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Selective Antiproliferative Activity of Caffeic Acid Phenethyl Ester Analogues on Highly Liver-Metastatic Murine Colon 26-L5 Carcinoma Cell Line

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Abstract—Caffeic acid phenethyl ester (CAPE, **2**) and its twenty analogues (**1**, **3**–**21**) were prepared. These esters were tested by MTT assay on growth of murine colon 26-L5 carcinoma, murine B16-BL6 malonoma, murine Lewis lung carcinoma, human HT-1080 fibrosarcoma, human lung A549 adenocarcinoma, and human cervix HeLa adenocarcinoma cell lines. It was found that CAPE analogues possessed selective antiproliferative activity toward highly liver-metastatic murine colon 26-L5 carcinoma cell line. Among them, 4-phenylbutyl caffeate (**4**), (*Z*)-8-phenyl-7-octenyl (**10a**) and (*E*)-8-phenyl-7-octenyl (**10b**) caffeate showed the most potent antiproliferative activity (EC_{50} value, 0.02 μM). In addition, CAPE (**2**) induced DNA fragmentation at concentrations of 1 to 10 μg/mL towards murine colon 26-L5 carcinoma cells.

Introduction

Caffeic acid phenethyl ester (CAPE), which was firstly isolated from Hungarian propolis,[1] is a well known constituent with interesting biological properties.[2] Grunberger et al. reported the significant cytotoxic effect of CAPE against various tumor cell lines.[3] Because of its simplicity in structure and of interesting cytotoxic property, its antitumor activity was further studied.[4–9] Besides cytotoxic and antitumor properties, CAPE also possessed inhibitory activity against HIV-1 integrase, cyclooxygenase and lipoxygenase.[10–12] It has also reported to block the activation of nuclear factor NF-κB by tumor necrosis factor and completely block the production of reactive oxygen species (ROS) in human neutrophils.[13,14]

In our recent work, we have isolated CAPE (**2**) together with benzyl caffeate (**1**) and cinnamyl caffeate (**9**) from The Netherlands propolis as potent antiproliferative agents.[15] They showed selective antiproliferative activity toward highly liver-metastatic murine colon 26-L5 carcinoma cells, which encouraged us to further investigate the structure–activity relationship on CAPE analogues. Thus, we synthesized CAPE (**2**) and twenty analogues, including benzyl and cinnamyl caffeates (**1**, **3**–**21**), and examined their antiproliferative activities toward six tumor cell lines.

Results and Discussion

Chemistry

Caffeic acid phenethyl ester (CAPE) has been synthesized previously by several groups.[3–5,14,16] Among the synthetic methods, we selected one-pot esterification of caffeic acid and alcohol with thionyl chloride ($SOCl_2$) in dioxane, reported by Lee et al.[5] to prepare CAPE and its analogues except for methyl (**13**) and ethyl caffeates (**14**). The percentage yield of individual compounds are given in Table 1. Methyl (**13**) and ethyl caffeates (**14**) were prepared by classical acid-catalyzed esterification of caffeic acid with methanol and ethanol, respectively.

Alcohols **23**–**26**, corresponding to esters **7**, **8**, **10** and **11**, are not commercially available, and thus we synthesized them from the corresponding bromoalcohol and benzaldehyde through Wittig reaction (Scheme 1). First, the hydroxyl group of commercially available 7-bromoheptanol (**22a**) and 11-bromoundecanol (**23a**) was protected as

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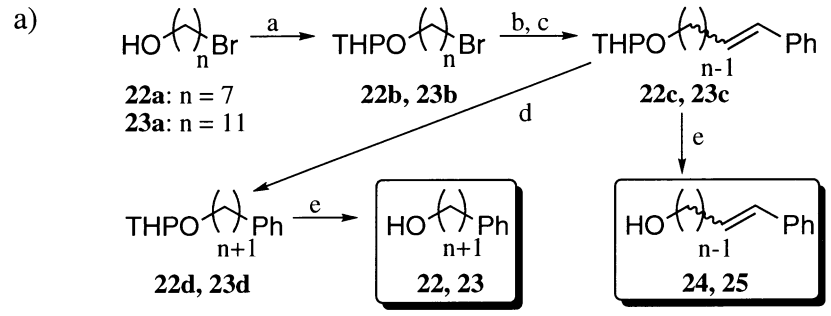
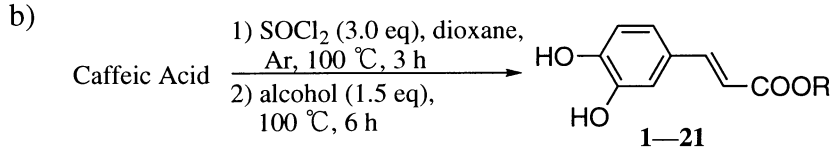


a: 2,3-dihydropyrane, *p*-TsOH, $Cl(CH_2)_2Cl$, r.t., 4h b: PPh_3, neat, 120 °C, 1 h
c: *n*-BuLi, benzaldehyde, THF, -15 °C—r.t., 2 h d: Pd/C, H_2, EtOH, r.t., 2 h
e: *p*-TsOH, MeOH, r.t., 1h



Scheme 1. (a) Synthetic procedure of alcohols **23**–**26**; (b) equation of esterificatin of caffeic acid and alcohols.

Table 1. Yields of CAPE analogues

Product	R	Yield (%)
1	$-CH_2Ph$	75
2	$-(CH_2)_2Ph$	55
3	$-(CH_2)_3Ph$	55
4	$-(CH_2)_4Ph$	57
5	$-(CH_2)_5Ph$	53
6	$-(CH_2)_6Ph$	56
7	$-(CH_2)_8Ph$	41
8	$-(CH_2)_{12}Ph$	54
9	$-CH_2CH=CHPh$	36
10	$-(CH_2)_6CH=CHPh$	40
11	$-(CH_2)_{10}CH=CHPh$	50
12	$-(CH_2)_2$-*c*-Hex	74
13	$-CH_3$	40
14	$-CH_2CH_3$	17
15	$-(CH_2)_2CH_3$	58
16	$-(CH_2)_3CH_3$	76
17	$-(CH_2)_7CH_3$	64
18	$-(CH_2)_9CH_3$	48
19	$-(CH_2)_{11}CH_3$	57
20	$-(CH_2)_{13}CH_3$	45
21	$-(CH_2)_{15}CH_3$	35

a tetrahydropyranyl (THP) ether. The protected bromides **22b** and **23b** were reacted with triphenylphosphine to yield phosphonium salts, followed by Wittig reaction with benzaldehyde to give olefins **22c** and **23c**, respectively. These alkenyl products were reduced by using Pd/C catalyst, and the products **22d** and **23d** were deprotected by *p*-toluenesulfonic acid (*p*-TsOH) to afford the saturated alcohols **22** and **23**, respectively. On the other hand, unsaturated alcohols **24** and **25** were prepared through deprotection of **22c** and **23c** as a 77/23 mixture of *cis*/*trans* isomers, which were used to prepare the esters **10** and **11** as a mixture. The *cis*/*trans* mixtures of **10** and **11** were separated by using HPLC and the antiproliferative activity of the individual compounds were examined.

