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ABSTRACT

The formation of foam cells in macrophages plays an essential role in the progression of early atherosclerotic lesions and therefore its prevention is considered to be a promising target for the treatment of atherosclerosis. We found that an extract of the marine sponge *Acanthostrongylophora ingens* inhibited the foam cell formation induced by acetylated low-density lipoprotein (AcLDL) in human monocyte-derived macrophages, as measured based on the accumulation of cholesterol ester (CE). Bioassay-guided purification of inhibitors from the extract afforded manzamines. Manzamine A was the most potent inhibitor of foam cell formation, and also suppressed CE formation in Chinese hamster ovary cells overexpressing acyl-CoA:cholesterol acyl-transferase (ACAT)-1 or ACAT-2. In addition, manzamine A inhibited ACAT activity. Next, we orally administered manzamine A to apolipoprotein E (apoE)-deficient mice for 80 days, and found that total cholesterol, free cholesterol, LDL-cholesterol, and triglyceride levels in serum were significantly reduced and the area of atherosclerotic lesions in the aortic sinus was also substantially diminished. These findings clearly suggest that manzamine A suppresses hyperlipidemia and atherosclerosis in apoE-deficient mice by inhibiting ACAT and is therefore a promising lead compound in the prevention or treatment of atherosclerosis. Although manzamine A has been reported to show several biological activities, this is the first report of a suppressive effect of manzamine A on atherosclerosis in vivo.

1. Introduction

The deposition of large clusters of foam cells derived from macrophages in the subendothelial spaces is a physiological feature of early atherosclerotic lesions.[1] Foam cells produce various endogenous signaling molecules, such as cytokines, growth factors, and proteases, which play important roles in the development and progression of atherosclerotic lesions.[1] Macrophages take up chemically modified low-density lipoproteins (LDL), including oxidized LDL, acetylated LDL (AcLDL), and glycated LDL, through scavenger receptors.[2] The scavenger receptors identified to date include the class A scavenger receptor (SR-A),[3] the class B scavenger receptor (CD36),[4] the class B scavenger receptor type-I (SR-BI),[5] and lectin-like oxidized LDL receptor-1 (LOX-1).[6] It is known that AcLDL is mainly recognized by SR-A and SR-BI. Since free cholesterol (FC), incorporated into cells as a constituent of modified LDL through scavenger receptors and released in a free form, is toxic to cells, FC should be esterified to cholesterol esters (CE) by an intracellular enzyme, designated acyl-coenzyme A:cholesterol acyl-transferase (ACAT), located in the rough endoplasmic reticulum.[7] Through these processes, the macrophages become foam cells, which are characterized by the intracellular accumulation of CE.

Two human ACAT isozymes (hACAT-1 and hACAT-2) have been identified to date.[8,9] ACAT-1 is highly expressed in foam cells in atherosclerotic lesions and is up-regulated during the differentiation of monocytes into macrophages.[10] In addition, ACAT-1 is located in the Kupffer cells of the liver, kidneys, and adrenal cortical cells, while ACAT-2 is mainly located in hepatocytes and intestinal mucosal cells. These observations are consistent with the notion that ACAT-1 plays a critical role in the formation of foam

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cells in macrophages, while ACAT-2 is responsible for the process of cholesterol absorption in intestinal mucosal cells.[10] Since foam cells are believed to play an essential role in the progression of early atherosclerotic lesions,[1] preventing the formation of foam cells is considered to be a major target in the treatment of atherosclerosis. Consequently, a number of anti-atherosclerotic approaches, such as the prevention of LDL-oxidation,[11] inhibition of scavenger receptor expression,[12] and inhibition of ACAT activity,[13] have been investigated. We previously reported that esculeoside A isolated from tomato suppressed hyperlipidemia and atherosclerosis in apolipoprotein E (apoE)-deficent mice by inhibiting ACAT activity.[14] During a further search for drug candidates for the treatment of atherosclerosis, we found that an extract of the marine sponge *Acanthostrongylophora ingens* collected in Indonesia inhibited the formation of foam cells in human monocyte-derived macrophages (HMDMs). Here, we report the isolation of manzamines from the sponge and their inhibitory effects on the formation of foam cells in HMDMs. In addition, we found that the oral administration of manzamine A, the strongest inhibitor of foam cell formation, diminished atherosclerosis in apoE-deficient mice.

