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Absolute Stereochemistry of Fungal Beauveriolide III and ACAT Inhibitory Activity of Four Stereoisomers

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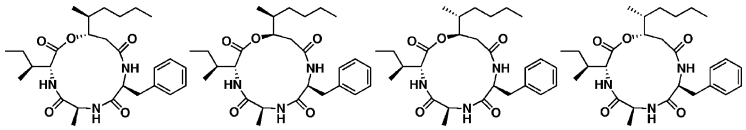

Fungal beauveriolide III (BeauIII, **1b**), a cyclodepsipeptide inhibiting acyl-CoA:cholesterol acyltransferase (ACAT) and showing antiatherogenic activity in mouse models, consists of L-Phe, L-Ala, D-*allo*-Ile, and 3-hydroxy-4-methyloctanoic acid (HMA) moieties, but the stereochemistry of the HMA part has not until now been fully defined. To determine it, four HMA stereoisomers were synthesized and labeled with (*S*)-(+)-2-(anthracene-2,3-dicarboximido)-1-propyl trifluoromethane sulfonate (AP-OTf), a chiral fluorescent reagent. The derivatives were separated by HPLC and compared with the natural HMA derivative, which was thereby identified as (3*S*,4*S*)HMA in BeauIII. Furthermore, the four beauveriolide III isomers ((3*S*,4*S*)BeauIII (**23a**), (3*R*,4*R*)BeauIII (**23b**), (3*R*,4*S*)BeauIII (**23c**), and (3*S*,4*R*)BeauIII (**23d)**) were synthesized, and it was shown that all the spectral data for **23a** were identical with those for natural **1b**. Isomers **23a** and **23d** showed potent inhibitory activity of lipid droplet accumulation in macrophages, while the other two isomers caused weak inhibition. Thus, the 3*S* configuration of BeauIII is important for this activity. Furthermore, **23a** and **23d** showed rather specific inhibition against the ACAT1 isozyme.

Introduction

The chiral discrimination of natural products has received considerable attention because the enantiomers usually have different biological properties.[1] Therefore, it is very important to determine the absolute stereochemistry of natural products in order to understand their biological function.

Cyclodepsipeptides beauveriolides I and III to IX produced by *Beauveria* sp. FO-6979 were discovered from a screening program for inhibitors of lipid droplet accumulation in mouse macrophages.[2,3] Among them, beauveriolides I (BeauI, **1a**) and III (BeauIII, **1b**) (Figure 1) showed very potent and specific inhibition. Studies on the mechanism of action revealed that

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(1) (a) Tomoda, H.; Kumagai, H.; Ogawa, Y.; Sunazuka, T.; Hashizume, H.; Nagashima, H.; Ōmura, S. *J. Org. Chem.* **1997**, *62*, 2161−2165. (b) Boruwa, J.; Kalita, B.; Barua, N. C.; Borah, J. C.; Mazumder, S.; Thakur, D.; Gogoi, D. K.; Bora, T. C. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 3571−3574. (c) Morohashi, A.; Satake, M.; Nagai, H.; Oshima, Y.; Yasumoto, T. *Tetrahedron* **2000**, *56*, 8995−9001.

(2) Namatame, I.; Tomoda, H.; Arai, H.; Inoue, K.; Ōmura, S. *J. Biochem.* **1999**, *125*, 319−327.

(3) (a) Namatame, I.; Tomoda, H.; Si, S.; Yamaguchi, Y.; Masuma, R.; Ōmura. S. *J. Antibiot.* **1999**, *52*, 1−6. (b) Namatame, I.; Tomoda, H.; Tabata, N.; Si, S.; Ōmura. S. *J. Antibiot.* **1999**, *52*, 7−12. (c) Matsuda, D.; Namatame, I.; Tomoda, H.; Kobayashi, S.; Zocher, R.; Kleinkauf, H.; Omura, S. *J. Antibiot.* **2004**, *57*, 1−9. (d) Mochizuki, K.; Ohmori, K.; Tamura, H.; Shizuri, H.; Nishiyama, S.; Miyoshi, E.; Yamamura, S. *Bull. Chem. Soc. Jpn.* **1993**, *66*, 3041−3046.

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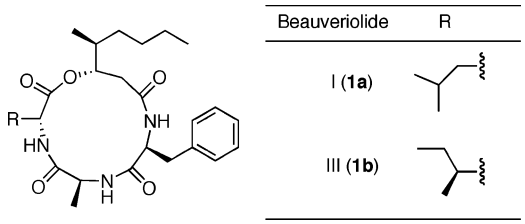


FIGURE 1. Structure of beauveriolides.

they inhibit cholesteryl ester (CE) synthesis by blocking acyl-CoA:cholesterol acyltransferase (ACAT) activity in macrophages, leading to inhibition of lipid droplet accumulation. More importantly, they proved orally active in atherogenic mouse models, reducing the atherogenic lesions in the artery and heart.[4] Therefore, they are expected to be novel agents for the prevention and treatment of atherosclerosis.[5]

BeauI/BeauIII are 13-membered cyclodepsipeptides consisting of L-Phe, L-Ala, D-Leu/D-*allo*-Ile and 3-hydroxy-4-methyloctanoic acid (HMA), respectively. Mochizuki et al. previously reported that the absolute stereochemistry of the HMA part is 3*S*,4*S*. However, the $[\alpha]^{20}_D$ values appeared different between natural-derived HMA (−19) and synthetic HMA (−44).[3d] On the other hand, our group reported that the $[\alpha]^{20}_D$ value (−29) of HMA prepared from BeauIII shows good agreement with that (−27) of HMA from BeauI, and we therefore concluded that the HMA stereochemistry in BeauIII is also 3*S*,4*S*.[3b] However, the $[\alpha]^{20}_D$ values from Mochizuki's report and our report still showed a discrepancy. Therefore, in this study we first defined the absolute stereochemistry by preparing the four HMA stereoisomers.

