Studies on the Constituents of the Leaves of *Baccharis dracunculifolia* (Asteraceae) and their Cytotoxic Activity

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A new sesquiterpene, named baccharisketone (1), and a new monoterpene, *p*-methoxythymol acetate (2), were isolated from the leaves of *Baccharis dracunculifolia* along with seventeen known compounds (3—19). The structures of the new compounds were determined by spectroscopic means. The growth inhibitory activity of the isolated compounds against leukemia cells (L 1210) was tested and three terpene phenols (4, 6, 17) and five sesquiterpene alcohols (8, 10, 11, 13, 16) were found to exhibit strong cytotoxic activity.

Key words *Baccharis dracunculifolia*; leukemia cell; Asteraceae; monoterpenoid; sesquiterpenoid; cytotoxic activity

Baccharis dracunculifolia (Asteraceae, local name in Brazil: Alecrim do campo) is a woody dioecious shrub (2—3 m in height) that is distributed throughout southern Brazil. It has been reported that this plant is a source of resin for Brazilian propolis (green propolis)[4,5] and leaf extracts have been used as an antipyretic, a stomachic, and a health tonic in Brazil. Our biological studies of the alcoholic extracts of the leaves found that the extracts exhibited potent cytotoxic activity against leukemia cells (L 1210). In this paper, we describe the isolation and structure elucidation of two new compounds, baccharisketone (**1**) and *p*-methoxythymol acetate (**2**), along with seventeen known compounds; caryophyllene oxide (**3**),[6] thymol (**4**),[7] carvacrol (**5**),[8] *p*-methoxythymol (**6**),[8] 4-isopropyl-2-methylphenol (**7**),[7] spathulenol (**8**),[9] *p*-cymene-2,3-diol (**9**),[10] bisacumol (**10**),[11] 2-methyl-6-(4′-methylphenyl)-3-hepten-2-ol (**11**),[12] 1β-hydroxyeudesma-4(15),7-diene (**12**),[13] cadinol (**13**),[14] an oppositane-type sesquiterpene (**14**),[15] germacra-1(11),5(12),6(*E*)-trien-2-ol (**15**),[16] a tricyclic sesquiterpene (**16**),[15] 3,4,3′,4′-tetrahydroxy-5,5′-diisopropyl-2,2′-dimethylbiphenyl (**17**),[17] 3-acetoxy-4′,5-dihydroxy-7-methoxyflavanone (**18**),[18] and naringenin (**19**),[19] and discuss their growth inhibitory activities against L 1210.

Results and Discussion

Isolation of Chemical Constituents from the Leaves of *B. dracunculifolia* The dried leaves of *B. dracunculifolia* were extracted with ethanol under ultrasonication and then the solvent was evaporated *in vacuo*. The residue was chromatographed on a HP-20 column eluted successively with 40% MeOH, 70% MeOH, MeOH, and acetone. The growth inhibitory activity of each fraction against L 1210 was tested and the MeOH fraction was found to show strong activity (Fig. 1). Therefore, the MeOH fraction was further separated on a silica gel column eluted with stepwise gradients of *n*-hexane–EtOAc and then purified by HPLC to give compounds (**1**—**19**) as shown in Fig. 2.

Chemical Structures of New Compounds (1, 2) Baccharisketone (**1**) was obtained as a colorless oil, $[\alpha]_D$ −13.2° (c=0.1, $CHCl_3$). Its high resolution (HR)-EI-MS spectrum showed the $[M]^+$ ion peak at m/z 218.1664 corresponding to the molecular formula $C_{15}H_{22}O$ (218.1669) and the [M−iso-

