

Oxygenated Hexylitaconates from a Marine Sponge-Derived Fungus *Penicillium* sp.

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In the course of our search for bioactive metabolites from marine organisms, new hexylitaconic acid derivatives (1–4), along with (3*S*)-hexylitaconic acid (5), were isolated from a sponge-derived fungus *Penicillium* sp. Based on the NMR and MS data, the structures of compounds 1–4 were defined as α,β -dicarboxylic acid derivatives, such as hexylitaconic acid and tenysuic acids which were previously reported as metabolite of *Aspergillus niger*, *Penicillium striatisporum*, or *Apiospora montagnei*. The isolated compounds were evaluated for cytotoxicity against a panel of five human solid tumor cell lines, and for anti-inflammatory activity gauged by their inhibitory effects on the production of major pro-inflammatory mediators (nitric oxide (NO), interleukin (IL)-6, tumor necrosis factor (TNF)- α , and IL-1 β) in murine macrophage cells. Compounds 1 and 4 showed weak inhibition of IL-1 β production at the concentration of 200 μ M.

Key words sponge-derived fungus; *Penicillium* sp.; dicarboxylic acid; hexylitaconate; cytotoxicity; anti-inflammatory

Although dicarboxylic acids do not occur in appreciable amount as components of animal or vegetal lipids, generally they are important metabolic products of fatty acids. Especially the α,β -dicarboxylic acid and its derivatives, such as itaconic acid^{1,2} and citraconic acid derivatives,³ have been of much interest for their biosynthesis, stereochemistry, and chemical relationship. Furthermore, some of them were reported to exhibit various interesting bioactivities, such as plant growth-stimulation,⁴ production of a cellulolytic active oxygen species, inhibition of the iron redox reaction,⁵ inhibition of p53-HDM2 interaction which can induce apoptosis in human cancer cells,⁶ and anti-trypanosomal activity.⁷

In the course of search for bioactive metabolites from marine organisms, five hexylitaconic acid derivatives including four new ones (1–4) were isolated from a sponge-derived fungus *Penicillium* sp. Herein we describe the structure elucidation and the biological evaluation of the compounds.

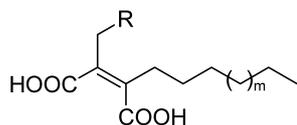
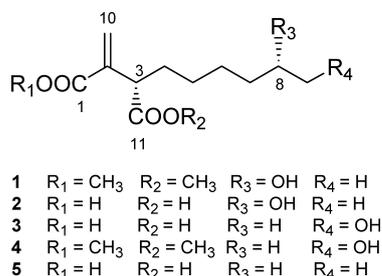
Results and Discussion

Accurate mass measurement (high resolution (HR)-FAB-MS) of compound 1 (m/z 259.1549 [M+H]⁺, Δ +0.4 mmu) revealed the molecular formula to be C₁₃H₂₂O₅ with three degrees of unsaturation. Thirteen carbon signals were evident in the ¹³C-NMR spectrum (Table 1) and deduced to be three methyl, five methylene, two methine, and three quaternary carbons from the distortionless enhancement by polarization transfer (DEPT)135 spectrum. According to ¹H- and ¹³C-NMR data, two carbonyl groups (δ_C 166.8, C-1, δ_C 174.2, C-11), and one exo-methylene group (δ_H 5.76/6.31, δ_C 126.8 and C-10, δ_C 138.2, C-2) were evident. Other structural elements obvious from 1D-NMR data were two methoxy groups (δ_H 3.64, δ_C 50.7, OCH₃-1, δ_H 3.71, δ_C 50.0, OCH₃-11) and an oxymethine carbon (δ_H 3.69, δ_C 67.3, C-8). Considering the molecular formula and proton signals evident from ¹H-NMR data, the remaining proton had to be present

