_2, O_3 mixture.

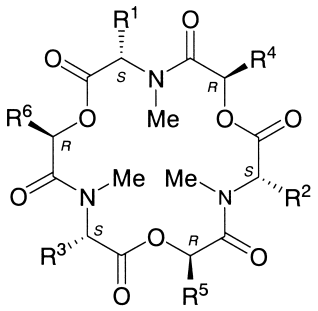
	position	^{13}C NMR (125 MHz, $CDCl_3$)[a,b,c]	^{1}H NMR (500 MHz, $CDCl_3$)[d]
*N*MeVal	1 *C*=O	170.57, 170.51×2, 170.43×3	-
(6 units)	2	62.91, 62.73, 62.64, 61.36, 61.33×2	4.95 (2H, d, ca 9), 4.93 (d, ca 9), 4.59 (d, 9.3), 4.57 (d, 8.0), 4.53 (d, 10.0)
	3	27.92, 27.84×2, 27.76, 27.68, 27.59	2.28-2.23 (6H, m)[e]
	4	20.16, 20.13, 20.68, 19.91×2, 19.85	1.06-1.01 (18H, m)
	4'	19.76, 19.62, 19.55, 19.24×2, 19.03	0.93-0.88 (18H, m)[f]
	N-*CH*$_3$	33.22×3, 32.54, 32.49, 32.36	3.18 (3H, s), 3.16 (6H, s), 3.15 (3H, s), 3.13 (6H, s)
*N*MeLeu	1 *C*=O	170.85×3	-
(3 units)	2	56.79×2, 56.63	4.83-4.78 (3H, m)
	3	37.79, 37.66×2	1.85-1.83 (3H, m), 1.75-1.73 (3H, m)
	4	25.35, 25.31, 25.27	1.53-1.52 (3H, m)
	5	23.29, 23.25, 23.25	0.96-0.92 (9H, m)[f]
	5'	21.33×3	0.96-0.92 (9H, m)[f]
	N-*CH*$_3$	31.71×2, 31.39	3.13 (3H, s), 3.12 (3H, s), 3.09 (3H, s)
Hiv	1 *C*=O	169.69, 169.55×2, 169.48, 169.37×2	-
(6 units)	2	75.31, 75.24, 75.17×3	5.22 (d, 8.5), 5.15 (d, ca.9), 5.04 (2H, d, ca 9), 5.02 (d, 7.0), 4.98 (d, 9.1)
	3	30.31, 30.28, 29.82, 29.81, 29.74, 29.70	2.31-2.25 (6H, m)[e]
	4	18.98, 18.90, 18.70, 18.65, 18.48, 18.46	1.00-0.94 (18H, m)[f]
	4'	18.37×2, 18.35, 18.34, 18.19, 18.15	1.00-0.94 (18H, m)[f]
Hmp	1 *C*=O	169.88×2, 169.80	-
(3 units)	2	73.88, 73.77×2	5.32 (d, 7.0), 5.18 (2H, d, 7.5)
	3	37.79, 37.66×2	2.03 (m), 1.97-1.96 (2H, m)
	4	25.37, 25.35, 25.22	1.45-1.44 (3H, m), 1.19-1.18 (3H, m)
	5	14.85, 14.60, 14.36	0.93-0.88 (9H, m)[f]
	3-*CH*$_3$	14.85, 14.60, 14.36	1.00-0.94 (9H, m)[f]

[a] Assignments of the carbonyl carbons can be interchanged between *N*MeVal and *N*MeLeu residues. [b] Assignments of the carbonyl carbons can be interchanged between Hiv and Hmp residues. [c] Assignments of the N-CH_3 carbons can be interchanged between the *N*MeVal and *N*MeLeu residues. [d] Assignments of the N-CH_3 protons can be interchanged between the *N*MeVal and *N*MeLeu residues. [e,f] ^{1}H signals are overlapping.