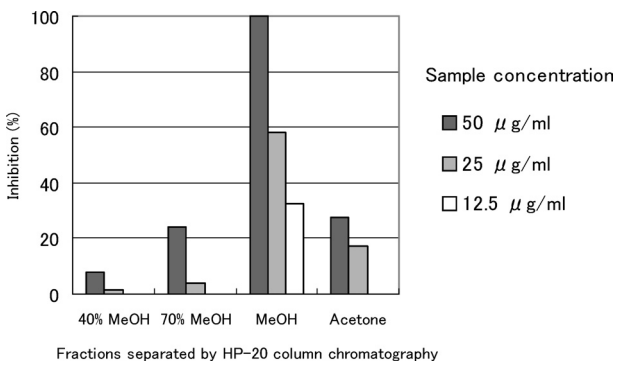

Fig. 1. Effects of the Fractions Obtained by HP-20 Column Chromatography on the Growth of Leukemia Cells (L 1210)

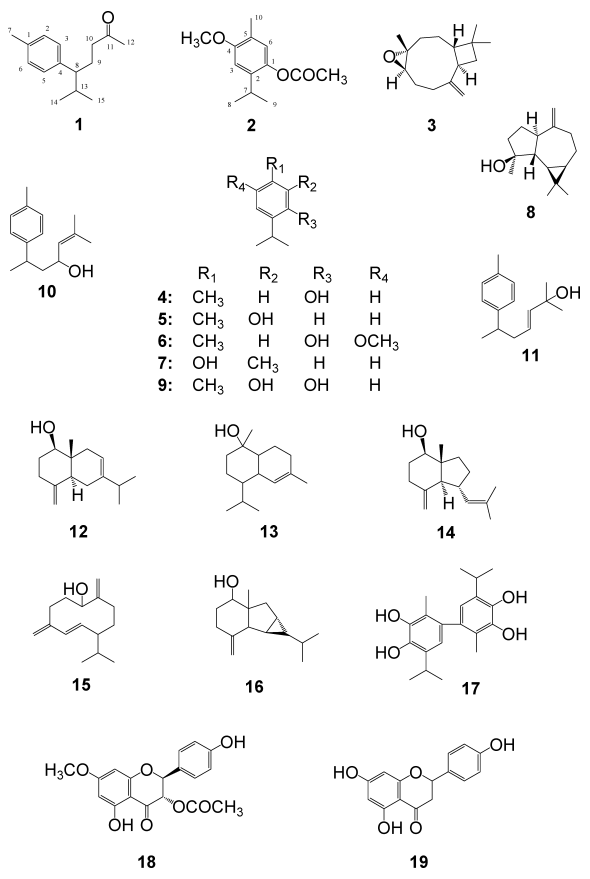

Fig. 2. Compounds Isolated from the Leaves of *B. dracunculifolia*

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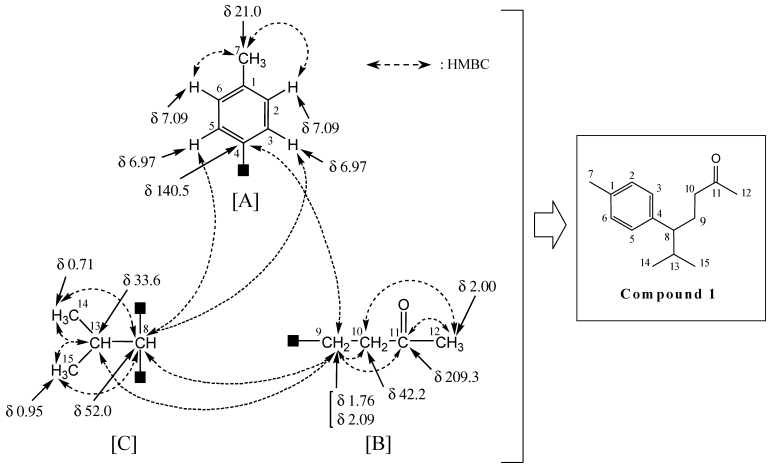

