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(3*Z*)-2-Acetylamino-3-octadecen-1-ol as a potent apoptotic agent against HL-60 cells

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Abstract—(2*R*,3*Z*)-, (2*R*,3*E*)-, (2*S*,3*Z*) and (2*S*,3*E*)-2-Acetylamino-3-octadecen-1-ol, and (2*R*)- and (2*S*)-2-acetylamino-octadecan-1-ol were prepared using the Wittig olefination of Garner's aldehyde (*N*-Boc-*N*,*O*-isopropylidene-L- or D-serinal) from L- or D-serine. The apoptotic activities of these saturated and unsaturated 2-acetylaminoalcohols were examined in human leukemia HL-60 cells using MTT assay. Among the newly synthesized compounds, the *cis*-isomers were the most potent. Despite their simple structures, (2*R*,3Z)- and (2*S*,3*Z*)-2-acetylamino-3-octadecen-1-ol showed high and comparable apoptotic activities compared with *N*-acetyl-D-*erythro*-sphingosine (D-*e*-C2-Cer, a well-known inducer of apoptosis). Their apoptotic activities were in the order D-*e*-C2-Cer≈L-*e*-C2-Cer≈(2*R*,3*Z*)-≈(2*S*,3*Z*)-> >(2*R*,3*E*)-≈(2*S*,3*E*)-≈(2*R*)-≈(2*S*)-derivative. Qualitative analysis of DNA fragmentation caused by these compounds was conducted using agarose gel electrophoresis, and typical DNA fragmentation was found in the cases of (2*R*,3*Z*)- and (2*S*,3*Z*)-isomers such as C2-Cer, but not *trans* and saturated isomers. The morphological features of the cells, the proteolytic processing of pro-caspase-3, and the cleavage of PARP as a result of exogenous treatment with (2*R*,3*Z*)- and (2*S*,3*Z*)-isomers indicated that cell death induced by these compounds was apoptosis. These observations suggest that these newly synthesized compounds, (*3Z*)-2-Acetylamino-3-octadecen-1-ol, have similar characteristics and apoptosis-inducing activities against HL-60 cells with C2-Cer.

1. Introduction

Ceramide (*N*-acyl-D-*erythro*-sphingosine) is an important lipid messenger involved in mediating a variety of cell functions including apoptosis, cell cycle arrest and cell senescence.[1] Apoptosis induced by a variety of inducers such as tumor necrosis factor-α (TNF-α), Fas ligation, chemotherapeutic agents, and environmental stress is associated with the hydrolysis of sphingomyelin accompanied by the accumulation of ceramide.[2–5] Although the downstream of ceramide signaling is still not known, ceramide-induced apoptosis has been well characterized, and a growing body of work is in progress to identify the targets of ceramide (ceramide binding proteins) that can mediate ceramide action. C2-Ceramide (*N*-acetyl-D-*erythro*-sphingosine, (2*S*,3*R*,4*E*)-2-acetylamino-4-octadecen-1,3-diol, **D-*e*-C2-Cer**) and C8-Ceramide (*N*-octanoyl) are cell-permeable ceramides, and they are used as apoptosis-inducing agents for a wide variety of cancer cells.[6] It has recently been reported that **C2-Cer** treatment activates a family of asparate-specific cysteine proteases, called caspases, which are intimately associated with apoptosis and which cleave a number of substrates including poly(ADP-ribose) polymerase (PARP).[7]

Many syntheses of ceramide and its isomers as well as of various analogues have been reported in the past few decades.[8] For examples, Chang et al. reported the synthesis and biological characterization of the ceramide library (528 ceramide analogues) against human leukemia U937 cells,[8a] and Kishida et al. studied the structural requirements of 4,5-*cis*-, 4,5-*trans*- or the 4,5-acetylene-type analogues of C6-ceramide (4 analogues) for apoptosis-inducing activity against HL-60 cells.[8b] In these studies, the fundamental structures inducing activities had 2-acylamino-4,5-unsaturated-1,3-alkandiol.[8a,b] Both the *cis* isomer and acetylene-type derivative of C6-ceramide analogues were more active than the *trans* isomer in HL-60 cells.[8b] Furthermore, synthetic C6 - phytoceramide

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(*N*-hexanoyl-D-*ribo*-phytosphingosine) induced higher levels of apoptosis than C6-Cer in SK-N-BE(2)C catecholaminergic neuroblastoma cells.[9]

We reported recently the apoptotic activities of C2-*homo*-ceramide and C2-*bishomo*-ceramide,[10] symbioramide derivatives[11] and *N*-lactyl-sphingosine[12] to elucidate structure–reactivity relationships in ceramide-mediated apoptosis.

From a preliminary screening in our laboratory, we found that a new categorical compound, (2*R*,3*Z*)-2-acetylamino-3-octadecen-1-ol, **RZ-2**, showed high apoptotic activity against human leukemia HL-60 cells. Surprisingly, this compound does not have a similar structure to (2*S*,3*R*,4*E*)-2-acetylamino-4-octadecen-1,3-diol (**D-*e*-C2-Cer**, a well-known inducer of apoptosis). Thus, in this paper, we report the syntheses and antileukemic activities of the stereo- and regio-isomers of **RZ-2** as well as their dihydro-analogues.

