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Cytotoxic and NF- κ B inhibitory sesquiterpene lactones from *Piptocomma rufescens*

Yulin Ren^a, Ulyana Muñoz Acuña^b, Francisco Jiménez^c, Ricardo García^c, Melciades Mejía^c, Heebyung Chai^a, Judith C. Gallucci^d, Norman R. Farnsworth^{e,†}, Djaja D. Soejarto^{e,f}, Esperanza J. Carcache de Blanco^{a,b}, and A. Douglas Kinghorn^{a,*}

^aDivision of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, The Ohio State University, Columbus, OH 43210, USA

^bDivision of Pharmacy Practice and Administration, College of Pharmacy, The Ohio State University, Columbus, OH 43210, USA

^cJardín Botánico Nacional “Dr. Rafael Ma. Moscoso”, Santo Domingo, Dominican Republic

^dDepartment of Chemistry and Biochemistry, The Ohio State University, Columbus, OH 43210, USA

^eProgram for Collaborative Research in the Pharmaceutical Sciences and Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, University of Illinois at Chicago, Chicago, Illinois 60612, USA

^fBotany Department, Field Museum of Natural History, Chicago, Illinois 60605, USA

Abstract

Six new (**1–6**) and eight known germacranolide-type sesquiterpene lactones, along with several known phenylpropanol coumarates and methylated flavonoids, were isolated from the leaves of *Piptocomma rufescens*, collected in the Dominican Republic. The new compounds were identified by analysis of their spectroscopic data, with the molecular structure of **3** being established by single-crystal X-ray diffraction. The absolute configurations of the sesquiterpene lactones isolated were determined from their CD and NOESY NMR spectra, together with the analysis of Mosher ester reactions. Bioassay screening results showed the majority of the sesquiterpene lactones isolated (**1–13**) to be highly cytotoxic toward the HT-29 human colon cancer cell line, with the most potent compound being 15-deoxygoyazensolid (**10**, IC₅₀, 0.26 μ M). In addition, several of the sesquiterpene lactones exhibited NF- κ B (p65) inhibitory activity.

*Corresponding author. Tel.: +1 614 247 8094; fax: +1 614 247 8081; kinghorn.4@osu.edu, kinghor@pharmacy.ohio-state.edu (A.D. Kinghorn).

[†]Deceased September 10, 2011.

Supplementary data

Copies of MS and ¹H and ¹³C NMR spectra of compounds **1–6** and ¹H NMR spectra of (*S*)- and (*R*)-MTPA esters (**1s/1r**) of **1**; diagrams of COSY and the key HMBC and NOESY correlations of compounds **2**, **4–13**, and eremantholide C; UV and CD spectra of compounds **1–13** and eremantholide C; structures of eremantholide C, phenylpropanol coumarates, and methylated flavonoids obtained from *P. rufescens*; crystallographic data and the CIF file of compound **3**; and analytical data of the known compounds. Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.tet.2012.01.061. These data include MOL files and InChIKeys of the most important compounds described in this article.

Keywords

Piptocoma rufescens, Goyazensolide-type sesquiterpene lactones; Germacranolide-type sesquiterpene lactones; Cytotoxicity for HT-29 human colon cancer cells; NF- κ B (p65) inhibition assay

1. Introduction

Piptocoma is a small genus of the plant family Asteraceae that occurs in tropical and subtropical regions of the Western Hemisphere.¹ There are no previous reports on the chemical constituents of any member of this genus, including the type species, *Piptocoma rufescens* Cass. As part of search for new natural product anticancer agents from diverse organisms,² a leaf crude methanol extract of *P. rufescens* collected in the Dominican Republic was found to be cytotoxic toward the HT-29 human colon cancer cell line. Using column chromatography, several new (**1–6**) and known cytotoxic sesquiterpene lactones, together with other known non-cytotoxic compounds, were isolated from this species. The isolation, structural characterization, and bioactivity evaluation of these isolates are reported herein.

2. Results and discussion

A methanol extract of the ground leaves of *P. rufescens* was partitioned with *n*-hexane and then chloroform, with these extracts subjected to cytotoxicity testing using the HT-29 human colon cancer cell line. The active *n*-hexane-soluble extract was separated by column chromatography over silica gel to afford two new compounds, rufesolides A (**3**) and B (**4**), and two known compounds, ereglomerulide (**7**)³ and 15-deoxygoyazensolide (**10**).⁴ In turn, separation of the chloroform-soluble extract of this plant part yielded four active fractions. Purification of the first fraction gave a new compound, rufesolide C (**5**), and five known compounds, 15-deoxygoyazensolide (**10**),⁴ 2',3'-dihydro-15-deoxygoyazensolide (**11**),⁵ lychnopholide (**12**),⁶ 4,5-dihydrolychnopholide (**13**),⁷ and eremantholide C.⁸ Separation of the second fraction produced three new compounds, rufescenolides A and B (**1** and **2**) and rufesolide D (**6**), and a known compound, 5-epiisogoyazensolide (**9**).⁹ Work up of the third fraction afforded three known compounds, goyazensolide (**8**),¹⁰ [3-(3,5-dimethoxy-4-hydroxyphenyl)]-1-propanol-*trans*-coumarate,³ and 4'-methoxy-5,7,3'-trihydroxyflavone.¹¹ Finally, separation of the fourth fraction yielded a further quantity of goyazensolide (**8**),¹⁰ in addition to 3-(4-hydroxyphenyl)-1-propanol-*trans*-coumarate,¹² 4'-methoxy-3,5,7,3'-tetrahydroxyflavone,¹¹ 3-methoxy-5,7-4'-trihydroxyflavone,¹¹ and 3,4'-dimethoxy-5,7-3'-trihydroxyflavone.¹¹ The structures of the cytotoxic sesquiterpene lactones isolated (**1–13**) are shown in Fig. 1, and those of the inactive compounds are presented in Fig. S10 (Supplementary Data).