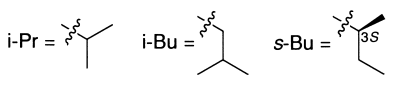
methylleucine, six 2-hydroxyisovaleric acid (Hiv), and three 2-hydroxy-3-methylpentanoic acid (Hmp) residues (Table 1). Considering also its HPLC retention time, eluted between enniatin G and enniatin I, the structures depicted as **8a**, **8b** and **8c** (Fig. 4), namely enniatins O_1, O_2 and O_3, respectively, were suggested. HMBC correlations from the *N*-methyl protons to the overlapping carbon signals at δ_C 169.3～169.9 indicated that these carbonyl carbons were assignable to those of the amides (Hiv and Hmp residues). The rest of the carbonyl carbons, situated at δ_C 170.4～170.9, were correlated from α-protons of both 2-amino acids and 2-hydroxyacids, and they were assigned to ester carbonyls (*N*MeVal and *N*MeLeu residues). Further structural analysis was conducted using the partial degradation procedure described by TOMODA *et al.* for the analysis of enniatin E.[6] $LiBH_4$ reduction of the mixture (THF, 0°C) followed by acetylation (Ac_2O, pyridine) gave a mixture of four amide fragments, **9**～**12**, in an approximate ratio of 4 : 2 : 2 : 1, and were separated by preparative HPLC. The authentic samples of **9**, **10** and **11** were obtained by the same reaction sequence from enniatins B (**1**), B_4 (**2**) and H (**5**), respectively. Structure of the fragment **12** was determined by NMR and mass spectral analysis. The ^1H and ^{13}C NMR data for the 2-hydroxy-3-methylpentanoic acid moiety of **12** were very similar to those of **11**, and the NMR data for the *N*-methylleucine-derived moiety of **12** were similar to those of **10**. Therefore, the absolute stereochemistry of **12** should be analogous to these analogs. These results are in good agreement with the proposed structures for the compounds **8a**, **8b** and **8c**.

Having established the fermentation condition for efficient enniatin production as well as the extracts profile for this standard fermentation, the fungus BCC 1449 is now well-suited for systematic studies on precursor-directed biosynthesis of unnatural enniatin analogs by feeding experiments with various amino acid precursors. It should also be reported that the established fermentation conditions have been shown to be efficient also for the production of diketopiperazines. While enniatins were obtained from the extracts from mycelia, large amounts of diketopiperazines, incluing several new analogs, have been found in the EtOAc extract from culture filtrate. Therefore, chemical investigation for such extracts are proceeding in our laboratory.

Fig. 4. Structures of enniatins isolated from *V. hemipterigenum* BCC 1449.



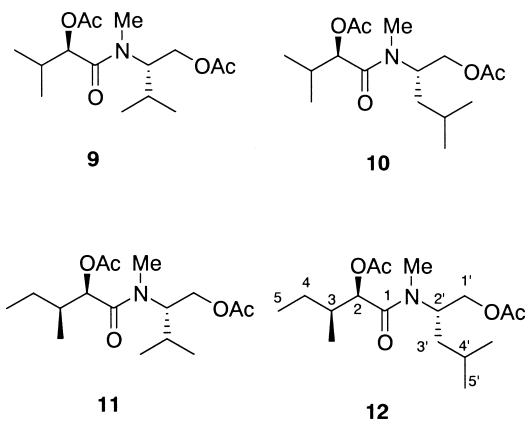
	R^1	R^2	R^3	R^4	R^5	R^6
enniatin B (**1**)	i-Pr	i-Pr	i-Pr	i-Pr	i-Pr	i-Pr
enniatin B_4 (**2**)	i-Bu	i-Pr	i-Pr	i-Pr	i-Pr	i-Pr
enniatin G (**3**)	i-Bu	i-Bu	i-Pr	i-Pr	i-Pr	i-Pr
enniatin C (**4**)	i-Bu	i-Bu	i-Bu	i-Pr	i-Pr	i-Pr
enniatin H (**5**)	i-Pr	i-Pr	i-Pr	*s*-Bu	i-Pr	i-Pr
enniatin I (**6**)	i-Pr	i-Pr	i-Pr	*s*-Bu	*s*-Bu	i-Pr
MK1688 (**7**)	i-Pr	i-Pr	i-Pr	*s*-Bu	*s*-Bu	*s*-Bu
enniatin O_1 (**8a**)	i-Bu	i-Pr	i-Pr	*s*-Bu	i-Pr	i-Pr
enniatin O_2 (**8b**)	i-Bu	i-Pr	i-Pr	i-Pr	*s*-Bu	i-Pr
enniatin O_3 (**8c**)	i-Bu	i-Pr	i-Pr	i-Pr	i-Pr	*s*-Bu



Biological Activities

The mixture of the three new enniatins O_1, O_2 and O_3, exhibited biological activities similar to those of enniatin B. Thus it inhibited the proliferation of the human malaria parasite (*Plasmodium falciparum* K1) with an IC_{50} value of 3.2 μg/ml (enniatin B: IC_{50} 3.4 μg/ml), and showed growth inhibitory activity against *Mycobacterium tuberculosis* $H_{37}Ra$ with a MIC value of 3.125 μg/ml (enniatin B: MIC 3.125 μg/ml). It was cytotoxic to cancer cell-lines, KB, BC-1 and NCI-185, with respective IC_{50} values of 2.4, 1.4 and 0.78 μg/ml.

