



Article

Structural Requirements of Alkylglyceryl-L-Ascorbic Acid Derivatives for Melanogenesis Inhibitory Activity

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Abstract: L-Ascorbic acid has multifunctional benefits on skin aesthetics, including inhibition of melanin production, and is widely used in cosmetics. It, however, has low stability and poor skin penetration. We hypothesize that alkylglyceryl-L-ascorbic acid derivatives, highly stable vitamin C-alkylglycerol conjugates, would have similar anti-melanogenic activity with better stability and penetration. We test 28 alkylglyceryl-L-ascorbic acid derivatives (1–28) on theophylline-stimulated B16 melanoma 4A5 cells to determine if they inhibit melanogenesis and establish any structure–function relationships. Although not the most potent inhibitors, 3-O-(2,3-dihydroxypropyl)-2-O-hexyl-L-ascorbic acid (**6**, IC₅₀ = 81.4 μM) and 2-O-(2,3-dihydroxypropyl)-3-O-hexyl-L-ascorbic acid (**20**, IC₅₀ = 117 μM) are deemed the best candidate derivatives based on their inhibitory activities and low toxicities. These derivatives are also found to be more stable than L-ascorbic acid and to have favorable characteristics for skin penetration. The following structural requirements for inhibitory activity of alkylglyceryl-L-ascorbic acid derivatives are also determined: (i) alkylation of glyceryl-L-ascorbic acid is essential for inhibitory activity; (ii) the 3-O-alkyl-derivatives (**2–14**) exhibit stronger inhibitory activity than the corresponding 2-O-alkyl-derivatives (**16–28**); and (iii) derivatives with longer alkyl chains have stronger inhibitory activities. Mechanistically, our studies suggest that L-ascorbic acid derivatives exert their effects by suppressing the mRNA expression of tyrosinase and tyrosine-related protein-1.

Keywords: alkylglyceryl-L-ascorbic acid; melanogenesis inhibitor; 3-O-(2,3-dihydroxypropyl)-2-O-hexyl-L-ascorbic acid; 2-O-(2,3-dihydroxypropyl)-3-O-hexyl-L-ascorbic acid; structural requirement; mechanism of action

1. Introduction

Melanin is a broad term for a group of natural pigments found in bacteria, fungi, plants, and animals. It is a heterogeneous, polyphenol-like biopolymer with a complex structure, and its color varies from yellow to black [1–5]. The color of mammalian skin and hair is determined by several factors, the most important one being the degree and distribution of melanin pigmentation [3–6]. Melanin is produced in the skin and hair [7,8]. Its role is to protect the skin from UV damage by absorbing UV light and removing reactive oxygen species [9,10]. However, excess production of melanin due to prolonged exposure to sunlight causes dermatologic disorders such as melasma, freckles, post-inflammatory melanoderma, and solar lentigines [9,11–13]. Melanin is secreted

from melanocytes distributed in the basal layer of the dermis. Melanocytes are known to be stimulated by various factors including UV radiation [14], POMC-derived α -melanocyte-stimulating hormone (α -MSH), and other neuropeptides [7,15–17], and phosphodiesterase inhibitors, such as theophylline [18]. Stimulation by these factors increases melanin production using L-tyrosine and L-3,4-dihydroxyphenylalanine (L-DOPA) as substrates through various mechanisms of action [7,19]. In addition to being substrates, L-tyrosine and L-DOPA act as bioregulatory agents [19].

In our previous investigation of compounds from several natural resources possessing melanogenesis inhibitory activity, we reported that dimeric pyrrolidinoinidoline- [20], aporphine- [21,22], benzyloquinoline- [22], and phenanthridine-type [23] alkaloids, as well as phenylethanoid glycosides [23], methoxyflavones [24], phenylpropanoids [25], neolignans [25], and diterpenes [26,27] exhibited significant positive effects against theophylline-stimulated melanogenesis in B16 melanoma 4A5 cells. As a continuing study on melanogenesis inhibitors from naturally occurring compounds and their related analogs, we focus on L-ascorbic acid (AsA), one of the most recognized sugar acids, and its highly stable derivatives. AsA has multifunctional benefits on parameters affecting skin aesthetics, such as the reduction in oxidative stress and increase in collagen production. Among them, the prevention and improvement in skin pigmentation by AsA and its derivatives are, collectively, one of the most important benefits for people [28–36]. However, it is well known that AsA is unstable in formulations, and has a low ability to penetrate the skin due to its hydrophilicity. In addition, existing water-soluble AsA derivatives, which were developed to improve its stability [30,32], have low skin penetration. To limit these disadvantages, we recently synthesized several alkylglyceryl-AsA derivatives (1–28) by introducing a glycerol group and an alkyl group to the 2,3-enediol positions in AsA [37]. In the present study, we examine the inhibitory effects of these amphiphilic AsA derivatives (1–28) on melanogenesis in theophylline-stimulated murine B16 melanoma 4A5 cells.

2. Results and Discussion

2.1. Syntheses of Alkylglyceryl AsA Derivatives (1–28)

As shown in Figure 1, a variety of alkylglyceryl-AsA derivatives were synthesized as described previously [37]: 3-O-(2,3-dihydroxypropyl)-AsA (1), 3-O-(2,3-dihydroxypropyl)-2-O-ethyl-AsA (2), 3-O-(2,3-dihydroxypropyl)-2-O-propyl-AsA (3), 2-O-butyl-3-O-(2,3-dihydroxypropyl)-AsA (4), 3-O-(2,3-dihydroxypropyl)-2-O-pentyl-AsA (5), 3-O-(2,3-dihydroxypropyl)-2-O-hexyl-AsA (6), 3-O-(2,3-dihydroxypropyl)-2-O-heptyl-AsA (7), 3-O-(2,3-dihydroxypropyl)-2-O-octyl-AsA (8), 3-O-(2,3-dihydroxypropyl)-2-O-nonyl-AsA (9), 2-O-decyl-3-O-(2,3-dihydroxypropyl)-AsA (10), 3-O-(2,3-dihydroxypropyl)-2-O-undecyl-AsA (11), 3-O-(2,3-dihydroxypropyl)-2-O-dodecyl-AsA (12), 3-O-(2,3-dihydroxypropyl)-2-O-tridecyl-AsA (13), 3-O-(2,3-dihydroxypropyl)-2-O-tetradecyl-AsA (14), 2-O-(2,3-dihydroxypropyl)-AsA (15), 2-O-(2,3-dihydroxypropyl)-3-O-ethyl-AsA (16), 2-O-(2,3-dihydroxypropyl)-3-O-propyl-AsA (17), 3-O-butyl-2-O-(2,3-dihydroxypropyl)-AsA (18), 2-O-(2,3-dihydroxypropyl)-3-O-pentyl-AsA (19), 2-O-(2,3-dihydroxypropyl)-3-O-hexyl-AsA (20), 2-O-(2,3-dihydroxypropyl)-3-O-heptyl-AsA (21), 2-O-(2,3-dihydroxypropyl)-3-O-octyl-AsA (22), 2-O-(2,3-dihydroxypropyl)-3-O-nonyl-AsA (23), 3-O-decyl-2-O-(2,3-dihydroxypropyl)-AsA (24), 2-O-(2,3-dihydroxypropyl)-3-O-undecyl-AsA (25), 2-O-(2,3-dihydroxypropyl)-3-O-dodecyl-AsA (26), 2-O-(2,3-dihydroxypropyl)-3-O-tridecyl-AsA (27), and 2-O-(2,3-dihydroxypropyl)-3-O-tetradecyl-AsA (28).

Considering general applications for cosmetics, these AsA derivatives (2–14 and 16–28) were designed to have both a hydrophilic glyceryl moiety and lipophilic alkyl chain to obtain high skin permeation and high stability in aqueous solution. Bos and Meinardi suggested that the development of new innovative compounds should be restricted to a molecular weight of less than 500 Dalton when topical dermatological therapy, percutaneous systemic therapy, or vaccination is the objective for pharmaceutical development purposes [38]. Therefore, we designed the AsA derivatives with molecular weights from 278 (2 and 16) to 446 (14 and 28) in accordance with their proposed “500 Dalton

rule". As plotted in Figure S1, excellent correlation was observed between $\text{clog } P$ values and the length of the alkyl chains in these AsA derivatives (2–14 and 16–28) ($R = 0.9998$).

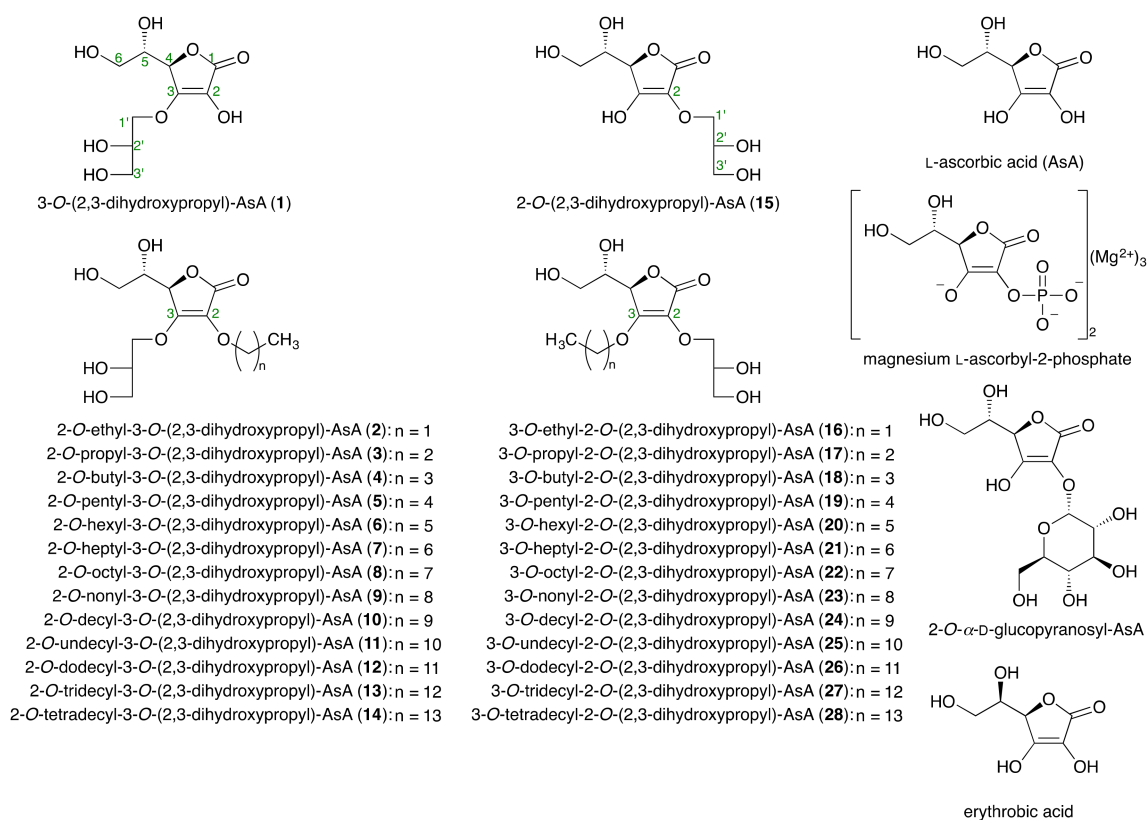


Figure 1. Structures of AsA derivatives (1–28). AsA: L-ascorbic acid.

2.2. Effects of the Alkylglyceryl AsA Derivatives (1–28) and Commercially Available AsA Derivatives on Theophylline-Stimulated Melanogenesis Inhibitory Activity

Since AsA and existing AsA derivatives are used in cosmetic materials as skin whitening and/or brightening agents [39,40], we compared their effects with those of the alkylglyceryl-AsA derivatives (1–28) on theophylline-stimulated melanogenesis in B16 melanoma 4A5 cells. As shown in Table 1, the commercially available derivatives, magnesium L-ascorbyl-2-phosphate and 2-O- α -D-glucopyranosyl-AsA, were found to significantly inhibit melanogenesis at a concentration of 3000 μM . However, these derivatives were weaker than a commercially used tyrosinase inhibitor, arbutin ($\text{IC}_{50} = 830 \mu\text{M}$) [40,41]. Among a series of 2-O-alkyl-3-O-glyceryl-AsA compounds (1–14), the derivatives having 6- to 14-carbon alkyl chains (6–14) exhibited the strongest inhibitory activities ($\text{IC}_{50} = 11.1\text{--}89.1 \mu\text{M}$). Likewise, for the 3-O-alkyl-2-O-glyceryl-AsA compounds (15–28), inhibitory activities were observed for derivatives with 4- to 14-carbon alkyl chains (18–28, $\text{IC}_{50} = 5.0\text{--}473 \mu\text{M}$). The derivative 2-O-(2,3-dihydroxypropyl)-3-O-tetradecyl-AsA (28, $\text{IC}_{50} = 5.0 \mu\text{M}$) exhibited the most potent inhibitory effect without notable cytotoxicity at effective concentrations. This melanogenesis inhibitory activity was found to be equivalent to that of the active metabolite of arbutin ($\text{IC}_{50} = 830 \mu\text{M}$), hydroquinone [40] ($\text{IC}_{50} = 8.7 \mu\text{M}$), and compound 28 was more than 166-fold more potent than arbutin (Figure 2). Plotting the alkyl chain length vs. the IC_{50} further revealed that the alkyl chain length is directly proportional to the inhibitory activity of the derivatives (Figure 3). Specifically, the potency of the derivative increased with increasing alkyl chain length. Compounds with longer alkyl chains (compounds 9–14 and 24–28), however, were also found to be cytotoxic at concentration ranges similar to those required for their melanogenesis inhibitory activities. Considering the need for both safety and effectiveness for cosmetic use, the derivatives having a hexyl chain, such as

3-*O*-(2,3-dihydroxypropyl)-2-*O*-hexyl-AsA (**6**) and 2-*O*-(2,3-dihydroxypropyl)-3-*O*-hexyl-AsA (**20**), were considered to be the most valuable candidates for study due to their low toxicity and relatively high potency. Compound **6** at 100 μ M significantly suppressed the inhibition of melanin production as compared with the control in normal melanocytes (Figure 4). Accordingly, for other compounds, it is necessary to determine whether the same trend is observed even in normal melanocytes.

