Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry Letters
Bioorganic & Medicinal Chemistry Letters 14 (2004) 4277–4280

Acyl-CoA: cholesterol acyltransferase inhibitory activities of fatty acid amides isolated from *Mylabris phalerate* Pallas

Ming-Zhe Xu,[a,c] Woo Song Lee,[b] Mi Jeong Kim,[b] Doo-Sang Park,[a] Hana Yu,[b] Guan-Rong Tian,[c] Tae-Sook Jeong[b,*] and Ho-Yong Park[a,*]

[a]*Insect Resources Laboratory, Korea Research Institute of Bioscience and Biotechnology, 52 Oun, Yusong, Daejeon 305-333, Republic of Korea*
[b]*Lipid Metabolism Research Laboratory, Korea Research Institute of Bioscience and Biotechnology, 52 Oun, Yusong, Daejeon 305-333, Republic of Korea*
[c]*Department of Chemistry, University of Yanbian, Yanji, China*

Received 11 May 2004; revised 28 May 2004; accepted 31 May 2004
Available online 19 June 2004

Abstract—Unsaturated fatty acid amides, 9(*Z*)-octadecenamide (**2**) and 9(*Z*),12(*Z*)-octadecadienamide (**4**) as inhibitors of acyl-CoA: cholesterol acyltransferase (ACAT) were isolated from the ethyl acetate extracts of the insect, *Mylabris phalerate* Pallas, and elucidated by their spectroscopic data analysis. Compounds **2** and **4** inhibited rat liver microsomal ACAT, hACAT-1, and hACAT-2 with IC_{50} values of 170, 85, and 63 μM for **2** and of 151, 53, and 45 μM for **4**, respectively.
© 2004 Elsevier Ltd. All rights reserved.

Atherosclerosis is a progressive disease characterized by the accumulation of lipids and fibrous elements in the large arteries.[1] In early atherogenesis, acyl-coenzyme A: cholesterol acyltransferase (ACAT, EC.2.3.1.26) accumulated cholesteryl esters with macrophages and smooth muscle cells to result in foam cell formation.[2] In both experimental and clinical atherosclerosis, the formation of foam cells derived from macrophages and smooth muscle cells is an important event.[3] Since the propagation of foam cells in the arterial walls is directly related to the increment of ACAT activity, the inhibitors of ACAT as new type of medicine are needed to treat or prevent atherosclerosis and hypercholesterolemia.[4] Inhibiting ACAT within the arterial wall may result in the inhibition of atherosclerotic lesion progression without lowering total plasma cholesterol levels.[5] Many of these inhibitors showed promise in animal studies inhibiting intestinal or hepatic ACAT and lowering plasma cholesterols levels.[6] In mammals, ACAT was found to exist as two isoforms, namely, ACAT-1 is more involved in macrophage foam cell formation and ACAT-2 is associated with intestinal cholesterol absorption.[3] ACAT-1 and ACAT-2 respond differently to ACAT inhibitors of differing structures and classes.[7]

Insects are the largest and most diverse groups of organisms. They are evolutionarily distant from vertebrates, plants, and microbial origin. For these reasons, the study of insects promises to reveal novel bioactive compounds. In order to search for human ACAT-1 (hACAT-1) and human ACAT-2 (hACAT-2) inhibitors from insect origin, especially traditional Asian medicinal insects, several solvent extracts of insects were screened and the ethyl acetate extracts of *M. phalerate* Pallas showed significant hACAT-1 and hACAT-2 inhibitory activities with 85% and 89% at 100 μg/mL, respectively. *M. phalerate* Pallas (order Coleopteran) is a dried beetle, which is abundant in China and Eastern India. As an aphrodisiac, an abortifacient, a vesicant, and a veterinary medicine diuretic,[8] *M. phalerate* Pallas has been used as traditional medicine in Korea, Japan, and China for hundreds of years. It has been found that *M. phalerate* Pallas possesses antitumor activity, increases the number of leucocytes, and has irritant effects on the urinary organs.[9] Activity-guided fractionation of the ethyl acetate extracts of *M. phalerate* Pallas led to the

Keywords: ACAT inhibitors; The insect; *Mylabris phalerate* Pallas.
* Corresponding authors. Tel.: +82-42-860-4650/4558; fax: +82-42-860-4659/861-2675; e-mail addresses: hypark@kribb.re.kr; tsjeong@kribb.re.kr