ACAT, an ER membrane protein that is the target of beauveriolides, is responsible for the esterification of cholesterol for storage inside cells. ACAT has been recognized as a target of inhibition for a new type of antiatherosclerotic agent. Thus far, many companies have attempted to develop synthetic ACAT inhibitors. However, almost none have been successfully developed. Recent molecular biological studies revealed the existence of two different isozymes, ACAT1 and ACAT2.[6,7] ACAT1 is ubiquitously expressed in tissues and cells including macrophages, while ACAT2 is expressed predominantly in the liver (hepatocytes) and intestine.[6−8] Therefore, for the development of a new antiatherosclerotic agent, it is important to understand the selectivity of an inhibitor against the two ACAT isozymes.[9] Recently, Rudel's group established sophisticated cell-based assays to evaluate ACAT1 and ACAT2 activities.[9a] Using this assay system, we evaluated the selectivity of BeauI and BeauIII in the inhibition of ACAT isozymes. We demonstrated that the stereochemistry of the HMA part of beauveriolides is important for the ability to strongly inhibit lipid droplet accumulation in mouse macrophages and for the selectivity toward the two ACAT isozymes.

Results

Absolute Stereochemistry of HMA in BeauI and BeauIII. To elucidate the absolute stereochemistry of the HMA in natural **1a** and **1b**, four isomers for HMA were synthesized as shown in Scheme 1.[10] Asymmetric hydrogenation of (*S*)-3-oxo-4-methyloctanoate **2** with $RuCl_2$[(*R*)-binap][11] under 90 atm of hydrogen at 45 °C provided *β*-hydroxyl ester **3** (94%, de 95%), while use of $RuCl_2$[(*S*)-binap] gave diastereomer **6** (94%, de 90%).[12] Hydrolysis of the ethyl ester provided carboxylic acids **4** and **7** which were converted to methyl esters **5** and **8**, respectively. *ent*-**4**, *ent*-**5**, *ent*-**7**, and *ent*-**8** were prepared by using the same method from ethyl (*R*)-3-oxo-4-methyloctanoate.

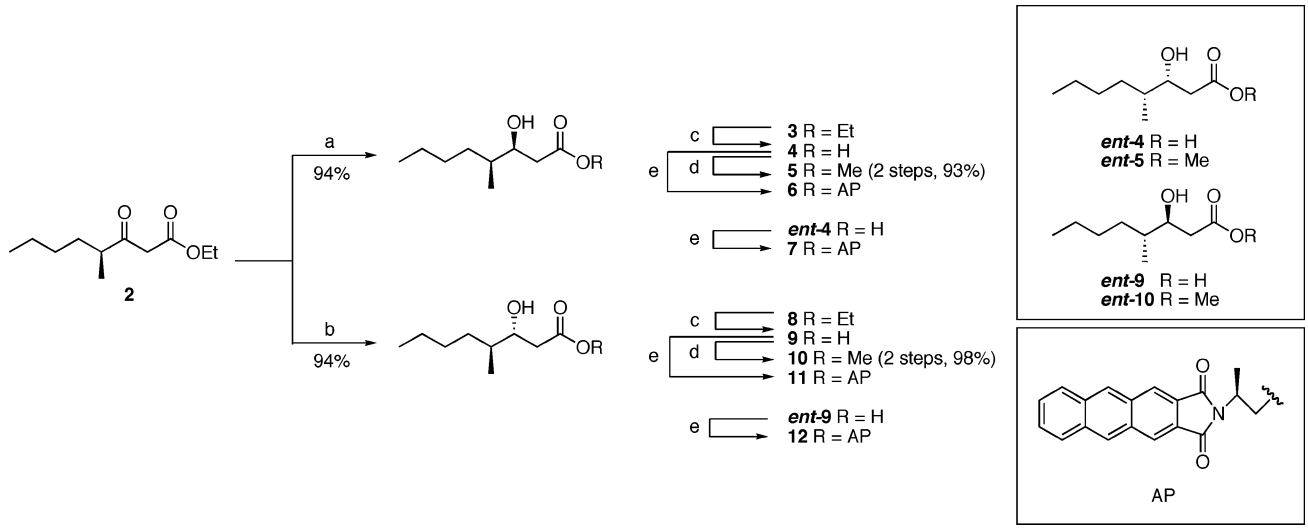
The structures of **5** and **8** were confirmed by the ^1H and ^{13}C NMR spectra, and the configurations were determined by comparison of the optical rotations of **5** {$[\alpha]^{28}_D$ −38 (*c* 0.98, $CHCl_3$), lit. $[\alpha]^{20}_D$ −44 (*c* 0.98, $CHCl_3$)} and **9** {$[\alpha]^{29}_D$ +18 (*c* 1.02, $CHCl_3$), lit. $[\alpha]^{22}_D$ +23 (*c* 0.62, $CHCl_3$)} with those previously reported.[3d]

Next, the hydrolytic products of natural **1a** and **1b** and the four HMA isomers **4**, **9**, *ent*-**4**, and *ent*-**9** were treated with (*S*)-(+)-2-(anthracene-2,3-dicarboximido)-1-propyl trifluoromethane sulfonate ((*S*)-(+)-AP-OTf)[13] to give HMA-(*S*)-(+)-AP derivatives **6**, **7**, **11**, and **12** (Scheme 1), which were analyzed by HPLC with a silica gel column. The derivatives **7**, **11**, **12**, and **6** were separated with retention times of 69, 71, 74, and 78 min, respectively (Figure 2). As shown in Figure 2B, the derivatives from natural **1a**- and **1b**-derived HMA were found to elute at 78 min, demonstrating that the absolute stereochemistry of HMA was 3*S*,4*S*.

Synthesis of Four Stereoismers in BeauIII. By using the four HMA isomers, (3*S*,4*S*)BeauIII, (3*R*,4*R*)BeauIII, (3*R*,4*S*)-BeauIII, and (3*S*,4*R*) were synthesized as summarized in Schemes 2 and 3.[10] In brief, after conversion of **4** to benzyl ester **13**, coupling of the secondary alcohol with Boc-D-*allo*-Ile-OH using DCC in the presence of DMAP followed by hydrogenolysis of the benzyl group produced ester unit **14** (Scheme 2). Synthesis of the four stereoisomers started with attachment of the carboxylic acid in Fmoc-L-Ala-OH onto 2-chlorotrityl chloride resin **18** (Scheme 3). The Fmoc group in **19** was removed with 20% piperidine in DMF, and subsequent condensation with Fmoc-L-Phe-OH afforded dipeptide **20**. After subsequent deprotection, coupling of the resulting amine with four ester units (**14**, **15**, **16**, and **17**) with use of PyBrop resulted in depsipeptides **21a**−**d**, respectively. Concurrently, removal of the Boc group and cleavage from the polymer support with 4 M HCl in 1,4-dioxane released the desired depsipeptides **22a**−**d** in parallel. Finally, the cyclization of **22a**−**d** with EDCI