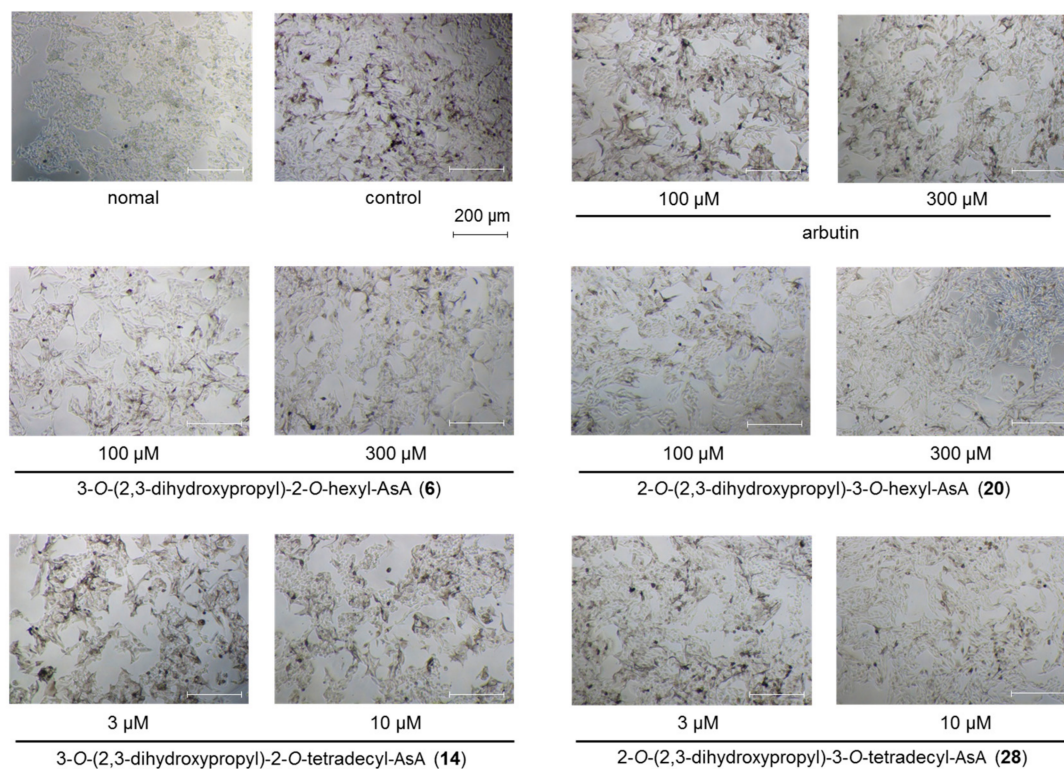


Figure 2. Theophylline-stimulated B16 melanoma 4A5 cells 72 h after treatment with **6** (100 μ M, 300 μ M), **20** (100 μ M, 300 μ M), **14** (3 μ M, 10 μ M), or **28** (3 μ M, 10 μ M). The images are representative of several experiments. normal: theophylline(-); control: theophylline(+).

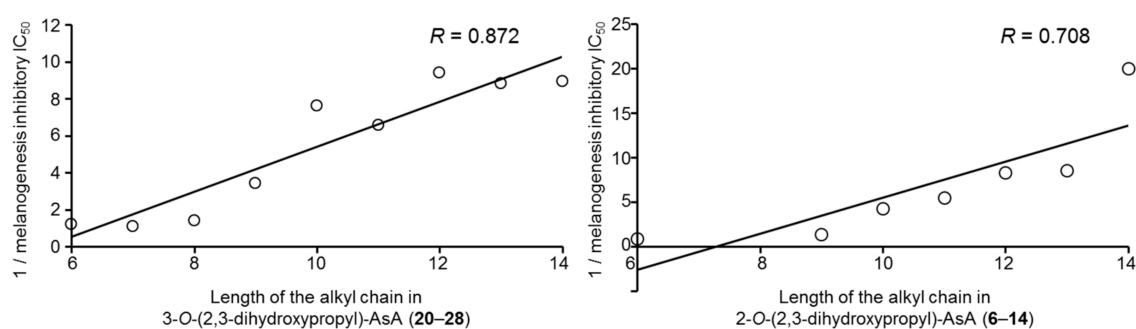


Figure 3. Correlation between melanogenesis inhibitory activity [$1/IC_{50}$ values (μ M)] and length of the alkyl chain in 2-*O*-alkyl-3-*O*-(2,3-dihydroxypropyl)-AsA compounds (**6–14**) and 3-*O*-alkyl-2-*O*-(2,3-dihydroxypropyl)-AsA compounds (**20–28**). AsA: L-ascorbic acid.

Table 1. Inhibitory effects of alkylglyceryl AsA (1–28) and commercially available AsA derivatives on theophylline-stimulated melanogenesis and viability of B16 4A5 cells.

Treatment	Inhibition (%)					IC ₅₀ (μ M)
	0 μ M	100 μ M	300 μ M	1000 μ M	3000 μ M	
3-O-(2,3-Dihydroxypropyl)-AsA (1)	0.0 \pm 4.1 (100.0 \pm 6.4)	−11.8 \pm 1.9 (100.0 \pm 1.0)	−10.7 \pm 1.1 (96.8 \pm 2.3)	−5.0 \pm 2.9 (100.4 \pm 0.4)	0.5 \pm 2.5 (107.9 \pm 1.0)	>3000
3-O-(2,3-Dihydroxypropyl)-2-O-ethyl-AsA (2)	0.0 \pm 5.9 (100.0 \pm 3.3)	−28.1 \pm 3.2 (101.3 \pm 3.3)	−2.8 \pm 3.6 (99.7 \pm 1.8)	−8.9 \pm 2.0 (101.3 \pm 1.5)	40.4 \pm 8.6 ** (103.8 \pm 4.1)	>3000
3-O-(2,3-Dihydroxypropyl)-2-O-propyl-AsA (3)	0.0 \pm 9.2 (100.0 \pm 1.3)	−2.1 \pm 5.3 (101.6 \pm 1.9)	8.2 \pm 3.2 (98.2 \pm 1.7)	2.5 \pm 7.9 (94.5 \pm 2.0)	35.9 \pm 3.4 * (95.0 \pm 1.5)	>3000
2-O-Butyl-3-O-(2,3-dihydroxy-propyl)-AsA (4)	0.0 \pm 6.0 (100.0 \pm 2.1)	−1.2 \pm 0.8 (94.5 \pm 2.3)	20.8 \pm 10.6 (97.4 \pm 0.8)	30.2 \pm 5.1 ** (93.9 \pm 0.8)	58.9 \pm 2.7 ** (88.7 \pm 1.1)	2220
3-O-(2,3-Dihydroxypropyl)-2-O-pentyl-AsA (5)	0.0 \pm 11.4 (100.0 \pm 2.1)	−14.0 \pm 9.7 (118.1 \pm 2.9)	29.8 \pm 8.1 (107.3 \pm 2.3)	45.9 \pm 1.2 ** (105.1 \pm 1.2)	81.9 \pm 3.3 ** (78.7 \pm 1.1 #)	931
2-O-(2,3-Dihydroxypropyl)-AsA (15)	0.0 \pm 4.4 (100.0 \pm 8.5)	−9.7 \pm 1.2 (101.2 \pm 2.2)	−4.8 \pm 1.0 (102.0 \pm 3.6)	−2.3 \pm 1.7 (101.6 \pm 3.2)	−6.1 \pm 2.7 (106.7 \pm 1.8)	>3000
2-O-(2,3-Dihydroxypropyl)-3-O-ethyl-AsA (16)	0.0 \pm 3.0 (100.0 \pm 6.2)	−4.2 \pm 2.3 (104.9 \pm 1.4)	−5.4 \pm 1.5 (95.9 \pm 1.2)	12.1 \pm 2.2 ** (95.9 \pm 3.1)	42.0 \pm 1.4 ** (84.0 \pm 1.1)	>3000
2-O-(2,3-Dihydroxypropyl)-3-O-propyl-AsA (17)	0.0 \pm 7.4 (100.0 \pm 4.3)	−5.9 \pm 3.3 (98.4 \pm 4.7)	−3.9 \pm 4.1 (95.6 \pm 3.5)	1.3 \pm 4.2 (92.0 \pm 2.3)	23.2 \pm 5.6 ** (82.9 \pm 2.3)	>3000
3-O-Butyl-2-O-(2,3-dihydroxy-propyl)-AsA (18)	0.0 \pm 2.7 (100.0 \pm 1.0)	0.2 \pm 4.4 (99.8 \pm 1.1)	36.3 \pm 2.8 ** (89.1 \pm 1.6)	68.0 \pm 2.1 ** (78.1 \pm 0.5 #)	84.1 \pm 1.4 ** (70.6 \pm 1.0 #)	473
2-O-(2,3-Dihydroxypropyl)-3-O-pentyl-AsA (19)	0.0 \pm 5.2 (100.0 \pm 0.4)	14.0 \pm 4.4 * (86.7 \pm 1.5)	53.0 \pm 1.1 ** (73.4 \pm 0.7 #)	83.1 \pm 1.4 ** (54.7 \pm 0.8 #)	97.1 \pm 0.9 ** (22.5 \pm 0.5 #)	283
2-O-(2,3-Dihydroxypropyl)-3-O-hexyl-AsA (20)	0.0 \pm 2.9 (100.0 \pm 1.0)	43.5 \pm 2.6 ** (95.3 \pm 0.6)	77.0 \pm 2.1 ** (83.9 \pm 0.4)	94.9 \pm 1.5 ** (60.8 \pm 0.6 #)	80.9 \pm 4.1 ** (44.7 \pm 1.9 #)	117
AsA	0.0 \pm 1.7 (100.0 \pm 2.2)	—	—	−17.4 \pm 4.0 (103.8 \pm 0.8)	3.8 \pm 4.7 (89.2 \pm 0.8)	>3000
Magnesium L-ascorbyl-2-phosphate	0.0 \pm 5.5 (100.0 \pm 0.5)	—	—	2.1 \pm 4.1 (125.6 \pm 3.6)	14.5 \pm 1.3 * (92.8 \pm 3.0)	>3000
2-O- α -D-Glucopyranosyl-AsA	0.0 \pm 3.0 (100.0 \pm 4.3)	—	—	−8.9 \pm 2.7 (106.8 \pm 2.2)	15.0 \pm 3.5 * (108.4 \pm 5.4)	>3000
Erythrobic acid	0.0 \pm 6.7 (100.0 \pm 5.7)	−22.9 \pm 6.5 (98.7 \pm 4.3)	−10.6 \pm 4.8 (94.8 \pm 3.6)	75.8 \pm 4.2 ** (44.5 \pm 2.0 #)	92.6 \pm 15.3 ** (29.8 \pm 2.2 #)	—
Arbutin	0.0 \pm 10.0 (100.0 \pm 3.7)	32.2 \pm 3.4 ** (92.2 \pm 0.6)	22.3 \pm 4.3 ** (96.0 \pm 2.0)	63.0 \pm 2.3 ** (96.2 \pm 2.7)	94.0 \pm 2.8 ** (105.8 \pm 2.9)	830

Table 1. Cont.

