Tensyuic Acids, New Antibiotics Produced by *Aspergillus niger* FKI-2342

Yoko HASEGAWA,[a] Takashi FUKUDA,[a] Keiichi HAGIMORI,[a] Hiroshi TOMODA,*[,b] and Satoshi ŌMURA*[,a,c]

[a] *Kitasato Institute for Life Sciences & Graduate School of Infection Control Sciences, Kitasato University;* [b] *School of Pharmacy, Kitasato University; 5–9–1 Shirokane, Minato-ku, Tokyo 108–8641, Japan: and* [c] *Kitasato Institute; 5–9–1 Shirokane, Minato-ku, Tokyo 108–8642, Japan.*

Received May 15, 2007; accepted June 29, 2007; published online July 3, 2007

Six new alkylitaconic acids, designated tensyuic acids A to F, were isolated from the culture broth of *Aspergillus niger* FKI-2342 by solvent extraction, silica gel column chromatography and HPLC. Their structures were elucidated by spectroscopic analysis including UV, NMR, and MS. They are all alkylitaconic acid derivatives. Only tesyuic acid C showed moderate antimicrobial activity against *Bacillus subtilis*.

Key words tensyuic acid; itaconic acid derivative; *Aspergillus niger*; fungal metabolite

During the course of screening for new antibiotics, we reported tensidoles A and B as the potentiators of miconazole activity from the culture broth of *Aspergillus niger* FKI-2342.[1)] Further investigation of the culture broth led to discovery of new six compounds designated tensyuic acids A to F. In this study, we describe the isolation, structure elucidation and biological properties of tensyuic acids. All tensyuic acids were found to be new alkylitaconic acid derivatives.

Experimental

General Experimental Procedures The strain FKI-2342 was isolated from soil collected at Oura Tensyudou, Nagasaki, Japan and was used for production of tensyuic acids. Optical rotations were recorded with a DIP-370 digital polarimeter (Jasco, Tokyo, Japan). FAB-MS spectrometry was conducted on a JMS-AX505H spectrometer (Jeol, Tokyo, Japan). UV and IR spectra were measured with a DU640 spectrophotometer (Beckman, California, U.S.A.) and an FT-210 Fourier transform infrared spectrometer (Horiba, Kyoto, Japan). The various NMR spectra were measured with a MERCURY plus 300 MHz spectrometer (Varian, California, U.S.A.).

Antimicrobial Activity Antimicrobial activity against 6 species of microorganisms was measured by the agar diffusion method using paper disks. The microorganisms were as follows; *Bacillus subtilis* PCI 219, *Micrococcus luteus* PCI 1001, *Escherichia coli* NIHJ, *Xanthomonas oryzae* KB 88, *Mucor racemosus* IFO 4581 and *Candida albicans* ATCC 64548. Media for microorganisms were as follows: Taiyo agar (Shimizu Syokuhin Kaisya Ltd.) for the bacteria; a medium composed of 1.0% yeast extract and 0.8% agar for fungi and yeasts. A paper disk (i.d. 6 mm, ADVANTEC) containing 25 or 50 μg of a sample was placed on an agar plate. Bacteria were incubated at 37 °C for 24 h. Yeasts and fungi were incubated at 27 °C for 24 h. Antimicrobial activity was expressed as diameter (mm) of the inhibitory zone.

Results

Isolation of Tensyuic Acids from the Culture Broth of *Aspergillus niger* FKI-2342 To the 6-d old culture broth (20 l) was added acetone (20 l). After the acetone extracts were filtered and concentrated, the resulting aqueous solution was extracted with ethyl acetate (20 l). The ethyl acetate layer was dried over Na_2SO_4 and concentrated *in vacuo* to dryness to yield an oily material (20.4 g). The material was dissolved in a small volume of $CHCl_3$, applied on a silica gel column (80 g, 3.4×18 cm, 70—230 mesh, Merck), and eluted stepwise with 100 : 0, 100 : 1, 75 : 1, 50 : 1, 25 : 1, 1 : 1, and 0 : 100 (v/v) of $CHCl_3$–CH_3OH solvents (1000 ml each). The antimicrobial activity was observed in the 50 : 1 fraction, which was concentrated to give a brown material (579.0 mg). The material was purified by HPLC; ODS column (20×250 mm, Pegasil, Senshu Sci. Co., Tokyo, Japan), a 50-min linear gradient from 20 to 55% CH_3CN in 0.05% CF_3COOH, 8.0 ml/min, and UV at 210 nm. Under the conditions, tensyuic acids A to F were eluted as peaks with retention times of 28.0, 28.4, 35.2, 40.8, 42.0 and 24.0 min, respectively (Fig. 2). Each peak was collected and concentrated to yield tensyuic acids A (7.3 mg), B (5.5 mg), C (7.3 mg), D (15.0 mg), E (3.8 mg) and F (6.8 mg) as pale yellow syrup.