(4) Namatame, I.; Tomoda, H.; Ishibashi, S.; Ōmura, S. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 737−742.
(5) *Chem. Eng. News* **2004**, *18*, 15.
(6) Chang, C. C.; Huh, H. Y.; Cadigan, K. M.; Chang, T. Y. *J. Biol. Chem.* **1993**, *268*, 20747−20755.
(7) (a) Anderson, R. A.; Joyce, C.; Davis, M.; Reagan, J. W.; Clark, M.; Shelness, G. S.; Rudel, L. L. *J. Biol. Chem.* **1998**, *273*, 26747−26754. (b) Cases, S.; Novak, S.; Zheng, Y. W.; Myers, H. M.; Lear, S. R.; Sande, E.; Welch, C. B.; Lusis, A. J.; Spencer, T. A.; Krause, B. R.; Erickson, S. K.; Farese, R. V., Jr. *J. Biol. Chem.* **1998**, *273*, 26755−26764. (c) Oelkers, P.; Behari, A.; Cromley, D.; Billheimer, J. T.; Sturley, S. L. *J. Biol. Chem.* **1998**, *273*, 26765−26771.
(8) Parini, P.; Davis, M.; Lada, A. T.; Erickson, S. K.; Wright, T. L.; Gustafsson, U.; Sahlin, S.; Einarsson, C.; Eriksson, M.; Angelin, B.; Tomoda, H.; Ōmura, S.; Willingham, M. C.; Rudel, L. L. *Circulation* **2004**, *110*, 2017−2023.
(9) (a) Lada, A. T.; Davis, M.; Kent, C.; Chapman, J.; Tomoda, H.; Ōmura, S.; Rudel, L. L. *J. Lipid. Res.* **2004**, *45*, 378−386. (b) Giovannoni, M. P.; Piaz, V. D.; Vergelli, C.; Barlocco, D. *Mini Rev. Med. Chem.* **2003**, *3*, 576−584.
(10) Nagai, K.; Doi, T.; Sekiguchi, T.; Namatame, I.; Sunazuka, T.; Tomoda, H.; Ōmura, S.; Takahashi, T. *J. Comb. Chem.* **2006**, *8*, 103−109
(11) Noyori, R.; Ohkuma, T.; Kitamura, M.; Takaya, H.; Sayo, N.; Kumobayashi, H.; Akutagawa, S. *J. Am. Chem. Soc.* **1987**, *109*, 5856−5858.
(12) Diastereoselectivity was determined by ^1H NMR analysis.
(13) Akasaka, K.; Ohrui, H.; Meguro, H.; Umestu, T. *Anal. Sci.* **1997**, *13*, 461−466.

SCHEME 1. Synthesis of Four Isomers of HMA[a]



[a] Reagents and conditions: (a) $RuCl_2$[(*R*)-binap], EtOH, H_2, 90 atm, 45 °C, 40 h; (b) $RuCl_2$[(*S*)-binap], EtOH, H_2, 90 atm, 45 °C, 40 h; (c) 1 M LiOH, THF, rt, 2.5 h; (d) cat. H_2SO_4, MeOH, CH_2Cl_2 40 °C, 15 h; (e) (S)-(+)-AP-OTf, TEAC, MeCN, rt, 30 min.

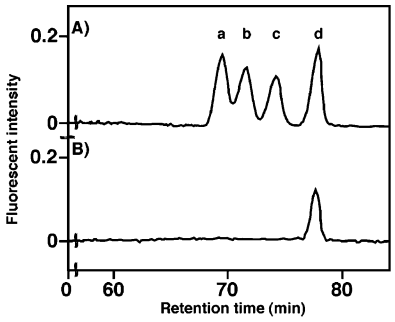
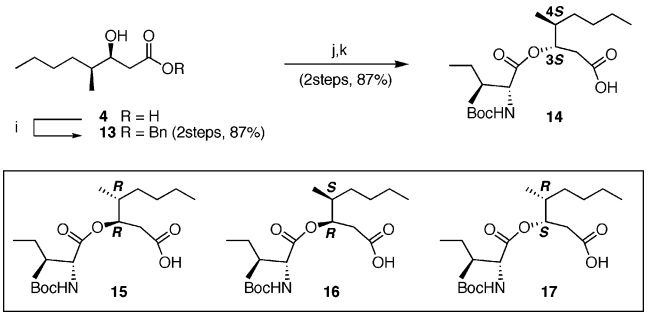


FIGURE 2. Chromatographic profiles of isolation of (*S*)-(+)-AP derivatives of four HMA isomers by preparative HPLC: (A) synthetic HMA, (a) (3*R*,4*R*)HMA (*ent*-**4**), (b) (3*R*,4*S*)HMA (**9**), (c) (3*S*,4*R*)HMA (*ent*-**9**), (d) (3*S*,4*S*)HMA (**4**), (B) HMA of BeauIII (**1b**). Column, PEGASIL Silica 120-5, 4.6 × 500 mm; hexanes−ethyl acetate (42:8); flow rate, 0.7 mL/min; fluorometer, excitation 298 nm, emission 462 nm.

SCHEME 2. Synthesis of Four Stereoisomers of HMA[a]



[a] Reagents and conditions: (i) Cs_2CO_3, BnBr, DMF, rt, 12 h; (j) Boc-D-*allo*-Ile-OH, DCC, DMAP, CH_2Cl_2, 0 °C to room temperature, 22 h; (k) 10% Pd/C, H_2, EtOH, rt, 12 h.