2. Results

2.1. Isolation of manzamines as inhibitors of CE accumulation

The formation of foam cells in HMDMs is known to be characterized by the intracellular accumulation of CE. We first found that, among the extracts of 29 marine sponges collected in Indonesia, an extract of *A. ingens* most strongly inhibited foam cell formation in HMDMs induced by AcLDL, measured on the basis of CE accumulation (Fig. S1). The marine sponge *A. ingens* (300 g, wet weight) was collected, the EtOH extract of the sponge was evaporated, and the aqueous residue was extracted with EtOAc. Bioassay-guided purification from the EtOAc soluble fraction (5.1 g) by repeated SiO_2 column chromatography followed by SiO_2 HPLC afforded manzamine A[15] (**1**, 323.0 mg), 6-hydroxymanzamine A[16] (**2**, 2.2 mg), 8-hydroxymanzamine A[17] (**3**, 104.97 mg), 12,34-oxamanzamine E[18] (**4**, 1.5 mg), and manzamine M[19] (**5**, 1.4 mg) (Fig. 1). The compounds **1**–**5** were identified based on their NMR and mass spectra. Among **1**–**5**, the major metabolite **1** showed the most significant inhibition of CE accumulation in HMDMs, induced by AcLDL (Fig. 2A). Interestingly, **1** is composed of β-carboline (**6**) and ircinal A (**7**),[20] and neither **6** nor **7** showed reduced inhibition, strongly suggesting that the presence of both **6** and **7** were essential for the inhibitory effect of **1** on CE accumulation. Since **2**–**5** were less effective than **1**, any structural modification of **1** seems to reduce the inhibitory activity. Among **2**–**5**, **2** and **3** were more effective than **4** and **5**, which suggested that the modification at the pentacyclic portion deteriorates the activity. As **1** showed the most significant inhibition of CE accumulation and was isolated in large amounts, detailed investigations were carried out with **1**. The inhibitory effect of **1** on CE accumulation was dose-dependent (IC_{50}, 4.1 μM) (Fig. 2B), but even at 50 μM, **1** induced little morphological change (data not shown) and exerted little cytotoxicity (Fig. S2A) in HMDMs, and had little inhibitory effect on the synthesis of triglyceride (TG) (Fig. S2B). Collectively, these results strongly suggested that **1** selectively inhibited CE accumulation in HMDMs induced by AcLDL.

2.2. Manzamine A (1) inhibits CE accumulation by inhibiting ACAT activity

We next examined the mechanisms underlying the inhibitory effects of **1** on CE accumulation in HMDMs. When ^{125}I-AcLDL (50 μg/mL) was incubated with the cells at 37 °C for 5 h, significant amounts of ^{125}I-AcLDL were found to be associated with the cells and subjected to lysosomal degradation, but neither the cell-associated nor degraded fraction remained constant in the presence of **1**, even at 50 μM (Fig. S3A), suggesting little inhibitory effect of **1** on the endocytic uptake of AcLDL by HMDMs. Furthermore, incubation of HMDMs for 24 h with **1** at various concentrations did not affect the protein level of SR-A

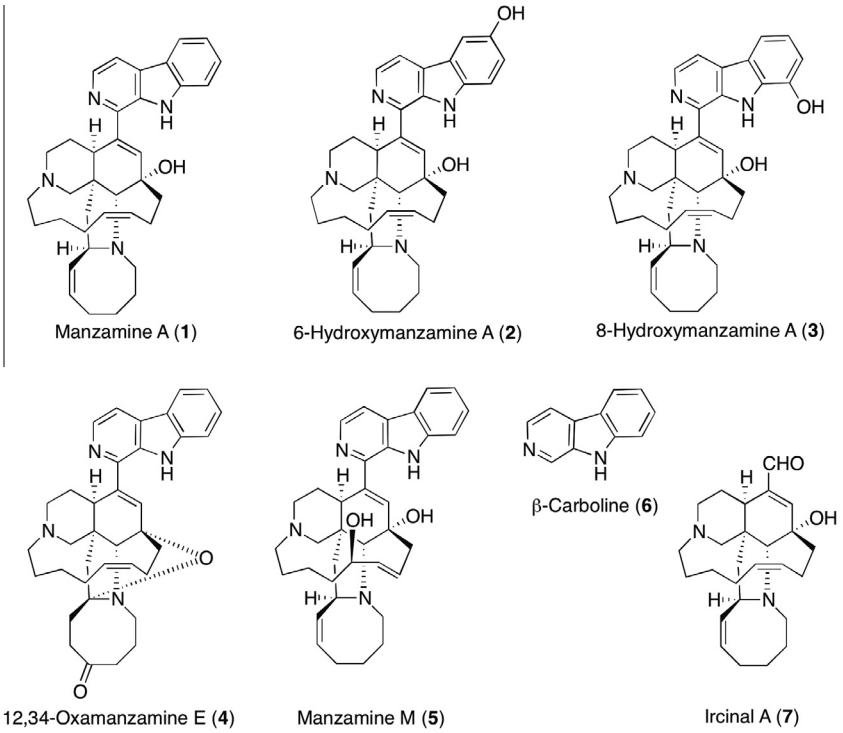

Figure 1. Structures of manzamines (**1**–**5**) isolated from *A. ingens*, β-carboline (**6**), and ircinal A (**7**).

