AMP-Activated Protein Kinase (AMPK) Activation by Benzofurans and Coumestans Isolated from *Erythrina abyssinica*

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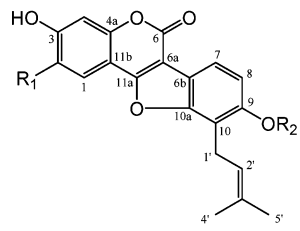
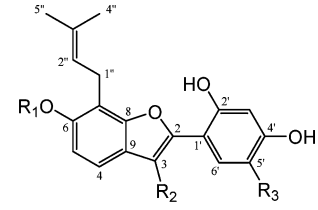
AMP-activated protein kinase (AMPK) has been proposed as a therapeutic target for the treatment of metabolic syndrome including obesity and type-2 diabetes. The bioassay-guided fractionation of an EtOAc-soluble extract of the stem bark of *Erythrina abyssinica* led to the isolation of a new coumestan, erythribyssin N (**1**), and two new benzofurans, erythribyssin F (**2**) and erythribyssin H (**3**), along with five known compounds (**4**−**8**). When tested for their stimulatory effects on AMPK activity at a concentration of 10 μM, compounds **4** and **5** showed potent activation, while compounds **1**, **2**, and **7** had moderate effects. These results suggest that benzofurans and coumestans may be new lead compounds for regulating the AMPK enzyme.

The recent upsurge in obesity due to high-calorie foods and sedentary lifestyles has led to an increase in the prevalence of type-2 diabetes and other metabolic disorders.[1a] AMP-activated protein kinase (AMPK), which is a heterotrimeric enzyme complex that works as a fuel gauge to regulate the cellular and whole-body energy homeostasis, may be a key player in metabolic control.[1b] The phosphorylation of Thr^{172} in the activation loop of the catalytic α subunit of AMPK is essential for AMPK activity.[2a] The activation of AMPK can be mediated by at least two upstream kinases, serine/threonine kinase (LKB1) and calmodulin-dependent protein kinase kinases (CaMKKs), as well as by an increase in the intracellular AMP/ATP ratio.[2b,3] Activated AMPK re-establishes the proper energy balance in the cell by switching off the ATP-consuming anabolic pathways and switching on the ATP-generating catabolic pathways.[3b]

Many downstream targets of AMPK have demonstrated the role of AMPK in glucose and lipid metabolism, such as stimulating glucose uptake in the muscle, suppressing hepatic glucose production in the liver, and reducing ectopic lipid accumulation for improving insulin sensitivity.[2b] The AMPK regulation of lipid homeostasis through acetyl-CoA carboxylases (ACCs) has also attracted considerable attention because the deregulation of the fatty acid metabolism is strongly associated with the development of insulin resistance and type-2 diabetes.[3b] The AMPK-mediated phosphorylation of ACC1 and ACC2 leads to the acute inhibition of fatty acid synthesis and increased fatty acid oxidation, respectively. Phosphorylated ACCs result in a decrease in the level of triglyceride storage and lower plasma fatty acid and triglyceride levels. Because AMPK plays an important role in mediating the whole-body glucose and lipid homeostasis and regulating food intake and energy expenditure, AMPK activators are considered promising candidates for the discovery of antiobesity and antidiabetes agents as well as drugs for the treatment of other metabolic diseases.[2,3]

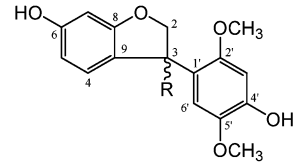
Plant extracts can be an important source for the development of better and safer drugs for the treatment of diabetes and obesity.[4] The *Erythrina* genus (Leguminosae) has been used traditionally as medicinal herbs to treat a variety of diseases, such as infections, cough, malaria, inflammation, bronchitis, asthma, and insomnia.[5,6] Previous phytochemical studies of these species revealed the presence of two major principle classes, including phenolic derivatives (flavonoids, pterocarpans, chalcones)[4,7] and alkaloids.[8] Our studies on the *Erythrina* species led to the isolation of a series of prenylated phenolics, which were found to show inhibitory activity against phospholipase Cγ1[9] and protein tyrosin phosphatase 1B (PTP1B).[4] As part of an ongoing investigation for discovering antidiabetes agents from plants, it was found that an EtOAc-soluble extract of the stem bark of *E. abyssinica* activated the AMPK enzyme in differentiated C2C12 cells. Bioassay-guided fractionation of the EtOAc extract resulted in the isolation of a new coumestan (**1**) and two new benzofurans (**2** and **3**), along with five known compounds (**4**−**8**). The present paper describes the isolation and structural elucidation of these compounds, as well as an evaluation of their stimulation effects on AMPK in differentiated C2C12 cells.