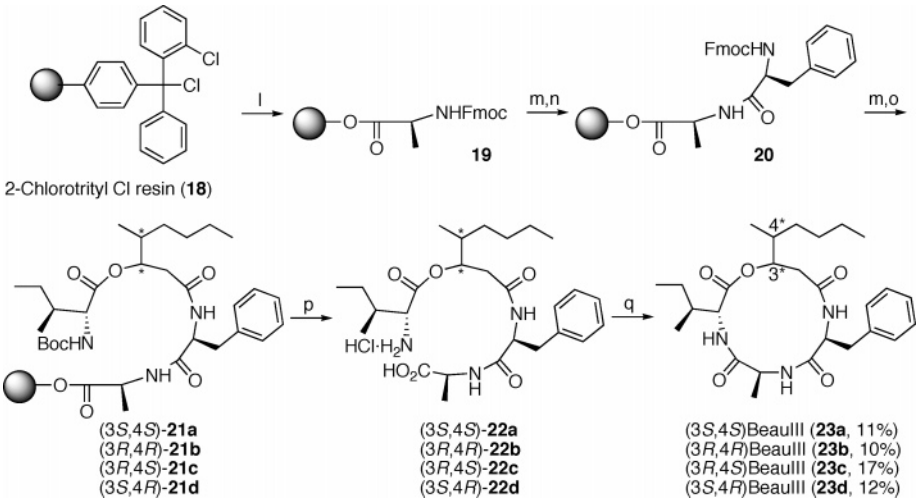
and *i*-Pr_2NEt under high dilution conditions proceeded to produce four stereoisomers, (3*S*,4*S*)BeauIII (**23a**, 11%), (3*R*,4*R*)-BeauIII (**23b**, 10%), (3*R*,4*S*)BeauIII (**23c**, 17%), and (3*S*,4*R*)-BeauIII (**23d**, 12%), respectively. The ^{1}H NMR spectra of the isomers are shown in Figure 3. The proton signals corresponding to 3-H were observed at 5.18 ppm for **23b** and 5.15 ppm for **23c**, and at 4.94 ppm for both **23a** and **23d**. In addition, the proton signals corresponding to 4-H were observed at 2.03 ppm for **23a**, which was close to those for **1b**, while they were observed at 2.13 ppm for **23d**. As is clearly shown, all the proton signals for **23a** were found to be identical with those for naturally occurring **1b**. Furthermore, other spectral data (^{13}C NMR, UV) were also identical between **23a** and **1b**. Thus, all the absolute stereochemistry of **1b** was finally defined as that of **23a**.

Effect of BeauIII Stereoisomers on Lipid Droplet Accumulation in Macrophages. In this macrophage assay, massive amounts of lipid droplets were accumulated in the cytosol when macrophages were incubated with liposomes, and were observed microscopically after oil red O staining (Figure 4E). Under the assay conditions, **23a** and **23d** inhibited lipid droplet accumulation in a dose-dependent fashion (2−20 μM, data not shown). As shown in Figure 4A,D, the size and the number of lipid droplets in macrophages were reduced in the presence of **23a** or **23d** (6 μM). By contrast, **23b** and **23c** showed no inhibition of lipid droplet accumulation even at 20 μM (Figure 4B,C). No cytotoxic effects were observed on macrophages upon addition of up to 20 μM of the four BeauIII stereoisomers.

Effect of BeauIII Stereoisomers on CE and TG Synthesis by Macrophages. Under the conditions of lipid droplet accumulation in mouse macrophages, about 40% of exogenously added [^{14}C]oleic acid was incorporated into [^{14}C]CE and [^{14}C]-triacylglycerol (TG), which are the main constituents of lipid droplets in the cytosol.[2] As shown in Figure 5A, **23a** selectively inhibited [^{14}C]CE synthesis in a dose-dependent fashion with an IC_{50} value of 1.19 μM. Isomer **23d** inhibited not only [^{14}C]-CE synthesis (IC_{50}, 0.69 μM) but also [^{14}C]TG synthesis (IC_{50}, 14.0 μM) although the TG inhibition was much weaker than the CE synthesis (Figure 5D). By contrast, **23b** and **23c** showed weak inhibition of [^{14}C]CE synthesis at the highest concentration (50 μM) (Figure 5B,C). These biochemical data are consistent with the morphological data of the lipid droplet accumulation in macrophages (Figure 4).

Selectivity in Inhibition of ACAT Isozymes by BeauIII Stereoisomers. Previously, we reported that BeauI and BeauIII

SCHEME 3. Synthesis of Beauveriolide III Isomers with Solid Support[a]



[a] Reagents and conditions: (l) Fmoc-L-Ala-OH, *i*-Pr_2NEt, CH_2Cl_2, rt, 2 h; (m) 20% piperidine/DMF, rt, 1 h; (n) Fmoc-L-Phe-OH, DIPCI, HOBt, CH_2Cl_2/DMF (4/1), rt, 2 h; (o) **14**, **15**, **16** or **17**, PyBrop, *i*-Pr_2NEt, CH_2Cl_2/DMF (4/1), rt, 1.5 h; (p) 4M HCl/1,4-dioxane, rt, 2 h; (q) EDCI·HCl, *i*-Pr_2NEt, CH_2Cl_2, rt, 2 h, 51%.

