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RUAKURIC ACID: A NATURAL PRODUCT FROM *ASPERGILLUS FUMIGATUS*

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Key Word Index—*Aspergillus fumigatus*; coral lichen; 6-acetyl-5-hydroxy-4-methoxychroman-2-carboxylic acid.

Abstract—A new chroman derivative, named ruakuric acid, was isolated from a strain of *Aspergillus fumigatus* growing in conjunction with a coral lichen. The structure was determined as 6-acetyl-5-hydroxy-4-methoxy-chroman-2-carboxylic acid (mixture of 2,4-*cis*,*trans* isomers). Published by Elsevier Science Ltd

INTRODUCTION

While examining fungi for natural products, we isolated a novel substance from a strain of *Aspergillus fumigatus* found growing in conjunction with a coral lichen in the hot, sulfurous springs environment of the area known as the Craters-of-the-Moon, in the North Island of New Zealand. We now report the structure of ruakuric acid (6-acetyl-5-hydroxy-4-methoxy-chroman-2-carboxylic acid) (**1**) and its derivatives.

RESULTS AND DISCUSSION

The strain of *A. fumigatus* was characterized by compactly columnar conidial heads, approximately 50 to 150 μm long; stipes were smooth subhyaline, 150–250 μm long, 5–6 μm wide, expanding into pyriform, light olive vesicles, 16–21 μm diameter aspergilli uniserate, phialides olive green, 6.2–7(7.5) × 2–2.5 μm, curving more or less parallel to the axis of the stipe; conidia were olive green, minutely roughened, subglobose to globose, 2.2–2.5 (2.7) μm diameter.

A bulk culture of the fungus grown on shredded wheat was extracted with solvent to give a viscous fluid which, after partition into EtOAc and column chromatography on silica gel, yielded crystals of ruakuric acid (**1**) from the benzene fraction. These were collected and washed with benzene to yield the pure material. The molecular formula, $C_{13}H_{14}O_6$, was deduced from HR-mass spectrometry with an exact mass at *m/z* 266.0779 allowing for 7° of unsaturation. The EI-mass spectra also showed prominent fragment ions corresponding to loss of methanol (*m/z* 234) and methanol plus carboxyl (*m/z* 189). The IR spectrum included bands at 3400 (broad, OH), 1741 (C=O), 1360 (CH_3) cm^{-1}. ^1H and ^{13}C NMR spectra established that the isolated material was a 13:12 mixture of the *cis*, *trans* forms of compound **1**. The groups identified by NMR were two aromatic protons (^3J coupled), a CH_2 group (^3J coupled to two -CHOR groups) and single COOH, OCH_3, CH_3CO, and OH (hydrogen bonded) groups (see Table 1). NOE-difference results and two-dimensional NMR data consisting of COSY, ^1J and long range ^{13}C–^1H correlated spectra (see Table 1), revealed the structural disposition of these groups. In particular, the COSY spectrum identified two independent systems (H2–H3′/H3″–H4 and H7–H8), while long-range ^{13}C–^1H correlation data (especially correlations between H2 and C9, H4 and C11 (and C2 in **1b**); the 5-OH group and C5, C6 and C10; H7 and C9, C5 and C13; H8 and C6 and C10; and H14 and C13 and C6), established the location of the pyranyl and aryl ring substituent groups. Structurally significant NOEs observed for the *cis*-isomer (**1a**) are depicted in Fig. 1. No NOEs were observed between H2 and H4 in the *trans*-isomer (**1b**).

Ruakuric acid (**1a, b**) showed unusual behaviour when analysed by HPLC. On a Zorbax ODS column with acetonitrile–water (36:64) mobile phase, a single broad peak at 6.3 min was produced. This is unusual because, in the absence of a pH modifier, carboxylic acids are usually poorly retained in reversed-phase systems. The UV spectra (identical over the full peak) showed maxima at 215 (l_1), 232 (shoulder) 274 (l_2) and 315. With a mobile phase of acetonitrile–aqueous 0.5% acetic acid (44:56), two peaks at 6.7 and 8.9 min connected by a well-defined shoulder (*ca* 30–50% of peak height) were produced. The UV spectra was

Table 1. ^{1}H and ^{13}C NMR spectral data determined in DMSO-d_6 for the *cis*- and *trans*-isomers of ruakuric acid (**1a, b**)