1 R_1 = H, R_2 = CH_3
4 R_1 = prenyl, R_2 = H
5 R_1 = R_2 = H

2 R_1 = H, R_2 = COOH, R_3 = prenyl
6 R_1 = H, R_2 = CHO, R_3 = prenyl
7 R_1 = CH_3, R_2 = CHO, R_3 = H



3 R = alpha-H
8 R = CHO

Results and Discussion

Repeated column chromatography (silica gel, RP-18, and semi-preparative HPLC) of the EtOAc-soluble extract of the stem bark of *E. abyssinica* resulted in the isolation of three coumestans (**1**, **4**, and **5**), three benzofurans (**2**, **6**, and **7**) and two 2,3-dihydrobenzofurans (**3** and **8**). The known compounds were identified as

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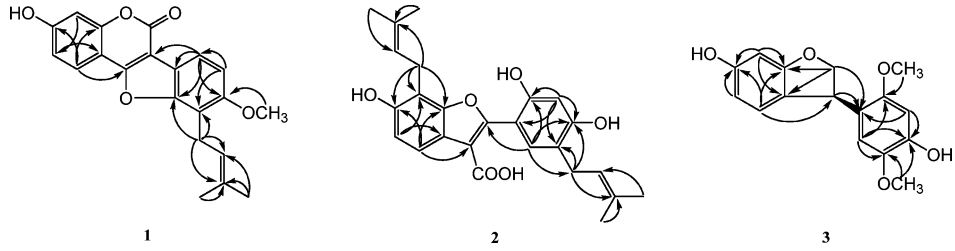

1 2 3

Figure 1. Selected HMBC correlations (H → C) for new compounds **1**−**3**.

Table 1. NMR Spectroscopic Data for New Compounds **1**−**3**

	1[a]		**2**[b]		**3**[b]	
position	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)
1	123.7	7.91, d (9.0)				
2	110.2	7.02, br d (7.8)	159.1		79.9	4.82, dd (4.0, 10.0)
						4.80, dd (6.0, 10.0)
3	162.9		111.1		41.5	4.21, dd (4.0, 6.0)
4	104.3	6.96, br s	120.4	7.70, d (7.5)	126.4	6.87, d (8.0)
4a	155.5					
5			113.6	6.92, d (7.5)	108.3	6.36, dd (2.5, 8.0)
6	158.6		153.6		159.0	
6a	103.5					
6b	117.8					
7	119.0	7.74, d (8.4)	111.9		98.2	6.31, d (2.5)
8	114.7	7.17, d (8.4)	154.6		162.9	
9	157.3		120.7		121.5	
10	115.1					
10a	156.5					
11a	161.4					
11b	105.7					
1′	23.4	3.68, d (7.2)	112.0		122.8	
2′	122.4	5.36, t-like (7.2)	156.5		152.9	
3′	132.9		105.5	6.56, s	101.0	6.57, s
4′	25.9	1.67, s	158.9		147.3	
5′	18.0	1.91, s	121.1		142.1	
6′			132.8	7.34, s	113.3	6.58, s
1″			23.3	3.59, d (7.0)		
2″			123.1	5.39, m		
3″			132.2			
4″			25.9	1.66, s		
5″			18.1	1.83, s		
1‴			28.4	3.31, d (7.0)		
2‴			124.0	5.36, m		
3‴			132.5			
4‴			26.0	1.72, s		
5‴			17.9	1.72, s		
9-OCH_3	57.1	3.96, s				
3-COOH			168.9			
2′-OCH_3					56.5	3.78, s
5′-OCH_3					57.3	3.64, s

[a] Spectra were recorded at 600 MHz (^{1}H NMR) and 150 MHz (^{13}C NMR). [b] Spectra were recorded at 500 MHz (^{1}H NMR) and 125 MHz (^{13}C NMR).

sigmoidin K (**4**),[10] isosojagol (**5**),[11] eryvarin Q (**6**),[12] erypoegin F (**7**),[13] and eryvarin R (**8**),[12] by comparison of the physicochemical and spectroscopic data (IR, UV, MS, 1D and 2D NMR) with reported data.

