Pseudosterins A–C, Three 1-Ethyl-3-formyl-β-carbolines from Pseudostellaria heterophylla and Their Cardioprotective Effects

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Abstract: Pseudostellaria heterophylla is used in China not only as a functional food but also as an herb to tonify the spleen, enhance immunity, and treat palpitation. Our previous investigation showed that a fraction enriched in glycosides obtained from the roots of P. heterophylla possessed pronounced protective effects on H9c2 cells against CoCl2-induced hypoxic injury. However, the active compounds responsible for the observed effects were still unknown. In the current investigation, pseudosterins A–C (1–3), three new alkaloids with a 1-ethyl-3-formyl-β-carbone skeleton, together with polydatin, have been isolated from the active fraction. Their structures were elucidated on the basis of spectroscopic analysis and quantum chemical calculations. The four compounds showed cardioprotective effects against sodium hydrosulfite-induced hypoxia-reoxygenation injury in H9c2 cells, with the three alkaloids being more potent. This is also the first report of alkaloids with a β-carboline skeleton isolated from P. heterophylla as cardioprotective agents.

Keywords: Pseudostellaria heterophylla; pseudosterins; cardioprotective agent

1. Introduction

Cardiovascular diseases (CVDs) are a leading cause of death and disability worldwide, with an estimate of 17.9 million deaths every year [1]. As one of the most serious CVDs, myocardial infarction (MI), which is defined by pathology as myocardial cell death due to prolonged ischemia, was reported with over 700,000 deaths every year in China [2,3]. Effective treatment of MI (e.g., reperfusion therapy) generally involves procedures to promote the return of blood flow to the ischemic zone of the myocardium. However, reperfusion itself may aggravate myocardial damage and may lead to further irreversible myocardial cell death (i.e., lethal myocardial reperfusion injury) [4,5]. Protection of myocardium against ischemia/reperfusion (I/R) injury is, therefore, crucial in the process of reperfusion. Unfortunately, no effective therapy is currently available for combined I/R injury on the market, yet clinical trials in the past 10 years demonstrated that some chemical entities (e.g., cyclosporine A and metoprolol) were effective in ameliorating the myocardial damage [6–8]. Therefore, the development of powerful cardioprotective agents or functional foods to limit the extent of infarcted tissue caused by I/R injury is of great clinical importance.
The roots of *Pseudostellaria heterophylla* (Taizisheng in Chinese) were used not only as an herb to tonify the spleen, enhance immunity, and treat palpitation in Chinese herbal medicine (CHM) and local ethnic medicines [9–12], but also as a tonic food. A number of functional foods prepared from *P. heterophylla* (e.g., Taizisheng Huangjing capsule and Taiziseng Tea (Hubei Zhenhao Biological Engineering Co., LTD, Yixing, China)) have been put into the market. Studies showed that *P. heterophylla* is rich in a variety of chemical components, including polysaccharides, glycosides, cyclic peptides, sterols, oils, and other volatile oily substances with a wide range of bioactivities [13–15]. Previous pharmacological investigations showed that the extracts of *P. heterophylla* possessed cardioprotective effects [16–19]. Recently, we found that pretreatment of H9c2 cells with a fraction enriched in glycosides markedly protected the cells from CoCl2-induced hyposic injury with effects comparable to that of the positive control N-Acetyl-L-Cysteine (NAC), and that the fraction may protect the cardiomyocytes from oxidative injury by preventing the increase of oxidative stress [20]. However, the active compounds responsible for the observed effects were still unknown. In the current investigation, three new alkaloids (1–3, Figure 1) with a 1-ethyl-3-formyl-β-carboline skeleton have been isolated from the active fraction. Their structures were elucidated on the basis of spectroscopic analysis and quantum chemical TDDFT calculations. Biological evaluations indicated that the three alkaloids showed more potent protective effects against sodium hydrosulfite-induced oxidative injury in H9c2 cells than that of polydatin, which was justified as an active principle in many fruits and vegetables [21–23]. These β-carboline alkaloids may be a promising type of compounds for the intervention of CVDs.