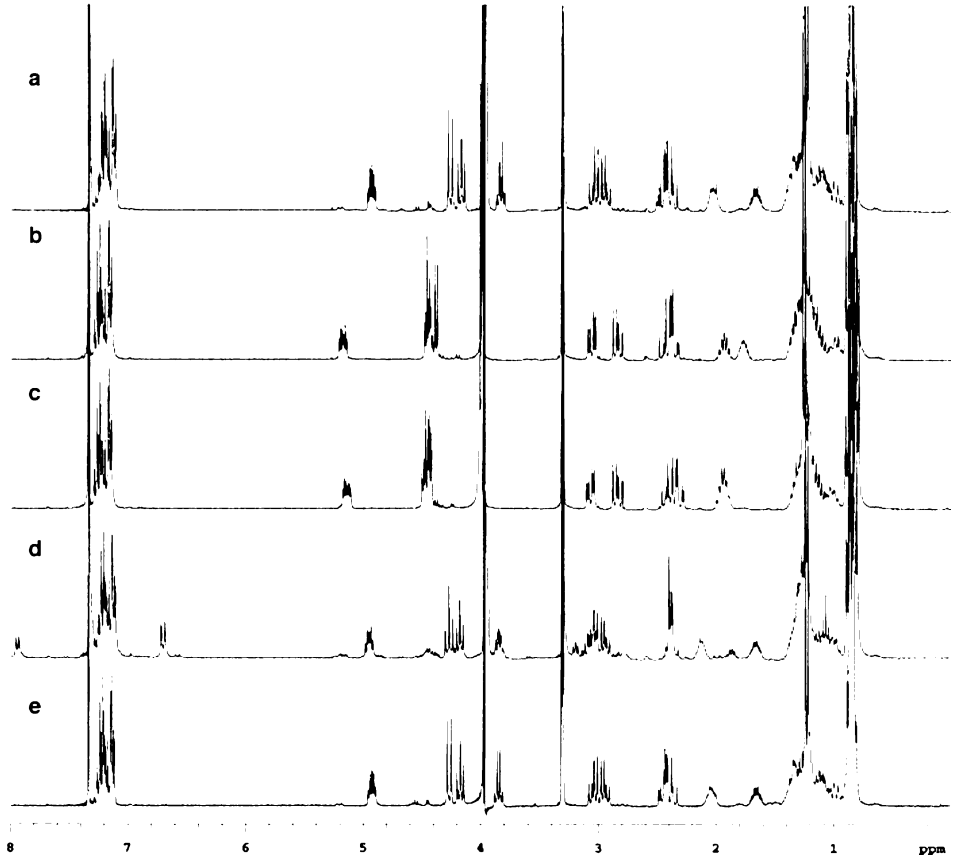


FIGURE 3. ^1H NMR spectra of (a) (3*S*,4*S*)BeauIII (**23a**), (b) (3*R*,4*R*)BeauIII (**23b**), (c) (3*R*,4*S*)BeauIII (**23c**), (d) (3*S*,4*R*)BeauIII (**23d**), and (e) naturally occurring beauIII (**1b**).

inhibited ACAT activity in enzyme-based assays using microsomes prepared from mouse macrophages (ACAT1 expression) and livers (ACAT2 expression), suggesting that they inhibited both the ACAT1 and ACAT2 isozymes.[4] To define the selectivity of the inhibition of four BeauIII isomers for the isozymes, a cell-based assay using ACAT1- and ACAT2-CHO cells was used.[9a]

As shown in Figure 6, **23a** and **23d** showed rather specific inhibition against ACAT1 compared to ACAT2. The selectivity was about 4, as shown by comparison of the IC_{50} values (Table 1). On the other hand, **23b** and **23c** showed almost no effect on CE synthesis at 10 μM in ACAT1- or ACAT2-CHO cells, although **23c** gave weak inhibition of both isozymes at 20 μM.

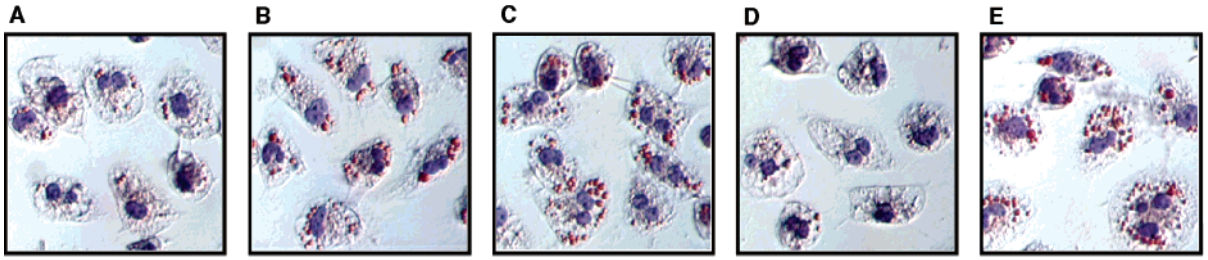


FIGURE 4. Effect of BeauIII stereoisomers on lipid droplet accumulation in macrophages: (A) (3*S*,4*S*)BeauIII (**23a**), 6 μM (B) (3*R*,4*R*)BeauIII (**23b**), 6 μM, (C) (3*R*,4*S*)BeauIII (**23c**), 6 μM, (D) (3*S*,4*R*)BeauIII (**23d**), 6 μM, and (E) control.

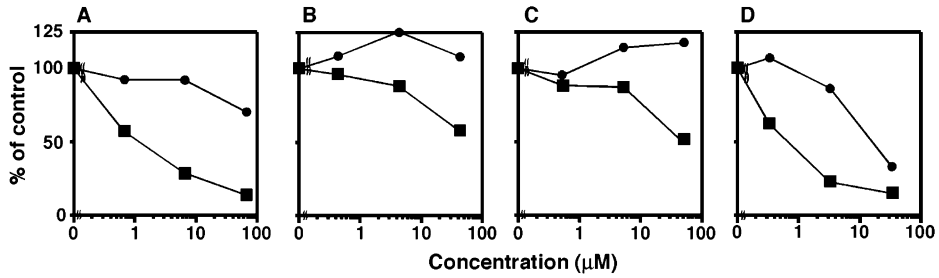


FIGURE 5. Effect of BeauIII stereoisomers on CE and TG synthesis by macrophages [^{14}C]CE (■) and [^{14}C]TG (●): (A) (3*S*,4*S*)BeauIII (**23a**), (B) (3*R*,4*R*)BeauIII (**23c**) and (3*S*,4*R*)BeauIII (**23d**), (C) (3*R*,4*S*)BeauIII (**23c,**) and (D) (3*S*,4*R*)BeauIII (**23d**).

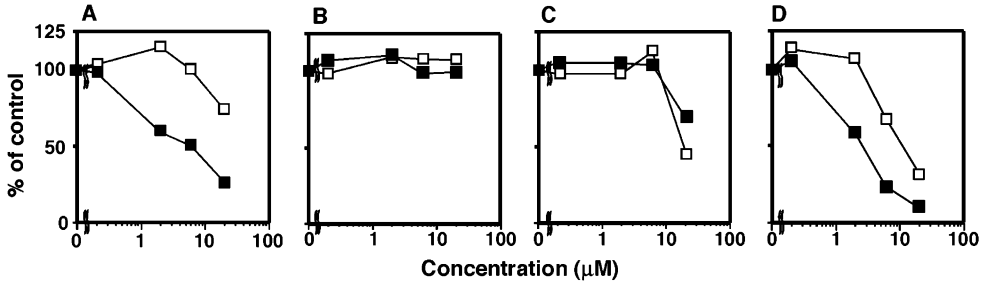


FIGURE 6. Effect of beauIII stereoisomers on CE synthesis by ACAT1- or ACAT2-CHO cells. [^{14}C]CE in ACAT1-CHO cells (■) or ACAT2-CHO cells (□): (A) (3*S*,4*S*)BeauIII (**23a**), (B) (3*R*,4*R*)BeauIII (**23b**), (C) (3*R*,4*S*)BeauIII (**23c**), and (D) (3*S*,4*R*)BeauIII (**23d**).

