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1-Deoxyrubralactone, a novel specific inhibitor of families X and Y of eukaryotic DNA polymerases from a fungal strain derived from sea algae

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Abstract—Talaroflavone (**1**) and 1-deoxyrubralactone (**2**) are natural compounds isolated from cultures of a fungal strain derived from sea algae, and their structures were determined by spectroscopic analyses. Compound **2** is a novel rubralactone derivative, 6-hydroxy-8-methoxy-1-methyl-1,2,3*a*,9*b*-tetrahydrocyclopenta[*c*]isochromene-3,5-dione. These compounds selectively inhibited the activities of families X and Y of eukaryotic DNA polymerases (pols), and compound **2** was a stronger inhibitor than compound **1**. The IC_{50} values of compound **2** on rat pol β, which is a pol of family X, and human pol κ, which is a pol of family Y, were 11.9 and 59.8 μM, respectively. On the other hand, compounds **1** and **2** did not influence the activities of the other families of eukaryotic pols, such as family A (i.e., pol γ) and family B (i.e., pols α, δ, and ε), and showed no effect even on the activities of plant pols α and β, prokaryotic pols, and other DNA metabolic enzymes, such as calf primase of pol α, human immunodeficiency virus type-1 (HIV-1) reverse transcriptase, human telomerase, T7 RNA polymerase, mouse IMP dehydrogenase (type II), human topoisomerase I and II, T4 polynucleotide kinase, and bovine deoxyribonuclease I. This is the first report about the selective inhibitors of families X and Y of eukaryotic pols.

1. Introduction

We have long been interested in the integrity of the genome of eukaryotes and its relation to cell differentiation. DNA replication, recombination, and repair in eukaryotes are key systems to maintain these processes,[1] and DNA polymerases (pols) have important roles. In this regard, we have concentrated our efforts on investigating eukaryotic pols associated with these processes.[2]

The human genome encodes at least 14 pols to conduct cellular DNA synthesis,[3,4] and pols have a highly conserved structure, which means that their overall catalytic subunits vary, on the whole, very little from species to species. Conserved structures usually indicate important, irreplaceable functions of the cell, the maintenance of which provides evolutionary advantages. Based on sequence homology, eukaryotic pols can be further subdivided into mainly four different families, A, B, X, and Y.[5] Family A of pols contain mitochondrial pol γ, and pol θ, and family B of pols mostly contain three replicative types: pols α, δ, and ε, and pol ζ. Family X of pols are pols β, λ, μ, and terminal deoxynucleotidyl transferase (TdT), and family Y of pols are pols η, ι, κ, and REV1, which differ from others in having low fidelity on undamaged templates and in their ability to replicate through damaged DNA; however, not all functions of eukaryotic pols have been fully elucidated. Selective inhibitors of pol families are useful tools for distinguishing pols and clarifying their biological functions. We have been searching for natural compounds that selectively inhibit each of these eukaryotic pols.[6–14]

Keywords: Talaroflavone; 1-Deoxyrubralactone; Polyketide family compound; DNA polymerase β; DNA polymerase κ; Families X and Y of DNA polymerases; Enzyme–inhibitor.
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In this study, we report newly found compounds **1** and **2** that selectively inhibit only the activity of families X and Y of eukaryotic pols. The natural compounds are the known compound **1** (talaroflavone) and new compound **2** (1-deoxyrubralactone) from a fungal strain derived from sea algae. To our knowledge, there have been no reports on such natural inhibitors specific to both families X and Y of pols, although we reported previously on selective inhibitors of only family X of pols; prunasin as a pol β-inhibitor,[8] solanapyrone A as a pols β- and λ-inhibitor,[11] and nodulisporol and nodulisporone as pol λ-inhibitors.[14] No such family Y of pol-selective inhibitors have been reported.