Table 1. ¹H- and ¹³C-NMR Data of Compounds 1–3 in CD₃OD

	1		2		3	
	¹³ C ^{a)}	¹ H	¹³ C	¹ H	¹³ C	¹ H
1	166.8		168.7		168.3	
2	138.2		140.0		140.7	
3	47.1	3.48, t (7.2)	47.8	3.50, t (7.5)	47.8	3.44, t (7.5)
4a	31.0	1.89, m	31.0	1.86, m	31.1	1.90, m
4b		1.66, m		1.66, m		1.72, m
5	27.4	1.33, m	27.5	1.33, m	27.7	1.40, m
6	25.8	1.33, m	25.4	1.33, m	29.3	1.40, m
7	38.9	1.42, m	38.8	1.41, m	26.0	1.40, m
8	67.3	3.69, m	67.3	3.70, m	33.1	1.53, m
9	22.2	1.12, d (6.0)	22.3	1.13, d (8.0)	61.3	3.51, t (6.0)
10a	126.8	6.31, s	125.5	6.29, s	125.8	6.31, s
10b		5.76, s		5.72, s	31.0	5.76, s
11	174.2		176.1		174.9	
1-OCH ₃	50.7	3.64, s				
11-OCH ₃	50.0	3.71, s				

¹H-NMR data of 1–3 were measured at 500 MHz, and ¹³C-NMR data of 2 and 3 were measured at 100 MHz. ^{a)} ¹³C assignments were supported by a HSQC experiment.



chaetomelic acid A	R = H	m = 9
chaetomelic acid C	R = H	m = 7
chaetomelic acid D	R = OH	m = 9
hexylcitraconic acid	R = H	m = 1

Fig. 1. Chemical Structures of Compounds 1–5 and Chaetomelic Acids

as a hydroxy group. In the ¹H-NMR spectrum, the terminal methyl proton showed coupling with the oxymethine proton (δ_{H} 3.69). The vicinity of H-7, H-6, and H-5 was proven from correlation spectroscopy (COSY) correlations. Further COSY correlations from H-5, H-4 and H-3 were observed. The partial structures were extended according to heteronuclear multiple bond connectivity (HMBC) correlations (Fig. 2). In the HMBC spectrum, another methine proton (δ_{H} 3.48, H-3) showed correlations with six carbons at δ_{C} 166.8 (C-1), 138.2 (C-2), 31.0 (C-4), 27.4 (C-5), 126.8 (C-10), and 174.2 (C-11). The two methoxyl groups (OCH₃-1, OCH₃-11) were defined as a part of esters. On the basis of the aforementioned observations, compound 1 was defined as methyl 8-hydroxy-3-methoxycarbonyl-2-methylenenonanoate, which is closely related to hexylitaconic acids from the same fungal genus.⁸ The stereochemistry of compound 1 could not be intensively studied due to the paucity of material. Compound 1 was defined as dimethyl ester of compound 2, presumably with the same stereochemistry at C-3 and C-8 (8*R*, *vide infra*). The same sign of optical rotation with that of 2 was also suggestive of the same configuration. The configurations at C-3 of compounds 1 and 2 were assumed to be the same (3*S*) as those of other co-isolated compounds (3–5).

A molecular formula of C₁₁H₁₈O₅ was determined for compound 2 by positive ion FAB-MS (m/z 229 [M+H]⁺) and NMR data. ¹H- and ¹³C-NMR analyses revealed that compound 2 is the free carboxylic acid form of compound 1. Two overlapped signals of the acid protons were observed at δ_{H} 12.7 (in dimethyl sulfoxide (DMSO)). The absolute configuration at C-3 was assumed to be the same (3*S*) as those of other co-isolated compounds (3–5). The absolute configuration at C-8 was defined by the modified Mosher's method.⁹ Positive $\Delta\delta$ ($\delta_{\text{S}} - \delta_{\text{R}}$) value for H-9 (+0.029 ppm) and negative $\Delta\delta$ ($\delta_{\text{S}} - \delta_{\text{R}}$) value for H-6 (−0.003 ppm) were observed, indicating the 8*R* configuration (Fig. 3).