Compound **1** was isolated as yellowish-white powder. A molecular formula of $C_{21}H_{18}O_5$ was determined for this compound from the quasimolecular ion peak at *m*/*z* 351.1153 [M + H]$^+$ (calcd for $C_{21}H_{18}O_5H$, 351.1154), obtained by HRESIMS. The IR spectrum of compound **1** suggested the presence of a hydroxy group at 3447, 2923 (C−C), 1716 (C(=O)-O), 1628 (C=O), 1423 (aromatic absorption), 1264, and 1160−1036 cm^{-1}. The UV spectrum showed absorption maxima at 242, 336, and 352 nm. The ^{1}H NMR spectrum of compound **1** showed the characteristic signals for a prenyl group [δ_H 3.68 (2H, d), 5.36 (1H, m), 1.67 (3H, s), and 1.91 (3H, s)] and ABX-type aromatic protons [δ_H 6.96 (1H, br s), 7.02 (1H, br d), and 7.91 (1H, d)]. This observation was further evidenced by an HMBC experiment (Figure 1), which identified a pair of *ortho*-coupled aromatic protons [δ_H 7.17 (1H, d, J = 8.4 Hz) and 7.74 (1H, d, J = 8.4 Hz)]. The aromatic proton at C-1 (δ_H 7.91) in the ABX system showed a HMBC correlation with the carbon at C-11a (δ_C 161.4), while H-7 (δ_H 7.74) correlated with C-6a (δ_C 103.5) (Figure 1). The remaining carbon at δ_C 158.6 did not show any long-range coupling with other protons. The chemical shifts at δ_C 158.6 (C-6), 103.5 (C-6a), and 161.4 (C-11a) and the UV maximum at 336−352 nm are indicative of a coumarin ring system.[10,11] The ^{1}H and ^{13}C NMR (Table 1) and physical data for compound **1** were similar to those of isosojagol (**5**).[11] However, an additional methoxy group [δ_H 3.96 (3H, s), δ_C 57.1] was found in compound **1**. The positions of the methoxy and prenyl groups were assigned by an HMBC experiment (Figure 1), where correlations of the methoxy protons at δ_H 3.96 with C-9 (δ_C 157.3) and of the methylene protons at C-1′ (δ_H 3.68) with C-9 (δ_C 157.3), C-10 (δ_C 115.1), and C-10a (δ_C 156.5) were observed. Therefore, the structure of compound **1** was determined to be

3-hydroxy-9-methoxy-10-(3-methylbut-2-enyl)coumestan and was named erythribyssin N.

