Isolation and Structure Elucidation of a Novel Antimalarial Macrocyclic Polylactone, Menisporopsin A, from the Fungus *Menisporopsis theobromae*

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An antimalarial macrocyclic polylactone, menisporopsin A (**1**), was isolated from a cell extract of the seed fungus *Menisporopsis theobromae*. The structure of **1** was elucidated on the basis of spectroscopic analysis and chemical transformations, with the absolute configuration established by application of the modified Mosher method and by using chiral HPLC. Menisporopsin A (**1**) possesses an unprecedented residue, 2,4-dihydroxy-6-(2,4-dihydroxy-*n*-pentyl)benzoic acid. This compound exhibited antimalarial activity, with an IC_{50} value of 4.0 μg mL^{-1}, and antimycobacterial activity (MIC value of 50 μg mL^{-1}).

As part of our continuing search for naturally occurring biologically active compounds from Thai bioresources,[1] we have screened the biological activities of extracts from plants and microorganisms. Routine biological activity screening revealed that a crude extract of the chemically unexplored seed fungus *Menisporopsis theobromae* BCC 4162 exhibited significant antimalarial activity. Chemical investigation led to the isolation of a novel macrocyclic polylactone, menisporopsin A (**1**), from a cell extract of *M. theobromae* BCC 4162. We report herein the isolation, structure elucidation, and biological activities of menisporopsin A (**1**), which possesses an unusual 2,4-dihydroxy-6-(2,4-dihydroxy-*n*-pentyl)benzoic acid residue.

A crude extract from mycelia of *M. theobromae* BCC 4162 was purified by repeated Sephadex LH-20 column chromatography to furnish menisporopsin A (**1**). The ESITOFMS of menisporopsin A (**1**) gave an accurate mass of *m*/*z* 797.2670 [(M − H)$^-$, Δ +1.3 mmu], establishing the molecular formula of **1** as $C_{40}H_{46}O_{17}$. The ^{1}H NMR spectrum (acetone-d_6) of menisporopsin A (**1**) showed the presence of three resorcylic acid derivatives (δ_H 6.25−6.37), five methyl groups (δ_H 1.21, 1.23, 1.41, 1.47, and 1.47), six methine protons (five with ester linkages (δ_H 5.16−5.60) and one attached to carbon bearing an oxygen atom at δ_H 3.95), and six methylenes (δ_H 1.97−3.57). The ^{13}C NMR spectrum of **1** revealed five carbonyl carbons (δ_C 169.8−171.3), and its IR spectrum showed absorption peaks at 1715 and 1651 cm^{-1}, confirming the presence of ester carbonyls. Methylation of menisporopsin A (**1**) with MeI/K_2CO_3 in DMF afforded a hexa-*O*-methyl derivative **2**, indicating the existence of three pairs of hydroxyl groups on three resorcylic acid rings. Extensive analysis of ^{13}C, DEPT, ^{1}H−^{1}H COSY, and HMQC spectral data of **1** allowed complete assignment of protons attached to their respective carbons (Table 1). The ^{1}H−^{1}H COSY and TOCSY spectra of **1** established the connectivities from H-8 to H-10, from H-12 to H-14, from H-22 to H-24, from H-26 to H-28, and from H-36 along the chain to H-40. Analysis of the ^{1}H−^{1}H COSY, TOCSY, and HMBC spectral data suggested that menisporopsin A (**1**) is a cyclic polylactone composed of two units of 2,4-dihydroxy-6-(2-hydroxy-*n*-propyl)benzoic acid, two units of 3-hydroxybutyric acid, and a residue of 2,4-dihydroxy-6-(2,4-dihydroxy-*n*-pentyl)benzoic acid. The linkage between these organic acids in **1** was readily assigned by HMBC spectral data which showed correlations of both H-9 and H-12 to C-11; H-13 to C-15; both H-23 and H-26 to C-25; H-27 to C-29; and H-39 to C-1. Other key HMBC correlations were observed from H-8 to C-2, C-6, C-7, and C-10; H-22 to C-16, C-20, C-21, and C-24; and H-36 to C-30, C-34, and C-35. On the basis of these spectral data, the

