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Bioactivity-guided isolation of cytotoxic sesquiterpenes of *Rolandra fruticosa*

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Abstract

Cytotoxicity-guided fractionation of a methanol extract of the leaves and twigs of *Rolandra fruticosa* using the HT-29 human colon cancer cell line led to the isolation of seven sesquiterpene lactones, including a new isorolandrolide, 13-methoxyisorolandrolide (**1**), a new bourbonenolide, 2 α ,13-diacetoxy-4 α -hydroxy-8 α -isobutyroxyloxybourbonen-12,6 α -olide (**2**), as well as five known compounds, 13-acetoxyrolandrolide (**3**), 8-desacyl-13-acetoxyrolandrolide-8-*O*-tiglate (**4**), 2-*epi*-glaucolide E (**5**), 2 α ,13-diacetoxy-4 α -hydroxy-8 α -methacryloxyloxybourbonen-12,6 α -olide (**6**), and 2 α ,13-diacetoxy-4 α -hydroxy-8 α -tigloyloxyloxybourbonen-12,6 α -olide (**7**). The structures of the two new sesquiterpenes were elucidated on the basis of spectroscopic methods. All isolates were evaluated for their cytotoxicity using the HT-29 cell line, and only 13-acetoxyrolandrolide (**3**) was found to possess a potent inhibitory effect against this cell line. Compounds **3**, **5** and **6** were also tested in a NF- κ B (p65) inhibition assay, and **3** was assessed in an *in vivo* hollow fiber assay.

Keywords

Rolandra fruticosa (Asteraceae); germacrane-type sesquiterpene lactones; isorolandrolide; rolandrolide; glaucolide; bourbonenolide; cytotoxicity; HT-29 human colon cancer cells; NF- κ B (p65) inhibition assay; *in vivo* hollow fiber assay

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1. Introduction

Rolandra fruticosa (L.) Kuntze (Asteraceae), the only species of the monotypic genus *Rolandra* Rottb., has been separated from the large subtribe Vernonieae and placed in the subtribe Rolandrinae (Robinson et al., 1980). This species, with the common name “yerba de plata”, is an evergreen shrub widespread throughout tropical areas of South America, and it has been introduced to Indonesia and Japan in Asia (Woodson et al., 1975; Smithsonian Tropical Research Institute Herbarium, 2009). This plant has been used in certain religious rituals by members of some indigenous populations living in Colombia and Panama (Duke, 1975; Woodson et al., 1975; Van Andel et al., 2007). According to the literature, species of the subtribe Vernonieae that are taxonomically closely related to the subtribe Rolandrinae have use as folk medicines for the treatment of various diseases such as arthritis (Latha et al., 1998), diabetes (Uhegbu et al., 2004; Okolie et al., 2008), edema (Awaad et al., 2000; Mazumder et al., 2003), HIV-related symptoms (Bessong et al., 2006; Njoroge and Bussmann, 2007), and malaria (Abosi and Raseroka, 2003; Walewa et al., 2003; Njan et al., 2008). Previous phytochemical studies on *R. fruticosa* have revealed the occurrence of germacrane-type sesquiterpene lactones including the bourbonenolides, glaucolides, isorolandrolides, and rolandrolides (Herz et al., 1981; Jakupovic et al., 1989). These sesquiterpenes are structurally close to germacrane-type sesquiterpenes that occur extensively in plants belonging to the tribe Vernonieae (Bohlmann and Jakupovic, 1990; Da et al., 2005). Rolandrolides and isorolandrolides have been identified only from *R. fruticosa* thus far. Information on the bioactivity of these principles is very limited, with the weak *in vivo* antitumor activity of 13-acetoxylolandrolide (**3**) against P-388 lymphocytic murine leukemia being the only report of this type (Herz et al., 1981).

