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ORIGINAL ARTICLE

Isolation and structure elucidation of tumescenamides A and B, two peptides produced by *Streptomyces tumescens* YM23-260

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Two peptides, tumescenamides A and B, were isolated from the fermentation broth of a marine bacterium, *Streptomyces tumescens* YM23-260. The structure of tumescenamide A was determined to be a cyclic depsipeptide consisting of α-amino-2-butenoic acid, tyrosine, valine, leucine and threonine, substituted with a 2,4-dimethylheptanoyl residue at the α-NH_2 position. Tumescenamide B possesses a 2,4,6-trimethylnonanoyl residue in place of the 2,4-dimethylheptanoyl substituent in tumescenamide A. Tumescenamide A induced reporter gene expression under the control of the insulin-degrading enzyme promoter.

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INTRODUCTION

Terpenoids are the largest family of compounds found in nature with over 24 000 known examples and are mainly produced by fungi and plants. Production of these compounds by actinomycetes including *Streptomyces*, however, was rather rare, and only limited numbers of compounds were reported to date.[1] In view of the excellent ability of *Streptomyces* to produce many kinds of bioactive secondary metabolites with structural diversity, this phenomenon seemed to be interesting for us.

Being stimulated in this phenomenon, we started screening for terpenoids produced by actinomycetes and succeeded in the isolation of oxaloterpins[2] and napyradiomycin analogs.[3] Our strategy for screening of terpenoids in these cases was as follows: (1) prepare a lipophilic fraction from the fermentation broth of actinomycetes by EtOAc extraction, (2) analyze by TLC development followed by colorization with vanillin-H_2SO_4. Select the spots that change to bright purple or violet, (3) analyze fractions corresponding to these spots by NMR and (4) select fractions showing methyl singlets at ca. δ_H l.0 as candidates for terpenoids.[2] This strategy, however, was not efficient as many of the selected fractions did not show the expected NMR features.

To carry out more efficient screening of terpenoids, we started using liquid chromatography NMR (LC-NMR) that enabled to detect compounds with several methyl groups without amenable to tedious procedures. During this screening process, we noticed that this method enabled to detect polyketides and peptides that showed strong methyl doublets at around δ_H l.0.

Here, we describe isolation aided by LC-NMR of the two new peptides, tumescenamides A and B, produced by *Streptomyces tumescens* YM23-260, and their structural elucidation.

RESULTS AND DISCUSSION

Fermentation, extraction and isolation

Several actinomycetes strains were cultured at 28 °C for 7 days by rotary shaking in 500 ml baffled Erlenmeyer flasks each containing 100 ml of the production medium. The filtrate of each fermentation broth was extracted with EtOAc and the residual oily material obtained after removal of the solvent was analyzed by LC-NMR (ODS column, 70% CH_3CN and 30% D_2O) to check for the presence of methyl signals at around δ_H 0 in the ^{1}H-NMR spectrum.

A sample prepared from *S. tumescens* YM23-260 showed an LC peak (t_R=5.2 min), which contained a substance showing the presence of nine methyl doublet signals at δ_H 0.4–1.6 (Figure 1). Additional signals at δ_H 4–5 ascribed to the α-methine signals of amino acids suggested that this LC peak was because of a peptide. In addition, a

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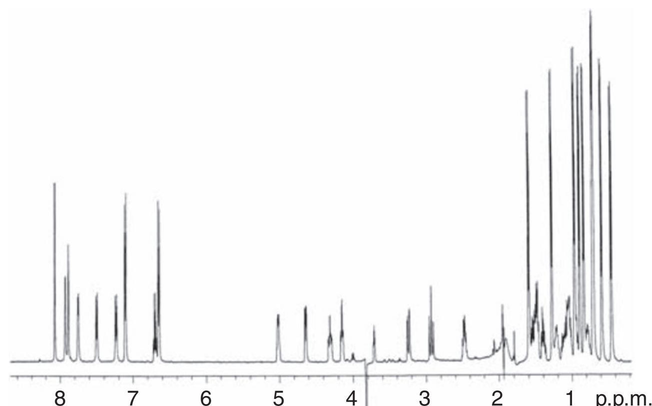

Figure 1 LC-NMR spectrum of EtOAc extract of the fermentation broth of *Streptomyces tumescens*. Retention time=5.2 min. ODS column, 70% CH_3CN and 30% D_2O. The strong peak at δ_H 1.9 is because of the residual suppression peak of the solvent.

