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Three acetylated flavonol glycosides from *Forsteronia refracta* that specifically inhibit p90 RSK

Ya-ming Xu,[a] Jeffrey A. Smith,[b] Deborah A. Lannigan[b] and Sidney M. Hecht[a,*]

[a]*Departments of Chemistry and Biology, University of Virginia, Charlottesville, VA 22901, USA*
[b]*Center for Cell Signaling, Department of Pathology and Microbiology, University of Virginia, Charlottesville, VA 22908, USA*

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Abstract—A survey of plant extracts for the presence of p90 ribosomal S6 kinase (RSK) inhibitors resulted in the isolation of three acetylated flavonol glycosides. Kaempferol 3-*O*-(2″,4″-*O*-diacetyl-α-L-rhamnopyranoside) (**1**), kaempferol 3-*O*-(3″,4″-*O*-diacetyl-α-L-rhamnopyranoside) (**2**), and kaempferol-3-*O*-(4″-*O*-acetyl-α-L-rhamnopyranoside) (**3**) were isolated from *Forsteronia refracta* as the first RSK inhibitors. Of these, compound **2** was found to be the best inhibitor with an IC_{50} value of 89 nM.

1. Introduction

The mitogen-activated protein kinases (MAPK) constitute a family of kinases involved in signal transduction in eukaryotic organisms.[1] The p90 ribosomal S6 kinases (RSK) are substrates for MAPK.[2,3] The human RSK kinases include four isozymes encoded by distinct genes. Studies of the mechanism of RSK activation have suggested the involvement of transcriptional regulators including c-Fos, estrogen receptor-α,[4] NFκB/IκBα,[5] cAMP-response element-binding protein (CREB) and CREB-binding protein,[6] and some kinases such as Myt1 protein kinase[7] and glycogen synthase kinase-3.[8] Because of the lack of a RSK-specific inhibitor, it is difficult to distinguish RSK functions from those of MAPK and other downstream regulators. The mechanism(s) by which proteins in the MAPK pathway control the growth and survival of a broad spectrum of human tumors is of considerable interest, and selective MAPK pathway inhibitors have already entered clinical trials.[9]

A survey of plant-derived extracts revealed that an extract prepared from *Forsteronia refracta* M. Arg. (Apocynaceae) specifically inhibited RSK2 without inhibiting FAK, a tyrosine kinase. The extract also failed to inhibit the archetypal serine/threonine kinase PKA and other two kinases related to RSK2, namely p70 S6K and MSK1. Bioassay-guided fractionation of the extract resulted in the isolation of three known flavonol glycosides as specific RSK inhibitors. This report details the isolation and structure identification of the active compounds. The chemical synthesis[10] and biological study[11] of these compounds have been described.

2. Results and discussion

A crude extract of *F. refracta*, exhibiting 76% p90 RSK inhibition at 2 μg/mL concentration, was applied to a polyamide 6S column to remove polyphenols. After washing successively with H_2O, 1:1 H_2O–MeOH, 9:1 CH_2Cl_2–MeOH, 1:1 CH_2Cl_2–MeOH, and 9:1 MeOH–NH_4OH, five fractions were obtained. The 1:1 CH_2Cl_2–MeOH fraction showed enhanced p90 RSK inhibition. The 1:1 CH_2Cl_2–MeOH fraction was fractionated further on a diol gel column. The column was washed successively with CH_2Cl_2, 99:1 CH_2Cl_2–MeOH, 95:5 CH_2Cl_2–MeOH, 90:10 CH_2Cl_2–MeOH, and MeOH to give five fractions. Among these, the 95:5 CH_2Cl_2–MeOH and 90:10 CH_2Cl_2–MeOH fractions exhibited enhanced inhibitory activity. After repeated fractionation by C_{18} reversed-phase HPLC, two compounds, **1** (IC_{50} 89 nM) and **2** (IC_{50} 580 nM), were obtained as amorphous pale yellow powders from the 95:5 CH_2Cl_2–MeOH fraction and compound **3** (IC_{50}

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* Corresponding author. Tel.: +1 434 924 3906; fax: +1 434 924 7856; e-mail: sidhecht@virginia.edu

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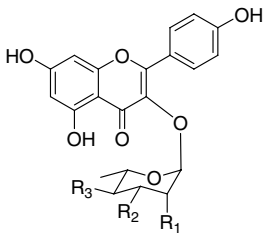
Table 1. RSK 2 inhibitory activities of compounds **1**–**3**

Compound	RSK inhibition (IC_{50}), nM
1	580
2	89
3	189

189 nM) was isolated from the 90:10 CH_2Cl_2–MeOH fraction, along with additional amounts of **1** and **2** (Table 1).