Atom	^{1}H δ ppm Hz	^{13}C	^{1}J ^{13}C–H	^{2}J, ^{3}J and/or ^{4}J-$(C)_n$-**C** correlations δ ppm (correlated signals)
cis-isomer (**1a**)				
2	5.95 (*t*, 8.4)	73.0 (*d*)	153.3	167.5 (C9)
3	2.72 (*m*)	36.4 (*t*)	136.2	178.8 (C11), 114.2 (C10), 79.8 (C4), 73.0 (C2)
4	4.55 (*t*, 9.8)	79.8 (*d*)	143.5	178.8 (C11), 61.2 (C12)
5	13.52 (*s*, OH)	167.0 (*s*)		167.0 (C5), 116.5 (C6), 114.2 (C10)
6		116.5 (*s*)		
7	7.87 (*d*, 8.9)	138.0 (*d*)	160.9	167.5 (C9), 167.0 (C5), 207.8 (C13)
8	6.56 (*d*, 8.9)	111.8 (*d*)	163.9	116.5 (CC6), 114.2 (C10)
9		167.5 (*s*)		
10		114.2 (*s*)		
11		178.8 (*s*)		
12	3.53 (*s*)	61.2 (*q*)	141.9	79.8 (C4)
13		207.8 (*s*)		
14	2.60 (*s*)	30.2 (*q*)	128.1	207.8 (C13), 116.5 (C6)
trans-isomer (**1b**)				
2	6.13 (*dd*, 9.3, 5.1) 74.5 (*d*)		156.1	166.8 (C9), 179.3 (C11)
3	2.53 (*m*), 2.72 (*m*)	37.0 (*t*)	135.2	179.3 (C11)*, 74.5 (C2)*
4	4.43 (*dd*, 8.5, 5.0)	80.4 (*d*)	152.3	61.3 (C12)
5	13.52 (*s*, OH)	166.4 (*s*)		166.4 (C5), 116.5 (C6), 115.9
6		116.5 (*s*)		
7	7.87 (*d*, 8.9)	137.8 (*d*)	160.9	166.8 (C9), 166.4 (C5), 207.7 (C13)
8	6.56 (*d*, 8.9)	111.9 (*d*)	164.0	116.5 (C6), 115.9 (C10)
9		166.8 (*s*)		
10		115.9 (*s*)		
11		179.3 (*s*)		
12	3.53 (*s*)	61.3 (*q*)	142.5	80.4 (C4)
13		207.7 (*s*)		
14	2.60 (*s*)	30.2 (*q*)	128.1	207.7 (C13), 116.5 (C6)

*Correlation observed for the H3″ signal (2.72 ppm).

identical at all parts of the pattern. This information suggests partially separable equilibrating isomers. Methylation of compound **1a, b** with diazomethane gave a mixture of methyl esters **2a, b**. This mixture gave poor chromatography with acetonitrile–water, but with acetonitrile–aqueous acetic acid gave two well-defined peaks. These were isolated in pure form by HPLC, but re-equilibrated during solvent evaporation to give identical isomeric mixtures. These methyl esters co-eluted on GC-mass spectrometry. Because of the solution re-equilibration characteristics of compound **2a, b** we did not attempt to assign the configuration (*cis* or *trans*) of this pair of compounds. Acetylation of the mixture **2a, b** gave, at earlier HPLC retention times, a mixture of methyl acetates **3a, b**. These could be separated in pure form by HPLC in acetonitrile–water and were also separable on GC-mass spectrometry. The production of monoacetates from this acetylation reaction indicates that the mixture **1a, b** exists principally in closed-ring form. Also, that the acetate is more polar

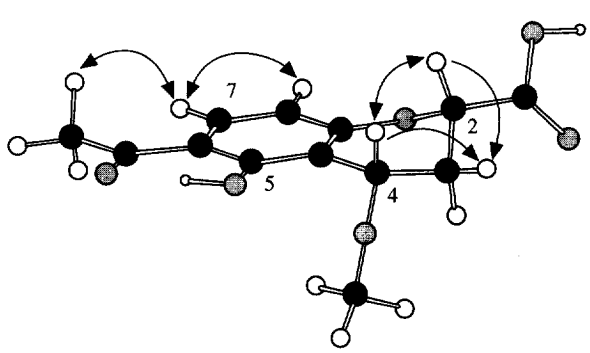


Fig. 1.