Compound **1** was isolated as an amorphous white powder with a molecular formula of C₁₉H₂₂O₇, as determined by HRESIMS (*m/z* 385.1253 [M + Na]⁺, calcd for 385.1263). It showed UV (λ_{max} 212 and 262 nm) and IR [ν_{max} 3466 (hydroxy), 1766 and 1634 (α,β -unsaturated γ -lactone), 1712 and 1634 (α,β -unsaturated ester), 1712 and 1588 (dihydrofuran-3-one ring) cm⁻¹] absorptions typical for a furan ring-containing germacranolide, with such compounds also known as furanoheliangolides or goyazensolides.¹³ This preliminary structural assignment was supported in the ¹H NMR spectrum by a three-proton methyl singlet at δ_{H} 1.47, a three-proton methyl doublet at δ_{H} 1.36, two doublets at δ_{H} 5.50 and 6.23 for two terminal olefinic protons (H-13), and a broad singlet at δ_{H} 5.78 for a vinylic proton H-2 (Table 1). In the ¹³C NMR spectrum, signals were observed for a carbonyl group at δ_{C} 204.7, an oxygen-substituted vinylic group at δ_{C} 106.2

and 192.0, a lactone carbonyl group at δ_C 168.2, and three oxygen-bearing carbons at δ_C 74.6, 80.7, and 71.0 (Table 2).¹⁰

An oxygen bridge between C-3 and C-10 was deduced from the HMBC correlations from H-2 to C-10, H-4 to C-2, and H-15 to C-3 (Fig. 2). In addition, the 1H and ^{13}C NMR spectra of **1** revealed the presence of a methacrylate group, characterized by a methyl singlet at δ_H 1.81 and two broad singlets at δ_H 5.53 and 5.99 in the 1H NMR spectrum, and four signals that appeared at δ_C 166.9 (C-1'), 135.3 (C-2'), 126.6 (C-3'), and 17.9 (C-4') in the ^{13}C NMR spectrum.¹⁰ This methacrylate group was assigned to C-8, as supported by a HMBC correlation between H-8 and C-1' (Fig. 2).

Comparison of the 1H and ^{13}C NMR data of **1** with those of **10**⁴ (Tables 1 and 2) indicated both compounds to be methacryloxy-bearing goyazensolide-type sesquiterpene lactones, with differences evident at their C-4 and C-5 positions. Replacement of the observed ^{13}C NMR signals at δ_C 130.4 (C-4) and 135.0 (C-5) for **10**⁴ with those at δ_C 37.5 (C-4) and 74.6 (C-5) for **1** (Table 2) indicated the presence of a 5-hydroxy substituent for the latter compound. This was evidenced by the molecular formula of $C_{19}H_{22}O_7$ for **1** ($C_{19}H_{20}O_6$ for **10**) indicated by the mass spectrum, and supported by HMBC correlations between H-2/C-10, H-4/C-2 and C-6, H-15/C-3 and C-5, and H-5/C-7, and the proton-proton spin system from H-4/H-5, H-5/H-6, H-6/H-7, H-7/H-8 to H-8/H₂-9 established from the 1H - 1H COSY NMR spectrum (Fig. 2).

The relative configurations at C-4, C-5, C-6, C-7, C-8, and C-10 of **1** were deduced by analysis of the NOESY 2D NMR spectrum (Fig. 3), and the absolute configuration at C-7 of **1** was determined by analysis of its CD spectrum. Negative Cotton effects at 232 and 264 nm observed in the CD spectrum (Fig. 4) supported a 7*R* absolute configuration, consistent with previous studies demonstrating that sesquiterpene lactones with a *trans*-fused α -methylene- γ -lactone ring connected between the C-6 and C-12 positions with an α -configuration for H-7 (7*R*) show negative Cotton effects at ca. 220 and 260 nm in their CD spectra, arising from the $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ transitions of the lactone ring, respectively.^{14,15}

To determine the absolute configuration at C-5, the modified Mosher ester NMR method was applied to **1**. Following a literature procedure, the (*S*)-MTPA ester of **1** (**1s**) was prepared by reaction of **1** and (*R*)-(-)- α -methoxy- α -(trifluoromethyl)phenylacetyl chloride, and the (*R*)-MTPA ester of **1** (**1r**) was prepared by reaction of **1** and (*S*)-(+)- α -methoxy- α -(trifluoromethyl)phenylacetyl chloride.¹⁶ Analysis of 1H NMR chemical shift differences ($\Delta\delta_{S-R}$) between **1s** and **1r** revealed negative values for H-2, H-5, H-9, H-13a, H-14, and H-15, and positive values for H-6, H-8, H-13b, H-3', and H-4' (Fig. 5), which indicated a *S* configuration for C-5.¹⁶ In turn, the absolute configurations at C-4, C-6, C-8, and C-10 were determined as *R*, *S*, *S*, and *R*, respectively, by analysis of the NOESY correlations between H-5/H-14, H-6/H-8 and H-15, H-7/Hb-9 and H-14, and H-8/H-15 (Fig. 3). Thus, the new compound **1** was determined as (4*R*,5*S*,6*S*,7*R*,8*S*,10*R*)-1-oxo-3,10-epoxy-5-hydroxy-8-methacryloyloxygermacra-2,11(13)-dien-6,12-olide,¹⁷ and has been assigned the trivial name, rufescenolide A.

Compound **2** was isolated as an amorphous white powder with a molecular formula of $C_{20}H_{24}O_7$, 14 (CH_2) units greater than that of **1**, as determined by HRESIMS (m/z 399.1404 [$M + Na$]⁺, calcd for 399.1420). The closely related UV and IR spectra of **1** and **2** indicated the latter compound to be also a goyazensolide-type sesquiterpene lactone. Direct comparison of the 1H and ^{13}C NMR spectra of **1** and **2** showed that both compounds contain identical sesquiterpene lactone units but different ester residues at their C-8 positions. Compound **2** was assigned as having an angelate group, which displayed a methyl singlet at δ_H 1.75, a methyl doublet at δ_H 1.87, and a vinylic quartet at δ_H 6.10 in the 1H NMR

spectrum (Table 1).⁶ In turn, five signals for this ester group appeared at δ_C 167.2 (C-1'), 126.3 (C-2'), 141.1 (C-3'), 20.0 (C-4') and 15.7 (C-5') in its ^{13}C NMR spectrum (Table 2).⁶ The NOESY correlations between H-4'/H-3' and H-3'/H-5' indicated a *trans* configuration for the C-4' and C-5' methyl groups (Fig. S8, Supplementary Data). The closely comparable 1H and ^{13}C NMR data (Tables 1 and 2), specific rotation values, and the CD and NOESY NMR spectra of **1** and **2** (Fig. 4 and Fig. S8, Supplementary Data) suggested that both compounds have the same absolute configurations at their C-4, C-5, C-6, C-7, C-8, and C-10 stereogenic centers. Therefore, compound **2** (rufescenolide B) was determined as (4*R*, 5*S*, 6*S*, 7*R*, 8*S*, 10*R*)-1-oxo-3,10-epoxy-5-hydroxy-8-angeloyloxygermacra-2,11(13)-dien-6,12-olide.¹⁷

