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Synthesis of cinnamic acid derivatives and their inhibitory effects on LDL-oxidation, acyl-CoA:cholesterol acyltransferase-1 and -2 activity, and decrease of HDL-particle size

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Abstract—A series of cinnamic acid derivatives were synthesized and their biological abilities on lipoprotein metabolism were examined. Among the tested compounds, 4-hydroxycinnamic acid (L-phenylalanine methyl ester) amide (**1**) and 3,4-dihydroxyhydrocinammic acid (L-aspartic acid dibenzyl ester) amide (**2**) inhibited human acyl-CoA:cholesterol acyltransferase-1 and -2 activities with apparent IC_{50} around 60 and 95 μM, respectively. Compounds **1** and **2** also served as an antioxidant against copper mediated low-density lipoproteins (LDL) oxidation with apparent $IC_{50} = 52$ and 3 μM, compound **1** and **2**, respectively. Additionally, decrease of HDL-particle size under presence of LDL was inhibited by the **1** at 307 μM of final concentration. Treatment of the **1** or **2** did not influence normal growth of RAW264.7 without detectable cytotoxic activity from a cell viability test. These results suggest that the new cinnamic acid derivatives possess useful biological activity as an anti-atherosclerotic agent with inhibition of cellular cholesterol storage and transport by the both ACAT, inhibition of LDL-oxidation, HDL particle size rearrangement.

Low-density lipoprotein (LDL) is a major cholesterol carrier in blood stream and concentration of LDL-cholesterol is directly correlated to incidence of coronary heart disease.[1,2] Numerous epidemiological studies revealed that increased oxidized-LDL (ox-LDL) acts as strong pro-atherogenic factor via triggering complex inflammation process, including macrophage proliferation, and adhesion process signaling.[3,4] Acyl-CoA:cholesterol acyltransferase (ACAT, EC 2.3.1.26) is an allosteric enzyme[5] and plays an important role in the esterification of cholesterol to facilitate intracellular storage[6] and intercellular circulation via incorporation into apolipoprotein-B (apo-B) containing lipoproteins.[7] For the last decade, in vitro and in vivo experiments have proved that the enzyme plays a critical role in the development of atherosclerosis under pathological conditions, via foam cell formation.[8–10] Since the enzyme was found to be present as two isoforms in mammals, ACAT-1 and -2, it has been required to develop an inhibitor against the both isotype to maximize therapeutic effect. High-density lipoprotein (HDL) plays a critical role in the reverse cholesterol transport in uptake and transport of excess cholesterol from peripheral (nonhepatic) cells to liver,[11] where cholesterol can be converted into bile acids for excretion. Cholesterol excretion that mediated by HDL is widely accepted as important step to suppress early pathogenesis of hypercholesterolemia and atherosclerosis.[12] Furthermore, HDL-particle size is found to be important to regulate cholesterol excretion since larger rHDL (10 nm) bound more efficiently to scavenger receptor B-I and delivered more cholesteryl esters (CE) and promoted free cholesterol (FC) efflux than small particles (8 nm).[13] Recently, 3,4-dihydroxy hydrocinnamides were reported as an anti-atherogenic agents in hypercholesterolemic rabbit by our research group,[14] especially 3,4-dihydroxycinnamic acid (L-alanine methyl ester) amide (**3**), although explanation for the in vitro mechanism was not provided in detail. On the basis of the previous result, we prepared a series of cinnamic acid derivatives from 3,4-dihydroxycinnamic acid or 4-hydroxycinnammic acid, and their relevant biological activities on lipid/lipoprotein metabolism were examined to prove the anti-atherogenic effect. Among several scores of the

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compound, **1** and **2** were selected with activity on various lipoprotein/lipid assay system including ACAT inhibition, and LDL-oxidation, and HDL-particle rearrangement.