Physico-chemical Properties Physico-chemical properties of tensyuic acids A to F are summarized in Table 1. Similarity in their data indicated that they are structurally related. All compounds showed the same absorptions at 201—207 nm in the UV spectra. Their IR spectra showed absorptions at 3482—3448 and 1737—1716 cm^{-1}, suggesting the presence of hydroxyl acid groups.

Structural Elucidation Tensyuic Acid A: The molecular formula of tensyuic acid A was determined to be $C_{11}H_{16}O_6$ on the basis of HR-FAB-MS measurement (Table 1). The ^{13}C-NMR spectrum (in $CDCl_3$) showed 11 resolved signals, which were classified into two methyl carbons, three

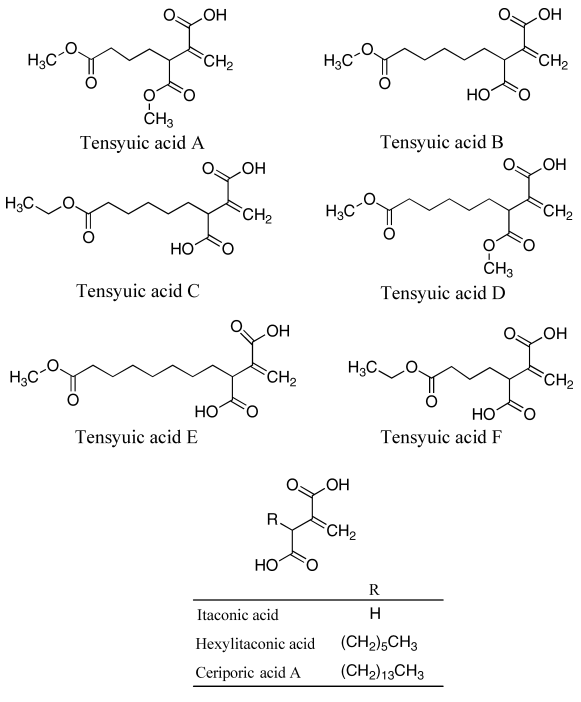

Fig. 1. Structures of Tensyuic Acids and Related Compounds

* To whom correspondence should be addressed. e-mail: tomodah@pharm.kitasato-u.ac.jp; omura-s@kitasato.or.jp

methylene carbons, one sp^2 methylene carbon, one methine carbon, one sp^2 quaternary carbon and three carbonyl carbons by analysis of DEPT spectra. The ^{1}H-NMR spectrum (in $CDCl_3$) showed three methylene signals, one methine signal, one sp^2 methylene signal and two methyl signals. The connectivity of proton and carbon atoms was established by the ^{13}C–^{1}H HMQC spectrum as shown in Table 2. Analysis of the ^{1}H–^{1}H COSY and HMBC spectra revealed the partial structure I (Fig. 3). The ^{13}C–^{1}H long range couplings of ^2J and ^3J observed in the HMBC experiments (Fig. 4) gave the following linkages. 1) The cross peaks from 8-H_2 (δ 5.88, 6.51) to C-1 (δ 170.8)), C-2 (δ 137.4) and C-3 (δ 46.1), from 3-H (δ 3.50) to C-1, C-2, C-8 (δ 129.5) and C-9 (δ 173.3), and from 5-H_2 (δ 1.28) and 6-H_2 (δ 2.34) to C-7 (δ 173.7) supported the partial structure I. 2) The cross peaks from 10-H_3 (δ 3.69) to C-9 and from 11-H_3 (δ 3.66) to C-7

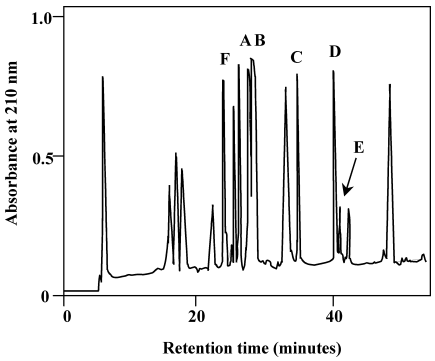

Fig. 2. A Chromatographic Profile of Purification of Tensyuic Acids by Preparative HPLC

