Novel Ca-ATPase Inhibitors from the Dried Root Tubers of Polygonum multiflorum

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NOVEL CA^{2+}-ATPASE INHIBITORS FROM THE DRIED ROOT TUBERS OF *POLYGONUM MULTIFLORUM*

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ABSTRACT.—The MeOH extract of the dried root tubers of *Polygonum multiflorum* yielded three bioactive compounds with an inhibitory activity on calmodulin-depleted erythrocyte calcium-dependent ATPase. These compounds were identified as *E*-2,3,5,4′-tetrahydroxystilbene 2-*O*-β-D-glucopyranoside [**2**] (IC_{50}=240 μM) and *cis*- and *trans*-*E*-3-butylidene-4,5,6,7-tetrahydro-6,7-dihydroxy-1(3H)-isobenzofuranone [**3** and **4**] (IC_{50}=160 and 260 μM, respectively). *E*-2,4,6,4′-Tetrahydroxystilbene 2-*O*-β-D-glucopyranoside [**1**] was also isolated but was found to have no inhibitory effect on the enzyme.

Plant preparations have long been used in the treatment of cardiovascular disease and in some countries, particularly the People's Republic of China, they are still currently used as a major form of treatment (1). The dried root tuber of *Polygonum multiflorum* Thunb. (Polygonaceae) has a number of applications in traditional Chinese medicine where it is known as "Heshowu." It is considered to be an herb affecting the cardiovascular system and is recognized as being therapeutically effective in the treatment of hyperlipemia (2). The mechanisms of cardiovascular effects of preparations of *P. multiflorum* are unknown but possibly involve regulation of calcium ions in cells.

It has long been known that calcium controls the contraction of heart muscle and the tone of blood vessels (3). Calcium efflux against a steep electrochemical gradient, to restore intracellular calcium toward its resting level, occurs via a number of pathways. One such mechanism is the calcium pump, a calcium- and magnesium-dependent ATPase (Ca^{2+}-ATPase), which uses energy directly from the hydrolysis of ATP to extrude calcium ions against its concentration gradient across the plasma membrane (4).

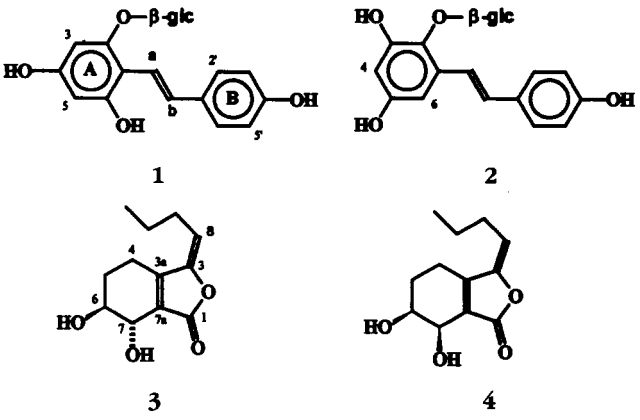
The Ca^{2+}-ATPase used for this study was prepared from human red blood cells. The suitability of red blood cells as a source of Ca^{2+}-ATPase is largely due to their lack of an active sodium/calcium exchange system and their similarity in basic function of calcium ion translocation with the plasma membrane calcium pump of cardiac muscle (4). In preliminary studies, a crude EtOH extract of the dried root tubers of *P. multiflorum* showed strong Ca^{2+}-ATPase inhibitory activity. This paper reports the isolation of some novel Ca^{2+}-ATPase inhibitors from this plant.

RESULTS AND DISCUSSION

Compounds **1–4** were tested for Mg^{2+}- and Ca^{2+}-ATPase inhibition. Compound **1** showed no inhibition of both ATPases at concentrations of 25, 50, 100, and 200 μM. For compounds **2–4**, dose response results (Table 1) were used to determine IC_{50} values for inhibition of Ca^{2+}-ATPase activity (Table 2).

(*E*)-2,4,6,4′-Tetrahydroxystilbene 2-*O*-β-D-glucopyranoside [**1**], which has not been previously reported, is isomeric with the reported (*E*)-2,3,5,4′-tetrahydroxystilbene 2-*O*-β-D-glucopyranoside [**2**] (5–7). Compound **1** differs from compound **2** only in the substitution pattern on ring A.