All reactions were conducted under a nitrogen atmosphere using oven-dried glassware unless otherwise noted. Analytical TLC was done on 0.25 mm E. Merck precoated silica gel 60 F_{254} plates. Visualization was accomplished with UV light at 254 nm and with phosphomolybdic acid, ninhydrin, or anisaldehyde stain. Flash chromatography was performed on silica gel 60 (E. Merck 9385, 230–400 mesh). ^{1}H and ^{13}C NMR spectra were recorded on a Varian Unity 300 or Varian Inova 400 spectrometer at ambient temperature. Chemical shifts are reported in ppm relative to tetramethylsilane/$CHCl_3$.

Biological activities of the compounds were measured including acyl-CoA:cholesterol acyltransferase assay,[15] copper ion mediated oxidation of LDL and antioxidant activity assay,[18] and HDL-particle rearrangement assay.[23] Cell viability and cytotoxicity of the compounds was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay and lactate dehydrogenase-release assay, respectively.[26]

4-Hydroxycinnamic acid (L-phenylalanine methyl ester) amide (**1**), 3,4-dihydroxyhydrocinnamic acid (L-aspartic acid dibenzyl ester) amide (**2**), and 3,4-dihydroxycinnamic acid (L-alanine methyl ester) amide (**3**) were prepared by condensation of the corresponding carboxylic acids with amino acid esters utilizing 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), 1-hydroxybenzotriazole (HOBT), and triethylamine in methylene chloride.[27–29] Structure of compounds **1**, **2**, and **3** are shown in Table 1.

The antioxidant activity of **1**, **2**, and **3** from thiobarbituric acid reactive substance (TBARS) assay was indicated in Table 1. At final 10 μM, **2** and **3** showed stronger antioxidant activity than probucol in the conjugated diene detection assay (Fig. 1A). In Figure 1B, compound **1**, **2**, and **3** showed similar relative electromobility (REM) on the 0.7% agarose gel, indicating good antioxidant activity. In the previous report, **3** showed a potent in vivo activity with significant reduction of atherosclerotic lesion formation in hypercholesterolemic rabbit without improvement of serum lipid profile.[14] The anti-atherosclerotic effect might be originated, at

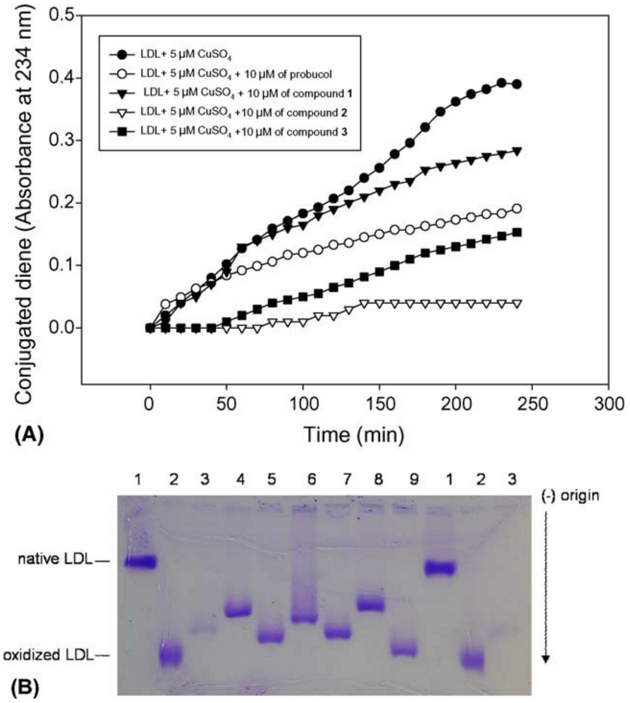
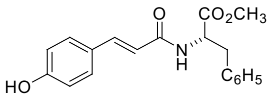
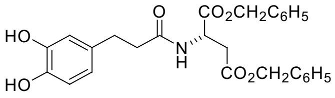
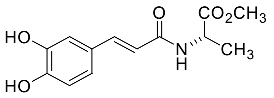