Treatment	Inhibition (%)					IC ₅₀ (μ M)
	0 μ M	3 μ M	10 μ M	30 μ M	100 μ M	
3-O-(2,3-Dihydroxypropyl)-2-O-hexyl-AsA (6)	0.0 \pm 5.7 (100.0 \pm 6.0)	-1.8 \pm 4.9 (102.1 \pm 4.9 [#])	5.6 \pm 2.0 (96.1 \pm 4.9)	26.2 \pm 6.0 ** (88.4 \pm 6.6)	53.1 \pm 3.1 ** (77.1 \pm 5.8 [#])	81.4
3-O-(2,3-Dihydroxypropyl)-2-O-heptyl-AsA (7)	0.0 \pm 9.5 (100.0 \pm 0.7)	-4.2 \pm 6.3 (100.6 \pm 3.0)	3.4 \pm 5.2 (95.4 \pm 3.6)	20.0 \pm 6.4 * (91.9 \pm 4.7)	52.2 \pm 5.0 ** (79.7 \pm 2.9 [#])	89.1
3-O-(2,3-Dihydroxypropyl)-2-O-octyl-AsA (8)	0.0 \pm 8.3 (100.0 \pm 5.1)	5.7 \pm 6.9 (108.4 \pm 6.2)	10.6 \pm 4.6 * (102.8 \pm 4.8)	22.4 \pm 4.4 ** (92.5 \pm 4.6)	64.1 \pm 8.2 ** (80.0 \pm 4.4)	68.8
3-O-(2,3-Dihydroxypropyl)-2-O-nonyl-AsA (9)	0.0 \pm 5.9 (100.0 \pm 2.3)	0.2 \pm 4.4 (99.2 \pm 0.5)	19.1 \pm 1.3 ** (88.9 \pm 4.2)	51.9 \pm 4.6 ** (77.6 \pm 3.6 [#])	91.6 \pm 1.2 ** (58.4 \pm 2.3 [#])	28.8
2-O-Decyl-3-O-(2,3-dihydroxy-propyl)-AsA (10)	0.0 \pm 6.7 (100.0 \pm 4.3)	3.7 \pm 7.8 (98.6 \pm 4.3)	39.2 \pm 4.1 ** (85.9 \pm 2.3)	78.1 \pm 4.8 ** (73.5 \pm 5.8 [#])	98.0 \pm 3.7 ** (27.9 \pm 2.8 [#])	13.0
3-O-(2,3-Dihydroxypropyl)-2-O-undecyl-AsA (11)	0.0 \pm 2.9 (100.0 \pm 4.8)	12.4 \pm 3.0 ** (106.2 \pm 2.5)	35.4 \pm 2.8 ** (90.8 \pm 7.1)	89.3 \pm 1.5 ** (69.0 \pm 1.9 [#])	100.3 \pm 4.7 ** (25.8 \pm 1.9 [#])	15.1
3-O-(2,3-Dihydroxypropyl)-2-O-dodecyl-AsA (12)	0.0 \pm 5.3 (100.0 \pm 7.3)	20.4 \pm 10.2 * (112.1 \pm 5.8)	50.0 \pm 5.7 ** (103.8 \pm 1.2)	96.0 \pm 2.9 ** (73.3 \pm 2.1 [#])	97.9 \pm 4.2 ** (24.6 \pm 3.4 [#])	10.6
3-O-(2,3-Dihydroxypropyl)-2-O-tridecyl-AsA (13)	0.0 \pm 5.2 (100.0 \pm 2.7)	0.1 \pm 8.6 (96.3 \pm 3.5)	46.0 \pm 7.9 ** (87.2 \pm 1.8)	97.1 \pm 2.3 ** (63.7 \pm 1.1 [#])	107.8 \pm 9.1 ** (23.5 \pm 2.1 [#])	11.3
3-O-(2,3-Dihydroxypropyl)-2-O-tetradecyl-AsA (14)	0.0 \pm 7.1 (100.0 \pm 2.3)	6.3 \pm 2.6 (101.2 \pm 1.8)	48.4 \pm 2.2 ** (89.1 \pm 4.5)	97.6 \pm 1.8 ** (56.3 \pm 3.2 [#])	100.0 \pm 18.2 ** (21.0 \pm 2.4 [#])	11.1
2-O-(2,3-Dihydroxypropyl)-3-O-heptyl-AsA (21)	0.0 \pm 2.8 (100.0 \pm 7.1)	-6.3 \pm 2.7 * (97.9 \pm 0.9)	0.9 \pm 8.2 (92.7 \pm 3.7)	21.0 \pm 5.0 ** (92.7 \pm 4.2)	44.6 \pm 6.2 ** (86.3 \pm 2.7)	>100
2-O-(2,3-Dihydroxypropyl)-3-O-octyl-AsA (22)	0.0 \pm 8.8 (100.0 \pm 2.0)	-10.4 \pm 6.0 (98.8 \pm 3.4)	1.0 \pm 7.0 (99.3 \pm 5.2)	2.3 \pm 5.9 (91.6 \pm 4.0)	34.8 \pm 8.2 ** (83.0 \pm 4.9)	>100
2-O-(2,3-Dihydroxypropyl)-3-O-nonyl-AsA (23)	0.0 \pm 14.8 (100.0 \pm 2.4)	-1.3 \pm 9.4 (96.6 \pm 5.5)	-2.6 \pm 8.0 (90.6 \pm 2.2)	14.9 \pm 5.6 (81.2 \pm 4.7)	77.1 \pm 2.4 ** (60.7 \pm 3.2 [#])	72.9
3-O-Decyl-2-O-(2,3-dihydroxy-propyl)-AsA (24)	0.0 \pm 5.0 (100.0 \pm 3.2)	-5.8 \pm 6.0 (92.5 \pm 5.7)	20.9 \pm 5.4 ** (87.1 \pm 1.6)	64.3 \pm 3.8 ** (68.5 \pm 2.6 [#])	102.7 \pm 2.4 ** (34.3 \pm 1.5 [#])	23.5
2-O-(2,3-Dihydroxypropyl)-3-O-undecyl-AsA (25)	0.0 \pm 2.0 (100.0 \pm 4.8)	-5.1 \pm 2.3 * (101.0 \pm 2.9)	26.6 \pm 3.0 ** (85.8 \pm 3.5)	85.0 \pm 3.8 ** (53.6 \pm 2.7 [#])	100.8 \pm 10.1 ** (24.0 \pm 0.5 [#])	18.1
2-O-(2,3-Dihydroxypropyl)-3-O-dodecyl-AsA (26)	0.0 \pm 7.9 (100.0 \pm 7.2)	14.1 \pm 8.5 * (99.8 \pm 4.2)	46.0 \pm 3.0 ** (87.3 \pm 2.8)	96.2 \pm 2.8 ** (45.5 \pm 1.1 [#])	89.6 \pm 24.4 ** (24.3 \pm 12.7 [#])	12.1
2-O-(2,3-Dihydroxypropyl)-3-O-tridecyl-AsA (27)	0.0 \pm 8.4 (100.0 \pm 5.5)	20.1 \pm 5.7 ** (104.9 \pm 4.1)	44.2 \pm 3.9 ** (84.3 \pm 2.8)	96.6 \pm 3.0 ** (40.2 \pm 2.5 [#])	112.9 \pm 10.1 ** (21.0 \pm 1.5 [#])	11.7
2-O-(2,3-Dihydroxypropyl)-3-O-tetradecyl-AsA (28)	0.0 \pm 7.6 (100.0 \pm 1.3)	32.8 \pm 7.2 ** (85.1 \pm 4.6)	75.7 \pm 3.9 ** (62.6 \pm 1.6 [#])	95.9 \pm 8.4 ** (23.0 \pm 2.1 [#])	95.2 \pm 20.7 ** (18.1 \pm 1.9 [#])	5.0
Hydroquinone	0.0 \pm 4.4 (100.0 \pm 1.6)	37.4 \pm 3.7 ** (94.1 \pm 1.7)	59.5 \pm 3.7 ** (85.2 \pm 1.4)	76.3 \pm 2.1 ** (64.3 \pm 0.8 [#])	—	8.7

Each value represents the mean \pm S.D. ($n = 4$); asterisks denote significant differences from the control group, * $p < 0.05$, ** $p < 0.01$; # cytotoxic effects were observed, and values in parentheses indicate cell viability (%). —: not measured; AsA: L-ascorbic acid.

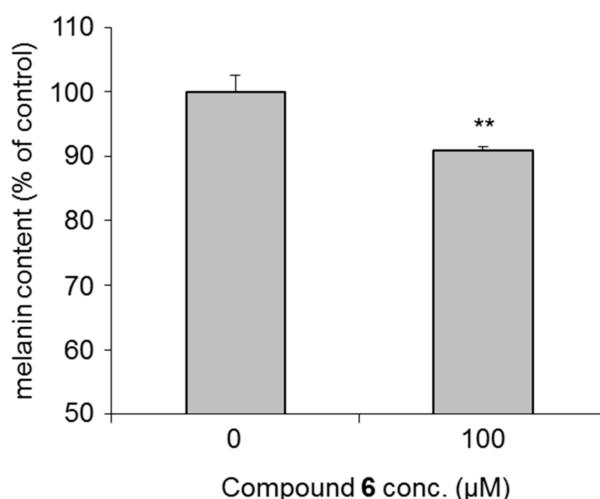


Figure 4. Inhibitory effect of 3-*O*-(2,3-dihydroxypropyl)-2-*O*-hexyl-AsA (**6**) on melanogenesis by normal melanocytes. Each value represents the mean \pm S.D. ($n = 3$); asterisks denote significant differences from the control group, ** $p < 0.01$.

2.3. Stability in Aqueous Solution

We examined the stability of 3-*O*-(2,3-dihydroxypropyl)-2-*O*-hexyl-AsA (**6**) and 2-*O*-(2,3-dihydroxypropyl)-3-*O*-hexyl-AsA (**20**) in aqueous solution at elevated temperature. As presented in Figure 5, residual AsA was found to decrease rapidly with only 9% remaining after 3 h. However, more than 80% of **6** (85%) and **20** (82%) remained after 3 h of treatment.

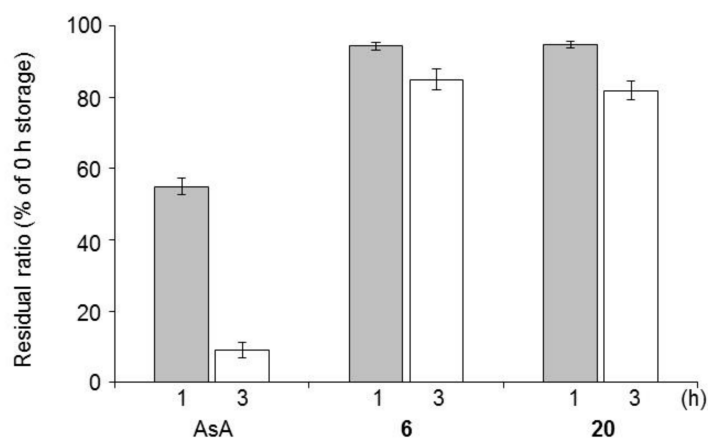


Figure 5. Residual ratio of **6**, **20**, and AsA in aqueous solution (20 mg/mL at 125 °C in the dark) after 1 h (gray bars) and 3 h (white bars). Each value represents the mean \pm S.D. ($n = 3$); AsA: L-ascorbic acid.

2.4. Effects on Tyrosinase

Tyrosinase, a copper-containing enzyme, plays a key role in melanin biosynthesis, which is involved in determining the color of skin and hair [42]. It catalyzes the oxidation of both L-tyrosine to L-DOPA, and L-DOPA to dopaquinone. Dopaquinone then undergoes a chain of oxidative polymerizations to yield melanin. Tyrosinase inhibitors are clinically used for the treatment of several dermatological disorders associated with melanin hyperpigmentation [43,44]. The tyrosinase inhibitor kojic acid is commonly used as an additive in cosmetics for skin whitening and/or depigmentation [10,45]. AsA reduces *o*-quinones to *o*-diphenols. Thus, AsA inhibits melanin production by reducing back quinone structures, such as L-DOPAquinone, to L-DOPA [45–48].

Therefore, to characterize the mode of action of melanogenesis inhibitory activity of the derivatives (6–14 and 20–28), their effects on mushroom tyrosinase enzymatic activity were examined. As shown in Table 2, none of the AsA derivatives showed inhibitory activity when using either L-tyrosine or L-DOPA as substrates. In addition, in mammalian tyrosinase testing, compound 6 did not show activity when using L-DOPA as substrates [49], and this result was similar to that observed in the mushroom tyrosinase test (Figure 6). This suggests that tyrosinase inhibition is not involved in the mechanisms of action of these melanogenesis inhibitors.

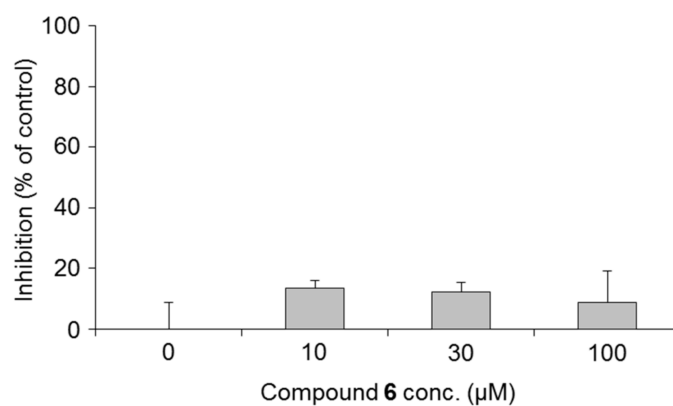


Figure 6. Effects of 3-*O*-(2,3-dihydroxypropyl)-2-*O*-hexyl-AsA (6) on the activity of mammalian tyrosinase. Each value represents the mean \pm S.D. ($n = 3$).

Table 2. Effects on activity of tyrosinase from mushroom.

Treatment	Inhibition (%)					
	Substrate: L-Tyrosine			Substrate: L-DOPA		
	0 μ M	30 μ M	100 μ M	0 μ M	30 μ M	100 μ M
3-O-(2,3-Dihydroxypropyl)-2-O-hexyl-AsA (6)	0.0 \pm 0.4	-1.5 \pm 6.7	0.9 \pm 1.4	0.0 \pm 6.3	-1.5 \pm 1.5	-1.0 \pm 0.7
3-O-(2,3-Dihydroxypropyl)-2-O-heptyl-AsA (7)	0.0 \pm 0.4	-1.7 \pm 4.0	1.0 \pm 2.4	0.0 \pm 6.3	-0.7 \pm 3.1	1.1 \pm 4.8
3-O-(2,3-Dihydroxypropyl)-2-O-octyl-AsA (8)	0.0 \pm 0.4	-0.3 \pm 2.3	-0.6 \pm 4.3	0.0 \pm 2.4	2.4 \pm 1.1	2.3 \pm 2.7
3-O-(2,3-Dihydroxypropyl)-2-O-nonyl-AsA (9)	0.0 \pm 0.4	4.0 \pm 6.1	-1.2 \pm 4.5	0.0 \pm 2.4	4.8 \pm 6.6	0.9 \pm 6.0
2-O-(2,3-Dihydroxypropyl)-3-O-hexyl-AsA (20)	0.0 \pm 10.7	1.4 \pm 4.7	-6.1 \pm 0.6	0.0 \pm 8.7	-5.7 \pm 5.1	-2.4 \pm 4.2
2-O-(2,3-Dihydroxypropyl)-3-O-heptyl-AsA (21)	0.0 \pm 10.7	-9.2 \pm 2.1	-9.8 \pm 3.9	0.0 \pm 8.7	-10.0 \pm 1.7	-11.3 \pm 2.4
2-O-(2,3-Dihydroxypropyl)-3-O-octyl-AsA (22)	0.0 \pm 10.7	-11.5 \pm 1.6	-14.4 \pm 1.8	0.0 \pm 11.2	-13.1 \pm 3.3	-4.5 \pm 2.0
2-O-(2,3-Dihydroxypropyl)-3-O-nonyl-AsA (23)	0.0 \pm 7.0	-5.0 \pm 3.1	-2.6 \pm 2.4	0.0 \pm 2.2	-4.1 \pm 2.2	-1.5 \pm 2.2
	0 μ M	10 μ M	30 μ M	0 μ M	10 μ M	30 μ M
2-O-Decyl-3-O-(2,3-dihydroxy-propyl)-AsA (10)	0.0 \pm 0.4	1.3 \pm 3.1	0.4 \pm 1.7	0.0 \pm 2.4	0.4 \pm 1.1	2.4 \pm 2.9
3-O-(2,3-Dihydroxypropyl)-2-O-undecyl-AsA (11)	0.0 \pm 0.4	2.9 \pm 3.9	1.0 \pm 2.5	0.0 \pm 3.2	-4.6 \pm 1.4	-5.1 \pm 2.2
3-O-(2,3-Dihydroxypropyl)-2-O-dodecyl-AsA (12)	0.0 \pm 6.7	-0.7 \pm 4.5	-7.1 \pm 1.4	0.0 \pm 3.2	-3.1 \pm 3.3	-4.6 \pm 2.2
3-O-(2,3-Dihydroxypropyl)-2-O-tridecyl-AsA (13)	0.0 \pm 6.7	-4.7 \pm 2.9	-7.1 \pm 1.7	0.0 \pm 3.2	-4.7 \pm 5.0	-4.4 \pm 1.8
3-O-(2,3-Dihydroxypropyl)-2-O-tetradecyl-AsA (14)	0.0 \pm 6.7	-7.0 \pm 6.7	-9.7 \pm 2.7	0.0 \pm 13.8	-8.9 \pm 3.5	-8.2 \pm 4.3
3-O-Decyl-2-O-(2,3-dihydroxy-propyl)-AsA (24)	0.0 \pm 7.0	-4.1 \pm 5.3	-3.1 \pm 4.1	0.0 \pm 4.2	0.7 \pm 3.8	-5.3 \pm 4.2
2-O-(2,3-Dihydroxypropyl)-3-O-undecyl-AsA (25)	0.0 \pm 7.0	-8.0 \pm 0.6	-8.7 \pm 1.5	0.0 \pm 4.2	-10.2 \pm 2.3	-10.7 \pm 3.8
2-O-(2,3-Dihydroxypropyl)-3-O-dodecyl-AsA (26)	0.0 \pm 7.0	-2.2 \pm 6.4	-0.5 \pm 4.3	0.0 \pm 3.0	-6.4 \pm 3.1	-6.6 \pm 8.7
2-O-(2,3-Dihydroxypropyl)-3-O-tridecyl-AsA (27)	0.0 \pm 7.0	-3.3 \pm 8.1	-5.4 \pm 1.9	0.0 \pm 3.0	-2.0 \pm 3.3	-3.2 \pm 2.7
2-O-(2,3-Dihydroxypropyl)-3-O-tetradecyl-AsA (28)	0.0 \pm 7.0	-4.4 \pm 4.6	-8.4 \pm 1.0	0.0 \pm 3.0	-5.7 \pm 9.5	-8.1 \pm 5.2
	Substrate: L-Tyrosine			Inhibition (%)		
Treatment	0 μ M	10 μ M	30 μ M	100 μ M	300 μ M	IC ₅₀ (μ M)
Kojic acid [20,22–27]	0.0 \pm 2.4	12.2 \pm 3.3	46.4 \pm 2.6 **	66.5 \pm 2.1 **	96.8 \pm 0.9 **	43.6
	Substrate: L-DOPA			Inhibition (%)		
Treatment	0 μ M	10 μ M	30 μ M	100 μ M	300 μ M	IC ₅₀ (μ M)
Kojic acid [20,22–27]	0.0 \pm 0.9	22.3 \pm 2.1 **	50.6 \pm 0.6 **	78.2 \pm 0.7 **	89.3 \pm 0.3 **	29.6

Each value represents the mean \pm S.D. ($n = 4$); asterisks denote significant differences from the control group, ** $p < 0.01$. AsA: L-ascorbic acid.