As part of an effort to discover naturally occurring anticancer agents from diverse organisms (Kingham et al., 2009), the chloroform-soluble partition of a methanol extract from the combined leaves and twigs of *R. fruticosa* collected in Indonesia was found to exhibit an inhibitory effect against the HT-29 human colon cancer cell line. In the present work, activity-guided fractionation of the chloroform-soluble extract using HT-29 cells led to the isolation of seven sesquiterpene lactones (**1-7**). Among these compounds, **1** and **2** were identified as two new sesquiterpenes, while compounds **3-7** were identified as the previously known 13-acetoxylolandrolide (**3**) (Herz et al., 1981; Jakupovic et al., 1989), 8-desacyl-13-acetoxylolandrolide-8-*O*-tiglate (**4**) (Jakupovic et al., 1989), 2-*epi*-glaucolide E (**5**) (Jakupovic et al., 1989), 2 α ,13-diacetoxy-4 α -hydroxy-8 α -methacryloyloxybourbonen-12,6 α -olide (**6**) (Jakupovic et al., 1989), and 2 α ,13-diacetoxy-4 α -hydroxy-8 α -tigloyloxybourbonen-12,6 α -olide (**7**) (Jakupovic et al., 1989), respectively, by comparison of their spectroscopic data with published values. Herein, we present the isolation and structure elucidation of new compounds **1** and **2**, as well as the *in vitro* biological evaluation of all isolates obtained in two bioassays, and the evaluation of the previously known compound, 13-acetoxylolandrolide (**3**) in an *in vivo* hollow fiber assay.

2. Results and discussion

2.1 Structure elucidation of new compounds

Compound **1** was obtained as a white amorphous powder. The HRESIMS analysis of compound **1** gave a sodiated molecular ion peak at m/z 443.1702 $[M+Na]^+$ (calcd for $C_{22}H_{28}O_8Na$), consistent with a molecular formula of $C_{22}H_{28}O_8$. The IR absorptions indicated the occurrence of hydroxy group (3468 cm^{-1}), double bond ($3040, 954, 795\text{ cm}^{-1}$), and ester carbonyl ($1750, 1731\text{ cm}^{-1}$) functionalities. The strong absorption at 211 nm ($\log \epsilon$ 4.60) and a broad band around 313 nm ($\log \epsilon$ 3.89) in the UV spectrum suggested the presence of an α,β -unsaturated lactone group. In the 1H NMR spectrum (Table 1), five tertiary methyl group signals (δ_H 1.71, 1.97, 2.00, 2.03, and 3.41, *s*, each 3H) were observed, and resonances for two

olefinic protons at δ_H 6.14 (1H, *br s*, H-3') and 5.66 (1H, *br s*, H-3') were ascribed to an exocyclic methylene, while another two olefinic proton signals at δ_H 4.76 (*br d*, $J = 10$ Hz, H-1) and 5.71 (*br s*, H-5) were attributed to two endocyclic double bonds. Moreover, the proton signals at δ_H 6.37 (*dd*, $J = 5.0, 2.0$ Hz, H-8) and δ_H 5.82 (*ddd*, $J = 10.8, 10.2, 3.0$ Hz, H-2) were consistent with the presence of two esterified oxymethine groups, and two signals at δ_H 4.36 and 4.27 (each 1H, H-13) with an AB coupling pattern ($J_{A,B} = 13.6$ Hz) suggested the presence of an isolated oxymethylene. Additional analysis of the ^{13}C NMR spectrum based on the ^1H NMR data mentioned above revealed the presence of an acetyl group, a methoxy group, as well as a methacrylate group (see Tables 1 and 2). After deduction of these three substituent residues, there were 15 carbon signals remaining in the ^{13}C NMR spectrum of **1**, which were classified by DEPT and HSQC into two methyls, two methylenes, four oxygenated carbons (including one primary, two secondary, and a tertiary), a carbonyl group, two trisubstituted double bonds, and a tetrasubstituted double bond. Analysis of these data suggested that compound **1** is an isorolandrolide analog. Isorolandrolide, a bicyclic germacranes sesquiterpene based on a carbon skeleton with a cyclodeca[*b*]furan moiety, having a 1(10)-*E* double bond and a 5-*Z* double bond in the ten-membered macrocyclic ring, was first isolated from *R. fruticosa* (Herz et al., 1981), and its structure was later revised (Jakupovic et al., 1989). Comparison of the ^1H -NMR data of compound **1** with those of isorolandrolide revealed that the proton signal of an acetate methyl group was absent and a methoxy group singlet appeared at δ_H 3.41 (*s*, 3H), consistent with upfield shifts of H-13 and H-13' of 0.23 ppm and 0.27 ppm, respectively. This evidence suggested that an acetyl group attached to C-13 in isorolandrolide was substituted by a methoxy group in compound **1**. In the HMBC spectrum, key correlations from H-13 to the methyl signal at δ_C 59.2 and from the methoxy protons to the oxygenated methylene at δ_C 63.9 (C-13) were found. Moreover, cross peaks between H-8 with the carbonyl of the methacrylate group at δ_C 166.4, and H-2 with the carbonyl of the acetyl group at δ_C 170.5 revealed the locations of these two ester residues. The positions of two endocyclic double bonds at C-1(10) and C-5(6) were confirmed from the HMBC correlations of H-1/C-14, H-1/C-9, H-2/C-1, H-9/C-10, H-5/C-15, H-5/C-4, and H-8/C-6.