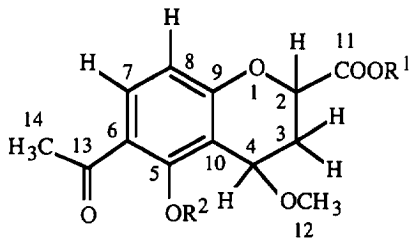
than the free phenol (less retention on reversed-phase media) indicates than the phenol must be strongly hydrogen-bonded.

The ^{1}H and ^{13}C NMR spectral features of compounds **3a** and **3b**, each of which were for comparative purposes determined in DMSO rather than CDl_3, were similar to those determined for compounds **1a** and **1b**. For example, irradiation of the H2 signal (5.86 ppm) of compound **3a** in an NOE-difference experiment, enhanced H4 (4.55 ppm) and vice versa, thereby identifying it as the 2,4-*cis* isomer, while irradiation of the H2 signal (5.98 ppm) of compound **3b** (the *trans*-isomer), while irradiation of the H2 signal (5.98 ppm) of compound **3b** (the *trans*-isomer) did not enhance H4 (4.39 ppm).

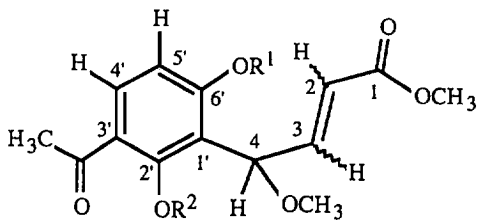
Ruakuric acid (**1a,b**) was unstable if left for prolonged periods in solution at room temperature. For example, when the metabolite was purified by HPLC and kept as a powder at room temperature under fluorescent light, it degraded over 25 days. In the impure state (purity +90%) the compound was stable under the same conditions. Another sample has remained fully stable, stored in a freezer, for over two years. In neat acetonitrile, ruakuric acid started to degrade at 4 h, in the light, at room temperature. Thus, a DMSO solution provided degradation products that were poorly retained on HPLC with acetonitrile–water, but which were retained with acetonitrile–aqueous acetic acid. This suggested the presence of a carboxylic acid group. These solution degradation products of compound **1a,b** proved useful for full structural elucidation. A partly decomposed solution in DMSO was taken to dryness and the residue methylated with diazomethane. After removal of solvent the methylated residue was washed with small amounts of methanol to selectively remove degradation products which were relatively concentrated in the methanol wash. These products showed longer retention on HPLC than the methyl esters **2a,b** and were separated by preparative HPLC using acetonitrile-aqueous acetic acid to give, in elution order, compounds **4b**, **4a** and **4c**. These products were also unstable in solution and were best stored dry at −15°. The ^{1}H NMR chemical shifts and signal multiplicities were consistent with presence in each of compounds **4a-c** of an aryl-$CH(OCH_3)$–CH=CH–COOMe group. Coupling constant analysis defined the disposition of H2 and H3 to be *trans* ($J = 16.3$ Hz) in compound **4a**, *cis* ($J = 11.4$ Hz) in **4b**, and *trans* ($J = 16.0$ Hz) in **4c**. The major product **4a** was acetylated to produce **4d**. Again, the acetylated product was more polar (less retained or reversed-phase media) than the free phenol. ^{1}H NMR analyses confirmed the presence in compound **4d** of an acetoxyl group, and the absence of a hydrogen-bonded hydroxyl proton signal.

Ruakuric acid did not exhibit biological activity in the etiolated wheat coleoptile assay [1]. However, the relative ease with which the metabolite converts to an open ring form makes it an interesting candidate for investigation in nonbiological areas.

Chromans are rare structures among the secondary metabolites of fungi. 3,7-Dimethyl-8-hydroxy-6-methoxyisochroman has been isolated from *Penicillium steckii* [2] and *P. corylophilum* [3]. It has been reported to have caused death in cattle [2] and it has shown marked plant growth regulatory activity [3]. The isochromanones 6,8-dihydroxy-1-methylisochroman-3-one, dihydrofuscin and deoxydihydrofuscin, and 6,8-dihydroxy-5-(3′-methylbut-2′-enyl)-1-methylisochroman-3-one, have been isolated from an unidentified fungus [4]. Dihydrofuscin has also been found in *Oidiodendron fuscum* [5]. Diplosporin (=diplodiol), (5*S*,6*X*)6-ethyl-5-hydroxy-3-hydroxymethyl-5,6,7,8-tetrahydrobenzo[*b*] pyran-4-one[6]{diplodiol,*trans*-6-ethyl-5-hydroxy-3-hydroxymethyl-5,6,7,8-tetrahydrochromone [7]} has been isolated from *Diplodia macrospora* and had an LD_{50} of 88 mg kg^{-1} against 1-day old chicks [7]. 5-Deoxydiplosporin has also been isolated from *D. macrospora* [8]. Other chroman relatives include the chromene series, 2,2-dimethylchromene from *Lactarius picinus*. Furthermore, 6-methoxy-2,2-