Compound 3 showed a [M+H]⁺ ion peak at m/z 229. The examination of the ¹H-NMR and FAB-MS spectrum revealed that it is isomeric to compound 2. In the DEPT spectrum, the signals of the terminal methyl group (CH₃-9, δ_{C} 22.3) and the methine group (−OCH-8, δ_{C} 67.3) were replaced by two

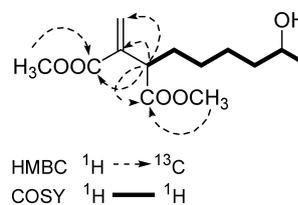


Fig. 2. Key COSY and HMBC Correlations of Compound 1

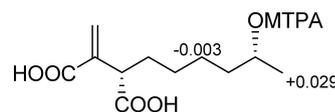


Fig. 3. $\Delta\delta$ ($\delta_{\text{S}} - \delta_{\text{R}}$) Values for the MTPA Esters of Compound 2

methylene groups (CH₂-9, δ_{C} 61.3 and CH₂-8, δ_{C} 33.1), indicating that the substitution position of the hydroxy group was changed to C-9 (δ_{C} 61.3). This was further confirmed by the HMBC and COSY correlations. The absolute configuration at C-3 was defined as *S* by comparison of the optical rotation ($[\alpha]_{\text{D}}^{23} +11$) with those of (3*S*)-3-methylitaconic acid ($[\alpha]_{\text{D}}^{23} +3.58$).¹⁰ It was also found that both stereoisomers of hexylitaconic acid are present in nature,^{4,11} and (*R*)-(−)/(*S*)-(+) relationship was revealed by synthesis and vibrational circular dichroism technique.¹² Therefore, compound 3 was defined as (3*S*)-9-hydroxy-3-carboxy-2-methylenenonanoate. Compound 4 was a dimethyl ester of compound 3, which was previously reported as an intermediate in the chemical synthesis of tenzyuic acid, anti-trypanosomal activity against *Trypanosoma brucei* was reported for compound 4.⁷ To the best of our knowledge, compounds 2–4 were first isolated from a natural source.

In addition to these new secondary metabolites, the strain of *Penicillium* sp. produced (3*S*)-hexylitaconic acid (5).^{4,12}

Itaconic acid has been found as a metabolite of filamentous fungi such as *Aspergillus itaconicus*,¹³ *Helicobasidium mompa*,¹⁴ *Ustilago zeae*,¹⁵ and *U. maydis*¹⁶ and some yeast belonging to the genus *Candida*.¹⁷ Whilst alkylated derivatives (alkylitaconic acids) have been isolated from lichens and several fungi. The other analogues of alkylitaconic acid are alkylcitraconic acids, called chaetomelic acids (Fig. 1). These metabolites are a viable target for the development of anticancer drugs owing to their strong effects on rat sarcoma (RAS) farnesyl-protein transferase.¹⁸ Because compounds 1–5 share close structural similarity to chaetomelic acids, they were evaluated for cytotoxicity against a panel of five human solid tumor cell lines (A549, lung cancer; SK-OV-3, ovarian cancer; SK-MEL-2, skin cancer; XF498, CNS cancer; and HCT15, colon cancer). However, they did not show significant activity, and the EC₅₀ was higher than 30 $\mu\text{g}/\text{ml}$. It might be suggested that the citraconate core in chaetomelic acids is crucial for the farnesyl transferase inhibitory effect. Or on the other hand, the alkyl chain length may modulate the binding affinity to the hydrophobic pocket of the enzyme. Compared to C₁₅ farnesylpyrophosphate, shorter alkyl chain of compounds 1–5 leads to poor binding and inhibitory activity. In fact, the chaetomelic acids with shorter alkyl side chain (hexylcitraconic acid) were completely inactive against FPTase,¹⁸ which is consistent with the molecular ruler hypothesis of substrate binding and specificity.¹⁹

