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Total Synthesis, Discovery and Biological Evaluation of Daldinin A Derivatives for Improving Hyperglycemia-Induced Endothelial Cell Death

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Daldinins are a novel type of naturally occurring tricyclic heterocycles isolated from Daldinia concentrica. In this study, four daldinin A derivatives with different alkyl side chains were synthesized using the same synthetic protocol. Bioactivity tests first indicated that the daldinin A derivatives showed significant protective effects on endothelial cells against damage caused by high glucose. The derivative compound with three carbon atoms on the alkyl side exhibited the best effect.

Key words daldinin; natural product derivative; total synthesis; bioactivity; endothelial cell

Introduction

Daldinins are a type of new 2,3-dihydrobenzofurans isolated from the culture medium of Daldinia concentrica. This type of naturally occurring tricyclic heterocycles have attracted pharmaceutical interest due to their novel structure and multiple bioactivities. For example, concentricolide isolated from the fruiting bodies of Daldinia concentrica has been previously reported as a hot-off press natural molecule, showing unique anti-human immunodeficiency virus (HIV)-1 activity. Annulatins isolated from the culture medium of Cordyceps annulata in the presence of a histone deacetylase (HDAC) inhibitor exhibited potent agonistic activity toward cannabinoid receptors. The families of naturally-occurring tricyclic heterocycles have been reported to exert a wide range of activities. Despite this, the bioactivity of daldinins has yet to be fully elucidated, indicating the magnitude of our lack of knowledge regarding these tricyclic heterocycles. In our previous study on the total synthesis of daldinins, we established a versatile and reliable protocol by which to obtain daldinins, in particular those with different alkyl side chains. These are important pharmaceutical groups in the development of therapeutic drugs and commonly show structure–activity relationships.

In the present study, we synthesized four daldinin A derivatives with different alkyl groups. Preliminary bioactivity screening showed that these compounds exerted obvious protective effects on endothelial cells induced by high glucose by maintaining the mitochondrial membrane potential, reducing mitochondrial oxidative stress, and reducing apoptosis. The structure–activity relationships of the daldinin A derivatives with different side chains were also analyzed. Our findings suggest that daldinin A derivatives are a promising drug with potential therapeutic effects on vascular complications caused by diabetes.

Results and Discussion

Total Synthesis of Daldinins

The total synthesis protocol is shown in Chart 1. This protocol provides a simple and reliable route for the preparation of daldinins. Four daldinins with different long side chains were prepared from the corresponding alkynols with different alkyl side chains. Under the same reaction conditions, the preparation route provided the target products in almost the same yield and optical purity. The reduction of 9 using the Noyori/Ikariya [RuCl(TsDPEN)] catalysts (p-cymene) in the aqueous phase was a key step and produced daldinins via dynamic kinetic resolution through asymmetric transfer hydrogenation (DKR-ATH) process.

Reduction of Cell Damage and Inhibition of Apoptosis on Human Umbilical Vascular Endothelial Cells (HUVECs) under High Glucose Conditions

HUVEC Cell Viability

The effect of daldinins (10a–10d) on cell viability was evaluated using Cell Counting Kit-8 (CCK8) with HUVECs. HUVECs were treated with compounds 10a–10c (10d could not be assessed due to its poor solubility in the safe dose of dimethyl sulfoxide (DMSO)). The optical density (OD) was measured 48 h post-treatment using a microplate reader (SpectraMax M5), and the cell viability was calculated as shown in Figs. 1A and 1B. Compared with the control group, the HUVEC cell viability of the high glucose group (HG) was reduced, however, the cell viability of the drug group (treated with 10a–10c) was higher than that of the HG. The experimental results revealed that daldinins have obvious protective effects on HUVEC cells damaged by high glucose (33 mM).

In addition, compound 10a showed the best protective effect on HUVEC cells induced by high glucose. Subsequent experiments were performed using compound 10a (200 µM) as the drug group.