The relative configuration of compound **1** was established by NOESY analysis as well as by chemical transformation. The configuration of the 1(10)-double bond was deduced as *E*, based on the observed NOE effects from H-14 to H-2, H-9 α , as well as from H-1 to H-3 β and H-9 β , and the *Z* configuration of 5(6)-double bond was suggested by NOE correlations between H-5 with H-14 and H-3 α . In order to confirm the configuration of these two double bonds, an intramolecular [2+2] addition reaction was induced by dissolving compound **1** in deuteriobenzene and keeping the solvent exposed to daylight for three days, to yield a known bourboneolide isomer (**1a**) as the major product. This chemical transformation also implied that the five-membered lactone ring and H-1 of compound **1** are β -oriented, while the methyl (C-14) on C-10 and H-5 are α -oriented relative to the main plane (see Fig. 1), which were consistent with deductions made by analysis of the NOESY spectrum. Furthermore, the β orientation of the acetyl group on C-2, as well as the α orientation of the methacrylate group on C-8, were deduced from NOE effects between H-2 with H-14 and H-3 α , and between H-8 with H-13, respectively. All of this information showed the relative configuration of compound **1** to be identical with that of known isorolandrolides. Thus, the structure of compound **1** was determined as 13-methoxyisorolandrolide.

In order to prove that compound **1** was not an artifact produced during extraction with methanol, a small amount of the plant material (10 g) was extracted with ethanol. Then, 10% water was added to the ethanol extract, and the latter was partitioned with hexane. The hexane solution was evaporated under reduced pressure to yield 200 mg of a hexane-soluble extract. A highly concentrated solution (100 mg/mL) of this hexane soluble extract was prepared and analyzed by HPLC using MeOH-H₂O (50:50; 1.5 ml/min). Compound **1** (t_R 24.7 min) was detected in

this hexane-soluble extract. Thus, compound **1** was determined to be a naturally occurring metabolite rather than a methylated artifact.

