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Cytotoxic and Antiherpetic Activity of Phloroglucinol Derivatives from *Mallotus japonicus* (Euphorbiaceae)

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The phloroglucinol derivatives isolated from *Mallotus japonicus* MUELL. ARG. (Euphorbiaceae) and their derivatives were evaluated for their capacity to produce cytotoxicity in HeLa cells and to inhibit the replication of herpes simplex virus type 1 (HSV-1). The characterizations of an isolated new acetophenone, mallophenone (16), and cyclization products of mallotojaponin (1), isomallotochromene (17), mallotochroman (18) and isomallotochroman (19), were also described. All tested derivatives inhibited the replication of HSV-1 with ED_{50} in the range of 88 ng—48 μg/ml. The derivatives 12 and 19 were found *in vitro* therapeutic index with 10.9 and 9.1, respectively, and they were considered to be active antivirals.

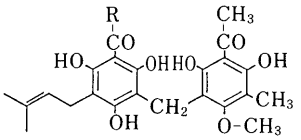
Keywords *Mallotus japonicus*; Euphorbiaceae; phloroglucinol derivative; cytotoxicity; antiherpetic activity

Introduction

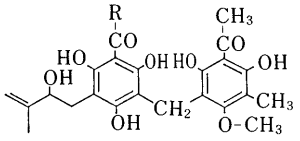
Many research efforts on antiviral agents from natural resources have been practiced in order to find useful drugs for the treatment of herpes virus infections. It's a well known fact that herpes viruses have been related to the cause of several carcinomas.[1-4] In recent years, the antiherpetic activity of phenolic derivatives from plant sources was described by some authors.[5,6]

As part of a program for biologically active constituents from natural resources, we have been studying cytotoxic constituents in the pericarps of *Mallotus japonicus* MUELL. ARG. (Euphorbiaceae) and have reported the isolation and structural elucidation of several new phloroglucinol derivatives, and the cytotoxicity and antitumor activity of the isolated compounds.[7-12]

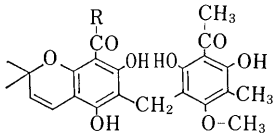
In this work, we have attempted to evaluate the capacity of phloroglucinol derivatives to produce cytotoxicity in HeLa cells and to inhibit the replication of herpes simplex virus type 1 (HSV-1). Compounds **1**—**16** were isolated from the pericarps and compounds **17**—**19** were derived from compound **1** with cyclization of its side chain. Compound **16** is a new acetophenone and its characterization is described together with the derivation of compounds **17**—**19** in this paper.



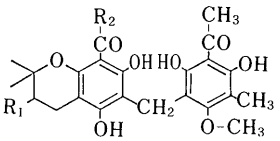
R=CH_3: mallotojaponin (**1**)
R=CH_2–CH_2–CH_3: butyrylmallotojaponin (**2**)
R=$CH(CH_3)_2$: isobutyrylmallotojaponin (**3**)



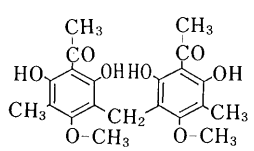
R=CH_3: mallotolerin (**4**)
R=CH_2–CH_2–CH_3: butyrylmallotolerin (**5**)
R=$CH(CH_3)_2$: isobutyrylmallotolerin (**6**)



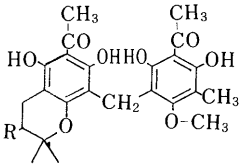
R=CH_3: mallotochromene (**7**)
R=CH_2–CH_2–CH_3: butyrylmallotochromene (**8**)
R=$CH(CH_3)_2$: isobutyrylmallotochromene (**9**)



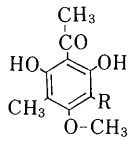
R_1=OH, R_2=CH_3: mallotochromanol (**11**)
R_1=OH, R_2=CH_2–CH_2–CH_3: butyrylmallotochromanol (**12**)
R_1=OH, R_2=$CH(CH_3)_2$: isobutyrylmallotochromanol (**13**)
R_1=H, R_2=CH_3: mallotochroman (**18**)