Flow Cytometric Analysis

To further evaluate the anti-apoptosis effect of the daldinin A derivatives on HUVEC cells, we performed an annexin V/propidium iodide (PI) dual staining assay via flow cytometry using compound 10a (Fig. 1C). The aim of this assay was to detect live cells (Q1; AV+/PI−), early apoptotic cells (Q2; AV+/PI−), late apoptotic cells (Q3; AV+/PI+), and necrotic cells (Q4; AV+/PI+). As shown in Table 1, HG induced apoptosis in 44.18% of HUVEC cells (3.48% early apoptosis, 13.14%...
(A): The compound 10a screened by CCK8 has obvious protective effect on HUVEC cells damaged by high glucose. Different grouping treatments: normal group (control); 33 mM high glucose model group (HG); drug group (HG +100 µM daldinin compounds). (C): HUVEC cells were treated with compound 10a and labeled with Annexin V/PI and analyzed for inhibition apoptosis using a flow cytometer. Cells in the upper left quadrant (Q1: AV−/PI−): necrotic cells; upper right quadrant (Q2: AV−/PI−): late apoptotic cells; lower right quadrant (Q3: AV+/PI+): early apoptotic cells and lower left quadrant (Q4: AV+/PI−): live cells. The results are expressed as the mean ± standard deviation (S.D.); *p < 0.05, **p < 0.001 versus control; †p < 0.05, ‡p < 0.001 versus HG.
late apoptosis, and 27.56% necrotic), which was significantly higher than the control, which induced apoptosis in 22.62% of the cells (2.32% early apoptosis, 8.56% late apoptosis, and 11.74% necrotic). However, when the cells were treated with compound 10a, apoptosis was induced in 24.26% of the cells (3.58% early apoptosis, 8.52% late apoptosis, and 12.16% necrotic). These results indicate that cell apoptosis induced by high glucose could be effectively inhibited by daldinin 10a.

**Reduction of Oxidative Stress Induced by HG via Daldinins and Maintenance of Mitochondrial Membrane Potential (ΔΨm) in HUVECs**

**Detection of Glutathione (GSH) and Reactive Oxygen Species (ROS) Levels**

Excessive OS can induce apoptosis by disrupting the normal function of mitochondria. An increased ROS content induces the opening of mitochondrial permeability transition pores and the decoupling of the mitochondrial electron transport chain, which leads to the initiation of the intrinsic apoptotic cascade. Eventually, this induces the rupture of the outer membrane of the mitochondria and apoptosis. Since compound 10a is able to inhibit HUVEC cell apoptosis, we evaluated the effect of 10a on ROS generation. The level of intracellular ROS was measured using 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA) staining and a microplate reader to detect the fluorescent intensity. DCFDA is a non-fluorescent dye that can be converted into fluorescent green DCF by intracellular esterase. The fluorescence intensity of DCF corresponds to the amount of ROS generated. As shown in Fig. 2A, the ROS level of the HG was considerably enhanced compared with the control. Compared with HG, the ROS level of daldinin 10a was markedly reduced. These results indicate that daldinin 10a can alleviate the increase in the level of oxidative stress in HUVECs caused by HG.

The decreased GSH content is potentially an early signal of apoptosis, and the subsequent generation of oxygen free radicals promotes apoptosis. Therefore, it is important to maintain GSH at relatively normal levels. As shown in Fig. 2B, compared with HG, the content of GSH in cells treated with 10a was clearly increased. These findings indicate that compound 10a can effectively alleviate the decrease in GSH and maintain the normal function of cells.