Compound **2** was obtained as a colorless resin. The molecular formula was determined as $C_{23}H_{30}O_9$, based on the sodiated molecular ion peak at m/z 473.1772 (calcd for $C_{23}H_{30}O_9Na$) in the HRESIMS. The IR spectrum showed absorptions for hydroxy (3479 cm^{-1}), carbonyl ($1768, 1740\text{ cm}^{-1}$), and methyl (1367 cm^{-1}) groups. In the 1H NMR spectrum (Table 1), two tertiary methyls (δ_H 1.24 and 1.31, *s*, each 3H), two acetyl groups (δ_H 2.04 and 2.07, *s*, each 3H), as well as an isobutyryl group (δ_H 1.20 and 1.21, each 3H, *d*, $J = 6.9\text{ Hz}$; δ_H 2.61, 1H, *hep*, $J = 6.9\text{ Hz}$) were recognized in the high-field region. Two proton signals at δ_H 5.38 (1H, *d*, $J = 7.0\text{ Hz}$, H-2) and δ_H 6.23 (1H, *dd*, $J = 9.2, 8.2\text{ Hz}$, H-8) suggested the presence of two esterified oxymethines, and resonances at δ_H 4.77 and 4.93 (each 1H, $J = 12.6\text{ Hz}$, H-13) were ascribed to an oxymethylene group. In the ^{13}C NMR spectrum, corresponding carbonyl groups of two acetyl groups and an isobutyrate group mentioned above were observed at δ_C 170.1, 170.4, and 176.0, respectively. Except for these three substituents, the 15 carbon signals that remained in the ^{13}C NMR spectrum were assigned as skeletal carbons with the aid of the DEPT and HSQC NMR data. On comparing the skeletal ^{13}C NMR signals of compound **2** with those of compound **1**, a similar α -oxymethylene- α,β -unsaturated- γ -lactone moiety was evident based on the chemical shifts of a carbonyl group at δ_C 170.1, a tetrasubstituted double bond at δ_C 169.0 and 123.6, and an oxymethylene at δ_C 55.2. Instead of ^{13}C NMR resonances belonging to two trisubstituted endocyclic double bonds in compound **1**, signals for two methines (δ_C 51.0, C-1; δ_C 59.5, C-5), an oxygenated carbon (δ_C 92.0, C-6), and a tertiary alkyl carbon (δ_C 47.8, C-10) were assigned for compound **2**. Furthermore, considering the unsaturation requirement from the molecular formula, a three-ring carbon skeleton was necessary to complete the molecular construction. Analysis of the above data indicated that compound **2** is a bourbonenoid sesquiterpene ester (Jakupovic et al., 1989). The position of the isobutyrate group was designated at C-8 based on a key HMBC correlation between H-8 with the carbonyl carbon that resonated at δ_C 176.0. The observed HMBC correlations between H-2 and the protons signal of the acetyl group at δ_H 2.07 with the carbonyl group at δ_C 170.1, and H-13 and the protons signal of the acetyl group at δ_H 2.04 with the carbonyl group at δ_C 170.4, led to the location of two acetyl groups on C-2 and C-13, respectively. The relative configuration of compound **2** was the same as that of known bourbonenoid analogs, as a result of the NOE effects between H-1 with H-14 and H-5, H-2 and H-8 with H-3 β and H-9 β , respectively, and H-15 with H-3 β (see Fig. 1). Therefore, the structure of compound **2** was determined as 2 α , 13-diacetoxy-4 α -hydroxy-8 α -isobutyroyloxybourbonen-12,6 α -olide.

2.2 Biological activity evaluation

All pure isolates obtained in the present investigation were evaluated for their cytotoxic activity against the HT-29 human colon cancer cell line (Table 2). Among these compounds, 13-acetoxyrolandrolide (**3**), was found to be a highly active principle with an $ED_{50} = 0.16\text{ }\mu\text{M}$, with compound **4** (8-desacyl-13-acetoxyrolandrolide-8-*O*-tiglate) being considerably less cytotoxic. The glaucolide analog **5** showed only marginal cytotoxicity, while the three bourbonenolides (**2**, **6** and **7**) were considered to be inactive ($ED_{50} > 10\text{ }\mu\text{M}$) (Table 2). An enzyme-based ELISA NF- κ B assay was employed to test the p65 (RelA) inhibitory activity of three major principles, **3**, **5** and **6**, and compounds **3** and **6** demonstrated moderate activities (Table 2). The major active compound, 13-acetylrolandrolide (**3**) was evaluated in the hollow fiber assay, which is used as a secondary discriminator for substances sharing promising *in vitro* activity, in order to prioritize such substances for possible further testing in a relevant *in vivo* xenograft system (Mi et al., 2009). The human cancer cell lines evaluated using ip administration included LNCaP (hormone-dependent human prostate cancer), MCF-7 (human breast cancer) and HT29 for the *in vivo* hollow fiber assay. Administration of the highest two doses (10 and 5 mg/kg/day) of **3** resulted in significant body weight loss (up to 20%) in the

host mice over the course of the study, and no inhibition of proliferation was observed in any of the cancer cell types. Therefore, 13-acetoxylolandrolide (**3**) was not found to represent a promising oncology lead for further development on the basis of the present study.