Fig. 5. Structures of compounds **9**～**12**.



Experimental

A Mixture of Enniatins O_1 (**8a**), O_2 (**8b**) and O_3 (**8c**)

Colorless gum, $[\alpha]_D^{29}$ −93° (*c* 0.155, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 206 (4.25) nm; IR (KBr) ν_{max} 2966, 1748 (s), 1659 (s), 1471, 1189, 1012 cm^{-1}; HRMS (ESI-TOF) *m/z* 668.4502 $[M+H]^+$ (calcd for $C_{35}H_{62}N_3O_9$ 668.4486); ^1H and ^{13}C NMR data, Table 1.

$LiBH_4$ Reduction of (**8a**, **8b**, **8c**)-Mixture and Acetylation with Ac_2O

To a solution of enniatins O_1 (**8a**), O_2 (**8b**) and O_3 (**8c**) mixture (10.0 mg) in THF (0.5 ml), $LiBH_4$ (10 mg) was added and stirred in an ice-water bath for 5 hours. The reaction was terminated by addition of H_2O (3 ml) and extracted three times with EtOAc (3 ml). The combined organic layer was concentrated *in vacuo*, the residue was dissolved in pyridine (0.3 ml), and Ac_2O (0.15 ml) was added. After standing 20 hours, the reaction mixture was diluted with EtOAc (10 ml) and washed with H_2O (4 ml). The organic layer was concentrated *in vacuo* to obtain a pale yellow oil (10.8 mg), which was subjected to preparative HPLC (ODS, MeOH/H_2O=55 : 45) to obtain compounds **9** (3.7 mg), **10** (1.8 mg), **11** (1.7 mg), and **12** (1.0 mg). Each compound was obtained as a mixture with its C-3 epimer (4 : 1～9 : 1).

Compound **9**: Colorless oil; HRMS (ESI-TOF) *m/z* 302.1965 $[M+H]^+$ (calcd for $C_{15}H_{28}NO_5$ 302.1967); ^1H NMR (400 MHz, $CDCl_3$) δ 5.02 (1H, d, *J*=6.5 Hz, H-2), 4.40 (1H, br, H-2=), 4.24～4.21 (2H, m, H-1′), 2.98 (3H, s, N-CH_3), 2.17 (1H, m, H-3), 2.12 (3H, s, acetyl), 2.03 (3H, s, acetyl), 1.88 (1H, m, H-3′), 1.01 (6H, d, *J*=6.8 Hz, H-4 and 3-CH_3), 1.00 (3H, d, *J*=6.4 Hz, H-4′), 0.87 (3H, d, *J*=6.7 Hz, 3′-CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.0 (s, CH_3*C*OO–), 170.8 (s, CH_3*C*OO–), 170.4 (s, C-1), 75.3 (d, C-2), 62.3 (t, C-1′), 59.5 (d, C-2′), 30.2 (q, N–*C*H_3), 29.6 (d, C-3), 27.0 (d, C-3′), 20.8 (q, *C*H_3COO–), 20.6 (q, *C*H_3COO–), 19.9×2 (q, C-4′ and 3′-*C*H_3), 18.8 (q, C-4), 17.5 (q, 3-*C*H_3).