**Detection of Mitochondrial Membrane Potential (ΔΨm)**

The maintenance of the mitochondrial membrane potential (ΔΨm) is essential for bio-energetic function and mitochondrial integrity. A fall in ΔΨm is an early event that occurs during apoptosis and chemical-hypoxia-induced necrosis. We used 5,5,6,6-tetrachloro-1,1,3,3-tetraethyl-imidacarbocyanineiodide (JC-1) staining and MitoTracker Red CMXRos to detect changes in ΔΨm. The protective effect of mitochondria by 10a was evaluated by measuring the increase in ΔΨm compared with HG. JC-1 is an ideal fluorescent probe that is widely used to detect ΔΨm. As shown in Fig. 2B, because healthy normal cells have a higher ΔΨm, most JC-1 were found to gather in the mitochondrial matrix in the form of JC-1 aggregates, and mainly emitted red fluorescence (Fig. 3A: HG). On the other hand, the JC-1 aggregates were converted into more JC-1 monomers in the case of cells damaged and emitted a green fluorescence (Fig. 3A: HG + 10a). Our results indicate a marked decrease in JC-1 monomers from normal polarized JC-1 aggregates, and that compound 10a transformed ΔΨm, in contrast to HG.

Next, MitoTracker Red CMXRos, a red fluorescent dye used to stain mitochondria in living cells, was used to evaluate the effect of 10a on ΔΨm. The accumulation of this dye depends on the level of ΔΨm: the higher the ΔΨm, the stronger the red fluorescence, and vice versa. The results shown in Fig. 3B indicate that the ΔΨm of the cells treated with 10a was notably enhancement compared to HG.

Taken together, these results indicate that daldinin 10a is able to effectively restore the decrease in ΔΨm caused by HG.
Conclusion
In this study, four natural daldinin A derivatives were synthesized using the same synthetic protocol. This protocol provides a reliable and versatile route by which to prepare daldinin A and its derivatives using cheap raw materials under mild reaction conditions and with high yields. It also conveniently allows for the preparation of daldinins with different alkyl side chains that belong to hydrophobic groups. Different lengths of these chains may affect their hydrophobicity, membrane permeability, and metabolic stability. Preliminary tests on the activity of those daldinins indicated that they exerted varying protective effects on endothelial cells against damage caused by high glucose (Bioactivity of daldinin A was also tested in this study, but it showed no effect on the cell protection, so it was not discussed in this paper). This indicates that they may be able to exert a significant protective effect on mitochondrial function to maintain the mitochondrial membrane potential and reduce mitochondrial oxidative stress and apoptosis. The daldinin A derivative containing three carbon atoms on the alkyl side (compound 10a) exhibited the best protective effect. This study is the first to associate a protective effect to daldinins, and suggests that daldinin A derivatives may exert a therapeutic effect against vascular complications in diabetes.

Experimental
General Information
All chemicals were purchased from Sigma-Aldrich (U.S.A.), Alfa Aesar (U.S.A.), or Acros (U.S.A.). Reactions involving non-aqueous conditions were carried out with freshly distilled solvents under an argon atmosphere. $^1$H-NMR and $^{13}$C-NMR spectra were recorded using a Bruker AV-400 MHz NMR spectrometer in CDCl$_3$ or CD$_3$OD using tetramethylsilane (TMS) (0.00 ppm) as the internal standard. Chemical shifts were reported as $\delta$ values (ppm). The high-resolution mass spectra (HRMS) of the new compounds were obtained on a Bruker microTOF-Q III spectrometer. Medium containing Dulbecco’s modified Eagle’s medium (DMEM), 1% penicillin–streptomycin solutions, and trypsin solutions were purchased from Hyclone, and 10% fetal bovine serum and glucose were purchased from Gibco (U.S.A.) and Sigma, respectively. Phosphate-buffered saline (PBS) contained 2.7 mM KCl, 137 mM NaCl, 2 mM KH$_2$PO$_4$, and 10 mM Na$_2$HPO$_4$. CCK8 was purchased from MedChem Express (U.S.A.). ROS and GSH assay kits were purchased from Nanjing Jiancheng Bioengineering Institute, China. 5,5,6,6-Tetrachloro-1,1,3,3-tetramethylimidacarbocyanine iodide (JC-1) and MitoTracker Red CMXRos were purchased from Beyotime Biotechnology, China. Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection Kit was purchased from KeyGEN BioTech, China. Human umbilical vascular endothelial cells (HUVECs) were obtained from Prof. Yu Song.
(College of Pharmacy, Xiangxiang Medical University, Henan, China). SpectraMax M5 microplate reader (ThermoFisher Scientific, U.S.A.) and fluorescence microscope were purchased from Nikon (Japan).

