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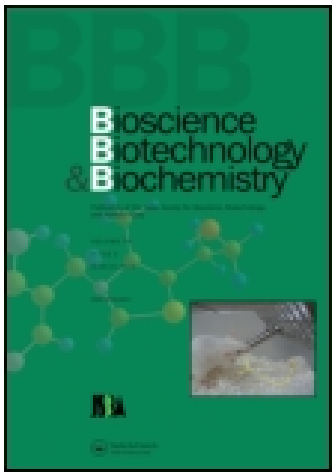

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Inhibitory Effect of Acylphloroglucinol Derivatives on the Replication of Vesicular Stomatitis Virus

Kazuhiro Chiba[a], Takako Takakuwa[a], Masahiro Tada[a] & Takao Yoshii[ab]
[a] Laboratory of Bio-organic Chemistry, Tokyo University of Agriculture and Technology, Fuchu, Tokyo 183, Japan
[b] Central Virus Diagnostic Laboratory, National Institute of Health, 4-7-1 Gakuen, Musashimurayama, Tokyo 190-12, Japan
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Inhibitory Effect of Acylphloroglucinol Derivatives on the Replication of Vesicular Stomatitis Virus

Kazuhiro CHIBA, Takako TAKAKUWA, Masahiro TADA,† and Takao YOSHII*

Laboratory of Bio-organic Chemistry, Tokyo University of Agriculture and Technology, Fuchu, Tokyo 183, Japan
** Central Virus Diagnostic Laboratory, National Institute of Health, 4–7–1 Gakuen, Musashimurayama, Tokyo 190–12, Japan*
Received April 15, 1992

The antiviral activity of natural phloroglucinols and of synthesized mono- and diacylphloroglucinols, and 2,6-diacyl-4,4-dialkylcyclohexa-1,3,5-triones was investigated. A correlation between the acyl chain length and inhibitory activity against vesicular stomatitis virus (VSV) was observed. Potent antiviral activity was found in di-isovalerylphoroglucinol. 2,6-Diacyl-4,4-dialkylcyclohexa-1,3,5-triones inhibited replication of the virus with low cytotoxicity.

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Acylphloroglucinols are found widely in higher plants. Some of these compounds inhibit the growth of bacteria[1] and the photosynthetic electron transport of plants.[2,3] Previously, we have isolated antimicrobial phloroglucinol derivatives from plants belonging to the Guttiferae family.[4,5] Chinesin I (**1**) and II (**2**) (Fig. 1), which were isolated from flowers of *Hypericum chinense* L., showed marked antagonistic activity against both thromboxane A_2 and leukotriene D_4.[6] Furthermore, we found that synthesized diacylphloroglucinols inhibited the replication of VSV and herpes simplex virus type 1 (HSV) which have the envelope.[7] On the other hand, the inhibitory activity of natural phloroglucinols and related compounds against HSV,[8,9] Epstein–Barr virus,[10,11] human immunodeficiency virus-reverse transcriptase,[12] and skintumor[13] has also been reported. Natural phloroglucinol derivatives have varied alkyl and acyl groups, and some of these compounds have no aromaticity by di-substitution of the alkyl groups on the same carbon of a six-membered ring. The correlation, however, between the antiviral activity, cytotoxicity and the structure of phloroglucinol derivatives is not yet known.

In the present investigation, the antiviral activity of chinesins and of twenty-three phloroglucinol derivatives, including five new compounds, was evaluated, and compounds which showed 10^{-6} yield reduction of VSV were obtained.

Results and Discussion

Chinesin I (**1**) and II (**2**) were taken from flowers of *Hypericum chinense* L. as a 3:1 mixture. 2-Acylphloroglucinols (**3**—**6**) and 2,4-diacylphloroglucinols (**7**—**17**)