0960-894X/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2004.05.086

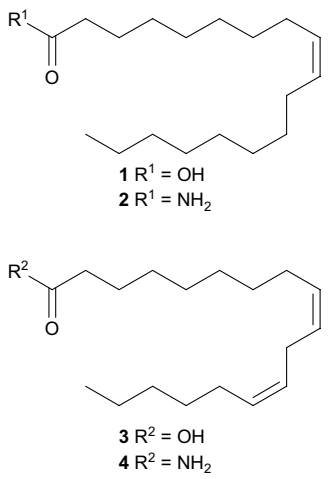


Figure 1. Chemical structures of commercial reagents **1** and **3**, and compounds **2** and **4** isolated from *M. phalerate* Pallas.

isolation of bioactive compounds **2** and **4** (Fig. 1) using silica gel column chromatography, thin layer chromatography, and HPLC.

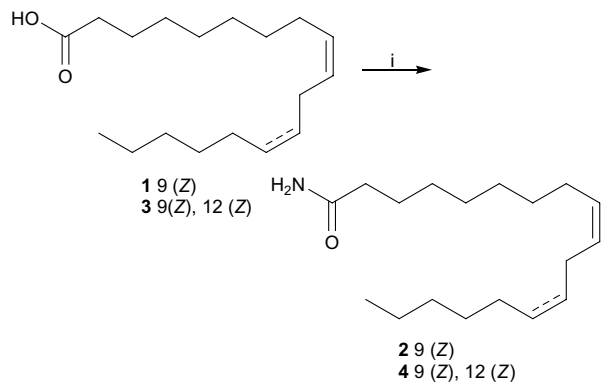
The powder of dried *M. phalerate* Pallas (200 g) was suspended twice with ethyl acetate (2 L) at room temperature. After stirring for 24 h and filtration using Whatman no. 2 filter papers, the ethyl acetate extracts was concentrated in vacuo to yield a brown oily material (24.0 g). This material was chromatographed on a silica gel column (E. Merck, Kieselgel 60, 230–400 mesh) with a gradient of chloroform–methanol (100:0–1:1, v/v). The active fractions were combined and concentrated in vacuo yielding an oily residue (2.0 g). The residue was subjected to flash silica gel column chromatography with a gradient of *n*-hexane–ethyl acetate (1:1–1:5, v/v). After combination of active fractions and concentration in vacuo, the active constituents were finally purified by preparative HPLC using an ODS column (Hydrosphere C-18, 250×20 mm, YMC, Kyoto, Japan; MeOH/H_2O = 9:1; 4.0 mL/min; λ = 220 nm) to obtain compounds **2** (12.0 mg) and **4** (10.0 mg).[10]

Compound **2**, white powder, showed a base peak at m/z 281 $[M]^+$ in the EIMS. The molecular formula of compound **2** was determined to be $C_{18}H_{35}NO$ on the basis of HREIMS (m/z $[M]^+$, 281.2719, calcd 281.2719 for $C_{18}H_{35}NO$) and has mp 72.0–73.0 °C ($CHCl_3$). The IR ($CHCl_3$) gave two signals at 3393 (symmetric stretching of N–H) and 3181 (H-bonded symmetric stretching of N–H) with a strong signal at 1650 (amide C=O) cm^{-1}, respectively. The ^1H NMR ($CDCl_3$, 500 MHz) showed two olefinic methines at δ_H 5.24 (2H, m, CH=CH), α-methylene protons of oleamide at δ_H 2.09 (2H, t, J = 7.4 Hz, CH_2CONH_2), and methyl protons at δ_H 0.80 (3H, t, J = 6.7 Hz), respectively. The ^{13}C NMR data indicated amide carbonyl carbon at 179.3 ppm and two olefinic carbons at 130.9 and 130.8 ppm, respectively. Then, the double bond of oleamide **2** was located on C-9, which was determined by the fragmentation patterns of EIMS data. Additionally, *cis*-geometry of **1** was showed peaks overlapped with triplet and triplet each other at δ_H 5.24, whereas *trans*-geometry exhibited a set of two apparent overlapping doublet split triplets at δ_H 5.28.[11] In the IR spectrum, *trans*-isomer exhibited an additional strong and characteristic absorption peak at 960 cm^{-1}.[12]