**Total Synthetic Procedures of 10**
The total synthesis of daldinins was initiated using commercially available hex-1-yn-1-ol 1 to produce hex-2-ynal 2 by an oxidant using the Jones reagent. A Michael addition-aldol reaction of 1,3-acetonedimethylidicarboxylate 11 and alkynal 2 produced phenol 3. Phenol 3 was then reacted with ethyl bromoacetate to obtain ester 4. The oxidation of the alkylbenzene side chain via the oxidation of CrO3 in AcOH/Ac2O produced compound 5. The reduction of compound 5 with NaBH4 in methanol produced racemic phthalide 6. The Dieckmann condensation of compound 6 produced compound 7, and the Krapcho reaction of 7 produced compound 8. The aldol reaction of compound 8 and acetone generated diasteromeres 9. The asymmetric transfer hydrogenation (ATH) of 10 using the Noyori/Ikariya catalysts in aqueous resulted in the production of daldinins via a dynamic kinetic resolution (DKR) process. Different from the daldinin A, the derivatives 4b – 8 were prepared using the Jones reagent.

**Spectroscopic Data**

- **1H-NMR (400 MHz, CDCl3):** δ ppm: 11.13 (s, 1H), 7.78 (d, J = 8.2 Hz, 1H), 6.76 (d, J = 8.2 Hz, 1H), 3.94 (s, 3H), 2.62 – 2.57 (m, 2H), 1.61 – 1.53 (m, 2H), 1.40 – 1.30 (m, 2H), 1.36 – 1.26 (m, 4H), 0.87 (t, J = 7.3 Hz, 3H).
- **13C-NMR (100 MHz, CDCl3):** δ ppm: 199.7, 168.8, 167.2, 164.6, 159.5, 140.2, 133.0, 130.5, 126.4, 124.7, 53.0, 52.9, 52.2, 39.5, 26.0, 22.3, 13.9; HRMS (ESI): Calcd for C18H22O3 [M + Na]+: 389.1207; Found: 389.1206.
- **1H-NMR (400 MHz, CDCl3):** δ ppm: 7.99 (d, J = 8.1 Hz, 1H), 7.67 (d, J = 8.2 Hz, 1H), 4.74 (s, 2H), 3.92 (s, 6H), 3.83 (s, 3H), 2.94 (t, J = 7.3 Hz, 2H), 1.73 – 1.64 (m, 2H), 1.44 – 1.34 (m, 2H), 0.94 (t, J = 7.3 Hz, 3H); **13C-NMR (100 MHz, CDCl3):** δ ppm: 199.7, 168.8, 167.2, 164.6, 155.5, 140.2, 133.0, 130.5, 126.4, 124.7, 53.0, 52.9, 52.2, 39.5, 26.0, 22.3, 13.9; HRMS (ESI): Calcd for C18H22O3 [M + Na]+: 389.1207; Found: 389.1206.
- **1H-NMR (400 MHz, CDCl3):** δ ppm: 7.73 (d, J = 8.1 Hz, 1H), 6.96 (d, J = 8.1 Hz, 1H), 4.56 (s, 2H), 3.78 (s, 3H), 3.73 (s, 3H), 3.68 (s, 3H), 2.49 – 2.43 (m, 2H), 1.48 – 1.39 (m, 2H), 1.26 – 1.16 (m, 2H), 0.77 (t, J = 7.3 Hz, 3H); **13C-NMR (100 MHz, CDCl3):** δ ppm: 168.9, 167.5, 164.9, 155.2, 146.7, 132.6, 130.5, 125.5, 121.7, 72.2, 52.3, 52.2, 51.9, 33.2, 32.8, 22.4, 13.7.