Column, Senshu Pak PEGASIL ODS (20×250 mm); solvent, 50-min linear gradient from 20 to 55% CH_3CN in 0.05% CF_3COOH; detection, UV at 210 nm; flow rate, 8.0 ml/min; sample, 10 mg of active materials dissolved in 10 ml of MeOH.

showed that the partial structure I was linked to two *O*-methyl moieties (Fig. 4). Furthermore, C-1 should be a free carboxylic acid from the IR spectrum and the molecular formula. Taken together, the structure of tensyuic acid A was elucidated as shown in Fig. 1.

Tensyuic Acid B: The molecular formula ($C_{12}H_{18}O_6$) of tensyuic acid B was CH_2 bigger than that of tensyuic acid A (Table 1). The ^{13}C-NMR spectrum (in $CDCl_3$) showed 12 resolved signals, which were classified into one methyl carbon, five methylene carbons, one sp^2 methylene carbon, one methine carbon, one sp^2 quaternary carbon and three carbonyl carbons by analysis of DEPT spectra. The ^{1}H-NMR spectrum (in $CDCl_3$) showed five methylene signals, one methine signal, one sp^2 methylene signal and one methyl signal. Analysis of the ^{1}H–^{1}H COSY and HMBC spectra revealed the partial structure II (Fig. 5). The cross peaks from 10-H_2 (δ 5.86, 6.51) to C-1 (δ 171.5), C-2 (δ 137.5) and C-3 (δ 46.8) and from 3-H (δ 3.46) to C-1, C-2, C-10 (δ 129.9) and C-11 (δ 179.2), from 7-H_2(δ 1.61) and 8-H_2 (δ 2.31) to C-9 (δ 174.6) and from 12-H_3 (δ 3.66) to C-9 showed proof of the partial structure II (Fig. 6). The chemical shift of C-11 (δ 179.2) was found to shift to the lower field than that of the corresponding methylated calboxylic acid C-9 (δ 173.3) of tensyuic acid A, resulting in its assignment as a free carboxylic acid. Thus, the structure of tensyuic acid B was elucidated as shown in Fig. 1.

Tensyuic Acid C: The molecular formula ($C_{13}H_{20}O_6$) of tensyuic acid C is CH_2 bigger than that of tensyuic acid B. The calboxylic acid C-9 (δ 173.9) was ethylated in tensyuic acid C, while methylated in tensyuic acid B (the partial structure III in Fig. 7). The cross peak from 12-H_2 (δ 4.05) to C-9 was observed in the HMBC experiments to confirm the position (Fig. 7). The molecular formula supported this structure.

Table 1. Phyisico-chemical Properties of Tensyuic Acids A to F

	A	B	C
Appearance	Yellow syrup	Yellow syrup	Yellow syrup
$[\alpha]_D^{26}$	+1.8 (c=0.1, CH_3OH)	−1.7 (c=0.1, CH_3OH)	−6.0 (c=CH_3OH)
Molecular formula	$C_{11}H_{16}O_6$	$C_{12}H_{18}O_6$	$C_{13}H_{20}O_6$
Molecular weight	244	258	272
HR-FAB-MS m/z $(M+H)^+$			
Calcd	245.1025 (for $C_{11}H_{17}O_6$)	259.1182 (for $C_{12}H_{19}O_6$)	273.1338 (for $C_{13}H_{21}O_6$)
Found	245.1024	259.1183	273.1335
UV $\lambda_{max}^{CH_3OH}$ nm (ε)	203 (6000), 205 (5600)	204 (8900), 207 (7700)	201 (7700), 206 (4500)
IR ν_{max}^{KBr} cm^{-1}	3450, 2956, 1735, 1438, 1261, 1207	3482, 2952, 1731, 1716, 1438, 1209	3448, 2931, 1731, 1716, 1261, 802
Solubility			
Soluble	CH_3OH, $CHCl_3$, EtOAc	CH_3OH, $CHCl_3$, EtOAc	CH_3OH, $CHCl_3$, EtOAc
Insoluble	H_2O	H_2O	H_2O