**Figure 1.** Structures of pseudosterins A–C (1–3) and polydatin.

### 2. Results and Discussion
#### 2.1. Structures Elucidation

Pseudosterin A (1) was obtained as a gum and possessed a molecular formula of C_{25}H_{29}N_{3}O_{11} as determined by the HR-ESI-MS peak at m/z 548.1868 [M + H]^+ (calcd 548.1875) (see Figure S3), corresponding to thirteen degrees of unsaturation. The IR absorptions suggested the presence of hydroxy (3086 cm\(^{-1}\)), carboxylic (ca. 3000–2500 cm\(^{-1}\)), carboxyl (1718 cm\(^{-1}\)), and aromatic (1594 and 1498 cm\(^{-1}\)) functionalities (see Figure S5 and Table S4). In the \(^{1}\)H-NMR spectrum data of 1 (CD\(_{3}\)OD, see Table 1 and Figure S6), signals for the following substructures were observed: A 1,2-disubstituted benzene [\(\delta_{\text{H}}\) 8.23 (1H, d, J = 7.8 Hz), 7.63 (1H, d, J = 8.2 Hz), 7.57 (1H, dd, J = 8.2, 7.4 Hz), and 7.30 (1H, dd, J = 7.8, 7.4 Hz)], and a CH\(_{2}\)CH(O-)- fragment [\(\delta_{\text{H}}\) 5.63 (1H, q, J = 6.6 Hz) and 1.81 (3H, d, J = 6.6 Hz)]. A combination of the remaining \(^{1}\)H-NMR signals with \(^{13}\)C-NMR and HSQC data (see Table 1 and Figures S7–S9) suggested the presence of a hexose [\(\delta_{\text{H}}\) 4.52 (1H, d, J = 7.7 Hz), 3.45 (1H, dd, J = 8.3, 7.7 Hz), 3.36–3.33 (2H, m), 3.28–3.25 (1H, m), 3.93 (1H, dd, J = 11.8, 2.2 Hz), 3.74 (1H, dd, J = 11.8, 5.8 Hz); \(\delta_{\text{C}}\) 102.9, 78.1, 78.1, 75.7, 71.7, 62.7]. However, one downfield singlet at \(\delta_{\text{H}}\) 8.74 (1H, s) as well as five upfield signals [\(\delta_{\text{H}}\) 4.71 (1H, br s), 2.48 (2H, m), 2.38 (1H, m), 2.18 (1H, m)] remained unassigned. In addition to signals for the above-mentioned subunits that contained a hexose and a 1,2-disubstituted benzene [\(\delta_{\text{C}}\) 122.6 (C-4b), 122.5 (C-5), 121.4 (C-6), 129.8 (C-7), 113.3 (C-8), 142.8 (C-8a)], the \(^{13}\)C-NMR and DEPT spectra showed additional signals for eight sp\(^{2}\) carbons [three acyl
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1-ethyl-3-formyl-β-carboline-3-yl-(piperidin-1-yl)methanone [24], ethyl 2-methyl-9H-pyrido[2,3-b]indole-3-carboxylate [25] and flazin [26], and taking into consideration the elemental constitution and carbon resonances, a β-carboline scaffold was supposed to be present in 1 through convergence of the 1,2-disubstituted benzene group (see Figure S4), two of the three nitrogen atoms, and the five non-acyl sp² carbons. Two-dimensional NMR experiments (HSQC, 1H-1H COSY, and HMBC) were further carried out to confirm the conclusion and to reveal the details of the structure. HSQC allowed the assignments of protons to their bonding carbons, while 1H-1H COSY enabled the confirmation of 1,2-disubstituted benzene, CH3CH(O-)-fragment, and hexose groups drawn in bold bonds (see Figure 2 and Figures S10–S12). Moreover, 1H-1H COSY correlations from H-3" (δH 2.38 and 2.18) to H-2" (δH 2.48) and H-4" (δH 4.71) indicated the presence of a spin system corresponding to C2"-C3"-C4", while a glutamic acid fragment was proposed for C-1" (δC 175.8) through C-5" (δC 176.8) by HMBC correlations H-2"/C-1" and H-3"/C-5" and on the basis of biosynthetic origin consideration. In addition, HMBC correlations between H-8/C-6 and C-4b, H-7/C-5 and C-8a, H-6/C-8 and C-4b, H-5/C-7, C-8a, and C-4a, and H-4/C-4a, C-4b, and C-9a confirmed the β-carboline structure, while correlations of H-11 with C-1 and C-9a suggested the ethoxyl group to be connected to C-1 via a carbon-carbon bond, and HMBC correlation between H-4/C-10 indicated that C-10 is located at C-3 of the β-carboline structure. A 1-ethyl-3-formyl-β-carboline skeleton was therefore established for 1. HMBC correlation between H-2"/C-10 indicated the glutamic acid fragment to be located at C-10 via an amide bond, while that between H-11/C-1" suggested the linkage of the hexosyl group at C-11. The planar structure of compound 1 was, therefore, established. The hexose obtained from HCl hydrolysate of 1 was confirmed by comparing its optical rotation data with that of an authentic D-glucose and by GC with their aldononitrile acetated derivatives (see Figures S13–S15). Coupling constant of the anomeric proton at δH 4.52 (1H, d, J = 7.7 Hz) suggested that the glucose had a β-configuration. Meanwhile, Marfey’s method was applied to identify the absolute configuration of glutamic acid in compound 1. The 1-fluoro-2,4-dinitrophenyl-5-1-leucinamide (l-FDLA) derivaties of glutamic acid in the acid hydrolyzate of 1 and standards of l- and D-glutamic acids were subjected to LC-ESI MS analysis (see Figures S16 and S17). The UHPLC retention time and molecular weight of the glutamic acid derivative of 1 were the same as those obtained for the derivative of l-glutamic acid. Thus, the configuration of C-2" was identified as S. The configuration of C-11 in 1 was, therefore, the only remaining task for its structural elucidation.