Antiproliferative activity

Caffeic acid phenethyl ester (CAPE) together with its twenty analogues (**1**, **3**–**21**) were tested for their antiproliferative activities toward six different tumor cell lines, that is, murine colon 26-L5 carcinoma (colon 26-L5),[17] murine B16-BL6 malonoma (B16-BL6),[18] murine Lewis lung carcinoma (LLC),[19] human HT-1080 fibrosarcoma (HT-1080),[20] human lung A549 adenocarcinoma (A549),[21] and human cervix HeLa adenocarcinoma (HeLa) cell lines.[22] The antiproliferative activities of **1**–**21** are summarized in terms of their EC_{50} values in Table 2. All these esters showed stronger antiproliferative activity than caffeic acid, and all the compounds except for **20** and **21** showed the strongest activity toward colon 26-L5 cell line. Interestingly, the CAPE analogues revealed no cytotoxic effect toward primary cultured mouse hepatocytes up to 100 μM concentration. This indicates that CAPE and its analogues possessed selective antiproliferative activity toward colon 26-L5 cell line. Especially, the activities of compounds **4**, **10a**, **10b** and **12** (EC_{50}: 0.02, 0.02, 0.02 and 0.03 μM, respectively) were stronger than those of 5-fluorouracil (EC_{50}: 0.06 μM) and doxorubicin (EC_{50}: 0.04 μM), which were used as positive controls. Antiproliferative activity of the *cis* (**10a** and **11a**) and *trans* (**10b** and **11b**) isomers of **10** and **11** possessed similar strength of activity toward all tested cell lines. Moreover, almost all esters possessed stronger antiproliferative activities than those of 5-fluorouracil in B16-BL6 and LLC cell lines, but less active than doxorubicin.

Considering the range of the potent cytotoxic agent ($EC_{50} < 4$ μg/mL) made by Geran et al.,[23] almost all CAPE analogues fall within the potent cytotoxic range against colon 26-L5, B16-BL6 and LLC cell lines. In addition, nearly 50% of the esters also possessed interesting antiproliferative activity toward human HT-1080 fibrosarcoma and HeLa cell lines ($EC_{50} < 4$ μg/mL) but

Table 2. Antiproliferative activities of caffeic acid esters

Compd	EC_{50} (μM)					
	Colon 26-L5	LLC	B16 BL-6	HT-1080	A549	HeLa
1	1.34	5.73	7.90	12.1	19.6	25.3
2	0.15	2.57	2.18	14.4	32.4	10.7
3	0.10	2.32	2.16	18.1	23.5	22.0
4	0.02	2.29	1.99	13.3	31.6	20.0
5	0.08	1.27	2.12	7.38	21.9	10.6
6	0.08	1.40	1.85	10.4	21.4	9.11
7	0.09	0.84	1.81	20.2	22.4	2.61
8	1.75	8.11	17.2	62.8	21.2	18.4
9	0.22	2.16	2.83	17.1	50.0	11.5
10a	0.02	0.88	1.77	10.51	22.3	1.93
10b	0.02	1.01	1.49	10.21	33.5	1.91
11a	1.17	1.93	7.20	37.3	38.1	7.30
11b	2.49	2.42	7.23	38.4	37.5	8.59
12	0.03	0.74	2.00	11.8	28.1	9.54
13	3.27	4.61	16.7	35.2	43.7	26.8
14	1.14	4.39	4.64	33.4	61.9	24.4
15	1.52	3.30	3.87	17.9	42.9	21.9
16	0.27	2.48	2.78	20.2	42.2	4.02
17	0.22	1.19	2.23	20.0	34.6	2.40
18	0.25	1.15	2.06	14.2	21.4	1.85
19	0.29	0.77	1.80	19.1	21.4	2.00
20	16.1	2.56	14.7	19.9	18.5	10.0
21	10.2	13.3	15.6	36.9	23.7	14.6
Caffeic acid	43.6	318	314	257	288	300
5-FU	0.06	4.69	8.76	5.00	3.61	0.76
Doxorubicin	0.04	0.09	0.22	0.06	0.20	0.12

showed less activity toward A549 cell line ($EC_{50} > 4\,\mu g/mL$). Colon 26-L5, B16-BL6 and LLC cell lines were derived from murine and the others (HT-1080, HeLa and A549) were derived from human. It indicates that CAPE analogues selectively inhibit murine tumor cell lines rather than human tumor cell lines. Colon 26-L5, B16-BL6, LLC and HT-1080 cell lines are highly metastatic cell lines and are frequently used for metastatic experiments.[24–26] In this study, CAPE analogues showed the significant activity toward these cell lines, except for HT-1080, suggesting that CAPE analogues can be used for anti-metastatic drugs.

In previous reports, CAPE and its analogues showed significant cytotoxic effect against B16, HCT116, A431, HL-60 and oral cancer cell lines.[4,5,27] The EC_{50} values of CAPE analogues toward these cell lines were at least 10 to 100 times higher than those against colon 26-L5 carcinoma cell line, in the present study. Thus, CAPE analogues are expected to be good candidates for anticancer agent for colon cancer. In fact, CAPE at a dietary level decreased tumor formation by 63% in C57BL/6J-Min/+ mice, which bear a germline mutation in the *Apc* gene and spontaneously develop numerous intestinal adenomas by 15 weeks of age.[7]

Structure–activity relationship

Considering the structure–activity relationship, neither caffeic acid alone nor the alcohol of an individual esters possessed any significant antiproliferative activity (data not shown), suggesting that the ester structure should be essential for the antiproliferative effect of CAPE analogues. In this study, the esters are mainly divided into four different groups according to the nature of their alcoholic part: esters having an alkyl group with a phenyl group at the end of the alkyl chain (**1**–**8**, group 1), esters having an alkyl group with a stylyl group at the end of the alkyl chain (**9**–**11**, group 2), an ester having an alkyl group with a cyclohexyl group at the end of the alkyl chain (**12**, group 3) and esters having a straight alkyl chain (**13**–**21**, group 4). Comparison of group 1 and group 2 compounds (**3**, **7**, **8** vs **9**, **10**, **11**) revealed that both group possessed almost equal extent of activity against all the tested cell lines. On the other hand, in a comparison of group 1 and group 3 compounds (**2** vs **12**), ester **12** showed stronger antiproliferative activity than ester **2** against all the tested cell lines, which indicates that conversion of a phenyl group into a cyclohexyl group may enhance antiproliferative activity. Moreover, group 1 compounds seem to be more potent than group 4 compounds.

On careful examination of the EC_{50} values of group 1 compounds (**1**–**8**) against colon 26-L5, B16-BL6 and LLC cell lines, it was concluded that the strength of antiproliferative activity was increased from benzyl caffeate (**1**) to 8-phenyloctyl caffeate (**7**). The EC_{50} values of 8-phenyloctyl caffeate (**7**) were 0.09, 0.84, 1.81 μM toward colon 26-L5, B16-BL6 and LLC cell lines, respectively. 12-Phenyldodecanyl caffeate (**8**) showed less antiproliferative activity than 8-phenyloctyl caffeate (**7**) toward all the tested cell lines, indicating further elongation of alkyl chain may decrease the antiproliferative activity.

In the EC_{50} values of group 4 compounds, having a straight chain alkyl group in the alcoholic part, gave more sharp structure–activity relationship in the tested cell lines. The EC_{50} values decreased from methyl caffeate (**13**) having only one carbon on the alcoholic part to *n*-dodecanyl caffeate (**18**) having a C_{10} alkyl chain, while further increase of the chain length of the alkyl part increased the EC_{50} values (Fig. 1). It should be noted here that there is only small differences in the EC_{50} values of **16**, **17** and **18**, having a C_4, C_8 and C_{10} alkyl chain, toward colon 26-L5, B16-BL6 and LLC cell lines. Similar trends were also observed for the other cell lines, although their EC_{50} values were high (Fig. 1). Etzenhouser et al. reported that *n*-octyl ester of caffeic acid is ten times more toxic than CAPE towards L1210 leukemia and MCF-7 breast cancer cell lines.[28] In the present study, however, we found the identical antiproliferative activities between CAPE (**2**) and *n*-octyl caffeate (**17**). These different results may be due to the difference in the cell lines taken in two different experiments. Interestingly, 8-phenyloctyl caffeate (**7**) possessed 5–10 times stronger antiproliferative activities than CAPE (**2**) in all the tested cell lines, except for HT-1080 cell line.