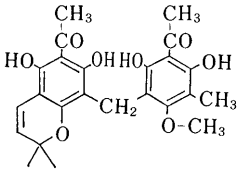
mallotophenone (**10**)



R=OH: isomallotochromanol (**14**)
R=H: isomallotochroman (**19**)



R=H: (**15**)
R=CH_3: mallophenone (**16**)



isomallotochromene (**17**)

Chart 1

Results and Discussion

The 15% EtOAc/*n*-hexane eluent from silica gel column chromatography of the C_6H_6 fraction[5] was further separated on a silica gel column by elution with C_6H_6/EtOAc mixture to give **16**.

Compound **16**, $C_{11}H_{14}O_4$, gave a positive $FeCl_3$ reaction. The molecular of **16** is 14 mass (CH_2) larger than that of compound **15**. The ultraviolet (UV) and infrared (IR) spectra were similar to those of **15**. The proton nuclear magnetic resonance (^{1}H-NMR) spectrum closely resembled that of **15**, except for appearance of the signal of a methyl group instead of a signal of an aromatic proton, and exhibited four singlets for the two methyls, acetyl, methoxyl and two hydroxyl groups at δ 2.11 (6H), 2.72 (3H), 3.71 (3H) and 9.49 ppm (2H), respectively. The carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectrum also closely resembled that of **15** with a slight difference in the signals and multiplicity of aromatic carbons, and showed eight signals at δ 8.18 ($CH_3 \times 2$), 33.36 ($CO\underline{C}H_3$), 60.31 (OCH_3), 107.28 (C-1), 108.49 (C-3 and 5), 158.38 (C-2 and 6), 162.85 (C-4) and 204.58 ppm (CO). From these spectral data, the structure of **16** was determined to be 2,6-dihydroxy-3,5-dimethyl-4-methoxyacetophenone, and it was named mallophenone (**16**).

Compound **17** was derived from **1** by cyclization of its side chain with a hydroxy group at *para*-position to an acetyl group in the presence of 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ).[13] The structure of **17** was determined to be 6-acetyl-5,7-dihydroxy-8-(3-acetyl-2,4-dihydroxy-5-methyl-6-methoxybenzyl) 2,2-dimethylchromene (**17**) by the comparison of ^{1}H-NMR data of **17** with those of the mallotochromene (**7**) cyclization compound of a side chain with a hydroxy group at *ortho*-position to the acetyl group on **1**, that is an isomer of **7**, and it was conveniently named isomallotochromene (**17**). Compounds **18** and **19** were also derived from **1** by cyclization of its side chain with a hydroxy group in the presence of formic acid,[14] and they were separated from the reaction mixture on preparative thin layer chromatography (PLC) described in the experimental section. The structures of **18** and **19** were determined to be 8-acetyl-5,7-dihydroxy-6-(3-acetyl-2,4-dihydroxy-5-methyl-6-methoxybenzyl) 2,2-dimethylchroman (**18**) and 6-acetyl-5,7-dihydroxy-8-(3-acetyl-2,4-dihydroxy-5-methyl-6-methoxybenzyl) 2,2-dimethylchroman (**19**), respectively, by the comparison of respective ^{1}H-NMR data of **18** and **19** with those of mallotochromanol (**11**), isomallotochromanol (**14**), **7** and **17**. Compound **18** is a cyclization product of a side chain with a hydroxy group at *ortho*-position to an acetyl group on **1**, while compound **19** is the cyclization with the *para* hydroxy group. As a matter of convenience, compounds **18** and **19** were named mallotochroman (**18**) and isomallotochroman (**19**), respectively.