Pseudosterin B (2) was obtained as a gum and the HR-ESI-MS peak at m/z 417.1302 [M−H]⁻ (calcd 417.1303) (see Figure S18) suggested its molecular formula to be C20H22N2O8 with 11 degrees of unsaturation. Its UV and IR spectra (see Figures S19 and S20) bore a resemblance to those of 1, indicating the presence of similar functionalities. Except signals for the glutamic acid moiety, the 13C-NMR data of 2 are very close to those of 1 (see Table 1 and Figures S22–S23), suggesting a possible β-carboline skeleton and a hexose moiety for 2. However, the poor solubility of 2 in CD3OD gave poor peak resolution, which retarded the full assignments of the NMR data, whereas 1H-NMR data recorded in pyridine-d5 are well-resolved and the structure was established by detailed 2D NMR (1H-1H COSY, HSQC, and HMBC) data analysis (see Figures S21, S24–S26). As described for 1, a 1-ethyl-3-formyl-β-carboline skeleton can be easily identified by 1H-1H COSY, HSQC, and HMBC correlation analyses (see Figure 2). Coupling constants between the 1,3-diaxial protons (H-2'/H-4' and H-1'/H-3'/H-5') suggested the presence of a β-glucopyranosyl group, the configuration of which was also consistent with the coupling constant (δH 5.19, 1H, d, J = 7.3 Hz) of the anomeric proton. HMBC correlation between H-1' and C-11 placed the β-glucopyranosyl group at C-11. Chemical shift (δC 168.8), molecular formula, and
IR analysis suggested a carboxylic acid group for C-10. The structure of 2, except the configuration of C-11, was, therefore, established.