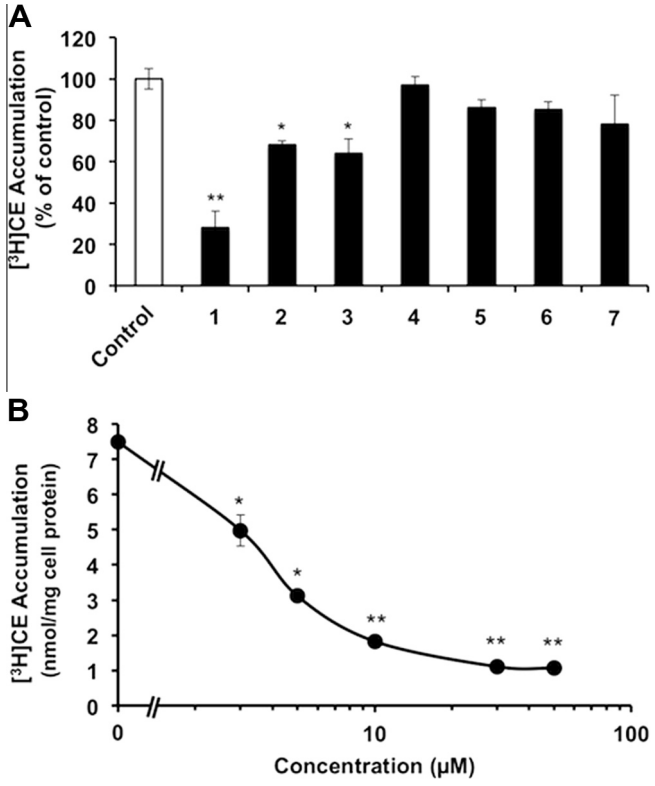

Figure 2. Inhibitory effects of **1**–**7** on CE accumulation in HMDMs induced by AcLDL. HMDMs were incubated with 50 μg/mL AcLDL and 0.1 mM [^{3}H]oleate conjugated with BSA in the absence or presence of **1**–**7** (20 μM) (A) or the indicated concentrations of **1** (B). After incubation for 24 h, [^{3}H]CE was separated by TLC and its radioactivity was measured with a radioscanner. Triple experiments were carried out and the error bars represent the standard deviation. Asterisks show significant differences at $^*P$ <0.05 and $^{**}P$ <0.005 (A) and at $^*P$ <0.01 and $^{**}P$ <0.005 (B).

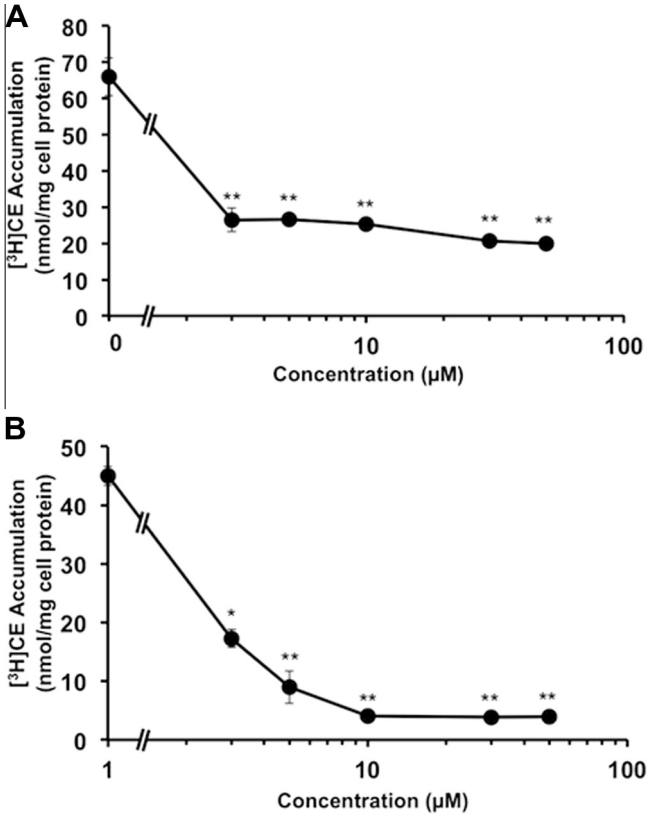

Figure 3. Inhibitory effect of **1** on CE accumulation in CHO cells overexpressing human ACAT-1 or ACAT-2. hACAT-1 CHO (A) and hACAT-2 CHO (B) cells were incubated with the medium containing 10% fetal calf serum in the presence of 0.1 mM [^{3}H]oleate-conjugated BSA and the indicated concentrations of **1**, and the accumulation of [^{3}H]CE was measured as in Figure 2. Triple experiments were carried out and the error bars represent the standard deviation. Asterisks show significant differences at $^*P$ <0.01 and $^{**}P$ <0.005.

or SR-BI (Fig. S3B), indicating that **1** has no effect on the expression of the scavenger receptor proteins SR-A and SR-BI, through which HMDMs take up AcLDL.

Next, we examined whether ACAT is relevant to the inhibitory action of **1** on CE accumulation since esculeoside A inhibited CE accumulation by inhibiting ACAT activity.[14] We used chinese hamster ovary (CHO) cells overexpressing human ACAT-1 (hACAT-1 CHO) and human ACAT-2 (hACAT-2 CHO). When the above two CHO cell lines were incubated with [^{3}H]oleate for 24 h, an increase in CE was detected. Under these conditions, **1** inhibited CE accumulation in both hACAT-1 (Fig. 3A) and hACAT-2 (Fig. 3B) CHO cells in a dose-dependent manner. Since **1** significantly inhibited CE accumulation in both HMDMs and hACAT CHO cells in a similar fashion, it can be inferred that **1** inhibits esterification of cholesterol, possibly by inhibiting ACAT activity and/or ACAT expression.

To confirm the above assumption, we first examined the effect of **1** on ACAT activity using microsomes prepared from HMDMs as the enzyme preparation. The microsomes were incubated with 250 μmol/L [^{14}C]oleoyl-CoA for 15 min in the presence or absence of **1**, and the formation of cholesteryl [^{14}C]oleate was measured. As shown in Figure 4A, **1** inhibited ACAT activity in a dose-dependent manner (IC_{50}, 6.4 μM) as a noncompetitive inhibitor. On the other hand, when HMDMs were incubated with **1** for 24 h, the protein levels of ACAT-1 remained almost constant independent of the concentration of **1** (Fig. 4B). Furthermore, ACAT activity, measured based on the formation of cholesteryl [^{14}C]oleate by microsomes prepared from either hACAT-1 or hACAT-2 CHO cells, was also inhibited by **1** dose-dependently with an IC_{50} value of 6.2 μM in the case of hACAT-1 CHO cells or 4.8 μM in hACAT-2 CHO cells through the noncompetitive inhibition mechanism (Fig. 4C and D). These results clearly suggested that **1** significantly prevented the formation of foam cells in HMDMs by inhibiting ACAT activity. The finding that **1** inhibited CE accumulation (IC_{50}, 6.2 μM) (Fig. S4A) and ACAT activity (IC_{50}, 5.8 μM) (Fig. S4B) in peritoneal macrophages prepared from apoE-deficient mice similarly as in HMDMs supported the above assumption.