	R_1	R_2	R_3
1	OAc	OH	OAc
2	OH	OAc	OAc
3	OH	OH	OAc

The ^{1}H NMR spectral data for compounds **1**–**3** are shown in Table 2. Proton signals typical of a 5,7,4′-trihydroxyflavon-3-ol moiety were observed at δ 6.12–6.20 (1H, $J = 2.0$ Hz, H-6), δ 6.30–6.38 (1H, $J = 2.0$ Hz, H-8), δ 6.94–6.95 (2H, $J = 8.8$–9.0 Hz, H-3′, 5′), and δ 7.74 (2H, 8.8–9.0 Hz, H-2′, 6′) for the three compounds. The proton signals of a sugar anomeric proton at δ 5.46–5.51 and a methyl group at δ 0.70–0.77, together with four other protons at δ 3.2–5.5, revealed the existence of a rhamnoside moiety. The low field shift of sugar proton signals and the acetyl proton signals at δ 1.85–2.03 suggested that some of the sugar OH groups were acetylated. Compared with normal rhamnose proton signals, the relatively low field proton signals at δ 5.37 (1H, dd, 3.0 and 1.2 Hz, H-2″ in rhamnose) and 4.66 (1H, dd, 10.0 and 10.0 Hz, H-4″ in rhamnose) indicated that for **1** the hydroxyl groups at positions 2″ and 4″ in rhamnose were acylated. The methyl signals of two acetyl groups were overlapped with the solvent signals at δ 1.8–2.1. Therefore, the structure of **1** was concluded to be kaempferol 3-*O*-(2″,4″-*O*-diacetyl-α-L-rhamnopyranoside), which has been reported previously.[12] Similar examination and analysis of the ^{1}H NMR data for **2** and **3** resulted in the elucidation of the known compounds kaempferol 3-*O*-(3″,4″-*O*-diacetyl-α-L-rhamnopyranoside) (**2**)[13] and kaempferol-3-*O*-(4″-*O*-acetyl-α-L-rhamnopyranoside) (**3**), respectively.[14] The structure of compound **3** was further confirmed from its ^{13}C NMR data (see Table 3) and by total synthesis.[10]

Table 3. ^{13}C NMR data for compound **3** (ppm)

Carbon	**3**	
	Isolated CD_3OD	Literature[14] $(CD_3)_2CO$
2	158.6	158.2
3	135.5	135.4
4	179.5	179.1
4a	105.8	105.4
5	163.3	163.2
6	100.0	100.0
7	166.4	166.3
8	94.9	94.8
8a	159.4	158.4
1′	122.7	122.4
2′,6′	131.9	131.7
3′,5′	116.5	116.4
4′	161.7	161.2
1″	102.5	102.3
2″	71.7	71.5
3″	70.0	69.8
4″	74.9	74.5
5″	69.6	69.0
6″	17.6	17.6
OAc	20.9,172.4	20.9, 170.8

Although these compounds have been isolated previously as natural products, there is a paucity of biological data on acetylated kaempferol-3-α-L-rhamnopyranosides. Several acetylated kaempferol-3-α-L-rhamnopyranosides were reported to inhibit the DNA-dependent DNA polymerase activity of HIV-1 reverse transcriptase.[15] The present isolation of three acetylated kaempferol-3-α-L-rhamnopyranosides as RSK-specific inhibitors provides an important tool for studying RSK function. Among the three active compounds, **2** exhibited RSK inhibition several times more potent than that of **1** although both are diacetylated kaempferols differing structurally only by the position of the acetyl group on rhamnose. Compound **3** is a monoacetylated kaempferol, but was found to be a more potent RSK inhibitor than **1**. These findings indicated that modification of rhamnose moiety may significantly affect the activity of these

Table 2. ^{1}H NMR data of the related flavonoids **1**–**3** (ppm)