DNA fragmentation

It was well documented that DNA fragmentation is one of the marker for induction of apoptosis.[29] Thus, we further examined DNA fragmentation to clarify whether CAPE analogues induced apoptosis or not. For this experiment, we selected CAPE (**2**) as a model compound and performed on murine colon 26-L5 cell line. Consequently, CAPE (**2**) at concentrations of 1–10 μg/

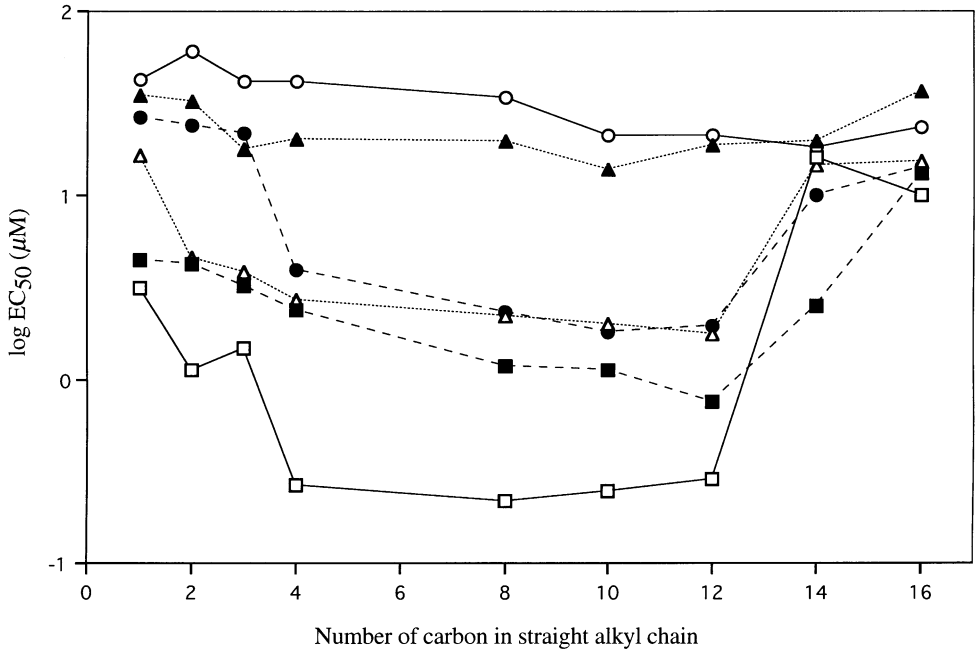


Figure 1. Structure–activity relationship of straight chain alkyl esters **13**–**21** on increasing alkyl chain. □ = murine colon 26-L5 carcinoma, ■ = murine B16-BL6 malonoma, △ = murine Lewis lung carcinoma, ▲ = human HT-1080 fibrosarcoma, ○ = human lung A549 adenocarcinoma, ● = human cervix HeLa adenocarcinoma.

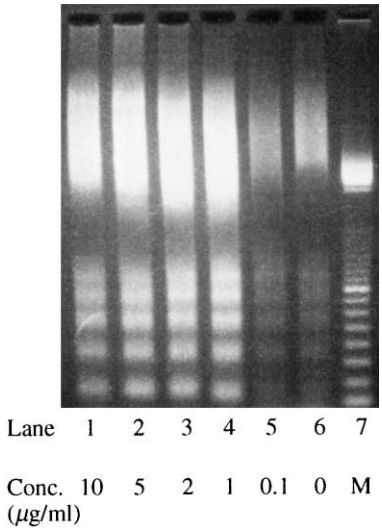


Figure 2. CAPE induced DNA fragmentation in murine colon 26-L5 carcinoma cells. After the cells were cultured for 24 h with various concentration of CAPE, the fragmented DNA was isolated, electrophoresed on 1.5% agarose gel, and then visualized by ethidium bromide staining. Lanes 1–5: treated with 10, 5, 2, 1, 0.1 μg/mL of CAPE, respectively; lane 6: normal; lane 7: 100 base pair ladder marker.

mL caused the DNA fragmentation, indicating the induction of apoptosis in tumor cells (Fig. 2).

In our previous study, we observed CAPE (**2**), benzyl caffeate (**1**) and cinnamyl caffeate (**9**) isolated from the Netherlands propolis possessed scavenging activities toward DPPH free radical equal to those of α-tocopherol and ascorbic acid, well known antioxidants.[15] Moreover, CAPE was reported to completely block the production of ROS in human neutrophils.[14] These reactive species are thought to act as second messengers for signal transduction pathways that regulate cell proliferation.[30] Thus, by the reducing intracellular peroxides, antioxidants are expected to inhibit cancer. Therefore, one of the possible mechanisms for inhibition of cell proliferation by CAPE analogues may be involvement of their antioxidative properties and induction of apoptosis.

Conclusion

In conclusion, we synthesized 21 CAPE analogues, which were tested by MTT assay on growth of six different tumor cell lines. Among the tested cell lines, CAPE analogues possessed selective antiproliferative activity toward colon 26-L5 cell line, especially, esters **4**, **10a**, **10b** and **12** showed stronger activity than positive controls, 5-fluorouracil and doxorubicin. Considering the structure–activity relationship, ester itself seems to be important for antiproliferative activity, and the structure of the alcoholic part, that is, phenyl group, conjugate double bond and cyclohexyl group, bring only small changes in their activity. However, the strength of antiproliferative activity was increased from benzyl caffeate (**1**) to 8-phenyloctyl caffeate (**7**) against colon 26-L5, B16-Bl6 and LLC cell lines, and further elongation of the alkyl chain decreased the antiproliferative activities. In esters with a straight alkyl chain, the C_8–C_{12} alcoholic chain appeared to be more effective structure for antiproliferative activity.

Experimental

All reagents and solvents were reagent grade or were purified by standard methods before use. IR spectra were

obtained on a Shimadzu IR-408 spectrophotometer. EI-MS and high-resolution FAB-MS (HRFABMS) data were obtained by a Jeol JMS-700T spectrometer. ^{1}H- and ^{13}C NMR spectra were taken on a Jeol JNM-LA400 spectrometer with tetramethylsilane (TMS) as an internal standard; chemical shifts are recorded in δ values. Column chromatography was performed by using Wakogel C-200 silica gel. TLC was carried out on precoated Merck silica gel F_{254} plates (0.25 or 0.5 mm).

General procedure for preparation of caffeic acid esters

A solution of caffeic acid (36 mg, 0.2 mmol) in dioxane (2 mL) under argon was treated with thionyl chloride (44 μL, 0.6 mmol). The mixture was stirred at 100 °C for 3 h. Then alcohol (0.3 mmol) was added dropwise to the mixture, and the mixture was stirred at 100 °C for 6 h. After removal of the solvent under reduced pressure, the residue was subjected to preparative TLC with $CHCl_3$–MeOH (90:10) to give the desired caffeic acid esters. The known esters **1**–**3**, **16** and **17** were identified by comparisons of their spectral data with those in literature.[28]

4-Phenylbutyl caffeate (4). IR (KBr, cm^{-1}): 3490, 3340, 1685, 1595, 1530, 1440, 1355, 1270, 1170, 975, 845, 810; ^{1}H NMR (CD_3OD) δ 7.52 (1H, d, J = 15.9 Hz), 7.30–7.10 (5H, m), 7.03 (1H, d, J = 1.9 Hz), 6.93 (1H, dd, J = 8.2, 1.9 Hz), 6.77 (1H, d, J = 8.2 Hz), 6.24 (1H, d, J = 15.9 Hz), 4.17 (2H, t, J = 6.8 Hz), 2.66 (2H, t, J = 6.8 Hz), 1.78–1.64 (4H, m); ^{13}C NMR (CD_3OD) δ 169.3 (s), 149.5 (s), 146.8 (d), 143.4 (s), 129.4 (d), 129.3 (d), 127.7 (s), 126.8 (s), 122.9 (d), 116.7 (d), 115.14 (d), 115.10 (d), 65.3 (t), 36.4 (t), 29.4 (t), 29.0 (t); HRFABMS calcd for $C_{19}H_{21}O_4$ $[M+H]^+$: 313.1440; found: 313.1440.

