HETEROCYCLES, Vol. 76, No. 2, 2008, pp. 1425 - 1438. © The Japan Institute of Heterocyclic Chemistry
Received, 30th April, 2008, Accepted, 12th June, 2008, Published online, 19th June, 2008. COM-08-S(N)108

SYNTHESIS AND EVALUATION OF ISOMERS OF MELLEUMIN B FROM THE MYXOMYCETE *PHYSARUM MELLEUM*; AN APPROACH FOR WNT SIGNAL INHIBITOR TRUSTED IN NATURE

Midori A. Arai, Yujiro Uchino, Shuwa Hanazawa, Xiaofan Li, Naoki Kimura, and Masami Ishibashi*

Graduate School of Pharmaceutical Science, Chiba University, Chiba 263-8522, Japan e-mail address: mish@p.chiba-u.ac.jp

Dedicated to Professor Ryoji Noyori on the occasion of his 70th birthday

Abstract – Two stereoisomers of melleumin B (**1**), *seco* acid methyl ester of melleumin A (**2**), which are novel peptide compounds isolated from the myxomycete *Physarum melleum*, were synthesized and evaluated for their inhibition of Wnt signal transcription. The 3*R*-epimer and (3*R*, 4*S*, 10*R*, 11*R*)-isomer of **1** showed moderate inhibition of the Wnt signal.

INTRODUCTION

Natural products have important meanings in biological systems. Isolation of new natural products and synthesis of small molecules based on their scaffolds would be of chemical relevance to living cells and organs, binding to proteins, absorption, distribution, metabolism, and excretion.[1] Recently, we focused on Wnt signaling, the pathway of which shows aberrant activation in many cancer cells, especially in colon cancers.[2] Efforts have been continuing to obtain a seed compound for a medicine that acts on certain molecular components in the Wnt signal pathway, such as β-catenin, T-cell factor/lymphoid enhancer factor (TCF/LEF), and glycogen synthase kinase 3 β (GSK3β) etc. Because in many cases there are mutated components upstream of this signal, it would be better for inhibitors to act on the transactivation event more downstream in this pathway. We previously reported that *cis*-dihydroarcyriarubin C, which is a synthesized small molecule based on bisindole alkaloid isolated from myxomycete by our group, showed moderate Wnt signal inhibitory activity.[3] We also found that a synthesized 10*R*-epimer of melleumin B (**1**), *seco* acid methyl ester of melleumin A (**2**), which are novel peptide compounds isolated

by our group from the cultured myxomycete *Physarum melleum*, showed moderate inhibition of Wnt signal transcription.[4] These compounds consist of four units, *p*-methoxy benzoic aid (pMBz), L-threonine (L-Thr), glycine (Gly), and an unusual amino acid, a tyrosine-attached acetic acid (TyrA). Herein, we report the synthesis of two isomers of melleumin B (**1**), the 3*R*-epimer and (3*R*, 4*S*, 10*R*, 11*R*)-isomer, and evaluated their inhibition of Wnt signal transcription.

RESULTS AND DISCUSSION

We previously determined the absolute stereochemistry of a novel peptide lactone, melleumin A (**2**) and its *seco* acid methyl ester, melleumin B (**1**), as shown in Figure 1, by an analytical method and total synthesis of **1**.[4] It was revealed that melleumin A (**2**) has 3*S*, 4*S*, 10*S*, 11*R* configuration. We synthesized **1** and 10*R*-epimer (**3**) to determine the absolute stereochemistry. In a previous report, we found that **3** has moderate Wnt signal inhibitory activity although a natural product **1** does not have activity. Because few Wnt signal inhibitors have been reported and peptide-based lead compounds seem to be good candidates to treat cancers, we planned to synthesize 3*R*-epimer of **1** (**4**) and (3*R*, 4*S*, 10*R*, 11*R*)-isomer of **1** (**5**) and evaluate their Wnt signal inhibitory activities.

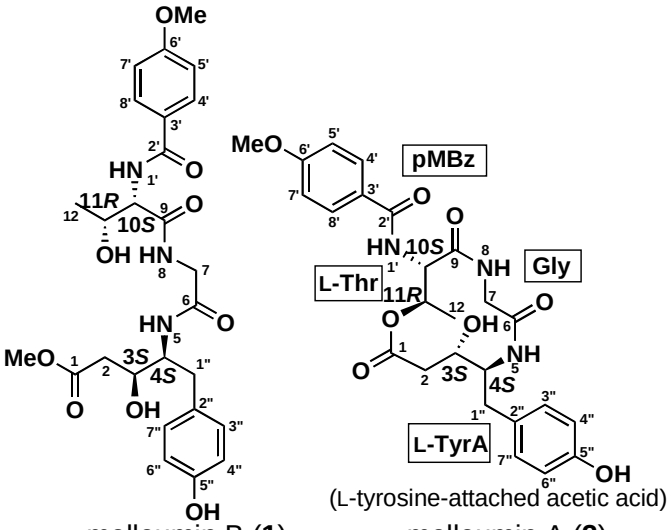
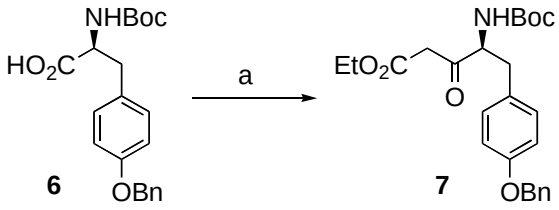


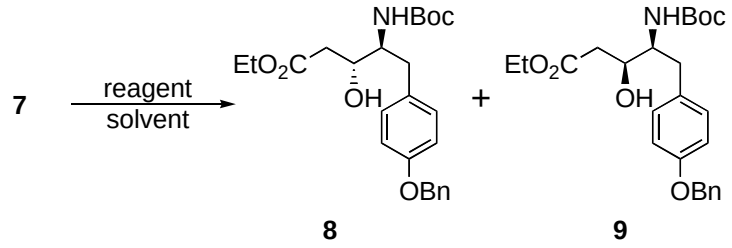
Figure 1. Structures of melleumin B (**1**), melleumin A (**2**) and synthesized isomers.

To obtain (3*R*, 4*S*)-TyrA unit steroselectively, the reduction of β-ketoester **7** was examined. β-Ketoester **7** was synthesized from *N*-Boc-*O*-benzyl-L-tyrosine **6** (Scheme 1). As shown in Table 1, although several typical conditions of chelating control were used, the desired (3*R*, 4*S*)-β-hydroxy ester **8** was obtained in



Scheme 1. Reagents and Conditions: (a) CDI, THF, rt, overnight, then EtOAc, LDA, -78 °C, 68%.

