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INHIBITION OF ACIDIC SPHINGOMYELINASE BY XANTHONE COMPOUNDS ISOLATED FROM *GARCINIA SPECIOSA*

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Sphingomyelinase is considered to be involved in the regulation of apoptosis and cell growth. In the course of our screening for acidic sphingomyelinase inhibitors we isolated three xanthone compounds, α-mangostin, cowanin, and cowanol, from the bark of *Garcinia speciosa*. These compounds competitively inhibited bovine brain-derived acidic sphingomyelinase with IC_{50} values of 14.1, 19.2, and 10.9 μM, respectively and inhibited the acidic sphingomyelinase more effectively than the neutral sphingomyelinase of bovine brain. α-Mangostin inhibited the acidic sphingomyelinase in the most selective manner. α-Mangostin was chemically modified and its structure–activity relationships are discussed.

Keywords: Sphingomyelinase; *Garcinia speciosa*; α-Mangostin; Cowanin; Cowanol

Abbreviations: TNF, tumor necrosis factor; NPD, Niemann–Pick disease; NBD-sphingomyelin, nitrobenzoxa-diazoylaminohexanoyl-sphingosylphosphocholine

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INTRODUCTION

Sphingomyelinases catalyse the breakdown of sphingomyelin to form ceramide and phosphorylcholine. Ceramide may function as a second messenger in the regulation of cell proliferation,[1,2] differentiation[1,3] and apoptosis.[4–10] There are several types of sphingomyelinases including acidic, acidic zinc-dependent, neutral magnesium-dependent, neutral magnesium-independent, and alkaline sphingomyelinases. At least three of these sphingomyelinases may be involved in the regulation of signal transduction. Firstly, acidic sphingomyelinase is mainly located in lysosomes or endosomes and requires an acidic pH environment of about pH 5. Acidic sphingomyelinase activity can be detected not only in lysosomes but also in caveolae of IL-1-treated normal human fibroblasts cells.[11] The second is the neutral sphingomyelinase located in the plasma membrane; it requires magnesium ion and a neutral pH environment of about pH 7.4. The third is the neutral sphingomyelinase located in the cytosol compartment; it requires a neutral pH environment but not magnesium ions.

It has also been reported that these sphingomyelinases can be activated by various cytokines. For example, tumor necrosis factor-α (TNF-α),[5,12,13] Fas stimulators[6–9] and ionizing radiation[14,15] activate the acidic sphingomyelinase; and TNF-α,[10,13] Fas stimulators,[6] and 1-α, 25-dihydroxyvitamin D_3,[3] activate the neutral sphingomyelinase. Adam *et al.* recently identified a small motif of 11 amino acid residues at position 309–319 in the cytoplasmic portion of the p55 TNF receptor that is necessary for activation of neutral sphingomyelinase.[16] This motif, termed NSD, was shown to specifically bind to a novel WD-repeat protein, FAN, and the TNF receptor/FAN complex activates neutral sphingomyelinase.[17] In contrast, activation of acidic sphingomyelinase is signalled by the C terminus region of the TNF receptor corresponding with the death domain.[8] This region binds an adaptor protein, TRADD, that recruits at least three proteins, TRAF2, FADD, and RIP.[18] In recent studies, neither TRAF2 nor RIP affected acidic sphingomyelinase activation but overexpression of TRADD and FADD enhanced TNF-induced acidic sphingomyelinase activation.[19] These observations suggest that neutral and acidic sphingomyelinase are activated through distinct molecular mechanisms and that acidic rather than neutral sphingomyelinase is likely to be involved in the mechanism of TNF-induced apoptosis.

Recently, definitive evidence for the critical role of acidic sphingomyelinase in initiating apoptotic signaling was provided by studies using genetic models of acidic sphingomyelinase deficiency. Santana *et al.*[15] reported that

lymphoblasts from patients with Niemann–Pick disease (NPD), an inherited deficiency of acidic sphingomyelinase, and acidic sphingomyelinase knock-out mice showed defects in the apoptotic response after irradiation. In contrast to the normal lymphoblasts, NPD-derived B-cell lines failed to respond to ionizing radiation for ceramide generation and apoptosis. These abnormalities were reversible upon restoration of acidic sphingomyelinase activity by retroviral transfer of human acidic sphingomyelinase cDNA. These results suggest that, at least in certain cases of apoptosis, acidic sphingomyelinase might be required.