Compound **4**, yellow oil, exhibited a molecular formula of $C_{18}H_{33}NO$ from its positive HREIMS data (m/z $[M]^+$, 279.2564, calcd 279.2531 for $C_{18}H_{33}NO$). The IR spectrum of **4** exhibited strong amide group at 3387 and 3186 cm^{-1} and amide carbonyl group at 1642 cm^{-1}. The ^1H NMR spectrum of **4** showed characteristic protons of two olefinic moieties at δ_H 5.24 (4H, m), methylene protons between two olefinic bonds at δ_H 2.68 (2H, t like, J = 6.5 Hz), α-methylene protons at δ_H 2.09 (2H, t, J = 7.5 Hz), and methyl protons at δ_H 0.81 (3H, t, J = 6.6 Hz), respectively. The ^{13}C NMR data indicated amide carbonyl carbon at 179.3 ppm and four olefinic carbons at 130.9, 130.8, 129.1, and 129.0 ppm, respectively.

In order to confirm more exact structures and activities of compounds **2** and **4**, commercial available oleic acids **1** and **3** were converted to compounds **2** and **4**, respectively. Namely, oxalyl chloride (3.0 equiv) was added slowly to a solution of **1** in CH_2Cl_2 at 0 °C and stirred for 4 h at room temperature. The reaction mixture was concentrated under reduced pressure, cooled down to 0 °C, treated with saturated aqueous NH_4OH for 5 min, and extracted with EtOAc. The organic layer was dried over anhydrous $MgSO_4$, filtered, concentrated, and chromatographed on SiO_2 (*n*-hexane–EtOAc = 2:1) to produce the fatty acid amide **2** in 97% yield. Linoleic acid **3** was treated with oxalyl chloride and NH_4OH under similar reaction condition to give compound **4** in 98% yield (Scheme 1). The synthetic compounds **2** and **4** were identical with spectroscopic data of compounds **2** and **4** that were isolated from *M. phalerate*. Therefore, the structure of compounds **2** and **4** was elucidated to be 9(*Z*)-octadecenamide (**2**) and 9(*Z*),12(*Z*)-octadecadienamide (**4**). Also, their structures were determined by comparing previously reported data.[11,13]

The compounds **1**–**4** were evaluated for ACAT inhibitory activities. The rate of incorporation of [1-^{14}C] oleoyl-CoA into cholesteryl ester was determined using the expressed hACAT-1 and hACAT-2 from Hi5 cells[14]



Scheme 1. Reagents and conditions: (i) $(COCl)_2$, NH_4OH, CH_2Cl_2, rt.

Table 1. ACAT inhibitory activities of compounds **1**–**4**

Compounds	IC_{50} (μM)[a]		
	Rat liver microsomal ACAT	hACAT-1	hACAT-2
1	>1000	>1000	>1000
2	170	85	63
3	>1000	>1000	>1000
4	150	53	45
Oleic acid anilide[b]	0.027	0.14	0.17
Pyripyropene A[b]	0.17	>1000	0.64

[a] In vitro ACAT inhibitory activity was measured using the rat liver microsomal ACAT, expressed hACAT-1 and -2. Data are shown as mean values of two independent experiments performed in duplicate.
[b] Oleic acid anilide and pyripyropene A were used as positive controls. Data from Ref. 14.

and rat liver microsomes.[15,16] Oleic acid anilide and pyripyropene A were used as positive controls.[16,17] In general, polyunsaturated fatty acid analogs have been known to exhibit anti-inflammatory activity,[18] in vitro or in vivo antioxidant activity,[19] and beneficial effects on risk factors of coronary heart disease (CHD).[20] Based on these results, oleic acid (**1**) and linoleic acid (**3**) were tested tentatively for their ACAT inhibitory activities. The unsaturated fatty acids **1** and **3** not inhibited hACAT-1 and hACAT-2, and rat liver microsomal ACAT, whereas the unsaturated fatty acid amides **2** and **4** showed ACAT inhibitory activities. Compounds **2** and **4** inhibited rat liver microsomal ACAT, hACAT-1, and hACAT-2 with IC_{50} values of 170, 85, and 63 μM for **2**, of 151, 53, and 45 μM for **4**, respectively (Table 1). Compounds **2** and **4** inhibited both isoforms with similar degree of inhibitory activities; whereas rat liver microsomal ACAT fraction was inhibited with two- to threefold lower activities compared to each human ACAT isoform. On the other hand, oleic acid anilide and pyripyropene A,[16,17] known ACAT inhibitors, showed more potent inhibitory activities against rat liver microsomal ACAT than both hACAT-1 and hACAT-2 as previously known.[14] These results suggest that the specificity of inhibitor was highly different, depending on enzyme sources.