2.3. Manzamine A (1) exhibits an in vivo suppressive effect on atherogenesis in ApoE-deficient mice

We next administered **1** to apoE-deficient mice to examine its effect on atherogenesis. The apoE-deficient mouse is known to undergo atherogenesis spontaneously. As shown in Figure 5, the total level of cholesterol was significantly decreased by approximately 40% after the administration of **1** at 30 mg/kg/day (A), and levels of FC (B), LDL cholesterol (C), and TG (D) were also decreased, by approximately 20%, 40% and 50%, respectively. In addition, as shown in Figure 6, **1** reduced the size of atherosclerotic lesions in apoE-deficient mice by 45% [A (manzamine A) and B], whereas cross sections of the aortic sinus in the mice not given **1** showed a marked thickening of the intima filled with foam cells stained by oil red O [A (control)]. The results led us to conclude that **1** exhibited a suppressive effect on atherogenesis in apoE-deficient mice.

3. Discussion

Marine invertebrates are well known sources of secondary metabolites with unique structures and significant biological

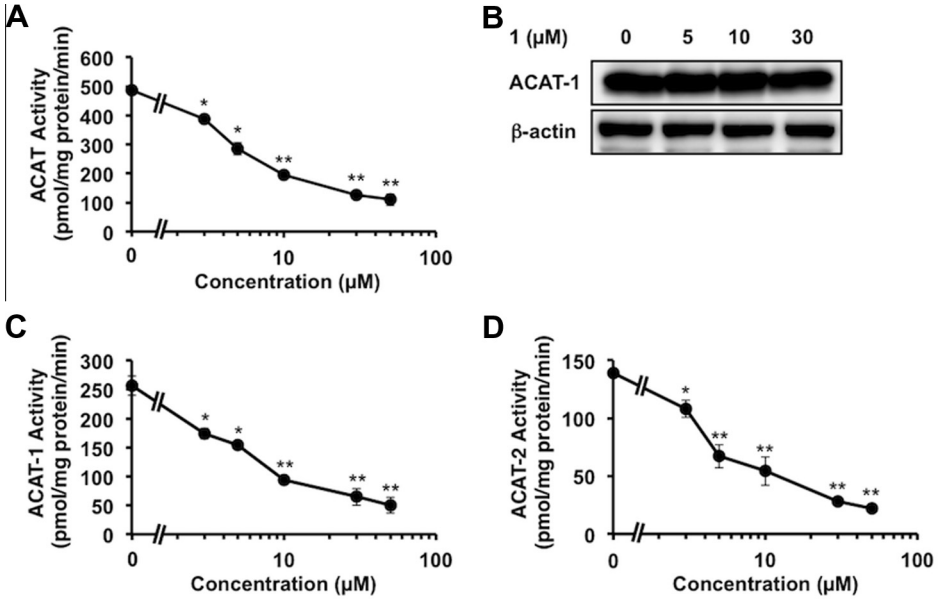

Figure 4. Inhibitory effects of **1** on ACAT activities in HMDMs and hACAT CHO cells. HMDMs (A), hACAT-1 CHO cells (C), or hACAT-2 CHO cells (D) were homogenized with buffer A and reconstituted with sodium taurocholate–cholesterol–PC mixed micelles together with the indicated concentrations of **1**. The resulting microsomes were used as an enzyme preparation and ACAT activity was measured with [^{14}C]oleoyl-CoA as a substrate. Triple experiments were carried out and the error bars represent the standard deviation. Asterisks show significant differences at $^{*}P$ <0.01 and $^{**}P$ <0.001. (B) Immunoblot analysis: HMDMs previously incubated with the indicated concentrations of **1** for 24 h were harvested and subjected to immunoblot analysis using antibodies against human ACAT-1 and human β-actin (control).