Compound **2** was isolated as a brown powder. A molecular formula of $C_{25}H_{26}O_6$ was determined for this compound from the quasimolecular ion peak at *m/z* 445.1636 $[M + Na]^+$ (calcd for $C_{25}H_{26}O_6Na$, 445.1627), obtained by HRESIMS. The IR spectrum of compound **2** suggested the presence of a hydroxy group at 3415, 1709 (C(=O)-O), 1621 (C=O), 1496, and 1159−1033 cm^{-1}. The UV spectrum of compound **2** was characteristic of a 2-arylbenzofuran, with maxima at 329 and 345 nm.[12,13] The observation of a characteristic olefinic oxygenated quaternary carbon at C-2 (δ_C 159.1) and an olefinic carbon at C-3 (δ_C 111.1) in the ^{13}C NMR spectrum further suggested that compound **2** is a 2-arylbenzofuran derivative. The ^1H NMR spectrum also showed *ortho*-coupled aromatic protons [δ_H 7.70 (1H, d, $J = 7.5$, 4-H) and 6.92 (1H, d, $J = 7.5$, 5-H)], two singlet aromatic protons [δ_H 6.56 (1H, s, 3′-H) and 7.34 (1H, s, 6′-H)], and two prenyl groups (Table 1). The ^1H and ^{13}C NMR spectra of compound **2** were similar to those of eryvarin Q (**6**).[12] However, the quaternary carbon [δ_C 188.3 (3-CHO)] in compound **6** was shifted upfield in the ^{13}C NMR spectrum of **2** (δ_C 168.9), and the aldehydic proton (δ_H 10.17, s) disappeared in the ^1H NMR spectrum of **2** (see Supporting Information). A comparison of the HRESIMS data of compound **2** with that of **6** suggested that compound **2** has one more oxygen atom in its quasimolecular formula ($C_{25}H_{26}O_6$; *m/z* 445.1636 $[M + Na]^+$) than compound **6** ($C_{25}H_{26}O_5$; *m/z* 429.1776 $[M + Na]^+$). All these observations indicated that the C-3 aldehyde group in compound **6** was changed to a carboxylic group in compound **2**. The placements of prenyl groups at C-7 and C-5′ were established by HMBC experiments, which showed that one prenyl group was located at C-7 by correlations from H-1″ to C-6, C-7, and C-8 and the other prenyl group at C-5′ by correlations from H-1‴ to C-4′ and C-5′ and from H-6′ to C-1‴. On the basis of the above data, compound **2** was found to be 2-[2′,4′-dihydroxy-5′-(3-methylbut-2-enyl)phenyl]-6-hydroxy-7-(3-methylbut-2-enyl)-1-benzofuran-3-oic acid and named erythribyssin F.

Compound **3** was obtained as a yellowish-green amorphous powder with absorption bands at 3419 cm^{-1} (OH) in its IR spectrum. A molecular formula of $C_{16}H_{16}O_5$ was determined for this compound from the quasimolecular ion peak at *m/z* 289.0997 $[M + H]^+$ (calcd for $C_{16}H_{16}O_5H$, 289.0998), obtained by HRESIMS. Its UV spectrum showed absorption bands at 206, 232, 288, and 316 nm. The ^1H NMR displayed an AMX-type spin system at δ_H 4.21 (1H, dd, $J = 4.0$, 6.0 Hz, H-3), 4.80 (1H, dd, $J = 6.0$, 10.0 Hz, H-2_{ax}), and 4.82 (1H, dd, $J = 4.0$, 10.0 Hz, H-2_{eq}), which appears to be characteristic of an isoflavanone skeleton.[10,13] However, the ^{13}C NMR spectrum did not show a signal assignable to the ketone carbon at C-4, while displayed a methylene carbon at C-2 (δ_C 79.9) and a methine carbon at C-3 (δ_C 41.5). The ^1H and ^{13}C NMR spectra of **3** also showed an ABX-type aromatic spin system at δ_H 6.87 (d, $J = 8.0$ Hz, H-4), 6.36 (dd, $J = 2.5$, 8.0 Hz, H-5), and 6.31 (d, $J = 2.0$ Hz, H-7), two singlet protons at δ_H 6.58 (1H, s) and 6.57 (1H, s), and two methoxy groups at δ_H 3.64 (3H, s, 5′-OMe) and 3.78 (3H, s, 2′-OMe) on a 4-hydroxy-3,6-dimethoxyphenyl-substituted B-ring. The heterocyclic oxymethylene group (δ_C 79.9) was further established by HMBC, showing correlations between the methylene proton (δ_H 4.80−4.82) and δ_C 121.5 (C-9), 122.8 (C-1′), and 162.9 (C-8) (Figure 1 and S.3.7, S.3.8 in the Supporting Information). The signals appearing in the ^1H and ^{13}C NMR spectra of **3** (Table 1) were similar to those of compound **8**[12] except for the absence of an aldehyde signal (see Supporting Information). The attachments of the two methoxy groups to C-2′ and C-5′ on the B-ring and the connection of the B-ring to C-3 on the benzofuran ring were established by a HMBC experiment (Figure 1), showing correlations between 5′-OMe (δ_H 3.64)/C-4′ and C-5′; 2′-OMe (δ_H 3.78)/C-2′; H-6′ (δ_H 6.58)/C-3, H-3 (δ_H 4.21)/C-1′; and H_2-2 (δ_H 4.80, 4.82)/C-1′. Because compound **3** with oxygenation at both the A- and B-rings displayed negative Cotton effects around 200 to 230 nm,[14] the absolute configuration of **3** was deduced to be 3*R*. Therefore, compound **3** was defined as 3(*R*)-2,3-dihydro-6-hydroxy-3-(4′-hydroxy-2′,5′-dimethoxyphenyl)-1-benzofuran, which is a new natural 3-aryl-2,3-dihydrobenzofuran derivative named erythribyssin H.