5-Phenylpentyl caffeate (5). IR (KBr, cm^{-1}): 3450, 3290, 1675, 1595, 1530, 1440, 1355, 1265, 1170, 975, 845, 810; ^{1}H NMR (CD_3OD) δ 7.52 (1H, d, J = 15.9 Hz), 7.26–7.08 (5H, m), 7.04 (1H, d, J = 1.9 Hz), 6.92 (1H, dd, J = 8.2, 1.9 Hz), 6.78 (1H, d, J = 8.2 Hz), 6.23 (1H, d, J = 15.9 Hz), 4.14 (2H, t, J = 6.6 Hz), 2.61 (2H, t, J = 7.6 Hz), 1.70 (2H, quintet, J = 7.3 Hz), 1.65 (2H, quintet, J = 7.6 Hz), 1.41 (2H, quintet, J = 7.6 Hz); ^{13}C NMR (CD_3OD) δ 169.3 (s), 149.5 (s), 146.7 (d), 143.6 (s), 129.4 (d), 129.2 (d), 127.7 (s), 126.7 (s), 122.9 (d), 116.5 (d), 115.2 (d), 115.1 (d), 65.4 (t), 36.7 (t), 32.2 (t), 29.62 (t), 26.58 (t); HRFABMS calcd for $C_{20}H_{23}O_4$ $[M+H]^+$: 327.1569; found: 327.1582.

6-Phenylhexyl caffeate (6). IR (KBr, cm^{-1}): 3480, 3250, 1675, 1595, 1530, 1445, 1360, 1270, 1175, 975, 850, 810; ^{1}H NMR (CD_3OD) δ 7.53 (1H, d, J = 15.9 Hz), 7.26–7.08 (5H, m), 7.04 (1H, d, J = 2.2 Hz), 6.93 (1H, dd, J = 8.2, 2.2 Hz), 6.77 (1H, d, J = 8.2 Hz), 6.24 (1H, d, J = 15.9 Hz), 4.14 (2H, t, J = 6.6 Hz), 2.60 (2H, t, J = 7.7 Hz), 1.68 (2H, quintet, J = 6.6 Hz), 1.63 (2H, quintet, J = 7.7 Hz), 1.48–1.32 (4H, m); ^{13}C NMR (CD_3OD) δ 169.4 (s), 149.5 (s), 146.8 (d), 143.8 (s), 129.4 (d), 129.2 (d), 127.7 (s), 126.6 (s), 123.0 (d), 122.9 (d), 116.5 (d), 115.2 (d), 115.1 (d), 65.5 (t), 36.8 (t), 32.6 (t), 29.9 (t), 29.8 (t), 26.6 (t); HRFABMS calcd for $C_{21}H_{25}O_4$ $[M+H]^+$: 341.1753; found: 341.1780.

8-Phenyloctyl caffeate (7). IR (KBr, cm^{-1}): 3500, 3340, 1675, 1595, 1535, 1445, 1360, 1270, 1170, 975, 850, 810; ^{1}H NMR (CD_3OD) δ 7.53 (1H, d, J = 15.9 Hz), 7.26–7.08 (5H, m), 7.04 (1H, d, J = 1.9 Hz), 6.92 (1H, dd, J = 8.2, 1.9 Hz), 6.77 (1H, d, J = 8.2 Hz), 6.24 (1H, d, J = 15.9 Hz), 4.14 (2H, t, J = 6.6 Hz), 2.57 (2H, t, J = 7.7 Hz), 1.67 (2H, quintet, J = 6.6 Hz), 1.60 (2H, quintet, J = 7.7 Hz), 1.44–1.24 (8H, m); ^{13}C NMR (CD_3OD) δ 169.3 (s), 149.5 (s), 146.7 (d), 143.9 (s), 129.3 (d), 129.2 (d), 127.7 (s), 126.6 (s), 122.9 (d), 116.5 (d), 115.2 (d), 115.1 (d), 65.5 (t) 36.9 (t), 32.6 (t), 30.4 (t), 30.3 (t), 30.2 (t), 29.8 (t), 27.0 (t); HRFABMS calcd for $C_{23}H_{29}O_4$ $[M+H]^+$: 369.2066; found: 369.2084.

12-Phenyldodecanyl caffeate (8). IR (KBr, cm^{-1}): 3460, 3300, 1685, 1600, 1525, 1445, 1385, 1270, 1175, 970, 855, 810; ^{1}H NMR (CD_3OD) δ 7.53 (1H, d, J = 15.9 Hz), 7.25–7.10 (5H, m), 7.03 (1H, d, J = 1.9 Hz), 6.92 (1H, dd, J = 8.2, 1.9 Hz), 6.77 (1H, d, J = 8.2 Hz), 6.24 (1H, d, J = 15.9 Hz), 4.15 (2H, t, J = 6.6 Hz), 2.57 (2H, t, J = 7.7 Hz), 1.68 (2H, quintet, J = 6.6 Hz), 1.58 (2H, quintet, J = 7.7 Hz), 1.44–1.22 (16H, m); ^{13}C NMR (CD_3OD) δ 169.3 (s), 149.5 (s), 146.8 (d), 143.9 (s), 129.4 (d), 129.3 (d), 127.7 (s), 126.6 (s), 122.9 (d), 116.5 (d), 115.2 (d), 115.1 (d), 65.5 (t) 36.1 (t), 32.7 (t), 30.7 (t), 30.6 (t), 30.30 (t), 30.28 (t), 29.8 (t), 27.0 (t); HRFABMS calcd for $C_{27}H_{37}O_4$ $[M+H]^+$: 425.2692; found: 425.2703.

Cinnamyl caffeate (9). IR (KBr, cm^{-1}): 3450, 3300, 1675, 1630, 1595, 1530, 1445, 1355, 1270, 1165, 965, 845, 810; ^{1}H NMR (CD_3OD) δ 7.59 (1H, d, J = 15.9 Hz), 7.42 (2H, br d, J = 7.2 Hz), 7.31 (2H, br t, J = 7.2 Hz), 7.23 (1H, br t, J = 7.2 Hz), 7.05 (1H, d, J = 1.9 Hz), 6.95 (1H, dd, J = 8.2, 1.9 Hz), 6.78 (1H, d, J = 8.2 Hz), 6.70 (1H, d, J = 15.9 Hz), 6.38 (1H, dt, J = 15.9, 6.2 Hz), 6.30 (1H, d, J = 15.9 Hz), 4.82 (2H, d, J = 6.2 Hz); ^{13}C NMR (CD_3OD) δ 169.0 (s), 149.6 (s), 147.1 (d), 146.8 (s), 137.8 (s), 135.0 (d), 129.6 (d), 129.0 (s), 127.7 (d), 124.6 (s), 123.0 (d), 116.5 (d), 115.1 (d), 114.9 (d), 66.0 (t); HRFABMS calcd for $C_{18}H_{17}O_4$ $[M+H]^+$: 297.1127; found: 297.1129.