**Conclusion**

The oxidation of the alkylbenzene side chain of ester 4 via the oxidation of CrO3 in AcOH/Ac2O produced compound 5. The reduction of compound 5 with NaBH4 in methanol produced racemic phthalide 6. The Dieckmann condensation of compound 6 produced compound 7, and the Krapcho reaction of 7 produced compound 8. The aldol reaction of compound 8 and acetone generated diasteromeres 9. The asymmetric transfer hydrogenation (ATH) of 10 using the Noyori/Ikariya catalysts in aqueous resulted in the production of daldinins via a dynamic kinetic resolution (DKR) process. Different from the daldinin A, the derivatives 4b – 8 were prepared using the Jones reagent.
1H-NMR (400 MHz, CDCl3): δ ppm: 8.06 (d, J = 8.0 Hz, 1H), 128.0 ppm: 6.8, 81.8, 34.4, 24.2, 22.5, 14.0; HRMS (ESI): Calcd for C_{17}H_{20}O_4 [M + Na]^+: 373.1298; Found: 373.1299.

Compound 6d: 1H-NMR (400 MHz, CDCl3): δ ppm: 8.06 ppm: 197.1, 170.7, 166.8, 161.0, 130.7, 115.5, 112.5, 82.1, 76.3, 34.4, 31.4, 24.4, 22.5, 14.0; HRMS (ESI): Calcd for C_{17}H_{20}O_4 [M + Na]^+: 283.0941; Found: 283.0941.

Compound 8d: 1H-NMR (400 MHz, CDCl3): δ ppm: 7.97 ppm: 198.3, 169.7, 166.7, 161.4, 130.8, 132.1, 115.8, 112.3, 112.3, 111.1, 112.4, 81.7, 82.0, 72.7, 36.8, 25.3, 18.9, 13.9. 13C-NMR (100 MHz, CDCl3): δ ppm: 198.3, 169.7, 166.7, 161.4, 130.8, 115.8, 112.3, 111.1, 112.4, 81.7, 82.0, 72.7, 36.8, 25.3, 18.9, 13.9. HRMS (ESI): Calcd for C_{17}H_{20}O_4 [M + Na]^+: 297.1097; Found: 297.1097.

Compound 9a: 1H-NMR (400 MHz, CDCl3): δ ppm: 7.94 ppm: 170.1, 176.8, 161.0, 130.7, 122.9, 115.6, 112.7, 82.2, 76.4, 34.6, 31.7, 29.0, 24.8, 22.6, 14.2; HRMS (ESI): Calcd for C_{17}H_{20}O_4 [M + Na]^+: 298.0941; Found: 297.1097.

Compound 8c: 1H-NMR (400 MHz, CDCl3): δ ppm: 7.96 ppm: 198.4, 169.7, 166.7, 161.4, 130.8, 123.7, 115.6, 112.3, 91.5, 82.0, 72.7, 36.8, 25.3, 18.9, 13.9.

Compound 10a: 1H-NMR (400 MHz, MeOD): δ ppm: 7.70 ppm: 198.3, 169.7, 166.8, 161.0, 130.7, 115.8, 112.4, 81.7, 82.0, 72.7, 34.7, 27.2, 25.2, 22.5, 22.5, 22.4, 14.1; HRMS (ESI): Calcd for C_{17}H_{20}O_4 [M + Na]^+: 341.1589; Found: 341.1365.

Compound 9c: 1H-NMR (400 MHz, CDCl3): δ ppm: 7.94 ppm: 170.1, 176.8, 161.0, 130.7, 122.9, 115.6, 112.7, 82.2, 76.4, 34.6, 31.7, 29.0, 24.8, 22.6, 14.2; HRMS (ESI): Calcd for C_{17}H_{20}O_4 [M + Na]^+: 298.0941; Found: 297.1097.

Compound 9b: 1H-NMR (400 MHz, CDCl3): δ ppm: 7.94 ppm: 198.4, 169.7, 166.7, 161.4, 130.8, 123.7, 115.6, 112.3, 112.3, 111.1, 112.4, 81.7, 82.0, 72.7, 34.7, 27.2, 25.2, 22.5, 22.5, 22.4, 14.1; HRMS (ESI): Calcd for C_{17}H_{20}O_4 [M + Na]^+: 298.0941; Found: 297.1097.