TABLE 1. IC_{50} Values of BeauIII Stereoismers in Cell-Based Assays

BeauIII isomer	IC_{50} (μM)		
	macrophages	ACAT1	ACAT2
(3*S*,4*S*)BeauIII (**23a** = **1b**)	1.19	5.5	>20
(3*R*,4*R*)BeauIII (**23b**)	>44.2	>20	>20
(3*R*,4*S*)BeauIII (**23c**)	>52.1	>20	19.0
(3*S*,4*R*)BeauIII (**23d**)	0.69	2.7	10.7

Discussion

Several research groups, including our group, reported that BeauI (**1a**) and BeauIII (**1b**) are composed of two L-amino acids, one D-amino acid, and HMA,[3] but the absolute stereochemistry of HMA was not fully defined. In this study, the four stereoisomers of HMA were synthesized and the (*S*)-(+)-AP derivatives were separated by HPLC and compared with the derivatives from natural HMA (Figure 2). This method proved effective to determine the absolute stereochemistry of the hydroxy fatty acid at the picomole level.[13,14] Thus, we defined clearly the stereochemistry of HMA in BeauI and BeauIII as 3*S*,4*S*. Furthermore, we succeeded in preparing a beauveriolide analogue library by combinatorial chemistry.[10] According to the same strategy (Scheme 3), the four BeauIII stereoisomers were synthesized by using the four HMA isomers as one of the building units. As a result, all the spectral data, including ^{1}H NMR (Figure 3), of synthetic (3*S*,4*S*)BeauIII (**23a**) were found to be consistent with those of natural BeauIII (**1b**), corroborating the absolute stereochemistry of BeauIII.

Fungal BeauIII was originally isolated as an inhibitor of lipid droplet accumulation in mouse macrophages.[3a,b] First, the four BeauIII stereoisomers were evaluated in this macrophage assay. Not only **23a** but **23d** inhibited lipid droplet accumulation (Figure 4), while the other isomers showed very weak or almost no inhibition even at higher concentration (20 μM). The microscopic observations were supported by the biochemical data (Figure 5); **23a** and **23d** inhibited CE synthesis strongly, but the other two isomers showed very weak inhibition at the highest concentration (50 μM). These results indicate that the 3*S* HMA configuration is responsible for the strong inhibition of lipid droplet accumulation in macrophages. Interestingly, **23d** was found to inhibit not only CE synthesis but also TG synthesis, although the TG inhibition was 20 times weaker than the CE inhibition, as shown by comparison of the IC_{50} values. Accordingly, inhibition of lipid droplet synthesis by **23d** appeared more effective than that by **23a** (Figure 4A,D). The

(14) Akasaka, K.; Ohrui, H. *Biosci. Biotechnol. Biochem.* **2004**, *68*, 153−158.

difference might be due to the fact that **23d** has multiple inhibition targets in macrophages.

Our previous experiments on the mechanism of action suggested that natural BeauIII (**1b**) inhibited both ACAT1 and ACAT2 isozymes in an enzyme-based assay using microsomes prepared from mouse macrophages and livers.[4] Second, we confirmed the selectivity of the four BeauIII stereoisomers for the inhibition of ACAT isozymes in sophisticated cell-based assays using ACAT1- and ACAT2-CHO cells. As shown in Figure 6, **23a** and **23d** inhibited ACAT1 activity strongly, while the other isomers, **23b** and **23c**, showed weak or no inhibition of ACAT1 activity. The results are consistent with those in macrophage assays. These findings are reasonable because mouse macrophages predominantly express ACAT1.[6] Furthermore, **23a** and **23d** also showed inhibition against ACAT2 activity, although the potency was several times weaker than that against ACAT1 (Table 1). Thus, we demonstrated that the 3*S* sterochemistry of HMA in BeauIII is responsible for potent ACAT inhibition and that BeauIII causes rather specific inhibition against ACAT1 compared to ACAT2.

In this study, we determined that the absolute stereochemistry of HMA in beauveriolide III is 3*S*,4*S* and that the 3*S* configuration in beauveriolide is responsible for potent inhibition of lipid droplet accumulation in macrophages and of ACAT activity.

Experimental Section

Materials. Reagents and solvents were purchased from commercial sources and used without further purification. BeauI and BeauIII were purified from the culture broth of *Beauveria* sp. FO-6979 as reported.[3a] 2-Chlorotrityl chloride resin 18 (100−200 mesh, 1% cross-linked, 1.25 mmol/g; batch, U1125032) and MicroKan microreactors were purchased from IRORI (now Biotage). The resin in the MicroKan microreactor was swelled in solvent for 1 h before use. CHO cells expressing African Green monkey ACAT1 (ACAT1-CHO) and ACAT2 (ACAT2-CHO) were established previously by Lada et al.[9a] ACAT1- or ACAT2- CHO cells were cultured by the method described previously.[9a] In brief, both cell lines were maintained at 37 °C in 5% CO_2 in Ham's F-12 medium supplemented with MEM vitamins, Geneticin (G418) (300 μg/mL), and 10% heat inactivated fetal bovine serum (FBS) (hereafter referred to as medium A).

^{1}H NMR (270 MHz) and ^{13}C NMR (67.9 MHz) spectra were recorded on a JEOL JNM-EX270 instrument with $CDCl_3$. HR-FABMS were measured with a JEOL JMS-AX505 HA mass spectrometer.

Synthesis of Four HMA Stereoisomers. Synthesis of the four HMA stereoisomers was carried out by the method described previously.[10] Details of the synthesis are available from the Supporting Information.

Hydrolysis of BeauIII. BeauIII (1 mg, 2 μmol) was degraded in a gas phase of 6 N HCl (500 μL) at 150 °C for 1 h, using a PICO TAG work station (Waters). The degradation products were dissolved in toluene (100 μL).