Compound **3**, obtained as colorless needles (mp 190–191°C), showed UV (λ_{max} 216 nm) and IR [ν_{max} 1769 (α,β -unsaturated γ -lactone), 1738 (ester) 1716 and 1698 (α,β -unsaturated ester) cm^{-1}] absorptions typical for a germacranolide.³ The positive HRESIMS exhibited a sodiated molecular ion peak at m/z 429.1890 (calcd 429.1889 for $C_{22}H_{30}O_7Na$), indicating eight degrees of unsaturation. Among these, four resulted from carbonyl groups, two from carbon-carbon double bonds, and the remaining two from a bicyclic system of a germacranolide.

A ketone group could be located at the C-1 position of **3**, as indicated by the HMBC correlations between H₂-2 and H₂-9/C-1 (Fig. 6). A saturated ten-membered ring was suggested from the long 1H - 1H COSY sequence represented by H₂-2/H₂-3/H-4 (H-15)/H₂-5/H-6/H-7/H-8/H₂-9. A cyclic lactone containing an exomethylene group could be proposed at the C-6 and C-7 positions, as supported by HMBC correlations between H-13/C-7 and C-12 (Fig. 6). An angelate group was linked to the C-8 position, as supported by the HMBC correlation between H-8/C-1' (Fig. 6). In addition, an acetoxy group was proposed at C-10, which was found to be the sole oxygen-substituted quaternary carbon in the molecule.

The complete structure of **3** was established by single-crystal X-ray diffraction analysis (Fig. 7), which confirmed the structure determined by analysis of the NMR spectroscopic data. The crystal structure of **3** showed that this molecule contains two rings, an almost planar *trans*-fused α -methylene- γ -lactone ring and a ten-membered ring adopting a half chair-boat-chair conformation, with C-1, C-2, C-3, C-4, C-5, C-9, C-10 forming a half chair conformation, C-1, C-3, C-4, C-5, C-6, C-8, C-9, and C-10 forming a boat conformation, and C-4, C-5, C-6, C-7, C-8, C-9, and C-10 comprising a chair conformation (Fig. 8). The axial proton H-7 is coupled with two *trans*-axial protons, H-6 and H-8, with Ha-2, H-6, H-8, H-14, and H-15 being β -configured, and Hb-2, H-4 and H-7 being α -configured, as indicated by its NOESY correlations (Fig. 9). An angelate residue is bonded to the C-8 position, and the carbonyl group is oriented *cis* to H-8 and in a *cis* relationship with the conjugated carbon-carbon double bond at the C-2' and C-3' positions. The two methyl groups of C-4' and C-5' are *trans* (Fig. 7).

Even though the conformation of the ring system and relative configuration of **3** have been established by X-ray analysis (Figs. 7 and 8), it is not possible to determine its absolute configuration in this manner. Refinement of the Flack parameter to determine the absolute configuration was inconclusive, as its value was 0.04(60).¹⁸ The small value of 0.04 indicates that the absolute configuration of the rufesolide A (**3**) molecule is probably correct, but the large error value of 0.60 gives little confidence in this number. As a result, it was not possible to determine the absolute configuration of **3** with the Flack parameter obtained. The Cotton effects displayed in the CD spectrum of **3** were sufficient to make such a determination. Negative Cotton effects at 223 and 272 nm in the CD spectrum (Fig. 4) of **3** implied a α -configuration for H-7 (7*S* configuration),^{14,15} and the 4*S*, 6*R*, 8*S*, and 10*R* configurations thus were established relative to 7*S* by X-ray crystallographic analysis and

supported by NOESY correlations between Ha-2/H-6, Hb-2/H-7, H-6/H-8 and H-15 (Fig. 9). Thus, the structure of **3** was determined as (4*S*,6*R*,7*S*,8*S*,10*R*)-1-oxo-8-angeloyloxy-10-acetoxygermacra-11(13)-en-6,12-olide.¹⁷ This germacranolide was accorded the trivial name, rufesolide A.

Compound **4** was isolated as an amorphous white powder with a molecular formula of C₂₂H₂₈O₇, from the HRESIMS sodiated molecular ion peak at *m/z* 427.1715, two hydrogen atoms less than that of **3**. This compound exhibited closely comparable ¹H and ¹³C NMR resonances to those of **3**, except for the signals for an extra carbon-carbon double bond at the C-3 and C-4 positions, as assigned in Tables 1 and 2 by HMBC correlations between H-2/C-1, H-5/C-3 and C-7, and H-15/C-3 and C-5 (Fig. S7, Supplementary Data). The closely comparable NOESY NMR and CD spectra of **4** with those of **3** (Figs. S8 and S9, Supplementary Data) indicated the same absolute configurations at the C-6, C-7, C-8, and C-10 stereogenic centers for both compounds. Therefore, compound **4** (rufesolide B) was determined as (6*R*,7*S*,8*S*,10*R*)-1-oxo-8-angeloyloxy-10-acetoxygermacra-3(4),11(13)-dien-6,12-olide.¹⁷

Compound **5** was isolated as an amorphous white powder with a molecular formula of C₂₃H₃₂O₈, 30 mass units more than that of **3**, consistent with the presence of a methoxy group at the C-3 position, as indicated by a singlet at δ_H 3.34 displayed in the ¹H NMR spectrum and a signal at δ_C 58.0 that appeared in the ¹³C NMR spectrum of **5**. This was supported by observed HMBC correlations between the protons of the methoxy group and C-3, H-5/C-3, and H-15/C-3 and C-5 (Fig. S7, Supplementary Data). The consistent NOESY and CD data of **5** with those of **3** (Figs. S8 and S9, Supplementary Data) suggested the same absolute configurations at the C-6, C-7, C-8, and C-10 stereogenic centers for both compounds, and 3*R* and 4*R* absolute configurations were determined by the NOESY correlations between H-3/H₃-15, H-6/Ha-2, H-8/Ha-2 and H-14 (Fig. S8, Supplementary Data). Therefore, compound **5** (rufesolide C) was determined as (3*R*,4*R*,6*R*,7*S*,8*S*,10*R*)-1-oxo-3-methoxy-8-angeloyloxy-10-acetoxygermacra-11(13)-en-6,12-olide.¹⁷