1a $R^1 = R^2 = H$; 2,4-*cis*
1b $R^1 = R^2 = H$; 2,4-*trans*
2a $R^1 = Me$, $R^2 = H$; 2,4-*cis*
2b $R^1 = Me$, $R^2 = H$; 2,4-*trans*
3a $R^1 = Me$, $R^2 = OAc$; 2,4-*cis*
3b $R^1 = Me$, $R^2 = OAc$; 2,4-*trans*



4a $R^1 = Me$, $R^2 = H$; 2,3-*trans*
4b $R^1 = Me$, $R^2 = H$; 2,3-*cis*
4c $R^1 = R^2 = H$; 2,3-*trans*
4d $R^1 = Me$, $R^2 = Ac$; 2,3-*trans*

Table 2. ^{1}H NMR signal assignments (δ ppm) for ruakuric acid derivatives

	2a, b			
	Major isomer ($CDCl_3$)	Minor isomer ($CDCl_3$)	**3a** (DMSO-d_6)	**3b** (DMSO-d_6)
H2	6.23	6.02	5.86	5.98
H3′	2.49	2.76	2.38	2.56
H3″	2.72	2.76	2.80	2.56
H4	4.30	4.28	4.55	4.39
H7	7.74	7.75	8.13	8.12
H8	6.48	6.59	7.20	7.20
2-$COOCH_3$	3.89	3.90	3.98	3.99
4-OCH_3	3.60	3.64	3.54	3.54
5-OH	13.10	13.08	–	–
5-$OCOCH_3$	–	–	2.31	2.37
6-$COCH_3$	2.57	2.57	2.56	2.56

dimethylchromene; 8,8′-bis(6-methoxy-2,2-dimethylchromene); 6-methoxy-8[2′-methoxy-4′(3′-methyl-2′-butenyl)phenoxy]-2,2-dimethylchromone; 6-methoxy-8[6′ - hydroxy - 2′ - methoxy - 4′(3″ - methyl - 2″ - butenyl)phenoxy]-2,2-dimethylchromene; 6-methoxy-8[6′ - hydroxy - 2′ - methoxy - 4′(3″ - methyl - 2″ - butenyl)phenyl]-2,2-dimethylchromene, and hydroxy-methoxy-2,2-dimethylchromene, have all been isolated from *L. picinus* and *L. fuliginosus* [9]. Quick references for these structures may be easily obtained [10].

EXPERIMENTAL

General. HPLC analyses were conducted on a gradient system equipped with a column oven and a rapid-scanning UV detector (generally set at 254 nm). Column: Zorbax ODS (4.6 mm id × 15 cm) at 35°. Mobile phase flow rate was 1 ml min^{-1}. One- and two-dimensional NMR spectra, at 300.13 MHz (^{1}H) or 75.47 MHz (^{13}C), were determined as $CDCl_3$, or DMSO-d_6 solutions, using a Bruker AC-300 spectrometer fitted with a 5 mm probe head. NOE-difference and one- and two-dimensional NMR spectra were determined using standard Bruker supplied software and pulse sequences. Three long-range correlation experiments were performed using the XHCORR sequence with D3 = 25, 35 or 40 msec respectively, and D4 = 35, 50 or 80 msec respectively. GC–MS with chemical ionization (NH_3) used a BP5 FSOT column (25 m × 0.32 mm i.d., 0.5 mm film) temperature programmed from 70° to 280° at 10° min^{-1}, 1 ml splitless injection at 250°. Accurate mass measurements were carried out at a resolution of 3500 with direct probe sample introduction.