Proton	**1**	**2**		**3**	
	Isolated $(CD_3)_2CO$	Isolated $(CD_3)_2CO$	Literature[13] $(CD_3)_2CO$	Isolated CD_3OD	Literature[14] $(CD_3)_2CO$
6	6.12 (1H, d, 2.0 Hz)	6.15 (1H, d, 1.9 Hz)	6.29 (1H, d, 2.0 Hz)	6.20 (1H, d, 2.0 Hz)	6.27 (1H, br s)
8	6.30 (1H, d, 2.0 Hz)	6.35 (1H, d, 1.9 Hz)	6.48 (1H, d, 2.0 Hz)	6.38 (1H, d, 2.0 Hz)	6.47 (1H, br s)
2′, 6′	7.74 (2H, d, 8.8 Hz)	7.74 (2H, d, 8.8 Hz)	7.87 (2H, d, 9 Hz)	7.74 (2H, d, 9.0 Hz)	7.82 (2H, d, 8.6 Hz)
3′, 5′	6.94 (2H, d, 8.8 Hz)	6.94 (2H, d, 8.8 Hz)	7.07 (2H, d, 9 Hz)	6.95 (2H, d, 9.0 Hz)	7.05 (2H, d, 8.6 Hz)
1″	5.51 (1H, d, 1.2 Hz)	5.46 (1H, d, 1.2 Hz)	5.57 (1H, d, 1.2 Hz)	5.51 (1H, d, 1.5 Hz)	5.54 (1H, br s)
2″	5.37 (1H, dd, 1.2, 3.0 Hz)	4.31 (1H, dd, 1.2, 3.0 Hz)	4.43 (1H, br s)	4.20 (1H, dd, 3.5, 1.5 Hz)	4.25 (1H, br s)
3″	3.93 (1H, dd, 10.0, 3.0 Hz)	5.07 (1H, dd, 10.0, 3.0 Hz)	5.18 (1H, dd, 10,3 Hz)	3.84 (1H, dd, 10.0, 3.5 Hz)	3.87 (1H, dd, 3.4, 9.8 Hz)
4″	4.66 (1H, dd, 10.0, 10.0 Hz)	4.96 (1H, dd, 10.0, 10.0 Hz)	5.08 (1H, dd, 9, 10 Hz)	4.82 (1H, dd, 10.0, 10.0 Hz)	4.83 (1H, dd, 9.8, 9.8 Hz)
5″	3.30 (1H, dq, 6.2 Hz)	3.39 (1H, dq, 6.2, 10.0 Hz)	3.50 (1H, dq, 6,9 Hz)	3.23 (1H, m)	3.42 (1H, dq, 6.1, 10.3 Hz)
6″	0.70 (3H, d, 6.2 Hz)	0.70 (3H, d, 6.2 Hz)	0.83 (3H, d, 6 Hz)	0.77 (3H, d, 6.5 Hz)	0.78 (3H, d, 6.1 Hz)
OAc	Overlap with acetone peak	1.85 (3H, s)	1.97 (3H, s)	2.03 (3H, s)	
OAc	Overlap with acetone peak	1.90 (3H, s)	2.02 (3H, s)		

compounds. Compound **2** is a selective cytostatic agent for human breast cancer cells and lacks cytotoxicity toward normal cells.[11] The accumulated data suggest that acetyl kaempferol-3-α-L-rhamnopyranosides may provide the basis for the development of useful anticancer agents.

3. Experimental

3.1. General experimental procedures

Polyamide 6S (pour density 0.25 g/mL, a product of Riedel-de Haen, Germany) was obtained from Crescent Chemical Co. Lichroprep diol (40–63 μm) was purchased from EM Industries, Inc. A Kromasil C_{18} reversed-phase column (250 × 10 mm, 5 μm) for HPLC was obtained from Higgins Analytical Inc. ^{1}H NMR spectra were measured on General Electric QE 300 and Varian unity INOVA-500 spectrometers. Mass spectra were recorded on a Finnigan MAT 4600 mass spectrometer.

3.2. Plant material

Stem bark of *F. refracta* was collected in April, 1973 in Brazil. A voucher specimen of *F. refracta* (ID 440458) is preserved at the New York Botanical Garden.