TABLE I. Cytotoxicity and Anti-HSV-1 Activity of Phloroglucinol Derivatives

Compound	Cytotoxicity ID_{50} (ng/ml)	Anti-HSV-1 activity ED_{50} (ng/ml)	Therapeutic index ID_{50}/ED_{50}
1	365	185	2.0
2	362	165	2.2
3	340	88	3.9
4	470	154	3.1
5	342	196	1.7
6	2450	890	2.8
7	5500	3180	1.7
8	3680	2080	1.8
9	2200	1140	1.9
10	25200	5600	4.5
11	21200	19200	1.1
12	2500	230	10.9
13	6900	6600	1.0
14	2640	655	4.0
15	34000	18600	1.8
16	14800	6180	2.4
17	285	116	2.5
18	49100	48000	1.0
19	8800	970	9.1

The nineteen phloroglucinols were evaluated for antiviral activity to produce cytotoxicity in HeLa cells and to inhibit replication of HSV-1. The cytotoxicity (ID_{50}) and antiherpetic activity (ED_{50}) were summarized in Table I and the therapeutic index that is the ratio between the cytotoxicity and the antiherpetic activity is also shown in Table I. All 19 compounds exhibited the cytotoxicity ranging from 285 ng to 49.1 μg/ml and the antiherpetic activity ranging from 88 ng to 48 μg/ml. The known selectivity of the antiviral agents described indicated that unless *in vitro* antiviral activity is separated from cytotoxicity by at least seven- to eight-fold, compounds probably do no merit additional consideration.[15] On this basis, compounds **12** and **19** were considered to be active antivirals and merit additional investigation.

Experimental

General Procedures All melting points were determined on a Yangaimoto micro melting point apparatus and are uncorrected. UV and IR spectra were recorded on a Hitachi 220 S double beam spectrophotometer and 260-10 infrared spectrometer with polystyrene calibration at 1601 cm^{-1}, respectively. ^{1}H- and ^{13}C-NMR spectra were taken on a JEOL JNM-GX 270 spectrometer at 270 MHz and a Varian XL-200 spectrophotometer at 50.3 MHz, with tetramethylsilane as an internal standard, respectively. The chemical shifts are recorded in δ (ppm) values. Electron impact mass spectra (EI-MS) were obtained on a JEOL JMS-D-200 mass spectrometer operating at 70 eV.

Compound 1—15 The extraction, separation and isolation of these compounds from the dried pericarps of *M. japonicus* have been described previously.[7-10,12]

Compound 16 The C_6H_6 eluent (47 g) was rechromatographed on a silica gel column by stepwise elution with *n*-hexane, EtOAc/*n*-hexane and EtOAc.[5] The 15% EtOAc/*n*-hexane eluent was further separated on a silica gel column by elution with C_6H_6/EtOAc (20 : 1) to give compound **16** (15 mg). Yellow needles, mp 131—132 °C (MeOH). UV λ_{max}^{EtOH} nm (log ε): 279 (3.71), 348 (3.05). IR ν_{max}^{KBr} cm^{-1}: 3350, 2930, 1630, 1600, 1420, 1370, 1280, 1115. ^{1}H-NMR ($CDCl_3$) δ: 2.11 (6H, s, Ar-$CH_3 \times 2$), 2.72 (3H, s, $COCH_3$), 3.71 (3H, s, OCH_3), 9.49 (2H, br s, OH × 2). ^{13}C-NMR ($CDCl_3$) δ: 8.18 (q, Ar-$CH_3 \times 2$), 33.36 (q, $COCH_3$), 60.31 (q, OCH_3), 107.28 (s, C-1), 108.49 (s, C-3 and 5), 158.38 (s, C-2 and 6), 162.85 (s, C-4), 204.58 (s, CO). EI-MS *m/z*: 210 (M^+), 195. EI-MS measurement *m/z* 210.0910 ($C_{11}H_{14}O_4$ requires 210.0888).

Cyclization of 1 with DDQ Compound **1** (100 mg) and DDQ (100 mg) in dry benzene were reacted at 50 °C for 20 min. The reaction mixture was filtered and evaporated to dryness. The residue was separated on PLC (silica gel, hexane : EtOAc = 10 : 3) to give compound **7** (20 mg) and **17** (22 mg) from the bands of *Rf* 0.5 and 0.33, respectively. Compound **7** was identified as mallotochromene by direct comparison with a natural authentic sample. Compound **17** was afforded as yellow needles, mp 164—165 °C (*n*-hexane/EtOAc). ^{1}H-NMR ($CDCl_3$) δ: 1.60 (6H, s, $-(CH_3)_2$), 2.14 (3H, s, Ar-CH_3), 2.69 (3H, s, $COCH_3$), 2.71 (3H, s, $COCH_3$), 3.71 (2H, s, Ar-CH_2-Ar), 4.00 (3H, s, OCH_3), 5.50 (1H, d, J = 10.0 Hz, =CH–), 6.71 (1H, d, J = 10.0 Hz, –CH=), 8.57 (1H, s, OH), 9.31 (1H, s,