In this paper, we would like to report on the isolation and structural determination of compounds **1** and **2**, which belong to a polyketide family. Similar compounds, such as talaroflavone and deoxytalaroflavone, have been isolated from *Talaromyces flavus*.[15] Compound **2** is the 1-deoxy derivative of rubralactone, which has been isolated from *Penicillium rubrum*.[16]

2. Results

2.1. Isolation and cultivation of fungus

The fungal strain, HJ33moB, was isolated from sea algae collected in Hatijou Island, Japan. The fungus was selected by culturing alga fragments on potato dextrose agar plates (Difco) and was transferred several times. The culture was incubated at 25 °C. A small agar plug was then transferred into a 2 L Erlenmeyer flask containing 1 L of a culture 24 g potato dextrose broth (Difco). Cultures of HJ33moB strain (4 L) were grown for 68 days without shaking in the dark.

2.2. Extraction and purification of compounds

Fungal mycelia were removed from the culture broth by filtering through cheesecloth. The filtrate was extracted with CH_2Cl_2. The organic layer was evaporated in vacuo to obtain a crude residue (97.3 mg). This crude extract was separated by silica gel column chromatography with $CHCl_3$–methanol (99:1–9:1) to give compound **1** (12.3 mg) as an orange solid. The other fraction was further purified by silica gel chromatography with toluene–EtOAc (8:1–2:1) to yield compound **2** (1.4 mg) as a colorless solid.

2.3. Structure determination of isolated compounds

The molecular formula of compound **1** was determined with a positive high resolution electrospray ionization mass spectrometer (HR-ESIMS) spectrum as $C_{14}H_{12}O_6$ (*m*/*z* found 299.0536 $[M+Na]^+$, calcd for $C_{14}H_{12}O_6Na$: 299.0531). From analyses of the NMR spectra and mass spectrum, the structure of compound **1** was identified as talaroflavone, which had been isolated from *Talaromyces flavus*, as described by Ayer and Racok (Fig. 1).[15]

The molecular formula of compound **2** was determined with a positive HR-ESIMS spectrum as $C_{14}H_{12}O_5$ (*m*/*z* found 283.0599 $[M+Na]^+$, calcd for $C_{14}H_{12}O_5Na$ 283.0582). The ^1H NMR spectrum indicated the presence of one methyl group at δ 1.46, one methoxy group at δ 3.94, methylene protons at δ 2.32 and 2.95, one aliphatic methine, and aromatic protons at δ 6.69 and 6.70 with meta coupling (J = 2.3 Hz) (Table 1). In the HMBC spectrum, the aromatic ring was assigned by characteristic correlations from the two aromatic protons H-7 and H-9 (δ 6.69 and 6.70, respectively) to C-6 at δ 165.3, C-8 at δ 166.8, C-5a at δ 100.8, C-7 at δ 103.2, and C-9 at δ 103.0. The phenolic proton at δ 11.34 (assigned as OH) showed correlations with C-6, C-5a, and C-7. The methoxy group at δ 3.94 also correlated with C-8. COSY and HMBC analyses suggested the partial structure of CH_3–CH–CH_2–CO to be due to 1-Me, C-1, C-2, and C-3, respectively. Judging from the HMBC correlation from H-1, H-2, and 1-Me to C-10a, C-1 connected to C-10a at δ 144.6. The HMBC spectrum showed correlation from H-9 to C-10a. The chemical shift of C-5 at δ 164.9 as well as IR absorption at 1714 cm^{-1} suggested the presence of the lactone moiety. NOESY correlations of H-1/H-9 and 1-Me/H-9 were also observed. ^1H and ^{13}C NMR spectra of **2** were similar to those of **1** except C-10a, C-1, C-2, C-3a, and H-2. Although the absolute configuration at C-1 was not determined, the structure of compound **2** was established to be 6-hydroxy-8-methoxy-1-methyl-1,2,3*a*,9*b*-