Compound **10**: Colorless oil; HRMS (ESI-TOF) *m/z* 316.2140 $[M+H]^+$ (calcd for $C_{16}H_{30}NO_5$ 316.2124); ^1H NMR (400 MHz, $CDCl_3$) δ 4.99 (1H, d, *J*=6.2 Hz, H-2), 4.97 (1H, m, H-2′), 4.12 (1H, dd, *J*=11.5, 8.9 Hz, Ha-1′), 4.01 (1H, dd, *J*=11.5, 4.01 Hz, Hb-1′), 2.93 (3H, s, N–CH_3), 2.15 (1H, m, H-3), 2.12 (3H, s, acetyl), 2.03 (3H, s, acetyl), 1.47 (1H, m, Ha-3′), 1.43 (m, H-4′), 1.18 (1H, m, Hb-3′), 1.01 (3H, d, *J*=6.7 Hz, 3-CH_3), 1.00 (3H, d, *J*=6.8 Hz, H-4), 0.93 (3H, d, *J*=6.3 Hz, H-5′), 0.91 (3H, d, *J*=6.4 Hz, 4′-CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.0 (s, CH_3*C*OO–), 170.8 (s, CH_3*C*OO–), 170.4 (s, C-1), 75.4 (d, C-2), 63.4 (t, C-1′), 49.9 (d, C-2′), 37.0 (t, C-3′), 29.7 (d, C-3), 29.2 (q, N-*C*H_3), 23.5 (q, C-5′), 21.6 (q, 4′-*C*H_3), 20.8 (q, *C*H_3COO–), 20.6 (q, *C*H_3COO–), 18.7 (q, C-4), 17.5 (q, 3-*C*H_3).

Compound **11**: Colorless oil; HRMS (ESI-TOF) *m/z* 316.2143 $[M+H]^+$ (calcd for $C_{16}H_{30}NO_5$ 316.2124); ^1H NMR (400 MHz, $CDCl_3$) δ 5.17 (1H, d, *J*=4.4 Hz, H-2), 4.42 (1H, br, H-2′), 4.24～4.21 (2H, m, H-1′), 2.94 (3H, s, N-CH_3), 2.12 (3H, s, acetyl), 2.04 (3H, s, acetyl), 1.86 (1H, m, H-3′), 1.84 (1H, m, H-3), 1.47 (1H, m, Ha-4), 1.30 (1H, m, Hb-4), 0.99 (3H, d, *J*=6.4 Hz, H-4′), 0.98 (3H, d, *J*=6.8 Hz, 3-CH_3), 0.96 (3H, t, *J*=7.4 Hz, H-5), 0.86 (3H, d, *J*=6.7 Hz, 3′-CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.0 (s, CH_3*C*OO–), 170.8 (s, CH_3*C*OO–), 170.3 (s, C-1), 73.3 (d, C-2), 62.2 (t, C-1′), 58.7 (d, C-2′), 35.9 (d, C-3), 30.3 (q, N-*C*H_3), 27.0 (d, C-3′), 26.1 (t, C-4), 20.8 (q, *C*H_3COO–), 20.6 (q, *C*H_3COO–), 19.9 (q, C-4′), 19.7 (q, 3′-*C*H_3), 14.1 (q, 3-*C*H_3), 11.7 (C-5).

Compound **12**: Colorless oil; HRMS (ESI-TOF) *m/z* 330.2285 $[M+H]^+$ (calcd for $C_{17}H_{32}NO_5$ 330.2280); ^1H NMR (500 MHz, $CDCl_3$) δ 5.17 (1H, d, *J*=4.4 Hz, H-1), 4.99 (1H, br, H-2′), 4.14 (1H, dd, *J*=11.6, 9.2 Hz, Ha-1′), 4.01 (1H, dd, *J*=11.6, 4.6 Hz, Hb-1′), 2.91 (3H, s, N–CH_3), 2.14 (3H, s, acetyl), 2.05 (3H, s, acetyl), 1.84 (1H, m, H-3), 1.47 (2H, m, Ha-4 and Ha-3′), 1.43 (1H, m, H-4′), 1.31 (H, m, Hb-4), 1.18 (1H, m, Hb-3′), 0.99 (3H, d, *J*=6.8 Hz, 3-CH_3), 0.97 (3H, t, *J*=7.4 Hz, H-5), 0.95 (3H, d, *J*=6.6 Hz, H-4′), 0.91 (3H, d, *J*=6.5 Hz, 3′-CH_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 171.0 (s, CH_3*C*OO–), 170.8 (s, CH_3*C*OO–), 170.3 (s, C-1), 73.4 (d, C-2), 63.3 (t, C-1′), 49.9 (d, C-2′), 36.9 (t, C-3′), 36.0 (d, C-3), 29.1 (q, N-*C*H_3), 26.1 (t, C-4), 24.9 (d, C-4′), 23.5 (q, C-5′), 21.6 (q, 4′-*C*H_3), 20.8 (q, *C*H_3COO–), 20.6 (q, *C*H_3COO–), 14.1 (q, 3-*C*H_3), 11.7 (C-5).

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