Table 1. $^1$H and $^{13}$C NMR data of pseudosterins A (1), B (2), and C (3).

| No. | $\delta_h$ (in Hz) | $\delta_c$ |
|-----|--------------------|-----------|
|     | 1 * | 2 * | 3 * | 1 * | 2 * | 3 * |
| 1   | 8.74 (1H, s) | 9.34 (1H, s) | 8.75 (1H, s) | 145.6 | 145.9 | 143.9 |
| 2   | 7.30 (1H, dd, 7.8, 7.4) | 7.35 (1H, br dd, 7.4, 7.3) | 7.27 (1H, br dd, 8.0, 8.0) | 121.4 | 120.6 | 120.0 |
| 3   | 7.57 (1H, dd, 8.2, 7.4) | 7.55 (1H, br dd, 7.7, 7.3) | 7.57 (1H, br dd, 8.0, 8.0) | 129.8 | 128.9 | 128.5 |
| 4   | 7.63 (1H, d, 8.2) | 7.77 (1H, br d, 7.7) | 7.66 (1H, br d, 8.0) | 113.3 | 112.9 | 112.4 |
| 5   | 5.63 (1H, q, 6.6) | 6.03 (1H, q-like) | 5.48 (1H, q, 6.5) | 78.4 | 77.4 | 76.9 |
| 6   | 1.81 (3H, d, 5.5) | 1.97 (3H, d, 5.5) | 1.74 (3H, d, 6.5) | 21.7 | 22.3 | 21.4 |
| 7   | 1.54 (1H, d, 7.7) | 1.94 (1H, d, 7.3) | 1.47 (1H, d, 7.7) | 102.9 | 102.9 | 101.9 |
| 8   | 4.52 (1H, d, 7.7) | 4.32 (1H, d, 7.7) | 3.25 (1H, d, 7.7) | 75.7 | 75.7 | 74.0 |
| 9   | 3.34 (1H, m) | 4.21 (1H, dd, 6.7, 7.9) | 3.16 (1H, d, 7.7) | 78.1 | 78.5 | 77.1 |
| 10  | 3.35 (1H, m) | 4.33 (1H, br d, 8.6) | 3.14 (1H, d, 7.7) | 71.7 | 71.7 | 70.2 |
| 11  | 3.27 (1H, m) | 3.92 (1H, br s) | 3.18 (1H, d, 7.7) | 78.1 | 78.8 | 76.5 |
| 12  | 3.03 (1H, dd, 11.8, 2.2) | 4.53 (1H, br d, 11.4) | 3.73 (1H, dd, 11.2, 5.1) | 62.7 | 62.6 | 61.1 |
| 13  | 3.74 (1H, dd, 11.8, 5.8) | 4.41 (1H, dd, 11.4, 4.6) | 3.30 (1H, m) | 175.8 | 79.8 | 79.8 |
| 14  | 175.8 | 79.8 | 79.8 |
| 15  | 4.71 (1H, br s) | 53.9 |
| 16  | 2.38 (1H, m) | 28.8 |
| 17  | 2.18 (1H, m) | 31.5 |
| 18  | 2.48 (2H, m) | 176.8 |
| 19  | 7.98 (1H, d, 2.0) |
| 20  | 7.45 (1H, d, 2.0) |

*a Recorded in CD$_3$OD; b Recorded in C$_5$D$_5$N; c Recorded in DMSO-d$_6$; o overlapped.

Figure 2. Key $^1$H-$^1$H COSY and HMBC correlations of pseudosterins A–C (1–3).