Table 1. Stereoselective synthesis of **8**

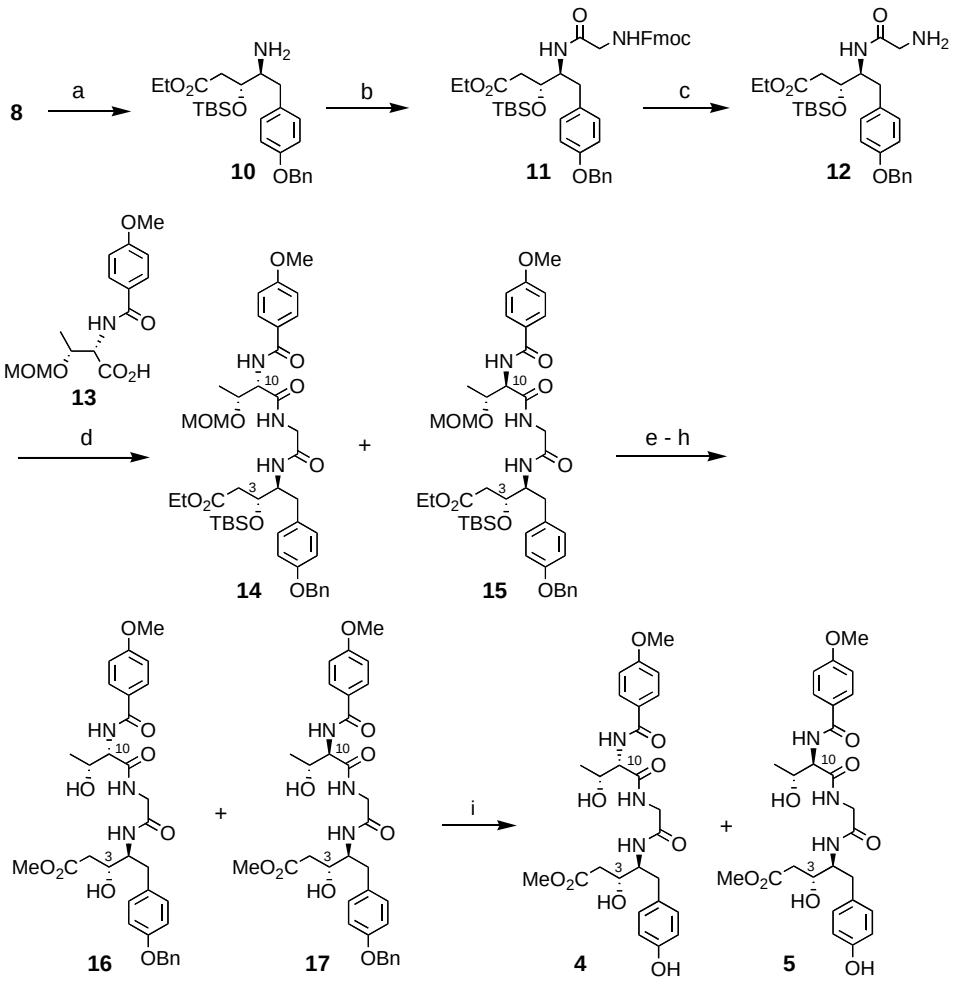


entry	reagent (eq.)	solvent	temp (C)	time (h)	**8** : **9**[a)]	yield (%)	**7** (%)
1	$Zn(BH_4)_2$ (10)	EtOH	-78	5	73 : 27	76	13
2	$Zn(BH_4)_2$ (10), $ZnCl_2$ (1.5)	EtOH	-78	5	78 : 22	44	35
3	$NaBH_4$ (1.5), $TiCl_4$ (1.5)	MeOH	-78	5	82 : 18	52	37
4	$NaBH_4$ (3.0), $CeCl_3$ (1.5)	EtOH	-78	5	57 : 43	90	0
5[b)]	BH_3-Py (1.5), $TiCl_4$ (1.5)	CH_2Cl_2	-78	1	87 : 13	72	0
6	$NaBH_4$ (1.5)	EtOH	0	1	80 : 20	95	0
7	$NaBH_4$ (1.5)	EtOH	-78	1	91 : 9	94	0
8	L-Selectride (3.0)	THF	0 to rt	216	- : -	no reaction	ND
9	K-Selectride (2.4)	THF	0	9	7 : 93	69	0
10	K-Selectride (2.4)	THF	-78	9	5 : 95	41	46

a) Diastereoselectivity was determined by HPLC; *b)* Diastereoselectivity was determined by ^{1}H NMR.
ND: Not determined.

94% yield with a ratio of **8**/**9** = 91/9, with only $NaBH_4$ (entry 7).[5] When K-Selectride was used, (3*S*, 4*S*)-TyrA unit **9** was predominately obtained as expected (entries 9, 10).Compound **8** was protected as the TBS ether in a one-pot reaction. Compound **8** was treated with TBSOTf for cleavage of the Boc group, and then 2,6-lutidine was added to give the TBS ether.[4] The reaction mixture, containing free amine **10**, was evaporated and the residue was subjected to coupling with Fmoc-Gly to give compound **11** in good yield (61%, 2 steps from **8**). Deprotection of the Fmoc group gave the desired TyrA-Gly unit **12**, which was used in the next reaction without purification. Coupling of the TyrA-Gly unit **12** with L-Thr-pMBz unit **13**[4] gave tripeptide **14** along with its 10*R*-epimer **15**, which was caused by racemization in the reaction (67%, 2 steps from **11**; **14**/**15** = 85/5, the ratio was determined by ^{1}H NMR). The structure of **14** was elucidated by H-H COSY, HMQC, and HMBC spectra, after purification by HPLC. Although we

examined several conditions, racemization occurs at the C10 position. Because 10*R*-isomer was also interesting to check a Wnt signal inhibition activity, we decided to separate the isomers at the final step. Deprotection of the TBS and MOM groups on the tripeptide, followed by hydrolysis of the ethyl ester with LiOH, afforded a mixture of *seco* acids.



Scheme 2. Reagents and Conditions: (a) TBSOTf, CH_2Cl_2, 0 °C, 3.5 h then 2,6-lutidine, 0.5 h; (b) Fmoc-Gly, EDC, HOBt, 4-DMAP, CH_2Cl_2, rt, overnight, 61% (2 steps from **8**); (c) piperidine, CH_2Cl_2, 0 °C, 1 h; (d) EDC, HOOBt, 4-DMAP, CH_2Cl_2, rt, overnight, 68% (2 steps from **11**), **14**/**15** = 85/15; (e) TBAF, THF, rt, 3 h, 83%; (f) HCl, EtOH, 60 °C, 45 min, 76%; (g) LiOH, THF/H_2O = 1/1, 0 °C, 45 min; (h) TMS-diazomethane, MeOH, rt, overnight, 84% in 2 steps, **16**/**17** = 83/14; (i) H_2, 10% Pd/C, EtOH, rt, overnight, 94%, **4**/**5** = 83/17.

Conversion to the methyl ester with TMS-diazomethane gave compounds **16** and **17** (84% in 2 steps, **16**/**17** = 83/14). The structure of **17** was determined by H-H COSY, HMQC, and HMBC spectra, after purification by HPLC. The benzyl group was finally removed by hydrogenolysis to give a mixture of 3*R*-epimer of **1** (**4**) and (3*R*, 4*S*, 10*R*, 11*R*)-isomer of **1** (**5**) in 94% yield (**4/5** = 83/17, the ratio was determined by reversed-phase HPLC). Compounds **4** and **5** were separated as a pure form by reversed-phase HPLC. These structures were carefully determined by using ^{1}H and ^{13}C NMR, H-H

COSY, HMQC, and HMBC spectra and comparison of the characteristic peak pattern with those of synthetic **1** and its 10*R*-epimer (**3**).