2-*cyclo*Hexylethyl caffeate (12). IR (KBr, cm^{-1}): 3460, 3290, 1675, 1595, 1530, 1445, 1360, 1260, 1160, 975, 850, 810; ^{1}H NMR (CD_3OD) δ 7.52 (1H, d, J = 15.9 Hz), 7.03 (1H, d, J = 1.9 Hz), 6.94 (1H, dd, J = 8.2, 1.9 Hz), 6.77 (1H, d, J = 8.2 Hz), 6.24 (1H, d, J = 15.9 Hz), 4.20 (1H, t, J = 6.7 Hz), 1.83–1.62 (6H, m), 1.58 (2H, q, J = 6.7 Hz), 1.48–1.35 (1H, m), 1.35–1.13 (2H, m), 1.04–0.91 (2H, m); ^{13}C NMR (CD_3OD) δ 169.4 (s), 149.5 (s), 146.8 (d), 127.7 (s), 122.9 (d), 116.5 (d), 115.2 (d), 115.1 (d), 63.6 (t), 37.3 (t), 35.9 (d), 34.3 (t), 27.6 (t), 27.3 (t); HRFABMS calcd for $C_{17}H_{23}O_4$ $[M+H]^+$: 291.1596; found: 291.1598.

***n*-Propyl caffeate (15).** IR (KBr, cm^{-1}): 3450, 1660, 1600, 1530, 1445, 1315, 1270, 1180, 1110, 1035, 980, 870, 810; ^{1}H NMR (CD_3OD) δ 7.53 (1H, d, J = 15.9 Hz), 7.04 (1H, d, J = 1.9 Hz), 6.94 (1H, dd, J = 8.2, 1.9 Hz), 6.78 (1H, d, J = 8.2 Hz), 6.25 (1H, d, J = 15.9 Hz), 4.12 (2H, t, J = 7.2 Hz), 1.71 (2H, sextet, J = 7.2 Hz), 0.99 (3H, t, J = 7.2 Hz); ^{13}C NMR (CD_3OD) δ 169.4 (s), 149.5 (s), 146.7 (d), 127.7 (s), 122.9 (d), 116.5

(d), 115.2 (d), 115.1 (d), 67.0 (t), 23.1 (t), 10.7 (q); HRFABMS calcd for $C_{12}H_{15}O_4$ $[M+H]^+$: 223.0970; found: 223.0980.

***n*-Decanyl caffeate (18).** IR (KBr, cm^{-1}): 3460, 3290, 1675, 1600, 1530, 1445, 1360, 1270, 1170, 1105, 1010, 975, 860, 810; ^1H NMR (CD_3OD) δ 7.53 (1H, d, $J=15.9$ Hz), 7.04 (1H, d, $J=1.9$ Hz), 6.93 (1H, dd, $J=8.2$, 1.9 Hz), 6.78 (1H, d, $J=8.2$ Hz), 6.24 (1H, d, $J=15.9$ Hz), 4.16 (2H, t, $J=6.6$ Hz), 1.68 (2H, quintet, $J=7.0$ Hz), 1.46–1.21 (14H, m), 0.89 (3H, t, $J=7.0$ Hz); ^{13}C NMR (CD_3OD) δ 169.4 (s), 149.5 (s), 146.8 (d), 127.7 (s), 122.9 (d), 116.5 (d), 115.2 (d), 115.1 (d), 65.6 (t), 33.0 (t), 30.6 (t), 30.4 (t), 30.3 (t), 29.8 (t), 27.1 (t), 23.7 (t), 14.4 (q); HRFABMS calcd for $C_{19}H_{29}O_4$ $[M+H]^+$: 321.2066; found: 321.2057.

***n*-Dodecanyl caffeate (19).** IR (KBr, cm^{-1}): 3490, 3310, 1680, 1605, 1535, 1445, 1360, 1275, 1175, 1110, 1025, 975, 860, 815; ^1H NMR (CD_3OD) δ 7.43 (1H, d, $J=15.9$ Hz), 6.93 (1H, d, $J=1.9$ Hz), 6.83 (1H, dd, $J=8.2$, 1.9 Hz), 6.67 (1H, d, $J=8.2$ Hz), 6.14 (1H, d, $J=15.9$ Hz), 4.06 (2H, t, $J=6.7$ Hz), 1.59 (2H, quintet, $J=6.7$ Hz), 1.37–1.02 (18H, m), 0.79 (3H, t, $J=6.9$ Hz); ^{13}C NMR (CD_3OD) δ 169.3 (s), 149.5 (s), 146.8 (d), 127.7 (s), 122.9 (d), 116.5 (d), 115.2 (d), 115.1 (d), 65.5 (t), 33.0 (t), 30.73 (t), 30.65 (t), 30.6 (t), 30.4 (t), 30.3 (t), 29.8 (t), 27.1 (t), 23.7 (t), 14.5 (q); HRFABMS calcd for $C_{21}H_{33}O_4$ $[M+H]^+$: 349.2379; found: 349.2381.

***n*-Tetradecanyl caffeate (20).** IR (KBr, cm^{-1}): 3480, 3300, 1675, 1600, 1530, 1445, 1355, 1275, 1175, 1105, 975, 855, 815; ^1H NMR (CD_3OD) δ 7.43 (1H, d, $J=15.9$ Hz), 6.93 (1H, d, $J=2.0$ Hz), 6.83 (1H, dd, $J=8.2$, 2.0 Hz), 6.67 (1H, d, $J=8.2$ Hz), 6.14 (1H, d, $J=15.9$ Hz), 4.06 (2H, t, $J=6.7$ Hz), 1.60 (2H, quintet, $J=6.7$ Hz), 1.40–1.10 (22H, m), 0.80 (3H, t, $J=6.8$ Hz); ^{13}C NMR (CD_3OD) δ 169.3 (s), 149.5 (s), 146.8 (d), 127.7 (s), 122.9 (d), 116.4 (d), 115.2 (d), 115.1 (d), 65.5 (t), 33.0 (t), 30.74 (t), 30.71 (t), 30.63 (t), 30.59 (t), 30.4 (t), 30.3 (t), 29.8 (t), 27.1 (t), 23.7 (t), 14.5 (q); HRFABMS calcd for $C_{23}H_{37}O_4$ $[M+H]^+$: 377.2692; found: 377.2678.

***n*-Hexadecanyl caffeate (21).** IR (KBr, cm^{-1}): 3490, 3300, 1680, 1605, 1530, 1445, 1360, 1280, 1175, 1110, 1020, 975, 860, 810; ^1H NMR (CD_3OD) δ 7.43 (1H, d, $J=15.9$ Hz), 6.93 (1H, d, $J=2.0$ Hz), 6.83 (1H, dd, $J=8.2$, 2.0 Hz), 6.67 (1H, d, $J=8.2$ Hz), 6.14 (1H, d, $J=15.9$ Hz), 4.06 (2H, t, $J=6.7$ Hz), 1.59 (2H, quintet, $J=6.7$ Hz), 1.38–1.10 (26H, m), 0.79 (3H, t, $J=6.8$ Hz); ^{13}C NMR (CD_3OD) δ 169.3 (s), 149.5 (s), 146.8 (d), 127.7 (s), 122.9 (d), 116.4 (d), 115.2 (d), 115.1 (d), 65.5 (t), 33.0 (t), 30.8 (t), 30.7 (t), 30.6 (t), 30.4 (t), 30.3 (t), 29.8 (t), 27.1 (t), 23.7 (t), 14.5 (q); HRFABMS calcd for $C_{25}H_{41}O_4$ $[M+H]^+$: 405.3005; found: 405.3003.