These structures of 2-acetylamino-3-alken-1-ols, **RZ-2**, **RE-3**, **SZ-2** and **SE-3** are simpler than that of **D-*e*-C2-Cer**. **RZ-2** is not structurally similar to **D-*e*-C2-Cer**, but the structure of **RE-3** seems to lack the hydroxymethylene moiety of the 3 position that is present in that of **D-*e*-C2-Cer**. In addition, these compounds have only one chiral center on the 2-acetylaminomethylene carbon and lack the secondary hydroxyl group. The antileukemic activities of these synthesized compounds were measured using HL-60 cells, and **RZ-2** and **SZ-2** indicate high and comparable apoptotic activities compared with **D-*e*-C2-Cer**. The characteristic behaviors of **RZ-2** and **SZ-2** against HL-60 cells indicate that cell death is caused by the apoptotic pathway.

2. Results and discussion

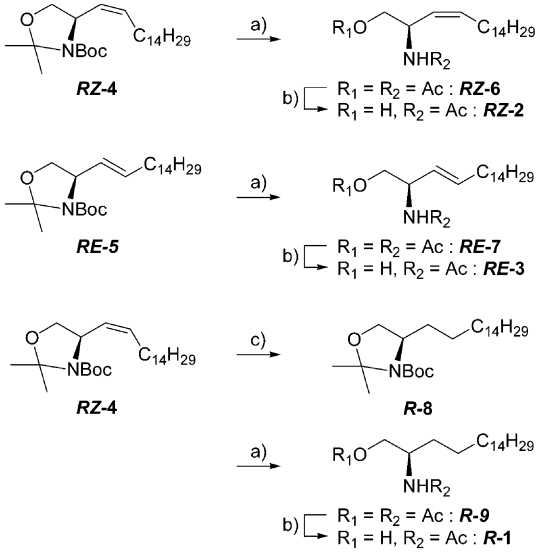
2.1. Chemistry

In this study, (2*R*,3*Z*)- **RZ-2**, (2*R*,3*E*)- **RE-3**, (2*S*,3*Z*)- **SZ-2**, and (2*S*,3*E*)-2-acetylamino-3-octadecen-1-ol **SE-3** and (2*R*)- **R-1** and (2*S*)-2-acetylaminooctadecan-1-ol **S-1** were prepared using our previous method.[13] A synthetic approach to *R*-derivatives is outlined in Scheme 1. From the Wittig olefination of Garner's aldehyde (*N*-Boc-*N*,*O*-isopropylidene-L-serinal) from L-serine with $C_{15}H_{31}PPh_3Br$/LiHMDS, we obtained an 88:12 mixture of (*Z*)- and (*E*)-isomers (**RZ-4** and **RE-5**, respectively) based on ^1H NMR analysis. **RZ-4** and **RE-5** were carefully separated using column chromatography. 2-Acetylamino compounds **RZ-2** and **RE-3** were obtained by the acidic hydrolyses of the corresponding acetonide derivatives, by acetylation with acetic anhydride in pyridine, followed by the alkaline hydrolysis of the primary acetoxy groups of **RZ-6** and **RE-7**, respectively. Saturated compound **R-1** was prepared as described above, after the catalytic hydrogenation of **RZ-4**.

Their corresponding (2*S*)-stereo-isomers, **S-1**, **SZ-2** and **SE-3** were also synthesized using similar methods from D-serine.

2.2. Biological properties

To examine the antileukemic activities of the analogues of C2-ceramide, we analyzed cell death by using HL-60 cells. The percentages of cell death were determined using MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] assay.[14] Time- and dose-dependent cell death induced by the incubation of HL-60 cells with these synthesized (2*R*)-compounds are shown in Figure 2. **RZ-2**- and **D-*e*-C2-Cer**- (positive control for the cell death) induced cell death depended on incubation time and concentration. Despite its simple structure, **RZ-2** showed comparable activity compared with **C2-Cer**. Its *trans*-isomer **RE-3** and saturated analogue **R-1** showed no cell death. These results indicate that *cis* configuration, not *trans*, of double bond on the 3,4-position of their structures is necessary for antileukemic activity.



Scheme 1. Reagents and conditions; (a) TFA-H_2O (20:1), rt, then Ac_2O, pyridine, rt; (b) K_2CO_3, EtOH–H_2O (4:1), rt; (c) PtO_2, H_2, EtOH, rt.

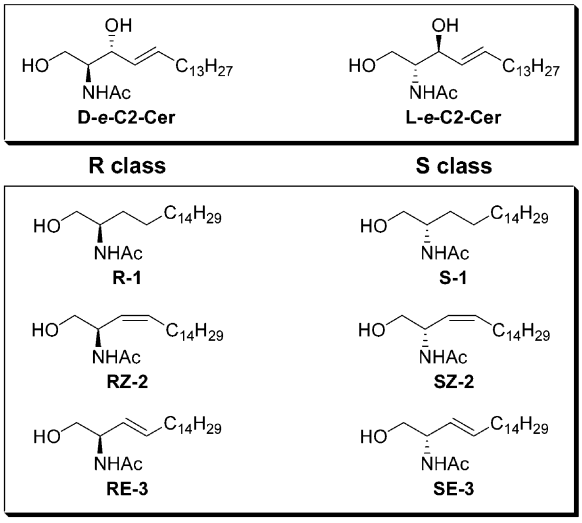


Figure 1.