Table 3. ^{1}H NMR signal assignments (δ ppm in $CDCl_3$) for open ring derivatives of ruakuric acid

	4a (*trans*)	**4b** (*cis*)	**4c** (*trans*)	**4d** (*trans*)
H2	7.08	6.51	7.06	6.71
H3	6.75	5.89	6.75	6.41
H4	4.41	4.33	4.42	4.38
H4′	7.66	7.73	7.65	7.78
H5′	6.47	6.50	6.50	6.82
1-OCH_3	3.78	3.72	3.78	3.78
4-OCH_3	3.45	3.29	3.45	3.44
2′-OH	13.34	12.90	13.34	–
2′-$OCOCH_3$	–	–	–	2.35
3′-$COCH_3$	2.58	2.60	2.59	2.51
6′-OCH_3	3.92	3.87	–	3.92

Accession and preparation of fungus. A coral lichen was collected in the sulfurous environment of the Craters-of-the-Moon, near Rotorua on the North Island, New Zealand and the material was plated out on potato-dextrose agar (PDA) to yield a mycelial mat that sporulated and was later identified as a strain of *As. fumigatus*. From this, a pure culture was maintained on PDA at 5° and used as a source of inoculum. Plugs were obtained from that source and placed in potato-dextrose broth at approximately 27° for 1 week then the mycelium and spore laden broth was transferred to 56 2.8-l Fernback flasks, each containing 100 g shredded wheat, 200 ml of Difco mycological broth (pH 4.8), 2% yeast extract and 20% sucrose [11], then incubated for 10 days at 24°.

Extraction and chromatography. Following incubation, approximately 300 ml of acetone was added to each flask and the mycelia and substrate were completely macerated with a Super Dispax homogenizer (Tekmar Co., Ohio). Following filtration through Whatman No. 1 filter paper under vacuum on a Buchner funnel, the filtrate was red. at 50° *in vacuo*, to give an aq. viscous syrup. This was extracted twice with an equal volume of EtOAc. Extracts were dried over anhydrous Na_2SO_4 then reduced in volume before CC on a silica gel 60 (70–230 mesh) column (10 × 13 cm, diameter, ×1—a tubby column) with stepwise elution of 1 l each of benzene, $(CH_3)_3COCH_3$, EtOAc, Me_2CO, and CH_3CN. Frs were red. in volume, under vacuum.

Ruakuric acid (**1**). Precipitated in the benzene fr. and recrystallized from benzene (1.3 g fine, needle-like crystals), mp 172–174° (Kofler block, uncorr.) $[\alpha]_D^{25}$ −47.2 (c = 0.10 Me_2CO). R_f on silica gel F-254 TLC

developed in toluene–EtOAc–HCO_2H (5:4:1) was 0.56–0.62. The compound gave a rust-coloured spot when sprayed with acidic *p*-anisaldehyde and heated (100°). UV λ_{max}^{ETOH} 229, 239, 285, and 332 (log e = 4.05, 3.96, 3.36, and 4.00). FTIR, ν_{max}^{KBr} cm^{-1}: 3300-2920, 1741, 1623, 1504, 1436, 1360, 1269, 1207, 1130, 1069, 897, 656. ^1H and ^{13}C NMR: Table 1. EI-MS (probe) 30 eV, *m/z* (rel. int.): 266 $[M]^+$ (8), 234, $[M - CH_3OH]^+$ (80), 219 $[M - CH_3OH - Me]$ (61), 189 $[M - C_2H_5O_3]^+$ (100), 171 $[M - 95]^+$ (52). HR-MS (probe), *m/z*: 266.0779 $[M]^+$ (calcd for $C_{13}H_{14}O_6$, 266.0790).