The Wnt signal transduction pathway has important functions in vertebrate development, and its deregulation is believed to occur early in human colorectal cancer.[2, 6] Wnt target genes have recently been found to be highly expressed in colon cancers; some of these genes are likely to contribute to cancer formation.[7] Small-molecule inhibitors of this pathway are desired because it is believed that their clinical use would suppress cancer cell growth. Several potent small-molecule inhibitors have been reported;[8] however, the development of therapies to specifically target the Wnt pathway in cancer cells is still in its infancy.

We examined Wnt signal inhibitory activity of the two synthesized compounds (**4** and **5**) using a luciferase reporter gene assay. Wnt signaling activates gene transcription with a complex between β-catenin and TCF/LEF, which is a DNA-binding protein. SuperTOP-Flash, a reporter plasmid with multiple TCF-binding sites (CCTTTGATC), was stably transfected in 293 cells.[9] SuperFOP-flash has eight mutated TCF-binding sites (CCTTTGGCC); therefore, a selective inhibitor would not show enhanced transcription in SuperFOP-Flash-transfected cells; thus, these cells provide a negative control for the assay and the TOP/FOP-Flash reporter activation ratio provides a measure of the selectivity of Wnt signal inhibition. It is also possible that a decrease of luciferase reporter activity in this assay is due to the cytotoxicity of the test samples. We therefore also examined the cytotoxicity of samples using a fluorimetric microculture cytotoxicity assay (FMCA). Compounds exhibiting both low luciferase reporter activity and high cell viability are "potent" compounds.

The results are shown in Figure 2, along with the cell viability. We have already found that 10*R*-epimer (**3**) of melleumin B is a moderate inhibitor of Wnt signal transcription, although melleumin B (**1**) did not show inhibition. We evaluated two newly synthesized isomers of **1**. For control experiments, the activities of **1** and **3** were also examined at the same time. The 10*R*-epimer (**3**) exhibited moderate dose-dependent SuperTOP-Flash-transfected cell inhibition, as reported previously.[4] The 3*R*-epimer (**4**) and (3*R*, 4*S*, 10*R*, 11*R*)-isomer (**5**) exhibited moderate Wnt signal inhibition in a dose-dependent manner with high cell viability. These results suggest that small molecules using a template of melleumin B (**1**) with unnatural stereocenters of amino acids might be lead compounds as peptide-based Wnt signal inhibitors.

Since few small molecules are known as Wnt signal inhibitors, and their clinical use has received great attention, we believe that peptide-based inhibitors would be worth synthesizing to evaluate their potential. Application using solid-phase reactions to construct small molecule libraries based on melleumins A and B, isolated from the myxomycete *Physarum melleum*, is in progress.

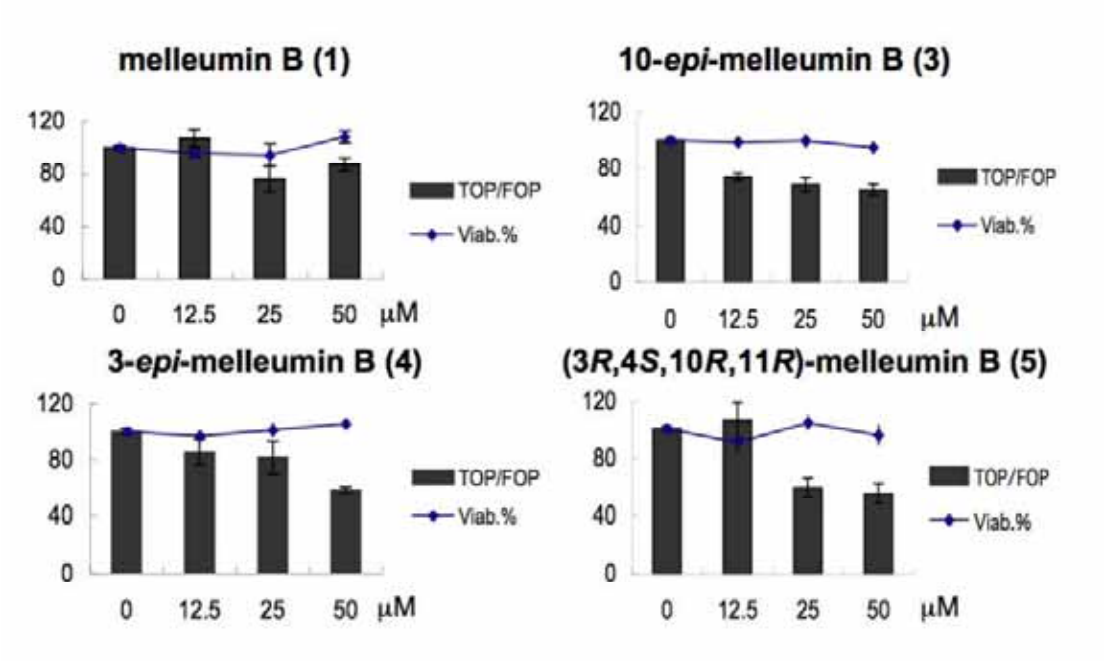


Figure 2. Wnt signal inhibitory activities of mellumins.

EXPERIMENTAL

3-(*tert*-Butoxycarbonylamino)-2-oxo-4-(benzyloxy)benzenepentanoic acid ethyl ester (7)

To a THF (24 mL) solution of (*S*)-*N*-(*tert*-butoxycarbonyl)-*O*-benzyltyrosine **6** (877 mg, 2.36 mmol) was added CDI (765 mg, 7.05 mmol) at 0 °C, and stirred for 0.5 h at 0 °C. The reaction mixture was allowed to warm to rt and stirred overnight. In another flask, to a stirred solution of diisopropylamine (1.60 mL, 11.3 mmol) in THF (24 mL) was added a 1.58 M *n*-hexane solution of *n*-butyllithium (7.02 mL, 11.1 mmol) at 0 °C under argon atmosphere. After stirring for 30 min, the reaction mixture was cooled to -78 °C, EtOAc (1.04 mL, 10.6 mmol) was added and the mixture was stirred for 15 min at -78 °C. The above reaction mixture of **6** was added to this mixture and the resulting mixture was stirred for 2 h at -78 °C. The mixture was warmed to rt, and acidified with 2 N HCl, then extracted with EtOAc. The organic layer was washed with brine, dried over Na_2SO_4, filtered, and concentrated in vacuo. The residue was chromatographed on silica gel (hexane:EtOAc = 5:1) to afford **7** (710 mg, 68% yield as white solid). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.27 (t, *J* = 7.1 Hz, 3H), 1.41 (s, 9H), 2.94 (dd, *J* = 7.1, 14.1 Hz, 1H), 3.07 (dd, *J* = 7.1, 14.1 Hz, 1H), 3.44 (d, *J* = 16.1 Hz, 1H), 3.50 (d, *J* = 16.1 Hz, 1H), 4.19 (q, *J* = 7.1 Hz, 2H), 4.52 (q, *J* = 7.1 Hz, 1H), 5.02 (s, 2H), 5.12 (m, 1H), 6.92 (brd, *J* = 8.7 Hz, 2H), 7.09 (d, *J* = 8.7 Hz, 2H) and 7.29-7.42 (m, 5H); ^{13}C NMR 100 MHz ($CDCl_3$) δ 13.9, 28.1 (3C), 35.9, 46.7, 60.5, 61.3, 69.8, 80.0, 114.9 (2C), 127.3 (2C), 127.8, 128.2, 128.4 (2C), 130.2 (2C), 136.8, 155.1, 157.7, 166.8, and 202.0; IR (ATR) 3336, 2983, 2926, 1747, 1718, 1686, 1512, 1313, 1242, 1143 and 1013 cm^{-1}; HRMS (EI) $C_{25}H_{31}NO_6$ $[M]^+$ calcd 441.2151, found 441.2146; $[\alpha]_D^{22}$ +0.3 (*c* 1.0, $CHCl_3$).

