Semisynthesis of Isetexane Diterpenoid Analogues and Their Cytotoxic Activity

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Isetexane diterpene analogues were semisynthesized from demethylsalvicanol isolated from *Perovskia abrotanoides* (Labiatae). The structure and cytotoxic activity relationships (SAR) of the natural parent diterpene, demethylsalvicanol, and its semisynthetic analogues were studied by using P388 murine leukemia cells.

Key words semisynthesis; isetexane diterpenoid; cytotoxic; microwave; structure activity relationships (SAR)

Perovskia abrotanoides (Labiatae) is an Iranian folk-medicinal herb, used for treatment of leishmaniasis.[1)] Sairafianpour *et al.* reported that the root of this plant, growing in the area between Natanz and Kashan, Isfahan, Iran of altitude 2100 m contained a large amount of tanshinones.[1)] In our studies on the chemical constituents of the aerial parts of this plant, purchased from Kordes Jungpflanzen (Germany) (place of produce not specified), we could not obtain tanshinones, and instead, we isolated carnosic acid and demethylsalvicanol (**1**) (Fig. 1) along with small amounts of rosmanols and carnosols.[2)] Of them, demethylsalvicanol (**1**), having a rearranged 9 (10→20)-abeoabietane skeleton, was found to be very rich in the plants (*ca.* 0.37% yield). There are two papers which refer to the cytotoxic activity of demethylsalvicanol analogues.[2,3)] Therefore, in the present paper, we report synthesis of demethylsalvicanol analogues, evaluation of their cytotoxic activity against P388 murine leukemia cells, and their structure and cytotoxic activity relationships (SAR) studies. Microwave-assisted biomimetic synthesis of brussonol (**8**) is also described.

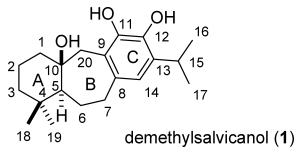
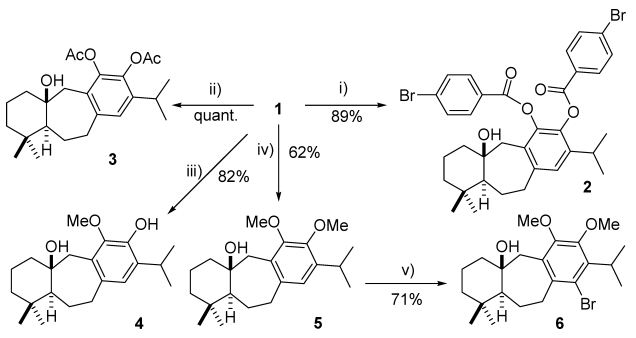


Fig. 1. Structure of Demethylsalvicanol (**1**)

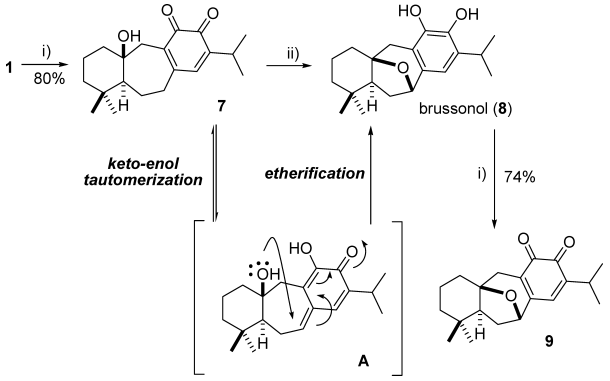
Silica gel column chromatography of a hot EtOAc extract of air dried aerial parts of *Perovskia abrotanoides* gave demethylsalvicanol (**1**). The absolute configuration of **1** was reported to be 5*S* and 10*S* by Gonzalez *et al.*, on the basis of chemotaxonomic consideration.[4)] We confirmed the absolute configuration of **1** to be as reported, by converting **1** to its bis(*p*-bromobenzoate) (**2**) and subjecting **2** to X-ray crystallographic analysis, which proved that the absolute configuration of **2** was 5*S* and 10*S* (Chart 1).[5,6)] Thus, the absolute configuration of **1** was established as 5*S* and 10*S*.