2.3 Conclusion

The cytotoxicity screening data obtained in this investigation implied that a 1(10)-Z double bond and a 5(6)-E double bond in the cyclodecane ring, as present in the structures of compounds **3** and **4**, is important for the cytotoxicity of the rolandrolide derivatives. The conversion of the configuration or the addition reaction of these two endocyclic double bonds will lead to the loss of cytotoxicity, as found for structures of the isorolandrolide analog **1**, the glaucolide analog **5**, and the bourbonenoid derivatives **2**, **6** and **7**. Moreover, the ester substituent on the α,β -unsaturated- γ -lactone moiety may have a significant impact on the cytotoxicity of rolandrolides. Thus, when the methacrylate group on C-8 in compound **3** was substituted by a tiglate group in compound **4**, the potency of the inhibitory effect on HT-29 cells decreased around ten times. It may be pointed out that the ten-membered macrocyclic ring with a 1(10) double bond and a 5(6) double bond occurred in the structures of rolandrolides was found to be unstable. As a major constituent of *R. fruticosa*, 13-acetoxylolandrolide (**3**) was partially transformed to compound **6** by a [2+2] addition, if kept in solvents such as CDCl_3 and deuteriobenzene at room temperature. This might be a possible reason for the lack of activity in the *in vivo* hollow fiber assay observed for this compound.

3. Experimental

3.1 General experimental procedures

Melting points were measured on a Fisher Scientific melting point apparatus and are uncorrected. Optical rotations were recorded on a Perkin-Elmer 343 automatic polarimeter. UV spectra were run on a Shimadzu 160U UV-vis recording spectrophotometer. IR spectra were obtained on a ATI Mattson Genesis Series FT-IR spectrometer. CD spectra were conducted on a JASCO J-810 spectropolarimeter. HRESIMS were acquired on a 3-Tesla Finnigan FTMS-2000 Fourier transform mass spectrometer. NMR spectroscopic data were obtained on a Bruker Avance DRX-600 MHz spectrometer (with TMS as an internal standard). Column chromatography was performed with 65-250 or 230-400 mesh silica gel (Sorbent Technologies, Atlanta, GA). Analytical thin-layer chromatography was conducted on precoated 250 μm thickness silica gel plates (UV₂₅₄, glass backed, Sorbent Technologies, Atlanta, GA), and preparative thin-layer chromatography was performed on precoated 20 cm $\times$ 20 cm, 500 μm thickness silica gel plates (UV₂₅₄, glass backed, Sorbent Technologies, Atlanta, GA). Analytical HPLC was conducted on a 150 mm $\times$ 4.6 mm i.d. Sunfire PrepC₁₈ column (Waters, Milford, MA), and semi-preparative HPLC was conducted on 150 mm $\times$ 10 mm i.d. or 150 mm $\times$ 19 mm i.d., 5 μm Sunfire PrepC₁₈ columns (Waters, Milford, MA), along with a Waters system equipped with a 600 controller, a 717 Plus autosampler, and a 2487 dual wavelength absorbance detector.

3.2. Plant material

The combined leaves and twigs of *Rolandra fruticosa* (500 g) were collected at Pangradin village, Jasinga, West Java by S. Riswan and the late Agus Ruskandi in August, 2005. A voucher specimen (acquisition no. 2285414) has been deposited in the John G. Searle Herbarium of the Field Museum of Natural History, Chicago, Illinois, where the identification of the species was confirmed.