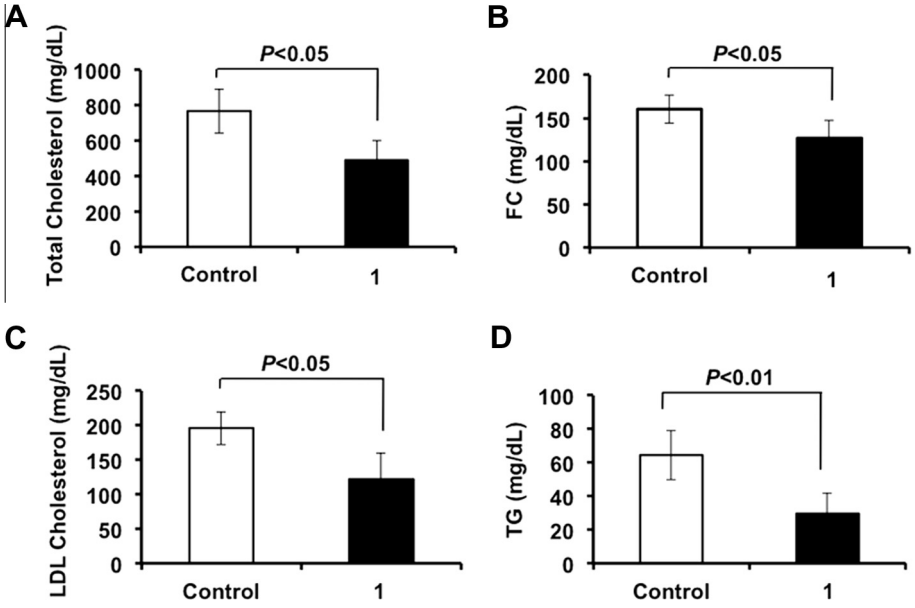

Figure 5. Suppressive effects of **1** on total cholesterol, FC, LDL cholesterol, and TG levels in plasma of apoE-deficient mice. ApoE-deficient mice were fed diets with or without **1** (30 mg/kg/day) for 80 days (n = 7, each group), and total cholesterol (A), FC (B), LDL cholesterol (C), and TG (D) levels were measured. The error bars represent the standard deviation.

activities, and therefore the metabolites are expected to be candidates of drug leads.[21] In this study, to search for lead compounds in the prevention and treatment of atherosclerosis, we first screened extracts of marine sponges for inhibitory effects on CE accumulation in HMDMs and found that an extract of *A. ingens* showed significant inhibition. Bioassay-guided purification from the sponge extract afforded five manzamines, **1**–**5**. Among them, manzamine A (**1**) was the most potent inhibitor. Since β-carboline (**6**) and ircinal A (**7**) showed reduced inhibition, the connection of **6** and **7** to **1** improves the activity (see Fig. 2A). As shown in the activities of **2**–**5**, any modification of **1** appears to reduce the inhibitory effect. In addition to the inhibitory activity against CE accumulation, characteristic of foam cells, we found that **1** inhibited ACAT activities in microsomes from HMDMs, hACAT-1 CHO cells, and hACAT-2 CHO cells with IC_{50} values of 6.4, 6.2, and 4.8 μM, respectively (see Fig. 4). NTE-122, a synthetic compound, inhibited microsomal ACAT activity in intestine of cholesterol-fed rabbits with an IC_{50} of 7.6 nM[22] and F-1394, a synthetic compound, inhibited macrophage ACAT activity with an IC_{50} value of 32 nM.[23] Pactimibe[24] and avasimibe,[25] clinically evaluated synthetic ACAT inhibitors (vide infra), inhibited ACAT-1 [IC_{50}, 4.9 μM (pactimibe) and 23.5 μM (avasimibe)] and ACAT-2 [IC_{50}, 3.0 μM (former) and

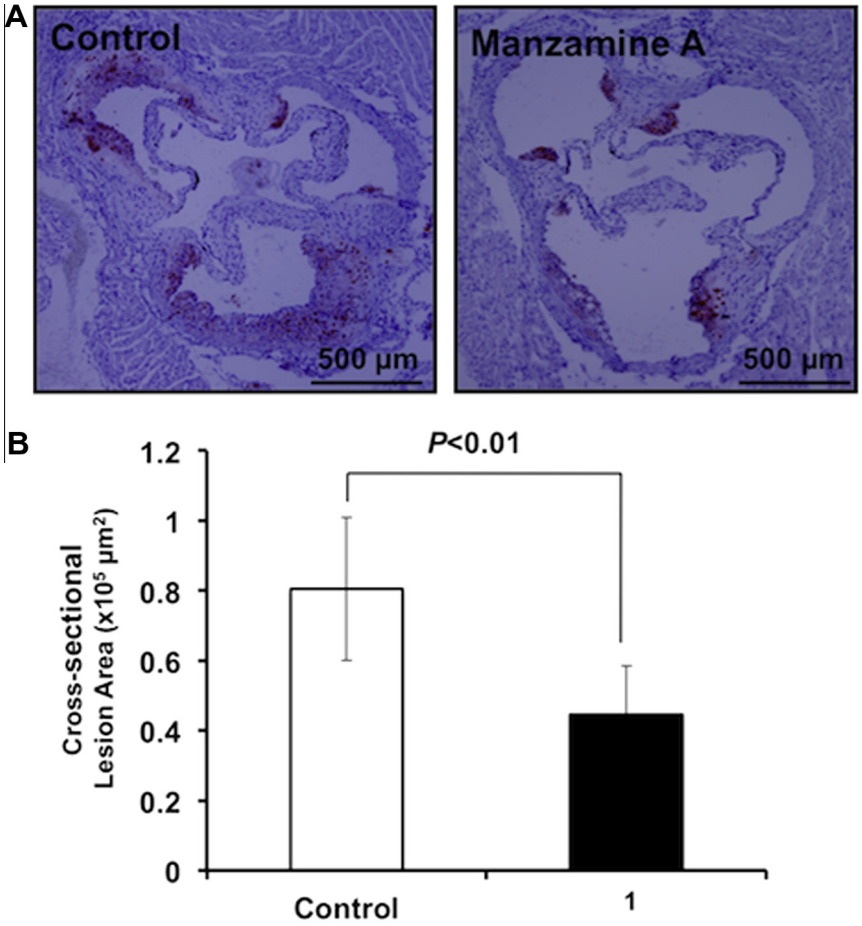

Figure 6. Inhibition of atherogenesis in apoE-deficient mice by administration of **1**. Administration of **1** reduced the atherosclerotic lesions in apoE-deficient mice. (A) Representative sections of aortic sinus stained with oil red O in apoE-deficient mice without (control) and with **1** (30 mg/kg/day) (manzamine A) treatment. (B) Quantitative evaluations of cross sectional lesions of the aortic sinus shown in (A). The error bars represent the standard deviation.