However, the mechanisms of ceramide-mediated apoptosis have not been elucidated, and moreover, no effective acidic sphingomyelinase inhibitor has been discovered. So, we first set up a new sphingomyelinase assay system and then screened tropical plant extracts and microbial secondary metabolites for acidic sphingomyelinase inhibitors. As a result we isolated the inhibitors α-mangostin, cowanin, and cowanol from the plant *Garcinia speciosa*.

MATERIALS AND METHODS

Materials

N-((6-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoyl) sphingosylphosphocholine (NBD-sphingomyelin) was obtained from Molecular Probes, Inc., Eugene, OR. Specimens of the bark of *Garcinia speciosa* were collected in Thailand near Khon Kaen in February 1998. The plant was identified by Prof. T. Kowithayakorn, Faculty of Agriculture, Khon Kaen University, where a voucher specimen is maintained.

Preparation of Acidic and Neutral Sphingomyelinase Fractions

Acidic and neutral sphingomyelinases were prepared from bovine brain. After having been chopped up on ice, bovine brain (5 g) was homogenized in 3 volumes of a buffer containing 10% sucrose and 10 mM HEPES (2-[4-(2-hydroxymethyl)-1-piperazinyl] ethanesulfonic acid)-KOH at pH 7.4. The homogenate was centrifuged at 7000× *g* for 10 min at 4°C to remove nuclei, mitochondria, and lysosomes. The supernatant was then ultracentrifuged at 100,000× *g* for 90 min at 4°C, and the supernatant after ultracentrifugation was regarded as the crude preparation of acidic sphingomyelinase. The microsomal pellet was suspended in 1 ml of the above buffer and used as the neutral sphingomyelinase fraction.

Sphingomyelinase Assays

The mixture for the acidic sphingomyelinase reaction consisted of 5 µl of the enzyme preparation (80 µg protein/ml), 20 µl of 250 mM acetate buffer (pH 5) containing 1 mM EDTA, and 5 µl of the test sample dissolved in MeOH. After preincubation of the mixture for 5 min at 37°C, 5 µl of 25 µM NBD-sphingomyelin suspended in 250 mM acetate buffer (pH 5) containing 1 mM EDTA was added to it. After incubation for 30 min at 37°C, the reaction mixture was treated with 200 µl of $CHCl_3$ and MeOH (2 : 1), vortexed, and centrifuged at 4000× *g* for 5 min. The organic layer was evaporated, resuspended in 9 µl of $CHCl_3$, then spotted on a TLC plate. The plate was developed in $CHCl_3$–MeOH (5 : 2). For quantification of the product, the preparative TLC was scraped off and the product eluted from the scrapings with $CHCl_3$–MeOH (2 : 1). The acidic sphingomyelinase activity was determined by measuring the fluorescence intensity of NBD (excitation at 465 nm and emission at 530 nm). In this assay system the product formation increased linearly up to 60 min. Linear correlation was observed between 0.02–1.0 nmol of NBD-sphingomyelin. The neutral sphingomyelinase was assayed in the same way with the pellet fraction (10 µg protein/ml).

Isolation of α-Mangostin, Cowanin, and Cowanol

The $CHCl_3$ extract (1 g) from the dried bark of *Garcinia speciosa* was applied to a silica gel column, which was eluted with $CHCl_3$–MeOH (100 : 0–100 : 2). The fractions of the first active peak were collected and concentrated under reduced pressure to give a brownish material (342.2 mg) containing α-mangostin (the Rf value was 0.49 on silica gel TLC with $CHCl_3$–MeOH, 10 : 1) and cowanin (the Rf value was 0.52 on silica gel TLC with $CHCl_3$–MeOH, 10 : 1). This brownish material was then chromatographed again over a silica gel column using hexane with increasing amounts of EtOAc. The active fractions eluted with hexane–EtOAc (7 : 1–2 : 1) were collected and evaporated to dryness to give a yellow oily residue (237.0 mg). A portion (35.8 mg) of this preparation was then rechromatographed over Sephadex LH-20 with MeOH to separate two active components, α-mangostin (10.7 mg) and cowanin (11.3 mg). The fractions of the second active peak from the first column were concentrated *in vacuo* to give a yellowish material (220.9 mg) containing cowanol. A portion (20 mg) of this material was applied on Sephadex LH-20 with MeOH to give cowanol (12.1 mg). These structures were elucidated by ^{1}H- and ^{13}C-NMR and mass spectroscopy.