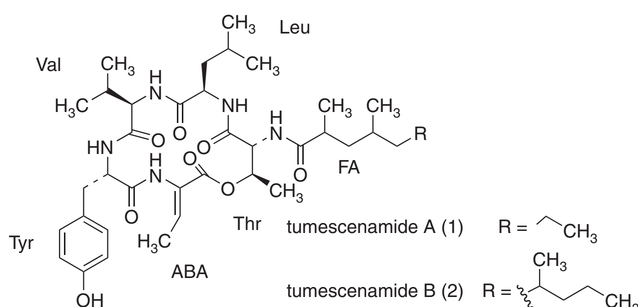

Figure 2 Structures of tumescenamides A (**1**) and B (**2**).

pair of aromatic doublet signals at δ_H 6.6 and 7.1 (each 2H) indicated the presence of a *p*-substituted aromatic ring presumably owing to a tyrosine residue.

These data implied that this LC peak might contain a new metabolite. In addition to this peak, another LC peak (t_R=7.9 min) showed similar NMR signals. Thus we carried out their large-scale purification as follows. The crude EtOAc extract prepared as described above from 4 l of the fermentation broth was subjected to silica gel column chromatography and C-18 reverse-phase HPLC yielding a major component (15 mg) and a minor component (0.6 mg).

Two-dimensional NMR studies of these compounds by COSY, heteronuclear single-quantum coherence and constant-time heteronuclear multiple bond correlation (CT-HMBC)[4] as well as by HR-MS and IR measurements proved them to be new peptides as expected, and they were designated as tumescenamides A (**1**) and B (**2**) (Figure 2).

Structure elucidation

Compound **1** was isolated as a white powder ($[\alpha]^{25}_D$+30, *c* 0.65, MeOH). The HRFAB-MS spectrum of **1** established its molecular formula as $C_{37}H_{57}N_5O_8$ (*m/z* 700.4248 $[M+Na]^+$, calcd. 700.4279). Its peptide structure was clarified by the ^{1}H-NMR, ^{13}C-NMR and heteronuclear single-quantum coherence spectra recorded in acetone-d_6 (Table 1) (Supplementary Figures S1–S6). Diagnostic resonances for five carbonyl carbons (δ_C 176.6 (Leu-1), 171.3 (Thr-1), 171.2 (Val-1), 169.5 (Tyr-1), 165.3 (α-amino-2-butenoic acid (ABA))) and four α-amino methine protons and carbons (δ_H 3.85/δ_C 61.7, 4.45/56.7, 4.92/55.5, and 4.29/54.4), indicated the peptide structure

Table 1 NMR data for tumescenamide A (1) (500 MHz, acetone-d_6)