Modification of C ring of **1** was accomplished as shown in Chart 1. The acetylation of **1** was carried out according to the reported manner.[7)] Methylation of OH-11 and OH-12 was reported by Gonzalez *et al.*[4)] and Kelecom *et al.*,[8)] but their yields were very poor. In the present scheme, **1** was treated with methyl iodide and potassium carbonate in dry acetone at 55 °C under light shielding. Then, methylation of both OH-11 and OH-12 proceeded apparently simultaneously to give the corresponding dimethylated compound **5**[8)] in 62% yield. Selective monomethylation of OH-11 was accomplished by the treatment of **1** with (trimethylsilyl)diazomethane in benzene–MeOH (9 : 1) to give a monomethylated compound **4**[9)] in 82% yield. Introduction of bromine atom at 14-C was carried out by the treatment of **5** with *N*-bromosuccinimide (NBS) in *N*,*N*-dimethylformamide (DMF) (Chart 1).



i) *p*-bromobenzoyl chloride/DMAP/pyridine/CH_2Cl_2/r.t.; ii) Ac_2O/pyridine/r.t.; iii) $TMSCHN_2$/benzene : MeOH (9 : 1), r.t.; iv) MeI/K_2CO_3/Me_2CO/55 °C; v) NBS/DMF/r.t.

Chart 1. Preparation of C-Ring Modified Demethylsalvicanol Analogues (**2**—**6**)



i) DDQ/dioxane/r.t.; ii) See Table 1.

Chart 2. Biomimetic Transformation of Demethylsalvicanol (**1**) to Brussonol (**8**) and its quinone (**9**)

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Table 1. Microwave-Assisted Etherification of **7** under Different Reaction Conditions

Entry[a]	Reaction conditions		Yields of **8** (%)
	Mineral supports	Temperature (°C)	
1[b]	Florisil	150	14
2	Aluminum oxide	150	—[c]
3	Florisil	100	19
4	Florisil	120	29
5	Florisil	150	37
6	Florisil	180	15

a) All reactions were conducted using microwave irradiation except for entry 1. *b*) Conventional heating (preheated oil bath) was employed. *c*) Complex mixture.

Table 2. Cytotoxic Activity of Demethylsalvicanol (**1**), and Its Semisynthetic Analogues (**2**—**9**)

Compounds	IC_{50} (μg/ml)
1	0.71
2	>100
3	7.5
4	7.3
5	9.9
6	70
7	0.57
8	1.9
9	2.2
Cryptotanshinone[a]	8.3
Mytomycin C[a]	0.035

a) Positive controls.

To modify the 3D-alignment of the B ring, we performed the biomimetic synthesis of brussonol (**8**), having a bicyclic ring system in B ring. Fraga *et al.* described a plausible biosynthetic route[3] from **1** to brussonol (**8**),[3,10] both being obtained from the roots of *Salvia brussonetii*. The proposed pathway involves production of a tautomer of **7**, and subsequent intramolecular bicyclization in **7** by attack of OH-10 on C-7 (Chart 2). As suggested in the proposed scheme, salvican-11,12-dione (**7**) was prepared by the treatment of **1** with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) in dry dioxane and the microwave heating-assisted etherification of **7** was performed to give **8**. The microwave heating-assisted etherification was studied under several reaction conditions (Table 1). When a chloroform solution of **7** was irradiated, the reaction did not proceed to recover only the starting material. When **7** supported on florisil® was irradiated by microwave apparatus (solvent-free system), the etherification proceeded to give brussonol (**8**), whose spectral data were identical with those of natural brussonol.[3,10] Of the irradiation temperatures (100—180 °C), 150 °C gave the best result. When the reaction was carried out in a preheated-oil bath, the yield was 14% (entry 1 in Table 1) and when the supporting material was aluminum oxide, the reaction gave complex products which were not separated (entry 2 in Table 1). Microwave irradiation is known to give rapid homogeneous heating to the substrate in the form of film over solid support particles,[11] and such efficient heating seems to be essential for the successful intramolecular etherification reaction of the present compound. Conventional heating from outside inevitably produces considerable temperature gradient, and non-homogeneous heat distribution in the reaction system, which may retard the expected reactions and cause possible degradation and side-reactions, resulting in the decrease in the yield of product. As shown in Table 1, another solid support particles, aluminum oxide, did not give the effect that florisil® gave, which is considered to be due to the alkalinity of aluminum oxide, inducing some complex reactions in the system. The oxidation of brussonol (**8**) with DDQ gave the corresponding *o*-quinone **9** in 74% yield.