2.5. Effects on Expression of Tyrosinase, TRP-1, and TRP-2 mRNA

The TRP enzyme family (tyrosinase, TRP-1, and TRP-2) catalyzes the major steps in melanin synthesis [50]. To clarify the mechanisms of action of the derivatives, we examined the effects of **6** and **20** on the expression of tyrosinase, TRP-1, and TRP-2 mRNAs in B16 melanoma 4A5 cells. As presented in Table 3, both **6** and **20** significantly downregulated the mRNA expression of tyrosinase and TRP-1 at 100 μM ; **14** and **28** significantly downregulated the mRNA expression of tyrosinase and TRP-2 at 10 μM .

Table 3. Effects of **6**, **14**, **20**, and **28** on expression of tyrosinase, TRP-1, and TRP-2 mRNA in B16 4A5 cells.

Treatment	Tyrosinase mRNA/ β -actin mRNA		
	0 μM	30 μM	100 μM
3-O-(2,3-Dihydroxypropyl)-2-O-hexyl-AsA (6)	1.00 \pm 0.05	0.60 \pm 0.07 **	0.42 \pm 0.03 **
2-O-(2,3-Dihydroxypropyl)-3-O-hexyl-AsA (20)	1.00 \pm 0.22	0.72 \pm 0.10	0.59 \pm 0.07 *
Treatment	TRP-1 mRNA/ β -actin mRNA		
	0 μM	30 μM	100 μM
3-O-(2,3-Dihydroxypropyl)-2-O-hexyl-AsA (6)	1.00 \pm 0.21	0.48 \pm 0.15 *	0.37 \pm 0.05 **
2-O-(2,3-Dihydroxypropyl)-3-O-hexyl-AsA (20)	1.00 \pm 0.21	0.67 \pm 0.12	0.50 \pm 0.15 *
Treatment	TRP-2 mRNA/ β -actin mRNA		
	0 μM	30 μM	100 μM
3-O-(2,3-Dihydroxypropyl)-2-O-hexyl-AsA (6)	1.00 \pm 0.32	0.53 \pm 0.18	0.70 \pm 0.06
2-O-(2,3-Dihydroxypropyl)-3-O-hexyl-AsA (20)	1.00 \pm 0.10	1.07 \pm 0.30	0.88 \pm 0.25
Treatment	Tyrosinase mRNA/ β -actin mRNA		
	0 μM	3 μM	10 μM
3-O-(2,3-Dihydroxypropyl)-2-O-tetradecyl-L-ascorbic acid (14)	1.00 \pm 0.12	0.78 \pm 0.10	0.58 \pm 0.09 **
2-O-(2,3-Dihydroxypropyl)-3-O-tetradecyl-L-ascorbic acid (28)	1.00 \pm 0.12	0.54 \pm 0.03 **	0.29 \pm 0.08 **
Treatment	TRP-1 mRNA/ β -actin mRNA		
	0 μM	3 μM	10 μM
3-O-(2,3-Dihydroxypropyl)-2-O-tetradecyl-L-ascorbic acid (14)	1.00 \pm 0.24	1.02 \pm 0.27	0.92 \pm 0.22
2-O-(2,3-Dihydroxypropyl)-3-O-tetradecyl-L-ascorbic acid (28)	1.00 \pm 0.24	0.88 \pm 0.20	0.83 \pm 0.27
Treatment	TRP-2 mRNA/ β -actin mRNA		
	0 μM	3 μM	10 μM
3-O-(2,3-Dihydroxypropyl)-2-O-tetradecyl-L-ascorbic acid (14)	1.00 \pm 0.11	0.58 \pm 0.06 **	0.50 \pm 0.08 **
2-O-(2,3-Dihydroxypropyl)-3-O-tetradecyl-L-ascorbic acid (28)	1.00 \pm 0.11	0.43 \pm 0.11 **	0.35 \pm 0.05 **

Each value represents the mean \pm S.D. ($n = 3$); asterisks denote significant differences from the control group, * $p < 0.05$, ** $p < 0.01$. AsA: L-ascorbic acid.

2.6. Effects on Expression of Tyrosinase Protein

We next examined the effects of **6** and **20** on the expression of tyrosinase protein since it is the rate-limiting enzyme in melanin synthesis [51]. As presented in Figure 7, both **6** and **20** suppressed tyrosinase protein expression in a concentration-dependent manner. Compound **6** decreased tyrosinase activity in cultured cells when using L-DOPA as substrates (Figure 8). This suggests that tyrosinase activity in cultured cells is decreased via suppression of the expression of tyrosinase.

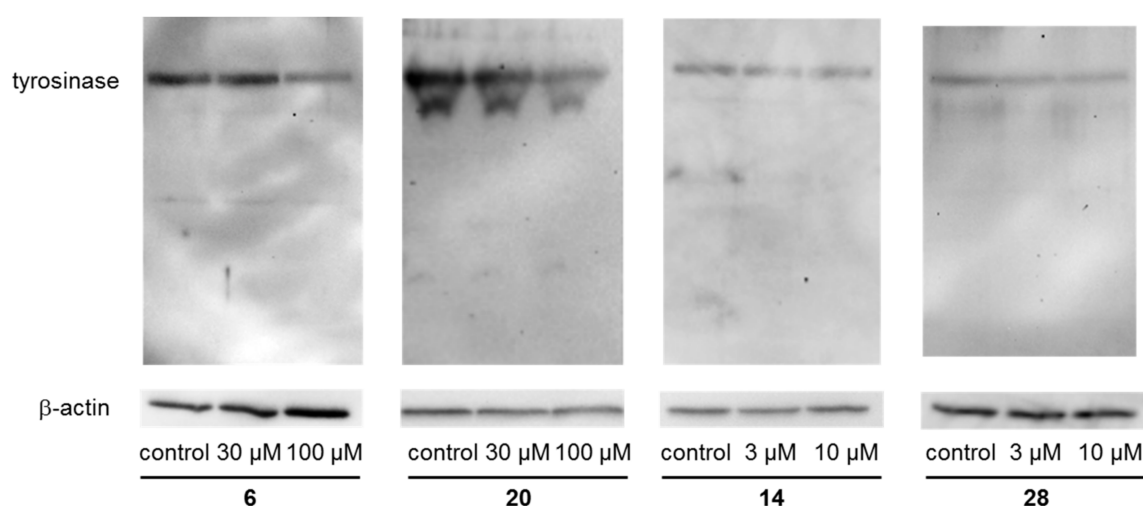


Figure 7. Effects of 6, 14, 20 and 28 on the expression of tyrosinase protein in B16 4A5 cells. The images are representative of several experiments.

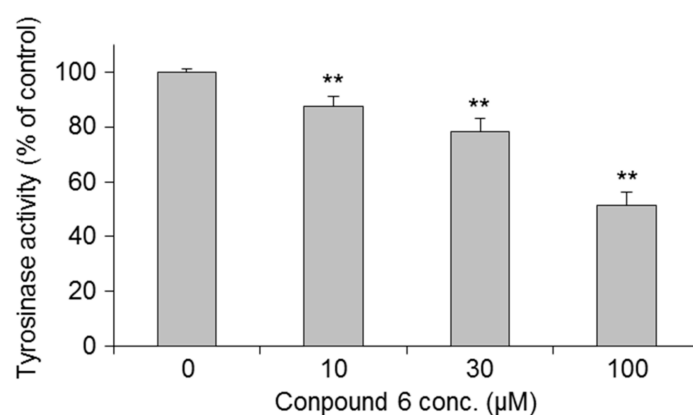


Figure 8. Effects of 3-*O*-(2,3-dihydroxypropyl)-2-*O*-hexyl-AsA (6) on tyrosinase activity in cultured cells. Each value represents the mean \pm S.D ($n = 3$); asterisks denote significant differences from the control group, ** $p < 0.01$.

3. Materials and Methods

3.1. General Experimental Procedures

The following instruments were used to obtain physical data: melting points, Yanagimoto micromelting point apparatus (Yanaco New Science Inc., Kyoto, Japan); specific rotations, JASCO P-2200 digital polarimeter (JASCO Corporation, Tokyo, Japan, $l = 5$ cm); UV spectra, UV-1600 spectrometer (Shimadzu Co., Kyoto, Japan); IR spectra, IRPrestige-21 spectrometer (Shimadzu Co.); high-resolution electrospray ionization mass spectrometry (HRESIMS), Exactive Plus mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA); $^1\text{H-NMR}$ spectra, JNM-ECA600 (600 MHz), JNM-ECA500 (500 MHz), and JNM-ECS400 (400 MHz) spectrometers (JEOL Ltd., Tokyo, Japan); $^{13}\text{C-NMR}$ spectra, JNM-ECA600 (150 MHz), JNM-ECA500 (125 MHz), and JNM-ECS400 (100 MHz) spectrometers (JEOL Ltd.) with tetramethylsilane as an internal standard; and HPLC detector, SPD-M20A PDA detector (Shimadzu Co.); HPLC column, Cadenza CD-C18 (Imtakt Co., Kyoto, Japan). The following experimental conditions were used for chromatography: ordinary-phase silica gel column chromatography, silica gel 60N (Kanto Chemical Co., Tokyo, Japan; 63–210 mesh, spherical, neutral); and normal-phase TLC, pre-coated TLC plates with silica

gel 60F₂₅₄ (Merck, Darmstadt, Germany; 0.25 mm), detection was carried out by spraying 2% H₃[PMo₁₂O₄₀]-nH₂O–5% aqueous H₂SO₄ on the plates, followed by heating. All chemicals were reagent grade, and were purchased from Wako Pure Chemical Industries, Ltd., Tokyo, Japan or Nacal Tesque Inc., Kyoto, Japan.

3.2. Syntheses of Alkylglyceryl Ascorbic Acid Derivatives

The alkylglyceryl AsA derivatives (1–28) were synthesized by following general procedure: a solution of **1:15** DMSO to alkyl bromide was stirred at 100 °C for 3 h. The reaction mixture was diluted with water, and extracted with EtOAc. The organic layer was washed with brine (saturated sodium chloride in water), dried, and evaporated. The residue was purified by silica gel chromatography with CHCl₃/MeOH/H₂O as the mobile phase. The detailed experimental procedures were described previously [37].

3-O-(2,3-dihydroxypropyl)-AsA (**1**): An amorphous powder; $[\alpha]_D^{26} + 15.4$ (*c* 3.70, MeOH); UV [MeOH, nm (log ϵ): 244 (3.32); IR (TlBr) ν_{\max} cm⁻¹: 3275, 1759, 1693, 1335, 1045; ¹H-NMR (600 MHz, CD₃OD): δ 3.59 (2H, m, H₂-3'), 3.66 (2H, m, H₂-6), 3.89 (1H, m, H-5), 3.92 (1H, m, H-2'), [4.45 (dd, *J* = 6.5, 11.0 Hz)/4.49 (dd, *J* = 6.5, 11.0 Hz), 4.59 (dd, *J* = 3.8, 11.0 Hz)/4.62 (dd, *J* = 3.8, 11.0 Hz), H₂-1'], 4.82 (1H, d, *J* = 1.7 Hz, H-4); ¹³C-NMR (150 MHz, CD₃OD): δ_C 63.4 (t, C-6), 63.7 (t, C-3'), 70.56/70.61 (d, C-5), 71.79/71.89 (d, C-2'), 73.4/73.6 (t, C-1'), 76.9 (d, C-4), 121.17/121.24 (s, C-2), 151.84/151.88 (s, C-3), 173.04/173.07 (s, C-1); HRESIMS *m/z*: 273.0577 [M + Na]⁺ (calcd for C₉H₁₄O₈Na, 273.0581).

3-O-(2,3-dihydroxypropyl)-2-O-ethyl-AsA (**2**): An amorphous powder; $[\alpha]_D^{26} + 37.0$ (*c* 0.32, MeOH); UV [MeOH, nm (log ϵ): 236 (3.91); IR (ATR) ν_{\max} cm⁻¹: 3316, 2934, 2889, 1748, 1667, 1321, 1169, 1111, 1026; ¹H-NMR (400 MHz, CD₃OD): δ 1.31 (3H, t, *J* = 7.4 Hz, H₃-2''), 3.60 (2H, brd, *J* = 5.5 Hz, H₂-3'), 3.65 (2H, dd-like, *J* = 1.8, 5.5 Hz, H₂-6), 3.90 (2H, m, H-5, 2'), 4.09 (2H, m, H₂-1''), [4.47/4.48 (1H, dd, *J* = 6.4, 10.5 Hz), 4.59/4.60 (1H, dd, *J* = 3.6, 10.5 Hz), H₂-1'], 4.88 (1H, brs, H-4); ¹³C-NMR (100 MHz, CD₃OD): δ_C 15.5 (q, C-2''), 63.2 (t, C-6), 63.5 (t, C-3'), 69.1 (t, C-1''), 70.46/70.50 (d, C-5), 71.49/71.55 (d, C-2'), 73.97/74.05 (t, C-1'), 76.7 (d, C-4), 123.87/123.91 (s, C-2), 159.53/159.58 (s, C-3), 172.3 (s, C-1); HRESIMS *m/z*: 301.0883 [M + Na]⁺ (calcd for C₁₁H₁₈O₈Na, 301.0894).