α-Mangostin ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 1.70, 1.78, 1.84, 1.85 (3H each, s, $CH_3 \times 4$), 3.46, 4.10 (2H each, d, $J = 7.0$ Hz, $J = 5.9$ Hz, C1′, C1″–H × 2), 3.81 (3H, s, C7–OCH_3), 5.26–5.31 (2H, complex, C2′, C2″–H), 6.30, 6.83 (1H each, s, C4, C5–H), 13.78 (1H, s, C1–OH); HR-EIMS *m/z* 410.1728 calcd. for $C_{24}H_{26}O_6$ (M^+), found *m/z* 410.1729.

Cowanin ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 1.55, 1.60, 1.77, 1.83, 1.84 (3H each, s, $CH_3 \times 5$), 2.04 (4H, complex, C4″–H × 2, C5″–H × 2), 3.46, 4.09 (2H each, d, $J = 7.0$ Hz, $J = 5.9$ Hz, C1′, C1″–H × 2), 3.80 (3H, s, C7–OCH_3), 5.03 (1H, t, $J = 5.9$ Hz, C6″–H), 5.27–5.31 (2H, complex, C2′, C2″–H), 6.29, 6.82 (1H each, s, C4, C5–H), 13.80 (1H, s, C1–OH); HR-EIMS *m/z* 478.2350 calcd. for $C_{29}H_{34}O_6$ (M^+), found *m/z* 478.2355.

Cowanol ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 1.55, 1.60, 1.79, 1.83 (3H, each, s, $CH_3 \times 4$), 2.02 (4H, complex, C4″, C5″–H × 2), 3.51, 4.09 (2H each, d, $J = 7.0$ Hz, $J = 5.9$ Hz, C1′, C1″–H × 2), 3.80 (3H, s, C7–OCH_3), 4.36 (2H, s, C3′–CH_2OH), 5.02 (1H, t, $J = 6.6$ Hz, C6″–H), 5.27, 5.47 (2H each, t, $J = 5.7$ Hz, $J = 7.3$ Hz, C2′, C2″–H × 2), 6.29, 6.81 (1H each, s, C4, C5–H), 13.86 (1H, s, C1–OH); HR-EIMS *m/z* 494.2299 calcd. for $C_{29}H_{34}O_7$ (M^+), found *m/z* 494.2304.

Catalytic Hydrogenation of α-Mangostin

A mixture of α-mangostin (1.2 mg, 0.0029 mmol) and 10% Pd/C (1.0 mg) in EtOH (1.0 ml) was stirred under a hydrogen atmosphere for 3 h. The reaction mixture was filtered through a Celite pad, washed with EtOH (2 ml × 2) and the filtrate and the washings combined. After evaporation of the solvent, the residue was purified by preparative TLC with $CHCl_3$–MeOH (10 : 1) to afford tetrahydro-α-mangostin (0.7 mg, 58%) as a yellow waxy solid. The structure was confirmed by ^{1}H-NMR and mass spectroscopy.

Tetrahydro-α-mangostin ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 0.97, 0.98, 0.99, 1.020 (3H each, s, $CH_3 \times 4$), 1.42–1.78 (6H, complex, C2′, C2″–H × 2, 3′, 3″-H), 2.66, 3.33 (2H, each, t, $J = 8.1$ Hz, $J = 8.1$ Hz, C1′, C1″–H × 2), 3.84 (3H, s, C7–OCH_3), 6.27, 6.81 (1H each, s, C4, C5–H), 13.84 (1H, s, C1–OH); HR-EIMS *m/z* 414.2048 calcd. for $C_{24}H_{30}O_6$ (M^+), found *m/z* 414.2042.