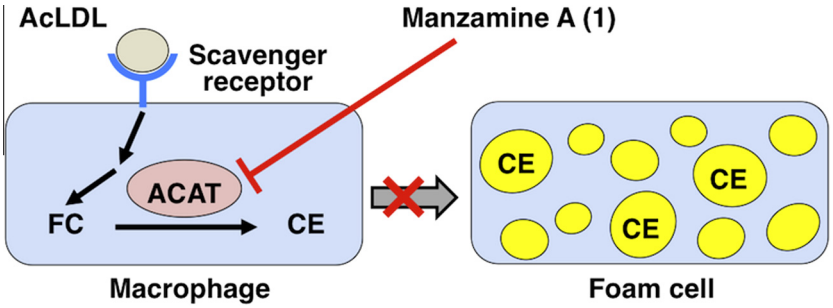

Figure 7. A proposed inhibitory mechanism of manzamine A (**1**) against CE accumulation. AcLDL, acetylated LDL; FC, free cholesterol; CE, cholesterol ester.

9.2 μM (latter)].[26] Thus, the potency of **1** as an ACAT inhibitor is comparable to those of known ACAT inhibitors such as pactimibe and avasimibe. A proposed inhibitory mechanism of **1** is shown in Figure 7. Most importantly, **1** exhibited an in vivo suppressive effect on atherosclerosis. To date, more than 80 congeners of manzamines have been isolated from marine sponges of the genera *Acanthostrongylophora*,[27] *Amphimedon*,[16] *Haliclona*,[15] *Ircinia*,[20] *Pachypellina*,[17] *Pellina*,[28] and *Xestospogia*[29] with complicated chemical structures as well as several biological activities such as cytotoxic,[15] antimicrobial,[18] antimalarial,[30] antiviral,[27] antineuroinflammatory,[27] and insecticidal[29] effects. Although several biological activities of **1** have been reported, our study provides the first evidence that **1** significantly suppresses hyperlipidemia and atherosclerosis in apoE-deficient mice by inhibiting ACAT activity. It should be noted that Hamann's group proposed GSK-3β as a possible common target of manzamines.[31] On the other hand, Coppens's group implied that *Pf*DGAT, acyl-CoA:diacylglycerol acyltransferase of malaria parasite *Plasmodium falciparum*, was a candidate of antimalarial target.[32] Taking into account that DGAT and ACAT are members of the membrane-associated transferase family, together with the fact that manzamines exhibit antimalarial activity, it can be inferred that **1**, which was found to inhibit ACAT in this study, would work as an antimalarial agent targeting *Pf*DGAT.

Acute coronary syndromes (ACS), including unstable angina, myocardial infarction, and sudden coronary death, are the major causes of cardiovascular diseases. Clinical studies reveal that the occurrence of ACS always correlates with the presence of vulnerable atherosclerotic plaques and subsequent thrombosis. In the

early stage of atherosclerogenesis, macrophages penetrate the intima, efficiently take up modified LDL, and store cholesterol and fatty acids as a foam of neutral lipids in cytosolic lipid droplets.[1,2] FC is esterified to CE by ACAT, leading to the formation of foam cells derived from macrophages in atherosclerotic lesions.[7] Therefore, preventing the formation of foam cells is believed to be important to preventing or treating atherosclerosis.

Clinical studies on a number of anti-atherosclerotic agents inhibiting ACAT activity have been conducted to date. Mice lacking ACAT-2 exhibit a reduced capacity to absorb cholesterol and resistance to diet-induced hypercholesterolemia and gallstone formation.[33] Non-selective ACAT inhibition is known to reduce atherosclerosis in apoE-deficient mice.[34] For example, NTE-122 and F-1394, prevent the progression of atherosclerosis in cholesterol-fed rabbits.[22,23] However, the administration of pactimibe, to the patients with atherosclerosis for 18 months failed to reduce plaque volume.[24] While avasimibe, reduces the number of macrophages and expression of matrix metalloproteinases in atherosclerotic lesions of hypercholesterolemic rabbits,[25] and also reduces atherosclerosis and the plasma concentration of cholesterol in apoE*3-Leiden mice.[35] However, the administration of avasimibe to the patients with atherosclerosis for 2 years did not reduce plaque volume.[36] ACAT-2 has been reported to be expressed in foam cells derived from macrophages in vitro and in vivo,[37] and **1** inhibits ACAT-1 and ACAT-2 activities. Therefore, it can be inferred that **1** reduces foam cell formation by inhibiting ACAT activity (see Fig. 7). In addition, it should be noted that **1** reduced the concentration of cholesterol or TG in serum in vivo. Our results are consistent with the previous reports that ACAT inhibitors decreased the serum cholesterol level,[26] blocked dietary cholesterol absorption in the intestines,[38] and reduced the level of serum triglycerides,[38,39] although the reason for the decrease of serum TG by administration of **1** remains unknown. Consequently, we propose that **1** is a good candidate for the prevention and treatment of atherosclerosis.

4. Experimental

4.1. General experimental procedures

Optical rotations were determined with a JASCO DIP-1000 polarimeter in $CHCl_3$. NMR spectra were recorded on a JEOL JNM-ECX-400 NMR spectrometer in $CDCl_3$. Chemical shifts were referenced to the residual solvent peak (δ_H 7.24). Mass spectra were measured on a JEOL JMS-700 mass spectrometer.