Position			δ_C	δ_H *(J in Hz)*
Thr	1	C	171.3	
	2	CH	55.5	4.92 (d, 9.0, 3.2)
	3	CH	75.1	5.15 (dq, 3.2, 6.1)
	4	CH_3	16.2	1.34 (d, 6.4)
	NH			7.04 (d, 9.0)
ABA	1	C	165.3	
	2	C	128.0	
	3	CH	134.8	6.57 (q, 7.1)
	4	CH_3	14.7	1.67 (d, 7.1)
	NH			7.98 (s)
Tyr	1	C	169.5	
	2	CH	56.7	4.45 (m)
	3	CH_2	36.6	2.99 (dd, 13.7, 13.7)
				3.29 (dd, 3.4, 13.9)
	4	C	130.0	
	5	CH	130.9	7.19 (d, 8.4)
	6	CH	115.8	6.71 (d, 8.4)
	7	C	156.8	
	NH			
	OH		7.48 (d, 8.6)	
			8.20 (br s)	
Val	1	C	171.2	
	2	CH	61.7	3.85 (dd, 3.9, 3.9)
	3	CH	29.2[a]	2.12 (m)
	4	CH_3	19.2	0.86 (d, 7.1)
	5	CH_3	17.0	0.58 (d, 7.1)
	NH			8.02 (d, 3.9)
Leu	1	C	176.6	
	2	CH	54.4	4.29 (dd, 7.6, 7.6)
	3	CH_2	40.7	1.52, 1.58 (m)
	4	CH	25.2	1.72 (m)
	5	CH_3	22.5	0.88 (d, 7.1)
	6	CH_3	22.9	0.98, d (6.6)
	NH			7.92 (br d, 2.0)
FA	1	C	177.3	
	2	CH	39.1	2.65 (m)
	3	CH_2	42.7	1.67, 1.02 (m)
	4	CH	31.0	1.38 (m)
	5	CH_2	39.9	0.92, 1.14 (m)
	6	CH_2	20.6	1.18, 1.20 (m)
	7	CH_3	14.6	0.78 (t, 7.1)
	2-Me	CH_3	19.1	1.04 (d, 6.8)
	4-Me	CH_3	20.2	0.76 (d, 6.6)

Abbreviations: ABA, α-amino-2-butenoic acid; FA, fatty acid; Leu, leucine; Thr, threonine; Tyr, tyrosine; Val, valine.
[a]Because of overlapping of the solvent peak, this value was obtained by analysis of the HSQC spectrum.

of **1**. Further structural information on **1** was obtained by analyzing heteronuclear single-quantum coherence, DEPT, CT-HMBC and COSY spectra (Supplementary Figures S7 and S8) resulting in the construction of six partial structures: threonine (Thr), valine (Val), leucine (Leu), tyrosine (Tyr), ABA and a fatty-acid (FA) side chain.

The structure of the FA moiety was straightforwardly determined to be 2,4-dimethylheptanoic acid by analysis of its CT-HMBC spectrum as shown in Figure 3. Interestingly, this simple branched FA has never been isolated from the nature. The amino-acid constituents of **1** and FA were then connected on the basis of COSY, CT-HMBC and NOESY correlations (Figure 4; Supplementary Figure S9).

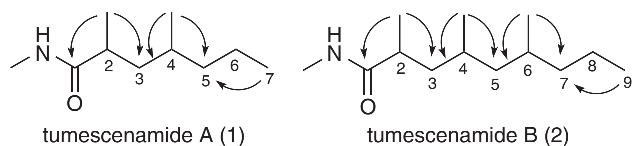

Figure 3 Structures of the FA moiety of tumescenamides A (**1**) and B (**2**).

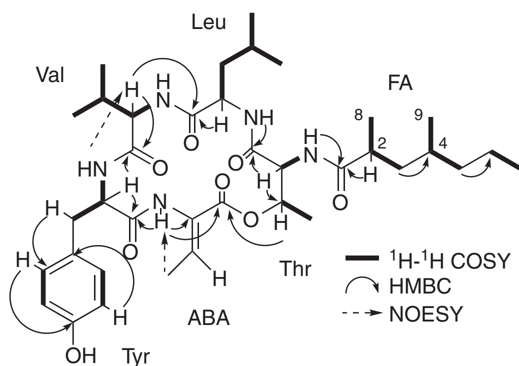

Figure 4 Important COSY, CT-HMBC and NOESY correlations observed for tumescenamide A (**1**).