3.3. Extraction and isolation

The dried and finely ground plant material (500 g) of *R. fruticosa* was extracted with methanol at room temperature three times (3×24 h). The solvent was evaporated under reduced pressure to yield 34 g of a crude extract, which was suspended in a methanol-water (9:1) mixture and then extracted with hexane (3×1 L) and chloroform (3×1 L), sequentially. The hexane-soluble extract (13 g) was obtained after evaporation of the hexane solution under reduced pressure. The chloroform partition was washed with 1% aqueous NaCl to yield 4.5 g of a partially detannified chloroform-soluble extract. Both the hexane-soluble and chloroform-soluble extracts were found to be active against the HT-29 cell line, with ED_{50} values of 4.5 μ g/ml and 1.6 μ g/ml, respectively. Part of the $CHCl_3$ extract (4.0 g) was subjected to separation over a Si gel column and eluted with a CH_2Cl_2 -acetone gradient solvent system (20:1 to pure acetone). Of the sub-fractions obtained, F01 and F02 were found to be the most active (both with ED_{50} values of 0.4 μ g/mL). These two fractions were combined (600 mg) and chromatographed on a Sephadex LH-20 column to remove chlorophylls, using MeOH- H_2O (90:10 to pure MeOH) for elution, then the residue (300 mg) was further purified repeatedly on a semi-preparative RP18 column (150 mm $\times$ 10 mm i.d.) by HPLC with MeOH- H_2O (50:50; 6 mL/min) as solvent system, to give compounds **3** (t_R 18.5 min; 50 mg), **5** (t_R 11.7 min; 25 mg), **6** (t_R 7.8 min; 10 mg), and a subfraction (F0104, t_R 13.2 min; 4.0 mg), which was further purified by HPLC using CH_3CN - H_2O (45:55; 6 mL/min) as mobile phase, to give compound **2** (t_R 11.4 min; 1.2 mg). The hexane-soluble extract was purified using a similar isolation procedure to the $CHCl_3$ -soluble extract. A portion of the hexane extract (12 g) was separated over a Si gel column with a gradient system of hexane-acetone for elution. Active subfractions F02 (ED_{50} 1.9 μ g/ml) and F03 (ED_{50} 1.5 μ g/ml) were combined (500 mg) and subjected to passage over a Sephadex LH-20 column, using MeOH- H_2O (90:10 to pure MeOH) for elution. The chlorophyll-free fraction (210 mg) was then purified on a semi-preparative RP18 column (150 mm $\times$ 19 mm i.d.) by HPLC using MeOH- H_2O (50:50; 10 mL/min) as solvent system. Compounds **3** (t_R 46.5 min; 10 mg), **5** (t_R 41.5 min; 12 mg), and **6** (t_R 30.2 min; 5 mg) were also found to be major constituents of the active fractions of the hexane-soluble extract. In addition to these three compounds, compounds **1** (t_R 60.7 min; 2.0 mg), **4** (t_R 67.2 min; 2.5 mg) and **7** (t_R 36.0 min; 1.5 mg) were obtained as three minor principles.

3.4. 13-Methoxyisorolandrolide (1)

White amorphous resin; $[\alpha]^{23}_D -7.0$ (c 0.1, MeOH); UV (MeOH) λ_{max} (log ϵ) 211 (4.60) nm, 313 (br, 3.89) nm; IR ν_{max} (film) 3468, 3040, 2934, 1750, 1731, 1671, 1452, 1371, 1244, 1157, 1020, 954, 795 cm^{-1} ; CD (c 1.2×10^{-4} M, MeOH) λ_{max} ($\Delta\epsilon$) 214 (+16.16), 240 (+14.14), 320 (−6.01) nm; HRESIMS m/z 443.1702 $[M+Na]^+$, calcd for $C_{22}H_{28}O_8Na$, 443.1682; 1H and ^{13}C NMR data, see Table 1.

3.5. 2 α ,13-Diacetoxy-4 α -hydroxy-8 α -isobutyroyloxybourbonen-12,6 α -olide (2)

Colorless resin; $[\alpha]^{23}_D -83$ (c 0.04, MeOH); UV (MeOH) λ_{max} (log ϵ) 218 (br, 4.53) nm; IR ν_{max} (film) 3479, 2966, 2923, 1768, 1740, 1455, 1367, 1246, 1148, 1025, 923 cm^{-1} ; CD (c 1.1×10^{-4} M, MeOH) λ_{max} ($\Delta\epsilon$) 205 (+11.2), 240 (−36.98), 320 (+1.86) nm; HRESIMS obsd m/z 473.1772 $[M+Na]^+$, calcd for $C_{23}H_{30}O_9Na$, 473.1788; 1H and ^{13}C NMR data, see Table 1.