Acetylation of α-Mangostin

α-mangostin (1.1 mg, 0.0027 mmol) was dissolved in pyridine (1.0 ml) and acetic anhydride (1.0 ml) at 0°C. The reaction mixture was stirred at room temperature for 1 h. After concentration of the reaction mixture to dryness,

purification by preparative TLC with $CHCl_3$–MeOH (10 : 1) gave diacetyl-α-mangostin (1.3 mg, quant.) as a yellow powder.

Diacetyl-α-mangostin ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 1.69 (6H, s, $CH_3 \times 2$), 1.78, 1.83 (3H each, s, $CH_3 \times 2$), 2.35, 2.40 (3H each, s, C3, C6–OAc), 3.32, 4.14 (2H, d, $J = 7.0$ Hz, $J = 6.6$ Hz, C1′, C1″–H × 2), 3.78 (3H, s, C7–OCH_3), 5.15–5.20 (2H, complex, C2′, C2″–H), 6.64, 7.13 (1H each, s, C4, C5–H), 13.4 (1H, s, C1–OH); HR-EIMS *m/z* 494.1937 calcd. for $C_{28}H_{30}O_8$ (M^+), found *m/z* 494.1940.

RESULTS AND DISCUSSION

A commercially available fluorescent analogue, NBD-sphingomyelin, was used as the substrate for the assay of acidic sphingomyelinase and a crude preparation of acidic sphingomyelinase from the bovine brain was used as the enzyme. After incubation for 30 min, the mixture was extracted with $CHCl_3$–MeOH (2 : 1), and the extract was applied on TLC to separate the product from the substrate. The spot corresponding to the product was scraped off, eluted with $CHCl_3$–MeOH (2 : 1), and measured for fluorescence. Using this assay system we tested about 200 plant extracts and 1500 microbial culture filtrates.

A chloroform extract of the bark of *Garcinia speciosa* inhibited the enzyme activity, and the active principles were isolated by successive silica gel and LH-20 column chromatography. Three active compounds were purified from *Garcinia speciosa*, and their structures were elucidated by NMR and mass spectroscopy. They were found to be three related xanthone compounds: α-mangostin, cowanin, and cowanol (Figure 1). The chloroform extract (1 g) gave 10.7 mg, 11.3 mg, and 12.1 mg of α-mangostin, cowanin, and cowanol, respectively.

α-Mangostin inhibited acidic sphingomyelinase with an IC_{50} value of 14.1 μM (5.8 μg/ml), whereas its IC_{50} for neutral sphingomyelinase was greater than 73.2 μM (30 μg/ml), as shown in Figure 2. Cowanin and cowanol also inhibited acidic sphingomyelinase, with IC_{50} values of 19.2 and 10.9 μM (9.2 and 5.2 μg/ml), respectively. Cowanol also inhibited the neutral sphingomyelinase at slightly higher concentrations with an IC_{50} value of 41.5 μM (20.5 μg/ml). Lineweaver–Burk plots showed that α-mangostin, cowanin, and cowanol all inhibited acidic sphingomyelinase in a competitive manner (Figure 3).

Since α-mangostin was the most potent and specific inhibitor against acidic sphingomyelinase amongst the three compounds, we examined its

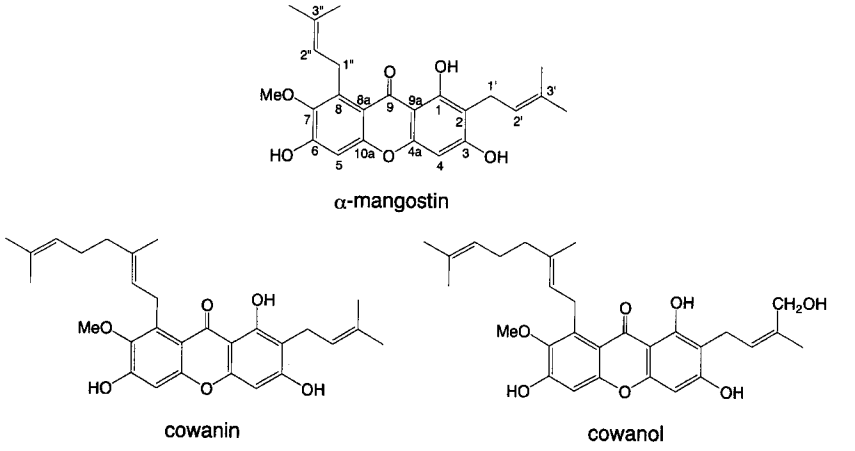

FIGURE 1 Plant-derived xanthone compounds that inhibit acidic sphingomyelinase.