The structure of **1** was determined from ^{1}H- and ^{13}C-nmr data by comparison with data for **2**. The H-5 resonance of **1** was observed in the ^{1}H-nmr spectrum at 6.17 ppm as it was shielded by two ortho hydroxyl groups. Proton H-3 of **1** at 6.25 ppm occurred between a hydroxyl group and the glycosidic *O*-sugar linkage and was, as a consequence, similarly shielded. The coupling constant, $J_{3,5}$ of 2.8 Hz, indicated a meta-relationship



1 2

3 4

TABLE 1. Mg^{2+}- and Ca^{2+}-ATPase Inhibitory Activity of Compounds **2–4**.

Concentration (μM)	% Inhibition[a]					
	2		**3**		**4**	
	Mg^{2+}	Ca^{2+}	Mg^{2+}	Ca^{2+}	Mg^{2+}	Ca^{2+}
25	14	8	21	0	0	0
50	25	8	13	3	17	0
100	28	15	32	8	36	27
200	41	44	32	36	52	62
400	56	70	50	72	67	79
800	62	95	71	87	85	90
1600	75	98	82	94	—	—

[a]The average difference between duplicate data was 12% for Mg^{2+}-ATPase and 3% for Ca^{2+}-ATPase.

between these protons. In **1**, the presence of oxygen-linked substituents at positions 2, 4, and 6 on ring A results in enhanced electron density at C-1 as a consequence of the π donor properties of these groups and the transfer of electron density by conjugation. By extended conjugation through the double bond to ring B, this effect was carried forward to H_b (6.52 ppm) and H-2′ and H-6′ (7.09 ppm), resulting in upfield chemical shifts compared with those observed in **2** for H_b (7.70 ppm) and H-2′ and H-6′ (7.44 ppm). The steric bulk of the aromatic substituents on the double bond would only allow this coplanar overlap to take place in the *E* configuration. The *E* configuration assignment was supported by the strong coupling (J=12.2 Hz) observed between H_a and H_b.

The ^{1}H-nmr spectra of compounds identified as *trans*- and *cis*-*E*-3-butylidene-4,5,6,7-tetrahydro-6,7-dihydroxy-1(3H)-isobenzofuranone, **3** and **4**, respectively, were consistent with those reported for the *Z* isomers (8–10). The carbon skeleton structure

TABLE 2. IC_{50} Values of Ca^{2+}-ATPase Activity of Compounds **2–4**.

Compound	IC_{50} (μM)
2	240
3	260
4	160

was also confirmed by a close similarity with the published ^{13}C-nmr spectra for the *Z* isomers (9,10). Couplings between H-5_a, H-5_b, H-6, and H-7 were measured with the aid of decoupling and COSY nmr experiments. These together with a comparison with published data for the *Z* isomers of **3** and **4** enabled the trans and cis stereochemistry of **3** and **4** to be determined.

The stereochemistry of the butylidene side-chain was determined by considering the chemical shifts of the olefinic proton. These have been reported as 5.5 and 5.3 ppm for *E*- and *Z*-butylidenephthalide, respectively, and as 5.46 and 5.25 ppm for *E*- and *Z*-ligustilide, respectively (11). The observed chemical shift of the olefinic protons in **3** and **4** of 5.49 ppm indicated that the butylidene side-chain in these compounds has the *E* configuration.

The presence of 6,7-dihydroxyligustilides has been reported in a number of plant species in the Umbelliferae (12–15), but the compounds have most often been reported to be present in *Ligusticum* species (8–10, 14, 15) from which they had been first isolated (16). No reference has been found in the literature of these compounds being present in *P. multiflorum*.

A comparison of the structural characteristics of the tetrahydroxystilbene glucopyranoside and dihydroxyligustilide with other known Ca^{2+}-ATPase inhibitors reveals a number of common traits. 4,4′-Diisothiocyanostilbene-2,2′-disulfonic acid, for example, the anion transport inhibitor which also has been shown to inhibit Ca^{2+}-ATPase (17), is also a stilbene, having substituents at positions 4′ and 2. *N*-(4-Azido-2-nitrophenyl)-2-aminoethanesulfonic acid selectively inhibits the erythrocyte plasma membrane Ca^{2+}-ATPase (18). The presence of a side-chain linked to an aromatic ring bears resemblance to that in dihydroxyligustilide and also to nonylphenol, which has been reported to inhibit sarcoplasmic reticulum Ca^{2+}-ATPase (19,20).