Compound **6** was isolated as an amorphous white powder with a molecular formula of C₂₁H₃₀O₇, 41 mass units (COCH₂) less than that of **5**, consistent with being a deacetyl derivative of the latter compound. This was supported by two signals at δ_C 21.0 and 169.6 for an acetyl group in the ¹³C NMR spectrum of **6** being absent in the analogous data for **5**. Comparison of the NMR data of **6** with literature values indicated this compound to be a new isomer of 3-methoxy-8β-angeloyloxyternifolin.¹⁹ The CD spectrum of **6** consistent with those of **3–5** (Fig. S9, Supplementary Data) indicated a 7*S* absolute configuration, and 3*R*, 4*R*, 6*R*, 8*S*, and 10*R* absolute configurations were supported by NOESY correlations between Ha-2/H-6 and H-8, H-3/H₃-15, H₃-15/Ha-5, and Ha-5/H-6 (Fig. S8, Supplementary Data). Therefore, compound **6** (rufesolide D) was determined as (3*R*,4*R*,6*R*,7*S*,8*S*,10*R*)-1-oxo-3-methoxy-8-angeloyloxy-10-hydroxygermacra-11(13)-en-6,12-olide.¹⁷

The structures of the known compounds (Fig. S10, Supplementary Data) isolated from *P. rufescens*, including several sesquiterpene lactones, phenylpropanol coumarates, and methylated flavonoids, were determined by analysis of their spectroscopic data and comparison of these data with reference values,^{3–12} and the assignments of their ¹H and ¹³C NMR spectroscopic data are listed in the Tables S1–S5 (Supplementary Data). The absolute configurations of the known sesquiterpene lactones were determined by comparison of their NOESY NMR and CD spectra with those of **1–6** (Figs. S8 and S9, Supplementary Data).

All isolated goyazensolides and germacranolides (**1–13**) displayed negative Cotton effects around 220 and 260 nm in their CD spectra and UV absorption maxima around 215 nm (Fig. S9, Supplementary Data), indicating an absolute configuration for the C-7 position.^{14,15}

Additionally, goyazensolides (**1–2** and **8–13**) all showed positive Cotton effects around 210 and 310 nm in their CD spectra and UV absorption maxima around 260 nm (Fig. S9, Supplementary Data), which disappeared in the CD and UV spectra of germacranolides (**3–7**), indicating that these positive Cotton effects are indicative of exciton coupling arising from the α,β -unsaturated carbonyl chromophores at C-10²⁰ for an α -configuration for H-14 (a 10*R* configuration for compounds **1–2** and **8–13**).

All compounds obtained in this study from *P. rufescens* were tested in terms of their cytotoxicity against the HT-29 human colon cancer cell line, using paclitaxel as the positive control. Both the goyazensolides (**1–2** and **8–13**) and germacranolides (**3–7**) showed potent cytotoxicity toward this cell line (Table 3), with 15-deoxygoyazensolide (**10**) being the most active compound with an IC₅₀ value of 0.26 μ M. However, all other compounds were inactive in this assay system. All the cytotoxic sesquiterpene lactones contain a α -methylene- γ -lactone ring at the C-6 and C-7 positions and a α,β -unsaturated ester group at the C-8 position, which have been reported as key structural requirements for sesquiterpene lactones to mediate cytotoxicity.²¹ To further investigate of the bioactivities of the isolates, all compounds obtained in sufficient quantity in this study, except for **1**, **2**, **9**, **11**, eremantholide C, and 4'-methoxy-5,7,3'-trihydroxyflavone, were tested in a NF- κ B (p65) inhibition assay, using rocaglamide as the positive control. Compounds **5**, **8**, **10**, and **12** were active (Table 3), with all other compounds tested being inactive (IC₅₀ value > 10 μ M).

Several goyazensolides and germacranolides, including goyazensolide (**8**), lychnopholide (**12**), and parthenolide, have been reported previously to exhibit cytotoxicity toward a panel of human cancer cell lines.²² Recently, goyazensolide (**8**) and 15-deoxygoyazensolide (**10**), together with other analogues, have been investigated for their NF- κ B inhibitory activity.²³ The NF- κ B inhibitory activity of compounds **5** and **12** has not been reported previously.

In summary, a number of germacranolide-type sesquiterpene lactones, phenylpropanol coumarates, and methylated flavonoids were isolated and characterized from the leaves of *P. rufescens* for the first time in this study. Based on the results obtained, both the goyazensolides and germacranolides can be regarded as characteristic chemical components and the cytotoxic principles of this species.

3. Experimental

3.1. General experimental procedures

Melting points were measured using a Fisher Scientific apparatus and are uncorrected. Specific rotation values were obtained on a Perkin-Elmer model 343 polarimeter. UV spectra were recorded on a Hitachi U2910 UV spectrophotometer. CD measurements were performed using a JASCO J-810 spectropolarimeter. IR spectra were recorded on a Nicolet 6700 FT-IR spectrometer. ¹H and ¹³C, DEPT, HSQC, HMBC, NOESY, and COSY NMR spectra were recorded at room temperature on Bruker Avance DPX-300, DRX-400, DRX-600, or DRX-800 MHz NMR spectrometers, with TMS as internal standard. ESIMS and HRESIMS were measured on a LCT-TOF mass spectrometer in the positive-ion mode. Column chromatography was conducted using silica gel (65 $\times$ 250 or 230 $\times$ 400 mesh, Sorbent Technologies, Atlanta, GA). Analytical and preparative thin-layer chromatograph (TLC) systems were performed on precoated silica gel 60 F254 plates (Sorbent Technologies, Atlanta, GA). Sephadex LH-20 was purchased from Amersham Biosciences, Uppsala, Sweden. For visualization of TLC plates, sulfuric acid reagent was used. All procedures were carried out using anhydrous solvents purchased from commercial sources and employed without further purification.