To confirm that cell death induced by these compounds was apoptosis, we analyzed DNA fragmentation. Figure 3 shows the electrophoretic analysis of extracted DNA from the cells cultured with 20 μM of *R*-compounds for 7 h. Treatments with **D-*e*-C2-Cer** and **RZ-2** showed the typical DNA ladder. Consistent with the results shown in Figure 2, **RZ-2** induced DNA fragmentation as large as **D-*e*-C2-Cer** did. Therefore, **RZ-2** showed comparable apoptotic activity compared with **D-*e*-C2-Cer**.

As these compounds such as **C2-Cer** have one chiral center on the 2-acetylaminomethylene position, we hypothesized that the stereo-chemistry of the 2 position is important for apoptotic activity. We synthesized **S-1**, **SZ-2**, **SE-3** and **L-*e*-C2-Cer**, and we analyzed the apoptotic activities of these compounds. Cell death was determined by treatment with 20 μM compounds against HL-60 cells after 8 h (Fig. 4), and nuclear fragmentation of HL-60 cells was determined after 6 h (Fig. 5). As **D-*e*-C2-Cer**, **L-*e*-C2-Cer**, **RZ-2** and **SZ-2** had almost equal apoptotic activities, cell death showed a similar trend to the *R*- or *S*-conformation of the 2 position. It is reported that the cell toxicity of **L-*e*-C2-Cer** is similar to **D-*e*-C2-Cer**.[8a,g] These results show that the stereo-chemistry of the 2 position of the C2-ceramide analogues, **RZ-2** and **SZ-2**, is not necessarily essential for apoptosis-inducing activity against HL-60 cells as well

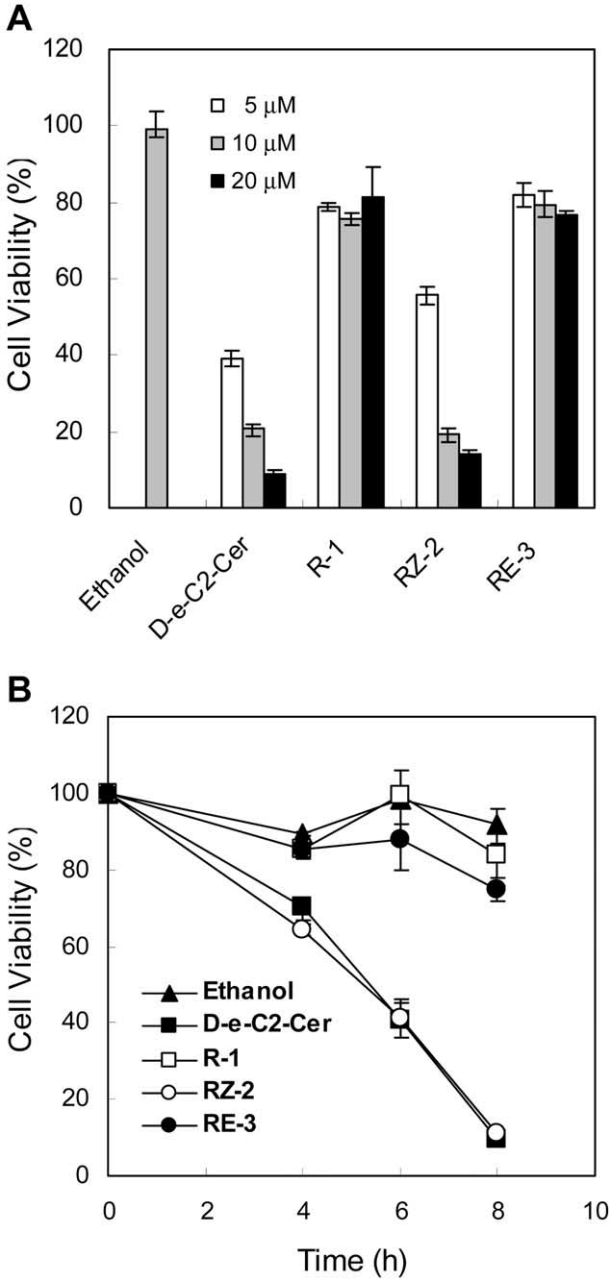


Figure 2. Dose- and time-dependent HL-60 cell death induced by *R*-analogues of C2-ceramide. (A) Cells were incubated with 5–20 μM concentrations for 7 h. (B) Cells were incubated with 20 μM of *R*-analogues of C2-ceramide and values are the average of at least three separate experiments.