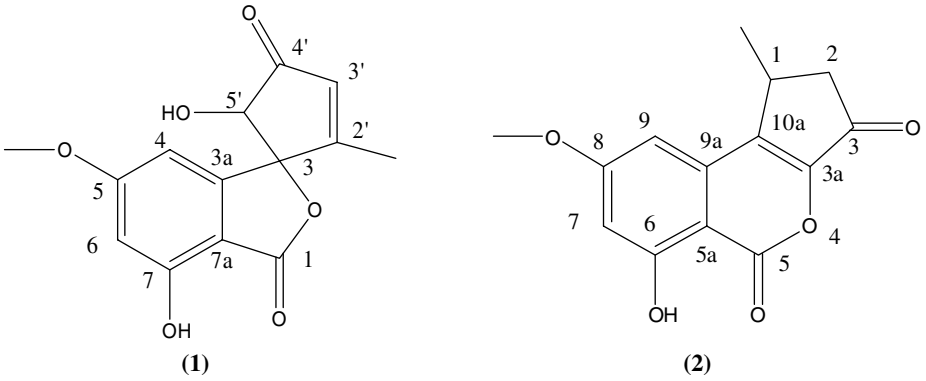

Figure 1. Structure of talaroflavone (**1**) and 1-deoxyrubralactone[6-hydroxy-8-methoxy-1-methyl-1,2,3*a*,9*b*-tetrahydrocyclopenta[*c*]isochromene-3,5-dione] (**2**).

Table 1. ^{1}H and ^{13}C NMR spectral data of compound **2**

Position	δ_c	δ_H (mult J = HZ)	NOESY
1	28.4	3.44 (m)	2, 9
1-Me	21.0	1.46 (d, 6.8)	1, 2, 9
2	42.8	2.95 (dd, 1.0, 19.0)	1
		2.32 (dd, 6.6, 19.0)	1
3	195.3		
3a	148.2		
5	164.9		
5a	100.8		
6	165.3		
6-OH		11.34 (s)	
7	103.2	6.69 (d,1.4)	8-Me
8	166.8		
8-OMe	56.0	3.94 (s)	7
9	103.0	6.70 (d, 1.4)	1,1-Me
9a	134.5		
10a	144.6		

Recorded in $CDCl_3$ for TMS as an internal standard and chemical shifts are expressed as δ ppm. s, singlet; d, doublet; dd, doublet of doublets; t, triplet.

tetrahydrocyclopenta[*c*]isochromene-3,5-dione (Fig. 2). Similar compounds, such as rubralactone and deoxytalaroflavone, have been isolated.[15,16] Since compound **2** was the deoxy derivative of rubralactone, **2** was named 1-deoxyrubralactone.[16]

2.4. Inhibition by isolated compounds of the activities of DNA polymerases and other DNA metabolic enzymes

First, isolated compounds **1** and **2** were investigated as to whether they inhibit the activities of the ten mammalian pols, such as families A (i.e., pol γ), B (i.e., pols α, δ, and ε), X (i.e., pols β and λ, and TdT), and Y (i.e., pols η, ι, and κ). As shown in Figre 3A, these compounds at 100 μM were found to significantly inhibit the activities of pols of families X and Y, and the inhibitory effects on pol β and pol κ were strongest in the family X and family Y, respectively. Compounds **1** and **2** inhibited the activities of pols β and κ dose-dependently, and 50% inhibition of pol β was observed at concentrations of 16.3 and 11.9 μM, respectively, and the IC_{50} values for pol κ were 86.5 and 59.8 μM, respectively (Fig. 4). These results suggested that compound **2** was an approximately 1.4-fold stronger inhibitor than compound **1**, and the inhibitory effect of these compounds on pols of family X, such as pol β, was stronger than that on pols of family Y, such as pol κ. On the other hand, families A and B of mammalian pols (Fig. 3A), a higher plant, cauliflower, pols α and β, and prokaryotic pols, for example, the Klenow fragment of *Escherichia coli* pol I, T4 pol, and *Taq* pol (Fig. 3B), did not influence the activities.