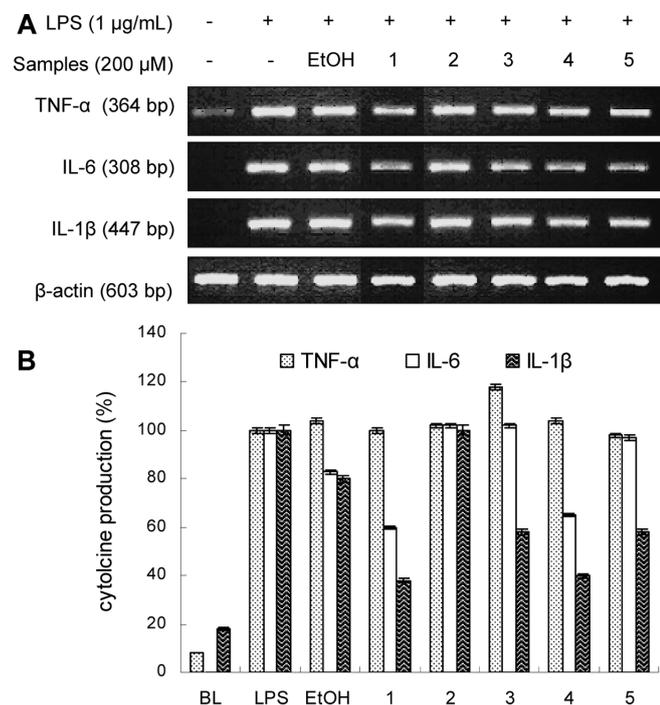


Fig. 4. (A) Inhibitory Effect of Compounds 1—5 on mRNA Expression of Cytokines (TNF- α , IL-6, and IL-1 β) in the LPS-Stimulated RAW264.7 Cells

RAW264.7 cells (1.5×10^5 cells/ml) were stimulated with LPS (1 $\mu\text{g}/\text{ml}$) alone or with test samples at the concentration of 200 μM for 18 h (BL, blank). Total RNA was isolated and mRNA expression of cytokines was determined by RT-PCR.

(B) Inhibitory Effect of Compounds 1—5 on the Production of Cytokines (TNF- α , IL-6, and IL-1 β) in the LPS-Stimulated RAW264.7 Cells

RAW264.7 cells (1.5×10^5 cells/ml) were stimulated with LPS (1 $\mu\text{g}/\text{ml}$) alone or with test samples at the concentration of 200 μM for 24 h (BL, blank). The production of cytokines was determined by Griess ELISA method. The data represent the mean \pm S.D. of triplicate experiments.

Compounds 1—5 were also evaluated for their anti-inflammatory activity gauged by their inhibitory effects on the production of major pro-inflammatory mediators (nitric oxide (NO), interleukin (IL)-6, tumor necrosis factor (TNF)- α , and IL-1 β) in murine macrophage cells. Compounds 1 and 4 showed weak inhibition of IL-1 β production at the concentration of 200 μM (Fig. 4). The ester forms (1, 4) showed higher inhibition on IL-6 and IL-1 β expression than free acid forms (2, 3, 5). Higher lipophilicity of the ester forms (1, 4) might deliver enhanced cell permeability.

Experimental

General Procedures 1D- and 2D-NMR spectra were recorded on Varian UNITY 400 and Varian INOVA 500 spectrometers. Chemical shifts were reported with reference to the respective residual solvent or deuterated solvent peaks (δ_{H} 3.30 and δ_{C} 49.0 for CD_3OD , δ_{H} 2.50 and δ_{C} 39.7 for DMSO). FAB-MS data were obtained on a JEOL JMS SX-102A. HR-FAB-MS data were obtained on a JEOL JMS SX-101A. HPLC was performed on a Gilson 370 pump with an YMC packed J'sphere ODS-H80 column (250×10 mm, 4 μm , 80 \AA) using a Shodex RI-71 detector.