(3*R*,4*S*)-5-[4-(Benzyloxy)phenyl]-4-(*tert*-butoxycarbonylamino)-3-hydroxypentanoic acid ethyl ester (8), (3*S*,4*S*)-5-[4-(benzyloxy)phenyl]-4-(*tert*-butoxycarbonylamino)-3-hydroxypentanoic acid ethyl ester (9)

To a stirred solution of **7** (50 mg, 0.11 mmol) in ethanol (0.29 mL) was added sodium tetrahydroborate (6.2 mg) at -78 °C under argon atmosphere. After 2 h, the reaction mixture was warmed to rt, and acidified with 2 N HCl, then extracted with EtOAc. The organic layer was dried over Na_2SO_4, filtered and concentrated in vacuo. Chlomatography of the residue on silica gel eluting with a gradient (hexane:EtOAc = 3:1 to 1:1) afforded a mixture of **8** and **9** (47.3 mg, 94%) (**8**:**9** = 91: 9, Diastereoselectivity was determined by reversed-phase HPLC; PEGASIL-ODS, 4.6 x 250 mm; UV detection at 274 nm; eluent, 60% MeOH; flow rate, 0.4 mL/min; retention time: **8** (19.3 min), **9** (22.1 min)). Recrystallization from EtOAc and *n*-hexane gave compound **8** as a pure form.

Spectral data of 8: ^{1}H-NMR (400 MHz, CD_3OD) δ 1.26 (t, *J* = 7.1 Hz, 3H,C*H*$_3$CH$_2$OCO), 1.31 (s, 9H, Boc), 2.40 (dd, *J* = 9.3, 15.3 Hz, 1H, H-2), 2.49 (dd, *J* = 10.5, 13.9 Hz, 1H, H-5), 2.62 (dd, *J* = 3.5, 15.3 Hz, 1H, H-2), 3.04 (dd, *J* = 3.7, 13.9 Hz, 1H, H-5), 3.59-3.64 (m, 1H, H-3), 3.91-3.96 (m, 1H, H-4), 4.09-4.19 (m, 2H, CH$_3$C*H*$_2$OCO), 5.04 (s, 2H, C$_6$H$_5$C*H*$_2$O), 6.47 (brd, *J* = 9.5 Hz, 1H), 6.88 (d, *J* = 8.5 Hz, 2H, H-3'and H-5'), 7.12 (d, *J* = 8.5 Hz, 2H, H-2'and H-6') and 7.29-7.42 (m, 5H, C$_6$*H*$_5$CH$_2$O); ^{13}C NMR (100 MHz, CD_3OD) δ 14.5, 28.7 (3C), 36.8, 40.5, 58.1, 61.6, 70.9, 71.9, 80.0, 115.7 (2C), 128.4, 128.4 (2C), 129.4 (2C), 131.4 (2C), 132.4, 138.9, 158.0, 158.7 and 173.7; IR (ATR) 3352, 2981, 2926, 1730, 1682, 1511, 1307 and 1233 cm^{-1}; HRMS (FAB) calcd for $C_{25}H_{34}NO_6$ 444.2386 $[M+H]^+$, found 444.2388; $[\alpha]_D^{23}$ +4.8 (*c* 1.0, $CHCl_3$).

Spectral data of 9: ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.24 (t, *J* = 7.0Hz, 3H), 1.39 (s, 9H), 2.37 (dd, *J* = 2.4, 16.8 Hz, 1H), 2.58 (dd, *J* = 10.4, 16.8 Hz, 1H), 2.85 (d, *J* = 7.8 Hz, 2H), 3.54 (s, 1H), 3.67 (q, *J* = 7.8 Hz, 1H), 3.98 (m, 1H), 4.13 (q, *J* = 7.0 Hz, 2H), 4.98 (m, 1H), 5.02 (s, 2H), 6.90 (d, *J* = 8.4 Hz, 2H), 7.16 (d, *J* = 8.4 Hz, 2H) and 7.27-7.44 (m, 5H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 14.1, 28.3 (3C), 37.7, 38.6, 55.4, 60.8, 66.9, 70.0, 79.3, 114.8 (2C), 127.4 (2C), 127.9, 128.5 (2C), 130.3 (2C), 130.5, 137.1, 155.8, 157.4, and 173.5; IR (ATR) 3368, 2978, 1717, 1679, 1509, 1366, 1242 and 1163 cm^{-1}; HRMS (FAB) calcd for $C_{25}H_{34}NO_6$ 444.2386 $[M+H]^+$, found 444.2361; $[\alpha]_D^{23}$ -12 (*c* 1.0, $CHCl_3$).

(3*R*,4*S*)-4-Amino-3-[(*tert*-butyldimethylsilyl)oxy]-5-[4-(benzyloxy)phenyl]pentanoic acid ethyl ester (10): A solution of **8** (585 mg, 1.32 mmol) in CH_2Cl_2 (13.2 mL) was treated with *tert*-butyldimethylsilyl trifluoromethansulfonate (0.91 mL, 3.96 mmol) at 0 °C under argon atmosphere. The mixture was stirred for 3.5 h at 0 °C, and then 2,6-lutidine (0.54 mL, 4.63 mmol) was added, and the mixture was further stirred at 0 °C for 0.5 h. After addition of water, the mixture was extracted with EtOAc, dried over Na_2SO_4, filtered, and concentrated in vacuo, to afford **10**, which was used without further purification in

the following reaction.