Figure 1. Antioxidant activity of the compounds (**1**, **2**, and **3**) against copper mediated LDL-oxidation. Panel A. Conjugated diene levels detection assay. Compounds **1**, **2**, and **3** (final 10–12 μM) were added to 3 mL of LDL (120 μg of apo-B) containing 5 μM of $CuSO_4$ in PBS, pH 7.4. Produced conjugated diene by the LDL-oxidation was monitored at 234 nm for 4 h. Panel B. Relative electrophoretic mobility (REM) profiles of the compounds. More oxidized LDL moved faster to the bottom of the gel (0.7% agarose). Lane 1, native LDL (2.7 mg/mL); lane 2, LDL + 5 μM $CuSO_4$; lane 3, 4 μM of Probucol; lane 4: 100 μM of **1**; lane 5, 10 μM of **1**; lane 6, 100 μM of **2**; lane 7, 10 μM of **2**; lane 8, 100 μM of **3**; lane 9, 10 μM of **3.**

Table 1. Structure and antioxidant activity of compound **1**, **2**, **3** by detection of malondialdehyde (MDA) generation from thiobarbituriacid reactive substances assay

Compound	Structure	nmol of MDA/mg of LDL[a]	% Inhibition
1		269.0	28
2		32.1	91
3		91.5	75
Probucol	As positive control (final 1 μM)	263.1	29
LDL + $CuSO_4$	As negative control	372.6	0

[a] At final 1 μg/mL concentration, approximately 3, 2, and 2 μM for **1**, **2**, and **3**, correspondingly, of each compound was treated.

least in part, from the virtue of strong antioxidant activity against the copper mediated LDL oxidation. It has been firmly established that inhibition of LDL-oxidation is a potent strategy to treat atherosclerosis with several beneficial effects to circulation.[30]

As shown in Figure 2, **1** and **2** exhibited strong inhibitory activity, more than 70% inhibition, against either ACAT-1 or -2 at final 30 μg/mL, approximately 61, 92, and 81 μM for **1**, **2**, **3**, correspondingly. However, **3** showed the lowest inhibition activity around 15% against only ACAT-1, whereas a positive control, final 125 nM of oleic acid anilide, showed 55% inhibitory activity against either ACAT-1 or ACAT-2. Since the **3** did not inhibit ACAT-2 activity, it is possible to speculate that the intestinal absorption and transfer of cholesterol was not inhibited in the hypercholesterolemic mice those had been fed **3** in our previous report.[14]

This result can explain the reason why that **3** did not induce cholesterol lowering effect in the previous report, because ACAT-2 plays important role in intestinal cholesterol absorption and hepatic storage.[5–7] The further determination revealed that **1** showed almost the same degree inhibitory activity against ACAT-1 and -2 with apparent $IC_{50} = 60\,\mu M$ (panel B of Fig. 2), while **2** revealed slightly higher inhibitory activity against ACAT-2 than ACAT-1 with $IC_{50} = 81\,\mu M$ and $IC_{50} = 95\,\mu M$, respectively (panel B of Fig. 4). This result showed interesting potential of the compounds that they can inhibit ACAT-1 and -2 simultaneously. The isotypes of ACAT are differently found in many biochemical and biophysical characteristics; substrate binding efficiency, membrane topology, and distribution of tissue location.[31] A few of ACAT inhibitors have markedly different inhibitory activity and sensitivity against ACAT-1 and -2 depends on the structure and solubility.[17,32] Therefore, it is generally accepted that a desirable ACAT inhibitor should have simultaneous inhibitory activity to both isotype of ACAT to maximize therapeutic effect and pharmaceutical potency.

In Figure 3, addition of compound **1** at final 307 μM inhibited the rHDL-particle rearrangement under presence of LDL at 37 °C, which resulted that 96 Å-rHDL of the original size (lane 2) was maintained during the reaction of apoA-I (lane 1) while 83 Å-rHDL appeared by treatment of **2** (lane 2). The result indicates that **1** can make a putative interaction with amino acids of apoA-I, those constitute hinge domain or transmembrane domain of apoA-I to regulate particle rearrangement activity. To our knowledge, this is the first report to screen a compound that can regulate interaction of LDL and HDL, and HDL-particle rearrangement even though the tested dosage is relatively higher than other application. Further investigation is necessary to elucidate more precise mechanism whether the compound interacts with apolipoproteins or phosphatidylcholines of the each lipoprotein.