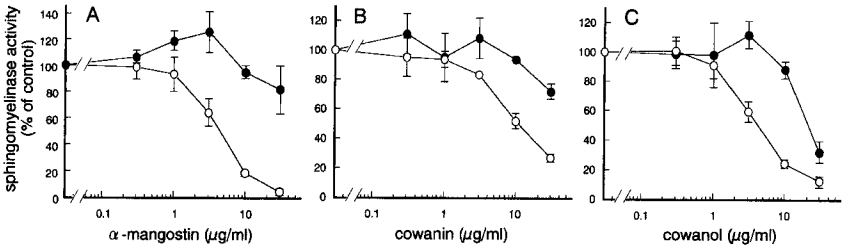

FIGURE 2 Inhibition of acidic sphingomyelinase by plant-derived xanthone compounds. Acidic (○) or neutral (●) sphingomyelinase reaction was carried out in the presence of α-mangostin (A), cowanin (B) or cowanol (C). Each sphingomyelinase activity was assayed quantitatively as described in Materials and Methods. The values are mean ± SD of triplicate determinations.

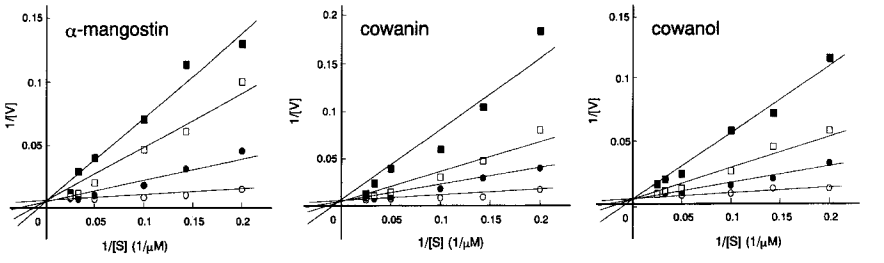

FIGURE 3 Lineweaver–Burk plots of acidic sphingomyelinase reactions with α-mangostin, cowanin, and cowanol. The enzymic reaction was carried out with 0 (○), 5 (●) or 10 (□) μg/ml of each inhibitor. (■): 20 μg/ml for α-mangostin and 30 μg/ml for cowanin or cowanol. The data are representatives of triplicate experiments.

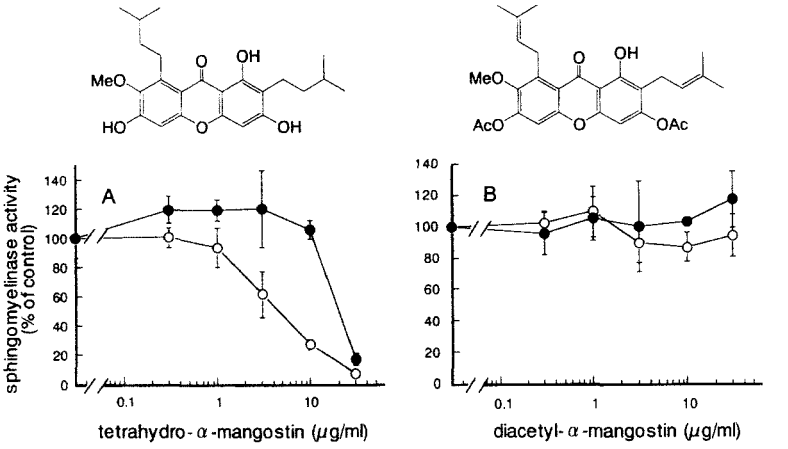

FIGURE 4 Effect of α-mangostin derivatives on sphingomyelinases. Acidic (○) or neutral (●) sphingomyelinase reaction was carried out in the presence of the (A) tetrahydro- or (B) diacetyl- derivative of α-mangostin (1). The values are mean ± SD of triplicate determinations.