3.2. Plant material

The leaves of *Piptocoma rufescens* Cass. (Asteraceae) were collected from a shrub 1.5 m tall with violet stamens, at Villa Mella (18° 39' N; 69° 58' E; 150 m), on the road to Yamasá, in a forest on serpentine rock, Sierra Prieta, Distrito Nacional, Dominican Republic, in January, 1996. A voucher herbarium specimen (*Jiménez & Mejía 2040*) was identified by F. J., R. G., and D. D. S. and has been deposited both at the Herbarium of the Jardín Botánico Nacional “Dr. Rafael Ma. Moscoso”, Santo Domingo, Dominican Republic, and at the John G. Searle Herbarium of the Field Museum of Natural History, Chicago, IL, under the accession number FM2169750.

3.3. Extraction and isolation

The ground leaves of *P. rufescens* (470 g) were extracted with MeOH (4 L × 5) at room temperature. The solvent was evaporated in vacuo. The resultant dried MeOH extract (90 g, 19.2%) was suspended in 10% H₂O in MeOH (700 mL) and partitioned with *n*-hexane (500, 500, and 400 mL) to yield a *n*-hexane-soluble residue (18.0 g, 3.8%). A volume of 100 mL of H₂O was added to the aqueous-MeOH layer and this was then partitioned with CHCl₃ (500, 500, and 400 mL) to afford a chloroform-soluble extract (8.4 g, 1.8%), which was washed with 1% aqueous solution of NaCl to partially remove any plant polyphenols present. Both the *n*-hexane- and chloroform-soluble residues showed inhibitory activity toward the HT-29 human colon cancer cell line in a cytotoxicity assay.

The active *n*-hexane-soluble extract (18.0 g, IC₅₀ <10 µg/mL) was subjected to passage over a silica gel column (4.5 × 45 cm), eluted with gradient mixtures of *n*-hexane–acetone (100:1→1:1; 500 mL each). The eluates were pooled by TLC analysis to give thirteen combined fractions. Of these, fraction 9 (IC₅₀ <2 µg/mL) was chromatographed further over a silica gel column (2.5 × 20 cm), eluted with gradient mixtures of *n*-hexane–acetone (20:1→3:1, 200 mL each), and then purified by separation over a Sephadex LH-20 column (2.5 × 25 cm), by elution with CH₂Cl₂–MeOH (1:1), affording rufesolide B (**4**, 6.0 mg) and 15-deoxygoyazensolide (**10**, 50 mg). Fractions 10–12 (IC₅₀ <1 µg/mL) were combined and separated by silica gel chromatography, eluted with *n*-hexane–acetone (5:1), and then purified by passage over a Sephadex LH-20 column, by elution with CH₂Cl₂–MeOH (1:1), to afford rufesolide A (**3**, 11.0 mg) and ereglomerulide (**7**, 45 mg). Compound **3** was recrystallized from a mixture of *n*-hexane and acetone to give fine needles suitable for single-crystal X-ray analysis.

The active chloroform-soluble extract (8.0 g, IC₅₀ <2 µg/mL) was subjected to passage over a silica gel column (4.5 × 45 cm), and eluted with gradient mixtures of *n*-hexane–acetone (100:1→1:1; 500 mL each). Fractions were pooled by TLC analysis to yield nine combined fractions. Of these, fractions 1–4 were deemed cytotoxic toward the HT-29 cell line (IC₅₀ <1 µg/mL). Fraction 1 was chromatographed over a silica gel column, and eluted with gradient mixtures of *n*-hexane–acetone (10:1→3:1), to yield two combined sub-fractions. The first sub-fraction was chromatographed over silica gel using *n*-hexane–acetone (5:1) as solvent, and then purified by separation over a Sephadex LH-20 column, eluted with CH₂Cl₂–MeOH (1:1), affording rufesolide C (**5**, 13.0 mg) and 15-deoxygoyazensolide (**10**, 550 mg). The second sub-fraction was separated by silica gel chromatography, eluted with a gradient mixture of *n*-hexane–acetone (5:1→1:1), and then purified by passage over a Sephadex LH-20 column, eluted with CH₂Cl₂–MeOH (1:1), to afford 2',3'-dihydro-15-deoxygoyazensolide (**11**, 3.0 mg), lychnopholide (**12**, 5.0 mg), 4,5-dihydrolychnopholide (**13**, 2.0 mg), and eremantholide C (1.0 mg).

Fraction 2 was chromatographed over a silica gel column, and eluted with gradient mixtures of *n*-hexane–acetone (10:1→1:1), to yield two combined sub-fractions. The first sub-

fraction was chromatographed over silica gel using *n*-hexane–acetone (3:1) as solvent, and then finally purified by passage over a Sephadex LH-20 column, eluted with CH₂Cl₂–MeOH (1:1), affording rufescenolide A (**1**, 10.0 mg) and rufescenolide B (**2**, 1.0 mg). The second sub-fraction was separated by silica gel chromatography, eluted by *n*-hexane–acetone (3:1), and then purified by passage over a Sephadex LH-20 column, eluted with CH₂Cl₂–MeOH (1:1), to afford rufesolide D (**6**, 8.0 mg) and 5-epiisogoyazensolide (**9**, 2.0 mg).

Fraction 3 was chromatographed over silica gel, eluted by *n*-hexane–acetone (3:1), and then purified over a Sephadex LH-20 column, with CH₂Cl₂–MeOH (1:1) as eluent, affording goyazensolide (**8**, 15 mg), [3-(3,5-dimethoxy-4-hydroxyphenyl)]-1-propanol-*trans*-coumarate (6.0 mg), and 4'-methoxy-5,7,3'-trihydroxyflavone (1.0 mg).

Fraction 4 was chromatographed over silica gel, eluted with a gradient mixture of *n*-hexane–acetone (50:1→1:1), to produce three combined sub-fractions. The precipitate from the first sub-fraction was triturated and purified over a Sephadex LH-20 column, using CH₂Cl₂–MeOH (1:1) for elution, to afford 3,4'-dimethoxy-5,7-3'-trihydroxyflavone (31 mg). The mother liquor was evaporated in vacuo, and the residue was chromatographed over silica gel, eluted with *n*-hexane–acetone (3:1), and then purified over a Sephadex LH-20 column, using CH₂Cl₂–MeOH (1:1) for elution, to furnish goyazensolide (**8**, 90 mg). The second sub-fraction was chromatographed over silica gel, eluted by *n*-hexane–acetone (3:1), and then separated over Sephadex LH-20, using CH₂Cl₂–MeOH (1:1) for elution, to yield 3-(4-hydroxyphenyl)-1-propanol-*trans*-coumarate (15.0 mg). The third sub-fraction was separated by silica gel, eluted by *n*-hexane–acetone (1:1), and then purified finally over a Sephadex LH-20 column, using CH₂Cl₂–MeOH (1:1) for elution, furnishing 4'-methoxy-3,5,7,3'-tetrahydroxyflavone (2.0 mg) and 3-methoxy-5,7-4'-trihydroxyflavone (13.0 mg).