It has been reported that 2,2′-methylenebis(4-methyl-6-*tert*-butylphenol) (*bis*-phenol) is a very potent inhibitor of sarcoplasmic reticulum Ca^{2+}-ATPase with an IC_{50} value of 0.7 μM (19). *bis*-Phenol is reminiscent of the stilbenes where two aromatic rings are linked by an aliphatic bridge, but is much less polar than the tetrahydroxystilbene glycosides.

The lack of inhibition on erythrocyte Ca^{2+}-ATPase by related compounds can also give information as to the nature of the structure-activity relationship. Compound **1**, which was shown to be an isomer of 2,3,5,4′-tetrahydroxystilbene 2-*O*-β-D-glucopyranoside [**2**], was inactive, thus showing that the substitution pattern on ring A of the stilbene glucopyranoside was important for its inhibitory effect. Similarly, the configurations of the hydroxyl groups on positions 6 and 7 in the 6,7-dihydroxyligustilides **3** and **4** also seem to be relevant to the magnitude of the inhibition, as seen by the slightly more potent effect of **4**.

The tetrahydroxystilbene glycoside, rhapontin, and the anthroquinones, rhein, emodin, and chrysophanol, were tested and found to be inactive against Ca^{2+}-ATPase at 1600 μM. The absence of inhibition from rhapontin may be attributed to the difference in the nature and positions of its substituents compared to those in 2,3,5,4′-tetrahydroxystilbene 2-*O*-β-D-glucopyranoside [**2**].

In conclusion, the identification of the tetrahydroxystilbene glucopyranoside [**2**] and dihydroxyligustilides [**3**] and [**4**] as Ca^{2+}-ATPase inhibitors serves as a basis for further structure-activity relationship studies with respect to the development of novel agents that act on this enzyme.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—ATP, EDTA, EGTA, ouabain, PMSF, and SDS were purchased from Sigma Chemical Co. (St. Louis, MO). Potassium HEPES was purchased from BDH (Victoria,

Australia) and dithiothreitol (DTT) was purchased from Boehringer (Mannheim, Germany). All other chemicals were of the highest purity commercially available. The tlc grade reversed-phase silanized 60H Si gel was purchased from Merck (Art. 7761, GmbH) and chrysophanic acid, emodin, rhapontin, and rhein were purchased from Aldrich Chemical Co. (Milwaukee, WI).

Analytical hplc experiments were carried out using a Beckman gradient system (System Gold) with an Activon (NSW, Australia) Goldpac Exsil 100/10 ODS (250×4.6 mm) hplc column connected to a Spectra-Physics Spectra 100 variable wavelength detector. Prep. hplc experiments were conducted using an Altex Model 100 Solvent Metering System together with an Altex Model 153 analytical uv detector and an Altex Analytical Optical Unit fitted with a preparative flow cell. The column used in this case was an Alltech (Bannockburn, IL) Econosil C_{18} 10 μm (250×22.5 mm) prep. hplc column. All hplc grade solvents were supplied by Rhone-Poulenc Chemicals (Australia) and were filtered and degassed before use. All hplc experiments were carried out using a wavelength of 280 nm for detection. Nmr experiments were carried out using a 300 MHz Varian Gemini instrument (Palo Alto, CA). The solvent was CD_3OD with the center of the residual proton signal at δ 3.34 used as the ^{1}H-nmr reference and the center of the carbon signal at δ 49.1 used as the ^{13}C-nmr reference. Cims were obtained with a Finnigan MAT TSQ46 MS/MS Instrument (San Jose, CA) using CH_4 or NH_3 as the reagent gas. The high-resolution eims determination of compound **3** was carried out on a Kratos MS9 high-resolution mass spectrometer upgraded to MS50 configuration.

PLANT MATERIAL.—The processed slices of the dried root tubers of *Polygonum multiflorum* were purchased from Everspring Chinese herbal and grocery store, Sydney, Australia. The specimen was identified by Professor Xu Guojun of China Pharmaceutical University and deposited at the Herbarium of Traditional Chinese Medicine, China Pharmaceutical University, Nanjing, People's Republic of China.