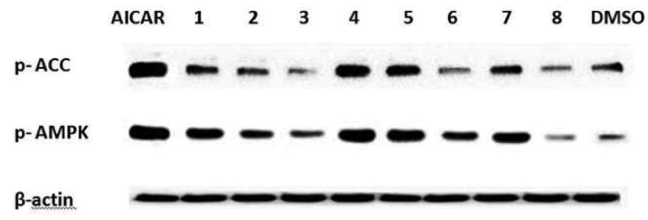

Figure 2. Stimulatory effects of the isolated compounds (**1**−**8**) on AMP-activated protein kinase (AMPK) and acetyl-CoA carboxylases (ACCs) activity at a concentration of 10 μM.

Although compound **4** was previously isolated from *Erythrina sigmoidea* by Nkengfack et al.[10] and from *Erythrina variegate* by Sato et al.,[15] the ^1H and ^{13}C NMR data of compound **4** were not reported. The present study provides the full spectroscopic data for compound **4** [3,9-hydroxy-2,10-(γ,γ-dimethylallyl)coumestan] based on 1D and 2D NMR analyses (see Figure S.5 and S.6 of the Supporting Information). The ^1H and ^{13}C NMR data for compound **5** are also reported for the first time[11] (Supporting Information). The known compounds **4**−**8** were isolated for the first time[10−13] from the stem bark of this species.

To examine whether the compounds induced AMPK activation, the phosphorylation of AMPK was measured on differentiated mouse C2C12 skeletal myoblasts. Western blot analysis showed that compounds **1**, **4**, **5**, and **7** markedly stimulated the phosphorylation of AMPK at a concentration of 10 μM, whereas the total level of AMPK and β-actin proteins did not change. The activated form of AMPK is responsible for metabolic changes via phosphorylation of downstream substrate acetyl-CoA carboxylase (ACC), which are directly related to fatty acid oxidation. Thus, phospho-ACC as detector of AMPK action was also measured using western blot analysis with phospho-ACC antibody. Compounds **4** and **5** showed the most potent activation on ACC, exhibiting strong stimulation at a concentration of 10 μM, which was comparable to AICAR as positive control. Compounds **1**, **2**, and **7** were less potent than compounds **4** and **5**, while compounds **3**, **6**, and **8** were inactive at the same concentration (Figure 2). The investigation of the chemical and biological properties of the isolates indicated that coumestans with a prenyl group had generally greater stimulatory effects on ACC than the benzofuran derivatives (compounds **2**, **6**, and **7**), which in turn displayed stronger effects than the nonprenylated benzofurans (compounds **3** and **8**). In benzofuranoids, the 2-arylbenzofuran derivatives with a prenyl moiety (compounds **2**, **6**, and **7**) displayed stronger activity than the 3-aryl-2,3-dihydrobenzofurans without a prenyl moiety (compounds **3** and **8**) (Figure 2). This suggests that prenyl groups play an important role in enhancing the activity of both coumestans and benzofurans, and substitution of a methoxy group in the B-ring may be responsible for the decrease in activation of the AMPK and ACC enzymes of the isolated coumestans.