Separation of compounds 10 and 11. Separations of *cis*/*trans* isomers of **10** and **11** were conducted by HPLC with a Shimadzu LC-5A system using a Discovery C18 column (21.2 mm i.d.×25 cm; Supelco, USA). The mobile phase was MeOH–H_2O (78:22) for the separation of **10** and MeOH–H_2O (87:13) for the separation of **11**, and UV (254 nm) was used for detection.

(*Z*)-8-Phenyl-7-octenyl caffeate (10a). Retention time: 34.5 min; IR (KBr, cm^{-1}): 3480, 3300, 1680, 1635, 1595, 1530, 1445, 1365, 1270, 1170, 975, 855, 810; ^1H NMR (CD_3OD) δ 7.52 (1H, d, $J=15.9$ Hz), 7.36–7.10 (5H, m), 7.03 (1H, d, $J=1.7$ Hz), 6.92 (1H, dd, $J=8.2$, 1.7 Hz), 6.77 (1H, d, $J=8.2$ Hz), 6.40 (1H, d, $J=11.7$ Hz), 6.23 (1H, d, $J=15.9$ Hz), 5.64 (1H, dt, $J=11.7$, 7.2 Hz), 4.13 (2H, t, $J=6.7$ Hz), 2.32 (2H, q, $J=7.2$ Hz), 1.68 (2H, quintet, $J=7.0$ Hz), 1.58–1.25 (6H, m); ^{13}C NMR (CD_3OD) δ 169.3 (s), 149.5 (s), 146.8 (d), 139.0 (s), 133.6 (d), 130.2 (d), 129.7 (d), 129.1 (d), 127.7 (s), 127.5 (d), 122.9 (d), 116.5 (d), 115.2 (d), 115.1 (d), 65.5 (t) 30.8 (t), 29.9 (t), 29.8 (t), 29.3 (t), 26.9 (t); HRFABMS calcd for $C_{23}H_{27}O_4$ $[M+H]^+$: 367.1909; found: 367.1918.

(*E*)-8-Phenyl-7-octenyl caffeate (10b). Retention time: 38.0 min; IR (KBr, cm^{-1}): 3480, 3300, 1680, 1635, 1595, 1530, 1445, 1365, 1270, 1170, 975, 855, 810; ^1H NMR (CD_3OD) δ 7.52 (1H, d, $J=15.9$ Hz), 7.36–7.10 (5H, m), 7.03 (1H, d, $J=1.7$ Hz), 6.92 (1H, dd, $J=8.2$, 1.7 Hz), 6.77 (1H, d, $J=8.2$ Hz), 6.36 (1H, d, $J=15.9$ Hz), 6.23 (1H, dt, $J=15.9$, 7.0 Hz), 6.23 (1H, d, $J=15.9$ Hz), 4.13 (2H, t, $J=6.7$ Hz), 2.21 (2H, q, $J=7.0$ Hz), 1.68 (2H, quintet, $J=7.0$ Hz), 1.58–1.25 (6H, m); ^{13}C NMR (CD_3OD) δ 169.4 (s), 149.5 (s), 146.8 (d), 139.2 (s), 131.6 (d), 131.3 (d), 129.4 (d), 127.8 (d), 127.7 (s), 126.9 (d), 122.9 (d), 116.5 (d), 115.2 (d), 115.1 (d), 65.5 (t) 33.9 (t), 30.4 (t), 29.9 (t), 29.8 (t), 27.0 (t); HRFABMS calcd for $C_{23}H_{27}O_4$ $[M+H]^+$: 367.1909; found: 367.1956.

(*Z*)-12-Phenyl-11-dodecenyl caffeate (11a). Retention time: 34.1 min; IR (KBr, cm^{-1}): 3440, 3290, 1685, 1630, 1595, 1525, 1445, 1365, 1270, 1170, 975, 855, 810; ^1H NMR (CD_3OD) δ 7.43 (1H, d, $J=15.9$ Hz), 7.24–7.00 (5H, m), 6.94 (1H, d, $J=1.9$ Hz), 6.83 (1H, dd, $J=8.2$, 1.9 Hz), 6.67 (1H, d, $J=8.2$ Hz), 6.29 (1H, d, $J=11.8$ Hz), 6.14 (1H, d, $J=15.9$ Hz), 5.53 (1H, dt, $J=11.8$, 7.2 Hz), 4.05 (2H, t, $J=6.7$ Hz), 2.19 (2H, q, $J=7.2$ Hz), 1.57 (2H, quintet, $J=7.0$ Hz), 1.45–1.00 (14H, m); ^{13}C NMR (CD_3OD) δ 169.4 (s), 149.5 (s), 146.8 (d), 139.1 (s), 133.8 (d), 130.0 (d), 129.7 (d), 129.1 (d), 127.7 (s), 127.5 (d), 122.9 (d), 116.5 (d), 115.2 (d), 115.1 (d), 65.5 (t) 30.9 (t), 30.5 (t), 30.4 (t), 30.3 (t), 29.8 (t), 29.4 (t), 27.0 (t); HRFABMS calcd for $C_{27}H_{35}O_4$ $[M+H]^+$: 423.2535; found: 423.2519.

(*E*)-12-Phenyl-11-dodecenyl caffeate (11b). Retention time: 36.9 min; IR (KBr, cm^{-1}): 3440, 3290, 1685, 1630, 1595, 1525, 1445, 1365, 1270, 1170, 975, 855, 810; ^1H NMR (CD_3OD) δ 7.43 (1H, d, $J=15.9$ Hz), 7.24–7.00 (5H, m), 6.94 (1H, d, $J=1.9$ Hz), 6.83 (1H, dd, $J=8.2$, 1.9 Hz), 6.67 (1H, d, $J=8.2$ Hz), 6.25 (1H, d, $J=15.9$ Hz), 6.14 (1H, dt, $J=15.9$, 7.0 Hz), 6.14 (1H, d, $J=15.9$ Hz), 4.05 (2H, t, $J=6.7$ Hz), 2.09 (2H, q, $J=7.0$ Hz), 1.57 (2H, quintet, $J=7.0$ Hz), 1.45–1.00 (14H, m); ^{13}C NMR (CD_3OD) δ 169.4 (s), 149.7 (s), 146.8 (d), 139.3 (s), 131.8 (d), 131.2 (d), 129.4 (d), 127.8 (d), 127.7 (s), 127.5 (d), 122.9 (d), 116.5 (d), 115.2 (d), 115.1 (d), 65.6 (t) 34.1 (t), 30.6 (t), 30.5 (t), 30.3 (t), 29.8 (t), 29.4 (t), 27.1 (t); HRFABMS calcd for $C_{27}H_{35}O_4$ $[M+H]^+$: 423.2535; found: 423.2504.

Preparation of 13 and 14. A solution of caffeic acid (180.16 mg, 1.0 mmol) in methanol or ethanol (30 mL)

containing *p*-TsOH (19.0 mg, 0.1 mmol) was stirred under reflux. After removal of the solvent under reduced pressure, the residue was subjected to silica gel column chromatography using $CHCl_3$–MeOH (90:10) to give methyl caffeate (**13**)[28] and ethyl caffeate (**14**).[28]

Preparation of tetrahydropyranyl ethers 22b and 23b. A solution of bromoalcohol (4.0 mmol) and dihydropyran (6.0 mmol) in dry 1,2-dichloroethane (20 mL) containing *p*-TsOH (0.4 mmol) was stirred for 4 h at room temperature. After removal of the solvent under reduced pressure, the residue was subjected to silica gel column chromatography using hexane–AcOEt (97:3) to give the tetrahydropyranyl ethers.