	D	E	F
Appearance	Yellow syrup	Yellow syrup	Yellow syrup
$[\alpha]_D^{26}$	+3.0 (c=0.1, CH_3OH)	+4.9 (c=0.1, CH_3OH)	−3.1 (c=0.1, CH_3OH)
Molecular formula	$C_{13}H_{20}O_6$	$C_{14}H_{22}O_6$	$C_{11}H_{16}O_6$
Molecular weight	272	286	244
HR-FAB-MS m/z $(M+H)^+$			
Calcd	273.1338 (for $C_{13}H_{21}O_6$)	287.1495 (for $C_{14}H_{12}O_6$)	245.1025 (for $C_{11}H_{17}O_6$)
Found	273.1342	287.1498	245.1034
UV $\lambda_{max}^{CH_3OH}$ nm (ε)	202 (17000), 205 (7700)	198 (13000)	199 (11000)
IR ν_{max}^{KBr} cm^{-1}	3455, 2952, 2861, 1737, 1438, 1207	3482, 2933, 2857, 1716, 1438, 1203	3442, 2956, 1716, 1444, 1243, 1203
Solubility			
Soluble	CH_3OH, $CHCl_3$, EtOAc	CH_3OH, $CHCl_3$, EtOAc	CH_3OH, $CHCl_3$, EtOAc
Insoluble	H_2O	H_2O	H_2O

Table 2. ^{1}H- and ^{13}C-NMR Chemical Shifts of Tensyuic Acids A to F

	A		B		C	
	^{13}C chemical shifts (ppm)[a]	^{1}H chemical shifts (ppm)[b]	^{13}C chemical shifts (ppm)[a]	^{1}H chemical shifts (ppm)[b]	^{13}C chemical shifts (ppm)[a]	^{1}H chemical shifts (ppm)[b]
C-1	170.8		171.5		171.2	
C-2	137.4		137.5		137.3	
C-3	46.1	3.50 (1H, t, J=7.0 Hz)	46.8	3.46 (1H, t, J=7.0 Hz)	46.8	3.37 (1H, t, J=7.5 Hz)
C-4	30.4	1.72 (1H, m)	30.3	1.71 (1H, m)	29.8	1.67 (1H, m)
		1.96 (1H, m)		1.92 (1H, m)		1.86 (1H, m)
C-5	22.7	1.62 (2H, m)	27.2	1.32 (2H, m)	27	1.28 (2H, m)
C-6	33.6	2.34 (2H, t, J=7.0 Hz)	29.0	1.32 (2H, m)	28.7	1.28 (2H, m)
C-7	173.7		24.9	1.61 (2H, m)	24.7	1.56 (2H, m)
C-8	129.5	5.88 (1H, s)	34.2	2.31 (2H, t, J=7.5 Hz)	34.2	2.24 (2H, t, J=7.5 Hz)
		6.51 (1H, s)				
C-9	173.3		174.6		173.9	
C-10	52.2	3.69 (3H, s)	129.9	5.86 (1H, s)	129.7	5.78 (1H, s)
				6.51 (1H, s)		6.45 (1H, s)
C-11	51.2	3.66 (3H, s)	179.2		179	
C-12			51.8	3.66 (3H, s)	60.3	4.05 (2H, q, J=7.0 Hz)
C-13					14.2	1.18 (3H, t, J=7.0 Hz)

	D		E		F	
	^{13}C chemical shifts (ppm)[a]	^{1}H chemical shifts (ppm)[b]	^{13}C chemical shifts (ppm)[a]	^{1}H chemical shifts (ppm)[b]	^{13}C chemical shifts (ppm)[a]	^{1}H chemical shifts (ppm)[b]
C-1	170.8		170.9		171.3	
C-2	137.7		137.3		136.8	
C-3	46.0	3.43 (1H, t, J=7.0 Hz)	47.0	3.41 (1H, t, J=7.0 Hz)	47.0	3.42 (1H, t, J=7.0 Hz)
C-4	30.8	1.63 (1H, m)	29.7	1.73 (1H, m)	29.0	1.80 (1H, m)
		1.84 (1H, m)		1.92 (1H, m)		1.98 (1H, m)
C-5	26.9	1.28 (2H, m)	27.2	1.31 (2H, m)	22.7	1.68 (2H, m)
C-6	28.6	1.28 (2H, m)	28.9	1.31 (2H, m)	33.8	2.34 (2H, m)
C-7	24.5	1.56 (2H, m)	29.0	1.31 (2H, m)	173.2	
C-8	33.8	2.24 (2H, t, J=7.0 Hz)	29.0	1.31 (2H, m)	130.2	5.88 (1H, s)
						6.54 (1H, s)
C-9	174.2		24.9	1.61 (2H, m)	178.7	
C-10	128.8	5.79 (1H, s)	34.1	2.30 (2H, t, J=7.5 Hz)	60.4	4.13 (2H, q, J=7.0 Hz)
		6.43 (1H, s)				
C-11	173.6		174.4		14.2	1.25 (3H, t, J=7.0 Hz)
C-12	52.0	3.63 (3H, s)	129.5	5.84 (1H, s)		
				6.52 (1H, s)		
C-13	51.4	3.60 (3H, s)	178.5			
C-14			51.5	3.67 (3H, s)		