Most of the 2-arylbenzofurans with prenyl groups have been isolated from a rather limited number of plant families, including the Leguminosae. There are a number of reports on isoprenylated 2-arylbenzofurans.[16] However, with the exception of the antimicrobial,[16b,17] antiplasmodial,[18] and estrogenic activities,[16a] little is known regarding the biological activities of these metabolites. Therefore, the coumestans and benzofurans with prenyl groups, particularly the 3-aryl-2,3-dihydrobenzofurans, were examined for their stimulation effects on the activity of AMPK enzyme. Further investigation and optimization of these derivatives may enable the

preparation of new AMPK activators with potential applications in the treatment of type-2 diabetes, obesity, and metabolic disorders.

Experimental Section

General Experimental Procedures. IR spectra (KBr) were recorded on a Nicolet 6700 FT-IR spectrometer (Thermo Electron Corp.), and the UV spectra in MeOH using a Shimadzu spectrometer. NMR spectra were obtained on Varian Unity Inova 500 and 600 MHz spectrometers at the Korea Basic Science Institute (KBSI, Gwangju Center, Korea). ESIMS and HRESIMS data were recorded on a Micromass QTOF2 (Micromass, Wythenshawe, UK) mass spectrometer. Silica gel (Merck, 63−200 μm particle size) and RP-18 (Merck, 75 μm particle size) were used for column chromatography. TLC was carried out using Merck silica gel 60 F_{254} and RP-18 F_{254} plates. HPLC was carried out using a Gilson system with a UV detector and Optima Pak C_{18} column (10 × 250 mm, 10 μm particle size, RS Tech, Korea). HPLC solvents were obtained from Burdick & Jackson, USA.

Plant Material. The stem bark of *E. abyssinica* was collected in Mukono, Uganda. The sample was botanically authenticated by Prof. John Silike-Muruumu, and a voucher specimen (No. 0001) was deposited at the Department of Botany, Makerere University, Uganda.

Extraction and Isolation. The dried stem bark (5 kg) was extracted with MeOH at room temperature for 3 days. After removing the solvent under reduced pressure, the residue was suspended in H_2O and partitioned successively with *n*-hexane, EtOAc, and *n*-BuOH. The EtOAc fraction was concentrated to dryness (105 g). A part (15 g) of this fraction was chromatographed on a silica gel column (10 × 60 cm; 63−200 μm particle size, Merck) using a stepwise gradient of *n*-hexane/acetone (10:1 to 0:10, each 2 L) to yield five fractions (Fr.1−Fr.5) according to their TLC profiles. Fraction 2 (2.4 g) was subjected to reversed-phase (ODS-A) column chromatography (6.0 × 60 cm; 150 μm particle size) and eluted with MeOH/H_2O (from 6:4 to 6:0, 2 L for each step) to afford five subfractions (Fr.2-1 to Fr.2-5). Further purification of Fr.2-3 by semipreparative Gilson HPLC [using an isocratic solvent system of 70% MeCN in H_2O (flow rate 2 mL/min) over 30 min; flow rate 2 mL/min; UV detection at 205 and 254 nm; RS Tech Optima Pak C_{18} column (10 × 250 mm, 10 μm particle size)] resulted in the isolation of compounds **1** (4.7 mg; t_R = 24.5 min) and **7** (27.8 mg; t_R = 26.8 min), respectively. Fraction 3 (4 g) was also subjected to reversed-phase (ODS-A) column chromatography (6.0 × 60 cm; 150 μm particle size) and eluted with MeOH/H_2O (from 5:5 to 5:0, 2 L for each step) to afford five subfractions (Fr.3-1 to Fr.3-5). Further purification of Fr.3-3 by semipreparative Gilson HPLC [using RS Tech Optima Pak C_{18} column (10 × 250 mm, 10 μm particle size); mobile phase MeCN/H_2O (60:40), isocratic; flow rate 2 mL/min; UV detection at 205 and 254 nm] resulted in the isolation of compounds **5** (3.7 mg, t_R = 14.5 min), **2** (10.8 mg; t_R = 20.8 min), and **6** (17.8 mg, t_R = 29.1 min), respectively. Compound **4** (15 mg) was isolated from subfraction F.3-5 by repeated column chromatography over silica gel (4.5 × 50 cm; 63−200 μm particle size, Merck) using an isocratic solvent system of *n*-hexane/acetone (4:1, 3 L). Using the same methods, fraction 4 (2.5 g) was also subjected to a reversed-phase (ODS-A) column chromatography (6.0 × 60 cm; 150 μm particle size) and eluted with MeOH/H_2O (from 4:6 to 4:0, 2 L for each step) to afford five subfractions (Fr.4-1 to Fr.4-5). Further purification of Fr.4-3 (eluted with MeOH/H_2O 1.5:1, 112 mg) by HPLC [Gilson system, RS Tech Optima Pak C_{18} column (10 × 250 mm, 10 μm particle size), UV detection at 205 and 254 nm] using an isocratic solvent system of 35% MeCN in H_2O (flow rate 2 mL/min) over 30 min yielded compounds **8** (12.8 mg, t_R = 25.4 min) and **3** (2.1 mg, t_R = 28.8 min), respectively.