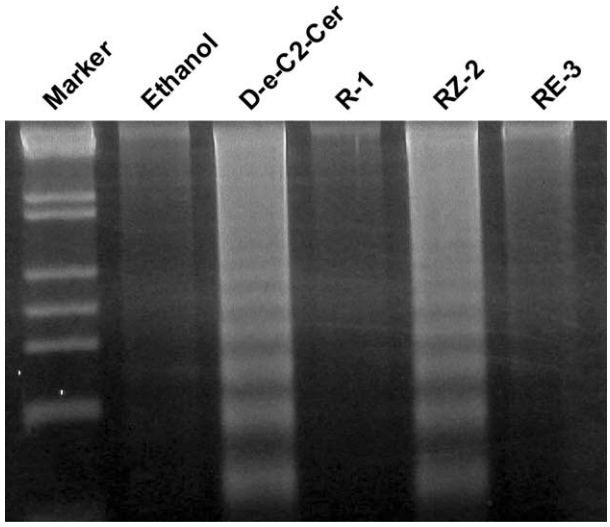


Figure 3. Agarose gel electrophoresis of DNA following treatment of HL-60 cells with ceramide analogues. HL-60 cells (1×10^6 cells/mL) were exposed to *R*-analogue of C2-ceramide (20 μM) or ethanol vehicle. After 7 h, genomic DNA was subjected to agarose gel electrophoresis.

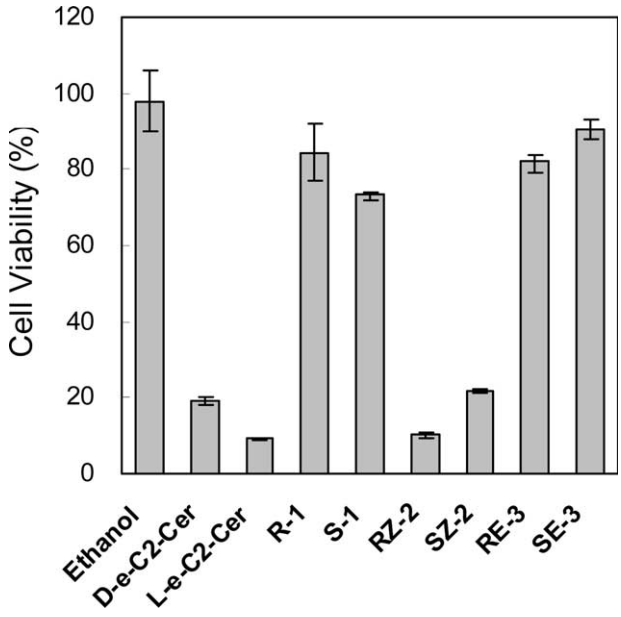


Figure 4. Percent of cell death after a 8 h treatment with 20 μM *R*- and *S*-analogue of C2-ceramide in HL-60 cells. Values are the average of at least three separate experiments.

as C2-Ceramide. The *trans* isomers, **RE-3** and **SE-3**, and the dihydro-isomers, **R-1** and **S-1**, show almost no activities.

These finding indicate that the ceramide-mediated apoptotic pathway includes caspase-3 activation. Caspase-3 is the most efficient PARP-cleaving caspase. This caspase is synthesized as a 32 kDa precursor that, after cleavage, results in the 17 kDa subunit (the active form).[15] PARP is cleaved to generate an 85 kDa fragment.[16] To elucidate the involvement of caspases in these compound-induced apoptotic processes, the activation of caspase-3 and the cleavage of PARP was analyzed in HL-60 cells. The proteolytic processing of pro-caspase-3 and the cleavage of PARP in response to the exogenous analogues of C2-ceramide was examined by Western blotting using a polyclonal anti-caspase-3 antibody or an anti-PARP antibody. The cells by treatments with **D-*e*-C2-Cer** and **RZ-2** generated the active form of caspase-3 and the cleaved form of PARP time dependently (Fig. 6). These results show that **RZ-2** induced apoptosis as well as C2-ceramide.