Table 1. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR Spectral Data (acetone-d_6) of Menisporopsin A (**1**)

unit	δ_C, multiplicity[a]	δ_H, multiplicity, *J* in Hz
	2,4-Dihydroxy-6-(2-hydroxy-*n*-propyl)benzoic Acid[b]	
1, 15	171.0, s; 171.3, s	
2, 16	105.8, s; 105.5, s	
3, 17	165.9, s; 165.7, s	
4, 18	102.3, d; 102.2, d	6.29, d, 2.2; 6.25, d, 2.0
5, 19	162.6, s; 162.6, s	
6, 20	112.5, d; 111.6, d	6.37, d, 1.9; 6.33, d, 2.3
7, 21	143.0, s; 143.0, s	
8, 22	41.6, t; 41.2, t	3.10, dd, 7.0, 13.0 (H-8a) and 3.43, dd, 7.4, 13.0 (H-8b); 2.85, m (H-22a) and 3.57, dd, 7.7, 13.9 (H-22b)
9, 23	72.4, d; 72.2, d	5.24, m; 5.16, m
10, 24	19.5, d; 19.4, d	1.23, d, 7.3; 1.21, d, 6.7
	3-Hydroxybutyric Acid[c]	
11, 25	169.8, s; 170.0, s	
12, 26	40.9, t; 40.7, t	2.85, m; 2.86, m
13, 27	69.8, d; 69.3, d	5.54, m; 5.56, m
14, 28	19.7, d; 19.6, d	1.41, d, 6.2; 1.47, d, 5.6
	2,4-Dihydroxy-6-(2,4-dihydroxy-*n*-pentyl)benzoic Acid	
29	171.2, s	
30	104.7, s	
31	166.3, s	
32	102.0, d	6.25, d, 2.0
33	162.9, s	
34	113.9, d	6.35, d, 2.1
35	144.8, s	
36	45.6, t	2.60, dd, 9.7, 12.8 (H-36a) 3.34, dd, 9.7, 13.0 (H-36b)
37	69.1, d	3.95, brt, 9.3
38	43.6, t	1.97, ddd, 4.3, 11.5, 11.5 (H-38a) 2.13, ddd, 4.3, 11.2, 11.8 (H-38b)
39	71.7, d	5.57, m
40	19.6, d	1.47, d, 5.6
OH		9.25, brs; 11.45 brs; 11.71 brs

[a] Multiplicity was determined by analysis of the DEPT spectra. [b,c] Protons and carbons may be exchangeable in the same acid unit.

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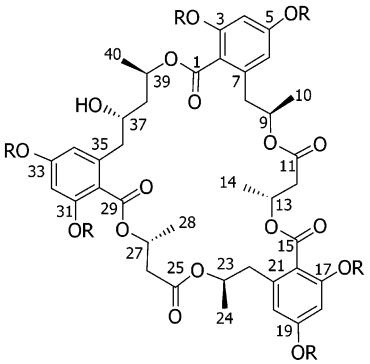
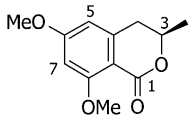
gross structure of menisporopsin A (**1**) could be established. Complete NMR assignments of the protons and carbons of **1** are shown in Table 1. The presence of a 2,4-dihydroxy-6-(2,4-dihydroxy-*n*-pentyl)benzoic acid residue in this macrocyclic polylactone makes menisporopsin A (**1**) structurally unique. The relative stereochemistry of the 2,4-dihydroxy-6-(2,4-dihydroxy-*n*-pentyl)benzoic acid residue in menisporopsin A (**1**) was assigned by analysis of the NOESY spectrum, in which correlations between H-37/H-36β, H-37/H-38β, H-37/H_3-40, H_3-40/H-38β, H-36α/H-38α, and H-38α/H-39α were observed, implying that H-37 and the H_3-40 methyl group are coplanar.

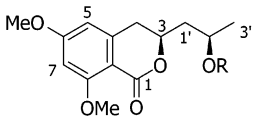
To establish the absolute stereochemistry of each individual acid residue in **1**, its methylated derivative (**2**) was hydrolyzed in an aqueous solution of NaOH (1 M) and purified by HPLC to yield 3,4-dihydro-6,8-dimethoxy-3-methylisocoumarin (**3**) and 3,4-dihydro-6,8-dimethoxy-3-(2-hydroxypropyl)isocoumarin (**4**). Cyclization of 2,4-dihydroxy-6-(2-hydroxy-*n*-propyl)benzoic acid and 2,4-dihydroxy-6-(2,4-dihydroxy-*n*-pentyl)benzoic acid to their respective corresponding lactones **3** and **4** took place spontaneously during acidic workup.