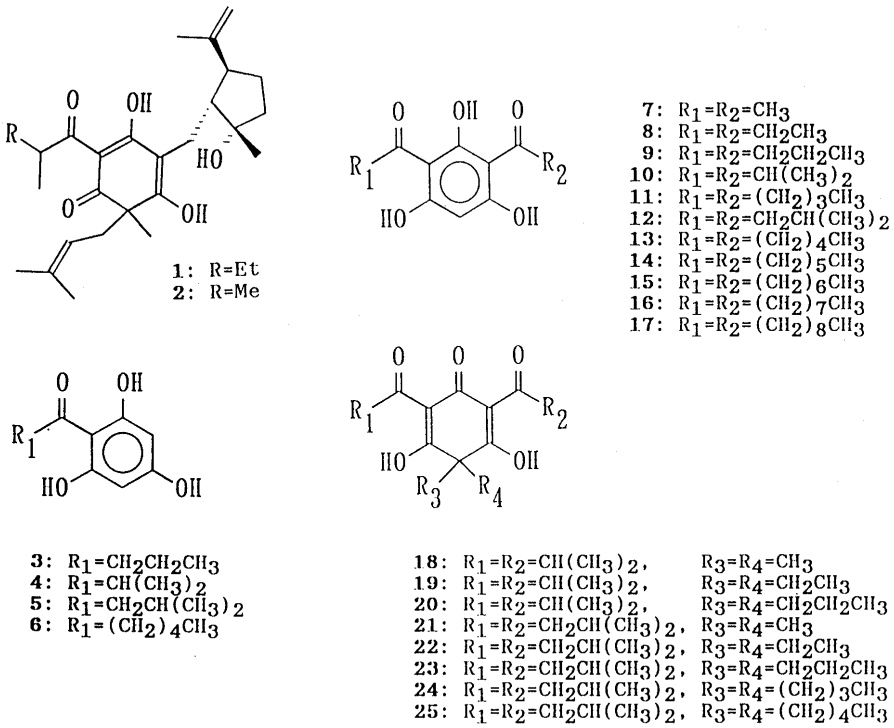

Fig. 1. Structures of Chinesin I (**1**), Chinesin II (**2**), Monoacylphloroglucinols (**3**—**6**), Diacylphloroglucinols (**7**—**17**), and 2,6-Diacyl-4,4-dialkylcyclohexa-1,3,5-triones (**18**—**25**).

† To whom correspondence should be addressed.

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were prepared by treating phloroglucinol with the corresponding carboxylic acid in the presence of BF_3–Et_2O (boron trifluoride diethyl ether) complex. 2,6-Diacyl-4,4-dialkylcyclohexa-1,3,5-triones (**18**—**25**) were obtained from **10** or **12** by alkylating with alkyl iodide in MeONa/MeOH (Fig. 1).

Table I shows the effect of chinesins and acylphlorogluciols on the replication of VSV in human embryonic lung fibroblast (HEL) cells. Monoacylphloroglucinols **3**, **4**, **5**, and **6** show a 10^{-3} yield reduction at concentrations of 32, 17, 14, and more than 10 μg/ml respectively; however, more potent inhibitory activity was found in corresponding diacylphloroglucinols **9**, **10**, **12**, and **13**, which show the same activity at concentrations of 1.2, 1.0, 0.4, and 0.6 μg/ml, respectively. Cytotoxicity was not observed for **3**, **4**, and **5** at a concentration of 50 μg/ml, nor for chinesins and **6** at a concentration of 25 μg/ml. On the other hand, compounds **9**, **10**, **12**, and **13** showed cytotoxicity in 2.5—10 μg/ml. Monoacylphloroglucinols **3** and **4** which possessed an acyl chain composed of four carbons showed lower antiviral activity in comparison with those possessing an acyl chain of five or six carbons (**5** and **6**). Potent antiviral activity was also found in diacylphloroglucinols with an acyl chain composed of five or six carbons (**12** and **13**).

Table I. Antiviral Activity and Cytotoxicity of Chinesins and Phloroglucinol derivatives Toward the Replication of VSV in HEL Cell Cultures

Compound	10^{-3} yield reduction[a] (μg/ml)	10^{-5} yield reduction[a] (μg/ml)	Minimum concn. for cytotoxicity (μg/ml)
1+**2** (3:1)	2.4	7.0	25
3	32	42	50
4	17	24	50
5	14	21	50
6	10<	—[b]	25
9	1.2	2.2	10
10	1.0	2.3	5
12	0.4	1.2	2.5
13	0.6	0.8	2.5
18	13	27	50
19	7.8	14	50
20	6>	11	25
21	3.8	10	25
22	3.1	6.2	25
23	3.0	4.6	25
24	3.0	4.6	25
25	2.2	4.5	25

[a] The virus yield reduction was measured at concentrations of 0.25, 0.5, 1.0, 2.5, 5.0, 10, 25, and 50 μg/ml. The concentrations for 10^{-3} and 10^{-5} yield reductions were calculated by the method of least squares. Virus yield control was at $\log_{10}$ p.f.u./ml = 9.6.