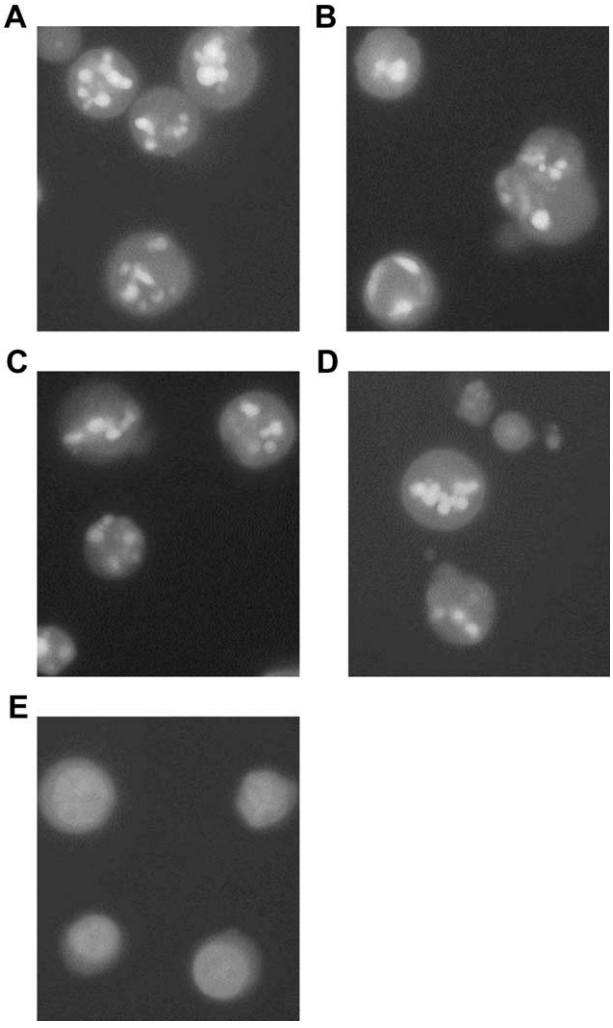


Figure 5. Morphological features of HL-60 cells after treatment with (A) **D-*e*-C2-Cer**; (B) **L-*e*-C2-Cer**; (C) **RZ-2**; (D) **SZ-2**; (E) ethanol vehicle after 6 h.

The structure–reactivity relationships for the biological action of ceramide are being elucidated. In the case of C6-ceramide, it has been clarified that the apoptosis activities of the *cis* isomer and triple bond-compound are stronger than the activity of the *trans* isomer, and it is important for activities to have unsaturated bonds.[8b] Furthermore, it has been reported that the direction of the 2-acetylamino group in **C2-Cer** is not always important for apoptotic activities.[6,10] This paper shows, we showed that newly synthesized compounds, (2*R*,3*Z*)- **RZ-2** and (2*S*,3*Z*)-2-acetylamino-3-octadecen-1-ol **SZ-2**, have similar characteristics and apoptotic activities to **C2-Cer**. As the synthesis of these compounds is simpler than that of **C2-Cer**, **RZ-2** and **SZ-2** may be used to create new anti-cancer agents and for the research of ceramide-induced apoptosis, although the reason for the high apoptotic activities of **RZ-2** and **SZ-2** and no activity of **RE-3** and **SE-3** are unclear at present.

Furthermore, 2-acetylamino-3-octadecen-1-ol is not an intermediate of the biosynthetic process, and neither is it a not the degraded product in the metabolic process of sphingolipids. Therefore, these compounds may be possible inhibitors of biosynthetic and/or metabolic enzymes of sphingolipids.

The findings of this study are summarized as follows:

(2*R*,3*Z*)- (**RZ-2**) and (2*S*,3*Z*)-2-Acetylamino-3-octadecen-1-ol (**SZ-2**), not ceramide, strongly induces cell death with similar activities to **D-*e*-C2-Cer** and **L-*e*-C2-Cer** at low concentrations. These *cis* isomers, **RZ-2** and **SZ-2** were more active than the *trans* isomers, **RE-3** and **SE-3**.

RZ-2 and **SZ-2**, which have only one chiral center at the 2 position showed almost the same apoptotic activities; therefore, the activity of this new class of compounds

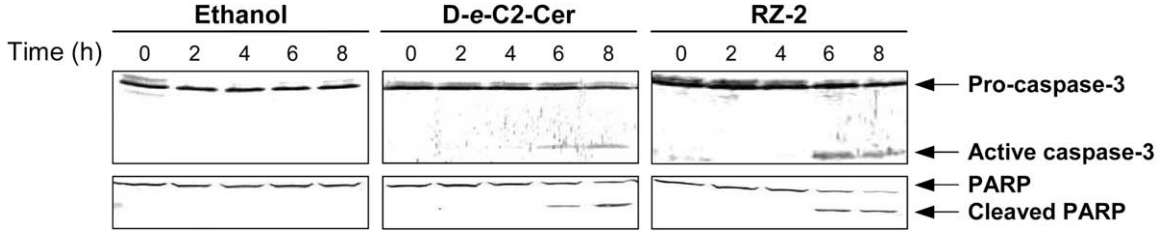


Figure 6. Time-dependent Western blot analysis of caspase-3 and PARP in HL-60 cells with the *R*-analogue of C2-ceramide. Cell extracts after treatment with each ceramide analogues (20 μM) were resolved by SDS-PAGE and probed with *anti*-caspase-3 or *anti*-PARP antibody.

depends on geometrical stereochemistry rather than optical stereochemistry.

The morphological changes in cells, DNA degradation, and the formation of the activated caspase-3 by treatment with **RZ-2** and **SZ-2** clearly indicates that cell death is induced by apoptosis as in the case of **D-*e*-C2-Cer**.

3. Experimental

All materials were obtained commercially (guaranteed reagent grade) and used without further purification. Column chromatography was performed on silica gel.