1 R = H
2 R = Me



3



4 R = H
4a R = (-)-MTPA
4b R = (+)-MTPA

Comparison of the optical rotation ($[\alpha]^{29}_D$ −91.95°, *c* 0.217, MeOH) and its NMR spectral data with those in the literature[2−5] indicated that compound **3** is (3*R*)-3,4-dihydro-6,8-dimethoxy-3-methylisocoumarin. The absolute configuration of **4** was addressed by the formation of Mosher esters.[6] Both the (*R*)- and (*S*)-methoxytrifluoromethylphenyl acetate (MTPA) esters of **4** (compounds **4a** and **4b**) were separately prepared and subjected to ^{1}H NMR analysis. The $\Delta\delta$ values [$\delta_{(-)} - \delta_{(+)}$] are shown in Figure 1, indicating that the absolute configuration at C-2′ of **4** is represented as *R*; hence the *R* configuration was established at C-39 in menisporopsin A (**1**). As mentioned earlier, H-37 and the H_3-40 methyl group in **1** are in the same plane; therefore the absolute stereochemistry at C-37 may be assigned with the *S* configuration. The absolute configuration at C-3 in 3-hydroxybutyric acid was determined to be *R* by chiral HPLC analysis. Direct analysis of 3-hydroxybutyric acid with chiral HPLC provided poor information of the acid identity due to its lack of chromophore. This problem was resolved by forming its derivative with Mosher reagent and generating diastereomers with aromatic chromophore. Mosher esters of (*R*)- and (*S*)-3-hydroxybutyric acid could be readily distinguished by chiral HPLC, and accordingly the acid in **1** was found to be (*R*)-3-hydroxybutyric acid. The absolute configurations of all six chiral centers in menisporopsin A (**1**) were finally established as 9*R*, 13*R*, 23*R*, 27*R*, 37*S*, and 39*R*.

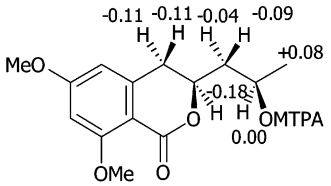


Figure 1. $\Delta\delta$ values [$\delta_{(-)} - \delta_{(+)}$] for the MTPA esters (**4a** and **4b**) of **4**.

Menisporopsin A (**1**) exhibited antimalarial activity, with an IC_{50} value of 4.0 μg mL^{-1}, and antimycobacterial activity with a MIC value of 50 μg mL^{-1}. Cytotoxicity of **1** against the BC-1 (breast cancer) and KB (nasopharyngeal carcinoma) cell lines was at IC_{50} values of 5.0 and 6.0 μg mL^{-1}, respectively. However, menisporopsin A (**1**) was inactive (at 20 μg mL^{-1}) toward *Candida albicans*. Interestingly, the methylated derivative **2** was inactive (at 20 μg mL^{-1}) toward both the malarial parasite and the BC-1 and KB cell lines. Compound **2** was also inactive against the mycobacterium tested (at MIC 200 μg mL^{-1}). This implies that the hydroxyl groups of menisporopsin A (**1**) might play an important role in mediating these biological activities.

Naturally occurring macrocyclic polylactones are rare, but they have been reported as fungal metabolites (*Penicillium verruculosum*, *Hypoxylon oceanicum*, *Periconia byssoides* isolated from the sea hare, *Aplysia kurodai*).[2−5] Macrocyclic polylactones have been previously reported to exhibit antibiotic and antifungal activities, as well as inhibition of cell adhesion.[2−5]

Experimental Section

General Experimental Procedures. IR spectra and optical rotations were measured on a Perkin-Elmer 2000 spectrometer and Jasco DIP 370 polarimeter, respectively. UV spectra were recorded on a Cary 1E UV−vis spectrophotometer. ^{1}H, ^{13}C, ^{1}H−^{1}H COSY, HMQC, HMBC, TOCSY, NOESY, and DEPT spectra were recorded on a Bruker DRX 400 NMR spectrometer, operating at 400 MHz for proton and 100 MHz for carbon. The ESITOFMS were obtained from a Micromass LCT mass spectrometer, and the lock mass calibration was applied for the determination of the accurate masses.[7]

Fungal Material. The seed fungus *M. theobromae* BCC 4162 was collected from Khao Yai National Park, Thailand, and identified by S. Somrithipol. The culture was deposited at the BIOTEC Culture Collection, Bangkok, Thailand (registration no. BCC 4162). The fungus was grown in a potato dextrose broth and incubated for 7 days at 25 °C, then transferred into 250 mL of peptone yeast glucose medium (PYGM). The culture was subsequently incubated (at 25 °C) for 19 days and harvested for further study.