The results of cytotoxicity assay are shown in Table 2. In our previous SAR study of carnosic acid analogues,[2] we found that among the carnosic acid analogues tested, the cytotoxic activity of the mother catechols tended to decrease when the phenolic hydroxy groups were acetylated or methylated, and that the activity of catechol often increased when the phenolic hydroxy groups were converted to an *o*-quinone system on C-ring. Our present studies on demethylsalvicanol series showed that, as in the case of carnosic acid series, the activity tended to decrease when the hydroxyls on C-ring were acetylated or methylated (**1** *vs.* **2**—**5**). However, unlike the case of the carnosic acid analogues, the oxidation of phenolic hydroxy group of catechol to *o*-quinone system on C-ring, apparently had little effect on the activity (**1** *vs.* **7** and **8** *vs.* **9**). Decreasing of the activity by the introduction of large groups (**2**) to the catechol substructure or a bromine atom at C-14 (**6**) may be ascribed to the resulting change in the steric or electrostatic field of the molecule, respectively, which are considered to be also important factors involved. Further, the lower cytotoxic activity of the analogues **8** and **9** may be due to their different 3D-alignment of B ring from those in **1** and **7**, respectively.

Experimental

General Experimental Procedures Melting points were determined on a Yanaco MP-3 apparatus and are recorded uncorrected. IR spectra were recorded on a JASCO FT/IR 620 spectrophotometer, optical rotation on a JASCO DIP-360 automatic digital polarimeter, and Mass spectra on a Micromass LCT (Manchester, U.K.) spectrometer. NMR spectra are recorded in $CDCl_3$ on a Brucker AM-400 and DRX-500 spectrometer at 300 K and the *J* values are given in Hz. The microwave-assisted synthesis was carried out in an Emrys Creator® single-mode microwave cavity producing controlled irradiation at 2.45 GHz (Biotage AB, Uppsala). The reaction times refer to the hold times at the temperature indicated. The temperature was monitored with an IR sensor equipped on the outside of the reaction vessel.

Plant Material Aerial parts of *Perovskia abrotanoides* (Labiatae) were purchased from Kordes Jungpflanzen (Bilsen, Germany, http://www.kordes-jungpflanzen.de). The botanical identification was made by Prof. K. Takeya, School of Pharmacy, Tokyo University of Pharmacy and Life Science, Tokyo, Japan. A voucher specimen has been deposited in the herbarium of Tokyo University of Pharmacy and Life Science (03JCP12).

Extraction and Isolation The air-dried aerial parts of *Perovskia abrotanoides* (2.9 kg) were extracted with hot AcOEt (45 l×3). The combined AcOEt extract was evaporated *in vacuo* to give a residue (238 g), which was applied to a silica gel column (Silica Gel 60N gel®, Kanto Chemical Co. Ltd., 63—210 μm). The column was eluted with toluene : AcOEt (1 : 0, 9 : 1, 9 : 1, 8 : 1, 4 : 1) and AcOEt : MeOH (5 : 1, 0 : 1) (each 3 l) to yield fractions I—VII. After removal of the solvent, fraction III (64 g) was further subjected to silica gel column chromatography (toluene : MeCN=19 : 1) to afford demethylsalvicanol (**1**) (9.6 g). Analogous silica gel column chromatography (toluene : MeCN=19 : 1) of fraction IV (12 g) also afforded demethylsalvicanol (**1**) (1.2 g).