3.1. (2*R*,3*Z*)-1-acetoxy-2-acetamino-3-octadecene (RZ-6)

To the oxazolidine compound **RZ-4**[13] (1 g, 2.36 mmol) was added a solution of trifluoroacetic acid (16 mL) and water (0.8 mL). After 1 h, the solvent was evaporated in vacuo and a saturated aqueous $NaHCO_3$ was added. The mixture was extracted with $CHCl_3$, dried with Na_2SO_4 and concentrated. To the residue dissolved in pyridine (10 mL) was added acetic anhydride (964 mg, 9.44 mmol). After a day of stirring at room temperature, the solvent was removed in vacuo. A saturated aqueous $NaHCO_3$ was added to the residue and the mixture was extracted with $CHCl_3$. The organic layer was dried with anhydrous Na_2SO_4 and concentrated. Purification by column chromatography with $CHCl_3$ and recrystallized from *n*-hexane–AcOEt to give **RZ-6** (0.77 g, 89%) as a solid; mp 64 °C; $[\alpha]_D^{25} = +8.95°$ (*c* 1.42, $CHCl_3$); IR (NaCl) 3273, 2918, 2849, 1746, 1647, 1558, 1537, 1466, 1373, 1304, 1259, 1043, 743, 721, 604 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$); δ 0.88 (t, 3H, $J = 6.8$ Hz), 1.25 (brs, 24H), 1.98 (s, 3H), 2.07 (s, 3H), 2.14 (q, 2H, $J = 6.8$ Hz), 4.05 (dd, 1H, $J = 4.9$, 11.2 Hz), 4.11 (dd, 1H, $J = 6.4$, 11.2 Hz), 4.95–5.04 (m, 1H), 5.25 (dd,1H, $J = 9.3$, 10.2 Hz), 5.60 (dt, 1H, $J = 10.2$, 7.8 Hz), 5.61 (d, 1H, $J = 7.3$ Hz); HRMS (FAB, direct) calcd for $C_{22}H_{42}O_3N$: $[M+H]^+$ 368.3165. Found: 368.3167 (100%); The enantiomer **SZ-6** was also prepared using compound **SZ-4**; $[\alpha]_D^{25} = -9.02°$ (*c* 1.27, $CHCl_3$); HRMS (FAB, direct) calcd for $C_{22}H_{42}O_3N$: $[M+H]^+$ 368.3165. Found: 368.3168 (100%).

3.2. (2*R*,3*E*)-1-acetoxy-2-acetamino-3-octadecene (RE-7)

The reaction was carried out as described above, using **RE-5** (1 g, 2.36 mmol) to give **RE-7** (0.73 g, 84%) as a solid; mp 100 °C; $[\alpha]_D^{25} = -7.45°$ (*c* 1.01, $CHCl_3$); IR (NaCl) 3285, 2918, 2851, 1723, 1653, 1558, 1474, 1464, 1391, 1373, 1370, 1165, 1042, 976, 762, 719, 600 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$); δ 0.88 (*t*, 3H, $J = 6.8$ Hz), 1.26 (brs, 22H), 1.30–1.40 (m, 2H), 1.98–2.06 (m, 2H), 1.99 (s, 3H), 2.06 (s, 3H), 4.06 (dd, 1H, $J = 4.4$, 11.2 Hz), 4.17 (dd,1H, $J = 6.3$, 11.2 Hz), 4.66–4.76 (m, 1H), 5.35 (dd,1H, $J = 6.3$, 15.6 Hz), 5.67 (dt, 1H, $J = 15.6$, 6.8 Hz), 5.68 (d, 1H, $J = 8.3$ Hz); HRMS (FAB, direct) calcd for $C_{22}H_{42}O_3N$: $[M+H]^+$ 368.3165. Found: 368.3168 (56%); The enantiomer **SE-7** was also prepared using compound **SE-5**; $[\alpha]_D^{25} = +7.86°$ (*c* 1.1, $CHCl_3$); HRMS (FAB, direct) calcd for $C_{22}H_{42}O_3N$: $[M+H]^+$ 368.3165. Found: 368.3169 (76%).

3.3. (2*R*,3*Z*)-2-acetamino-3-octadecen-1-ol (RZ-2)

The diacetate **RZ-6** (650 mg, 1.77 mmol) was stirred with anhydrous K_2CO_3 (294 mg, 1.2 equiv) in EtOH (12 mL) and water (3 mL) for 24 h at room temperature. The reaction mixture was diluted with water and extracted with $CHCl_3$. The organic phase was dried with Na_2SO_4, and concentrated in vacuo. The residue was purified by column chromatography with $CHCl_3$–MeOH (10:1) and recrystallized from *n*-hexane–AcOEt to give **RZ-2** (490 mg, 85%) as a powdery solid; mp 74 °C; $[\alpha]_D^{25} = +7.68°$ (*c* 1.61, $CHCl_3$); IR (NaCl) 3283, 2918, 2851, 1645, 1589, 1464, 1375, 1310, 1271, 1101, 1061, 1028, 1061, 752, 605 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$); δ 0.88 (t, 3H, $J = 6.8$ Hz), 1.26 (brs, 22H), 1.34–1.42 (m, 2H), 2.01 (s, 3H), 2.12 (dt, 2H, $J = 7.3$, 6.8 Hz), 3.25 (t, 1H, $J = 5.9$ Hz), 3.56–3.70 (m, 2H), 4.72–4.82 (m, 1H), 5.30 (dd,1H, $J = 9.3$, 10.7 Hz), 5.61 (dt, 1H, $J = 10.7$, 7.8 Hz), 5.86 (d, 1H, $J = 5.9$ Hz); HRMS (FAB, direct) calcd for $C_{20}H_{40}O_2N$: $[M+H]^+$ 326.3059. Found: 326.3058 (100%). Anal. calcd: C, 73.79; H, 12.08; N, 4.3. Found: C, 73.85; H, 12.13; N, 4.25; The enantiomer **SZ-2** was also prepared using compound **SZ-6**; $[\alpha]_D^{25} = -7.51°$ (*c* 1.4, $CHCl_3$); HRMS (FAB, direct) calcd for $C_{20}H_{40}O_2N$: $[M+H]^+$ 326.3059. Found: 326.3056 (100%). Anal. calcd: C, 73.79; H, 12.08; N, 4.3. Found: C, 73.76; H, 12.06; N, 4.29.