Extraction and Isolation. The culture (5 L) of *M. theobromae* BCC 4162 was filtered to separate cells and broth. Mycelia were macerated in MeOH for 2 days at room temperature, and the extract was concentrated and then H_2O (100 mL) was added. The mixture was washed with hexane (300 mL × 2). The aqueous solution was then extracted twice with an equal volume of EtOAc. The EtOAc layers were combined

and evaporated to dryness, yielding 1.2 g of a crude extract. The crude EtOAc extract was fractionated using a Sephadex LH-20 column (eluted with MeOH) to provide 15 fractions (A−O), each with a volume of 100 mL. Fractions M and N were combined and further rechromatographed on Sephadex LH-20 (eluted with MeOH) to furnish menisporopsin A (**1**) (173.5 mg).

Bioassays. Antimalarial activity was evaluated against the parasite *Plasmodium falciparum* (K1, multidrug-resistant strain), which was cultured continuously according to the method of Trager and Jensen.[8] Quantitative assessment of antimalarial activity in vitro was determined by means of the microculture radioisotope technique based upon the method described by Desjardins et al.[9] The inhibitory concentration (IC_{50}) represents the concentration that causes 50% reduction in parasite growth as indicated by the in vitro uptake of [^{3}H]-hypoxanthine by *P. falciparum*. An IC_{50} value of 1 ng mL^{-1} was observed for the standard compound, artemisinin, in the same test system. The cytotoxicity of compound **1** was determined, employing the colorimetric method as described by Skehan and co-workers.[10] The reference substance, ellipticine, exhibited activities toward BC-1 (breast cancer) and KB (nasopharyngeal carcinoma) cell lines (both with an IC_{50} of 0.3 μg mL^{-1}). The antimycobacterial activity was assessed against *Mycobacterium tuberculosis* H37Ra using the Microplate Alamar Blue Assay (MABA).[11] The standard drugs, isoniazid and kanamycin sulfate, used as reference compounds for the antimycobacterial assay showed minimum inhibitory concentrations (MIC) of 0.040−0.090 and 2.0−5.0 μg mL^{-1}, respectively.

Menisporopsin A (1): amorphous solid; $[\alpha]^{29}_D$ −42.37° (*c* 0.236, MeOH); UV (MeOH) λ_{max} (log ϵ) 216 (4.8), 264 (4.3), 304 (4.2) nm; IR (KBr) ν_{max} 3392, 2985, 2938, 1715, 1651, 1621, 1456, 1384, 1353, 1315, 1262, 1197, 1173, 1108, 1053 cm^{-1}; ^{1}H and ^{13}C NMR, see Table 1; ESITOFMS *m*/*z* 797.2670 [M − H]$^-$, calcd for $[C_{40}H_{46}O_{17} - H]^-$ 797.2657.