3-O-(2,3-dihydroxypropyl)-2-O-propyl-AsA (**3**): An amorphous powder; $[\alpha]_D^{26} + 41.7$ (*c* 0.33, MeOH); UV [MeOH, nm (log ϵ): 235 (3.94); IR (ATR) ν_{\max} cm⁻¹: 3325, 2940, 2880, 1748, 1669, 1323, 1165, 1113, 1040; ¹H-NMR (400 MHz, CD₃OD): δ 0.99 (3H, t, *J* = 7.4 Hz, H₃-3''), 1.72 (2H, m, H₂-2''), 3.60 (2H, brd, *J* = 6.0 Hz, H₂-3'), 3.65 (2H, dd-like, *J* = 1.4, 6.4 Hz, H₂-6), 3.91 (2H, m, H-5, 2'), 3.99 (2H, m, H-1''), [4.47/4.48 (1H, dd, *J* = 6.4, 10.5 Hz), 4.58/4.59 (1H, dd, *J* = 4.1, 11.0 Hz), H₂-1'], 4.87 (1H, brs, H-4); ¹³C-NMR (100 MHz, CD₃OD): δ_C 10.7 (q, C-3''), 24.0 (t, C-2''), 63.2 (t, C-6), 63.5 (t, C-3'), 69.1 (t, C-1''), 70.49/70.53 (d, C-5), 71.5/71.6 (d, C-2'), 74.0/74.1 (t, C-1'), 75.55/75.58 (t, C-1'), 76.7 (d, C-4), 123.24/123.26 (s, C-2), 159.27/159.30 (s, C-3), 172.3 (s, C-1); HRESIMS *m/z*: 315.1046 [M + Na]⁺ (calcd for C₁₂H₂₀O₈Na, 315.1050).

2-O-butyl-3-O-(2,3-dihydroxypropyl)-AsA (**4**): An amorphous powder; $[\alpha]_D^{26} + 47.4$ (*c* 0.34, MeOH); UV [MeOH, nm (log ϵ): 236 (3.93); IR (TlBr) ν_{\max} cm⁻¹: 3393, 1749, 1674, 1331, 1167, 1117, 1049; ¹H-NMR (500 MHz, CD₃OD): δ 0.96 (3H, t, *J* = 7.5 Hz, H₃-4''), 1.45 (2H, m, H₂-3''), 1.68 (2H, m, H₂-2''), 3.60 (2H, brd, *J* = 5.8 Hz, H₂-3'), 3.65 (2H, m, H₂-6), 3.90 (1H, m, H-5), 3.91 (1H, m, H-8), 4.03 (1H, m, H-1''), [4.47 (dd, *J* = 6.0, 10.6 Hz)/4.48 (dd, *J* = 6.3, 10.7 Hz), 4.57 (dd, *J* = 3.8, 10.7 Hz)/4.59 (dd, *J* = 4.0, 10.6 Hz), H₂-1'], [4.858 (d, *J* = 1.5 Hz)/4.859 (d, *J* = 1.5 Hz), H-4]; ¹³C-NMR (125 MHz, CD₃OD): δ_C 14.1 (q, C-4''), 20.1 (t, C-3''), 32.9 (t, C-2''), 63.2 (t, C-6), 63.56/63.59 (t, C-3'), 70.5/70.6 (d, C-5), 71.5/71.6 (d, C-2'), 73.69/73.72 (d, C-1'), 74.0/74.1 (t, C-1''), 76.7 (d, C-4), 123.28/123.30 (s, C-2), 159.23/159.25 (s, C-3), 172.2 (s, C-1); HRESIMS *m/z*: 329.1203 [M + Na]⁺ (calcd for C₁₃H₂₂O₈Na, 329.1207).

3-O-(2,3-dihydroxypropyl)-2-O-pentyl-AsA (**5**): An amorphous powder; $[\alpha]_D^{26} + 40.9$ (*c* 0.32, MeOH); UV [MeOH, nm (log ϵ): 235 (3.98); IR (ATR) ν_{\max} cm⁻¹: 3304, 2934, 2874, 1750, 1670, 1321, 1165, 1115, 1032; ¹H-NMR (400 MHz, CD₃OD): δ 0.93 (3H, t, *J* = 6.8 Hz, H₃-5''), 1.39 (4H, m, H₂-3'', 4''), 1.70 (2H,

m, H₂-2''), 3.60 (2H, brd, *J* = 5.5 Hz, H₂-3'), 3.65 (2H, dd-like, *J* = 1.4, 6.4 Hz, H₂-6), 3.91 (2H, m, H-5, 2'), 4.02 (1H, m, H-1''), [4.47/4.48 (1H, dd, *J* = 6.4, 10.6 Hz, *J* = 6.0, 10.6 Hz), 4.58/4.59 (1H, dd, *J* = 4.1, 10.6 Hz), H₂-1'], 4.86 (1H, brs, H-4); ¹³C-NMR (100 MHz, CD₃OD): δ_C 14.4 (q, C-5''), 23.5 (t, C-4''), 29.1 (t, C-3''), 30.5 (t, C-2''), 63.2 (t, C-6), 63.5/63.6 (t, C-3'), 70.48/70.52 (d, C-5), 71.5/71.6 (d, C-2'), 74.0/74.1 (t, C-1', 1''), 76.7 (d, C-4), 123.2/123.3 (s, C-2), 159.2/159.3 (s, C-3), 172.3 (s, C-1); HRESIMS *m/z*: 343.1357 [M + Na]⁺ (calcd for C₁₄H₂₄O₈Na, 343.1363).

3-*O*-(2,3-dihydroxypropyl)-2-*O*-hexyl-AsA (6): An amorphous powder; [α]_D²⁶ + 42.2 (*c* 0.32, MeOH); UV [MeOH, nm (log ε)]: 236 (3.90); IR (TlBr) *v*_{max} cm⁻¹: 3379, 2934, 1751, 1674, 1330, 1167, 1117, 1051; ¹H-NMR (400 MHz, CD₃OD): δ 0.91 (3H, t, *J* = 6.9 Hz, H₃-6''), 1.33 (4H, m, H₂-4'', 5''), 1.41 (2H, m, H₂-3''), 1.70 (2H, m, H₂-2''), 3.60 (2H, brd, *J* = 5.5 Hz, H₂-3'), 3.65 (2H, dd-like, H₂-6), 3.90 (2H, m, H-5, 2'), 4.03 (2H, m, H-1''), [4.47/4.48 (1H, dd, *J* = 6.4, 10.6 Hz/*J* = 6.0, 10.6 Hz), 4.57/4.59 (1H, dd, *J* = 4.1, 10.7 Hz), H₂-1'], 4.86 (1H, brs, H-4); ¹³C-NMR (100 MHz, CD₃OD): δ_C 14.4 (q, C-6''), 23.7 (t, C-5''), 26.6 (t, C-3''), 30.7 (t, C-2''), 32.7 (t, C-4''), 63.2 (t, C-6), 63.6 (t, C-3'), 70.5 (d, C-5), 71.5/71.6 (d, C-2'), 74.01/74.04/74.09 (t, C-1', 1''), 76.7 (d, C-4), 123.2/123.3 (s, C-2), 159.2/159.3 (s, C-3), 172.3 (s, C-1); HRESIMS *m/z*: 357.1519 [M + Na]⁺ (calcd for C₁₅H₂₆O₈Na, 357.1520).

3-*O*-(2,3-dihydroxypropyl)-2-*O*-heptyl-AsA (7): An amorphous powder; [α]_D²⁶ + 29.8 (*c* 0.35, MeOH); UV [MeOH, nm (log ε)]: 236 (3.87); IR (TlBr) *v*_{max} cm⁻¹: 3389, 2932, 2507, 1751, 1674, 1331, 1169, 1119, 1051; ¹H-NMR (400 MHz, CD₃OD): δ 0.90 (3H, t, *J* = 6.4 Hz, H₃-7''), 1.34 (6H, m, H₂-4'', 5'', 6''), 1.42 (2H, m, H₂-3''), 1.70 (2H, m, H₂-2''), 3.60 (2H, brd, *J* = 5.5 Hz, H₂-3'), 3.65 (2H, dd-like, *J* = 1.8, 6.4 Hz, H₂-6), 3.90 (1H, m, H-5, 2'), 4.04 (1H, m, H₂-1''), [4.47 (1H, dd, *J* = 6.4, 10.6 Hz), 4.57/4.59 (dd, *J* = 4.1, 10.6 Hz), H₂-1'], 4.86 (1H, brd, *J* = 2.3 Hz), H-4]; ¹³C-NMR (100 MHz, CD₃OD): δ_C 14.4 (q, C-7'), 23.7 (t, C-6'), 26.9/30.2/30.8/33.0 (t, C-2', 3', 4', 5'), 63.2 (t, C-6), 63.5/63.6 (t, C-3'), 70.49/70.52 (d, C-5), 71.52/71.59 (d, C-2'), 74.0/74.1 (t, C-1', 1''), 76.7 (d, C-4), 123.2/123.3 (s, C-2), 159.2/159.3 (s, C-3), 172.3 (s, C-1); HRESIMS *m/z*: 371.1673 [M + Na]⁺ (calcd for C₁₆H₂₈O₈Na, 371.1676).

3-*O*-(2,3-dihydroxypropyl)-2-*O*-octyl-AsA (8): An amorphous powder; [α]_D²⁶ + 32.6 (*c* 0.32, MeOH); UV [MeOH, nm (log ε)]: 236 (3.91); IR (KBr) *v*_{max} cm⁻¹: 3368, 2855, 1751, 1676, 1339, 1169, 1115, 1071; ¹H-NMR (400 MHz, CD₃OD): δ 0.90 (3H, t, *J* = 6.9 Hz, H₃-8''), 1.31 (10H, m, H₂-4'', 5'', 6'', 7''), 1.42 (2H, m, H₂-3''), 1.70 (2H, m, H₂-2''), 3.59 (2H, brd, *J* = 6.0 Hz, H₂-3'), 3.65 (2H, dd-like, *J* = 1.8, 6.4 Hz, H₂-6), 3.90 (2H, m, H-5, 2'), 4.03 (1H, m, H₂-1''), [4.47/4.48 (1H, dd, *J* = 6.4, 10.6 Hz), 4.58/4.59 (1H, dd, *J* = 4.6, 10.6 Hz/*J* = 4.1, 10.6 Hz), H₂-1'], 4.86 (d, *J* = 1.8 Hz, H-4); ¹³C-NMR (100 MHz, CD₃OD): δ_C 14.5 (q, C-8''), 23.7 (t, C-7''), 27.0/30.4/30.5/30.8/33.0 (t, C-2', 3', 4', 5', 6'), 63.2 (t, C-6), 63.51/63.55 (t, C-3'), 70.49/70.52 (d, C-5), 71.52/71.59 (d, C-2'), 74.02/74.09 (t, C-1', 1''), 76.7 (d, C-4), 123.2/123.3 (s, C-2), 159.26/159.29 (s, C-3), 172.3 (s, C-1); HRESIMS *m/z*: 385.1829 [M + Na]⁺ (calcd for C₁₇H₃₀O₈Na, 385.1833).

3-*O*-(2,3-dihydroxypropyl)-2-*O*-nonyl-AsA (9): An amorphous powder; [α]_D²⁶ + 33.3 (*c* 0.31, MeOH); UV [MeOH, nm (log ε)]: 236 (3.93); IR (KBr) *v*_{max} cm⁻¹: 3327, 2924, 2853, 1761, 1684, 1333, 1169, 1117, 1046; ¹H-NMR (400 MHz, CD₃OD): δ 0.89 (3H, t, *J* = 6.8 Hz, H-9''), 1.30 (10H, m, H₂-4'', 5'', 6'', 7'', 8''), 1.42 (2H, m, H₂-3''), 1.70 (2H, m, H₂-2''), 3.60 (2H, brd, *J* = 5.5 Hz, H₂-3'), 3.65 (2H, dd-like, *J* = 1.4, 6.4 Hz, H₂-6), 3.91 (2H, m, H-5, 2'), 4.03 (1H, m, H₂-1'), [4.47/4.48 (1H, dd, *J* = 6.4, 10.6 Hz), 4.58/4.59 (1H, dd, *J* = 4.6, 10.6 Hz, *J* = 4.1, 10.6 Hz), H₂-1'], 4.86 (d, *J* = 0.9 Hz, H-4); ¹³C-NMR (100 MHz, CD₃OD); δ_C 14.5 (q, C-9''), 23.7 (t, C-8''), 27.0/30.4/30.5/30.7/30.8/33.1 (t, C-2'', 3'', 4'', 5'', 6'', 7''), 63.2 (t, C-6), 63.5/63.6 (t, C-3'), 70.48/70.52 (d, C-5), 71.5/71.6 (d, C-2'), 74.01/74.05/74.09 (t, C-1', 1''), 76.7 (d, C-4), 123.2/123.3 (s, C-2), 159.2/159.3 (s, C-3), 172.3 (s, C-1); HRESIMS *m/z*: 399.1988 [M + Na]⁺ (calcd for C₁₈H₃₂O₈Na, 399.1989).

2-*O*-decyl-3-*O*-(2,3-dihydroxypropyl)-AsA (10): An amorphous powder; [α]_D²⁶ + 31.4 (*c* 0.30, MeOH); UV [MeOH, nm (log ε)]: 236 (3.88); IR (KBr) *v*_{max} cm⁻¹: 3317, 2959, 2922, 2924, 2849, 1759, 1682, 1331, 1165, 1113, 1043; ¹H-NMR (600 MHz, CD₃OD): δ 0.89 (3H, t, *J* = 7.2 Hz, H₃-10''), 1.30 (12H, brs, H₂-4'', 5'', 6'', 7'', 8'', 9''), 1.41 (2H, m, H₂-3''), 1.69 (2H, m, H₂-2''), 3.60 (2H, brd, *J* = 5.6 Hz, H₂-3'), 3.65 (2H, m,

H₂-6), 3.90 (1H, m, H-5), 3.91 (1H, m, H-2'), 4.02 (1H, m, H₂-1''), [4.47 (dd, *J* = 6.1, 10.7 Hz)/4.48 (dd, *J* = 6.2, 10.7 Hz), 4.57 (dd, *J* = 3.8, 10.7 Hz)/4.59 (dd, *J* = 3.9, 10.7 Hz), H₂-1'], [4.860 (d, *J* = 1.1 Hz)/4.862 (d, *J* = 1.5 Hz), H-4]: ¹³C-NMR (150 MHz, CD₃OD); δ_C 14.4 (q, C-10''), 23.7 (t, C-9''), 26.9 (t, C-3''), 30.48 (t, C-2''), 30.44/30.48/30.7/30.8 (t, C-4'', 5'', 6'', 7''), 33.1 (t, C-8''), 63.2 (t, C-6), 63.5/63.6 (t, C-3'), 70.5/70.6 (d, C-5), 71.5/71.6 (d, C-2'), 74.02/74.05 (t, C-1'), 74.11 (t, C-1''), 76.7 (d, C-4), 123.26/123.29 (s, C-2), 159.24/159.27 (s, C-3), 172.2 (s, C-1); HRESIMS *m/z*: 413.2145 [M + Na]⁺ (calcd for C₁₉H₃₄O₈Na, 413.2146).