The same concentration (i.e., 100 μM) of these compounds also did not suppress the activities of other DNA metabolic enzymes, such as calf primase pol α, human immunodeficiency virus type-1 (HIV-1) reverse transcriptase, human telomerase, T7 RNA polymerase, mouse IMP dehydrogenase (type II), human topoisomerase I and II, T4 polynucleotide kinase, and bovine deoxyribonuclease I. Since these compounds have tricyclic ring systems, the backbone structure of the tricyclic rings might be important for the selective inhibition of pols of families X and Y in vitro.

3. Discussion

As described in this report, we found novel potent inhibitors specific to families X and Y of eukaryotic pols from the fungal strain derived from sea algae. The natural compounds were found to be a polyketide family, talaroflavone (**1**) and 1-deoxyrubralactone (**2**).

Family X contains the well-known pol β as well as other pols such as pols λ and μ, and TdT.[3,5] Pol β is required for short-patch base excision repair, a DNA repair pathway that is essential for repairing abasic sites.[3] Pols λ and μ are involved in non-homologous end joining, a mechanism for rejoining DNA double-strand breaks. TdT is only expressed in lymphoid tissue and adds '*n* nucleotides' to double-strand breaks formed during V(D)J recombination to promote immunological diversity. The yeast *Saccharomyces cerevisiae* has only one pol of family X, pol4, which is involved in non-homologous end joining.[3] The inhibitors of family X pols, especially TdT, could be immunosuppressive agents.

Family Y of pols differs from other families in having low fidelity on undamaged templates and in their ability to replicate through damaged DNA. Members of this family are hence called translesion synthesis (TLS) pols.[17] Depending on the lesion, TLS pols can bypass the damage in an error-free or error-prone fashion, the

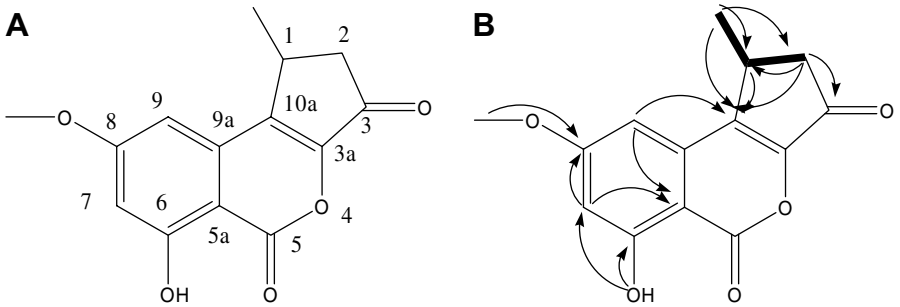

Figure 2. (A) Structure of compound **2** (1-dehydroxyrubralactone). (B) Connectives among the partial structures of compound **2** by HMBC. Bold lines show proton spin systems, arrows indicate ^{1}H–^{13}C long-range correlations.