Methylation of Menisporopsin A (1). To a solution of menisporopsin A (**1**) (30.0 mg) in DMF (1 mL) were added K_2CO_3 (100 mg) and MeI (0.3 mL), and the mixture was left stirring at room temperature for 20 h. After the usual aqueous workup, a crude reaction mixture (32.9 mg) was subjected to Sephadex LH-20 column chromatography (eluted with MeOH) to obtain the methylated derivative **2** (20.2 mg): $[\alpha]^{29}_D$ −94.11° (*c* 0.136, MeOH); ^{1}H NMR ($CDCl_3$) δ_H 6.43 (1H, d, J = 1.9 Hz, H-32), 6.38 (1H, brs, H-20), 6.38 (1H, brs, H-34), 6.34 (1H, brs, H-6), 6.34 (1H, brs, H-18), 6.29 (1H, d, J = 1.8 Hz, H-4), 5.50 (1H, m, H-13), 5.39 (1H, m, H-39), 5.17 (1H, m, H-9), 5.17 (1H, m, H-23), 4.65 (1H, m, H-37), 4.12 (1H, m, H-27), 3.55−3.92 (18H, s, 6 × OMe), 3.03 (1H, dd, J = 2.9, 16.0 Hz, H-36a), 2.88−2.97 (3H, m, H-8a, H-22a, H-36b), 2.67−2.77 (3H, m, H-8b, H-12a, H-22b), 2.50 (1H, dd, J = 6.6, 15.6 Hz, H-12b), 2.43 (1H, dd, J = 3.7, 16.1 Hz, H-26a), 2.38 (1H, dd, J = 3.7, 16.0 Hz, H-26b), 2.35 (1H, m, H-38a), 1.97 (1H, m, H-38b), 1.43 (3H, d, J = 6.2 Hz, H-40), 1.33 (3H, d, J = 6.3 Hz, H-14), 1.23 (3H, d, J = 6.7 Hz, H-10), 1.23 (3H, d, J = 6.7 Hz, H-24), 1.16 (3H, d, J = 6.8 Hz, H-28); ESITOFMS *m*/*z* 906.3618 [M + Na]$^+$, calcd for $[C_{46}H_{58}O_{17} + Na]^+$ 906.3650.

Hydrolysis of 2. The methylated derivative (**2**) (9.4 mg) was dissolved in an aqueous solution of NaOH (1 M) and stirred overnight at room temperature. After workup with dilute HCl solution, the reaction mixture was purified with HPLC on a C_{18} reversed-phase column, eluted with H_2O−MeCN (50:50), yielding (3*R*)-3,4-dihydro-6,8-dimethoxy-3-methylisocoumarin (**3**) (2.3 mg) and (3*S*,2*R*)-3,4-dihydro-6,8-dimethoxy-3-(2-hydroxypropyl)isocoumarin (**4**) (1.3 mg).

(3*R*)-3,4-Dihydro-6,8-dimethoxy-3-methylisocoumarin (3): $[\alpha]^{29}_D$ −91.95° (*c* 0.217, MeOH); UV (MeOH) λ_{max} (log ϵ) 216 (4.4), 267 (4.2), 301 (3.9) nm; ^{1}H NMR ($CDCl_3$) δ_H 6.43 (1H, d, J = 2.3 Hz, H-5), 6.32 (1H, d, J = 2.0 Hz, H-7), 4.53 (1H, m, H-3), 3.94 (3H, s, OMe), 3.88 (3H, s, OMe), 2.90 (1H, dd, J = 11.0, 16.0 Hz, H-4a), 2.81 (1H, dd, J = 3.3, 16.0 Hz, H-4b), 1.48 (3H, d, J = 6.4 Hz, Me-3); ESITOFMS *m*/*z* 245.0806 [M + Na]$^+$, calcd for $[C_{12}H_{14}O_4 + Na]^+$ 245.0790.

(3*S*,2*R*)-3,4-Dihydro-6,8-dimethoxy-3-(2-hydroxypropyl)isocoumarin (4): $[\alpha]^{28}_D$ −97.39° (*c* 0.0575, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 216 (4.6), 268 (4.4), 301 (4.1) nm; ^{1}H NMR ($CDCl_3$) δ_H 6.43 (1H, d, J = 2.2 Hz, H-5), 6.33 (1H, d, J = 2.1 Hz, H-7), 4.58 (1H, m, H-3), 4.16 (1H, m, H-2′), 3.94 (3H, s, OMe), 3.88 (3H, s, OMe), 2.98 (1H, dd, J = 11.3, 16.0 Hz, H-4a), 2.86 (1H, dd, J = 2.9, 16.1 Hz, H-4b), 2.07 (1H, m, H-1′a), 1.80 (1H, m, H-1′b), 1.29 (3H, d, J = 6.2 Hz, H-3′); ESITOFMS *m*/*z* 289.1061 [M + Na]$^+$, calcd for $[C_{14}H_{18}O_5 + Na]^+$ 289.1052.