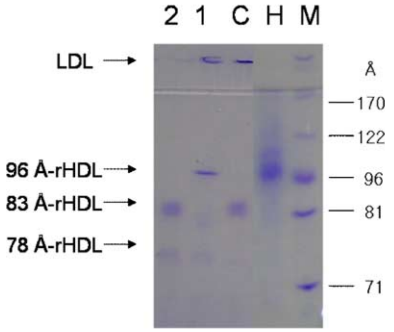


Figure 3. Electrophoretic patterns of rHDL products after incubation with human LDL and compound **1** or **2** (8–25% native gel, Pharmacia Phastgel system). Under presence of LDL for 24 h incubation, **1** treated rHDL (lane 1) maintained original size of the rHDL (96 Å), while **2** treated rHDL showed reduced size of rHDL species (83 Å). Lane M, high molecular weight marker (Amershampharmacia); lane H, native rHDL (96 Å) only; lane C, rHDL + LDL.

Treatment of **1** or **2** to the mouse RAW264.7 macrophage cell (1×10^5 cell/100 μL) was appeared that they were not cytotoxic as shown in Figure 4. Treatment of 50 μM/1×10^5 cell of **1** and **2** showed no detectable cytotoxicity with less than 10% reduced cell viability from both 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay (panel A) and lactate dehydrogenase (LDH)-release assay (panel B). When the highest concentration (final 100 μM/1×0^5 cell) was treated, **1** and **2** showed around 80% of cell viability from both assay.

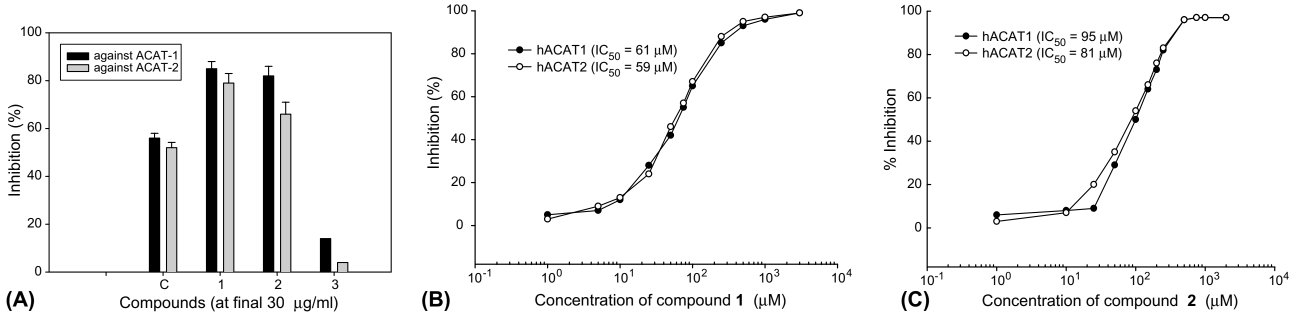


Figure 2. (A) Inhibitory activity of compounds **1**, **2**, and **3** against ACAT-1 and -2 activities. Compounds were tested in the final 30 μg/mL, approximately 61, 92, and 81 μM for **1**, **2**, **3**, correspondingly and oleic acid anilide (final 0.125 μM) was used as a positive control. The number of *X*-axis is compound number, correspondingly, and C is oleic acid anilide as a positive control. Data are shown as mean ± SD from three independent experiments performed in triplicate. ACAT-inhibitory activity of the **1** (B) and **2** (C) increased with concentration dependent manner.

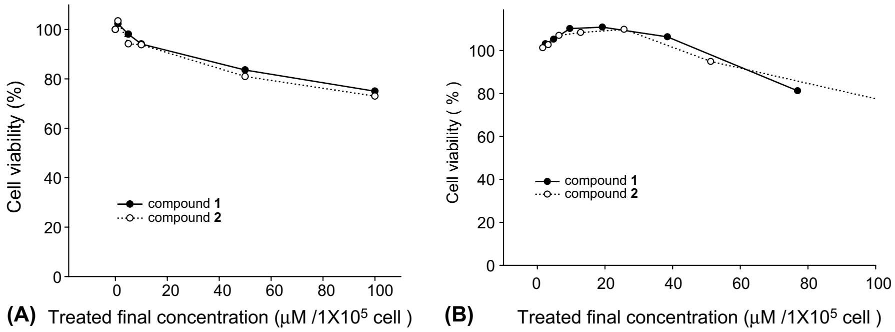


Figure 4. Cell viability assay (panel A) and cytotoxicity assay (panel B) using RAW264.7 cell. Cell viability and cytotoxicity were determined by MTT assay and LDH-release assay, respectively.