3-O-(2,3-dihydroxypropyl)-2-O-undecyl-AsA (11): An amorphous powder; [α]_D²⁶ + 34.7 (*c* 0.34, MeOH); UV [MeOH, nm (log ε)]: 236 (3.88); IR (KBr) *v*_{max} cm⁻¹: 3300, 2916, 2851, 1761, 1684, 1329, 1171, 1119, 1063, 1030; ¹H-NMR (600 MHz, CD₃OD): δ 0.89 (3H, t, *J* = 6.9 Hz, H₃-11''), 1.29 (14H, brs, H₂-4'', 5'', 6'', 7'', 8'', 9'', 10''), 1.41 (2H, m, H₂-3''), 1.70 (2H, m, H₂-2''), 3.60 (2H, brd, *J* = 5.8 Hz, H₂-3''), 3.65 (2H, m, H₂-6), 3.90 (1H, m, H-5), 3.91 (1H, m, H-2'), 4.02 (1H, m, H₂-1''), [4.47 (dd, *J* = 6.3, 10.6 Hz)/4.48 (dd, *J* = 6.3, 10.6 Hz), 4.57 (dd, *J* = 4.0, 10.6 Hz)/4.59 (dd, *J* = 4.0, 10.6 Hz), H₂-1'], [4.860 (d, *J* = 1.8 Hz)/4.861 (d, *J* = 1.8 Hz), H-4]: ¹³C-NMR (150 MHz, CD₃OD); δ_C 14.4 (q, C-11''), 23.7 (t, C-10''), 26.9 (t, C-3''), 30.70 (t, C-2''), 30.47/30.49/30.70/30.74/30.8 (t, C-4'', 5'', 6'', 7'', 8''), 33.1 (t, C-9''), 63.2 (t, C-6), 63.5/63.6 (t, C-3'), 70.5/70.6 (d, C-5), 71.5/71.6 (d, C-2'), 74.02/74.05 (t, C-1'), 74.11 (t, C-1''), 76.7 (d, C-4), 123.27/123.29 (s, C-2), 159.2/159.3 (s, C-3), 172.4 (s, C-1); HRESIMS *m/z*: 427.2299 [M + Na]⁺ (calcd for C₂₀H₃₆O₈Na, 427.2302).

3-O-(2,3-dihydroxypropyl)-2-O-dodecyl-AsA (12): An amorphous powder; [α]_D²⁶ + 38.4 (*c* 0.31, MeOH); UV [MeOH, nm (log ε)]: 236 (3.86); IR (KBr) *v*_{max} cm⁻¹: 3422, 2918, 2851, 1749, 1676, 1319, 1115, 1070; ¹H-NMR (500 MHz, CD₃OD): δ 0.89 (3H, t, *J* = 6.6 Hz, H₃-12''), 1.29 (16H, brs, H₂-4'', 5'', 6'', 7'', 8'', 9'', 10'', 11''), 1.41 (2H, m, H₂-3''), 1.70 (2H, m, H₂-2''), 3.60 (2H, brd, *J* = 5.7 Hz, H₂-3''), 3.65 (2H, m, H₂-6), 3.90 (1H, m, H-5), 3.91 (1H, m, H-2'), 4.02 (1H, m, H₂-1''), [4.47 (dd, *J* = 6.4, 10.7 Hz)/4.48 (dd, *J* = 6.4, 10.7 Hz), 4.57 (dd, *J* = 4.0, 10.7 Hz)/4.59 (dd, *J* = 4.0, 10.7 Hz), H₂-7], 4.86 (1H, m, *J* = 6.6 Hz, H-2'): ¹³C-NMR (125 MHz, CD₃OD); δ_C 14.4 (q, C-12''), 23.7 (t, C-11''), 27.0 (t, C-3''), 30.5 (t, C-4''), 30.76 (t, C-2''), 30.72/30.76/30.79/30.81 (t, C-5'', 6'', 7'', 8'', 9'', 10''), 33.1 (t, C-10''), 63.2 (t, C-6), 63.56/63.58 (t, C-3'), 70.5/70.6 (d, C-5), 71.5/71.6 (d, C-2'), 74.0 (t, C-7), 74.1 (t, C-1''), 76.7 (d, C-4), 123.27/123.29 (s, C-2), 159.2/159.3 (s, C-3), 172.2 (s, C-1); HRESIMS *m/z*: 441.2457 [M + Na]⁺ (calcd for C₂₁H₃₈O₈Na, 441.2459).

3-O-(2,3-dihydroxypropyl)-2-O-tridecyl-AsA (13): An amorphous powder; [α]_D²⁶ + 35.8 (*c* 0.30, MeOH); UV [MeOH, nm (log ε)]: 231 (4.02); IR (KBr) *v*_{max} cm⁻¹: 3289, 2916, 2849, 1761, 1684, 1329, 1119, 1171, 1119, 1063, 1030; ¹H-NMR (500 MHz, CD₃OD): δ 0.89 (3H, t, *J* = 6.9 Hz, H₃-13''), 1.28 (18H, brs, H₂-4'', 5'', 6'', 7'', 8'', 9'', 10'', 11'', 12''), 1.41 (2H, m, H₂-3''), 1.70 (2H, m, H₂-2''), 3.60 (2H, brd, *J* = 5.8 Hz, H₂-3'), 3.65 (2H, m, H₂-6), 3.90 (1H, m, H-5), 3.91 (1H, m, H-2'), 4.02 (1H, m, H₂-1''), [4.47 (dd, *J* = 6.3, 10.7 Hz)/4.48 (dd, *J* = 6.3, 10.7 Hz), 4.57 (dd, *J* = 4.0, 10.7 Hz)/4.59 (dd, *J* = 4.0, 10.7 Hz), H₂-1'], [4.859 (d, *J* = 1.8 Hz)/4.861 (d, *J* = 1.8 Hz), H-4]: ¹³C-NMR (125 MHz, CD₃OD); δ_C 14.4 (q, C-13''), 23.7 (t, C-12''), 27.0 (t, C-3''), 30.81 (t, C-2''), 30.48/30.50/30.71/30.78/30.81 (t, C-4'', 5'', 6'', 7'', 8'', 9'', 10'', 11''), 33.1 (t, C-12''), 63.2 (t, C-6), 63.56/63.58 (t, C-3'), 70.5/70.6 (d, C-5), 71.5/71.6 (d, C-2'), 74.0 (t, C-1'), 74.1 (t, C-1''), 76.7 (d, C-4), 123.27/123.29 (s, C-2), 159.2/159.3 (s, C-3), 172.2 (s, C-1); HRESIMS *m/z*: 455.2612 [M + Na]⁺ (calcd for C₂₂H₄₀O₈Na, 455.2615).

3-O-(2,3-dihydroxypropyl)-2-O-tetradecyl-AsA (14): An amorphous powder; [α]_D²⁶ + 24.4 (*c* 0.31, MeOH); UV [MeOH, nm (log ε)]: 230 (3.99); IR (KBr) *v*_{max} cm⁻¹: 3326, 2920, 2849, 1759, 1680, 1466, 1329, 1165, 1115, 1034; ¹H-NMR (500 MHz, CD₃OD): δ 0.89 (3H, t, *J* = 7.2 Hz, H₃-14''), 1.28 (20H, brs, H₂-4'', 5'', 6'', 7'', 8'', 9'', 10'', 11'', 12'', 13''), 1.41 (2H, m, H₂-3''), 1.70 (2H, m, H₂-2''), 3.60 (2H, brd, *J* = 5.8 Hz, H₂-3'), 3.65 (2H, m, H₂-6), 3.90 (1H, m, H-5), 3.91 (1H, m, H-2'), 4.02 (1H, m, H₂-1''), [4.47 (dd, *J* = 6.4, 10.7 Hz)/4.48 (dd, *J* = 6.4, 10.7 Hz), 4.57 (dd, *J* = 3.8, 10.7 Hz)/4.59 (dd, *J* = 3.8, 10.7 Hz), H₂-1'], [4.858 (d, *J* = 1.5 Hz)/4.860 (d, *J* = 1.5 Hz), H-4]: ¹³C-NMR (125 MHz, CD₃OD); δ_C 14.4 (q, C-14''), 23.7 (t, C-13''), 27.0 (t, C-3''), 30.80 (t, C-2''), 30.48/30.51/30.72/30.76/30.80 (t, C-4'', 5'', 6'', 7'', 8'', 9'', 10'', 11''),

33.1 (t, C-12''), 63.2 (t, C-6), 63.56/63.59 (t, C-3'), 70.5/70.6 (d, C-5), 71.5/71.6 (d, C-2'), 74.0 (t, C-1'), 74.1 (t, C-1''), 76.7 (d, C-4), 123.27/123.30 (s, C-2), 159.23/159.25 (s, C-3), 172.2 (s, C-1); HRESIMS m/z : 469.2769 [M + Na]⁺ (calcd for C₂₃H₄₂O₈Na, 469.2772).

2-O-(2,3-dihydroxypropyl)-AsA (**15**): Colorless needles, mp.153.0-153.2; $[\alpha]_D^{26} + 55.7$ (c 0.31, MeOH); UV [MeOH, nm (log ϵ): 238 (3.89); IR (KBr) ν_{\max} cm⁻¹: 3326, 2920, 2849, 1759, 1680, 1466, 1329, 1165, 1115, 1034; ¹H-NMR (400 MHz, CD₃OD): δ 3.61 (2H, m, H₂-3'), 3.67 (2H, m) (2H, m, H₂-6), 3.90 (1H, m, H-2'), 3.92 (1H, dt-like, $J = 1.8, 6.4$ Hz, H₃-5), [4.07 (1H, dd, $J = 4.1, 10.4$ Hz)/4.09 (1H, d, $J = 3.6, 10.4$ Hz), H-1'], 4.86 (1H, d, $J = 1.8$ Hz, H-4); ¹³C-NMR (100 MHz, CD₃OD) ; δ_C 63.3 (t, C-6), 63.7 (t, C-3'), 70.4 (d, C-5), 72.0 (d, C-2'), 74.6 (t, C-1'), 76.8 (t, C-4'), 122.2 (s, C-2), 161.6 (s, C-3), 172.9 (s, C-1); HRESIMS m/z : 273.0576 [M + Na]⁺ (calcd for C₉H₁₄O₈Na, 273.0581).

2-O-(2,3-dihydroxypropyl)-3-O-ethyl-AsA (**16**): Pale yellow oil; $[\alpha]_D^{26} + 34.3$ (c 0.33, MeOH); UV [MeOH, nm (log ϵ): 236 (3.86); IR (ATR) ν_{\max} cm⁻¹: 3337, 2938, 2881, 1744, 1665, 1325, 1173, 1109, 1038; ¹H-NMR (400 MHz, CD₃OD): δ 1.38 (3H, t, $J = 6.8$ Hz, H₃-2''), 3.58 (2H, m, H₂-3') 3.64 (2H, dd-like, $J = 1.4, 6.4$ Hz, H₂-6), 3.87 (2H, m, H-5, 2'), [3.97 (1H, m), 4.13 (1H, m), 4.68 (2H, m, H₂-1''), 4.83(d, $J = 0.9$ Hz, H-4): ¹³C-NMR (100 MHz, CD₃OD) ; δ_C 15.5 (q, C-2''), 63.3 (t, C-6), 63.98/63.48 (t, C-3'), 69.6 (t, C-1''), 70.6 (d, C-5), 72.0 (d, C-2'), 75.19/75.23 (t, C-1'), 76.7 (d, C-4), 122.60/122.62 (s, C-2), 159.7 (s, C-3), 175.6 (s, C-1); HRESIMS m/z : 301.0887 [M + Na]⁺ (calcd for C₁₁H₁₈O₈Na, 301.0894).

2-O-(2,3-dihydroxypropyl)-3-O-propyl-AsA (**17**): Pale yellow oil; $[\alpha]_D^{26} + 49.8$ (c 0.31, MeOH); UV [MeOH, nm (log ϵ): 237 (3.94); IR (ATR) ν_{\max} cm⁻¹: 3339, 2938, 2882, 1744, 1665, 1327, 1173, 1040; ¹H-NMR (400 MHz, CD₃OD): δ 1.01 (3H, t, $J = 7.3$ Hz, H₃-3''), 1.78 (2H, m, H₂-2''), 3.58 (2H, m, H₂-3'), 3.64 (2H, dd-like, $J = 0.9, 6.4$ Hz, H₂-6), 3.87 (2H, m, H-5, 2'), [3.96/3.97 (1H, dd, $J = 6.4, 10.1$ Hz), 4.11/4.13 (1H, dd, $J = 4.1, 10.1$ Hz), H₂-1'], 4.50 (2H, m, H₂-1''), 4.83(1H, d, $J = 1.4$ Hz, H-4): ¹³C-NMR (100 MHz, CD₃OD) ; δ_C 10.3 (q, C-3''), 23.9 (t, C-2''), 63.3 (t, C-6), 64.0 (t, C-3'), 70.6 (d, C-5), 72.0 (d, C-2'), 75.1 (t, C-1''), 75.19/75.23 (t, C-1'), 76.7 (d, C-4), 122.4 (s, C-2), 159.9 (s, C-3), 172.6 (s, C-1); HRESIMS m/z : 315.1042 [M + Na]⁺ (calcd for C₁₂H₂₀O₈Na, 315.1050).