Fungal Strain The fungal strain *Penicillium* sp. (J05B-3-F-1) was isolated from a sponge *Stelletta* sp. collected from the coast of Jeju island, Korea, in October 2005. The microorganism was identified by Dr. K. S. Bae. The culture collection is deposited at the Marine Natural Product Laboratory, PNU. The fungus was cultured in MEA medium (prepared with 75% sea water) containing glucose (20 g/l), malt extract (20 g/l), agar (20 g/l) and peptone (1 g/l). The fungus was cultured at 30 $^{\circ}\text{C}$ on a shaker platform at 160 rpm for 28 d, in total of 201.

Extraction and Isolation Culture medium and mycelia of *Penicillium* sp. (J05B-3-F-1) were separated by filtration, and extracted with EtOAc and

MeOH, respectively, the combined crude extract (3.8 g) was partitioned between CH_2Cl_2 and water. The CH_2Cl_2 layer was further partitioned between aqueous 90% MeOH and *n*-hexane. The 90% MeOH layer (2.6 g) was subjected to a stepped-gradient MPLC (ODS-A, 120 \AA , S-30/50 mesh) eluting with 20 to 100% MeOH to afford 25 fractions. Fraction 10 (30.1 mg), which showed some interesting signals in $^1\text{H-NMR}$ spectrum, was subjected to a reversed-phase HPLC (YMC packed J'sphere ODS-H80 column, 250×10 mm, 4 μm , 80 \AA) eluting with 65% MeOH to afford compounds 1 (1.4 mg) and 4 (1.1 mg). Compound 2 (5.4 mg) was obtained by a separation of the fraction 5 (38.8 mg) on a reversed-phase HPLC eluting with 50% MeOH. Compound 3 (7.0 mg) was obtained by a separation of the fraction 6 (55.3 mg) on a reversed-phase HPLC eluting with 45% MeOH. Fraction 11 (51.4 mg) was subjected to a reversed-phase HPLC eluting with 65% MeOH to afford compound 5 (10.2 mg).

(3*S*,8*R*)-Methyl 8-Hydroxy-3-methoxycarbonyl-2-methylenonanoate (1): Colorless oil; $[\alpha]_{\text{D}}^{25} +17$ ($c=1.0$, MeOH), $^1\text{H-NMR}$ data (CD_3OD , 500 MHz), see Table 1; $^{13}\text{C-NMR}$ data (CD_3OD , 100 MHz), see Table 1; HR-FAB-MS m/z 259.1549 $[\text{M}+\text{H}]^+$ (Calcd for $\text{C}_{13}\text{H}_{23}\text{O}_5$, 259.1545); FAB-MS m/z 259 $[\text{M}+\text{H}]^+$; 241 $[\text{MH}^+-\text{H}_2\text{O}]^+$, 209 $[\text{MH}^+-\text{H}_2\text{O}-\text{OCH}_3]^+$.

(3*S*,8*R*)-8-Hydroxy-3-carboxy-2-methylenonanoic Acid (2): Colorless oil; $[\alpha]_{\text{D}}^{25} +7.5$ ($c=1.0$, MeOH), $^1\text{H-NMR}$ data (CD_3OD , 500 MHz), see Table 1; $^{13}\text{C-NMR}$ data (CD_3OD , 100 MHz), see Table 1; FAB-MS m/z 231 $[\text{M}+\text{H}]^+$; 213 $[\text{MH}^+-\text{H}_2\text{O}]^+$.

(3*S*)-9-Hydroxy-3-carboxy-2-methylenonanoic Acid (3): Colorless oil; $[\alpha]_{\text{D}}^{25} +11$ ($c=1.0$, MeOH), $^1\text{H-NMR}$ data (CD_3OD , 500 MHz), see Table 1; $^{13}\text{C-NMR}$ data (CD_3OD , 100 MHz), see Table 1; FAB-MS m/z 231 $[\text{M}+\text{H}]^+$; 213 $[\text{MH}^+-\text{H}_2\text{O}]^+$.