Some cinnamic acid-based thiazolidinedione derivatives (TZDs) revealed an anti-hyperglycemic activity with in vitro peroxisome proliferator-activated receptor gamma (PPARγ) transactivation and strong glucose-lowering activity in type 2 diabetes animal model.[33] Huff and colleagues reported that the TZDs also effective to attenuate atherosclerosis in mice by inhibition of foam cell formation through enhanced cholesterol efflux despite increased oxidized LDL uptake.[34] The anti-atherogenic effect from the in vivo result strongly suggests that it might be mediated by enhanced HDL-function and/or apoA-I, since the transactivation of PPARγ/retinoid X receptor (RXR) resulted increased cholesterol efflux to apolipoproteinA-I.[26] Similarly, 4-hydroxy cinnamate was effective to lower the plasma cholesterol and hepatic lipids in cholesterol-fed rats.[35] A few of 3,4-dihydroxycinnamic acid (caffeic acid) derivatives displayed a hepatoprotective activity with inhibition of 5-lipoxygenase activity and putative radical scavenging or antioxidant activity.[36,37] It is necessary that in vivo test or preclinical evaluation with the compound to develop more potent agent. Taken together the others report and our current findings, the compounds are expected to contribute treatment of hypercholesterolemia via stimulation of cholesterol excretion with their useful activities on lipoprotein metabolism and no cytotoxic effect.