Derivatization of Four HMA Stereoisomers by (*S*)-(+)-2-(Anthracene-2,3-dicarboximido)-1-propyl Trifluoromethane Sulfonate (AP-OTf). The degradation products of beauIII **1b** (800 nmol) and the four HMA stereoisomers (**4**, **9**, *ent*-**4**, *ent*-**9,** 188 nmol) were reacted with (*S*)-(+)-2-(anthracene-2,3-dicarboximido)-1-propyl trifluoromethane sulfonate (AP-OTf)[13] and tetraethylammonium carbonate (TEAC) in dry CH_3CN for 30 min at room temperature. The reaction mixture were purified by preparative TLC. Active bands were collected and extracted with $CHCl_3-CH_3OH$ (10:1). The extracts were concentrated in vacuo to yield (*S*)-(+)-AP derivatives **6**, **7**, **11**, and **12** (375 pmol).

Analysis of (*S*)-(+)-AP Derivatives of HMA by HPLC. To determine the HMA configuration, (*S*)-(+)-AP derivatives of HMA were analyzed by HPLC (column, PEGASIL Silica 120-5 (4.6 × 500 mm); solvent, hexane/ethyl acetate (42:8); excitation, 298 nm, emission, 462 nm; flow rate, 0.7 mL/min; column temperature, 40 °C). HPLC was carried out with use of the SSC flow system 3100 (Senshu). Fluorometry was carried out with a JASCO FP-210 spectrofluorometer (JASCO).

Synthesis of BeauIII Stereoisomers. Synthesis of BeauIII stereoisomers was carried out by the method described previously.[10] Details of the synthesis are available from the Supporting Information.

Selected Spectral Data of BeauIII Stereoisomers: BeauIII (natural product, 1b). HRFABMS: calcd for $C_{27}H_{42}N_3O_5$ [M + H]$^+$ 488.3124, found 488.3127. ^{1}H NMR (400 MHz, $CDCl_3$:CD_3OD 4:1): δ (ppm) 7.23 (ddd, J = 8.0, 7.0, 1.0 Hz, 2H), 7.0.18 (ddd, J = 7.0, 1.5 Hz, 1H), 7.14 (ddd, J = 8.0, 1.5, 1.0 Hz, 2H), 4.93 (ddd, J = 10.0, 4.5, 4.0 Hz, 1H), 4.27 (d, J = 10.0 Hz, 1H), 4.19 (dd, J = 8.5, 7.5 Hz, 1H), 3.80 (q, J = 7.0 Hz, 1H), 3.04 (dd, J = 13.5, 7.5 Hz, 1H), 2.95 (dd, J = 13.5, 8.5 Hz, 1H), 2.46 (dd, J = 14.0, 4.5 Hz, 1H), 2.38 (dd, J = 14.0, 10.0 Hz, 1H), 2.04 (m, 1H), 1.67 (m, 1H), 1.36 (m, 1H), 1.32 (m, 1H), 1.32 (m, 1H), 1.22 (d, J = 7.0 Hz, 3H), 1.21 (m, 2H), 1.20 (m, 2H), 1.11 (m, 1H), 0.99 (m, 1H), 0.88 (d, J = 7.0 Hz, 3H), 0.86 (t, J = 7.0 Hz, 3H), 0.85 (d, J = 6.0 Hz, 3H), 0.84 (t, J = 7.0 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$:CD_3OD 4:1): δ (ppm) 172.4, 171.8 (×2), 169.7, 136.7, 129.3 (×2), 128.8 (×2), 127.2, 76.8, 59.6, 57.2, 49.7, 37.4, 36.0, 35.9, 35.8, 30.7, 29.8, 26.1, 23.2, 15.7, 15.1, 14.8, 14.1, 11.1.

(3*S*,4*S*)BeauIII (23a). HRFABMS: calcd for $C_{27}H_{42}N_3O_5$ [M + H]$^+$ 488.3124, found 488.3117. ^{1}H NMR (300 MHz, $CDCl_3$:CD_3OD 4:1): δ (ppm) 7.28−7.11 (5H, m), 4.94 (1H, dt, J = 10.0, 4.5 Hz), 4.26 (1H, d, J = 10.0 Hz), 4.17 (1H, dd, J = 8.5, 7.5 Hz), 3.82 (1H, q, J = 7.0 Hz), 3.02 (1H, dd, J = 13.5, 8.5 Hz), 2.94 (1H, dd, J = 13.5, 7.5 Hz), 2.46 (1H, dd, J = 14.0, 5.0 Hz), 2.38 (1H, dd, J = 14.0, 10.0 Hz), 2.03 (1H, m), 1.67 (1H, m), 1.44−1.26 (3H, m), 1.22 (3H, d, J = 7.0 Hz), 1.21−1.13 (5H, m), 1.00 (1H, m), 0.87 (3H, t, J = 7.0 Hz), 0.86 (3H, d, J = 7.0 Hz), 0.85 (3H, d, J = 7.0 Hz), 0.83 (3H, t, J = 7.0 Hz). ^{13}C NMR (75.4 MHz, $CDCl_3$:CD_3OD 4:1): δ (ppm) 172.1, 171.5, 171.4, 169.4, 136.5, 129.0 (×2), 128.5 (×2), 126.9, 76.6, 59.4, 57.0, 49.3, 37.2, 35.9, 35.7 (×2), 30.6, 29.7, 26.0, 23.1, 15.7, 15.0, 14.8, 14.1, 11.1.

(3*R*,4*R*)BeauIII (23b). HRFABMS: calcd for $C_{27}H_{42}N_3O_5$ [M + H]$^+$ 488.3124, found 488.3108. ^{1}H NMR (300 MHz, $CDCl_3$:CD_3OD 4:1): δ (ppm) 7.30−7.14 (5H, m), 5.18 (1H, dd, J = 11.0, 5.0, 4.0 Hz), 4.45 (1H, dd, J = 9.0, 5.5 Hz), 4.44 (1H, q, J = 7.0 Hz), 4.39 (1H, d, J = 6.0 Hz), 3.06 (1H, dd, J = 14.0, 5.5 Hz), 2.84 (1H, dd, J = 14.0, 9.0 Hz), 2.44 (1H, dd, J = 15.5, 11.0 Hz), 2.35 (1H, dd, J = 15.5, 4.0 Hz), 1.94 (1H, m), 1.77 (1H, m), 1.39−1.26 (3H, m), 1.25 (3H, d, J = 7.0 Hz), 1.24−1.05 (5H, m), 1.00 (1H, m), 0.88 (3H, t, J = 7.0 Hz), 0.84 (3H, d, J = 7.0 Hz), 0.83 (3H, t, J = 7.0 Hz), 0.82 (3H, d, J = 7.0 Hz). ^{13}C NMR (75.4 MHz, $CDCl_3$:CD_3OD 4:1): δ (ppm) 172.1, 171.8, 171.7, 170.3, 136.0, 128.9 (×2), 128.7 (×2), 127.2, 76.0, 56.7, 55.9, 48.3, 36.6, 36.1 (×2), 35.9, 31.3, 29.5, 26.6, 22.9, 15.7, 15.4, 14.4, 14.0, 11.6.