Fig. 3. Selected HMBC Correlations of **1**

propyl]$^+$ ion peak at *m/z* 175.1111 (Calcd 175.1121 for $C_{12}H_{15}O$). The ^{1}H-NMR spectrum showed two secondary methyl signals [δ 0.71 (3H, d, J=7.3 Hz), 0.95 (3H, d, J=7.3 Hz)] and two tertiary methyl signals [δ 2.00 (3H, s), 2.32 (3H, s)]. The downfield proton signals at δ 6.97 (2H, d, J=8.1 Hz) and 7.09 (2H, d, J=8.1 Hz) exhibited the presence of a 1,4-disubstituted benzene ring. The distortionless enhancement by polarization transfer (DEPT) spectrum of **1** indicated the presence of four methyl carbons (δ 20.8, 20.9, 21.0, 30.0), two methylene carbons (δ 26.8, 42.2), six methine carbons (δ 33.6, 52.0, 128.3×2, 128.9×2), and a carbonyl carbon (δ 209.3). The ^{13}C–^{1}H shift correlation spectrum (^{13}C–^{1}H COSY) of **1** showed non-equivalent methylene proton signals [δ 1.76, 2.09 (1H each, m)] correlated with the carbon signal at δ 26.8. In the ^{1}H-detected heteronuclear multiple bond connectivity (HMBC) spectrum of **1**, the aromatic protons at δ 7.09 (H-2, H-6) exhibited a long-range correlation with the methyl carbon signal at δ 21.0 (C-7), suggesting the presence of the partial structure [A] as shown in Fig. 3. Both the non-equivalent methylene protons (H-9, δ 1.76 and 2.09) and the methyl proton signal at δ 2.00 (H-12) showed long-range correlations with the carbon signals at δ 42.2 (C-10) and 209.3 (C-11), therefore, the presence of partial structure [B] was confirmed. Furthermore, the partial structure [C] was deduced since the methyl protons at δ 0.71 (H-14) and 0.95 (H-15) showed cross peaks with the carbon signals at δ 33.6 (C-13) and 52.0 (C-8). The connectivities of these partial structures [A—C] were further investigated by analysis of the HMBC spectrum of **1**. As shown in Fig. 3, the aromatic proton signals at δ 6.97 (H-3, H-5) in the partial structure [A] exhibited a long-range correlation with the methine carbon signal at δ 52.0 (C-8) in [C], and the non-equivalent proton signals (H-9, δ 1.76 and 2.09) in [B] showed cross peaks with the quaternary aromatic carbon at δ 140.5 (C-4) in [A], and the methine carbons at δ 33.6 (C-13) and 52.0 (C-8) in [C]. The structure of **1** was confirmed to be that shown in Fig. 3.

From the viewpoint of the biogenesis of various sesquiterpenoids, it is presumed that the baccharisketone (**1**) may be biosynthesized in the plant from a bisabolane type sesquiterpenoid *via* an acorane type sesquiterpenoid (Fig. 4).

p-Methoxythymol acetate (**2**) was obtained as an oily material. Its EI-MS spectrum showed the [M]$^+$ ion peak at *m/z*

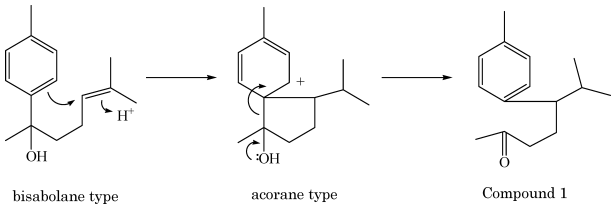

Fig. 4. Presumed Biosynthetic Pathway for **1**

222. The ^{1}H- and ^{13}C-NMR spectra suggested the presence of an isopropyl group [δ_H 1.20 (6H, d, J=7.3 Hz) and 2.96 (1H, m), δ_C 23.0×2 ($\underline{C}H_3$) and 27.6 ($\underline{C}H$)], a vinyl methyl group [δ_H 2.16 (3H, s), δ_C 15.8 ($\underline{C}H_3$)], an acetoxyl group [δ_H 2.30 (3H, s), δ_C 20.6 ($\underline{C}H_3COO$) and 170.2 ($CH_3\underline{C}OO$)], a methoxyl group [δ_H 3.82 (3H, s), δ_C 55.6 ($\underline{C}H_3O$)], and a tetra-substituted benzene ring [δ_H 6.71 (1H, s) and 6.76 (1H, s), δ_C 107.8, 124.1, 125.1, 137.9, 140.8, 155.8].