[b] Because of the potent cytotoxicity at 25 μg/ml, an exact determination was difficult in the range of 10 to 25 μg/ml by the method of least squares.

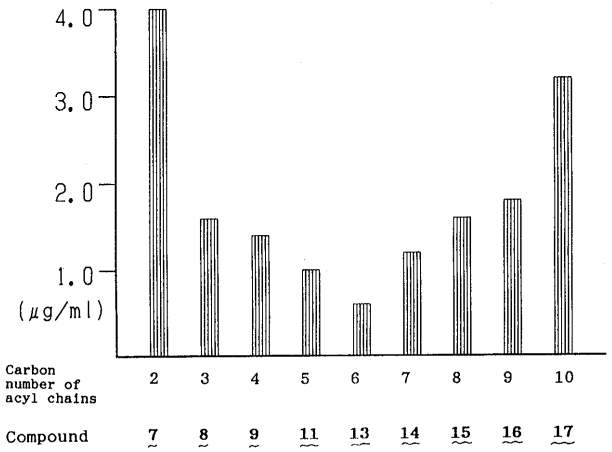

Fig. 2. Effect of the Acyl Chain Length of Diacylphloroglucinols on the 10^{-3} Yield Reduction of VSV in HEL Cell Cultures.

Compounds **7**—**9**, **11**, and **13**—**17** were tested.

Figure 2 shows the change in concentration of each derivative for a 10^{-3} yield reduction of VSV with the acyl chain length of diacylphloroglucinols. These acyl groups were composed of straight chains. Compound **13** had the strongest activity among the diacylphloroglucinols with straight chains so far tested. The antiviral activity of the compounds was decreased by the addition or reduction of the number of acyl-chain carbons. Moreover, compound **10** and **12** with branched acyl chains show a 10^{-3} yield reduction at a lower concentration than that of **9** and **11** (Table I and Fig. 2). This result suggested that the chain length and branching of acyl groups were closely correlated with the antiviral activity.

Diacylphloroglucinols **9**, **10**, **12**, and **13** showed potent antiviral activity, although their cytotoxicity was also strong. On the other hand, the cytotoxicity of chinesins was weaker than that of these compounds. As chinesins have no aromaticity by the di-substituted alkyl groups on the same carbon of the ring, the introduction of 4,4-dialkyl groups in the diacylphloroglucinols is suggested as being effective to reduce the cytotoxicity. Consequently, the antiviral activity of 2,6-diacyl-4,4-dialkyl-cyclohexa-1,3,5-triones which were derived from the potent antiviral diacylphloroglucinols (**10** and **12**), was also evaluated (Table I).

By introducing geminal methyl groups at C-4 of the benzene ring (**18** and **21**), antiviral activity was observed at approximately one tenth the concentration for the corresponding diacylphloroglucinols (**10** and **12**); in addition, this activity was stronger than that of the corresponding monoacylphloroglucinols (**4** and **5**). Furthermore, the antiviral activity of 2,6-diacyl-4,4-dialkylcyclohexa-1,3,5-triones (**22**, **23**, **24**, and **25**) became stronger with increasing carbon number of the 4,4-dialkyl chains.