7-Bromoheptyl tetrahydropyranyl ether (22b). Yield: 94%; ^{1}H NMR ($CDCl_3$) δ 4.57 (1H, dd, J = 4.5, 2.8 Hz), 3.91–3.83 (1H, m), 3.73 (1H, dt, J = 9.6, 6.6 Hz), 3.54–3.47 (1H, m), 3.40 (2H, t, J = 6.7 Hz), 3.38 (1H, dt, J = 9.6, 6.6 Hz), 1.86 (2H, quintet, J = 7.0 Hz), 1.84–1.30 (14H, m); HREIMS calcd for $C_{12}H_{22}O_2Br$ [M^+-H, ^{79}Br]: 277.0803; found: 277.0786, calcd for $C_{12}H_{22}O_2Br$ [M^+-H, ^{81}Br]: 279.0784; found: 277.0777.

11-Bromoundecanyl tetrahydropyranyl ether (23b). Yield: 96%; ^{1}H NMR ($CDCl_3$) δ 4.57 (1H, dd, J = 4.6, 2.4 Hz), 3.89 (1H, ddd, J = 11.2, 7.5, 3.5 Hz), 3.73 (1H, dt, J = 9.6, 6.8 Hz), 3.54–3.46 (1H, m), 3.40 (2H, t, J = 6.8 Hz), 3.38 (1H, dt, J = 9.6, 6.8 Hz), 1.85 (2H, quintet, J = 7.0 Hz), 1.84–1.20 (22H, m); HREIMS calcd for $C_{16}H_{30}O_2Br$ [M^+-H, ^{79}Br]: 333.1429; found: 333.1435, calcd for $C_{16}H_{30}O_2Br$ [M^+-H, ^{81}Br]: 335.1410; found: 335.1412.

Preparation of phenylalkenyl tetrahydropyranyl ethers 22c and 23c. A mixture of THP ethers (3.0 mmol) and triphenylphosphine (3.0 mmol) was stirred for 1 h at 120 °C. After cooling, the reaction mixture was decanted into hexane and ether. Then, to a stirred suspension of the decanted residue (ca. 1.7 mmol) in THF (10 mL) under argon, *n*-BuLi (2 mmol) was added at −10 °C, and the mixture was stirred at −10 °C for 5 min. Benzaldehyde (2 mmol) in THF (10 mL) was added to the mixture and stirred at −10 °C for 2 h. After poured into 1 N HCl at 0 °C, the product was extracted with CH_2Cl_2, and the CH_2Cl_2 layer was dried over Na_2SO_4, and evaporated. The residue was subjected to silica gel column chromatography using hexane–AcOEt (95:5) to give phenylalkenyl tetrahydropyranyl ethers.

8-Phenyl-7-octenyl tetrahydropyranyl ether (22c). Yield: 25% (*cis*/*trans* = 77/23); ^{1}H NMR ($CDCl_3$) δ 7.40–7.15 (5H, m), 6.40 (1H, d, J = 11.6 Hz, *cis*), 6.37 (1H, d, J = 15.9 Hz, *trans*), 6.21 (1H, dt, J = 15.9, 6.8 Hz, *trans*), 5.65 (1H, dt, J = 11.6, 7.2 Hz, *cis*), 4.57 (1H, dd, J = 4.4, 2.8 Hz), 3.86 (1H, ddd, J = 11.1, 7.2, 3.6 Hz), 3.72 (1H, dt, J = 9.4, 6.8 Hz), 3.54–3.46 (1H, m), 3.37 (1H, dt, J = 9.6, 6.8 Hz), 2.32 (2H, q, J = 7.2 Hz, *cis*), 2.21 (2H, q, J = 6.8 Hz, *trans*), 1.90–1.30 (14H, m); HREIMS calcd for $C_{19}H_{28}O_2$ [M^+]: 288.2089; found: 288.2072.

12-Phenyl-11-dodecenyl tetrahydropyranyl ether (23c). Yield: 23% (*cis*/*trans* = 77/23); ^{1}H NMR ($CDCl_3$) δ 7.38–7.15 (5H, m), 6.40 (1H, d, J = 11.6 Hz, *cis*), 6.37 (1H, d, J = 15.9 Hz, *trans*), 6.22 (1H, dt, J = 15.9, 6.8 Hz, *trans*), 5.66 (1H, dt, J = 11.6, 7.2 Hz, *cis*), 4.57 (1H, dd, J = 4.4, 2.6 Hz), 3.87 (1H, ddd, J = 11.1, 7.2, 3.6 Hz), 3.73 (1H, dt, J = 9.6, 6.8 Hz), 3.53–3.46 (1H, m), 3.38 (1H, dt, J = 9.6, 6.8 Hz), 2.32 (2H, q, J = 7.2 Hz, *cis*), 2.20 (2H, q, J = 6.8 Hz, *trans*), 1.90–1.20 (22H, m); HREIMS calcd for $C_{23}H_{36}O_2$ [M^+]: 344.2715; found: 344.2696.

Reduction of 22c and 23c

The ether (0.8 mmol) was dissolved in MeOH (10 mL) and hydrogenated over 10% palladium on charcoal (100 mg) at room temperature for 2 h. After the catatyst was filtrated off and the solvent was removed under reduced pressure, the residue was subjected to silica gel column chromatography using hexane–AcOEt (97:3) to give phenylalkyl tetrahydropyranyl ethers.

8-Phenyloctyl tetrahydropyranyl ether (22d). Yield: 95%; ^{1}H NMR ($CDCl_3$) δ 7.22–7.06 (5H, m), 4.49 (1H, dd, J = 4.1, 2.6 Hz), 3.79 (1H, ddd, J = 11.3, 7.2, 3.6 Hz), 3.65 (1H, dt, J = 9.6, 6.8 Hz), 3.45–3.38 (1H, m), 3.30 (1H, dt, J = 9.6, 6.8 Hz), 2.52 (2H, t, J = 7.7 Hz), 1.80–1.40 (10H, m), 1.32–1.20 (8H, m); HREIMS calcd for $C_{19}H_{30}O_2$ [M^+]: 290.2246; found: 290.2244.

12-Phenyldodecyl tetrahydropyranyl ether (23d). Yield: 95%; ^{1}H NMR ($CDCl_3$) δ 7.31–7.12 (5H, m), 4.57 (1H, dd, J = 4.4, 2.6 Hz), 3.87 (1H, ddd, J = 11.3, 7.2, 3.6 Hz), 3.73 (1H, dt, J = 9.6, 6.8 Hz), 3.53–3.46 (1H, m), 3.38 (1H, dt, J = 9.6, 6.8 Hz), 2.60 (2H, t, J = 7.7 Hz), 1.88–1.45 (10H, m), 1.40–1.20 (16H, m); HREIMS calcd for $C_{23}H_{38}O_2$ [M^+]: 346.2872; found: 346.2889.

Deprotection of 22c, 23c, 22d and 23d. A solution of ether (0.5 mmol) in MeOH (10 mL) containing *p*-TsOH (0.5 mmol) is stirred for 1 h at room temperature. After removal of the solvent under reduced pressure, the residue was subjected to preparative TLC with $CHCl_3$–MeOH (95:5) to give each alcohol.

8-Phenyloctanol (22). Yield: 92%; ^{1}H NMR ($CDCl_3$) δ 7.30–7.15 (5H, m), 3.61 (1H, t, J = 6.8 Hz), 2.60 (2H, t, J = 7.2 Hz), 1.61 (2H, quintet, J = 7.2 Hz), 1.55 (2H, quintet, J = 6.8 Hz), 1.40–1.26 (8H, m); HREIMS calcd for $C_{14}H_{22}O$ [M^+]: 206.1671; found: 206.1675.