Oxidation of Demethylsalvicanol (1) with DDQ DDQ (7.0 mg, 0.03 mmol) was added to a solution of **1** (10.1 mg, 0.03 mmol) in dry dioxane (0.5 ml) in one portion. After stirring at r.t. for 30 min under an argon atmosphere, the reaction mixture was passed through a pack of Celite® and the Celite® was washed with AcOEt (50 ml). The eluate was evaporated *in vacuo* to give an oily residue, which was subjected to silica gel column chromatography (hexane : Me_2CO=9 : 1) to give **7** (8.0 mg, 80%). Pale red amorphous solid ($CHCl_3$), mp 115—116 °C; $[\alpha]_D$ −115.9° (*c*=0.10, $CHCl_3$); ^{1}H-

NMR (400 MHz, 300 K) δ: 6.58 (1H, s), 3.05 (1H, d, 14.7), 2.92 (1H, sept, 6.8), 2.62 (1H, dd, 14.1, 12.1), 2.47 (1H, dd, 7.0, 7.0), 2.16 (1H, d, 14.7), 1.86 (1H, m), 1.79 (1H, m), 1.71 (1H, m), 1.55 (1H, m), 1.52 (1H, m), 1.42 (1H, m), 1.41 (1H, m), 1.26 (1H, m), 1.25 (1H, m), 1.11 (3H, d, 6.3), 1.10 (3H, d, 6.3), 0.91 (3H, s), 0.91 (3H, s); ^{13}C-NMR (100 MHz, 300 K) δ: 180.4, 180.2, 154.2, 146.8, 138.4, 134.4, 70.6, 58.0, 42.4, 42.2, 40.0, 36.6, 34.4, 32.1, 27.2, 21.6, 21.5, 21.4, 20.8, 18.2; IR (film): ν_{max} 3495 (OH), 1674, 1648 (C=O) cm^{-1}; HR-MS (ESI): Calcd for $C_{20}H_{29}O_3$ (M^++H): 317.2117. Found: 317.2105.

Preparation of 11-*O*,12-*O*-Bis(*p*-bromobenzoyl)demethylsalvicanol (2) *p*-Bromobenzoyl chloride (69.4 mg, 0.32 mmol) and cat. amount of 4-dimethylaminopyridine (DMAP) were added to a solution of **1** (20.1 mg, 0.063 mmol) in a mixture of pyridine (0.5 ml) and CH_2Cl_2 (0.5 ml). After stirring at r.t. for 24 h under an argon atmosphere, the reaction mixture was poured into ice-cooled water (10 ml) and the aqueous phase was extracted with AcOEt (10 ml×3). The combined organic phase was washed with 5% HCl (10 ml×3), sat. aq. $NaHCO_3$ (10 ml×3), and sat. aq. NaCl (10 ml×3), successively, dried over $MgSO_4$, filtered, and evaporated *in vacuo*. The oily residue was subjected to medium pressure liquid chromatography (MPLC) (hexane : Me_2CO=49 : 1) to give **2** (38.5 mg, 89%). Colorless needles (CH_3CN–MeOH), mp 112—115 °C, $[\alpha]_D$ +61.8° (c=0.13, $CHCl_3$); ^{1}H-NMR (500 MHz, 300 K) δ: 7.86 (4H, d, 8.4), 7.49 (2H, d, 8.4), 7.46 (2H, d, 8.4), 7.07 (1H, s), 2.99 (1H, sept, 6.9), 2.93 (1H, dd, 7.3, 7.3), 2.78 (1H, dd, 12.4, 13.6), 2.72 (2H, s), 1.97 (1H, m), 1.86 (1H, m), 1.26—1.50 (5H, m), 1.24 (3H, d, 6.9), 1.22 (1H, m), 1.20 (3H, d, 6.9), 0.934 (3H, s), 0.927 (3H, s); ^{13}C-NMR (125 MHz, 300 K) δ: 165.0, 163.9, 143.2, 141.5, 139.7, 138.1, 131.9, 131.6, 131.5, 129.4, 129.0, 128.4, 127.6, 127.1, 124.0, 70.6, 58.5, 43.4, 42.6, 42.4, 36.8, 34.4, 32.2, 27.7, 23.2, 23.0, 22.9, 21.7, 18.6; IR (film): ν_{max} 3515 (OH), 1740 (C=O) cm^{-1}; HR-MS (ESI): Calcd for $C_{34}H_{35}O_4$ (MH^+–H_2O): 665.0902. Found: 665.0909.