Pseudosterin C (3) was obtained as white amorphous powder. Its molecular formula was determined to be C$_{20}$H$_{22}$N$_2$O$_8$ from the quasi-molecular ion peak at $m/z$: 418.1609 [M + H]$^+$ (calcd: 418.1614) in the HR-ESI-MS (see Figure S27), indicating 11 degrees of unsaturation. The IR absorptions suggested the presence of hydroxy or/and amino (3443–3210 cm$^{-1}$), -NH$_2$ (1649 and 1562 cm$^{-1}$), and aromatic (1625 and 1508 cm$^{-1}$) groups (see Figure S29). The $^1$H-NMR data (see Table 1 and Figure S30) of 3 was very close to those of 2 in DMSO-d$_6$ (due to poor coupling splitting in pyridine-d$_5$). 3 was detected in DMSO-d$_6$; these observations suggested that compound 3 also possessed a similar 11-β-glucopyranosyl-β-carboline structure, which was also supported by a UV, $^{13}$C NMR, $^1$H-$^1$H
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H+ (calcd. 418.1614) in the HR-ESI-MS (see Figure S27), indicating 11 degrees of unsaturation analyses (see Figure 2). Coupling constants between the 1,3-diaxial protons rationalization. The IR absorptions suggested the presence of hydroxy or/and amino (3443–3210 mixture of enantiomers, the theoretical and experimental ECD data qualitatively allowed the determination of the absolute configuration at C-11 of pseudosterins A–C (data all supported the assignment of compounds has already been determined to be D-glucose with GC analysis, NMR and optical rotation positive first, negative second and third, and positive fourth Cotton effect. Since the glucose Similarly, the calculated ones for the corresponding model compounds exhibited a positive first, negative ones around 270 and 235 nm, and ended by a positive one near 200 nm. Similarly, the calculated ones for the corresponding model compounds exhibited a positive first, negative second and third, and positive fourth Cotton effect. Since the glucose showed a small positive first Cotton effect around 320 nm (see Figure 3, Tables S2 and S3), two negative ones around 270 and 235 nm, and ended by a positive one near 200 nm. Similarly, the calculated ones for the corresponding model compounds exhibited a positive first, negative second and third, and positive fourth Cotton effect. Since the glucose 

![](image.png)

Figure 3. Experimental ECD spectra of 1 (black dash line), 2 (black solid line), 3 (black dash dot line), and B3LYP/6-311++G(2d,2p)//B3LYP/6-311++G(2d,2p) calculated ECD spectra (σ = 0.5, red shifted by 10 nm and σ = 0.4, red shifted by 15 nm) of 1a (red dash lines) and 2a (red solid lines), respectively, and calculated ECD spectra of enantiomers 1a’ and 2a’ (blue dash line and blue solid line, respectively).

2.2. Hypoxia/Reoxygenation Injury Protective Activity

Since these compounds were obtained from the cardioprotective fraction, they were further evaluated for cardioprotective effects in H9c2 cells according to the method reported [28]. All the compounds showed no cytotoxicity at concentrations up to 500 μM (see Figure S2), whereas compounds 1–3 exhibited protective effects against Na2S2O4-induced hypoxia-reoxygenation injury in a concentration-dependent manner in the range of 100–400 μM (Figure 4), with a potency better than that of polydatin, a well-known natural cardioprotective agent that significantly decreased apoptosis rate in ischemia/reperfusion (I/R)-induced myocardial injury of rats [29,30] and that was also isolated as an active compound in the current investigation. The structure-activity relationship analysis showed that compounds 2 and 3, which carry a free carboxylic or a primary amide group at C-3, are more active than compound 1, which carries a secondary amide group. However, a negligible effect was observed when changing a free carboxylic group (as in 2) to a primary amide one (as in 3).
3. Materials and Methods