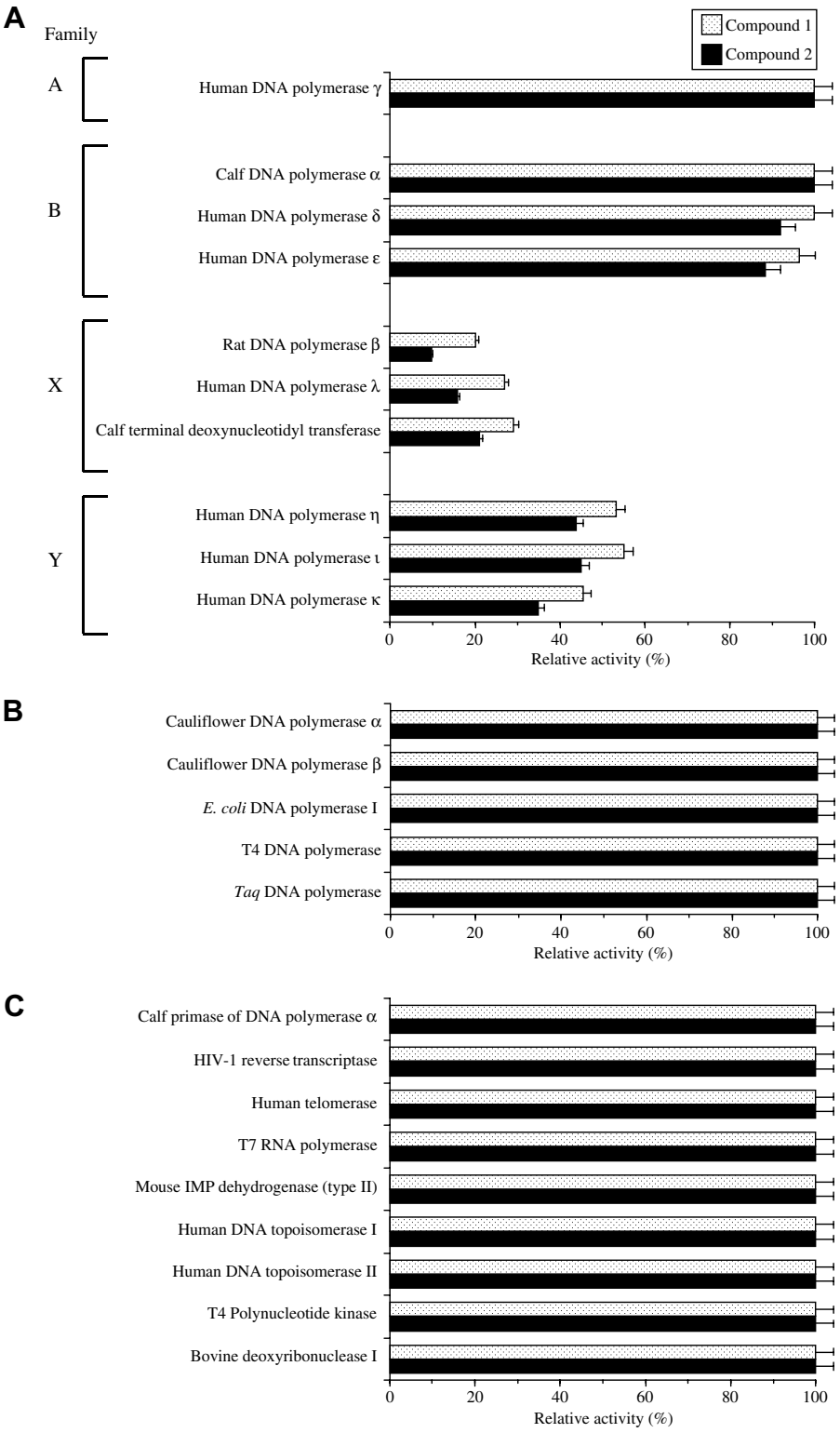

Figure 3. Effect of compounds **1** (talaroflavone) and **2** (1-deoxyrubralactone) on the activities of various DNA polymerases and other DNA metabolic enzymes. (A) Mammalian pols, (B) plant and prokaryotic pols, and (C) other DNA metabolic enzymes. Compound **1** (gray bars) and compound **2** (black bars) (100 μM each) were incubated with each enzyme (0.05 units). % of relative activity. Enzymatic activity was measured as described previously.[6,7,11] Enzyme activity in the absence of the compounds was taken as 100%. Data are shown as means ± SEM of four independent experiments.

latter resulting in elevated mutagenesis. Xeroderma pigmentosum variant (XPV) patients, for instance, have mutations in the gene encoding pol η, which is error-free for UV lesions. In XPV patients, alternative error-prone pols, for example, pol ζ (a pol of family B), are thought to be involved in mistakes which result in the cancer pre-