BIOASSAYS.—Human packed red blood cells for use in the Ca^{2+}-ATPase assay were obtained from the New South Wales Red Cross Transfusion Service, Sydney. Calmodulin-depleted erythrocyte membranes were prepared using a method that involved the circulation of a hemolysate mixture through a hollow fiber plasma separator and filtering off unwanted intracellular components (21). The Protein Estimation Kit was purchased from Bio-Rad (Richmond, CA) and the bovine serum albumin used in the protein determination was obtained from Pierce (Rockford, IL). The protein content was determined (22) using serum albumin as a standard. The free calcium concentration levels were calculated by a computer program which solves equations from equilibria between calcium, magnesium, and hydrogen ions and EGTA, EDTA, and ATP (23), using a Ca^{2+}:$EGTA^{4-}$ association constant of $10^{10.97}$ and other constants published (24).

The Ca^{2+}-ATPase assay was performed in duplicate by a published method (21). Calmodulin-depleted erythrocyte membranes (2.5 mg/ml) were incubated at 37° for one h in a total volume of 0.4 ml of 65 mM KCl, 20 mM potassium-HEPES, 5 mM $MgCl_2$, 50 μM $CaCl_2$ (50.4 μM free Ca^{2+}), 0.1 μM calmodulin, and 0.1 mM EGTA. The reaction was started by adding 2 mM ATP (pH 7.4) and the phosphate liberated to the medium was determined spectrometrically (25,26). The activity of the Mg^{2+}-ATPase (assayed in the absence of added $CaCl_2$) was subtracted from the total activity assayed in the presence of Ca^{2+}. Control-specific activities of the ATPase measured in units of nmoles of phosphate per mg of protein per min were 8±1 (n=10) for Mg^{2+}-ATPase and 52±3 (n=10) for Ca^{2+}-ATPase. Test substances were dissolved in DMSO; the final concentration of DMSO in the assay mixture was 2.5%. DMSO itself had no effect on ATPase activity. The solutions of test substances were added to the reaction buffer before adding ATP.

EXTRACTION AND ISOLATION.—The ground plant material (500 g) was soaked in MeOH (4 liters), protected from light, for a period of three days. The plant material was then filtered off and exposed to a fresh volume of MeOH (4 liters) for another three days. The MeOH extracts were combined, filtered, concentrated under reduced pressure, and the residue partitioned between H_2O (800 ml) and EtOAc (400 ml). After separation of the EtOAc, the aqueous phase was extracted twice with EtOAc (2×400 ml). The combined EtOAc phase was concentrated under reduced pressure to give a brown-yellow oily substance (11.09 g).

A short-column vacuum chromatography apparatus was set up using a Buchner funnel with a sintered glass disk of diameter 13.7 cm and porosity 3. Tlc grade reversed-phase silanized Si gel (80 g) was packed under suction into a uniform compact silica bed with a maximum depth of 2.5 cm. The mobile phase used in this experiment consisted of a range of solvents made up of various amounts of MeOH and a H_2O/CH_3COOH mixture (H_2O* designated as 99% H_2O/1% CH_3COOH). The Si gel was first prewashed under suction with MeOH (300 ml) and portions (100 ml) of each of the following: H_2O*-MeOH (1:3, 1:1, 3:1, 9:1). The EtOAc extract was dissolved in the H_2O*-MeOH (9:1) solvent mixture (100 ml) and transferred to the column under suction. The same volume of solvent (100 ml) was used to elute this and all subsequent fractions. Fractions were collected using a step-wise gradient from H_2O*-MeOH (9:1) to (1:1). Bioactive fractions eluting with solvent mixtures containing 50% and 55% MeOH were combined and evaporated to dryness (660 mg).

A second reversed-phase short column of the same dimensions was set up with a different set of solvents.

In this case the Si gel bed was prewashed under suction with portions (100 ml) of each of the following: H_2O-CH_3CN (1:3, 1:1, 3:1, 9:1).

The combined fractions (660 mg) obtained from the first reversed-phase short column were dissolved in the H_2O-CH_3CN (9:1) solvent mixture (80 ml) and transferred to the column and the eluent collected. Further fractions were collected using a stepwise gradient of H_2O and CH_3CN. Bioactive fractions eluting with aqueous mixtures containing 30%, 35%, 40%, and 45% CH_3CN were evaporated under reduced pressure to give a residue (376 mg). Further fractionation by reversed-phase prep hplc using an Econosil C_{18} preparative column with H_2O-CH_3CN (4:1) solvent mixture as mobile phase and a flow rate of 9 ml/min (ca. 30 mg/injection) yielded **1** (2.9 mg), **2** (48 mg), **3** (16.6 mg), and **4** (5.7 mg).