Erythribyssin N (1): yellowish-white powder; IR (KBr) ν_{max} 3447, 2923, 1716, 1628, 1423, 1264, 1160−1036 cm^{-1}; UV (*c* 0.02, MeOH) λ_{max} 210, 214, 242, 299, 336, 352 nm; ^{1}H (600 MHz) and ^{13}C (150 MHz, acetone-d_6) NMR data, see Table 1; HRESIMS *m/z* 351.1153 $[M + H]^+$ (calcd $C_{21}H_{18}O_5H$ 351.1154).

Erythribyssin F (2): brown powder; IR (KBr) ν_{max} 3415, 2904, 1709, 1621, 1496, 1159−1033 cm^{-1}; UV (*c* 0.025, MeOH) λ_{max} 329, 345 nm; ^{1}H (500 MHz) and ^{13}C (125 MHz, acetone-d_6) NMR data, see Table 1; HRESIMS *m/z* 445.1636 $[M + Na]^+$ (calcd $C_{25}H_{26}O_6Na$ 445.1627).

Erythribyssin H (3): yellowish-green, amorphous powder; IR (KBr) ν_{max} 3419, 2926, 1664, 1606, 1468, 1280−960 cm^{-1}; UV (*c* 0.025, MeOH) λ_{max} 206, 232, 288, 316 nm; CD (*c* 0.04, MeOH) $[\theta]_{205}$ −0.50, $[\theta]_{220}$ −2.18; ^{1}H (500 MHz) and ^{13}C (125 MHz, acetone-d_6) NMR data, see Table 1; HRESIMS *m/z* 289.0997 $[M + H]^+$ (calcd $C_{16}H_{16}O_5H$ 289.0998).

Cell Culture. Mouse C2C12 skeletal myoblasts were maintained in DMEM supplemented with 10% fetal bovine serum in an atmosphere containing 95% air and 5% CO_2 at 37 °C. To prepare for each assay, the cells were seeded in 12-well plates at 10^5 cells/well in 2 mL of growth medium. The differentiation of C2C12 myoblasts was induced by replacing the growth medium with DMEM containing 5% horse serum when the cells were confluent. The medium was changed every 48 h until the formation of myotubes was observed. The cells were used in the experiments after differentiation.

AMPK Assay (Immunoblot Analysis). The C2C12 myotubes were incubated with the appropriate concentration of the compounds for 30 min and then lysed in an EBC lysis buffer [50 mM Tris-HCl (pH 7.6), 120 mM NaCl, 1 mM EDTA (pH 8.0), 0.5% nonidet-P40 (NP-40), and 50 mM NaF]. The cell debris was removed by centrifugation at 12 000 rpm for 15 min, at 4 °C. The protein concentrations in the cell lysates were determined using a Biorad protein assay kit. Approximately 30 μg of proteins from the total cell extracts was subjected to western blot analysis using antiphosphospecific AMPKα Thr^{172} and antiphosphospecific ACC Ser^{79}. The β-actin protein levels were used as a control for equal protein loading. The immunoreactive antigen was then recognized using a horseradish peroxidase-labeled anti-rabbit IgG and an enhanced chemiluminescence detection kit.[19]

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Supporting Information Available: 1D and HMBC NMR spectra for the new compounds **1**−**3** and 1D, HMBC, and HSQC NMR spectra for compound **4**. These materials are available free of charge via the Internet at http://pubs.acs.org.

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