(3*R*,4*S*)-4-[2-(9-Fluorenylmethoxycarbonylamino)acetylamino]-3-[(*tert*-butyldimethylsilyl)oxy]-5-[4-(benzyloxy)phenyl]pentanoic acid ethyl ester (**11**): A solution of Fmoc-glycine (544 mg, 1.83 mmol), EDC·HCl (351 mg, 1.83 mmol), HOBt·H_2O (247 mg, 1.83 mmol) and 4-DMAP (223 mg, 1.83 mmol) in CH_2Cl_2 (6.1 mL) were mixed at 0 °C under argon atmosphere. The mixture was stirred for 10 min, and then a solution of **10** in CH_2Cl_2 (6.1mL) was added. The reaction mixture was allowed to warm to rt and stirred overnight. The solvent was evaporated under reduced pressure and the residue was dissolved in EtOAc. The organic layer was washed with water, saturated aqueous $NaHCO_3$, 10% aqueous citric acid, water, and brine. The organic layer was dried over Na_2SO_4, filtered and concentrated in vacuo. The residue was chromatographed on silica gel (hexane:EtOAc = 3:1) to afford **11** (592 mg, 0.80 mmol, 61%) as a yellow powder. ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.04 (s, 3H), 0.07 (s, 3H), 0.89 (s, 9H), 1.26 (t, *J* = 7.1 Hz, 3H), 2.51-2.59 (m, 2H), 2.65 (dd, *J* = 9.6, 14.3 Hz, 1H), 2.95 (dd, *J* = 4.7, 14.3 Hz, 1H), 3.64 (dd, *J* = 5.4, 16.5 Hz, 1H), 3.72 (dd, *J* = 5.6, 16.5Hz, 1H), 4.08-4.82 (m, 5H), 4.40 (d, *J* = 6.6 Hz, 2H), 4.98 (s, 2H), 5.13 (brs, 1H), 5.96 (brd, *J* = 8.5 Hz, 1H), 6.84 (d, *J* = 8.5 Hz, 2H), 7.05 (d, *J* = 8.3 Hz, 2H), 7.27-7.42 (m, 9H), 7.58 (brd, *J* = 6.6 Hz, 2H,), and 7.76 (d, *J* = 7.6 Hz, 2H); ^{13}C NMR 100 MHz ($CDCl_3$) δ -4.9, -4.7, 14.1, 18.0, 25.7 (3C), 34.6, 39.9, 44.4, 47.0, 55.1, 60.7, 67.1, 69.8, 70.6, 114.8 (2C), 120.0 (2C), 125.0 (2C), 127.1 (2C), 127.4 (2C), 127.7, 127.8, 128.5 (4C), 130.0 (2C), 136.9 (2C), 141.2, 141,3, 143.7, 156.3, 157.3, 168.1, and 171.2; IR (ATR) 2952, 2928, 1728, 1664, 1509, 1449, 1240, 1175, 1081, 1025, 831, 737, and 695 cm^{-1}; HRMS (FAB) calcd for $C_{43}H_{53}N_2O_7Si$ 737.3970 $[M+H]^+$, found 737.3544; $[\alpha]_D^{20}$ -12.6 (*c* 1.0, $CHCl_3$).

(3*R*,4*S*)-4-[(2-Aminoacetyl)amino]-3-[(*tert*-butyldimethylsilyl)oxy]-5-[(4-benzyloxy)phenyl]-pentanoic acid ethyl ester (12): A solution of **11** (592 mg, 0.80 mmol) in DMF (8.0 mL) was treated with piperidine (0.8 mL, 8.1 mmol) for 1 h at 0 °C under argon atmosphere. The reaction mixture was concentrated in vacuo. The mixture was used without further purification in the following reaction.

(3*R*,4*S*)-5-(4-Benzyloxyphenyl)-3-[(*tert*-butyldimethylsilyl)oxy]-4-[*N*-(4-methoxybenzoyl)-3-methoxy-methyl-L-threonylglycyl]amino]pentanoic acid ethyl ester (14) and its 10*R*-epimer (15): A solution of **12** (287 mg, 1.01 mmol), EDC·HCl (171 mg, 0.894 mmol), HOOBt (146 mg, 0.894 mmol) and 4-DMAP (109 mg, 0.894 mmol) in CH_2Cl_2 (2.8 mL) were mixed at 0 °C under argon atmosphere. The mixture was stirred for 30 min, and then **13** in CH_2Cl_2 (2.8 mL) was added. The reaction mixture was allowed to warm to rt and stirred overnight. The solvent was evaporated under reduced pressure and the residue was dissolved in EtOAc. The organic layer was washed with water, saturated aqueous $NaHCO_3$, 10% aqueous citric acid, water, and brine, dried over Na_2SO_4, filtered and concentrated in vacuo. The residue was chromatographed on silica gel (hexane:EtOAc = 2:3) to afford a mixture of **14** and **15** (491 mg, 77%, two steps from **12**) (**14**:**15** = 85:15, determined by reversed-phase HPLC).

Purification of 14: The mixture (**14** and **15**) was separated by preparative HPLC (Develosil C-30 UG-5, 10 x 250 mm; eluent, 89% MeOH; UV detection at 254 nm; flow rate, 2.5 mL/min). Retention time: **14** (42.0 min), **15** (46.5 min).

Spectral data of 14: ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.04 (s, 3H, Si(C*H*$_3$)$_2$C(CH$_3$)$_3$), 0.08 (s, 3H, Si(C*H*$_3$)$_2$C(CH$_3$)$_3$), 0.89 (s, 9H, Si(CH$_3$)$_2$C(C*H*$_3$)$_3$), 1.19 (d, *J* = 6.3 Hz, 3H, H-12), 1.25 (t, *J* = 7.1 Hz, 3H, C*H*$_3$CH$_2$OCO), 2.50-2.60 (m, 2H, H-2), 2.64 (dd, *J* = 9.6, 14.2 Hz, 1H, H-1''), 2.92 (dd, *J* = 4.6, 14.2 Hz, 1H, H-1''), 3.39 (s, 3H, C*H*$_3$OCH$_2$-), 3.79 (dd, *J* = 5.2, 16.8 Hz, 1H, H-7), 3.85 (s, 3H, PhOC*H*$_3$), 3.90 (dd, *J* = 6.1, 16.8 Hz, 1H, H-7), 4.06-4.26 (m, 4H, H-3 and H-4 and -COOC*H*$_2$CH$_3$), 4.34-4.37 (m, 1H, H-11), 4.60 (dd, *J* = 4.1, 5.9 Hz, 1H, H-10), 4.74 (d, *J* = 6.5Hz, 2H, CH$_3$OC*H*$_2$O-), 4.99 (s, 2H, PhC*H*$_2$O), 6.36 (brd, *J* = 8.8 Hz, 1H, H-5), 6.85 (d, *J* = 8.5 Hz, 2H, H-4'' and H-6''), 6.93 (d, *J* = 9.0 Hz, 2H, H-5' and H-7'), 6.99 (m, 1H, H-8), 7.04 (d, *J* = 8.5 Hz, 2H, H-3'' and H-7''), 7.20 (brd, *J* = 5.9 Hz, 1H, H-1'), 7.29-7.52 (m, 5H, C$_6$*H*$_5$CH$_2$O), and 7.82 (d, *J* = 8.8 Hz, 2H, H-4'and H-8'); ^{13}C NMR 100 MHz ($CDCl_3$) δ -4.8, -4.6, 14.1, 16.5, 18.0, 25.8 (3C), 35.2, 39.9, 43.0, 55.4, 55.57, 55.61, 57.9, 60.7, 69.9, 70.7, 73.4, 77.2, 113.8 (2C), 114.8 (2C), 125.6, 127.5 (2C), 127.9, 128.5 (2C), 129.0 (2C), 130.0 (2C), 130.2, 137.1, 157.4, 162.6, 167.0, 167.9, 169.9 and 171.6; IR (ATR) 3270, 2930, 1734, 1629, 1542, 1507, 1463, 1383, 1252, 1176, 1111, 1029, 838, 777, and 696 cm^{-1} ; HRMS (FAB) calcd for $C_{42}H_{60}N_3O_{10}Si$ 794.3970 $[M+H]^+$, found 794.4039; $[\alpha]_D^{20}$ -8.2 (*c* 1.0, $CHCl_3$).