3.3. Extraction and isolation

Dried stem bark of *F. refracta* was soaked three times with methanol at room temperature. The resulting methanol extracts were combined and concentrated under diminished pressure. The crude extract (888 mg, 24% activity at 2 μg/mL) was applied to a 40 g polyamide 6S column, which was washed successively with 150 mL each of H_2O, 1:1 H_2O–MeOH, 9:1 CH_2Cl_2–MeOH, 1:1 CH_2Cl_2–MeOH, and 9:1 MeOH–NH_4OH. The 1:1 CH_2Cl_2–MeOH fraction (127 mg, 20% RSK activity at 2 μg/mL and 28% RSK activity at 0.2 μg/mL) exhibited stronger inhibition of p90RSK than the starting original extract. The 1:1 CH_2Cl_2–MeOH fraction was fractionated further on a 30 g diol gel column. The column was washed successively with 150 mL each of CH_2Cl_2, 99:1 CH_2Cl_2–MeOH, 95:5 CH_2Cl_2–MeOH, 90:10 CH_2Cl_2–MeOH, and MeOH. Among these, the 95:5 CH_2Cl_2–MeOH (38.1 mg, 7% RSK activity at 2 μg/mL and 17% activity at 0.2 μg/mL) and 90:10 CH_2Cl_2–MeOH (42.1 mg, 14% activity at 2 μg/mL and 26% activity at 0.2 μg/mL) fractions exhibited enhanced RSK inhibitory activity. The 95:5 CH_2Cl_2–MeOH fraction (4 mg) was fractionated repeatedly on a C_{18} reversed-phase HPLC column (250 × 10 mm); elution was with 65:35 MeOH–H_2O at a flow rate of 3 mL/min and UV detection at 265 nm. Two compounds, **2** (2 mg, IC_{50} 89 nM) and **1** (0.8 mg, IC_{50} 580 nM), were obtained as amorphous pale yellow powders. Compound **2**: see Table 2 for ^{1}H NMR data; positive APCI-MS *m/z* 516 $[M]^+$. Compound **1**: see Table 2 for ^{1}H NMR data.

The 90:10 CH_2Cl_2–MeOH fraction (7 mg) from the above diol column was also fractionated repeatedly on a C_{18} reversed-phase HPLC column using 45:55 H_2O–MeOH as eluant at a flow rate of 4 mL/min and UV detection at 275 nm. Compound **3** (4.1 mg, IC_{50} 189 nM) was obtained as an amorphous powder. The ^{1}H NMR and ^{13}C NMR data are given in Tables 2 and 3, respectively.

3.4. Kinase assay

Kinase assays were performed as described previously.[16] Glutathione-*S*-transferase (GST)-fusion protein (1 μg) containing the sequence RRRLASTNDKG was adsorbed in the wells of LumiNunc 96-well polystyrene plates (MaxiSorp surface treatment). The wells were blocked with sterile 3% tryptone in phosphate-buffered saline and stored at 4 °C for up to six months. Kinase (5 nM) in 70 μL of kinase buffer (5 mM β-glycerophosphate, pH 7.4, 25 mM Hepes, pH 7.4, containing 1.5 mM DTT, 30 mM $MgCl_2$, and 0.15 M NaCl) was dispensed into each well. Vehicle, test compounds or extracts at the indicated concentrations were added and reactions were initiated by the addition of 30 μL of ATP to a final ATP concentration of 10 μM. Reactions were terminated after 30 min by the addition of 75 μL of 500 mM EDTA, pH 7.5. All assays measured the initial velocity of reaction. After extensive washing of wells, anti-phospho-p140 antibody, a polyclonal phosphospecific antibody developed against the phosphopeptide CGLA(pS)TND, and HRP-conjugated anti-rabbit antibody (211-035-109, Jackson ImmunoResearch Laboratories, West Grove, PA) were used to detect substrate phosphorylation on serine. HRP activity was measured using Western Lightning Chemiluminescence Reagent (NEL102, Perkin-Elmer Life Sciences). Maximum and minimum activity was the relative luminescence detected in the presence of vehicle and 200 mM EDTA, respectively. His-tagged active RSK was expressed in Sf9 cells and purified using Ni NTA resin (Qiagen, Valencia, CA) as previously described.[16]

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