The fatty acid amides **2** and **4** were isolated firstly from the EtOAc extracts of *M. phalerate* Pallas and moderately inhibited rat liver microsomal ACAT, hACAT-1, and hACAT-2. Further studies on confirmation of the inhibitory activity of ACAT using cells stably expressing hACAT-1 or -2 and the in vivo efficacy test of cholesterol-lowering and anti-atherogenic activities of compounds **2** and **4** are underway.

Acknowledgements

This work is supported by grants from KRIBB Research Initiative Program (KGS 3220311) and the Ministry of Science and Technology (M1-0302-00-0033), Korea.

References and notes

1. Lusis, A. J. *Nature* **2000**, *407*, 233.
2. Ross, R. *Nature* **1993**, *362*, 801.
3. Chang, T. Y.; Chang, C. C. Y.; Lin, S.; Yu, C.; Li, B. L.; Miyazaki, A. *Curr. Opin. Lipidol.* **2001**, *12*, 289.
4. Sliskovic, D. R.; Picard, J. A.; Krause, B. R. *Prog. Med. Chem.* **2002**, *39*, 121.
5. Chang, T. Y.; Chang, C. C. Y.; Cheng, D. *Annu. Rev. Biochem.* **1997**, *66*, 613.
6. (a) Krause, B. R.; Anderson, M.; Bisgaier, C. L.; Bocan, T.; Bousley, R.; Dehart, P.; Essenburg, A.; Hamelehle, K.; Homan, R.; Kieft, K.; Ncnally, W.; Stanfield, R.; Newton, R. S. *J. Lipid. Res.* **1993**, *34*, 279; (b) Carr, T. P.; Hamiltton, R. L.; Rudel, L. L. *J. Lipid. Res.* **1995**, *36*, 25; (c) Lee, H. T.; Sliskovic, D. R.; Picard, J. A.; Roth, B. D.; Wierenga, W.; Hicks, J. L.; Boualey, R. F.; Hamelehle, K. L.; Homan, R.; Speyer, C.; Stanfield, R. L.; Krause, B. R. *J. Med. Chem.* **1996**, *39*, 5031.
7. Burnett, J. R.; Wilcox, L. J.; Huff, M. W. *Clin. Chim. Acta* **1999**, *286*, 231.
8. Wang, C. C.; Wu, C. H.; Hsieh, K. J.; Yen, K. Y.; Yang, L. L. *Toxicology* **2000**, *147*, 77.
9. Wang, G. S. *J. Ethnopharmacol.* **1989**, *26*, 147.
10. Physical and spectroscopic data: *9(Z)-octadecenamide*: white powder; it has mp 72–73 °C ($CHCl_3$); IR ($CHCl_3$) ν_{max} 3393, 3181, 2914, 2840, 1650, 1462, 1396, 1146, 810, 664 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 5.24 (2H, m), 2.09 (2H, t, $J = 7.4$ Hz), 1.93 (4H, m), 1.50 (2H, m), 1.23–1.19 (20H, m), 0.81 (3H, t, $J = 6.6$ Hz); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 179.3, 130.9, 130.8, 36.5, 33.1, 30.84, 30.81, 30.62, 30.64, 30.61, 30.45, 30.35, 30.24, 28.1, 28.1, 26.9, 14.5; EIMS m/z 281 $[M]^+$, 264 (25), 246 (12), 126 (30), 98 (25), 83 (20), 72 (90), 59 (100); HREIMS m/z calcd for $C_{18}H_{35}NO$ 281.2719, found 281.2719. *9(Z), 12(Z)-Octadecadienamide*: a yellow oil; IR ($CHCl_3$) ν_{max} 3387, 3186, 2927, 2583, 1642, 1468, 1410, 1289, 1124, 829, 664 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 5.24 (4H, m), 2.67 (2H, t, $J = 6.5$ Hz), 2.09 (2H, t, $J = 7.5$ Hz), 1.96 (4H, m), 1.50 (2H, m), 1.28–1.19 (14H, m), 0.81 (3H, t, $J = 6.5$ Hz); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 179.3, 130.9, 130.8, 129.1, 129.0, 36.5, 32.7, 30.7, 30.5, 30.4, 30.3, 30.2, 28.1, 28.1, 26.9, 26.5, 23.5, 14.4; EIMS m/z 279 $[M]^+$ 262 (11), 244 (10), 220 (20), 150 (25), 109 (35), 95 (60), 81 (80), 67 (90), 59 (100); HREIMS m/z calcd for $C_{18}H_{33}NO$ 279.2562, found 279.2531.
11. Cravatt, B. F.; Lerner, R. A.; Boger, D. L. *J. Am. Chem. Soc.* **1996**, *118*, 580.
12. Griffiths, L.; Horton, R. *Magn. Reson. Chem.* **1998**, *36*, 104.
13. Jain, M. K.; Ghomashchi, F.; Yu, B. Z.; Bayburt, T.; Murphy, D.; Houck, D.; Brownell, J.; Reid, J. C.; Solowiej, J. E.; Wong, S. M.; Mocek, U.; Jarrell, R.; Sasser, M.; Gelb, M. H. *J. Med. Chem.* **1992**, *35*, 3584.
14. Cho, K. H.; An, S. J.; Lee, W. S.; Paik, Y. K.; Jeong, T. S. *Biochem. Biophys. Res. Commun.* **2003**, *309*, 864.
15. (a) Jeong, T. S.; Kim, S. U.; Son, K. H.; Kwon, B. M.; Kim, Y. K.; Choi, M. U.; Bok, S. H. *J. Antibiot.* **1995**, *48*, 751; (b) Lee, C. H.; Jeong, T. S.; Choi, Y. K.; Hyun, B. W.; Oh, G. T.; Kim, E. H.; Kim, J. R.; Han, J. I.; Bok, S. H. *Biochem. Biophys. Res. Commun.* **2001**, *284*, 681.
16. ACAT activity assay: microsomal fractions of Hi5 cells containing baculovirally expressed hACAT-1 or -2 and rat liver microsomes were used as sources of the enzyme. The activity of the hACAT-1 and hACAT-2 was measured according to the method of Brecher and Chan[21] with slight modification.[15] The reaction mixture, containing 4 μL of microsomes (8 mg/mL protein), 20 μL of 0.5 M potassium–phosphate buffer (pH 7.4) with 10 mM dithiothreitol, 15 μL of bovine serum albumin (fatty acid free, 40 mg/mL), 2 μL of cholesterol in acetone (20 μg/mL, added last),