The substitution pattern of the benzene ring was determined by difference nuclear Overhauser effect (NOE) experiments on **2**. As shown in Fig. 5, irradiation of the proton signal (H-10, δ 2.16) due to the aromatic methyl group produced the significant enhancement of an aromatic proton (H-6, δ 6.76) signal and the methoxyl proton (δ 3.82) signal. Furthermore, the NOEs were observed on the aromatic proton (H-6, δ 6.76), two methyl proton (H-8, H-9, δ 1.20), and methine proton (H-7, δ 2.96) signals due to the isopropyl group by irradiation of the acetoxyl proton signal (δ 2.30). In addition, irradiation of the methoxyl proton (δ 3.82) enhanced the signal intensities of the aromatic proton (H-3, δ 6.71) and the methyl proton (H-10, δ 2.16) signals. Based on these results, the chemical structure of **2** was elucidated to be that shown in Fig. 5.

Inhibition of Leukemia Cell (L 1210) Growth (IC_{50}) The cytotoxicities of the isolated compounds were studied using L 1210 (Table 1). While the new compounds (**1**, **2**) exhibited moderate cytotoxic activities (**1**: IC_{50}=114.5 μM, **2**: IC_{50}=90.0 μM), compounds **8** (spathulenol) and **17** (3,4,3′,4′-tetrahydroxy-5,5′-diisopropyl-2,2′-dimethylbiphenyl) showed strong cytotoxicity (**8**: IC_{50}=13.6 μM, **17**: IC_{50}=9.1 μM), as did compounds **4** (IC_{50}=33.3 μM), **6** (IC_{50}=27.7 μM), **10** (IC_{50}=22.9 μM), **11** (IC_{50}=34.4 μM), **13** (IC_{50}=22.5 μM), and **16** (IC_{50}=22.7 μM). These findings indicated that the monoterpene phenols and the sesquiterpene al-

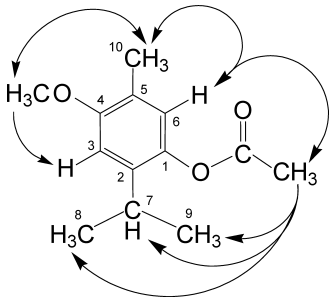

Fig. 5. NOE Correlations on **2** and its Chemical Structure

Table 1. Inhibition of L 1210 Cell Growth (IC_{50}) by the Compounds Isolated in the Present Study

Compound	IC_{50} (μM)	Compound	IC_{50} (μM)
1	114.5	**10**	22.9
2	90.0	**11**	34.4
3	113.5	**12**	45.4
4	33.3	**13**	22.5
5	66.6	**14**	45.4
6	27.7	**16**	22.7
7	66.6	**17**	9.1
8	13.6	**18**	58.1
9	45.1	**19**	36.7

cohols potently inhibited the growth of L 1210.

Conclusion

Two new compounds (**1**, **2**) and seventeen known compounds (**3**—**19**) were isolated from the leaves of *B. dracunculifolia* and the chemical structures were determined by spectroscopic means. Cytotoxicity testing of the compounds isolated in this study revealed the monoterpene phenols like compounds **4**, **6**, and **17** (dimer) and the sesquiterpene alcohols such as compounds **8**, **10**, **11**, **13**, and **16** exhibited strong cytotoxicity. Since the major components of this plant are compounds **3**, **4**, **5**, **6**, and **9**, it was concluded that the growth inhibitory activity of this plant against L 1210 was attributable to the monoterpene phenols, such as thymol (**4**: IC_{50}=33.3 μM), carvacrol (**5**: IC_{50}=66.6 μM), *p*-methoxythymol (**6**: IC_{50}=27.7 μM), and *p*-cymene-2,3-diol (**9**: IC_{50}=45.1 μM).