3.3.1. Rufescenolide A (1)—Amorphous colorless powder (*n*-hexane); [α]_D²⁰ +95 (*c* 0.12, MeOH); [α]_D²⁰ +132.0 (*c* 0.1, CH₂Cl₂); UV (MeOH) $\lambda_{\max}$ (log ϵ) 212 (3.87), 262 (3.81) nm; CD (MeOH, nm) $\lambda_{\max}$ ($\Delta\epsilon$) 210 (+7.8), 232 (−1.8), 264 (−4.0), 312 (+5.3); IR (dried film) $\nu_{\max}$ 3466, 1766, 1712, 1634, 1588, 1453 cm^{−1}; ¹H and ¹³C NMR data, see Tables 1 and 2; HRESIMS *m/z*: 385.1253 [M+Na]⁺ (calcd for C₁₉H₂₂O₇Na, 385.1263).

3.3.1.1. Preparation of the (S)-MTPA Ester (1s) of Rufescenolide A (1): Compound **1** (1.5 mg) was transferred to a NMR tube and dried under vacuum in an oil pump for 8 h. Deuterated pyridine (0.5 mL) and (*R*)-(−)- α -methoxy- α -(trifluoromethyl)phenylacetyl chloride (5 μ L) were transferred into the NMR tube under argon. Then, the NMR tube was shaken and kept at room temperature for 8 h. The ¹H NMR spectrum was measured directly at room temperature, and the data were assigned based on comparison of the ¹H NMR spectrum of the product with that of **1**. ¹H NMR data of **1s** (400 MHz, pyridine-*d*₅): δ 6.38 (1H, d, *J* = 2.78 Hz, H-13a), 6.05 (1H, brs, H-3'), 6.01 (1H, s, H-2), 5.97 (1H, dd, *J* = 6.70, 8.70, H-5), 5.67 (1H, d, *J* = 2.33, H-13b), 5.48 (1H, brs, H-3'), 4.83 (1H, dd, *J* = 4.00, 9.03 Hz, H-6), 4.68 (1H, d, *J* = 11.51 Hz, H-8), 3.88 (2H, m, H-7 and H-4), 2.73 (1H, t, *J* = 13.29 Hz, H-9), 2.49 (1H, d, *J* = 12.85 Hz, H-9), 1.76 (3H, s, H-4'), 1.55 (3H, s, H-14), 1.17 (3H, d, *J* = 6.92 Hz, H-15).

3.3.1.2. Preparation of the (R)-MTPA Ester (1r) of Rufescenolide A (1): Using the same procedure as described for **1s**, the (*R*)-MTPA ester derivative of **1** (**1r**) was produced by the reaction of **1** (1.5 mg), (*S*)-(+)- α -methoxy- α -(trifluoromethyl)phenylacetyl chloride (5 μ L), and deuterated pyridine (0.5 mL). ¹H NMR data of **1r** (400 MHz, pyridine-*d*₅): δ 6.38 (1H, d, *J* = 2.81 Hz, H-13a), 6.05 (1H, brs, H-3'), 6.04 (1H, s, H-2), 6.00 (1H, dd, *J* = 6.67 and 8.86 Hz, H-5), 5.65 (1H, d, *J* = 2.35 Hz, H-13b), 5.47 (1H, brs, H-3'), 4.77 (1H, dd, *J* = 4.02 and 8.82 Hz, H-6), 4.67 (1H, d, *J* = 12.45 Hz, H-8), 3.88 (2H, m, H-7 and H-4), 2.74 (1H, t,

$J = 13.08$ Hz, H-9), 2.50 (1H, d, $J = 12.74$ Hz, H-9), 1.75 (3H, s, H-4'), 1.56 (3H, s, H-14), 1.35 (3H, d, $J = 6.94$ Hz, H-15).

3.3.2. Rufescenolide B (2)—Amorphous colorless powder (*n*-hexane); $[\alpha]_D^{20} +40$ (c 0.06, MeOH); $[\alpha]_D^{20} +33.3$ (c 0.06, CH₂Cl₂); UV (MeOH) $\lambda_{\max}$ (log ϵ) 216 (3.94), 262 (3.79) nm; CD (MeOH, nm) $\lambda_{\max}$ ($\Delta\epsilon$) 209 (+1.1), 229 (−4.1), 270 (−3.3), 312 (+2.1); IR (dried film) $\nu_{\max}$ 3473, 1770, 1710, 1589, 1454 cm^{−1}; ¹H and ¹³C NMR data, see Tables 1 and 2; HRESIMS m/z : 399.1404 [M+Na]⁺ (calcd for C₂₀H₂₄O₇Na, 399.1420).

3.3.3. Rufesolide A (3)—Colorless needles (*n*-hexane/acetone); mp 190–191 °C; $[\alpha]_D^{20} +7.7$ (c 0.09, MeOH); $[\alpha]_D^{20} +1.0$ (c 0.1, CH₂Cl₂); UV (MeOH) $\lambda_{\max}$ (log ϵ) 216 (3.92) nm; CD (MeOH, nm) $\lambda_{\max}$ ($\Delta\epsilon$) 223 (−4.1), 272 (−0.7), 292 (−1.1); IR (dried film) $\nu_{\max}$ 1769, 1738, 1716, 1698, 1461 cm^{−1}; ¹H and ¹³C NMR data, see Tables 1 and 2; HRESIMS m/z : 429.1890 [M+Na]⁺ (calcd for C₂₂H₃₀O₇Na, 429.1889).