41 μL of water, and 10 μL of test sample in a total volume of 92 μL, was preincubated for 20 min at 37 °C with brief vortexing and sonication. The reaction was initiated by the addition of 8 μL of [1-^{14}C] oleoyl-CoA solution (0.05 μCi, final concn 10 μM). After 25 min of incubation at 37 °C, the reaction was stopped by the addition of 1.0 mL of isopropanol–heptane (4:1; v/v) solution. A mixture of 0.6 mL of heptane and 0.4 mL of 0.1 M potassium–phosphate buffer (pH 7.4) with 2 mM dithiothreitol was then added to the terminated reaction mixture. The above solution was mixed and allowed to phase separation under gravity for 2 min. Cholesterol oleate was recovered in the upper heptane phase (total volume 0.9~1.0 mL). The radioactivity in 100 μL of the upper phase was measured in a 3 mL liquid scintillation viral with 3 mL of scintillation cocktail (Lipoluma, Lumac Co.) using a liquid scintillation counter (1450 Microbeta Trilux Wallac Oy, Turku, Finland). Background values were obtained by preparing heat inactivated microsomes or normal insect cell lysate microsomes, usually background value was 200–250 cpm, while 8000 cpm of ACAT reaction. The ACAT activity was expressed as a defined unit, cholesteryl oleate pmol/min/mg protein.

17. (a) Roth, B. D.; Blankley, J.; Hoefle, M. L. *J. Med. Chem.* **1992**, *35*, 1609; (b) Kim, Y. K.; Tomoda, H.; Nishida, H. *J. Antibiot.* **1994**, *47*, 154.
18. Bassaganya-riera, J.; Hontecillas, R.; Beitz, D. C. *Clin. Nutr.* **2002**, *21*, 451.
19. Kisun, N. L.; David, K.; Michael, W. P. *Atherosclerosis* **1994**, *108*, 19.
20. Ghafoorunissa; Vani, A.; Laxmi, R.; Sesikeran, B. *Lipids* **2002**, *37*, 1077.
21. Brecher, P.; Chan, C. T. *Biochem. Biophys. Acta* **1980**, *617*, 458.