3.1. General Experimental Procedures

High-resolution electrospray ionization mass spectra (HR-ESI-MS) were carried out on a Bruker Daltonics microTOF-Q II mass spectrometer (Bruker Daltoniks GmbH, Bremen, Germany) equipped with an ESI interface. UV spectra were obtained on a Perkin-Elmer S2 Lambda 35 UV/VIS spectrometer (PerkinElmer, Boston, MA, USA). IR spectra were detected by the Perkin-Elmer Spectrum One FT-IR spectrometer (as KBr pieces; in cm⁻¹) (PerkinElmer, Boston, MA, USA). Optical rotations were measured on a Perkin-Elmer 341 polarimeter (PerkinElmer, Boston, MA, USA). NMR spectra were recorded on a Bruker AVANCE NEO 600 M (¹H: 600 MHz; ¹³C: 150 MHz) (Bruker, Karlsruhe, Germany) or JEOL ECX 500/400 NMR spectrometer (¹H: 500/400 MHz; ¹³C: 125/100 MHz) (JEOL, Germany) or with an ESI interface. UV spectra were obtained on a Perkin-Elmer S2 Lambda 35 UV/VIS spectrometer (PerkinElmer, Boston, MA, USA). IR spectra were detected by the Perkin-Elmer Spectrum One FT-IR spectrometer (as KBr pieces; in cm⁻¹) (PerkinElmer, Boston, MA, USA). Optical rotations were measured on a Perkin-Elmer 341 polarimeter (PerkinElmer, Boston, MA, USA). NMR spectra were recorded on a Bruker AVANCE NEO 600 M (¹H: 600 MHz; ¹³C: 150 MHz) (Bruker, Karlsruhe, Germany) or JEOL ECX 500/400 NMR spectrometer (¹H: 500/400 MHz; ¹³C: 125/100 MHz) (JEOL, Germany).
Akishima-shi, Japan). Thin-layer chromatography (TLC) was executed on silica gel GF$_{254}$ from Qingdao Haiyang Chemical Co., Ltd, Qingdao, China (QHCC), detected under a UV lamp at 254 or 365 nm, and visualized by spraying with 10% sulfuric acid/ethanol (v/v) solution followed by heating. Column chromatography (CC) was performed on columns with silica gel (QHCC), Sephadex LH-20 (Pharmacia, Stockholm, Sweden), and semi-preparative high-performance liquid chromatography with a UV detector (Beijing Guopu Technology Co., LTD, Beijing, China) ($\lambda$ = 254 nm) and a Kromasil C$_{18}$ column (250 mm × 10 mm; 5 µm) (Akzo Nobel N.V, Stockholm, Sweden). All solvents were of analytical grade.

3.2. Plant Material

The roots of Pseudostellaria heterophylla (Miq.) Pax were collected in October 2011 from the Radix Pseudostellariae cultivation base, Shibing County, Guizhou province, China and were identified by Prof. Qing-De Long of Guizhou Medical University. A voucher specimen (accession No. 20111101) was deposited at the Herbarium of the School of Pharmacy, Guizhou Medical University.

Extraction and Isolation. The dried roots of P. heterophylla (30 kg) were powdered and extracted by boiling water (70 L, 1.5 h × 2 times). The extract was evaporated under reduced pressure to yield a dark brown residue and then re-dissolved in H$_2$O. The obtained solution was then fractionated into five fractions, Fr. A–E, over a D101 macroporous resin column eluted with 0, 30%, 50%, 70%, and 95% ethanol/water, respectively. According to the previously reported procedure [42], fraction B was re-dissolved/dispersed in water and successively extracted with ethyl acetate and n-butyl alcohol to give three fractions (ethyl acetate fraction, n-butyl alcohol fraction, and water fraction). Purification of the n-butyl alcohol fraction sequentially by silica gel (petroleum ether:acetone = 3:1→0:1) afforded subfractions B1–B4. Fraction B2 was then subjected to Sephadex LH-20 eluted with MeOH to produce fractions B2a–B2c. Compound 1 (11 mg) was obtained from fraction B2b by Octadecylsilyl (ODS) column chromatography (CC) using MeOH–H$_2$O (50:50, v/v) as a mobile phase. Subfraction B4 was also submitted to ODS CC eluted with MeOH–H$_2$O (32:68, v/v) to yield compound 2 (5 mg). Fraction C was subjected to successive ethyl acetate and n-butyl alcohol extraction to give three fractions (Fr. C1–C3). Fr. C3 was sequentially separated by silica gel CC (CHCl$_3$:MeOH = 10:1→1:1) to give Fr.C3a, Fr.C3b, and Fr.C3c. Purification of Fr.C3b by a Sephadex LH-20 CC (MeOH) yielded polydatin (12.1 mg), while reversed phase silica gel CC of Fr.C3c eluted with MeOH in water gave compound 3 (5 mg).