The linkage of ABA to Tyr was established by CT-HMBC correlations from the NH amide proton of ABA (δ_H: 7.98) and the α-amino proton of Tyr (H-2, δ_H: 4.45) to the Tyr amide carbonyl carbon (C-1, δ_C: 169.5). The Z configuration of ABA was clarified by an NOE between the methyl and amide protons of ABA. The α-amino proton of Tyr (H-2, δ_H: 4.45) and the α-amino proton of Val (H-2, δ_H: 3.85) were correlated to the carbonyl carbon of Val (C-1, δ_C: 171.2) by the CT-HMBC spectrum. A NOESY cross-peak between the NH of Tyr (δ_H: 7.48) and the α-amino proton of Val (H-2) also supported the sequence of Tyr–Val. The connection between Val and Leu was determined through interpretation of CT-HMBC data, which showed correlations from the α-amino proton of Val (H-2, δ_H: 3.85) and the α-amino proton of Leu (H-2, δ_H: 4.29) to the Leu amide carbonyl carbon (C-1, δ_C: 176.6). The NOESY correlation between the amide NH proton of Val (δ_H: 8.02) and the α-amino proton of Leu (H-2) also confirmed this connectivity. The subsequent connection of Leu to Thr was achieved on the basis of CT-HMBC correlations from the NH of Leu (δ_H: 7.92) and the α-amino proton of Thr (H-2, δ_H: 4.92) to the Thr amide carbonyl (C-1, δ_C: 171.3). This connectivity was also supported by the presence of a NOESY correlation between the amide NH proton of Leu (δ_H: 7.92) and the α-amino proton of Thr (H-2, δ_H: 4.92). Further, CT-HMBC correlations from the FA proton (H-2, δ_H: 2.65) and the amide NH proton of Thr (δ_H: 7.04) to the FA carbonyl carbon (C-1, $\ddot{a}_C$: 177.3) led to the assignment of Thr to FA linkage. A NOESY correlation was also observed between these protons. Finally, location of the ester linkage was established by the observation of CT-HMBC correlation from Thr (H-3, δ_H: 5.15) to ABA (C-1, δ_C: 165.3) completing the full planar structure of **1**.

The relative stereochemistry of FA was determined by *J*-based configurational analysis using $^3J_{H-H}$ and $^3J_{C-H}$ (Figure 5).[5,6] For this experiment, we used *J*-resolved HMBC[7] for efficient measurements (Supplementary Figure S10). Both the methyl carbon at C-2 and H-2 exhibited small three-bond couplings with H_b-3 ($J_{CH3(2)-Ha3}$=3.3 Hz, J_{H2-H3b}=5.4 Hz) and thus they were required to be gauche to H_b-3. Anti orientations between H_a-3 and H-2 and between H_b-3 and C-1 were determined from the large three-bond couplings (J_{Ha3-H2}=8.8 Hz, J_{Hb3-C1}=6.3 Hz). Both H-4 and C-5 exhibited small three-bond couplings with H_a-3 (J_{H4-Ha3} =5.9 Hz, J_{C5-Ha3}=2.2 Hz) and were required to be gauche to H_a-3. Anti-orientations between H_a-3 and methyl at C-4 and between H_b-3 and H-4 were determined from the large three-bond couplings ($J_{Ha3-CH3\ (4)}$=5.3 Hz, J_{Hb3-H4}=8.3 Hz). These relationships assigned the relative stereochemistry of FA to 2*R**, 4*R** as shown in Figure 5.

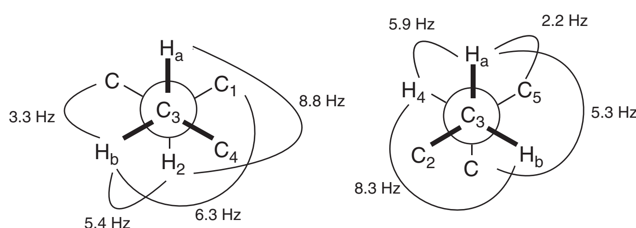

Figure 5 *J*-based configurational analysis of FA at C-2 and C-4.