Spectral data of 15^{1}H NMR (400 MHz, $CDCl_3$) δ 0.00 (s, 3H), 0.06 (s, 3H), 0.86 (s, 9H), 1.22 (t, *J* = 7.2 Hz, 3H), 1.36 (d, *J* = 6.4 Hz, 3H), 2.46 (dd, *J* = 6.0, 15.2 Hz, 2H), 2.56 (dd, *J* = 5.4, 15.4 Hz, 1H), 2.64 (dd, *J* = 9.6, 14.0 Hz, 1H), 2.95 (dd,*J* = 4.5, 14.0 Hz, 1H), 3.39 (s, 3H), 3.81 (dd, *J* = 3.0, 5.4 Hz, 2H), 3.85 (s, 3H), 4.01-4.18 (m, 5H), 4.54 (dd, *J* = 5.2, 6.6 Hz, 1H), 4.69 (dd, *J* = 7.7, 11.0 Hz, 2H), 5.02 (s, 2H), 6.24 (brd, *J* =8.3 Hz, 1H), 6.58 (brt, *J* = 5.4 Hz, 1H), 6.88 (d, *J* = 8.5 Hz, 2H), 6.94 (d, *J* = 8.8 Hz, 2H), 7.10 (d, *J* = 8.5 Hz, 2H), 7.29-7.44 (m, 5H) and 7.82 (d, *J* = 8.8 Hz, 2H).

Removal of TBS protective group.

(3*R*,4*S*)-5-(4-Benzyloxyphenyl)-4-[[*N*-(4-methoxybenzoyl)-3-methoxymethyl-L-threonylglycyl]-amino]pentanoic acid ethyl ester and its 10*R*-epimer: To a stirred solution of a mixture of **14** and **15** (109.5 mg, 0.138 mmol) in THF (0.41 mL) was added a 1.0 M THF solution of tetrabutylammonium fluoride (0.41 mL, 0.41 mmol) at 0 °C under argon atmosphere. After 10 min, the reaction mixture was allowed to warm to rt and stirred for 5 h. The reaction mixture was quenched with H_2O and extracted with EtOAc. The organic layer was dried over Na_2SO_4, filtered and concentrated in vacuo. The residue was chromatographed on silica gel (hexane:EtOAc = 1:5) to afford a mixture of alchols (78.2 mg, 0.115 mmol, 84%), which was used without further purification in the following reaction.

Spectral data of mixture: ^{1}H-NMR (400 MHz, $CDCl_3$) δ 1.19-1.27 (m, 5.5H), 1.34 (d, *J* = 9.9 Hz, 0.5H),

2.06 (brs, 2H), 2.45-2.57 (m, 2H), 2.70-2.94 (m, 2H), 3.36 (s, 2.5H), 3.38 (s, 0.5H), 3.74-3.81 (m, 2H), 3.83 (s, 3H), 3.84-4.00 (m, 2H), 4.05-4.30 (m, 6H), 4.45 (t, *J* = 4.9 Hz, 0.2H), 4.49 (t, *J* = 5.1 Hz, 0.8H), 4.64-4.77 (m, 2H), 4.96 (s, 1.6H), 5.00 (s, 0.4H), 6.80-7.16 (m, 8H), 7.26-7.42 (m, 6H), and 7.78-7.83 (m, 2H).

Removal of MOM protective group.

(3*R*,4*S*)-5-(4-Benzyloxyphenyl)-4-[[*N*-(4-methoxybenzoyl)-L-threonylglycyl]amino]pentanoic acid ethyl ester and its 10*R*-epimer: The above mixture (69.3 mg, 0.10 mmol) was dissolved in EtOH (10 mL) containing conc. aq HCl (0.2 mL), and the mixture was heated to 60 °C and stirred for 4 h. The reaction mixture was cooled to rt, quenched with saturated aqueous $NaHCO_3$ and extracted with EtOAc. The organic layer was dried over Na_2SO_4, filtered and concentrated in vacuo. The residue was chromatographed on silica gel ($CHCl_3$/MeOH = 20/1) to afford a mixture of diols (48.6 mg, 0.08 mmol, 76%), which was used without further purification in the following reaction.

Spectral data of mixture: ^{1}H-NMR (400 MHz, $CDCl_3$) δ 1.16-1.38 (m, 6H), 2.43-2.84 (m, 4H), 3.61-3.69 (m, 1H), 3.78 (s, 3H), 3.92 (dd, *J* = 6.3, 16.6 Hz, 0.8H), 3.95-4.19 (m, 4.2H), 4.38-4.53 (m, 2H), 4.93-4.99 (m, 2H), 6.80-6.89 (m, 4H), 7.03-7.06 (m, 1.6H), 7.09 (d, *J* = 8.5 Hz, 0.5H), 7.16 (d, *J* = 8.5 Hz, 1H), 7.26-7.44 (m, 6H), and 7.69-7.90 (m, 3H).

(3*R*,4*S*)-5-(4-Benzyloxyphenyl)-4-[[*N*-(4-methoxybenzoyl)-L-threonylglycyl]amino]pentanoic acid methyl ester (16) and its 10*R*-epimer (17):

A solution of the above mixture of protected tripeptide compounds (48.6 mg, 0.08 mmol) in solvent (THF/H_2O = 1/1, 0.76 mL) was treated with LiOH-H_2O (6.4 mg, 0.153 mmol) at 0 °C, and the mixture was stirred for 40 min. The mixture was acidified with 2 N HCl, and then extracted with EtOAc. The organic layer was dried over $MgSO_4$, filtered, and concentrated in vacuo to afford a mixture of *seco* acid compounds, which was used without further purification in the following reaction. A solution of the mixture of *seco* acid compounds in MeOH (2.6 mL) was treated with 10% hexane solution of TMS-diazomethane (0.58 mL, 0.34 mmol) at rt under argon atmosphere. After stirring overnight, the reaction mixture was concentrated in vacuo. Then, the residue was chromatographed on silica gel ($CHCl_3$:MeOH = 20:1) to afford **16** as a mixture with its 10*R*-epimer **17** (39.5 mg, 0.06 mmol, 84%) (**16**:**17** = 83:17, determined by reversed-phase HPLC), which was used without further purification in the following reaction. A solution of the mixture (11.1 mg) in MeOH (0.36 mL) was treated with 10% hexane solution of TMS-diazomethane (1.8 mL) at rt under argon atmosphere. The mixture was stirred overnight. The reaction mixture was concentrated in vacuo. Then, the residue was chromatographed on silica gel ($CHCl_3$:MeOH = 10:1) to afford a mixture of **16** and **17** (8.3 mg, 0.06 mmol, 74%) (**16**:**17** = 83:17, the ratio was determined by reversed-phase HPLC).

Purification of 16: The mixture (**16** and **17**) was separated by preparative HPLC (Develosil C-30 UG-5; 10 x 250 mm; eluent, 70% MeOH; UV detection at 254 nm; flow rate, 3.0 mL/min). Retention time: **16** (16.2 min), **17** (20.4 min).