Acknowledgements

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15. Acyl-CoA:cholesterol acyltransferase assay: Microsomal fraction of Hi-5 cell that is expressing ACAT-1 or -2 was prepared by ultracentrifugation[16] using Beckman L8-M (Palo Alto, CA) and SW55.1 rotor. The microsomal fraction of Hi5 cell lysate (4 μL, 10 mg/mL) was used as the enzyme source, $[^{14}C]$-oleoyl-CoA (specific activity = 56 mCi/mmol) and cholesterol was used as substrate. In vitro inhibition activity of the compounds against ACAT-1 or ACAT-2 was determined as described previously.[17]
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26. Cell morphology and viability assay: Mouse macrophage cell line (RAW264.7) was obtained from American Type Culture Collection (ATCC #TIB-71, Rockville, MD) and was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Viability of the RAW264.7 cell was determined by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) to detect functioning mitochondria. Cytotoxicity of the compound was determined using commercially available Lactate dehydrogenase cytotoxicity detection kit (Takara cat# MK401, Shiga, Japan).
27. Typical procedure for the preparation of 3,4-dihydroxyhydrocinnamic acid (L-aspartic acid dibenzyl ester) amide (**2**): To a solution of 3,4-dihydroxyhydrocinnamic acid (3.0 g, 16.5 mmol), L-aspartic acid dibenzyl ester *p*-toluenesulfonate (8.0 g, 16.5 mmol), and 1-hydroxybenzotriazole (2.3 g, 17.0 mmol) in *N*,*N*-dimethylformamide (50 mL) was added triethylamine (6.9 mL, 49.5 mmol) at 0 °C. After 10 min, 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (3.2 g, 16.7 mmol) was added at 0 °C and the resultant reaction mixture was stirred for 18 h at room temperature. The mixture was poured into water (200 mL), extracted with ethyl acetate (4 × 200 mL), washed with water (2 × 100 mL) and brine (100 mL), dried over $MgSO_4$, and concentrated in vacuo. The residue was purified by flash chromatography on silica gel [hexane/EtOAc (1:1)] to afford 6.0 g (76%) of 3,4-dihydroxyhydrocinnamic acid (L-aspartic acid dibenzyl ester) amide as a pale yellow oil: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.36–7.24 (10H, m), 6.73 (1H, d, $J = 8.0$ Hz), 6.66 (d, $J = 2.0$ Hz, 1H), 6.55 (1H, dd, $J = 8.0, 2.0$ Hz), 6.49 (1H, d, $J = 7.6$ Hz), 5.10 (2H, s), 5.05 (1H, d, $J = 12.0$ Hz), 5.00 (1H, d, $J = 12.0$ Hz), 4.87 (1H, m), 3.01 (1H, dd, $J = 16.8, 4.4$ Hz), 2.83–2.77 (3H, m), 2.43 (2H, m); ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.9, 171.0, 170.6, 143.8, 142.8, 135.4, 135.1, 132.9, 128.9, 128.8, 128.7, 128.6, 128.5, 120.6, 115.6, 115.4, 67.9, 67.2, 48.8; MS (EI^{+}) *m*/*z* (rel intensity) 477 (M^{+}, 5), 181 (5), 178 (5), 163 (6), 123 (18), 91 (100); HRMS Calcd for $C_{27}H_{27}NO_7$ (M^{+}): 477.1788. Found: 477.1784.
28. Typical procedure for the preparation of 4-hydroxycinnamic acid (L-phenylalanine methyl ester) amide (**1**): Compound **1** was prepared from 4-hydroxycinnamic acid and L-phenylalanine methyl ester hydrochloride as described for **2**, which was purified by flash chromatography on silica gel [hexane/EtOAc (1:1)] in 98% yield as a white solid: mp 153–154 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.53 (1H, d, $J = 15.2$ Hz), 7.31 (2H, d, $J = 8.8$ Hz), 7.27 (3H, m), 7.11 (2H, m), 6.82 (2H, d, $J = 8.8$ Hz), 6.20 (1H, d, $J = 15.2$ Hz), 6.16 (1H, s), 5.02 (1H, dd, $J = 13.6, 6.0$ Hz), 3.74 (3H, s), 3.22 (1H, dd, $J = 14.0, 6.0$ Hz), 3.16 (1H, dd, $J = 14.0, 6.0$ Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.3, 166.2, 158.1, 142.1, 135.7, 129.7, 129.2, 128.6, 127.2, 126.7, 116.7, 115.9, 53.4, 52.4, 37.8; MS (EI^{+}) *m*/*z* (rel intensity) 325 (M^{+}, 10), 163 (29), 162 (32), 147 (100), 119 (17), 91 (25); HRMS Calcd for $C_{19}H_{19}NO_4$ (M^{+}) 325.1314. Found: 325.1323.
29. Typical procedure for the preparation of 3,4-dihydroxyhydrocinnamic acid (L-alanine methyl ester) amide (**3**): Compound **3** was prepared from 3,4-dihydroxyhydrocinnamic acid and L-alanine methyl ester hydrochloride as described for **2**, which was purified by flash chromatography on silica gel [hexane/EtOAc (1:1)] in 88% yield as a white solid: mp 98–99 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.69 (1H, s), 8.61 (1H, s), 8.23 (1H, d, $J = 7.2$ Hz), 6.58 (1H, d, $J = 8.0$ Hz), 6.54 (1H, d, $J = 1.6$ Hz), 6.40 (1H, dd, $J = 8.0, 1.6$ Hz), 4.22 (quint, $J = 7.2$ Hz, 1H), 3.58 (3H, s), 2.59 (2H, m), 2.28 (2H, m), 1.22 (3H, d, $J = 7.2$ Hz); ^{13}C NMR (100 MHz, DMSO-d_6) δ 173.9, 172.2, 145.6, 143.9, 132.7, 119.3, 116.3, 116.0, 52.4, 48.1, 37.7, 31.0, 17.6; MS (EI^{+}) *m*/*z* (rel intensity) 267 (M^{+}, 72), 163 (25), 136 (100), 123 (78), 104 (89); HRMS Calcd for $C_{13}H_{17}NO_5$ (M^{+}) 267.1107. Found: 267.1107.
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