Hydrolysis of **1** using 6 N HCl yielded a mixture of free amino acids. The hydrolysis product was derivatized using the Marfey reagent[8] and analyzed by HPLC. Comparison with the retention times of authentic Marfey standards of L- and D-forms of Thr, Tyr, Leu and Val showed that the absolute configurations of these amino acids were L-Thr, D-Tyr, L-Leu and L-Val. The absolute stereochemistry of the FA remained to be determined.

Tumescenamide B (**2**) was obtained as a white powder, which analyzed for the molecular formula $C_{40}H_{63}N_5O_8$ by the HRFAB-MS ($[M+H]^+$ *m/z* 742.4756, calcd. 742.4755). The ^{1}H- and ^{13}C-NMR spectral data of the amino-acid moiety of **2** were completely identical with those of **1** (see Experimental procedure) suggesting that the structural difference between them resided in the FA moiety (Supplementary Figures S11–S13). The molecular formula of **2** and its ^{13}C-NMR spectral data indicated that **2** has additional carbons, CH_3 (δ_C 21.0), CH_2 (40.0) and CH (28.2). Its COSY and CT-HMBC spectra proved the structure of the FA moiety to be 2,4,6-trimethylnonanoic acid as shown in Figure 3 (Supplementary Figures S14–S16).

Biological activity

Tested so far, **1** showed weak activity only in the luciferase-reporter assay system, which enabled to observe the expression of insulin-degrading enzyme (IDE). This enzyme, a metalloprotease enzyme responsible for insulin degradation, had been shown to have a key role in β-amyloid (Aβ) peptide degradation both *in vitro* and *in vivo*, and was selective for Aβ monomer.[9–11] Thus, substances which induce the upregulation of IDE enzyme are expected to be promising drugs for the treatment of Alzheimer's disease. Compound **1** induced reporter gene expression under the control of the *IDE* promoter more than three times at a concentration of 100 μM.

EXPERIMENTAL PROCEDURE

General experimental procedures

Optical rotations were recorded on a Jasco DIP-140 polarimeter (Jasco, Tokyo, Japan). UV and IR spectra were recorded on a Hitachi U-3310 spectrophotometer (Hitachi, Ibaraki, Japan) and a Shimadzu 8300 FTIR spectrometer (Shimadzu, Kyoto, Japan), respectively. LC-NMR was carried out on a Varian Inova 500 equipped with a cryo probe (Varian, Palo Alto, CA, USA). The HPLC condition of LC-NMR was as follows: Senshu Pak Pegasil ODS column (4.6×250 mm, flow rate of 0.7 ml min^{-1}; Senshu, Tokyo, Japan), linear gradients started with 70% CH_3CN containing D_2O and finished 100% CH_3CN in 30 min, flow rate of 1 ml min^{-1}).

Both one-dimensional ^{1}H- and ^{13}C-NMR spectra were obtained on a Jeol A-400 (Jeol, Akishima, Tokyo, Japan) or a Varian Inova 500 NMR spectrometer

(Varian), and two-dimensional COSY, NOESY, heteronuclear single-quantum coherence, CT-HMBC and *J*-resolved HMBC spectra were recorded on a Varian Inova 500 NMR spectrometer. Samples were dissolved in acetone-d_6 and the solvent peak was used as an internal standard (δ_H 2.16 and δ_C 30.8). High-resolution FAB mass spectra were obtained using an LCT-premier XE mass spectrometer (Waters, Milford, MA, USA).

HPLC purifications were carried out using a Senshu Pak Pegasil ODS column (20ϕ×250 mm, at a flow rate of 14 ml min^{-1}; Senshu) equipped with a Hitachi High Technologies L-2450 Diode array detector (Hitachi, Ibaraki, Japan). Merck silica gel 60 F_{254} plastic-backed sheets (Merck, Darmstadt, Germany) were used for TLC analysis. Preparative TLC was performed using Merck Si gel 60 F_{254} plastic-backed sheets.