E-*2,4,6,4′-Tetrahydroxystilbene 2*-O-β-D-*glucopyranoside* [**1**].—^{1}H nmr (CD_3OD, 300 MHz) δ 7.09 (2H, d, J=8.5 Hz, H-2′ and H-6′), 6.75 (1H, d, J=12.2 Hz, H_a), 6.64 (2H, d, J=8.5 Hz, H-3′ and H-5′), 6.52 (1H, d, J=12.2 Hz, H_b), 6.25 (1H, d, J=2.8 Hz, H-3), 6.17 (1H, d, J=2.8 Hz, H-5), 4.60 [1H, d, J=7.7 Hz, H-1″ (β-glc)], 3.79 (2H, m, H-6″), 3.45 (3H, m, H-3″, H-4″, and H-5″), 3.25 (1H, m, H-2″); ^{13}C nmr (CD_3OD, 75.4 MHz) δ 157.6 (C-4′), 155.2 (C-6), 151.7 (C-4), 137.8 (C-2), 133.9 (C_a), 131.4 (C_b), 131.2 (C-2′ and C-6′), 129.7 (C-1′), 125.5 (C-1), 115.9 (C-3′ and C-5′), 108.1 (C-5), 107.6 (C-3), 103.7 (C-1″), 78.4 (C-3″), 77.9 (C-5″), 75.4 (C-2″), 71.1 (C-4″), 62.3 (C-6″); cims (NH_3) m/z $[M+18]^+$ 424 (41), $[M+1]^+$ 407 (56), 236 (73), 184 (100).

trans-E-*3-Butylidene-4,5,6,7-tetrahydro-6,7-dihydroxy-1(3H)-isobenzofuranone* [**3**].—^{1}H nmr (CD_3OD, 300 MHz) δ 5.49 (1H, t, J=7.9 Hz, H-8), 4.30 (H, d, $J_{6,7}$=3.4 Hz, H-7), 3.98 (H, m, H-6), 2.56 (2H, m, H-4), 2.39 (2H, q, J=7.5 Hz, H-9), 1.98 (2H, m, H-5), 1.56 (2H, sext, J=7.4 Hz, H-10), 1.00 (3H, t, J=7.4 Hz, H-11); ^{13}C nmr (CD_3OD, 75.4 MHz) δ 170.6 (C-1), 155.2 (C-3), 149.9 (C-3a), 126.5 (C-7a), 114.4 (C-8), 71.1 (C-6), 65.4 (C-7), 29.2 (C-9), 25.1 (C-5), 23.4 (C-10), 18.3 (C-4), 14.2 (C-11), cims (CH_4) m/z $[M+41]^+$ 265 (4), $[M+29]^+$ 253 (10), $[M+1]^+$ 225 (23), 207 (100); hreims m/z found 224.101, calcd for $C_{12}H_{16}O_4$, $[M]^+$ 224.105.

cis-E-*3-Butylidene-4,5,6,7-tetrahydro-6,7-dihydroxy-1(3H)-isobenzofuranone* [**4**].—^{1}H nmr (CD_3OD, 300 MHz) δ 5.48 (1H, t, J=7.9 Hz, H-8), 4.45 (H, d, $J_{6,7}$=4.0 Hz, H-7), 3.80 (1H, m, H-6), 2.69 (2H, m, H-4), 2.38 (2H, q, J=7.5 Hz, H-9), 2.01 (2H, m, H-5), 1.56 (2H, sext, J=7.5 Hz, H-10), 1.00 (3H, t, J=7.4 Hz, H-11); ^{13}C nmr (CD_3OD, 75.4 MHz) δ 170.1 (C-1), 155.3 (C-3), 149.7 (C-3a), 127.7 (C-7a), 114.7 (C-8), 70.4 (C-6), 63.2 (C-7), 29.2 (C-9), 25.8 (C-5), 23.4 (C-10), 21.1 (C-4), 14.2 (C-11); cims (CH_4) m/z $[M+41]^+$ 265 (3), $[M+29]^+$ 253 (8), $[M+1]^+$ 225 (17), 207 (100).

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