Desirable antiviral compounds should inhibit virus replication at less than the minimum concentration for cytotoxicity. Diacylphloroglucinol **12** showed potent antiviral activity in reducing the virus yield to 10^{-5} at a concentration of 1.2 μg/ml; however, concentrations for 10^{-6} and less than 10^{-6} yield reduction could not be determined because of the cytotoxicity which became apparent at a concentration of 2.5 μg/ml. On the other hand, although the activity of compounds **22**—**25** was weaker than that of **12**, cytotoxicity was not apparent for compounds **22**—**25** at a concentration of 25 μg/ml. These results suggest that the di-substituted alkyl groups at C-4 were effective for the selectivity in activity and cytotoxicity. This selectivity between the antiviral activity and cytotoxicity from introducing geminal alkyl groups on the six-membered ring enabled compounds **22**—**25** to reduce the virus yield to less than 10^{-6}. The activity of chinesins was

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similar to that of 2,4-diacyl-4,4-dialkylcyclohexa-1,3,5-triones, and 2-acyl-4,4,6-tri-substituted phloroglucinol is throught to have played an important role in their activity with lower cytotoxicity.

Although the mechanism for this selectivity is not clear, the difference between the diacylphloroglucinols and 2,6-diacyl-4,4-dialkylcyclohexa-1,3,5-triones for their antiviral activity and cytotoxicity is interesting, and these results may give important information for the development of suitable drugs for clinincal use.

Experimental

NMR spectra were measured with a JEOL GX-270 spectrometer in a $CDCl_3$ solution containing tetramethylsilane as an internal standard. IR and UV spectra were measured with JASCO IR-810 and JASCO UVDEC-460 UV-VIS spectrophotometers, and MS spectra with a JEOL JMS DX-300 spectrometer with a direct inlet system at 70 eV. Melting point (mp) was determined with micro hot-stage appartus, and the values are uncorrected.

Acylaction of phloroglucinol. The corresponding carboxylic acid (10 mmol) was dissolved in BF_3–Et_2O complex (5.0 ml) at room temperature. Anhydrous phloroglucinol (4.0 mmol) was added to this complex, and the mixture heated over a steambath for 2 h. After cooling, the reaction mixture was added dropwise to aqueous potassium acetate (2.6 g/50 ml). After filtration, the filtrate was dissolved with AcOEt and dried over $MgSO_4$. Evaporation of the dried AcOEt and purification by silica-gel column chromatography (hexane-AcOEt) gave 2-acyl and 2,4-diacylphloroglucinol, respectively. These compounds were identified by their ^{1}H-, and ^{13}C-NMR, IR and UV spectra.[14]