4.2. Materials

Oil red O, cholesterol oleate, phenylmethylsulfonyl fluoride, L-α-phosphatidylcholine, and oleoyl CoA were purchased from Sigma–Aldrich Japan (Osaka, Japan). Leupeptin and pepstatin A were purchased from Peptide Institute (Osaka, Japan). Penicillin G and streptomycin sulfate were purchased from Invitrogen (Tokyo, Japan). Ficoll-Paque, Na[^{125}I] (17 Ci/mg), [9,10-^{3}H]oleate (4 Ci/mg), [^{14}C]oleoyl CoA (50 μCi/mg), and [1α, 2α(*n*)-^{3}H]cholesteryl oleate (1 mCi/mg) were purchased from GE Healthcare Bio-Sciences (Tokyo, Japan). G418 was purchased from Gibco (Grand Island, NY). All other chemicals were of the best grade available from commercial sources. β-Carboline (**6**) was purchased from Tokyo Chemical Industry (Tokyo, Japan). Ircinal A (**7**) was previously isolated from another marine sponge *A. ingens* in our laboratory.

4.3. Extraction and isolation of manzamines

The marine sponge was collected at a depth of 10 m in North Sulawesi, Indonesia, in December 2006 and soaked in EtOH immediately. The sponge was identified as *Acanthostrongylophora ingens* and a voucher specimen (RMNH POR 3991) has been deposited in the Netherlands Centre for Biodiversity Naturalis, The Netherlands. The marine sponge (300 g, wet weight) was extracted with EtOH. The EtOH extract was evaporated, and the aqueous residue was extracted with EtOAc. The EtOAc soluble fraction (5.1 g) was subjected to SiO_2 column chromatography with $CHCl_3$/MeOH to afford fractions A and B. Fraction A (3.7 g) eluted with $CHCl_3$/MeOH (99:1 and 98:2) was fractionated by SiO_2 column chromatography with *n*-hexane/EtOAc to afford fractions A1 and A2. Fraction A1 (1.1 g) eluted with *n*-hexane/EtOAc (3:1) was purified by SiO_2 column chromatography with *n*-hexane/EtOAc/Et_3N (75:25:2) to afford manzamine A[15] (**1**, 323.0 mg) and a fraction containing 12,34-oxamanzamine E[18] (**4**). The latter was subjected to purification by SiO_2 HPLC with *n*-hexane/EtOAc/Et_3N (70:29:1) to afford **4** (1.5 mg). Fraction A2 (71 mg) eluted with *n*-hexane/EtOAc (1:1) was purified by SiO_2 HPLC with *n*-hexane/EtOAc/Et_3N (50:50:1.5) to afford 8-hydroxymanzamine A[17] (**3**, 104.97 mg). Fraction B (172 mg) eluted with $CHCl_3$/MeOH (95:5) was purified by SiO_2 HPLC with *n*-hexane/EtOAc/Et_3N (48.5:50:1.5) to afford 6-hydroxymanzamine A[16] (**2**, 2.2 mg) and manzamine M[19] (**5**, 1.4 mg). Compounds **1**–**5** were identified on the basis of their spectral data.

4.4. Preparation and modification of lipoproteins

Human LDL (d = 1.019–1.063 g/mL) was isolated by sequential ultracentrifugation from the plasma of normolipidemic subjects as described previously.[40] AcLDL was prepared by chemical modification of LDL with acetic anhydride as described.[40] AcLDL was labeled with ^{125}I according to the procedure described by McFarlane[41] to give a specific radioactivity of 600 cpm/ng.

4.5. Cell culture

Human monocytes were purified from the blood of healthy volunteers by Ficoll density gradient centrifugation.[42] Purified monocytes were suspended in RPMI 1640 medium and seeded onto 24-well plates (4×10^5/well) or 6-cm dishes (2×10^6/dish). After a 1-h incubation for adherence, the medium was replaced by Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% pooled human serum, streptomycin (100 μg/mL), and penicillin G (100 U/mL). The adherent monocytes were incubated for 7 days to induce their differentiation into macrophages. Under these conditions, the cells contained >95% macrophages and were >98% viable as determined by trypan blue staining. CHO cells stably overexpressing human ACAT-1 (hACAT-1 CHO cells) or human ACAT-2 (hACAT-2 CHO cells)[43] were cultured in Nutrient mixture F-12 HAM medium (Sigma–Aldrich, Japan) supplemented with 10% fetal calf serum, streptomycin (50 μg/mL), penicillin G (50 U/mL), and G418 (800 μg/mL). Mouse peritoneal macrophages were cultured in DMEM supplemented with 10% pooled human serum, streptomycin (100 μg/mL), and penicillin G (100 U/mL). All experiments using the cells were performed under a humidified atmosphere containing 5% CO_2 at 37 °C.

4.6. Measurement of AcLDL uptake

The differentiated HMDMs seeded onto 24-well plates were washed with 1.0 mL of phosphate-buffered saline (PBS) and cultured in DMEM containing 3% bovine serum albumin (BSA), streptomycin (100 μg/mL), and penicillin G (100 U/mL) (medium A). The cells in each well were incubated in 0.5 mL of medium A with 50 μg/mL ^{125}I-AcLDL in the presence of the indicated concentrations of samples at 37 °C for 5 h, and then 375 μL of the culture medium was taken from each well and mixed with 150 μL of 40% trichloroacetic acid (TCA) in a vortex mixer. To this solution,

100 μL of 0.7 M $AgNO_3$ was added, followed by centrifugation. The resulting supernatant (250 μL) was used to determine the TCA-soluble radioactivity, an index of intracellular degradation. Each well was washed with ice-cold PBS containing 1% BSA, and the cells were lysed with 0.1 M NaOH to determine the radioactivity associated with cells and also the protein concentration.