11-*O*-Monomethylation of 1 1.0 M (trimethylsilyl)diazomethane ($TMSCHN_2$) in hexane (1.5 ml, 1.5 mmol) was added to a solution of **1** (100 mg, 0.3 mmol) in a mixture of benzene (4.5 ml) and MeOH (0.5 ml) at r.t. After stirring at r.t. for 3 h, the solvent was evaporated *in vacuo* to give an oily residue, which was purified by MPLC (hexane : AcOEt=9 : 1) to yield **4** as a colorless amorphous solid (0.085 g, 82%), whose spectral data were identical with those reported.[9]

11-*O*,12-*O*-Dimethylation of 1 **1** (100 mg, 0.31 mmol) was dissolved in dry Me_2CO (5.0 ml), and the solution was treated with powdered potassium carbonate (1.0 g, 7.2 mmol) and methyl iodide (1.0 ml, 16.1 mmol). After the reaction mixture was stirred at 55 °C for 15 h under an Ar atmosphere and light-protecting, the solvent was evaporated under reduced pressure. Ice-cooled H_2O (20 ml) was added to the residue and the aqueous phase was extracted with AcOEt (20 ml×3). The combined organic phase was washed with sat. aq. NaCl (15 ml×3), dried over $MgSO_4$, filtered, and evaporated *in vacuo*. The oily residue obtained was subjected to MPLC (hexane : AcOEt= 49 : 1) to give **5** (67.1 mg, 62%), whose spectral data were identical with those reported previously.[4,8]

Bromination of 5 A solution of **5** (12.7 mg, 0.037 mmol) and NBS (7.0 mg, 0.039 mmol) in dry DMF (0.5 ml) was stirred at r.t. for 24 h under an argon atmosphere. The reaction mixture was poured into ice-cooled water (10 ml) and the aqueous phase was extracted with AcOEt (10 ml×3). The combined organic phase was washed with sat. aq. NaCl (15 ml×3), dried over $MgSO_4$, filtered, and evaporated *in vacuo* to give an oily residue, which was subjected to silica gel column chromatography (hexane : AcOEt=49 : 1) to give **6** (11 mg, 71%). Colorless amorphous solid ($CHCl_3$), mp 53—56 °C, $[\alpha]_D^{25}$ +25.0° (c=0.13, $CHCl_3$); ^{1}H-NMR (500 MHz, 300 K) δ: 3.84 (3H, s), 3.77 (3H, s), 3.71 (1H, m), 3.29 (1H, d, 14.0), 2.56 (1H, d, 14.0), 2.53 (1H, m), 2.01 (1H, m), 1.90—1.77 (2H, m), 1.55 (1H, m), 1.44 (2H, m), 1.35 (6H, t, 6.8), 1.34—1.14 (3H, m), 0.97 (1H, d, 1.7), 0.92 (3H, s), 0.89 (3H, s); ^{13}C-NMR (100 MHz, 300 K) δ: 151.9, 151.3, 139.4, 139.1, 129.8, 120.6, 70.9, 60.5, 60.3, 58.3, 42.4, 42.1, 41.8, 35.2 (overlapped), 34.3, 32.1, 22.6, 21.6, 21.3, 21.0, 18.7; IR (film): ν_{max} 3475 (OH) cm^{-1}; HR-MS (ESI): Calcd for $C_{22}H_{33}O_3BrLi$ (M^++Li): 431.1773. Found: 431.1775.