3-O-butyl-2-O-(2,3-dihydroxypropyl)-AsA (**18**): Pale yellow oil; $[\alpha]_D^{26} + 46.6$ (c 0.31, MeOH); UV [MeOH, nm (log ϵ): 237 (3.89); IR (ATR) ν_{\max} cm⁻¹: 3341, 2936, 2876, 1746, 1665, 1329, 1171, 1115, 1036; ¹H-NMR (400 MHz, CD₃OD): δ 0.97 (3H, t, $J = 7.3$ Hz, H₃-4''), 1.47 (2H, m, H₂-3''), 1.74 (2H, m, H₂-2''), 3.58 (2H, m, H₂-3'), 3.64 (2H, dd-like, $J = 0.8, 6.4$ Hz, H₂-6), 3.86 (2H, m, H-5, 2'), [3.96/3.97 (1H, dd, $J = 6.4, 10.1$ Hz), 4.11/4.13 (1H, dd, $J = 4.1, 10.1$ Hz), H₂-1'], 4.55 (2H, m, H₂-1''), 4.83(1H, d, $J = 1.4$ Hz, H-4): ¹³C-NMR (100 MHz, CD₃OD) ; δ_C 14.1 (q, C-4''), 19.9 (t, C-3''), 32.7 (t, C-2''), 63.3 (t, C-6), 63.28/64.01 (t, C-3'), 70.6 (d, C-5), 71.9 (d, C-2'), 73.4 (t, C-1''), 75.2/75.3 (t, C-1'), 76.7 (d, C-4), 122.60/122.62 (s, C-2), 159.9 (s, C-3), 172.62/172.63 (s, C-1); HRESIMS m/z : 329.1200 [M + Na]⁺ (calcd for C₁₃H₂₂O₈Na, 329.1207).

2-O-(2,3-dihydroxypropyl)-3-O-pentyl-AsA (**19**): Pale yellow oil; $[\alpha]_D^{26} + 44.4$ (c 0.30, MeOH); UV [MeOH, nm (log ϵ): 236 (3.93); IR (ATR) ν_{\max} cm⁻¹: 3358, 2934, 2872, 1748, 1665, 1331, 1169, 1115, 1040; ¹H-NMR (400 MHz, CD₃OD): δ 0.94 (3H, t, $J = 6.9$ Hz, H₃-5''), 1.40 (4H, m, H₂-3, 4''), 1.76 (2H, m, H₂-2''), 3.58 (2H, m, H₂-3'), 3.64 (2H, dd-like, $J = 1.4, 6.4$ Hz, H₂-6), 3.86 (2H, m, H-5, 2'), [3.96/3.98 (1H, dd, $J = 6.4, 10.1$ Hz), 4.11/4.13 (1H, dd, $J = 4.1, 10.1$ Hz), H₂-1'], 4.54 (2H, m, H₂-1''), 4.84(1H, brs, H-4): ¹³C-NMR (100 MHz, CD₃OD) ; δ_C 14.3 (q, C-5''), 23.4 (t, C-4''), 28.9 (t, C-3''), 30.3 (t, C-2''), 63.3 (t, C-6), 63.97/64.00 (t, C-3'), 70.6 (d, C-5), 72.0 (d, C-2'), 73.7 (t, C-1''), 75.21/75.25 (t, C-1'), 76.7 (d, C-4), 122.57/122.60 (s, C-2), 159.9 (s, C-3), 172.6 (s, C-1); HRESIMS m/z : 343.1357 [M + Na]⁺ (calcd for C₁₄H₂₄O₈Na, 343.1363).

2-O-(2,3-dihydroxypropyl)-3-O-hexyl-AsA (**20**): Pale yellow oil; $[\alpha]_D^{26} + 42.5$ (c 0.35, MeOH); UV [MeOH, nm (log ϵ): 237 (3.96); IR (ATR) ν_{\max} cm⁻¹: 3367, 2930, 2859, 1748, 1667, 1331, 1167, 1117, 1041; ¹H-NMR (400 MHz, CD₃OD): δ 0.92 (3H, t-like, $J = 6.9$ Hz, H₃-6''), 1.34 (4H, m, H₂-4'', 5''), 1.44 (2H, m, H₂-3''), 1.75 (2H, m, H₂-2''), 3.57 (2H, m, H₂-3') 3.63 (2H, dd-like, $J = 1.4, 6.4$ Hz, H₂-6), 3.86 (2H, m, H-5, 2'), [3.95/3.97 (1H, dd, $J = 6.4, 10.1$ Hz), 4.10/4.12 (1H, dd, $J = 4.1, 10.1$ Hz), H₂-1'], 4.53 (2H,

m, H₂-1''), 4.84 (1H, brs, H-4): ¹³C-NMR (100 MHz, CD₃OD) ; δ_C 14.3 (q, C-6''), 23.6 (t, C-5''), 26.3 (t, C-4''), 30.6 (t, C-3''), 32.6 (t, C-2'') 63.3 (t, C-6), 64.00/64.03 (t, C-3'), 70.6 (d, C-5), 72.0 (d, C-2'), 73.7 (t, C-1''), 75.2/75.3 (t, C-1'), 76.7 (d, C-4), 122.60/122.63 (s, C-2), 159.9 (s, C-3), 172.6 (s, C-1); HRESIMS *m/z*: 357.1515 [M + Na]⁺ (calcd for C₁₅H₂₆O₈Na, 357.1520).

2-O-(2,3-dihydroxypropyl)-3-O-heptyl-AsA (**21**): Pale yellow oil; [α]_D²⁶ + 40.4 (c 0.34, MeOH); UV [MeOH, nm (log ε)]: 235 (3.91); IR (ATR) *v*_{max} cm⁻¹: 3341, 2926, 2857, 1748, 1669, 1331, 1167, 1115, 1040; ¹H-NMR (400 MHz, CD₃OD): δ 0.90 (3H, t-like, *J* = 6.9 Hz, H₃-7''), 1.36 (6H, m, H₂-4'', 5'', 6''), 1.44 (2H, m, H₂-3''), 1.75 (2H, m, H₂-2''), 3.57 (2H, m, H₂-3') 3.64 (2H, dd-like, *J* = 1.8, 6.0 Hz, H₂-6), 3.86 (2H, m, H-5, 2'), [3.94/3.97 (1H, dd, *J* = 6.4, 10.1 Hz), 4.11/4.13 (1H, dd, *J* = 4.1, 10.1 Hz), H₂-1'], 4.54 (2H, m, H₂-1''), 4.83 (1H, d, *J* = 1.8 Hz, H-4): ¹³C-NMR (100 MHz, CD₃OD) ; δ_C 14.4 (q, C-7''), 23.7 (t, C-6''), 26.7 (t, C-5''), 30.1/30.7 (t, C-3'', 4''), 32.9 (t, C-2''), 63.3 (t, C-6), 63.97/64.01 (t, C-3'), 70.6 (d, C-5), 71.9 (d, C-2'), 73.7 (t, C-1''), 75.21/75.25 (t, C-1'), 76.7 (d, C-4), 122.58/122.60 (s, C-2), 159.9 (s, C-3), 172.6 (s, C-1); HRESIMS *m/z*: 371.1674 [M + Na]⁺ (calcd for C₁₆H₂₈O₈Na, 371.1676).

2-O-(2,3-dihydroxypropyl)-3-O-octyl-AsA (**22**): Pale yellow oil; [α]_D²⁶ + 42.3 (c 0.34, MeOH); UV [MeOH, nm (log ε)]: 236 (3.92); IR (ATR) *v*_{max} cm⁻¹: 3364, 2924, 2857, 1748, 1667, 1331, 1165, 1115, 1036; ¹H-NMR (400 MHz, CD₃OD): δ 0.90 (3H, t, *J* = 6.8 Hz, H₃-8''), 1.32 (8H, m, H₂-4'', 5'', 6'', 7''), 1.42 (2H, m, H₂-3''), 1.75 (2H, m, H₂-2''), 3.58 (2H, m, H₂-3') 3.63 (2H, dd-like, *J* = 1.4, 6.4 Hz, H₂-6), 3.87 (2H, m, H-5, 2'), [3.96/3.97 (1H, dd, *J* = 6.4, 10.1 Hz), 4.11/4.12 (1H, dd, *J* = 3.9, 10.1 Hz), H₂-1'], 4.53 (2H, m, H₂-1''), 4.83 (1H, d, *J* = 1.4, H-4): ¹³C-NMR (100 MHz, CD₃OD) ; δ_C 14.4 (q, C-8''), 23.7 (t, C-7''), 26.7 (t, C-6''), 30.33/30.39 (t, C-4'', 5''), 30.6 (t, C-3''), 32.9 (t, C-2''), 63.3 (t, C-6), 63.99/64.02 (t, C-3'), 70.6 (d, C-5), 72.0 (d, C-2'), 73.7 (t, C-1''), 75.2/75.3 (t, C-1'), 76.68/76.70 (d, C-4), 122.60/122.64 (s, C-2), 159.9 (s, C-3), 172.63/172.64 (s, C-1); HRESIMS *m/z*: 385.1827 [M + Na]⁺ (calcd for C₁₇H₃₀O₈Na, 385.1833).

2-O-(2,3-dihydroxypropyl)-3-O-nonyl-AsA (**23**): Pale yellow oil; [α]_D²⁶ + 39.1 (c 0.30, MeOH); UV [MeOH, nm (log ε)]: 237 (3.92); IR (ATR) *v*_{max} cm⁻¹: 3358, 2924, 2855, 1750, 1669, 1333, 1163, 1115, 1040; ¹H-NMR (400 MHz, CD₃OD): δ 0.90 (3H, t, *J* = 7.3 Hz, H₃-9''), 1.30 (10H, brs, H₂-4'', 5'', 6'', 7'', 8''), 1.42 (2H, m, H₂-3''), 1.75 (2H, m, H₂-2''), 3.58 (2H, m, H₂-3') 3.64 (2H, dd-like, *J* = 1.4, 6.4 Hz, H₂-6), 3.86 (2H, m, H-5, 2'), [3.96/3.97 (1H, dd, *J* = 6.4, 10.1 Hz), 4.11/4.13 (1H, dd, *J* = 4.1, 10.1 Hz), H₂-1'], 4.53 (2H, m, H₂-1''), 4.83 (1H, d, *J* = 1.4, H-4): ¹³C-NMR (100 MHz, CD₃OD) ; δ_C 14.5 (q, C-9''), 23.7 (t, C-8''), 26.7 (t, C-7''), 30.4/30.5/30.6 (t, C-3'', 4'', 5'', 6''), 30.6 (t, C-3''), 33.1 (t, C-2''), 63.2 (t, C-6), 63.97/64.00 (t, C-3'), 70.6 (d, C-5), 71.9 (d, C-2'), 73.7 (t, C-1''), 75.2/75.3 (t, C-1'), 76.7 (d, C-4), 122.57/122.60 (s, C-2), 159.9 (s, C-3), 172.6 (s, C-1); HRESIMS *m/z*: 399.1984 [M + Na]⁺ (calcd for C₁₈H₃₂O₈Na, 399.1989).

3-O-decyl-2-O-(2,3-dihydroxypropyl)-AsA (**24**): Pale yellow oil; [α]_D²⁶ + 36.7 (c 0.33, MeOH); UV [MeOH, nm (log ε)]: 237 (4.02); IR (ATR) *v*_{max} cm⁻¹: 3364, 2924, 2855, 1749, 1666, 1333, 1165, 1119, 1030; ¹H-NMR (400 MHz, CD₃OD): δ 0.89 (3H, t, *J* = 6.9 Hz, H₃-10''), 1.30 (12H, brs, H₂-4'', 5'', 6'', 7'', 8'', 9''), 1.43 (2H, m, H₂-3''), 1.75 (2H, m, H₂-2''), 3.59 (2H, m, H₂-3') 3.66 (2H, dd-like, *J* = 1.4, 6.4 Hz, H₂-6), 3.86 (2H, m, H-5, 2'), [3.96/3.97 (1H, dd, *J* = 6.4, 10.1 Hz), 4.10/4.12 (1H, dd, *J* = 4.1, 10.1 Hz), H₂-1'], 4.53 (2H, m, H₂-1''), 4.83 (1H, brs, H-4): ¹³C-NMR (100 MHz, CD₃OD) ; δ_C 14.4 (q, C-10''), 23.7 (t, C-9''), 26.7 (t, C-8''), 30.4/30.7 (t, C-3'', 4'', 5'', 6'', 7''), 33.0 (t, C-2''), 63.3 (t, C-6), 64.0 (t, C-3'), 70.6 (d, C-5), 72.0 (d, C-2'), 73.7 (t, C-1''), 75.2/75.3 (t, C-1'), 76.7 (d, C-4), 122.61/122.64 (s, C-2), 159.9 (s, C-3), 172.6 (s, C-1); HRESIMS *m/z*: 413.2146 [M + Na]⁺ (calcd for C₁₉H₃₄O₈Na, 413.2146).

2-O-(2,3-dihydroxypropyl)-3-O-undecyl-AsA (**25**): Pale yellow oil; [α]_D²⁶ + 35.9 (c 0.31, MeOH); UV [MeOH, nm (log ε)]: 237 (3.95); IR (ATR) *v*_{max} cm⁻¹: 3358, 2922, 2853, 1750, 1669, 1333, 1163, 1115, 1040; ¹H-NMR (400 MHz, CD₃OD): δ 0.89 (3H, t, *J* = 6.9 Hz, H₃-11''), 1.29 (14H, brs, H₂-4'', 5'', 6'', 7'', 8'', 9'', 10''), 1.44 (2H, m, H₂-3''), 1.75 (2H, m, H₂-2''), 3.58 (2H, m, H₂-3') 3.64 (2H, dd-like, *J* = 1.4, 6.4 Hz, H₂-6), 3.86 (2H, m, H-5, 2'), [3.96/3.97 (1H, dd, *J* = 6.4, 10.1 Hz), 4.10/4.13 (1H, dd, *J* = 4.1, 10.1 Hz), H₂-1'], 4.53 (2H, m, H₂-1''), 4.83 (1H, d, *J* = 1.4 Hz, H-4): ¹³C-NMR (100 MHz, CD₃OD) ; δ_C 14.4 (q, C-11''), 23.8 (t, C-10''), 26.7 (t, C-9''), 30.46/30.48/30.69/30.73/30.77 (t, C-3'', 4'', 5'', 6'', 7'', 8''), 33.1 (t, C-2''), 63.2 (t, C-6), 64.0 (t, C-3'), 70.6 (d, C-5), 71.9 (d, C-2'), 73.7 (t, C-1''), 75.2/75.3 (t, C-1'), 76.7 (d,

C-4), 122.58/122.60 (s, C-2), 159.9 (s, C-3), 172.6 (s, C-1); HRESIMS m/z : 427.2301 [M + Na]⁺ (calcd for C₂₀H₃₆O₈Na, 427.2302).