Ruakuric acid, methyl ester (**2a,b**). A partly decomposed sample of compound **1** (20 mg) recovered as a yellow oil from solution in DMSO was treated with diazomethane in EtOAc (1 ml),left at room temp. for 1 hr, then 'blown' to dryness (N_2). Addition of MeOH (0.5 ml) gave crystals with a yellow supernatant. Crystals (6.5 mg) were gathered, washed with MeOH (0.5 ml), then analysed and purified by HPLC. The combined supernatant plus wash (12 mg) was also analysed and sepd into components by HPLC. The crystals (6.5 mg) were purified using acetonitrile–aq. 0.5% HOAc (36:64) to give, in elution order, **2a,b** (specific identity not determined because peaks re-equilibrated on work up) (2.5 mg and 2.8 mg) and **4a** (1 mg). In $CDCl_3$ solution **2a,b** existed as an equilibrating mixture (*c* 4:1). The major isomer had ^1H NMR ($CDCl_3$): Table 2, δ 2.49 (1H, *ddd*, J = 13.8, 9.3, 4.7 Hz, H3′), 2.57 (3H, *s*, 6-$COCH_3$), 2.72 (1H, *ddd*, J = 13.8, 8.6, 5.3 Hz, H3″), 3.60 (3H, *s*, 4-OCH_3), 3.89 (3H, *s*, 2-$COOCH_3$), 4.30 (1H, *dd*, J = 8.6, 4.7 Hz, H4), 6.23 (1H, *dd*, J = 9.3, 5.3 Hz, H2), 6.48 (1H, *d*, J = 8.9 Hz, H8), 7.74 (1H, *d*, J = 8.9 Hz, H7), 13.10 (1H, *s*, 5-OH); the minor isomer had ^1H NMR ($CDCl_3$): δ 2.57 (3H, *s*, 6-$COCH_3$), 2.76 (2H, m, H3′ and H3″), 3.64 (3H, *s*, 4-OCH_3), 3.90 (3H, *s*, 2-$COOCH_3$), 4.28 (1H, *t*, J = 9.6 Hz, H4), 6.02 (1H, *t*, J = 8.5 Hz, H2), 6.50 (1H, *d*, J = 8.9 Hz, H8), 7.75 (1H, *d*, J = 8.9 Hz, H7), 13.08 (1H, *s*, 4-OH). GC-CIMS (NH_3) for **2a,b** (co-eluted), *m/z* (rel. int.): 298 $[M + NH_4]^+$ (13), 281 $[M + H]^+$ (100), 249 $[M + H - CH_3OH]^+$ (37), 204 $[M + H - 77]^+$ (41).

Open-ring compounds **a**, **b** *and* **4c**. The supernatant oil (12 mg) from above was sepd by HPLC using acetonitrile–aq. 0.5% HOAc (38:62) to give, in elution order, **2a,b** (1.5 mg and 1 mg), **4b** (0.25 mg), **4a** (1 mg) and **4c** (0.7 mg). **4a** gave ^1H NMR ($CDCl_3$): Table 3, δ 2.58 (3H, *s*, 3′-$COCH_3$), 3.45 (3H, *s*, 4-OCH_3), 3.78 (3H, *s*, 1-OCH_3), 3.92 (3H, *s*, 6′-OCH_3), 4.41 (1H, *d*, J = 7.3 Hz, H4), 6.47 (1H, *d*, J = 9.0 Hz, H5′), 6.75 (1H, *dd*, J = 16.3, 7.3 Hz, H3), 7.08 (1H, *d*, J = 16.3 Hz, H2), 7.66 (1*H*, *d*, J = 9.0 Hz, H4′), 13.34 (1H, *s*, 2-OH), GC-CIMS (NH_3), *m/z* (rel. int.): 312 $[M + MH_4]^+$ (0.3), 295 $[M + H]^+$ (9), 263 $[M + H - CH_3OH]^+$ (100), 203 $[M + H - 92)^+$ (57). **4b** gave ^1H NMR ($CDCl_3$): δ 2.60 (3H, *s*, 3-$COCH_3$), 3.29 (3H, *s*, 4-OCH_3), 3.72 (3H, *s*, 1-OCH_3), 3.87 (3H, *s*, 6′-OCH_3), 4.33 (1H, *dd*, J = 8.9, 1.0 Hz, H4), 5.89 (1H, *dd*, J = 11.4, 8.9 Hz, H3), 6.50 (1H, *d*, J = 7.8 Hz, H5′), 6.51 (1H, *d*, J = 11.4 Hz, H2), 7.73 (1H, *d*, J = 7.8 Hz, H4′), 12.90 (1H, *s*, 2′-OH). **4c** gave ^1H NMR ($CDCl_3$): δ 2.59 (3H, *s*, 3′-$COCH_3$), 3.45 (3H, *s*, 4-OCH_3), 3.78 (3H, *s*, 1-OCH_3), 4.42 (1H, *dd*, J = 7.4, 1.0 Hz, H4), 6.50 (1H, *d*, J = 8.9 Hz, H5′), 6.75 (1H, *dd*, J = 16.0, 7.4 Hz, H3), 7.06 (1H, *d*, J = 16.0 Hz, H2), 7.65 (1H, *d*, J = 8.9 Hz, H4′), 13.34 (1H, *s*, 2′-OH).