3.3.3.1. X-ray Crystal Data of Rufesolide A (3): Data for a small colorless needle from **3** were collected by synchrotron radiation at the Advanced Light Source at Lawrence Berkeley National Laboratory.²⁴ Crystal data: molecular formula C₂₂H₃₀O₇, MW=406.46, orthorhombic, space group *P*2₁2₁2₁, $a = 5.5228(2)$ Å, $b = 17.4103(7)$ Å, $c = 22.1641(10)$ Å, $V = 2131.2(2)$ Å³, $Z = 4$, $D_{\text{calc}} = 1.267$ gm/cm³, 150K, size 0.01 × 0.02 × 0.09 mm³, Bruker APEXII CCD diffractometer with $\lambda = 0.8856$ Å, $5.42^\circ \leq 2\theta \leq 67.36^\circ$ (Table S6, Supplementary Data). Data were collected using the *APEX2* software and processed with *SAINT* within *APEX2*.²⁵ Corrections for absorption and beam corrections are based on the multi-scan technique as implemented in *SADABS*.²⁵ The structure was solved by direct methods in *SHELXS-97*.²⁶ Full-matrix least-squares refinements based on F^2 were performed in *SHELXL-97*,²⁶ as incorporated in the *WinGX* package.²⁶ The final refinement cycle was based on 4356 intensities and 267 variables and resulted in agreement factors of $RI(F) = 0.060$ and $wR2(F^2) = 0.088$. For the subset of data with $I > 2\sigma(I)$, the $RI(F)$ value is 0.040 for 3464 reflections. Refinement of the Flack parameter¹⁸ to determine the absolute configuration was not conclusive, as its value was 0.04 (60). The CIF file of the X-ray data of **3** has been deposited in the Cambridge Crystallographic Data Centre (deposition no.: CCDC 852357).

3.3.4. Rufesolide B (4)—Amorphous colorless powder (*n*-hexane); $[\alpha]_D^{20} -46.6$ (c 0.09, MeOH); $[\alpha]_D^{20} -68.0$ (c 0.1, CH₂Cl₂); UV (MeOH) $\lambda_{\max}$ (log ϵ) 216 (3.94) nm; CD (MeOH, nm) $\lambda_{\max}$ ($\Delta\epsilon$) 206 (+10.3), 226 (−7.7), 279 (−3.8), 289 (−4.3); IR (dried film) $\nu_{\max}$ 1770, 1715, 1669, 1641, 1581, 1455 cm^{−1}; ¹H and ¹³C NMR data, see Tables 1 and 2; HRESIMS m/z : 427.1715 [M+Na]⁺ (calcd for C₂₂H₂₈O₇Na, 427.1733).

3.3.5. Rufesolide C (5)—Amorphous colorless powder (*n*-hexane); $[\alpha]_D^{20} +29.3$ (c 0.08, MeOH); $[\alpha]_D^{20} +10$ (c 0.1, CH₂Cl₂); UV (MeOH) $\lambda_{\max}$ (log ϵ) 219 (4.41) nm; CD (MeOH, nm) $\lambda_{\max}$ ($\Delta\epsilon$) 221 (−4.3), 275 (−2.1), 292 (−2.5); IR (dried film) $\nu_{\max}$ 1768, 1716, 1461 cm^{−1}; ¹H and ¹³C NMR data, see Tables 1 and 2; HRESIMS m/z : 459.2031 [M+Na]⁺ (calcd for C₂₃H₃₂O₈Na, 459.1995).

3.3.6. Rufesolide D (6)—Amorphous colorless powder (*n*-hexane); $[\alpha]_D^{20} +66.6$ (c 0.09, MeOH); $[\alpha]_D^{20} +65.0$ (c 0.1, CH₂Cl₂); UV (MeOH) $\lambda_{\max}$ (log ϵ) 215 (3.95) nm; CD (MeOH, nm) $\lambda_{\max}$ ($\Delta\epsilon$) 223 (−3.8); IR (dried film) $\nu_{\max}$ 3494, 1770, 1699, 1647, 1456 cm^{−1}; ¹H and ¹³C NMR data, see Tables 1 and 2; HRESIMS m/z : 417.1875 [M+Na]⁺ (calcd for C₂₁H₃₀O₇Na, 417.1889).

3.5. Cytotoxicity Assay

Cytotoxicity of the samples was performed against HT-29 human colon cancer cells by a previously reported procedure.²⁷ Paclitaxel was used as a positive control, and the IC₅₀ values of the test samples in serial dilutions were calculated using nonlinear regression analysis (Table Curve2Dv4; AISN Software, Inc., Mapleton, OR). Measurements were performed in triplicate and are representative of two independent experiments in which the values generally agreed within 10%.

3.6. Enzyme-Based ELISA NF-κB p65 Inhibition Assay

A NF-κB p65 inhibition assay was carried out using a published procedure, with an EZ-Detect Transcription Factor Assay System ELISA kit (Pierce Biotechnology, Rockford, IL).²⁷ Rocaglamide was used as a positive control, and measurements were performed in duplicate and are representative of two independent experiments, with the values generally agreed within 10%. The dose response curve was calculated for IC₅₀ determinations using non-linear regression analysis (Table Curve2DV4; AISN Software Inc., Mapleton, OR).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.
Structures of the cytotoxic sesquiterpene lactones (1–13) isolated from *P. rufescens*.

Fig. 2.
COSY and the key HMBC correlations of **1**.

Fig. 3.
Selected NOESY correlations of **1**.

Fig. 4.
CD and UV spectra of **1**, **2**, and **3**.

Fig. 5.
 $\Delta\delta_{S-R}$ values for the Mosher esters of **1**.

Fig. 6.
COSY and the key HMBC correlations of **3**.

Fig. 7. ORTEP plot for the molecular structure of **3** drawn with 50% probability displacement ellipsoids.

Fig. 8.
View of the central portion of compound **3** showing the conformation of the 10-membered ring.

3 R = OAc

Fig. 9.
Selected NOESY correlations of **3**.