structure–activity relationships. α-Mangostin was reduced by catalytic hydrogenation or acetylated with acetic anhydride and the structures of the derivatives were confirmed by NMR and mass spectroscopy. α-Mangostin has three hydroxy groups but only two of them were acetylated. This is because the third hydroxy group was stabilized by hydrogen bonding with the proximate carbonyl moiety. The hydrogenated derivative inhibited the enzyme with an IC_{50} of 10.9 μM (4.5 μg/ml), but the specificity for acid sphingomyelinase decreased. The IC_{50} value for neutral sphingomyelinase was 47.1 μM (19.5 μg/ml). On the other hand, the diacetylated derivative completely lost its inhibitory activity, as shown in Figure 4.

Neutral sphingomyelinase inhibitors of low molecular weight, such as F-11263[20] and S-12792A ~ E,[21] have been reported in the patent literature. On the other hand, tricyclic antidepressants, desipramine and imipramine, were reported to decrease acidic sphingomyelinase activity.[22,23] These drugs were used as indirect acidic sphingomyelinase inhibitors in cell culture studies.[2,24] Hurwitz *et al.* reported that in cultured human fibroblasts acidic sphingomyelinase is synthesized as a propolypeptide with a molecular mass of 75 kDa, yielding the next precursor form of 72 kDa after proteolytic cleavage which is then further processed to the lysosomal mature form of 70 kDa. The 70-kDa protein is concomitantly converted into another lysosomal form of 50 kDa.[25] Further, they reported that the decrease in the acidic sphingomyelinase activity in desipramine-treated cells was due to proteases

that degraded the 70-kDa and presumably the 50-kDa acidic sphingomyelinase.[26] Thus, no inhibitor of acidic sphingomyelinase had been previously demonstrated until we showed that α-mangostin, cowanin and cowanol were competitive inhibitors of acidic sphingomyelinase in the present study.

Acetylation of the two hydroxy groups of α-mangostin completely diminished the inhibitory activity against both acidic and neutral sphingomyelinase. As a competitive inhibitor, α-mangostin has a biphasic structure with hydrophilic and hydrophobic portions as does the substrate sphingomyelin. Acetylation would modify the biphasic structure to influence the inhibitory activity. On the other hand, the olefinic bonds of the isoprene units present in α-mangostin are not essential for the inhibitory activity, although they may contribute to the selective inhibition of acidic sphingomyelinase.

α-Mangostin was first isolated by Schmidt in 1865.[27] Several biological activities of α-mangostin have been reported. α-Mangostin is a competitive antagonist of the histamine H1 receptor in smooth muscle cells.[28] Tosa *et al.* reported that α-mangostin inhibited topoisomerase I (IC_{50}: 55 μg/ml) and II (IC_{50}: 38 μg/ml) in *in vitro* experiments.[29] α-Mangostin was also tested for its antibacterial activity. It exhibited moderate activity against *Trichophyton mentagrophytes* and *Microsporum gypseum*.[30] α-Mangostin also showed antibacterial activity against *Helicobacter pylori* and it has been suggested that α-mangostin would be useful for treatment of *Helicobacter pylori*-associated upper digestive tract disorders, including peptic ulcer, gastritis, and gastric carcinomas.[31]

Shankarayan *et al.* reported that α-mangostin produced pronounced anti-inflammatory activity by either intraperitoneal or oral administration to adrenalectomized rats as tested by carrageenin-induced hind paw edema, cotton pellet implantation, and granuloma pouch techniques.[32] Moreover, Williams *et al.* reported that α-mangostin inhibited the oxidative modification of human low-density lipoprotein (LDL) that may play an important role in atherosclerosis. They concluded that α-mangostin acts as a free radical scavenger to protect LDL from oxidative damage in their *in vitro* system.[33] It is also possible that inhibition of acidic sphingomyelinase might be involved in the mechanism of these anti-inflammatory activities of α-mangostin.

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