Ruakuric acid, methyl ester, acetate (**3a,b**). Acetylation of the methyl ester mixture **2a,b** (2.5 mg) gave a mixture (2.8 mg) of two main products which were separated by HPLC using acetonitrile-water (30:70) to give, in elution order, **3a** (1.1 mg) and **3b** (1 mg). **3a** had ^1H NMR (DMSO-d_6): δ 2.31 (3H, *s*, 5-$OCOCH_3$), 2.38 (1H, *m*, H3′), 2.56 (1H, *s*, 6-$COCH_3$), 2.80 (1H, *m*, H3″), 3.54 (3H, *s*, 4-OCH_3), 3.98 (3H, *s*, 2-$COOCH_3$), 4.55 (1H, *t*, J = 9.6 Hz, H4), 5.86 (1H, *dd*, J = 10.1, 6.8 Hz, H2), 7.20 (1H, *d*, J = 8.8 Hz, H8), 8.13 (1H, *d*, J = 8.8 Hz, H7). ^{13}C NMR (DMSO-d_6): δ 24.8 (*q*), 32.8 (*q*), 36.7 (*t*), 61.3 (*q*), 73.2 (*d*), 79.3 (*d*), 113.0 (*d*), 123.3 (*s*), 127.1 (*s*), 137.3 (*d*), 152.2 (*s*), 165.3 (*s*), 172.8 (*s*), 178.6 (*s*), 200.0 (*s*), GC–CIMS (NH_3) (second eluting component), *m/z* (rel. int.): 340 $[M + NH_4]^+$ (46), 323 $[M + H]^+$ (11), 298 $[M + NH_4 - 42]^+$ (29), 281 $[M + H - 42]^+$ (100), 236 $[M + H - 87]^+$ (44), 204 $[M + H - 119]^+$ (48), 203 $[M + H - 120]^+$ (38). Compound **3b** gave ^1H NMR (DMSO-d_6): δ 2.37 (3H, *s*, 5-$OCOCH_3$), 2.56 (3H, *s*, 6-$COCH_3$), 2.56 (2H, *m*, H3′ and H3″), 3.54 (3H, *s*, 4-OCH_3), 3.99 (3H, *s*, 2-$COOCH_3$], 4.39 (1H, *dd*, J = 8.1, 5.3 Hz, H4), 5.98 (1H, *dd*, J = 8.8, 5.9 Hz, H2), 7.20 (1H, *d*, J = 8.9 Hz, H8), 8.12 (1H, *d*, J = 8.9 Hz, H7). ^{13}C NMR (DMSO-d_6): δ 24.7 (*q*), 32.9 (*q*), 37.4 (*t*), 60.5 (*q*), 61.2 (*q*), 74.9 (*d*), 79.7 (*d*), 113.1 (*d*), 124.5 (*s*), 126.9 (*s*), 137.2 (*d*), 151.7 (*s*), 165.0 (*s*), 173.0 (*s*), 178.8 (*s*), 200.0 (*s*). GC–CIMS (NH_3) (first eluting component), *m/z* (rel. int.): 340 $[M + NH_4]^+$ (43), 323 $[M + H]^+$ (21), 298 $[M + NH_4 - 42]^+$ (20), 281 $[M + H - 42]^+$ (100), 236 $[M + H - 87]^+$ (52), 204 $[M + H - 119]^+$ (51), 203 $[M + H - 120]^+$ (50).

Compound **4d**. Acetylation of compound **4a** (0.7 mg) gave a crude residue (0.7 mg) which was purified by HPLC using acetonitrile–H_2O (34:66) to give pure material (0.7 mg). ^1H NMR ($CDCl_3$): Table 3, δ 2.35 (3H, *s*, 2′-$OCOCH_3$), 2.51 (3H, *s*, 3′-$COCH_3$), 3.44 (3H, *s*, 4-OCH_3), 3.78 (3H, *s*, 1-OCH_3), 3.92 (3H, *s*, 6′-OCH_3), 4.38 (1H, *dd*, J = 7.0, 1.2 Hz, H4), 6.41 (1H, *dd*, J = 16.2, 7.0 Hz, H3), 6.71 (1H, *dd*, J = 16.2, 1.2 Hz, H2), 6.82 (1H, *d*, J = 8.9 Hz, H5′), 7.78 (1H, *d*, J = 9.0 Hz, H4′).

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