(3*S*)-Methyl 9-Hydroxy-3-methoxycarbonyl-2-methylenonanoate (4): Colorless oil; $[\alpha]_{\text{D}}^{25} +13$ ($c=1.0$, MeOH), FAB-MS m/z 259 $[\text{M}+\text{H}]^+$; 241 $[\text{MH}^+-\text{H}_2\text{O}]^+$, 228 $[\text{MH}^+-\text{OCH}_3]^+$. ^1H - and ^{13}C -NMR data were in good agreement with literature values.⁷⁾

(3*S*)-Hexylitaconic Acid (5): Orange oil; $[\alpha]_{\text{D}}^{25} +13$ ($c=1.0$, MeOH), FAB-MS m/z 209 $[\text{M}+\text{H}]^+$; ^1H - and ^{13}C -NMR data were in good agreement with literature values.^{4,12)}

α -Methoxy- α -(trifluoromethyl)phenylacetic acid (MTPA) Esters of (+)-8-Hydroxyhexylitaconic Acid (2) Compound 2 (1.0 mg) was treated with (*R*)-(-) and (*S*)-(+)-MTPA chloride (4 μl) in dry pyridine (50 μl) to yield (*S*)-MTPA ester and (*R*)-MTPA ester, respectively. After being kept at room temperature for 24 h, the reaction mixtures were evaporated to dryness under vacuum. The MTPA Esters were obtained by the separation of the residues on a reversed-phase HPLC (YMC packed J'sphere ODS-H80 column, 250×10 mm, 4 μm , 80 \AA) eluting with 50% MeOH at a flow of 1.0 ml/min.

Evaluation of Cytotoxicity The rapidly growing cells were harvested, counted, and inoculated at the appropriate concentrations [(1—2) $\times 10^4$ cells/well] into 96-well microtiter plates. After incubation for 24 h, the compounds dissolved in culture medium (RPMI 1640, Gibco; 10% fetal bovine serum (FBS), Gibco) were applied to the culture wells in triplicate followed by incubation for 48 h at 37 $^{\circ}\text{C}$ under a 5% CO_2 atmosphere. The culture was fixed with cold trichloroacetic acid (TCA) and was stained by 0.4% sulforhodamine B (SRB), (Sigma) dissolved in 1% acetic acid. After solubilizing the bound dye with 10 mM unbuffered Tris base by a gyrotatory shaker, the absorbance at 520 nm was measured with a microplate reader (Dynatech Model MR 700). Fifty percent inhibitory concentration (EC_{50}) was defined as the concentration that reduced absorbance by 50% compared to the control level in the untreated wells.

Nitrite Assay The production of nitric oxide (NO) was measured, as previously described by Ryu *et al.*,²⁰⁾ by using the Griess reagent (Sigma, MO, U.S.A.). Briefly, the RAW 264.7 cells were stimulated with lipopolysaccharide (LPS) (1 $\mu\text{g}/\text{ml}$), and 100 μl of the supernatant was mixed with 100 μl of the Griess reagent (0.1% naphthylene diamine dihydrochloride, 1% sulfanilamide, 2.5% H_3PO_4). The mixture was incubated for 10 min at room temperature. Absorbance at 540 nm was measured using an ELISA reader (Amersham Pharmacia Biotech, U.K.), and the results were compared against a calibration curve using sodium nitrite as the standard.

Measurement of Production of Pro-inflammatory Cytokines (IL-6, IL-1 β and TNF- α) The inhibitory effects of the isolated compounds on IL-6, IL-1 β , and TNF- α production were determined by the method previously described by Cho *et al.*²¹⁾ The samples were dissolved with EtOH and diluted with DMEM. The final concentration of chemical solvents did not exceed 0.1% in the culture medium. At these conditions, none of the solubilized solvents altered IL-6, IL-1 β , and TNF- α production in RAW 264.7 cells. Before stimulation with LPS (1 $\mu\text{g}/\text{ml}$) and testing samples, RAW 264.7 cells were incubated for 18 h in 24-well plates under the same condi-

tions. Stimuli and the testing samples were then added to the culture cells for 6 h. Supernatants were then collected and assayed for IL-6, IL-1 β and TNF- α content using mouse enzyme-linked immunosorbent assay (ELISA) kits (R & D Systems Inc., MN, U.S.A.).