3.4. (2*R*,3*E*)-2-acetamino-3-octadecen-1-ol (RE-3)

The reaction was carried out as described above, using **RE-7** (500 mg, 1.36 mmol) to give **RE-3** (400 mg, 90%) as a powdery solid; mp 85–86 °C; $[\alpha]_D^{25} = -15.67°$ (*c* 1.862, $CHCl_3$); IR (NaCl) 3270, 2918, 2851, 1647, 1614, 1564, 1470, 1373, 1086, 1067, 1043, 978, 719, 625 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$); δ 0.88 (t, 3H, $J = 6.8$ Hz), 1.26 (brs, 22H), 1.34–1.42 (m, 2H), 1.97–2.07 (m, 2H), 2.03 (s, 3H), 3.03 (t, 1H, $J = 5.9$ Hz), 3.57–3.65 (m,1H), 3.65–3.72 (m, 1H), 4.45–4.52 (m, 1H), 5.39 (dd,1H, $J = 5.9$, 15.6 Hz), 5.69 (dt, 1H, $J = 15.6$, 6.4 Hz), 5.99 (d, 1H, $J = 7.3$ Hz); HRMS (FAB, direct) calcd for $C_{20}H_{40}O_2N$: $[M+H]^+$ 326.3059. Found: 326.3056 (100%). Anal. calcd: C, 73.79; H, 12.08; N, 4.3. Found: C, 73.68; H, 12.10; N, 4.30; The enantiomer **SE-3** was also prepared using compound **SE-7**; $[\alpha]_D^{25} = +15.0°$ (*c* 1.12, $CHCl_3$); HRMS (FAB, direct) calcd for $C_{20}H_{40}O_2N$: $[M+H]^+$ 326.3059. Found: 326.3053 (100%). Anal. calcd: C, 73.79; H, 12.08; N, 4.3. Found: C, 73.80; H, 12.09; N, 4.27.

3.5. (2*R*)-1-acetoxy-2-acetamino-3-octadecane (R-9) via (2*R*)-2-[(*tert*-Butoxycarbonyl)amino]-1,2-*O*,*N*-isopropylideneoctadecan-1-ol (RZ-4)

To the solution of **RZ-4** (1 g, 2.36 mmol) in EtOH (20 mL) was added a catalytic amount of PtO_2 (25 mg, 0.11 mmol) and the mixture was stirred overnight at room temperature under H_2. The reaction mixture was filtered over Celite and concentrated in vacuo. The obtained crude **R-8** was used without further purification. The next reaction was carried out as described above to give **R-9** (690 mg, 79%) as a powdery solid; mp 109.5 °C; $[\alpha]_D^{25} = +22°$ (*c* 1.09, $CHCl_3$); IR (KBr) 3287, 2918,

2851, 1784, 1651, 1558, 1462, 1373, 1254, 1159, 1043, 719 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$); δ 0.88 (t, 3H, $J = 6.8$ Hz), 1.25 (brs, 28H), 1.36–1.54 (m, 2H), 1.99 (s, 3H), 2.08 (s, 3H), 4.03 (dd, 1H, $J = 3.4$, 10.7 Hz), 4.15 (dd, 1H, $J = 5.4$, 10.7 Hz), 4.14–4.21 (m, 1H), 5.45 (d, 1H, $J = 8.3$ Hz); HRMS (FAB, direct) calcd for $C_{22}H_{44}O_3N$: $[M+H]^+$ 370.3321. Found: 370.3318 (22%); The enantiomer **S-9** was also prepared using compound **SZ-4**; $[\alpha]_D^{25} = -20.3°$ (*c* 1.18, $CHCl_3$); HRMS (FAB, direct) calcd for $C_{22}H_{44}O_3N$: $[M+H]^+$ 370.3321. Found: 370.3325 (67%).