4.7. Measurement of CE and TG formation

HMDM monolayers were incubated with AcLDL (50 μg/mL) in the presence of 0.1 mM [^{3}H]oleate conjugated with BSA for 24 h, and cellular lipids were extracted to determine the radioactivity of cholesteryl-[^{3}H]oleate and TG-[^{3}H]oleate as described previously.[44]

4.8. Immunoblot analyses

HMDMs were solubilized with 1% Triton X-100, as determined using the BCA protein assay reagent, followed by pretreatment by boiling in sample buffer (60 mM Tris–HCl, pH 7.4, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.01% bromophenol blue) for 3 min. These samples were incubated with 3 units of *N*-glycosidase (Roche Applied Science) at 37 °C for 24 h, and then 10 μg of protein was run on a 10% SDS–polyacrylamide gel and transferred to a PVDF (polyvinylidine fluoride) transfer membrane (Millipore, Bedford, MA). The membranes were exposed to an anti-human SR-A antibody (E5) (Trans Genic Inc., Kumamoto, Japan) and visualized using a horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibody with the ECL Western blotting detection reagent. SR-BI and ACAT-1 were detected by immunoblot analyses with an anti-human SR-BI antibody (Novus Biologicals, Littleton, CO) and an anti-human ACAT-1 antibody (Cayman Chemical Company, Ann Arbor, MI), respectively. These membranes were re-blotted with an anti-β-actin antibody as a control. The density of the bands was measured with the Imaging Gauge software program in LAS 4000 (Fujifilm, Tokyo).

4.9. ACAT assay

The cultured cells were treated with 1 mM Tris–HCl, pH 7.4, containing 1 mM EDTA for hypotonic shock followed by homogenization with buffer A (50 mM Tris–HCl, pH 7.8, containing 1 mM EDTA and three protease inhibitors, phenylmethylsulfonyl fluoride, leupeptin, and pepstatin A). The homogenates were mixed with 2 M KCl and 10% 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate (CHAPS) in buffer A to obtain the final concentrations of 1 M KCl and 2% CHAPS, respectively. This sample (80 μg in 20 μL) was reconstituted with 140 μL of sodium taurocholate–cholesterol–phosphatidylcholine (PC) mixed micelles (0.2 as cholesterol/PC molar ratio). The ACAT reaction with the resulting microsomes was initiated by adding 20 μL of 250 μM [^{14}C]oleoyl-CoA (20 dpm/pmol) as a substrate followed by incubation at 37 °C for 15 min. Lipids were extracted and the radioactive cholesteryl-[^{14}C]oleate was determined by TLC as described previously.[45]

4.10. Cytotoxic assay

HMDMs (1×10^4 cells) were cultured in a 96-well plate and treated with **1**. Cell viability was determined by using the WST assay (WST-8 cell counting kit; Dojin Chemical, Kumamoto, Japan) according to the manufacturer's directions.

4.11. Measurement of in vivo anti-atherosclerotic activity

All experiments on animals were approved by the Ethics Committee for Animal Experiments of Kumamoto University and performed in accordance with the Guidelines for Animal Experiments of the laboratories. Six-week-old apoE-deficient mice (C57BL/6.KOR-*Apoe*^{shl}) were purchased from SLC (Shizuoka, Japan). The mice were housed in a pathogen-free barrier facility under a light/dark (12 h/12 h) cycle and fed a normal rodent chow diet (Clea, Japan) for 1 week after purchase and then a diet containing **1** (30 mg/kg of body weight). The dose was determined based on the following experiments. Oral administration of berberine (50 and 100 mg/kg/day) to hamsters, which were fed a high-fat and high-cholesterol diet, significantly decreased their serum cholesterol and serum LDL cholesterol.[46] Furthermore, oral administration of beauveriolide I and III (50 mg/kg/day) to apoE knockout mice decreased the pathogenesis of atherosclerosis.[13] Fourteen mice (seven for controls and seven for administration of **1**) were used for in vivo evaluation. Blood samples were collected from the abdominal aorta at the end of the administration. Total cholesterol, FC, LDL-cholesterol, and TG concentrations in serum were determined on an Olympus AU5200 automatic analyzer (Olympus, Tokyo) using standard enzymatic methods. For the analyses of atherosclerotic lesions, the mice were sacrificed, and their hearts were perfused with PBS containing 4% (w/v) paraformaldehyde and embedded in OCT (optimal cutting temperature) compound (Sakura Tissue-Tek, Tokyo). Serial sections (6 μm thick) were cut by using a Cryostat (Leica, Tokyo) and counterstained with oil red O and hematoxylin. The extent of atherosclerosis was determined as the average lesion area in the stained aortas by analysis with the IPAP-WIN software package (Sumika Technoservice, Hyogo, Japan) as described previously.[47]

4.12. Statistical analysis

All experimental data are expressed as the mean ± SD. Differences between groups were examined for statistical significance using the Mann–Whitney *U*-test and the non-repeated measures ANOVA. A P value <0.05 denoted the presence of a statistically significant difference.

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Supplementary data

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