(3*R*,4*S*)BeauIII (23c). HRFABMS: calcd for $C_{27}H_{42}N_3O_5$ [M + H]$^+$ 488.3124, found 488.3119; ^{1}H NMR (300 MHz, $CDCl_3$:CD_3OD 4:1): δ (ppm) 7.29−7.14 (5H, m), 5.15 (1H, dd, J = 11.0, 5.0, 4.0 Hz), 4.48 (1H, dd, J = 9.0, 5.5 Hz), 4.46 (1H, q, J = 7.0 Hz), 4.43 (1H, d, J = 6.0 Hz), 3.07 (1H, dd, J = 14.0, 5.5 Hz), 2.84 (1H, dd, J = 14.0, 9.0 Hz), 2.42 (1H, dd, J = 15.5, 11.0 Hz), 2.31 (1H, dd, J = 15.5, 4.0 Hz), 2.00−1.89 (2H, m), 1.38−1.26 (3H, m), 1.25 (3H, d, J = 7.0 Hz), 1.24−1.04 (5H, m), 1.00 (1H, m), 0.88 (3H, t, J = 7.0 Hz), 0.84 (3H, d, J = 7.0 Hz), 0.82 (3H, d, J = 7.0 Hz), 0.81 (3H, t, J = 7.0 Hz); ^{13}C NMR (75.4 MHz, $CDCl_3$:CD_3OD 4:1) δ (ppm) 172.3, 172.0 (×2), 170.6, 136.2, 129.0 (×2), 128.9 (×2), 127.3, 76.1, 56.6, 55.6, 48.2, 36.6, 36.0, 35.3, 34.8, 32.8, 29.4, 26.6, 22.9, 15.6, 14.4, 13.9 (×2), 11.7.

(3*S*,4*R*)BeauIII (23d). HRFABMS: calcd for $C_{27}H_{42}N_3O_5$ [M + H]$^+$ 488.3124, found 488.3122. ^{1}H NMR (300 MHz, $CDCl_3$:

CD_3OD 4:1): δ (ppm) 7.28−7.12 (5H, m), 6.71 (1H, d, J = 9.5 Hz), 4.94 (1H, ddd, J = 9.0, 5.5, 5.0 Hz), 4.28 (1H, t, J = 9.5 Hz), 4.19 (1H, dd, J = 8.5, 7.5 Hz), 3.85 (1H, q, J = 7.0 Hz), 3.05 (1H, dd, J = 13.5, 8.5 Hz), 2.94 (1H, dd, J = 13.5, 7.5 Hz), 2.40 (1H, dd, J = 13.5, 5.0 Hz), 2.37 (1H, dd, J = 13.5, 9.0 Hz), 2.13 (1H, m), 1.67 (1H, m), 1.39−1.26 (3H, m), 1.23 (3H, d, J = 7.0 Hz), 1.21−1.02 (5H, m), 1.00 (1H, m), 0.89 (3H, d, J = 7.0 Hz), 0.85 (3H, d, J = 7.0 Hz), 0.85−0.84 (6H, m); ^{13}C NMR (75.4 MHz, $CDCl_3$:CD_3OD 4:1): δ (ppm) 172.0, 171.5, 171.4, 169.3, 136.4, 129.0 (x 2), 128.5 (x 2), 126.9, 75.9, 59.4, 57.0, 49.3, 37.4, 35.8, 35.3, 35.1, 32.8, 29.3, 26.0, 23.0, 15.0, 14.8, 14.0, 13.5, 11.0.

Assay for [^{14}C]Neutral Lipid Synthesis by Mouse Peritoneal Macrophages. The assay for [^{14}C]CE and [^{14}C]TG synthesis from [^{14}C]oleic acid in mouse peritoneal macrophages was carried out by the method described previously.[2] Details of the assay are available from the Supporting Information.

Cellular Neutral Lipid Staining. Cellular neutral lipid staining was carried out by the method described previously.[2] Details of the assay are available from the Supporting Information.

Assay for [^{14}C]CE Synthesis in ACAT1- or ACAT2-CHO Cells. ACAT1- or ACAT2-CHO cells (1.25×10^5 cells/0.25 mL medium A) were plated in a 48-well plastic microplate and allowed to recover overnight at 37 °C in 5% CO_2. The assay was done with cells that were at least 80% confluent. Following the overnight recovery, 2.5 μL of a sample (methanol solution) and 5 μL of [^{14}C]-oleic acid (1 nmol, 1.85 KBq, 10% ethanol/PBS solution) were added to each culture at 37 °C in 5% CO_2. Following a 6 h incubation, the medium was removed, and the cells in each well were washed two times with PBS. The cells were lysed by adding 0.25 mL of 10 mM Tris-HCl (pH 7.5) containing 0.1% (w/v) sodium dodecyl sulfate, and the cellular lipids were extracted by the method of Bligh and Dyer.[15] The organic solvent was reduced by centrifugation under vacuum, and [^{14}C]CE was separated on a thin-layer chromatography (TLC) plate (silica gel) and analyzed with a bioimaging analyzer (BAS 2000, Fuji Film).

Assay for Cell Viability. The numbers of viable macrophages, ACAT1-CHO cells, and ACAT2-CHO cells were measured in the presence of various inhibitors with use of alamar Blue.

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Supporting Information Available: Materials, synthesis procedures, characterization of compounds, and biological assay procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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(15) Bligh, E. G.; Dyer. W. *Can. J. Biochem. Physiol.* **1959**, *37*, 911−917.