Alkylation of 2,4-diacylphloroglucinols. Anhydrous di-isobutyryl or di-isovalerylphloroglucinol (7.5 mmol) was dissolved in a solution of sodium methoxide (1.0 g of sodium/33 ml of methanol) and alkyl iodide (185 mmol) was slowly added. After the complete addition, the mixture was stirred for 15 min at room temperature, 2 M hydrochloric acid was added, and the reaction mixture was extracted with AcOEt. The combined AcOEt extracts were washed with water, dried over $MgSO_4$ and concentrated. Purification by column chromatography over silica-gel (hexane–AcOEt–AcOH 5–20 : 1 : 0.1) gave compounds **18**—**25** respectively. Compound **18** was identified from its ^{1}H- and ^{13}C-NMR, IR and UV spectra.[14] **19** was obtained as an oil. MS *m/z* (% int.): 322 (9, M^+), 293 (100), 275 (15), 223 (15), 69 (19). ^{1}H-NMR δ: 0.73 (6H, t, J=7.3 Hz), 1.21 (12H, d, J=7.1 Hz), 2.06 (4H, q, J=7.3 Hz), 4.13 (2H, sep, J=7.1 Hz), 18.41 (s), 19.07 (s), and 19.72 (s) (2 : 2 : 3, 2H, chelated OH, contribution from tautomers). ^{13}C-NMR δ: 9.41, 19.03, 19.12, 32.14, 32.79, 35.62, 36.73, 37.35, 56.17, 61.49, 107.83, 107.96, 112.62, 188.67, 193.07, 194.04, 200.00, 208.02, 209.92, 210.76. IR cm^{-1} (neat): 2950, 1650, 1550 br., 1460, 1100, 880. UV λ_{max} (EtOH): 224 nm (log ε=4.06), 245 nm (log ε=4.07), 294 nm (log ε=4.13). **20** was obtained as an oil. MS *m/z* (% int.): 307 (19, $M-C_3H_7$), 293 (16), 238 (13), 223 (31), 151 (10), 69 (59). ^{1}H-NMR δ: 0.83 (6H, t, J=6.5 Hz), 1.05 (4H, m), 1.22 (12H, m), 1.86 (m), and 1.99 (m) (1 : 2, 4H), 4.05 (2H, m), 18.43 (s), 19.09 (s), and 19.77 (s) (1 : 1 : 1, 2H, chelated OH, contribution from tautomers). ^{13}C-NMR: 14.06, 14.28, 18.03, 18.23, 18.97, 19.00, 19.09, 19.26, 21.94, 23.80, 35.61, 36.66, 37.30, 39.41, 41.34, 42.15, 42.24, 55.09, 60.37, 107.48, 112.05, 182.09, 188.53, 193.57, 194.09, 200.27, 208.07, 209.89, 210.68. IR cm^{-1} (neat): 2950, 1650, 1550 br., 1460, 1100, 880. UV λ_{max} (EtOH): 230 nm (log ε=3.69), 240 nm (log ε=3.72), 282 nm (log ε=3.89). **21** was obtained as an oil. MS *m/z* (% int.): 322 (82, M^+), 307 (100), 289 (23), 279 (62), 265 (51), 251 (49), 195 (41), 85 (52), 69 (51). ^{1}H-NMR δ: 0.98 (6H, d, J=7.1 Hz) 1.02 (6H, d, J=7.1 Hz), 1.46 (6H, s), 2.20 (2H, m), 2.87 (2H, d, J=7.6 Hz), 3.01 (2H, d, J=7.6 Hz), 18.40 (s), 18.53 (s), 19.06 (s),and 19.48 (s) (1 : 1 : 5 : 5, 2H, chelated OH, contribution from tautomers). ^{13}C-NMR δ: 22.60, 22.66, 22.71, 23.80, 24.21, 25.86, 26.16, 47.14, 47.45, 49.21, 52.35, 105.54, 106.57, 187.93, 194.85, 201.38, 202.80, 204.63. IR cm^{-1} (neat): 2950, 1662, 1550 br., 1460, 1216, 1092, 843. UV λ_{max} (EtOH): 245 nm (log ε=3.88), 292 nm (log ε=4.02). **22**, mp 28°C; MS *m/z* (% int.): 321 (69, M−Et), 293 (33), 223 (50), 69 (60), 53 (100). ^{1}H-NMR δ: 0.73 (6H, t, J=7.6 Hz), 1.02 (12H, d, J=7.4 Hz), 2.1 (6H, m), 3.0 (4H, m), 18.49 (s), 19.12 (s), and 19.61 (s) (1 : 1 : 1, 2H, chelated OH, contribution from tautomers). ^{13}C-NMR δ: 9.22, 9.42, 22.70, 22.73, 25.55, 25.86, 25.98, 32.20, 32.86, 48.20, 49.47, 49.94, 61.34, 109.06, 113.75, 188.50, 193.14, 194.23, 200.09, 203.18, 205.05. IR cm^{-1} (KBr): 2950, 1650, 1550 br., 1450, 1097, 830. UV λ_{max} (EtOH): 249 nm (log ε=3.92), 288 (log ε=3.99). **23** was obtained as an oil. MS *m/z* (% int.): 335 (18, $M-C_3H_7$), 307 (43), 279 (16), 85 (32), 69 (41), 56 (100). ^{1}H-NMR δ: 0.81 (6H, m), 1.02 (12H, d, J=7.0 Hz), 1.9 (4H, m), 2.19 (2H, m), 3.0 (4H, m), 18.53 (s), 19.13 (s), and 19.63 (s) (1 : 1 : 1, 2H, chelated OH, contribution from tautomers). ^{13}C-NMR δ: 14.28, 18.22, 22.70, 25.83, 42.28, 48.23, 49.39, 49.91, 60.27, 108.57, 108.80, 188.34, 194.30, 200.38, 203.26, 205.03. IR cm^{-1} (neat): 2950, 1650, 1550 br., 1458, 1160, 1095, 850. UV λ_{max} (EtOH): 290 nm (log ε=3.88). **24** was obtained as an oil. MS *m/z* (% int.): 349 (33, $M-C_4H_9$), 307 (33), 293 (15), 85 (30), 69 (42), 56 (100). ^{1}H-NMR δ: 0.80 (6H, t, J=7.1 Hz), 1.0 (4H, m), 1.21 (4H, m), 1.9 (4H, m), 2.2 (2H, m), 3.0 (4H, m), 18.46 (s), 19.03 (s), and 19.60 (s) (1 : 1 : 1, 2H, chelated OH, contribution from tautomers). ^{13}C-NMR δ: 13.74, 22.74, 22.94, 25.64, 25.93, 26.82, 27.03, 39.93, 48.20, 49.45, 49.97, 60.19, 108.97, 200.42, 203.27, 205.09. IR cm^{-1} (neat): 2950, 1650, 1550 br., 1450, 1100. UV λ_{max} (EtOH): 290 nm (log ε=3.79). **25** was obtained as an oil. MS *m/z* (% int.): 434 (3, M^+), 364 (50), 363 (44), 307 (42), 85 (31), 69 (45), 56 (100). ^{1}H-NMR δ: 0.82 (6H, t, J=7.0), 1.0 (4H, m), 1.02 (12H, d, J=7.3 Hz), 1.18 (8H, m), 1.9 (4H, m), 2.2 (2H, m), 3.0 (4H, m), 18.47 (s), 19.07 (s), and 19.59 (s) (1 : 1 : 1, 2H, chelated OH, contribution from tautomers). ^{13}C-NMR δ: 13.86, 22.21, 22.24, 22.73, 24.31, 24.50, 25.67, 25.95, 31.73, 31.94, 39.26, 40.10, 48.18, 49.42, 49.94, 60.25, 108.93, 113.35, 188.36, 193.69, 194.36, 200.43, 203.26, 205.05, 205.95. IR cm^{-1} (neat): 2950, 1650, 1550 br., 1450, 1100, 880. UV λ_{max} (EtOH): 290 nm (log ε=3.86).