3.6. (2*R*)-2-acetamino-octadecan-1-ol (R-1)

The reaction was carried out as described above for **RZ-2**, using compound **R-9** (440 mg, 1.19 mmol) to give **R-1** (360 mg, 92%) as a powdery solid; mp 104 °C; $[\alpha]_D^{25} = +13.0°$ (*c* 2.37, $CHCl_3$); IR (KBr) 3280, 2910, 2849, 1649, 1622, 1556, 1472, 1443, 1373, 1323, 1153, 1086, 721 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$); δ 0.88 (t, 3H, $J = 6.8$ Hz), 1.26 (brs, 28H), 1.36–1.57 (m, 2H), 2.00 (s, 3H), 2.62–2.68 (m, 1H), 3.52–3.59 (m, 1H), 3.64–3.71 (m, 1H), 3.87–3.96 (m, 1H) 5.62 (d, 1H, $J = 5.9$ Hz). Anal. calcd: C, 73.34; H, 12.62; N, 4.28. Found: C, 73.26; H, 12.57; N, 4.25; HRMS (FAB, direct) calcd for $C_{20}H_{42}O_2N$: $[M+H]^+$ 328.3216. Found: 328.3216 (100%). Anal. calcd: C, 73.34; H, 12.62; N, 4.28. Found: C, 73.34; H, 12.57; N, 4.25; The enantiomer **S-1** was also prepared using compound **S-9**; $[\alpha]_D^{25} = -13.15°$ (*c* 1.68, $CHCl_3$); HRMS (FAB, direct) calcd for $C_{20}H_{42}O_2N$: $[M+H]^+$ 328.3216. Found: 328.3219 (100%). Anal. calcd: C, 73.34; H, 12.62; N, 4.28. Found: C, 73.13; H, 12.69; N, 4.25.

3.7. Cell culture

Human pronyelocytic leukemia HL-60 cells were grown in RPMI 1640 medium (Sigma) containing 5% heat-incubated fetal bovine serum supplemented with 100 units/mL penicillin and 100 μg/mL streptomycin. On the day of the experiment, cells were washed once in serum-free RPMI 1640 and resuspended in the serum-free medium ($0.4–1 \times 10^6$ cells/mL). The cells were treated with various compounds at 37 °C, and an MTT assay was run or DNA fragmentation was observed. All the compounds were dissolved in ethanol. Control experiments were performed with ethanol (0.1%) as the vehicle.

3.8. MTT assay

The cell death was assessed by the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). MTT is a water-soluble tetrazolium salt that is reduced by metabolically viable cells to a colored, water insoluble formazan salt. 4×10^4 cells/100 μL were plated in 96-well dishes. The cells were treated with various compounds dissolved in ethanol. The final concentration of ethanol was 0.1%. 10 μL of 5 mg/mL MTT was added to each well before 2 h from the end of the culture, and reactions were stopped by adding 100 μL of 0.04 N HCl in isopropanol. The absorbance at a wavelength of 570 nm was measured. All results were determined in triplicate.

3.9. DNA Fragmentation

HL-60 cells (2×10^6 cells/2 mL) were plated in 6-well dishes. Compounds were dissolved in ethanol at stock concentration of 20 mM and then diluted with serum free medium. After an 7 h incubation, the cells were collected by centrifugation at 2500 rpm for 5 min at 4 °C. Cells were lysed in 100 μL of lysis buffer (10 mM Tris–HCl; pH 7.4, 10 mM EDTA; pH 8.0, and 0.5% Triton X-100). Soluble cell lyses were collected by centrifugation at 15,000 rpm for 5 min. Cell lysates were treated for 1 h at 37 °C with RNase A (0.2 mg/mL). Proteinase K (0.2 mg/mL) was added, and sample was incubated at 50 °C for 30 min. 5 M NaCl (20 μL) and isopropanol (120 μL) were added and the sample was incubated at −20 °C for one night. DNA pellets were collected by centrifugation at 15,000 rpm for 15 min, and dissolved in 20 μL of a TE buffer (10 mM Tris–HCl; pH 7.4, 1 mM EDTA; pH 8.0). The DNA was then electrophoresed at 50 V through 2.0% agarose gel. The DNA bands were visualized under UV light after staining with ethidium bromide.

3.10. Staining of apoptotic nuclei

Morphological changes in the nuclear chromatin of cells undergoing apoptosis were detected by staining with the 4′,6-diamidino-2-phenylindole (DAPI). HL-60 cells were treated with compounds as described above. After an 6 h incubation, cells were fixed with 3.7% formaldehyde in phosphate buffered saline (PBS) for 30 min at room temperature. Thereafter, the cells were stained with the DAPI (1 μg/mL) in PBS for 30 min at room temperature. Condensed nuclei were detected by Olympus IX-71 fluorescence microscopy.

3.11. Immunoblot analysis

HL-60 cells were treated with compounds as described 'DNA Fragmentation'. After an incubation, cells were collected by centrifugation at 4000 rpm for 5 min at 4 °C, washed twice with PBS and lysed in 100 μL of SDS sample buffer (62.5 mM Tris–HCl; pH 6.8, 6 M urea, 10% glycerol, 2% SDS, 0.00125% bromophenol blue, 5% β-mercaptoethanol). Cell lysates were boiled for 5 min and separated on 12% SDS-polyacrylamide gels, transferred to Immobilon-P transfer membrane (Millipore), and probed with rabbit polyclonal anti-caspase-3 antibody (Santa Cruz Biotechnology), followed by goat anti-rabbit antibody coupled to alkaline phosphatase (Sigma).

For PARP immnoblots, cell lysates were separated on 8% SDS-polyacrylamide gels, transferred to Immobilon-P transfer membrane, and probed with monoclonal mouse anti-human PARP (Trevigen), followed by goat anti-mouse antibody coupled to alkaline phosphate (Sigma).

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