OH), 13.64 (1H, s, OH), 14.00 (1H, s, OH). EI-MS *m/z*: 442 (M^+), 247, 246, 231, 219, 213, 196, 181.

Cyclization of 1 with Formic Acid Compound **1** (130 mg) in 10 ml of absolute EtOH and HCOOH (20 ml) were reacted at 90 °C for 30 min. The reaction mixture was evaporated *in vacuo* and separated on PLC (silica gel, *n*-hexane : EtOAc = 5 : 1) to give compounds **18** (22 mg) and **19** (6 mg) from the bands of *Rf* 0.34 and 0.23, respectively. Compound **18** was afforded as yellow needles, mp 206—207 °C (*n*-hexane/EtOAc). ^{1}H-NMR ($CDCl_3$) δ: 1.36 (6H, s, $-(CH_3)_2$), 1.76 (2H, t, J = 6.9 Hz, $-CH_2-$), 2.12 (3H, s, Ar-CH_3), 2.59 (2H, t, J = 6.9 Hz, $-CH_2-$), 2.65 (3H, s, $COCH_3$), 2.72 (3H, s, $COCH_3$), 3.75 (2H, s, Ar–CH_2–Ar), 3.98 (3H, s, OCH_3), 9.15 (1H, s, OH), 9.40 (1H, s, OH), 13.69 (1H, s, OH), 15.99 (1H, s, OH). EI-MS *m/z*: 444 (M^+), 249, 236, 196, 193, 181. Compound **19** was also afforded as yellow needles, mp 167—168 °C (*n*-hexane/EtOAc). ^{1}H-NMR ($CDCl_3$) δ: 1.51 (6H, s, $-(CH_3)_2$), 1.88 (2H, t, J = 6.9 Hz, $-CH_2-$), 2.14 (3H, s, Ar-CH_3), 2.66 (2H, t, J = 6.9 Hz, $-CH_2-$), 2.70 (3H, s, $COCH_3$), 2.72 (3H, s, $COCH_3$), 3.69 (2H, s, Ar–CH_2–Ar), 3.99 (3H, s, OCH_3), 8.83 (1H, s, OH), 9.00 (1H, s, OH), 13.63 (1H, s, OH), 14.07 (1H, s, OH). EI-MS *m/z*: 444 (M^+), 249, 236, 233, 209, 196, 193, 181.

Assay for Antiviral Activity in Cell Culture Monolayers of HeLa 229 cells in 24-well culture plates were washed, infected with HSV-1 at a multiplicity of infection of 0.5, absorbed for 1.5 h at room temperature, and refed with maintenance medium (MEM plus 2% FCS) containing various amounts of the test compound. Cultures were incubated at 34 °C for 24 h in 5% CO_2, harvested and disrupted by three cycles of freezing and thawing. Virus yields were determined by plaque assay. Each test compound was assayed at least twice. The ED_{50} was determined as the least drug concentration which reduced plaque numbers by 50% in the treated cultures compared to untreated infected cultures.[16]

Cytotoxicity Assay For growth inhibition studies on HeLa cells, 5×10^4 cells in 0.5 ml MEM supplemented with 5% FCS were seeded into each well of 24-well plates, cultured for 24 h at 37 °C, and allowed to grow for an additional 24 h in the presence of increasing amounts of the test compound. After the medium was removed, cell monolayers were detached with 0.05% trypsin and the viable cells were calculated by the trypan blue-exclusion method. The inhibition data were plotted as dose-effect curves from which the 50% inhibitory doses (ED_{50}) were obtained. The ED_{50} is the average of three assays with four concentrations within inhibitory range of the compounds (duplicate cultures).[16]

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