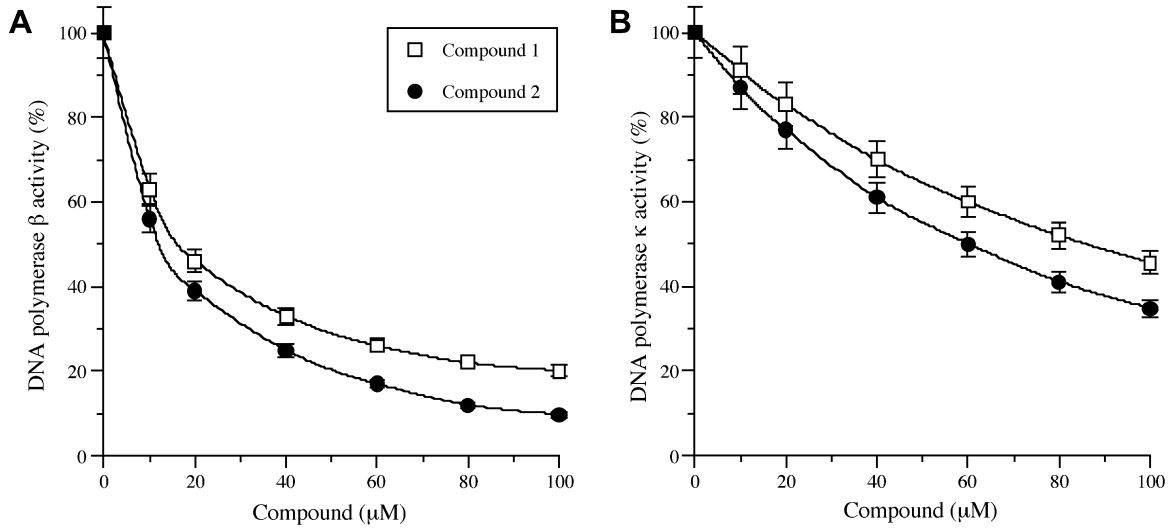

Figure 4. Mammalian DNA polymerase inhibition dose–response curves of compounds **1** and **2**. Compounds **1** (open square) and **2** (closed circle) were incubated with rat pol β (A) and human pol κ (B) (0.05 units of each). Pol activities were measured as described in the Experimental section. Pol activity in the absence of the compounds was taken as 100%. Data are shown as means ± SEM of three independent experiments.

disposition of these patients. Other members in humans are pols ι, κ, and Rev1.[5,17] In *E. coli*, two TLS pols, pol IV (DINB) and pol V (UMUC), are known.[17] The inhibitors of family Y pols may be anti-cancer drugs for clinical radiation therapy or cancer chemotherapy.

In conclusion, this is the first report about the potent inhibitors of family Y of eukaryotic pols. Since compounds **1** and **2** have extremely high specificity for families of pols, these compounds could be useful molecular tools as pols X and Y families-specific inhibitors in studies to determine the precise roles of pol family in vitro, and also might be useful to develop a drug design strategy for immunosuppressive and/or anti-cancer chemotherapy agents.

4. Experimental

4.1. Materials

Nucleotides and chemically synthesized template primers such as poly(dA), oligo(dT)$_{12–18}$, and [^{3}H]-deoxythymidine 5′-triphosphate (dTTP) (43 Ci/mmol) were purchased from GE Healthcare Bio-Sciences (Little Chalfont, UK). All other reagents were of analytical grade and were purchased from Wako Chemical Industries (Osaka, Japan).