Spectral data of 16: ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.23 (d, *J* = 6.3 Hz, 3H, H-12), 1.87 (brs, 2H, -O*H*), 2.51 (d, *J* = 2.2 Hz, 1H, H-2), 2.52 (s, 1H, H-2), 2.71 (dd, *J* = 9.0, 14.3 Hz, 1H, H-1''), 2.83 (dd, *J* = 4.9, 14.3 Hz, 1H, H-1''), 3.64 (s, 3H, C*H*$_3$OCO-), 3.69 (dd, *J* = 5.2, 6.5 Hz, 1H, H-7), 3.81 (s, 3H, C*H*$_3$OC$_6$H$_4$-), 3.93 (dd, *J* = 6.5, 16.5 Hz, 1H, H-7), 4.11 (brs, 1H, H-3), 4.16-4.21 (m, 1H, H-4), 4.26 (brs, 1H, H-5), 4.42 (brd, *J* = 3.9 Hz, 1H, H-11), 4.47 (brdd, *J* = 2.2, 7.1 Hz, 1H, H-10), 4.97 (s, 2H, C$_6$H$_5$C*H*$_2$-), 6.83 (d, *J* = 8.8 Hz, 2H, H-4'' and H-6''), 6.88 (d, *J* = 9.0 Hz, 2H, H-5' and H-7'), 6.95 (brd, *J* = 9.0 Hz, 1H, H-5), 7.05 (d, *J* = 8.5 Hz, 1H, H-3'' and H-7''), 7.24 (m, 1H, H-1'), 7.29-7.52 (m, 5H, C$_6$*H*$_5$CH$_2$O), 7.54 (brs, 1H, H-8), and 7.80 (d, *J* = 8.8 Hz, 2H, H-4' and H-8'); ^{13}C NMR 100 MHz ($CDCl_3$) δ 19.2, 34.2, 37.8, 43.1, 52.0, 54.8, 55.4, 58.9, 66.9, 69.8, 69.9, 113.8 (2C), 114.8 (2C), 125.1, 127.5 (2C), 127.9, 128.5 (2C), 129.3 (2C), 129.8, 130.1 (2C), 137.0, 157.4, 162.8, 168.1, 169.4, 172.2 and 173.1; HRMS (FAB) calcd for $C_{33}H_{39}N_3NaO_9$ 644.2584 $[M+Na]^+$, found 644.2552; IR (ATR) 3296, 2926, 2852, 1730, 1637, 1606, 1540, 1508, 1455, 1439, 1410, 1379, 1298, 1253, 1176, 1111, 1074, 1024, 844, 768, 738, and 696 cm^{-1}; $[\alpha]_D^{23}$ +4.1 (*c* 1.0, MeOH).

Spectral data of 17: ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.32 (d, *J* = 6.3 Hz, 3H), 1.87 (brs, 2H), 2.46 (d, *J* = 6.3 Hz, 2H), 2.74 (dd, *J* = 8.8, 14.4 Hz, 1H), 2.86 (dd, *J* = 4.6, 14.4 Hz, 1H), 3.63 (s, 3H), 3.76 (dd, *J* = 5.2, 5.6 Hz, 1H), 3.82 (s, 3H), 3.89 (dd, *J* = 5.6, 16.6 Hz, 1H), 4.00-4.21 (m, 4H), 4.41 (t, *J* = 6.7 Hz, 1H), 5.00 (s, 2H), 6.87 (d, *J* = 8.5 Hz, 2H), 6.89 (d, *J* = 5.8 Hz, 2H), 7.02 (brd, *J* = 7.6 Hz, 1H), 7.10 (d, *J* = 8.6 Hz, 2H), 7.29-7.42 (m, 6H), and 7.79 (d, *J* = 8.8 Hz, 2H); ^{13}C NMR 100 MHz ($CDCl_3$) δ : 20.2, 34.1, 37.8, 43.1, 52.0, 54.9, 55.4, 59.1, 68.8, 69.9, 70.0, 113.9, 114.8, 125.1, 127.5, 127.9, 128.3, 128.5, 129.2, 130.2, 137.0, 157.4, 162.8, 167.7, 169.2, 171.8 and 173.1; HRMS (FAB) calcd for $C_{33}H_{40}N_3O_9$ 622.2765 $[M+H]^+$, found 622.2768; IR (ATR) 3296, 2927, 2852, 1733, 1636, 1606, 1541, 1507, 1456, 1438, 1376, 1297, 1252, 1176, 1112, 1065, 1025, 845, 768, 738, and 696 cm^{-1}; $[\alpha]_D^{23}$ -27.9 (*c* 0.5, MeOH).

3-*epi*-Melleumin B (4) and (3*R*,4*S*,10*R*,11*R*)-melleumin B (5):

A mixture of **16** and **17** (3.8 mg, 6.1 μmol) was dissolved in EtOH (0.1 mL), and then 10% palladium on carbon (0.4 mg) was added. The reaction mixture was stirred overnight under hydrogen and then filtered through celite and concentrated in vacuo. The residue was chromatographed on silica gel ($CHCl_3$:MeOH = 10:1) to afford **4** as a mixture with its 10*R*-epimer **5** (3.1 mg, 5.8 μmol, 94%) (**4**:**5** = 83:17, The ratio was determined by reversed-phase HPLC).

Purification of 4: The mixture (**4** and **5**) was separated by preparative HPLC (Develosil C-30 UG-5; 10 x 250 mm; eluent, 50% MeOH; UV detection at 254 nm; flow rate, 3.0 mL/min). Retention time: **4** (13.5 min), **5** (18.6 min).

Spectral data of 3-*epi*-melleumin B (4): ^{1}H NMR (400 MHz, CD_3OD) δ 1.25 (d, *J* = 6.3 Hz, 3H, H-12), 2.43 (dd, *J* = 9.5, 15.5 Hz, 1H, H-2), 2.58 (dd, *J* = 9.8, 14.2 Hz, 1H, H-1"), 2.68 (dd, *J* = 2.9, 15.5 Hz, 1H, H-2), 3.00 (dd, *J* = 3.5, 14.1 Hz, 1H, H-1"), 3.67 (s, 3H, -COOC*H*$_3$), 3.69 (d, *J* = 16.8 Hz, 1H, H-7), 3.81 (d, *J* = 16.8 Hz, 1H, H-7), 3.85 (s, 3H, -C_6H_4OC*H*$_3$), 3.94-4.08 (m, 2H, H-3 and H-4), 4.22-4.28 (m, 1H, H-11), 4.39 (d, *J* = 4.6 Hz, 1H, H-10), 6.59-6.65 (m, 2H, H-4'' and H-6''), 6.92-6.96 (m, 2H, H-3'' and H-7''), 6.99-7.03 (m, 2H, H-5' and H-7'), and 7.87-7.91 (m, 2H, H-4' and H-8'); ^{13}C NMR 100 MHz (CD_3OD) δ 20.2, 36.4, 40.1, 43.6, 52.1, 56.0, 57.1, 61.7, 68.4, 71.5, 114.8 (2C), 116.1 (2C), 126.8, 130.4 (2C), 130.5, 131.2 (2C), 156.8, 164.3, 170.0, 171.4, 173.6 and 174.1; HRMS (FAB) calcd for $C_{26}H_{34}N_3O_9$ 532.2295 $[M+H]^+$, found: 532.2253; IR (ATR) 3852, 3649, 3291, 2940, 1717, 1635, 1606, 1541, 1492, 1457, 1436, 1254, 1177, 1108, 1026, 844, 757, and 701 cm^{-1}; $[\alpha]_D^{23}$ +22.9 (*c* 0.5, MeOH).