a) Chemical shifts are shown with reference to $CDCl_3$ as 77.0 ppm.
b) Chemical shifts are shown with reference to $CDCl_3$ as 7.26 ppm. J values are given in Hz in parentheses.

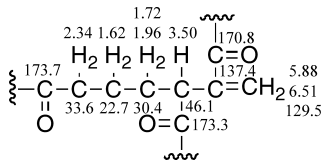

Fig. 3. Partial Structure I of Tensyuic Acid A

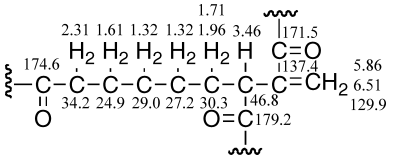

Fig. 5. Partial Structure II of Tensyuic Acid B

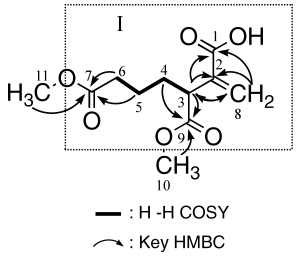

Fig. 4. Key Cross Peaks Observed in ^{1}H–^{1}H COSY and ^{13}C–^{1}H HMBC Experiments of Tensyuic Acid A

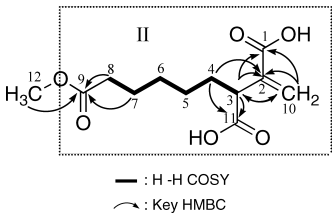

Fig. 6. Key Cross Peaks Observed in ^{1}H–^{1}H COSY and ^{13}C–^{1}H HMBC Experiments of Tensyuic Acid B

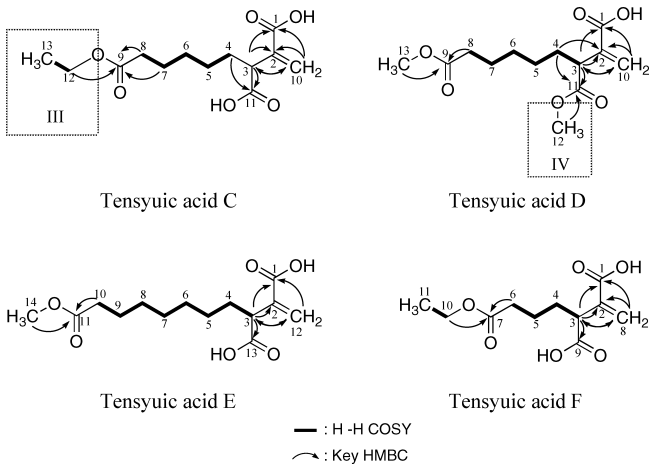

Fig. 7. Key Cross Peaks Observed in ^{1}H–^{1}H COSY and HMBC Experiments of Tensyuic Acids C to F

Thus, the structure of tensyuic acid C was elucidated as shown in Fig. 1.

Tensyuic Acid D: The molecular formula ($C_{13}H_{20}O_6$) of tensyuic acid D is CH_2 bigger than that of tensyuic acid B. The different part was that the calboxylic acid C-11 (δ 173.6) is methylated in tensyuic acid D (the partial structure IV in Fig. 7). The cross peak from 12-H_3 (δ 3.63) to C-11 was observed in the HMBC experiments to confirm the position (Fig. 7). The molecular formula supported this structure. Thus, the structure of tensyuic acid D was elucidated as shown in Fig. 1.