4.2. DNA polymerase and other DNA metabolic enzymes assays

Pols from mammals and plants were purified, and prokaryotic pols and other DNA metabolic enzymes were purchased as described in our previous report.[6,7,11] The activities of all pols and other DNA metabolic enzymes were measured as described in previous reports.[6,7,11,18] The substrates of the pols were poly(dA)/oligo(dT)$_{12–18}$ and dTTP as the DNA template-primer and dNTP (2′-deoxyribonucleoside 5′-triphosphate) substrate, respectively. Compounds **1** and **2** were dissolved in dimethylsulfoxide (DMSO) at various concentrations and sonicated for 30 s. The sonicated samples (4 μl) were mixed with 16 μl of each pol enzyme (final amount, 0.05 units) in 50 mM Tris–HCl (pH 7.5) containing 1 mM dithiothreitol, 50% glycerol, and 0.1 mM EDTA, and kept at 0 °C for 10 min. These inhibitor–enzyme mixtures (8 μl) were added to 16 μl of each standard enzyme reaction mixture, and incubation was carried out at 37 °C for 60 min, except for *Taq* pol, which was incubated at 74 °C for 60 min. Activity without the inhibitor was considered to be 100%, and the remaining activity at each concentration of the inhibitor was determined relative to this value. One unit of pol activity was defined as the amount of enzyme that catalyzed the incorporation of 1 nmol of dNTP (i.e., dTTP) into the synthetic DNA template-primer (i.e., poly(dA)/oligo(dT)$_{12–18}$, A/T = 2/1) in 60 min at 37 °C under normal reaction conditions for each enzyme.[6,7]

4.3. Instrumental analyses

All reactions were monitored by TLC, which was carried out on Silica Gel 60F$_{254}$ plates (Merck, Germany).

^{1}H and ^{13}C NMR spectra were recorded on a Bruker 400 MHz spectrometer (Avance DRX-400), using $CDCl_3$ (with TMS for ^{1}H NMR and chloroform-*d* for ^{13}C NMR as an internal reference) solution, unless otherwise noted. Chemical shifts were expressed in δ (ppm) relative to Me_4Si or residual solvent resonance, and coupling constants (*J*) were expressed in Hz.

Optical rotations were recorded on a JASCO P-1010 digital polarimeter at room temperature.

Infrared spectra (IR) were recorded on a JASCO FT/IR-410 spectrometer using NaCl (neat), and were reported as wave numbers (cm^{-1}).

Mass spectra (MS) were obtained on an Applied Biosystems mass spectrometer (APIQSTAR pulsar i) under conditions of high resolution, using poly (ethylene glycol) as the internal standard.

4.4. Structure determination

4.4.1. Talaroflavone (1). Orange solid; HR-ESIMS *m/z* found 299.0536 $[M+Na]^+$, calcd for $C_{14}H_{12}O_6Na$: 299.0531; ^1H NMR (400 MHz, $CDCl_3$): 6.45 (1H, d, $J = 1.9$ Hz), 6.30 (1H, q, $J = 1.5$ Hz), 6.01 (1H, d, $J = 1.9$ Hz), 4.77 (1H, s), 3.81 (3H, s), 1.90 (3H, d, $J = 1.5$ Hz); ^{13}C NMR (400 MHz, $CDCl_3$): 200.6, 170.1, 169.5, 166.9, 158.3, 148.4, 129.9, 104.7, 102.4, 100.0, 93.0, 78.2, 55.6, 13.3.

4.4.2. 1-Deoxyrubralactone (6-hydroxy-8-methoxy-1-methyl-1,2,3*a*,9*b*-tetrahydrocyclopenta[*c*]isochromene-3,5-dione) (2). Colorless solid; $[\alpha]_D^{22}$ −8.33 (*c* 0.06, $CHCl_3$); IR (film) 3020, 2926, 1715, 1610, 1566, 1513, 1428, 1390, 1217, 1107, 881, 770, 669 cm^{-1}; HR-ESIMS *m/z* found 283.0599 $[M+Na]^+$, calcd for $C_{14}H_{12}O_5Na$ 283.0582; ^{13}C and ^1H data, see Table 1.

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