Experimental

General Experimental Procedures The ^{1}H- and ^{13}C-NMR spectra were measured on a JEOL GSX-400 spectrometer in either $CDCl_3$, CD_3OD or acetone-d_6 each containing tetramethylsilane (TMS) as the internal standard. The MS spectra were recorded on a Hitachi M-2000 double-focusing mass spectrometer. Optical rotations were recorded using a JASCO DIP-360 digital polarimeter. Column chromatography was carried out on Diaion HP-20 column (Mitsubishi Kasei) and silica gel (Wakogel C-200). HPLC was conducted with either a Spectra Physics SP 8800, Senshu SSC 3160, Hitachi pressure pump 635-5002, or Hitachi L-7100 pump, equipped with either an ERMA ERC-7520 (RI), ERMA ERC-7522 (RI), Hitachi 635-5004 (UV), Hitachi 655A-21 (UV), or Hitachi L-4000H (UV) as a detector. TLC was conducted using a silica gel 60 F_{254} (Merck) precoated TLC plate and the detection was carried out by spraying 10% sodium phosphomolybdate methanol solution followed by heating. Leukemia cells (L 1210) were obtained from the National Cancer Center Research Institute, Japan.

Plant Material The leaves of *B. dracunculifolia* were purchased from Laboratorio Farmaervas Ltda. in Sao Paulo, Brazil and a voucher specimen was deposited in the herbarium of the College of Pharmacy, Nihon University.