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References

- 1) Li N., Lin G., Kwan Y. W., Min Z. D., *J. Chromatogr. A*, **849**, 349—355 (1999).
- 2) Amirta R., Fujimori K., Shirai N., Honda Y., Watanabe T., *Chem. Phys. Lipids*, **126**, 121—131 (2003).
- 3) Singh S. B., Zink D. L., Liesch J. M., Goetz M. A., Jenkins R. G., Nallin-Omstead M., Silverman K. C., Bills G. F., Mosley R. T., Gibbs J. B., Albers-Schonberg G., Lingham R. B., *Tetrahedron*, **49**, 5917—5926 (1993).
- 4) Isogal A., Washizu M., Kondo K., Murakoshi S., Suzuki A., *Agric. Biol. Chem.*, **48**, 2607—2609 (1984).
- 5) Enoki M., Honda Y., Kuwahara M., Watanabe T., *Chem. Phys. Lipids*, **120**, 9—20 (2002).
- 6) Tsukamoto S., Yoshida T., Hosono H., Ohta T., Yokosawa H., *Bioorg. Med. Chem. Lett.*, **16**, 69—71 (2006).
- 7) Matsumaru T., Sunazuka T., Hirose T., Ishiyama A., Namatame M., Fukuda T., Tomoda H., Otaguro K., Ōmura S., *Tetrahedron*, **64**, 7369—7377 (2008).
- 8) Stewart M., Capon R. J., Lacey E., Tennant S., Gill J. H., *J. Nat. Prod.*, **68**, 581—584 (2005).
- 9) Ohtani I., Kusumi T., Kashman Y., Kakisawa H., *J. Am. Chem. Soc.*, **113**, 4092—4096 (1991).
- 10) Günther H., Wolfgang B., *Eur. J. Biochem.*, **156**, 301—304 (1986).
- 11) Klemke C., Kehraus S., Wright A. D., Höning G. M., *J. Nat. Prod.*, **67**, 1058—1063 (2004).
- 12) Nakahashi A., Miura N., Monde K., Tsukamoto S., *Bioorg. Med. Chem. Lett.*, **19**, 3027—3030 (2009).
- 13) Bonnarme P., Gillet B., Sepulchre A. M., Role C., Beloeil J. C., Ducrocq C., *J. Bacteriol.*, **177**, 3573—3578 (1995).
- 14) Araki T., Yamazaki Y., Suzuki N., *Bull. Natl. Inst. Agric. Sci. Jpn. Ser.*, **C8**, 53—55 (1957).
- 15) Haskins R. H., Thom J. A., Boothroyd B., *Can J. Microbiol.*, **1**, 749—756 (1955).
- 16) Guevarra E. D., Tabuchi T., *Agric. Biol. Chem.*, **54**, 2353—2358 (1990).
- 17) Tabuchi T., Sugisawa T., Ishidori T., Nakahara T., Sugiyama J., *Agric. Biol. Chem.*, **45**, 475—479 (1981).
- 18) Singh S. B., Jayasuriya H., Silverman K. C., Bonfiglio C. A., Williamson J. M., Lingham R. B., *Bioorg. Med. Chem.*, **8**, 571—580 (2000).
- 19) Long S. B., Casey P. J., Beese L. S., *Biochemistry*, **37**, 9612—9618 (1998).
- 20) Ryu S. Y., Oak M. H., Yoon S. K., Cho D. I., Yoo G. S., Kim T. S., Kim K. M., *Planta Med.*, **66**, 358—360 (2000).
- 21) Cho J. Y., Baik K. U., Jung, J. H., Park M. H., *Euro. J. Pharm.*, **398**, 399—407 (2000).