Compound 9c: 1H-NMR (400 MHz, CDCl3): δ ppm: 7.94 ppm: 170.1, 176.8, 161.0, 130.7, 122.9, 115.6, 112.7, 82.2, 76.4, 34.6, 31.7, 29.0, 24.8, 22.6, 14.2; HRMS (ESI): Calcd for C_{17}H_{20}O_4 [M + Na]^+: 298.0941; Found: 297.1097.

Compound 10b: 1H-NMR (400 MHz, CDCl3): δ ppm: 7.94 ppm: 198.4, 169.7, 166.7, 161.0, 130.7, 115.8, 112.4, 81.7, 82.0, 72.7, 34.7, 27.2, 25.2, 22.5, 22.5, 22.4, 14.1; HRMS (ESI): Calcd for C_{17}H_{20}O_4 [M + Na]^+: 298.0941; Found: 297.1097.
Compound 10b: 1H-NMR (400 MHz, MeOD): δ ppm: 7.68 (d, J = 7.6 Hz, 1H), 7.04 (d, J = 7.6 Hz, 1H), 5.52 (dd, J1 = 7.6 Hz, J2 = 3.8 Hz, 1H), 5.38 (t, J = 5.8 Hz, 1H), 4.46 (d, J = 6.4, 2.4 Hz, 1H), 2.13–2.02 (m, 1H), 1.78–1.62 (m, 1H), 1.50 (s, 3H), 1.48 (s, 3H), 1.45–1.27 (m, 4H), 0.91 (t, J = 7.0 Hz, 3H). 13C-NMR (100 MHz, MeOD): δ ppm: 169.0, 157.3, 153.2, 132.1, 132.0, 113.8, 108.6, 92.6, 82.1, 71.8, 70.4, 34.1, 26.5, 25.6, 25.0, 22.1, 12.9. HRMS (ESI): Calcd for C19H26O2Na+: 357.1673; Found: 357.1673.

Cell Culture and Group Processing HUVECs were cultured in DMEM low-glucose medium containing 10% fetal bovine serum and 1% penicillin–streptomycin solution in a 5% CO2 atmosphere at 37°C. After culturing at 37 °C for 48 h according to the treatment protocols for each group. After collecting the cells incubated under standard conditions, part of the cell suspension was sonicated to determine the protein concentration, while another part was mixed with 2,7-dichlorofluorescin diacetate (DCFH-DA) (10μmol/L) working solution and incubated at 37°C for 30 min in the dark. Then, the ROS level of each group of cells was detected. The results were expressed as fluorescence value/mg protein.

Detection of GSH Levels Intracellular GSH levels were measured using Glutathione Assay Kit, according to the manufacturer’s instructions. An appropriate amount of PBS buffer was added to resuspend the HUVECs of each group after collecting the cells incubated under standard conditions. Then, the cells were broken by sonication (using ultrasonic disrupter) and bicinchoninic acid (BCA) Protein Assay Kit was used to detect the protein content of the samples. The cells were then centrifuged, and 200 μL of the supernatant was used to measure the absorbance of each group at 420 nm using a microplate reader.

Detection of Mitochondrial Membrane Potential After 48 h of treatment, the mitochondrial membrane potential was detected using JC-1 and MitoTracker Red CMXRos kits, according to the manufacturer’s instructions. Using a fluorescence microscope, red fluorescence indicated JC-1 aggregates in the matrix of mitochondria and a higher mitochondrial membrane potential, while green fluorescence indicated a decreased mitochondrial membrane potential after depolarization. The change in the mitochondrial membrane potential was evaluated based on the changes in fluorescence. In normal cells, MitoTracker Red CMXRos stains mitochondria bright red. However, when the mitochondrial membrane potential drops, the intensity of the red fluorescence of the mitochondria is gradually reduced. Thus, the intensity of fluorescence can be used to detect changes in the mitochondrial membrane potential.

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Conflict of Interest The authors declare no conflict of interest

Supplementary Materials The online version of this article contains supplementary materials.

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