12-Phenyldodecanol (23). Yield: 91%; ^{1}H NMR ($CDCl_3$) δ 7.35–7.10 (5H, m), 3.62 (1H, t, J = 6.8 Hz), 2.59 (2H, t, J = 7.2 Hz), 1.61 (2H, qu, J = 7.2 Hz), 1.55 (2H, quintet, J = 6.8 Hz), 1.40–1.20 (16H, m); HREIMS calcd for $C_{18}H_{30}O$ [M^+]: 262.2297; found: 262.2274.

8-Phenyl-7-octenol (24). Yield: 97%; ^{1}H NMR ($CDCl_3$) δ 7.36–7.17 (5H, m), 6.41 (1H, d, J = 11.6 Hz, *cis*), 6.37 (1H, d, J = 15.9 Hz, *trans*), 6.21 (1H, dt, J = 15.9, 6.8 Hz, *trans*), 5.65 (1H, dt, J = 11.6, 7.2 Hz, *cis*), 3.60 (1H, t, J = 6.8 Hz), 2.33 (2H, q, J = 7.2 Hz, *cis*), 2.21 (2H, q, J = 6.8 Hz, *trans*), 1.62–1.26 (8H, m); HREIMS calcd for $C_{14}H_{20}O$ [M^+]: 204.1514; found: 204.1521.

12-Phenyl-11-dodecenol (25). Yield: 94%; ^{1}H NMR ($CDCl_3$) δ 7.28–7.07 (5H, m), 6.32 (1H, d, $J=11.6$ Hz, *cis*), 6.29 (1H, d, $J=15.9$ Hz, *trans*), 6.14 (1H, dt, $J=15.9$, 6.8 Hz, *trans*), 5.58 (1H, dt, $J=11.6$, 7.2 Hz, *cis*), 3.54 (1H, t, $J=6.8$ Hz), 2.24 (2H, q, $J=7.2$ Hz, *cis*), 2.12 (2H, q, $J=6.8$ Hz, *trans*), 1.48 (2H, quintet, $J=7.2$ Hz), 1.37 (2H, quintet, $J=6.8$ Hz), 1.32–1.12 (12H, m); HREIMS calcd for $C_{18}H_{28}O$ [M^+]: 260.2140; found: 260.2142.

Cells

Highly liver metastatic murine colon 26-L5 carcinoma cell line was established by one of the authors (I. Saiki).[17] Highly liver metastatic murine B16-BL6 melanoma cell line, obtained by an in vivo selection procedure for invasion,[18] was kindly provided by Dr. I. J. Fidler (M.D. Anderson Cancer Center, Houston, TX, USA). Highly lung metastatic murine Lewis lung carcinoma (LLC) cell line, originated spontaneously from murine lung,[19] was kindly provided by Dr. K. Takeda (Juntendo University, Tokyo, Japan). Highly metastatic human HT-1080 fibrosarcoma cell line (ATCC # CCL-121)[20] was obtained from American Type Culture Collection (Rockville, MD, USA). Human lung A549 adenocarcinoma (RCB0098)[21] and human cervix HeLa adenocarcinoma (RCB0007)[22] cell lines were purchased from Riken Cell Bank (Tsukuba, Japan).

B16-BL6, LLC, HT-1080, A549 and HeLa cell lines were maintained in 75-cm^2 cell culture flasks in Eagle's minimum essential medium (EMEM) supplemented with 10% heat inactivated fetal calf serum (FCS), 2 mM L-(+)-glutamine and 0.1% sodium hydrogen carbonate at 37 °C under a humidified 5% carbon dioxide. Colon 26-L5 cell line was maintained in RPMI 1640 medium containing the same supplement under the same conditions.

Antiproliferative activity

Viability of cells other than LLC, in the presence or absence of compounds, was determined using the standard MTT assay[31] as described previously.[32] In brief, exponentially growing cells were harvested and 100 μL medium per well with 2×10^3 cells suspended was plated in 96-well plate. After 24 h incubation at 37 °C under a humidified 5% carbon dioxide to allow cell attachment, the cells were treated with varying concentrations of test specimens in their respective medium (100 μL) and incubated for 72 h under the same conditions. After 2 h of the MTT (0.4–0.5 mg/mL, 100 μL) addition, the formazan formed was extracted with dimethyl sulfoxide (DMSO) and its amount was measured spectrophotometrically at 550 nm with Perkin-Elmer HTS-7000 Bio Assay Reader (Norwalk, CT, USA).

In the case of LLC cells, standard crystal violet staining assay was used in following the literature procedure.[25] In brief, exponentially growing cells were harvested and 100 μL medium per well with 1×10^3 cells suspended was plated in 96-well plate. After 24 h of incubation at 37 °C under a humidified 5% carbon dioxide atmosphere, 100-μL medium containing various concentration of test specimen was added to each well and incubated for 72 h under the same conditions. After fixation with 25% glutaraldehyde solution (20 μL), the cells were stained with 0.5% crystal violet in 20% methanol/water for 30 min. After gentle rinsing with water, the retained crystal violet was extracted with 30% acetic acid and measured spectrophotometrically at 590 nm.

Each esters was dissolved in a bit of DMSO, and then diluted by the medium; final concentration of DMSO was less than 0.25%. 5-FU and doxorubicin were used as positive controls, and EC_{50} values were calculated from the mean values of data from four wells.

Cytotoxicity of CAPE analogues towards primary cultured mouse hepatocytes

Mouse liver parenchymal cells were isolated according to the procedure described previously.[33,34] In brief, the liver was perfused with Ca^{2+}-free Hank's balanced salt solution (HBSS) containing 0.5% bovine serum albumin (BSA) and 5 mM ethyleneglycol-*O*,*O*-bis(2-aminoethyl)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), then recirculated with collagenase solution composed of Ca^{2+}-free free HBSS, 0.075% collagenase, 4 mM $CaCl_2$, and 0.005% trypsin inhibitor. Isolated hepatocytes were cultured in William's E medium supplemented with 10% FCS, 100 IU/mL penicillin G, 100 μg/mL streptomycin, 100 mM dexamethasone and 50 ng/mL insulin and incubated in 96-well plastic plate (2×10^4 cells/well). After 2 h pre-incubation, the medium was replaced with fresh medium containing test specimens at various concentrations. After 72 h incubation, the hepatocytes viability was assessed by MTT method.

DNA fragmentation

DNA was isolated and detected by the procedure described previously.[35,36] Briefly, murine colon 26-L5 cell ($>2\times10^6$ cells) was preincubated in RPMI medium for 24 h, and then cultured with various concentrations of test specimen in serum free Dulbecco's modified MEM (DMEM/F-12) medium containing 0.1% BSA, 100 IU/mL penicillin G and 80 IU/mL streptomycin for 24 h. At end of the incubation, cells were pelleted and lysed in 600 μL of lysis buffer (10 mM Tris–HCl buffer, pH 8.0, 10 mM EDTA and 0.2% Triton X-100) for 10 min on ice. After the lysate was centrifuged at 14,000 rpm for 10 min, the supernatant was extracted with TE buffer (10 mM Tris–HCl buffer, pH 8.0, 1 mM EDTA)-saturated phenol, and then centrifuged at 14,000 rpm for 10 min. After the upper layer was extracted with CIAA solution (chloroform–isoamylalcohol = 24:1), DNA in the upper layer (500 μL) was precipitated with 3 M NaCl (50 μL) and cold ethanol (1000 μL) at −20 °C overnight. After drying, DNA was dissolved in TE buffer. Contamination of RNA was removed by incubation with 1 mg/mL RNase at 37 °C for 30 min. Following the addition of loading buffer, fragmented DNA was electrophoresed on 1.5% agarose gel in TAE (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) at 100 V for 30 min and visualized by ethidium bromide staining.

References and Notes

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