2-O-(2,3-dihydroxypropyl)-3-O-dodecyl-AsA (26): An amorphous powder; $[\alpha]_D^{26} + 41.7$ (c 0.33, MeOH); UV [MeOH, nm (log ϵ): 237 (3.96); IR (ATR) ν_{\max} cm⁻¹: 3341, 2922, 2853, 1748, 1668, 1335, 1165, 1115, 1028; ¹H-NMR (400 MHz, CD₃OD): δ 0.89 (3H, t, $J = 6.8$ Hz, H₃-12''), 1.29 (16H, brs, H₂-4'', 5'', 6'', 7'', 8'', 9'', 10'', 11''), 1.44 (2H, m, H₂-3''), 1.75 (2H, m, H₂-2''), 3.57 (2H, m, H₂-3') 3.64 (2H, dd-like, $J = 1.8$, 6.4 Hz, H₂-6), 3.86 (2H, m, H-5, 2'), [3.96/3.97 (1H, dd, $J = 6.4$, 10.1 Hz), 4.11/4.13 (1H, dd, $J = 4.1$, 10.1 Hz), H₂-1'], 4.53 (2H, m, H₂-1''), 4.83 (1H, d, $J = 1.4$ Hz, H-4): ¹³C-NMR (100 MHz, CD₃OD); δ_C 14.4 (q, C-12''), 23.7 (t, C-11''), 26.7 (t, C-10''), 30.47/30.67/30.70/30.74/30.78 (t, C-3'', 4'', 5'', 6'', 7'', 8'', 9''), 33.1 (t, C-2''), 63.3 (t, C-6), 64.00/64.03 (t, C-3'), 70.6 (d, C-5), 71.9 (d, C-2'), 73.7 (t, C-1''), 75.22/75.27 (t, C-1'), 76.7 (d, C-4), 122.61/122.64 (s, C-2), 159.9 (s, C-3), 172.6 (s, C-1); HRESIMS m/z : 441.2458 [M + Na]⁺ (calcd for C₂₁H₃₈O₈Na, 441.2459).

2-O-(2,3-dihydroxypropyl)-3-O-tridecyl-AsA (27): An amorphous powder; $[\alpha]_D^{26} + 37.8$ (c 0.30, MeOH); UV [MeOH, nm (log ϵ): 233 (4.02); IR (ATR) ν_{\max} cm⁻¹: 3358, 2922, 2853, 1750, 1670, 1333, 1167, 1115, 1042; ¹H-NMR (400 MHz, CD₃OD): δ 0.89 (3H, t, $J = 6.4$ Hz, H₃-13''), 1.29 (18H, brs, H₂-4'', 5'', 6'', 7'', 8'', 9'', 10'', 11'', 12''), 1.44 (2H, m, H₂-3''), 1.75 (2H, m, H₂-2''), 3.58 (2H, m, H₂-3') 3.64 (2H, dd-like, $J = 1.8$, 6.0 Hz, H₂-6), 3.86 (2H, m, H-5, 2'), [3.96/3.97 (1H, dd, $J = 6.9$, 10.1 Hz/6.4, 10.1 Hz), 4.11/4.13 (1H, dd, $J = 3.7$, 10.1 Hz), H₂-1'], 4.54 (2H, m, H₂-1''), 4.83 (1H, d, $J = 1.4$ Hz, H-4): ¹³C-NMR (100 MHz, CD₃OD); δ_C 14.5 (q, C-13''), 23.7 (t, C-12''), 26.7 (t, C-11''), 30.48/30.69/30.73/30.78/30.81 (t, C-3'', 4'', 5'', 6'', 7'', 8'', 9'', 10''), 33.1 (t, C-2''), 63.2 (t, C-6), 63.96/63.99 (t, C-3'), 70.6 (d, C-5), 71.9 (d, C-2'), 73.7 (t, C-1''), 75.19/75.25 (t, C-1'), 76.7 (d, C-4), 122.6 (s, C-2), 159.9 (s, C-3), 172.6 (s, C-1); HRESIMS m/z : 455.2616 [M + Na]⁺ (calcd for C₂₂H₄₀O₈Na, 455.2615).

2-O-(2,3-dihydroxypropyl)-3-O-tetradecyl-AsA (28): An amorphous powder; $[\alpha]_D^{26} + 31.1$ (c 0.30, MeOH); UV [MeOH, nm (log ϵ): 237 (3.93); IR (ATR) ν_{\max} cm⁻¹: 3379, 2922, 2853, 1750, 1669, 1333, 1167, 1117, 1040; ¹H-NMR (400 MHz, CD₃OD): δ 0.89 (3H, t, $J = 6.4$ Hz, H₃-14''), 1.28 (20H, brs, H₂-4'', 5'', 6'', 7'', 8'', 9'', 10'', 11'', 12'', 13''), 1.44 (2H, m, H₂-3''), 1.75 (2H, m, H₂-2''), 3.58 (2H, m, H₂-3') 3.64 (2H, dd-like, $J = 1.4$, 6.4 Hz, H₂-6), 3.86 (2H, m, H-5, 2'), [3.96/3.97 (1H, dd, $J = 6.9$, 10.1 Hz/6.4, 10.1 Hz), 4.11/4.13 (1H, dd, $J = 4.1$, 10.1 Hz), H₂-1'], 4.54 (2H, m, H₂-1''), 4.83 (1H, d, $J = 0.9$ Hz, H-4): ¹³C-NMR (100 MHz, CD₃OD); δ_C 14.5 (q, C-14''), 23.7 (t, C-13''), 26.7 (t, C-12''), 30.48/30.69/30.73/30.78/30.81 (t, C-3'', 4'', 5'', 6'', 7'', 8'', 9'', 10'', 11''), 33.1 (t, C-2''), 63.2 (t, C-6), 63.97/64.00 (t, C-3'), 70.6 (d, C-5), 71.9 (d, C-2'), 73.7 (t, C-1''), 75.20/75.25 (t, C-1'), 76.7 (d, C-4), 122.6 (s, C-2), 159.9 (s, C-3), 172.6 (s, C-1); HRESIMS m/z : 469.2773 [M + Na]⁺ (calcd for C₂₃H₄₂O₈Na, 469.2772).

3.3. Reagents for Bioassays

Dulbecco's modified Eagle's medium (DMEM, 4.5 g/L glucose) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Gibco (Invitrogen, Carlsbad, CA, USA). All other chemicals used in this study were purchased from Wako Pure Chemical Co., Ltd. (Osaka, Japan). The 48- and 96-well microplates (Sumilon) were purchased from Sumitomo Bakelite Co., Ltd. (Tokyo, Japan).

3.4. Cell Culture

Murine B16 melanoma 4A5 cells (RCB0557) were obtained from Riken Cell Bank (Tsukuba, Japan). The cells were grown in DMEM supplemented with 10% FBS, penicillin (100 units/mL), and streptomycin (100 μ g/mL) at 37 °C in 5% CO₂/air. The cells were harvested by incubation in phosphate-buffered saline (PBS) containing 0.05% ethylenediaminetetraacetic acid (EDTA) and 0.02% trypsin for ~5 min at 37 °C and used for the subsequent bioassays.

Normal human epidermal melanocytes (NHEMs; Black donor) were obtained from Kurabo (Osaka, Japan), and were cultured in DermaLife Basal Medium (Kurabo, Osaka) supplemented with DermaLife M LifeFactors (Kurabo, Osaka) at 37 °C in 5% CO₂/air.

3.5. Melanogenesis and Cell Viability

The effects on theophylline-stimulated melanogenesis and viability of B16 melanoma 4A5 cells were examined according to previously described protocols [21–27]. Briefly, murine B16 melanoma 4A5 cells were seeded into 48-well plates in DMEM. After 24 h of culture, a test compound and theophylline (1 mM) were added and incubated for 72 h. The melanin content and cell viability were then measured. IC₅₀ values were determined graphically.

Inhibition (%) was calculated using the following formula, where A and B indicate the optical density of the vehicle- and test compound-treated groups, respectively, and C indicates cell viability (%) (see below): $\text{Inhibition (\%)} = [(A - B) / A] / (C / 100) \times 100$.

3.6. Melanogenesis in Normal Melanocytes

Effects on melanogenesis in NHEMs were examined according to a protocol described previously [21–27,34] with modifications. The cells (3.0×10^5 cells/2 mL/well) were seeded into 6-well multiplates. After 24 h of culture, a test compound was added and incubated for 6 days. The cells were harvested using DermaLife Basal Medium supplemented with DermaLife M LifeFactors, and then treated with 1 M NaOH (50 μ L/tube, 80 °C, 30 min) to yield a lysate. An aliquot (100 μ L) of the lysate was transferred to a 96-well microplate, and the optical density of each well was measured with a microplate reader at 405 nm (reference 655 nm). The test compound was dissolved in DMSO, and the final concentration in the medium was 0.1%. The production rates of melanin were corrected based on the viability of melanoma cells.

3.7. AsA Derivative Stability

To determine stability, the samples were stored at 125 °C, and the residual ratio was calculated by measuring the remaining compound by HPLC. HPLC mobile phase conditions were as follows: AsA was described previously, 6 and 20 were 40% methanol containing 0.1% formic acid at flow rate of 0.2 mL/min [52,53].

3.8. Mushroom Tyrosinase

Tyrosinase activities using L-tyrosine or L-DOPA as a substrate were determined according to the protocols described previously [20,22–27]. Briefly, samples, L-tyrosine (2.5 mM, or L-DOPA, 3.0 mM), mushroom tyrosinase (46 units/mL), and phosphate buffer were mixed in a 96-well plate and incubated at 25 °C. (L-tyrosine; 30 min, L-DOPA; 5 min). The absorbance was then measured at 492 nm.

3.9. Mammalian Tyrosinase

Tyrosinase activity using L-DOPA as a substrate was determined according to protocols described previously [50]. Briefly, B16 cells were seeded into 6-well plates in DMEM. After preculture with theophylline (1 mM) for 72 h, the cells were solubilized using 0.1% Triton X-100. Fifty microliters of each lysate and sample was then mixed with 100 μ L 2 mM L-DOPA. After the mixtures were incubated for 30 min at 37 °C, the absorbance of each solution at 492 nm was measured.

3.10. Expression of Tyrosinase, TRP-1, and TRP-2 mRNA

The expression of tyrosinase, TRP-1, and TRP-2 mRNA was assessed according to previously reported methods [20,23–26]. Briefly, murine B16 melanoma 4A5 cells were seeded into 96-well plates in DMEM. After 24 h of culture, a test compound and theophylline (1 mM) were added and incubated for 72 h. Then, the amount of each mRNA was quantified by quantitative reverse transcription PCR.

3.11. Expression of Tyrosinase Protein

The expression of tyrosinase protein was assessed according to a previously reported method [54]. Briefly, murine B16 melanoma 4A5 cells were seeded into 6-well plates in DMEM. After 24 h of culture,

a test compound and theophylline (1 mM) were added and incubated for 72 h. The expression of tyrosinase protein then was measured by western blotting.

3.12. Tyrosinase Activity in B16 Cells

Tyrosinase activity in cultured cells was determined according to protocols described previously [34] with modifications. Briefly, B16 melanoma 4A5 cells (4×10^3 cells/100 μL /well) were seeded into 96-well plates in DMEM. After pre-culture with sample and theophylline (1 mM) for 72 h, the cells were solubilized using 0.1% Triton X-100, and then each lysate was mixed with 50 μL 2 mM L-DOPA. After the mixtures were incubated for 1 h at 37 $^\circ\text{C}$, the absorbance of the solution at 492 nm was measured using the microplate reader.

3.13. Statistics

Values are expressed as means \pm S.D. One-way analysis of variance (ANOVA) followed by Dunnett's test was used for statistical analysis. Probability (p) values less than 0.05 were considered significant.

4. Conclusions

We examine the inhibitory effects of 28 alkylglyceryl-AsA derivatives (1–28) on theophylline-stimulated murine B16 melanoma 4A5 cells. We find the following structural requirements to be important for the inhibitory activity of alkylglyceryl-AsA derivatives toward melanogenesis: (i) alkylation of glyceryl-AsA is essential for the activity; (ii) the 3-*O*-alkyl-2-*O*-(2,3-dihydroxypropyl)-AsA compounds (16–28) exhibit stronger activities than those of the corresponding 2-*O*-alkyl-3-*O*-(2,3-dihydroxypropyl)-AsA compounds (2–14); and (iii) derivatives with longer alkyl chains have significantly stronger inhibitory activity. Using these guidelines along with our cytotoxicity data, 3-*O*-(2,3-dihydroxypropyl)-2-*O*-hexyl-AsA (6, $\text{IC}_{50} = 81.4 \mu\text{M}$) and 2-*O*-(2,3-dihydroxypropyl)-3-*O*-hexyl-AsA (20, $\text{IC}_{50} = 117 \mu\text{M}$) are deemed the best candidate derivatives for use in cosmetics. However, although it has a limited effective concentration range, 2-*O*-(2,3-dihydroxypropyl)-3-*O*-tetradecyl-AsA (28, $\text{IC}_{50} = 5.0 \mu\text{M}$) demonstrates high melanogenesis inhibitory activity. These derivatives are also found to be more stable than AsA and to have favorable characteristics for skin penetration. Mechanistic studies reveal that the mechanisms of action of 6 and 20, with respect to their melanogenesis inhibitory activities, are the inhibition of tyrosinase and TRP-1 mRNA expression, as well as tyrosinase protein expression, but not the direct inhibition of tyrosinase enzymatic activity (Figure 9). Further skin brightening effects of these candidates, such as those involving intracellular melanosome transport, require further study.

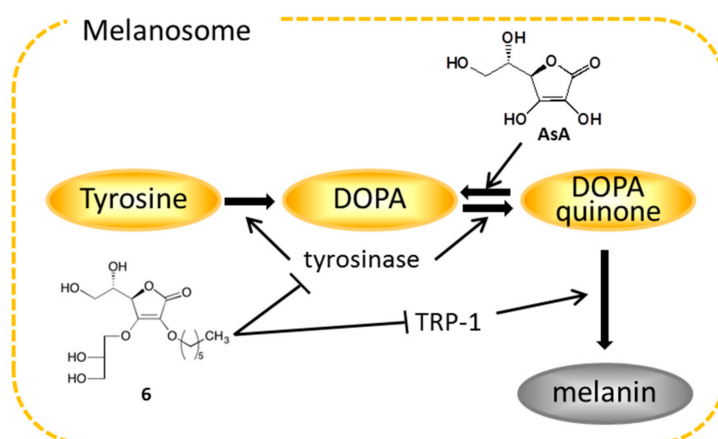


Figure 9. Plausible mechanisms of action of alkylglyceryl-AsA derivatives on melanogenesis inhibitory activity. AsA: L-ascorbic acid.

Supplementary Materials: Supplementary Materials are available at <http://www.mdpi.com/1422-0067/19/4/1144/s1>.

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