3.6. 13-Acetoxyrolandrolide (3)

Colorless crystals; mp 169–170 $^{\circ}C$ (lit. 174–175 $^{\circ}C$); $[\alpha]^{23}_D +63.0$ (c 0.1, MeOH) (lit. +60.6); HRESIMS obsd m/z 471.1643 $[M+Na]^+$, calcd for $C_{23}H_{28}O_9Na$ 471.1631; the spectroscopic data (1H and ^{13}C NMR) were comparable with published values (Herz et al., 1981; Jakupovic et al., 1989).

3.7. 8-Desacyl-13-acetoxyrolandrolide-8-O-tiglate (4)

Colorless gum; $[\alpha]_D^{23} +52.0$ ($c = 0.04$, MeOH); HRESIMS obsd m/z 485.1773 $[M+Na]^+$, calcd for $C_{24}H_{30}O_9Na$ 485.1788; the spectroscopic data (1H and ^{13}C NMR) were comparable with published values (Jakupovic et al., 1989).

3.8. 2-epi-Glaucolide E (5)

Colorless crystals; mp 156-157 °C (lit. 159 °C); $[\alpha]_D^{23} -19.0$ ($c = 0.1$, $CHCl_3$) (lit. -25.0); HRESIMS obsd m/z 471.1646 $[M+Na]^+$, calcd for $C_{23}H_{28}O_9Na$ 471.1631; the spectroscopic data (1H and ^{13}C NMR) were comparable with published values (Jakupovic et al., 1989).

3.9. 2 α ,13-Diacetoxy-4 α -hydroxy-8 α -methacryloyloxybourbonen-12,6 α -olide (6)

White amorphous powder; $[\alpha]_D^{23} -60.0$ ($c = 0.1$, MeOH) (lit. -70.0); HRESIMS obsd m/z 471.1637 $[M+Na]^+$, calcd for $C_{23}H_{28}O_9Na$ 471.1631; the spectroscopic data (1H and ^{13}C NMR) were comparable with published values (Jakupovic et al., 1989).

3.10. 2 α ,13-Diacetoxy-4 α -hydroxy-8 α -tigloyloxybourbonen-12,6 α -olide (7)

White amorphous powder; $[\alpha]_D^{23} -12.0$ ($c = 0.05$, MeOH); HRESIMS obsd m/z 485.1789 $[M+Na]^+$, calcd for $C_{24}H_{30}O_9Na$ 485.1788; the spectroscopic data (1H and ^{13}C NMR) were comparable with those published values (Jakupovic et al., 1989).

3.11. 2 β -Acetoxy-4 α -hydroxy-13-methoxy-8 α -methacryloyloxycompa-7(11)-en-12,6 α -olide (1a)

Compound **1** (0.8 mg) was dissolved in 2 mL of deuteriobenzene, and the solution was kept in a small glass vial and exposed to daylight for 72 hours at room temperature. Deuteriobenzene was evaporated to give 0.8 mg of residue, with bourboneolide isomer **1a** obtained as a major compound (yield >90%). Pale yellow oil; $[\alpha]_D^{23} -40.0$ ($c = 0.03$, MeOH); HRESIMS m/z 443.1698 $[M+Na]^+$, calcd for $C_{22}H_{28}O_8Na$, 443.1682; the spectroscopic data (1H and ^{13}C NMR) were comparable with those published values (Jakupovic et al., 1989).

3.12. Evaluation of cytotoxic activity

The cytotoxic activity of extracts, chromatographic fractions of extracts, and all pure compounds were evaluated against the HT-29 (human colon cancer) cell line, according to an established protocol (Seo et al., 2001).

3.13. Evaluation of NF- κ B inhibitory activity

The NF- κ B p65 subunit inhibitory activity of three major compounds (**3**, **5**, and **6**) was tested in an enzyme-based ELISA NF- κ B assay, which was carried out according to an established protocol (Renard et al., 2001; Deng et al., 2009). Rocaglamide was used as a positive control.

3.14 Evaluation in the *in vivo* hollow fiber assay

The potential *in vivo* anticancer activity of 13-acetoxyrolandrolide (**3**) against HT-29, LNCaP, and MCF-7 human cancer cells was evaluated in the murine hollow fiber model, according to a procedure developed at the U.S. National Cancer Institute (NCI) (Hollingshead et al., 1995; Mi et al., 2009). Based on our previous MTT assay results, a dose range from 0.5 to 10 mg 13-acetylrolandrolide (**3**) per kg per day for four days was used. The vehicle was 5% EtOH and 5% Tween 80 in physiologic saline, and paclitaxel was used as the positive control (2.0 mg/kg per day for four days).