3.3. Acid Hydrolysis, Derivatization, and GC Analysis

Pseudosterins A–C (1–3), 4.0, 3.0, and 3.0 mg, respectively) were hydrolyzed by 2 N HCl at 80 °C in wedge bottles. The sugar solutions were obtained after removal of impurity with dichloromethane, and the sugar was subsequently purified by the Sephadex LH-20 column. The obtained sugars were determined to be D-glucose by direct optical rotation comparison and co-TLC (BuOH/acetone/H$_2$O, 4/3/1, Rf = 0.45) with that of an authentic D-glucose. Further derivatization and identification were conducted according to previous procedures [43,44]. Briefly, 1.2 mg hydroxylamine-HCl and 0.4 mg sugar samples (or standard D-glucose and D-mannose) were dissolved with 2 mL pyridine in batches in wedge bottles to react for 30 min at 90 °C. The solutions were then cooled to room temperature. After addition of 1 mL of acetic anhydride to react at 90 °C for 30 min, the mixture was cooled to room temperature. Chloroform (1 mL) was then added and the organic phase was washed twice with 1 mL of water. The obtained organic phase was dried with rotary evaporator, and then the residue was dissolved in 100 µL of ethyl acetate–hexane (1:1, v/v) for GC analysis. The GC analysis was performed on a 7890–5975C system equipped with flame ionization detector (FID) (Agilent Technologies Inc., Santa Clara, CA, USA) and HP-5 capillary column (30 m × 320 µm i.d., 0.25 µm film thickness).
Samples (1 µL) were injected with a split ratio of 60:1 by the Agilent auto-injector. Helium was used as the carrier gas at a constant flow rate of 2 mL/min. The temperature program was set as follows: the initial column temperature of 180 °C was increased at 2 °C min⁻¹ to 250 °C, and held for 2 min. The inlet temperature was 240 °C and the temperature of detector was 280 °C. All the sugar substructures of pseudosterins A–C (1–3) were finally identified as D-glucose via a comparison with an authentic substance.

3.4. Acid Hydrolysis, Derivatization, and LC-ESI MS Analysis

Compound 1 (1 mg) with 6 N HCl (200 µL) were heated at 115 °C for 16 h according to report to release glutamic acid [45]. This solution was evaporated to dryness, and the residue was dissolved in 50 µL of water. To the glutamic acid-containing solution was added 40 µL of 1 M NaHCO₃ and then 120 µL of 1% L-FDLA in acetone. The solution was stirred and incubated at 40 °C for 2 h, then quenched by 40 µL of 1 N HCl. Finally, the solution was evaporated and re-dissolved with 500 µL of HPLC grade methanol for LC-ESI MS analysis. The corresponding L- and D-glutamic acids were modified in the same way. LC-ESI MS analysis was performed with an Ultimate 3000 UHPLC system (Thermo Fisher Scientific, Waltham, MA, USA) coupled with a TSQ endure mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). The chromatographic column used was Agilent Eclipse Plus C18 (2.10 mm × 100 mm, 1.8 µm) maintained at 40 °C. Acetonitrile–0.1% formic acid aqueous solution was used as mobile phase under a linear gradient elution program (acetonitrile, 20%–40%) for 45 min with photodiode array detection. The flow rate was 0.2 mL/min and the injection volume was 5 µL. The following MS parameters were employed: the capillary voltage was set at 2.5 kV with the sheath and auxiliary nebulizing gas (N₂) pressure set at 2 psi, respectively, and the vaporizer temperature was 275 °C. The scan range was set at m/z 50–1000. MS analysis was operated in the negative ion mode of electrospray ionization.