Preparation of MTPA Ester Derivatives of 4. A reaction mixture consisting of **4** (ca. 1.3 mg), pyridine (300 μL), and (*R*)-(−)-α-methoxy-α-trifluoromethylphenylacetyl chloride (40 μL) was left standing at room temperature for 6 h. The mixture was dried under vacuum, then dissolved in 5 mL of EtOAc and subsequently washed with H_2O. The EtOAc layer was dried, yielding the (*S*)-(−)-MTPA ester of **4** (ca. 1.6 mg). Preparation of the (*R*)-(+)-MTPA ester of **4** from (*S*)-(+)-α-methoxy-α-trifluoromethylphenylacetyl chloride was also conducted in the same manner as that of the (*S*)-(−)-MTPA ester derivative. The *S*-(−)-MTPA ester (**4a**) was obtained as a colorless oil: ^{1}H NMR ($CDCl_3$) δ_H 7.53 (2H, m, aromatic protons of MTPA), 7.43 (3H, m, aromatic protons of MTPA), 6.44 (1H, s, H-5), 6.25 (1H, s, H-7), 5.43 (1H, m, H-2′), 4.22 (1H, m, H-3), 3.94 (3H, s, OMe), 3.89 (3H, s, OMe), 3.59 (3H, s, OMe of MTPA), 2.73 (2H, m, H-4), 2.24 (1H, m, H-1′a), 1.92 (1H, m, H-1′b), 1.46 (3H, d, J = 6.3 Hz, H-3′); ESITOFMS *m*/*z* 483.1636 [M + H]$^+$, calcd for $[C_{24}H_{25}O_7F_3 + H]^+$ 483.1630. The *R*-(+)-MTPA ester (**4b**) was obtained as a colorless oil: ^{1}H NMR ($CDCl_3$) δ_H 7.53 (2H, m, aromatic protons of MTPA), 7.43 (3H, m, aromatic protons of MTPA), 6.44 (1H, s, H-5), 6.29 (1H, s, H-7), 5.43 (1H, m, H-2′), 4.40 (1H, m, H-3), 3.95 (3H, s, OMe), 3.89 (3H, s, OMe), 3.51 (3H, s, OMe of MTPA), 2.84 (2H, m, H-4), 2.33 (1H, m, H-1′a), 1.96 (1H, m, H-1′b), 1.38 (3H, d, J = 6.2 Hz, H-3′); ESITOFMS *m*/*z* 483.1627 [M + H]$^+$, calcd for $[C_{24}H_{25}O_7F_3 + H]^+$ 483.1630.

Preparation of MTPA Ester Derivatives of Authentic (*R*)- and (*S*)-3-Hydroxybutyric Acid. A reaction mixture consisting of (*S*)- or (*R*)-3-hydroxybutyric acid (1 mg), pyridine (300 μL), and (*S*)-(+)-α-methoxy-α-trifluoromethylphenylacetyl chloride (40 μL) was left standing at room temperature for 6 h. The mixture was dried under vacuum, then dissolved in 5 mL of EtOAc and subsequently washed with H_2O. The EtOAc layer was dried, yielding the corresponding MTPA ester of (*R*)- or (*S*)-3-hydroxybutyric acid. Each individual product was analyzed by HPLC on a chiral column (ChiraDex, Merck), using H_2O−MeOH (60:40) as eluent. The standard MTPA ester derivatives of (*R*)- and (*S*)-3-hydroxybutyric acid had retention times of 5.0 and 6.4 min, respectively.

Determination of the Stereochemistry of the 3-Hydroxybutyric Acid Residue in 1. Menisporopsin A (**1**) (15.2 mg) was dissolved in an aqueous solution of NaOH (1 M) and stirred overnight at room temperature. After neutralization with a dilute HCl solution, the reaction mixture was dried under vacuum. The mixture was dissolved in dry pyridine (0.5 mL), and (*S*)-(+)-α-methoxy-α-trifluoromethylphenylacetyl chloride (40 μL) was added. The mixture was left standing at room temperature for 6 h and dried under vacuum. The reaction mixture was washed with H_2O (equal volume, 6 times) and then subjected to HPLC analysis with a chiral column (ChiraDex, Merck), using H_2O−MeOH (60:40) as eluent. Co-injection of the sample with a standard derivative of (*R*)-3-hydroxybutyric acid (t_R 5.0 min) or (*S*)-3-hydroxybutyric acid (t_R 6.4 min) readily indicated the presence of (*R*)-3-hydroxybutyric acid in the hydrolysate of menisporopsin A (**1**).

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Supporting Information Available: The ^{1}H, ^{13}C, ^{1}H−^{1}H COSY, HMQC, and HMBC NMR spectra (acetone-d_6) of menisporopsin A (**1**). This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

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