Cultivation of *S. tumescens* YM23-260

S. tumescens YM23-260 was isolated from the sediments of Big drop-off of the Republic of Palau and is maintained at Marine Biotechnology Institute. It showed 99% homology to the type strain of *S. tumescens*. This strain was cultivated in 15 ml test tubes each containing 5 ml of a preliminary seed medium consisting of starch 1.0%, polypeptone 1.0%, molasses 1.0% and meat extract 1.0% (pH 7.2). The test tubes were shaken on a reciprocal shaker (200 r.p.m.) at 28 °C for 2 days. A total of 1 ml aliquot of the seed culture was inoculated into each of the 500 ml baffled Erlenmeyer flasks containing 100 ml of the production medium consisting of starch 2.5%, soybean meal 1.5%, yeast extract 0.2% and $CaCO_3$ 0.4% (pH 6.2). The fermentation was carried out at 28 °C with agitation at 170 r.p.m. After 7 days, the fermentation broth was separated into mycelial cake and filtrate by suction filtration. The mycelial cake was dipped in 60% acetone for 2 h and the mycelium residue was removed by filtration. The extract was evaporated *in vacuo* to remove the acetone and the aqueous residue was extracted with EtOAc. The organic layer was dried over anhydrous Na_2SO_4 and evaporated to afford a brown oil (ca. 3 g from 10 l of the fermentation broth).

Purification of tumescenamides A (1) and B (2)

The brown oil was subjected to Si gel column chromatography developed with $CHCl_3$ and MeOH at 20:1 to give fractions containing **1** and **2**. Both the fractions were combined and dried under reduced pressure. The dried material was finally purified by ODS HPLC. Detection of the compounds was carried out at UV 254 nm. The column was eluted with MeCN in H_2O (70%) to give **1** and **2** in this order. These fractions were separately concentrated *in vacuo* to give pure **1** (white powder, 30.0 mg) and **2** (white powder, 2.5 mg).

Determination of the amino acids and FA stereochemistry

A sample of **1** (6.0 mg) was hydrolyzed in 6 N HCl at 140 °C for 4 h. After concentration to dryness, the residue was dissolved in 10 ml of EtOAc-H_2O (7:3). An amino-acid mixture was recovered in the aqueous layer and FA in the organic layer.

Preparation and HPLC analysis of Marfey derivatives

The aqueous layer was dried *in vacuo* and was added with 5% $NaHCO_3$ (500 μl) and FDAA (Marfey's reagent, 10 mg) in acetone (500 μl). The mixture was heated on a bath at 70 °C for 3 h. The reaction product was analyzed by HPLC as follows: Senshu Pak Pegasil ODS column (20ϕ×250 mm, flow rate of 0.7 ml min^{-1}), TFA mobile phase (0.05%) consisting of 1% methanol in both A and B with 5% MeCN in A and 60% MeCN in B (linear gradients started with 0% B and finished 100% B in 45 min, flow rate of 1 ml min^{-1}). Retention times of the standard FDAA derivatives were as follows: L-Thr 23.8 min, D-Thr 27.8 min, L-Tyr 29.2 min, D-Tyr 30.9 min, L-Leu 31.4 min, D-Leu 40.7 min, L-Val 32.6 min and D-Val 36.9 min. The chromatograms of the hydrolysate derivatized with FDAA showed peaks corresponding to L-Thr, D-Tyr, L-Leu and L-Val.

Tumescenamide A (1). White powder; $[\alpha]^{25}_D$+30 (*c* 0.65, MeOH); UV (MeOH) λ_{max} (ε) 277 (1850), 224 (18900), IR (KBr) 3300, 1720, 1650, 1520 cm^{-1}; ^{1}H-NMR (500 MHz, acetone-d_6) and ^{13}C-NMR (125 MHz, acetone-d_6), see Table 1; HRFAB-MS *m/z* 700.4248 (calcd. for $C_{37}H_{58}N_5O_8$, $[M+H]^+$ 700.4279).