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Fig. 1.
Key NOESY correlations of **1** and **2**

1 **2** R = Isobutyryloyl**6** R = Methacryloyl**7** R = Tigloyl **3** R = Methacryloyl**4** R = Tigloyl **5** **1a**

Table 1¹H and ¹³C NMR chemical shifts of compounds **1** and **2**^a

position	1		2	
	δ_C , mult. ^b	δ_H , (J in Hz) ^c	δ_C , mult. ^b	δ_H , (J in Hz) ^c
1	129.1	4.76 (<i>br d</i> , 10.0)	51.0	2.71 (<i>d</i> , 10.8)
2	68.7	5.82 (<i>ddd</i> , 10.8, 10.2, 3.0)	77.2	5.38 (<i>d</i> , 7.0)
3 α	51.3	2.06 (<i>dd</i> , 13.0, 3.0)	49.2	2.22 (<i>d</i> , 16.7)
3 β		2.23 (<i>dd</i> , 13.0, 11.0)		2.53 (<i>dd</i> , 16.7, 7.0)
4	73.0		80.6	
5	133.2	5.71 (<i>br s</i>)	59.5	3.43 (<i>dd</i> , 10.2, 1.8)
6	144.1		92.0	
7	152.4		169.0	
8	70.2	6.37 (<i>dd</i> , 5.0, 2.0)	70.7	6.23 (<i>dd</i> , 9.2, 8.2)
9 α	44.5	2.81 (<i>dd</i> , 14.0, 5.4)	45.5	1.74 (<i>dd</i> , 14.0, 9.2)
9 β		2.31 (<i>br d</i> , 14.0)		2.89 (<i>dd</i> , 14.0, 8.2)
10	135.6		47.8	
11	123.6		123.6	
12	169.0		170.1	
13a	63.9	4.36 (<i>d</i> , 13.6)	55.2	4.93 (<i>d</i> , 12.6)
13b		4.27 (<i>d</i> , 13.6)		4.77 (<i>d</i> , 12.6)
14	21.5	2.03 (<i>s</i>)	22.6	1.31 (<i>s</i>)
15	29.1	1.71 (<i>s</i>)	23.2	1.24 (<i>s</i>)
1'	166.4		176.0	
2'	135.6		33.8	2.61 (<i>hep</i> , 6.9)
3'	127.4	6.14 (<i>br s</i>)	19.0	1.20 (<i>d</i> , 6.9)
		5.66 (<i>br s</i>)		
4'	18.7	1.97 (<i>br s</i>)	18.5	1.21 (<i>d</i> , 6.9)
OAc-2	170.5		170.1	
	20.4	2.00 (<i>s</i>)	21.3	2.07 (<i>s</i>)
OAc-13			170.4	
			20.6	2.04 (<i>s</i>)
OMe-13	59.2	3.41 (<i>s</i>)		

^a¹H NMR measured at 600 MHz, ¹³C NMR measured at 150 MHz; obtained in CDCl₃ with TMS as internal standard. Assignments are based on HSQC and HMBC NMR spectra.

^bMultiplicity obtained from the DEPT spectra.

^cJ values (Hz) are given in parentheses.

Table 2*In vitro* cytotoxicity and NF- κ B inhibitory activity of compounds isolated from *R. fruticosa*.^a

Compound	Cytotoxicity ^b	NF- κ B Inhibition ^c
3	0.16	7.1
4	1.5	-
5	9.4	> 10
6	>10	2.8
Paclitaxel ^d	0.0006	-
Camptothecin ^d	0.06	-
Rocaglamide ^d	-	0.08

^aResults are expressed as ED₅₀ values (μ M).^bCompounds **1**, **2**, **6**, and **7** were considered to be inactive against HT-29 cells (ED₅₀ >10 μ M).^cCompounds **1**, **2**, **4**, and **7** were not tested due to the limited amount isolated.^dUsed as positive control substances.