Table 1

¹H NMR data of compounds 1–6 in CDCl₃.^a

Position	1 ^b	2 ^b	3 ^b	4 ^b	5 ^c	6 ^c
2a	5.78 brs	5.78 s	2.74 m	3.07 m	2.79 dd (9.9, 19.8)	2.45 dd (2.9, 16.6)
2b			2.59 m	3.38 m	3.22 dd (4.5, 19.0)	2.66 dd (3.9, 16.6)
3a			2.01 m	5.74 brs	3.67 m	3.70 m
3b			1.58 m			
4	3.39 m	3.36 m	2.01 m		2.14 m	2.13 m
5a	4.34 m	4.32 m	1.73 m	2.60 brs	2.03 m	2.19 dd (4.2, 16.5)
5b			1.67 m		1.45 m	1.12 m
6	4.49 dd (5.0, 9.5)	4.51 dd (5.0, 9.4)	4.30 m	4.19 m	4.45 m	4.68 m
7	3.35 m	3.36 m	2.97 m	3.27 m	2.88 m	2.86 m
8	4.34 m	4.32 m	4.94 m	5.22 m	4.98 m	4.88 dt (3.1, 11.4)
9a	2.34 dd (1.5, 14.2)	2.33 m	2.21 m	2.00 m	2.14 m	2.86 m
9b	2.43 dd (11.9, 12.9)	2.45 t (13.4)	2.59 m	2.43 t (12.0)	2.61 dd (9.0, 17.1)	3.00 m
13a	6.23 d (3.2)	6.23 d (3.2)	6.27 d (2.2)	6.35 br s	6.29 br s	6.24 br s
13b	5.50 d (2.7)	5.47 d (2.7)	5.63 d (1.5)	5.74 br s	5.67 br s	5.64 br s
14	1.47 s	1.47 s	1.77 s	1.79 s	1.88 s	1.34 s
15	1.36 d (7.0)	1.38 d (7.7)	1.02 d (6.4)	1.86 s	1.13 d (7.0)	1.06 d (6.3)
3'a	5.53 br s	6.10 q (8.6)	6.14 q (6.5)	6.13 q (6.0)	6.17 q (7.0)	6.09 dq (1.3, 7.2)
3'b	5.99 br s					
4'	1.81 s	1.75 s	1.84 s	1.77 s	1.82 s	1.86 d (1.3)
5'		1.87 d (8.6)	1.98 d (6.5)	1.98 d (6.0)	1.97 d (7.0)	1.98 dd (1.3, 7.2)
OMe-3					3.34 s	3.37 s
OAc-10			2.03 s	2.08 s	2.01 brs	
OH-10						3.81 s

^a Assignments based on analysis of 2D NMR spectra.

^b Data (6) measured at 400 MHz.

^c Data (6) measured at 300 MHz; s = singlet, br s = broad singlet, d = doublet, dd = double doublet, t = triplet, dt = double triplet, q = quartet, dq = double quartet, m = multiplet. *J* values are presented in Hz and are omitted if the signals overlapped as multiplets. The overlapped signals were assigned from ¹H–¹H COSY, HSQC, and HMBC spectra without designating multiplicity.

Table 2

¹³C NMR data of compounds **1–6** in CDCl₃.^a

Position	1 ^b	2 ^b	3 ^b	4 ^b	5 ^c	6 ^b
1	204.7 C	204.8 C	208.5 C	207.2 C	207.6 C	214.5 C
2	106.2 CH	106.2 CH	36.0 CH ₂	38.6 CH ₂	41.7 CH ₂	38.5 CH ₂
3	192.0 C	192.0 C	29.6 CH ₂	122.3 CH	79.7 CH	79.4 CH
4	37.5 CH	37.5 CH	29.6 CH	133.6 C	29.5 CH	30.8 CH
5	74.6 CH	74.7 CH	38.6 CH ₂	36.1 CH ₂	33.0 CH ₂	36.6 CH ₂
6	80.7 CH	80.7 CH	78.0 CH	78.4 CH	78.4 CH	76.8 CH
7	51.8 CH	51.8 CH	47.1 CH	47.7 CH	46.4 CH	44.3 CH
8	71.0 CH	70.7 CH	67.1 CH	65.5 CH	66.3 CH	70.0 CH
9	45.3 CH ₂	45.4 CH ₂	43.8 CH ₂	43.5 CH ₂	43.9 CH ₂	38.8 CH ₂
10	89.7 C	89.8 C	84.0 C	84.8 C	83.3 C	78.8 C
11	132.8 C	133.1 C	134.5 C	133.9 C	134.2 C	135.0 C
12	168.2 C	168.2 C	169.7 C	169.9 C	169.0 C	168.6 C
13	125.2 CH ₂	125.1 CH ₂	124.8 CH ₂	122.8 CH ₂	124.1 CH ₂	126.7 CH ₂
14	20.9 CH ₃	21.0 CH ₃	21.1 CH ₃	21.3 CH ₃	21.9 CH ₃	28.6 CH ₃
15	9.4 CH ₃	9.4 CH ₃	24.0 CH ₃	25.8 CH ₃	20.4 CH ₃	20.5 CH ₃
1'	166.9 C	167.2 C	166.2 C	166.3 C	166.4 C	166.3 C
2'	135.3 C	126.3 C	126.7 C	126.7 C	126.7 C	126.9 C
3'	126.6 CH ₂	141.1 CH	140.7 CH	140.8 CH	140.9 CH	139.9 CH
4'	17.9 CH ₃	20.0 CH ₃	20.4 CH ₃	20.4 CH ₃	20.4 CH ₃	20.5 CH ₃
5'		15.7 CH ₃	15.9 CH ₃	16.1 CH ₃	16.0 CH ₃	15.8 CH ₃
OAc			169.1 C	169.1 C	169.6 C	
			21.1 CH ₃	21.9 CH ₃	21.0 CH ₃	
OMe-3					58.0 CH ₃	58.2 CH ₃

^a Assignments based on analysis of 2D NMR spectra.

^b Data (δ) measured at 100.6 MHz.

^c Data (δ) measured at 75.5 MHz; CH₃, CH₂, CH, and C multiplicities determined by DEPT 90, DEPT 135, and HSQC experiments.

Table 3Cytotoxicity and NF- κ B p65 inhibition of **1–13**^a

Compound	Cytotoxicity ^b	NF- κ B p65 inhibition ^c
1	1.9	NT ^d
2	6.6	NT ^d
3	3.0	>10
4	4.0	>10
5	1.4	2.1
6	1.0	>10
7	1.2	>10
8	0.56	3.8
9	0.28	NT ^d
10	0.26	3.2
11	0.58	NT ^d
12	1.4	2.9
13	0.56	>10

^aAll data presented as 95% confidence intervals obtained by non-linear regression in these assays.^bData presented as IC₅₀ values (μ M) toward HT-29 cells, with paclitaxel used as a positive control (IC₅₀, 1 nM).^cData presented as IC₅₀ values (μ M), with rocaglamide used as positive control (IC₅₀, 0.075 μ M).^dNot tested due to sample limitations.