Extraction and Isolation The dried leaves of *B. dracunculifolia* (1028 g) were extracted with EtOH (2.0 l×4) under ultrasonication. The EtOH extract was concentrated *in vacuo* to give a brown syrup (120.0 g). The crude extracts were chromatographed on a Diaion HP-20 column eluted successively with stepwise gradients of 2.0 l each of 40% MeOH, 70% MeOH, MeOH, and acetone, and then each eluate was concentrated *in vacuo* to afford four fractions [40% MeOH (46.2 g), 70% MeOH (43.7 g), MeOH (23.4 g), and acetone (10.8 g)]. The cytotoxicity of each fraction against L 1210 was tested and the MeOH fraction was found to be most active. Therefore, the MeOH fraction (23.4 g) was chromatographed on a silica gel column eluted successively with solvents of increasing polarity [*n*-hexane : EtOAc=20 : 1 (2.0 l), 10 : 1 (2.0 l), 7 : 1 (1.0 l), 5 : 1 (1.0 l), 3 : 1 (1.0 l), 1 : 1 (2.0 l), and EtOAc (3.0 l)] to afford eleven fractions (Fr. 1—10 and EtOAc). Fraction 4 (3.0 g) was separated by HPLC (Senshu Pak Silica-4251-N, 10 mm×250 mm, *n*-hexane : EtOAc=30 : 1, flow rate: 3.0 ml/min) to afford thirteen fractions (Fr. 4-1—4-13). Fraction 4-5 (retention time: 29.4—30.6 min) was further purified by HPLC (Diachroma ODS M-20 5 μm, 10×250 mm, MeOH : H_2O=8 : 2, flow rate: 3.0 ml/min) to give **2** (7.0 mg, retention time: 10.0 min). Fraction 4-7 (retention time: 32.0—34.0 min) was purified using HPLC (Senshu Pak PEGASIL ODS, 10×250 mm, MeOH : H_2O=85 : 15, flow rate: 3.0 ml/min) to give **3** (46.0 mg, retention time: 16.6 min). Fraction 4-8 (retention time: 37.2—42.0 min) was purified by HPLC (Senshu Pak PEGASIL ODS, 10×250 mm, MeOH : H_2O=8 : 2, flow rate: 3.0 ml/min) to afford **4** (48.8 mg, retention time: 9.2 min) and **1** (5.5 mg, retention time: 21.0 min). Fraction 4-10 (retention time: 44.8—52.0 min) was purified by HPLC (Diachroma ODS M-20 5 μm, 10×250 mm, MeOH : H_2O=65 : 35, flow rate: 3.0 ml/min) to give **5** (312.0 mg, retention time: 17.2 min). Fraction 4-11 (retention time: 59.2—63.6 min) was purified by HPLC (Senshu Pak PEGASIL ODS, 10×250 mm, MeOH : H_2O=8 : 2, flow rate: 3.0 ml/min) to give **6** (20.0 mg, retention time: 7.2 min) and **7** (7.0 mg, retention time: 8.2 min). Fraction 5 (1.4 g) was separated by HPLC (Shodex SIL-5E, 10×250 mm, *n*-hexane : EtOAc=15 : 1, flow rate: 3.0 ml/min) to afford thirteen fractions (Fr. 5-1—5-13). Fractions 5-9 (retention time: 22.6—25.6 min) and 5-11 (retention time: 31.8—34.8 min) were each subjected to HPLC separation (Diachroma ODS M-20 5 μm, 10×250 mm, MeOH : H_2O=75 : 25, flow rate: 3.0 ml/min) to give **6** (15.3 mg, retention time: 34.0 min) and **8** (5.0 mg, retention time: 7.4 min), respectively. Fraction 6 (1.5 g) was separated using HPLC (Senshu Pak Silica-4251-N, 10×250 mm, *n*-hexane : EtOAc=15 : 1, flow rate: 3.0 ml/min) to afford six fractions (Fr. 6-1—6-6). Fraction 6-2 (retention time: 23.6—28.6 min) was further purified by HPLC (Senshu Pak PEGASIL ODS, 10×250 mm, MeOH : H_2O=8 : 2, flow rate: 3.0 ml/min) to give **9** (35.0 mg, retention time: 6.8 min). Fraction 6-3 (retention time: 28.8—31.6 min) was purified by HPLC (Senshu Pak PEGASIL ODS, 10×250 mm, MeOH : H_2O=75 : 25, flow rate: 3.0 ml/min) to afford **10** (4.0 mg, retention time: 24.8 min). Fraction 6-4 (retention time: 31.6—40.0 min) was purified using HPLC (Diachroma ODS M-20 5 μm, 10×250 mm, MeOH : H_2O=8 : 2, flow rate: 3.0 ml/min) to give **11** (11.0 mg, retention time: 14.8 min), **12** (10.9 mg, retention time: 21.2 min), and **13** (3.0 mg, retention time: 23.6 min). Fraction 6-6 (retention time: 45.6—54.8 min) was separated by HPLC (Diachroma ODS M-20 5 μm, 10×250 mm, MeOH : H_2O=8 : 2, flow rate: 3.0 ml/min) to afford three fractions (Fr. 6-6-1—6-6-3). Fraction 6-6-1 (retention time: 14.6—15.4 min) was further purified by HPLC (Senshu Pak Silica-4251-N, 10×250 mm, *n*-hexane : EtOAc=10 : 1, flow rate: 3.0 ml/min) to give **14** (3.0 mg, retention time: 48.9 min). Fraction 6-6-2 (retention time: 16.0—16.8 min) was purified using HPLC (Senshu Pak Silica-4251-N, 10×250 mm, *n*-hexane : EtOAc=7 : 1, flow rate: 3.0 ml/min) to give **15** (10.0 mg, retention time: 26.8 min). Fraction 6-6-3 (retention time: 18.0—19.6 min) was purified by HPLC (Senshu Pak Silica-4251-N, 10×250 mm, *n*-hexane : EtOAc=10 : 1, flow rate: 3.0 ml/min) to afford **16** (3.0 mg, retention time: 35.4 min). Fraction 9 (2.1 g) was separated by HPLC (Shodex SIL-5E, 10×250 mm, *n*-hexane : EtOAc=3 : 1, flow rate: 3.0 ml/min) to afford seven fractions (Fr. 9-1—9-7). Fraction 9-3 (retention time: 15.0—16.2 min) was further purified by HPLC (Diachroma ODS M-20 5 μm, 10×250 mm, MeOH : H_2O=7 : 3, flow rate: 3.0 ml/min) to give **17** (2.0 mg, retention time: 13.4 min). Fraction 9-4 (retention time: 20.2—22.0 min) was purified using HPLC (Diachroma ODS M-20 5 μm, 10×250 mm, MeOH : H_2O=65 : 35, flow rate: 3.0 ml/min) to afford **18** (30.0 mg, retention time: 21.4 min). Fraction 9-6 (retention time: 24.7—26.0 min) was purified by HPLC (Diachroma ODS M-20 5 μm, 10×250 mm, MeOH : H_2O=6 : 4, flow rate: 3.0 ml/min) to give **19** (3.0 mg, retention time: 9.5 min).