Microwave-Assisted Etherification of 2 (Biomimetic Synthesis of Brussonol (8)) **7** (21.9 mg, 0.07 mmol) was dissolved in dry Et_2O (2 ml), to which florisil® (1.0 g) was added and the solvent was evaporated completely *in vacuo* on a rotary evaporator at room temperature. The florisil®-supported **7** thus prepared was irradiated by using the microwave apparatus described in the General Experimental Procedures, at 150 °C for 5 min. After the reaction, the florisil® particles were washed with $CHCl_3$: MeOH (9 : 1) and the combined washings were evaporated *in vacuo*. The oily residue thus obtained was subjected to silica gel column chromatography (hexane : Me_2CO=17 : 3) to give **8** (8.1 mg, 37%), whose spectral data were identical with those of natural brussonol.[3,10]

Oxidation of 8 with DDQ DDQ (6.8 mg, 0.03 mmol) was added to a solution of **8** (9.5 mg, 0.03 mmol) in dry dioxane (0.5 ml) in one portion. After stirring at r.t. for 25 min under an argon atmosphere, the reaction mixture was passed through a pack of Celite® and the Celite® was washed with AcOEt (50 ml). The filtrate and washings were evaporated *in vacuo* to give an oily residue, which was subjected to short silica gel column chromatography (hexane : Me_2CO=9 : 1) to give **9** (7.0 mg, 74%). Pale red amorphous solid ($CHCl_3$), mp 60—63 °C; $[\alpha]_D$ −86.5° (c=0.15, $CHCl_3$); ^{1}H-NMR (400 MHz, 300 K) δ: 6.46 (1H, s), 4.48 (1H, d, 6.9), 2.95 (1H, sept, 6.9), 2.48 (1H, d, 18.7), 2.18 (1H, d, 18.7), 2.15—1.9 (3H, m), 1.82—1.72 (3H, m), 1.65—1.45 (2H, m), 1.16 (1H, m), 1.113 (3H, d, 6.9), 1.109 (3H, d, 6.9), 0.97 (3H, s), 0.85 (3H, s); ^{13}C-NMR (100 MHz, 300 K) δ: 180.6, 179.8, 152.9, 148.0, 131.9, 129.5, 80.4, 74.9, 51.6, 38.1, 37.9, 32.0, 31.7, 30.4, 29.9, 27.3, 26.7, 21.6, 21.5, 15.9; IR (film): ν_{max} 1660 (C=O) cm^{-1}; HR-MS (ESI): Calcd for $C_{20}H_{27}O_3$ (M^++H): 315.1960. Found: 315.1989.

Assay for Cytotoxic Activity The cytotoxic assay was performed by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay method. The murine P388 leukemia cells were precultured in RPMI 1640 medium (Nissui Co. Ltd., Japan) supplemented with 5% heat-inactivated fetal bovine serum (FBS) and kanamycin (5.3 ml/l) in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. The cell suspension (3×10^4 cels/ml, 100 μl) was added to each well (3×10^3 cells/well) of a 96-microwell plate (flat bottom, polystyrene treated) and incubated for 24 h. Test compound solutions in dimethyl sulfoxide (DMSO) in various concentrations (100, 10, 1, 0.1 μg/ml) were prepared and 10 μl of the test solution or DMSO (control) was added to each well. The plate was kept in an incubator for 48 h. After termination of the cell culture by adding 5% MTT in PBS (20 μl) to each well, the plate was kept in the incubator for 4 h. After addition of 100 μl of 10% SDS–0.01 N HCl to each well, the plate was read on a microplate reader (MPR A4i, Toso) at 550 nm. A dose-response curve was plotted for each compound, and the concentrations giving 50% inhibition of the cell growth (IC_{50}) were recorded.

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