Tensyuic Acid E: The molecular formula ($C_{14}H_{22}O_6$) of tensyuic acid E is C_2H_4 bigger than that of tensyuic acid B. The difference lay in the alkyl chain length. It was C_2H_4 longer than that of tensyuic acid B. The structure was confirmed by the ^{1}H–^{1}H COSY and HMBC experiments (Fig. 7). Thus, the structure of tensyuic acid E was elucidated as shown in Fig. 1.

Tensyuic Acid F: The molecular formula ($C_{11}H_{16}O_6$) of tensyuic acid F is C_2H_4 smaller than that of tensyuic acid C. The difference lay in the alkyl chain length between them. It was C_2H_4 shorter than that of tensyuic acid C. The structure was confirmed by the ^{1}H–^{1}H COSY and HMBC experiments (Fig. 7). Thus, the structure of tensyuic acid E was elucidated as shown in Fig. 1.

Biological Properties Tensyuic acid C showed moderate antimicrobial activity only against *B. subtilis* (inhibition zone: 10 mm at 50 μg/disk). All the other tensyuic acids showed no inhibition zone against the microorganisms tested at 50 μg/disk.

Discussion

The structures of tensyuic acids A to F were elucidated in this study, and they were found to belong to the itaconic acid family. Several alkylitaconic acids have been reported. Hexylitaconic acid was isolated from *Aspergillus niger* K-88 as a plant growth regulator.[2] In addition, it was also isolated from a saltwater culture of sponge-derived *Asperpillus niger*,[3] from marine endophytic fungus *Apiospora montagnei*[4] and from *Penicillium striatisporum*.[5] Ceriporic acids from white rot fungus *Ceriporiopsis subvermispora*, were reported as the inhibitor of the production of a cellulolytic active oxygen species and of the iron redox reactions.[6,7] All these structures were itaconic acids having a long alkyl or alkenyl side chain, but no alkylic acid chain in the R position (Fig. 1). To our knowledge, tensyuic acids are the first itaconic acid derivatives having the ester carboxyl moieties at the end of the alkyl side chain. As shown in Table 1, all the tensyuic acids have low $[\alpha]_D$ values (−6.0—+4.9). The $[\alpha]_D$ values of structurally related hexylitaconic acid and ceriporic acid A have not been reported. Therefore, the stereochemistry of these compounds remains to be studied.

Among them, tensyuic acid C showed moderate antimicrobial activity only against *B. subtilis*. When compared the activity with other itaconic acid related compounds, tensyuic acid C showed much weaker activity. Recently, hexylitaconic acid was isolated from *Arthrinium* sp. as an inhibitor of p53-HDM2 interaction.[8] However, all tensyuic acids showed no inhibitory activity in the p53-HDM2 interaction even at 250 μg/ml (Dr. Tsukamoto, personal communication).

Acknowledgments We wish to thank Ms. Noriko Sato, Ms. Akiko Nakagawa and Ms. Chikako Sakabe, School of Pharmaceutical Sciences, Kitasato University, for measurements of NMR and mass spectra. We wish to thank Dr. Sachiko Tsukamoto, Graduate School of Natural Science and Technology, Kanazawa University, for testing the activity of p53-HDM2 interaction. This study was supported in part by the 21st Century COE Program and Kakenhi 16073215, Ministry of Education, Culture, Sports, Science and Technology, Japan.

References and Notes

1) Fukuda T., Hasegawa Y., Hagimori K., Yamaguchi Y., Masuma R., Tomoda H., Ōmura S., *J. Antibiot.*, **59**, 480—485 (2006).
2) Nanjo K., Kora S., Suzuki A., Isogai A. J., Jpn. Kokai Tokkyo Koho JP 62132804 (1985).
3) Varoglu M., Crews P., *J. Nat. Prod.*, **63**, 41—43 (2000).
4) Klemke C., Kehraus S., Wright D. A., Konig M. G., *J. Nat. Prod.*, **67**, 1058—1063 (2004).
5) Stewart M., Capon J. R., Lacey E., Tennant S., Gill H. J., *J. Nat. Prod.*, **68**, 581—584 (2005).
6) Watanabe T., Teranishi H., Honda Y., Kuwahara M., *Biochem. Biophys. Res. Commum.*, **297**, 918—923 (2002).
7) Enoki M., Honda Y., Kuwahara M., Watanabe T., *Chem. Phys. Lipids*, **120**, 9—21 (2002).
8) Tsukamoto S., Yoshida T., Hosono H., Ohta T., Yokosawa H., *Bioorg. Med. Chem. Lett.*, **16**, 69—71 (2006).