Baccharisketone (**1**): Colorless oil. $[\alpha]_D$ −13.2° (*c*=0.1, $CHCl_3$). TLC: *Rf* 0.26 (Merck, silica gel, solvent; *n*-hexane : EtOAc=20 : 1). ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 0.71 (H-14; 3H, d, *J*=7.3 Hz), 0.95 (H-15; 3H, d,

J=7.3 Hz), 1.76 (H-9a; 1H, m), 1.78 (H-13; 1H, m), 2.00 (H-12; 3H, s), 2.09 (H-9b; 1H, m), 2.16 (H-8; 1H, m), 2.18 (H-10; 2H, m), 2.32 (H-7; 3H, s), 6.97 (H-3 and H-5; 2H, d, J=8.1 Hz), 7.09 (H-2 and H-6; 2H, d, J=8.1 Hz). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 20.8 (q, C-14), 20.9 (q, C-15), 21.0 (q, C-7), 26.8 (t, C-9), 30.0 (q, C-12), 33.6 (d, C-13), 42.2 (t, C-10), 52.0 (d, C-8), 128.3 (d, C-3 and C-5), 128.9 (d, C-2 and C-6), 135.5 (s, C-1), 140.5 (s, C-4), 209.3 (s, C-11). HR-EI-MS m/z: 218.1664 $[M]^+$ (Calcd for $C_{15}H_{22}O$: 218.1669), 175.1111 [M−isopropyl]$^+$ (Calcd for $C_{12}H_{15}O$: 175.1121).

p-Methoxythymol acetate (**2**): Oily material. ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 1.20 (H-8 and H-9; 6H, d, J=7.3 Hz), 2.16 (H-10; 3H, s), 2.30 ($OCOCH_3$; 3H, s), 2.96 (H-7; 1H, m), 3.82 (OCH_3; 3H, s), 6.71 (H-3; 1H, s), 6.76 (H-6; 1H, s). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 15.8 (q, C-10), 20.9 (q, $OCO\underline{C}H_3$), 23.0 (q, C-8 and C-9), 27.6 (d, C-7), 55.6 (q, OCH_3), 107.8 (d, C-3), 124.1 (d, C-6), 125.1 (s, C-5), 137.9 (s, C-2), 140.8 (s, C-1), 155.8 (s, C-4), 170.2 (s, $O\underline{C}OCH_3$). EI-MS m/z: 222 $[M]^+$.

Cytotoxicity Assay The effects of isolated compounds on the growth of leukemia cells (L-1210) were investigated as follows. Cells were suspended in RPMI 1640 medium (NISSUI) containing 100 μg/l kanamycin sulfate and 12% fetal bovine serum, supplemented respectively with L-glutamine and 10% $NaHCO_3$. Aliquots (2 ml) of cell suspension (approximately 1×10^5 cells/ml) were transferred into vials. After the addition of a compound to be tested at the concentration indicated, the cells were incubated under an atmosphere containing 5% CO_2 at 37 °C for 3 d and then the number of viable cells was counted after the colorization of dead cells with 0.5% trypan blue in order to distinguish between the cells. The percent inhibition of the compound in the L 1210 cells was calculated as follows,

$$\text{percent inhibition (\%)} = 100 - Sn/Cn \times 100$$

where Sn is the number of live cells in the sample medium after incubation and Cn is the number of live cells in the control medium after incubation. From the percent inhibition at the different concentrations of the compound, the median inhibitory concentration (IC_{50}) that caused a 50% reduction in the growth of L 1210 was calculated and reported.

References and Notes

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