Cell culture and virus. RL-33 cells derived from rabbit lung were grown in Eagle's minimum essential medium (MEM) containing 10% calf serum, 0.11% $NaHCO_3$ and antibiotics (100 U/ml of penicillin and 100 μg/ml of streptomycin).[15,16] The maintenance medium was MEM supplemented with 0.15% $NaHCO_3$, 2% fetal bovine serum and antibiotics. For the growth of HEL cells, the concentration of $NaHCO_3$ was reduced to 0.075%. The Indiana serotype of VSV was propagated in HEL cells with the maintenance medium and stored at −70°C until needed for use.

Infectivity assay. Plaque titration was performed in RL-33 cells. Briefly, conflunt monolayers of the cells in 60-ml prescription bottles inoculated with an appropriately diluted virus in 0.2 ml amounts. After virsu adsorption for 1 h at 37°C, the infected cell cultures were overlaid with 5 ml amounts of agar medium, and incubated at 37°C. The agar overlay medium consisted of MEM containing 0.8% BiTek-agar (Difco), 0.15% $NaHCO_3$, 2% fetal bovine serum, and antibiotics (as in the growth medium). A second overlay medium containing 0.006% neutral red was added in 4 ml amounts to the cultures on the second day post infection (p.i.). The plaques were counted on day 3 p.i.

Replication of VSV in the presence or absence of the compounds. A multicycle growth method was employed in order to observe the growth characteristics of VSV. Confluent monolayers of HEL cells grown in 60-ml prescription bottles were infected with VSV at an input m.o.i. (multiplicity of infection) of 0.01 p.f.u. (plaque forming unit)/cell, and the virus was allowed to adsorb for 1 h at 37°C. The infected cell cultures were then washed 3 times with MEM to remove the unadsorbed virus, and 5 ml amounts of the maintenance medium in the presence or absence of various concentrations of phloroglucinol derivatives were immediately added. Thereafter, the cell cultures were incubated at 37°C for 24 h. These cultures were disrupted by 3 cycles of freezing and thawing, and the supernatant fluid obtained by low speed centrigugation was tested by an infectivity assay. Cytotoxicity was evalutated by the minimum concentration of the compounds to produce morphological transformation in the HEL cells.

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