3.5. Hypoxia/Reoxygenation (H/R) Model and Experimental Protocols

The H/R model was established according to literature with minor modification [34]. Briefly, H9c2 cells were cultured in high glucose DMEM media supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), including penicillin (100 U/mL) and streptomycin (100 µg/mL). Cells grown at logarithmic growth stage were inoculated in a 96-well plate with a density of 4 × 10⁴ cells/mL (100 µL for each well) and cultured in an incubator with 5% CO₂ at 37 °C for 24 h. The cells were then randomly divided into 14 groups: control group (with culture medium only), model group (with 20 mM of sodium hydrosulfite in the culture medium), and test groups (each tested compound had three groups with concentrations of 100, 200, and 400 µM of the compound in the culture medium, respectively). After removal of the supernatant, 100 µL of culture medium without (for blank and model groups) or with the tested compounds (for groups of polydatin and pseudosterins A–C, all purities above 95%) were added, and the mixtures were then cultured in an incubator for 24 h. Except for the blank group, the supernatant was removed and the cells were washed twice with sugar-free DMEM medium. The Na₂S₂O₄ solution (20 mM) prepared with sugar-free DMEM medium was then added to the cells and incubated for 15 min to induce hypoxic condition in vitro. After removal of the Na₂S₂O₄ solution from cells, high glucose DMEM medium was added and the cells were cultured for another 15 min of reoxygenation to mimic reperfusion. Cell viability was then determined by the MTS method. The OD values were measured with microplate reader at a wavelength of 490 nm. The cell survival rate was calculated as ODₜᵉˢᵗ/ODₜמעות × 100%.

3.6. Statistical Analysis

Experimental data were shown as mean ± SD. The experiments were repeated three times. The difference between the mean values of two groups was assessed by the Student’s t-test. Multiple group comparisons were performed using a Dunnett’s test. The accepted
level of significance for the test was $p < 0.05$. All statistical tests were carried out using the SPSS 19.0 for Windows.

4. Conclusions

In the current investigation, three $\beta$-carbolines with a 1-ethyl-3-formyl-$\beta$-carboline skeleton have been isolated as cardioprotective agents from *P. heterophylla* in the treatment of cardiovascular diseases and in its use as a tonic food. $\beta$-Carbolines are an important subclass of carboline alkaloids with prominent biological effects. Diverse bioactivities, including antimicrobial, antitumor, antiparasitic, anticonvulsant, and vasorelaxant activities, have intrigued chemists and pharmacologists over centuries since the isolation of harmalin in 1841, and nine $\beta$-carboline drugs have been commercialized [46–49]. Thus, the discovery of 1-ethyl-3-formyl-$\beta$-carbolines as a new type of cardioprotective agent would not only stimulate efforts toward their structure–activity relationship, pharmacological application, and/or chemical synthesis, but also promote the use of *P. heterophylla* as a functional food for patients with cardiovascular diseases.

**Supplementary Materials:** Supplementary Materials are available online. Tables S1–S3, and Figure S1: ECD calculation details; Table S4: Optical rotation, UV and IR data of compounds 1–3; Figure S2: Cytotoxicity of compounds 1–3 and polydatin; Figures S3–S12: HR-MS, UV, IR, and 1D and 2D NMR of compound 1; Figures S13–S15: Glucose fragment of 1–3 identification; Figures S16 and S17: Glutamic acid fragment of 1 identification; Figures S18–S26: HR-MS, UV, IR, and 1D and 2D NMR of compounds 1–3. References [50–52] are cited in the Supplementary Material.

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**Sample Availability:** Samples of the compounds 1–3 are available from the authors.

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