Spectral data of (3*R*,4*S*,10*R*,11*R*)-melleumin B (5): ^{1}H-NMR (400 MHz, CD_3OD): δ 1.33 (d, *J* = 6.1 Hz, 3H, H-12), 2.31-2.38 (m, 1H, H-2), 2.60-2.63 (m, 1H, H-2), 2.60-2.66 (m, 1H, H-1''), 3.05 (dd, *J* = 2.7, 14.2 Hz, 1H, H-1"), 3.62 (s, 3H, -COOC*H*$_3$), 3.71 (d, *J* = 17.1 Hz, 1H, H-7), 3.80 (d, *J* = 17.1 Hz, 1H, H-7), 3.86 (s, 3H, -C_6H_4OC*H*$_3$), 3.96-4.10 (m, 2H, H-3 and H-4), 4.12-4.18 (m, 1H, H-11), 4.29 (d, *J* = 7.6 Hz, 1H, H-10), 6.68 (d, *J* = 8.5 Hz, 2H, H-4'' and H-6''), 7.01 (d, *J* = 8.9Hz, 2H, H-5' and H-7'), 7.04 (d, *J* = 8.5 Hz, 2H, H-3'' and H-7''), and 7.88 (d, *J* = 8.9 Hz, 2H, H-4' and H-8'); ^{13}C NMR 125 MHz (CD_3OD) δ 20.7, 36.4, 40.0, 43.6, 52.0, 56.0, 57.2, 61.8, 68.7, 71.6, 114.8 (2C), 116.1 (2C), 127.0, 130.60, 130.63 (2C), 131.3 (2C), 156.8, 164.3, 170.1, 171.5, 173.7 and 174.0; HRMS (FAB) calcd for $C_{26}H_{34}N_3O_9$ 532.2295 $[M+H]^+$, found 532.2253; IR (ATR) 3820, 3302, 2925, 2852, 1726, 1639, 1607, 1541, 1505, 1441, 1256, 1179, 1111, 1065, 1028, 847, and 770 cm^{-1}; $[\alpha]_D^{16}$ +25.0 (*c* 0.1, MeOH).

Luciferase Assays (Wnt signal inhibition assay)

TOPflash reporter assay

STF/293 cells ($3x10^4$) were split into 96-well plates and 24 h later cells were treated with 15 mM LiCl and testing samples (DMSO solution). After incubation for 24 h, cells were lysed with CCLR (cell culture lysis reagent; 20 μL/well, Promega) and luciferase activities were measured with a Luciferase Assay System (Promega). We checked this system worked collect by using quercetin as a standard positive compound. Assays were performed in triplicate.

FOPflash reporter assay

293T cells ($1x10^5$) were split into 24-well plates and were transfected 24 h later with 1 μg/well superFop-flash reporter plasmid using Lipofectamine2000 (Invitrogen; 2.5 μL/well). 3 h after transfection, cells were treated with 15 mM LiCl and testing samples (DMSO solution). After incubation for 24 h, cells were lysed with Reporter Lysis buffer (Promega; 50 μL/well) and luciferase activities were measured with a Dual-Glo Luciferase Assay System (Promega). Assays were performed in triplicate.

Assay of cell viability

STF/293 cells ($3x10^4$) were split into 96-well plates and incubated for 24 h. Cells were treated with testing samples (DMSO solution) and incubated for 24 h. Cells were treated with fluorescein diacetate (Wako) in PBS buffer (3.5 μg/mL), after 1 h incubation, fluorescence were detected. Assays were performed in triplicate.

ACKNOWLEDGEMENTS

We thank Prof. Jeremy Nathans (John Hopkins Medical School) for kindly providing STF cells (293 cell line stably transfected with SuperTOP-Flash by Prof. R.T. Moon) and Prof. Randall T. Moon (University of Washington) for the SuperFOP-Flash reporter plasmid. This work was supported by Grants-in Aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (No. 18032020 and 19310136 to M.I. and No. 18790012 to M.A.A.). M.A.A. also gratefully acknowledges financial support from the Uehara Memorial Foundation.

REFERENCES

1. For a structural classification of natural products and excellent works of biology-oriented synthesis (BIOS), see M. A. Koch, A. Schuffenhauer, M. Scheck, S. Wetzel, M. Casaulta, A. Odermatt, P. Ertl, and H. Waldmann, *Proc. Natl. Acad. Sci. U. S. A.*, 2005, **102**, 17272; I. R. Corrêa Jr., A. Nören-Müller, H.-D. Ambrosi, S. Jakupovic, K. Saxena, H. Schwalbe, M. Kaiser, and H. Waldmann, *Chem. Asian J.*, 2007, **2**, 1109.
2. N. Barker and H. Clevers, *Nat. Rev. Drug Disc.*, 2006, **5**, 997.
3. K. Kaniwa, M. A. Arai, X. Li, and M. Ishibashi, *Bioorg. Med. Chem. Lett.*, 2007, **17**, 4254.
4. S. Hanazawa, M. A. Arai, X. Li, and M. Ishibashi, *Bioorg. Med. Chem. Lett.*, 2008, **18**, 95.
5. T. Shioiri, K. Hayashi, and Y. Hamada, *Tetrahedron*, 1993, **49**, 1913.
6. K. W. Kinzler and B. Vogelstein, *Cell*, 1996, **87**, 159.
7. M. van de Wetering, E. Sancho, C. Verweij, W. de Lau, I. Oving, A. Hurlstone, K. van der Horn, E. D. Batlle, A.-P. Coudreuse, M. Haramis, P. Tjon-Pon-Fong, M. Moerer, G. van den Born, Soete, S. Pals, M. Eilers, R. Medema, and H. Clevers, *Cell*, 2002, **111**, 241.
8. M. Lepourcelet, Y.-N. P. Chen, D. S. France, H. Wang, P. Crews, F. Petersen, C. Bruseo, A. W. Wood, and R. A. Shivdasani, *Cancer Cell*, 2004, **5**, 91; J.-Y. Trosset, C. Dalvit, S. Knapp, M. Fasolini, M. Veronesi, S. Mantegani, L. M. Gianellini, C. Catana, M. Sundström, P. F. W. Stouten, and J. K. Moll, *Proteins*, 2006, **64**, 60; K. H. Emami, C. Nguyen, H. Ma, D. H. Kim, K. W. Jeong, M. Eguchi, R. T. Moon, J.-L. Teo, S. W. Oh, H. Y. Kim, S. H. Moon, J. R. Ha, and M. Kahn, *Proc. Natl. Acad. Sci. U.S.A.*, 2004, **101**, 12682; J. Shan, D. -L. Shi, J. Wang, and J. Zheng, *Biochemistry*, 2005, **44**, 15495.

9. V. Korinek, N. Barker, P. J. Morin, D. van Wichen, R. de Weger, K. W. Kinzler, and B. Vogelstein, *Science*, 1997, **275**, 1784; Q. Xu, Y. Wang, A. Dabdoub, P. M. Smallwood, J. Williams, C. Woods, M. W. Kelley, L. Jiang, W. Tasman, K. Zhang, and J. Nathans, *Cell*, 2004, **116**, 883.