Tumescenamide B (2). White powder; HRFAB-MS *m/z* 742.4756 (calcd. for $C_{40}H_{63}N_5O_8$, $[M+H]^+$ 742.4755). ^{1}H-NMR (400 MHz, acetone-d_6) δ_H 4.93 (d, 9.0, 3.2, Thr-2), 5.13, (dq, 3.2, 6.1, Thr-3), 1.34 (d, 6.1, Thr-4), 7.01 (d, 9.0, Thr NH), 6.59 (d, 7.0, ABA-3), 1.67 (d, 7.0, ABA-4), 8.01 (s, ABA NH), 4.43 (m, Tyr-2), 2.99 (dd, 13.7, 13.7, Tyr-3), 3.29 (dd, 3.4, 13.9, Tyr-3), 7.20 (d, 8.4, Tyr-5), 6.71 (d, 8.40, Tyr-6), 7.46 (d, 8.6, Tyr NH), 3.65 (dd, 3.9, 3.9, Val-2), 2.12 (m, Val-3), 0.86 (d, 7.1, Val-4), 0.58 (d, 7.1, Val-5), 8.02 (d, 3.9, Val NH), 4.30 (dd, 7.6, 7.6, Leu-2), 1.52 and 1.58 (m, Leu-3), 1.72 (m, Leu-4), 0.88 (d, 7.1, Leu-5), 0.98 (d, 6.6, Leu-6), 7.95 (br d, 2.0, Leu NH), 2.66 (m, FA-2), 0.97 and 1.72 (m, FA-3), 1.28 and 1.42 (m, FA-4), 0.78 and 1.12 (m, FA-5), 1.50 (CH, m, FA-6), 0.99 and 1.20 (CH_2, m, FA-7), 1.22 and 1.31 (CH_2, m, FA-8), 0.84 (CH_3, d, 7.0, FA-9), 1.08 (CH_3, d, 6.8, FA-2-Me), 0.78 (CH_3, d, 6.3, FA-4-Me), 0.76 (CH_3, d, 6.5, FA-6-Me). ^{13}C NMR δ_c 171.3 (Thr-1), 55.5 (Thr-2), 75.1 (Thr-3), 16.2 (Thr-4), 165.2 (ABA-1), 128.3 (ABA-2), 134.8 (ABA-3), 14.7 (ABA-4), 169.3 (Tyr-1), 56.7 (Tyr-2), 36.6 (Tyr-3), 130.2 (Tyr-4), 130.9 (Tyr-5), 115.8 (Tyr-6), 156.8 (Tyr-7), 171.2 (Val-1), 61.7 (Val-2), 29.2 (Val-3), 19.2 (Val-4), 17.0 (Val-5), 176.6 (Leu-1), 54.4 (Leu-2), 40.7 (Leu-3), 25.2 (Leu-4), 22.3 (Leu-5), 22.9 (Leu-6), 177.1 (C, FA-1), 38.8 (CH, FA-2), 42.1 (CH_2, FA-3), 28.2 (CH, FA-4), 45.6 (CH_2, FA-5), 30.2 (CH, FA-6), 40.0 (CH_2, FA-7), 20.5 (CH_2, FA-8), 14.6 (CH_3, FA-9), 19.2 (CH_3, FA-2-Me), 21.0 (CH_3, FA-4-Me) and 20.3 (CH_3, FA-6-Me).

Cell culture and bioassay for luciferase activity

We used the reporter plasmid vector (pGL3 basic vector, Promega, Madison, WI, USA) in which the *IDE* promoter gene is inserted.[12] COS7 cells were transfected with the *IDE* reporter gene and maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 50 μg per ml streptomycin and 5 units per ml penicillin. For screening, 1 μl of sample containing 4×10^4 cells was added into 96 multi-well plates and 100 μl of the medium into each well. After incubation for 18 h, the productivity of luciferase in each well was measured with Picagene BrillianStar-LT Luciferase assay system (Toyo B-Net, Tokyo, Japan) and a 2103 EnVision Multilabel Reader (Perkin-Elmer, Waltham, MA, USA).

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