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Separation of 25*R*/*S*-ergostane triterpenoids in the medicinal mushroom *Antrodia camphorata* using analytical supercritical-fluid chromatography

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ABSTRACT

Ergostanes are major bioactive constituents of the medicinal mushroom *Antrodia camphorata*. These tetracyclic triterpenoids usually occur as 25*R*/*S* epimeric pairs, which renders their chromatographic separation difficult. In this study, we used analytical supercritical-fluid chromatography (SFC) to separate seven pairs of 25*R*/*S*-ergostanes from *A. camphorata*. The (*R*)- and (*S*)-forms for each of the seven pairs could be well resolved ($R_s > 1.3$) on a Chiralcel OJ-H column (4.6 × 250 mm, 5 μm, chiral), eluted by 10% MeOH in CO_2 at 2 mL/min with a back pressure of 120 bar and a column temperature of 40 °C. Particularly, this chiral-SFC method could rapidly and efficiently separate low-polarity epimers like antcin A and antcin B, which were very difficult for RP-HPLC. A 3-min preparative-scale method was established to purify (25*S*)- and (25*R*)-antcin A for the first time. However, OJ-H column suffered from peak overlapping of different pairs of ergostanes. We found that Princeton 2-ethylpyridine column (2-EP, 4.6 × 250 mm, 3 μm, achiral) could effectively separate different pairs, although the resolutions for 25-*R*/*S* forms of each epimeric pair were not as good as OJ-H column. Meanwhile, all the (25*S*)-forms showed stronger retentions than the corresponding (25*R*)-epimers on the 2-EP column. These results demonstrated different selectivity of chiral- and achiral-SFC in separating 25*R*/*S*-ergostane epimers. Aside from high separation efficiency, SFC also showed advantage over HPLC in short analysis time and low consumption of organic solvents. Finally, both OJ-H and 2-EP columns were used on analytical SFC to separate 25*R*/*S*-ergostanes in an extract of *A. camphorata*.

1. Introduction

Ergostanes are a class of tetracyclic triterpenoids commonly seen as secondary metabolites of fungi [1]. Recently, we have isolated a series of 25*R*/*S*-ergostanes from *Antrodia camphorata*, a precious medicinal mushroom [2]. *A. camphorata* is popularly used in Taiwan for cancer treatment and liver protection, and ergostanes are its major bioactive constituents [3]. Interestingly, most ergostanes in *A. camphorata* are present as (25*R*)- and (25*S*)-epimeric pairs, which renders their separation difficult. Up to date, only four pairs of ergostane epimers from *A. camphorata* have been separated by reversed-phase HPLC [4]. However, these methods suffered from poor separation of low-polarity epimers like antcin A and antcin B. Particularly, (25*S*)- and (25*R*)-antcin A have never been isolated in pure optical form, so far [5].

Supercritical-fluid chromatography (SFC) is a powerful technique for chiral and achiral separation [6]. Due to low viscosity and high diffusivity of supercritical fluid CO_2, SFC is operated at a higher flow rate with lower back pressure, and is generally faster and more efficient than HPLC [7]. Rapid column equilibration and convenient mobile phase removal also make SFC attractive for preparative scale separation [8]. Recently, SFC has been widely used to separate chiral mixtures of pharmaceutical products and natural products, including triterpenoids, steroids, and bile acids [9–13]. Different types of packing materials such as 2-ethylpyridine, cyano, phenyl-hexyl, and silica showed diverse chromatographic selectivity [14–19]. However, few reports are available on the separation of triterpenoid epimers by SFC.

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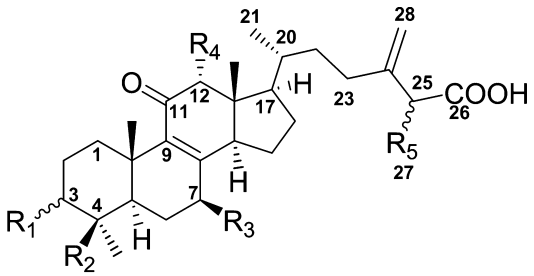

	R_1	R_2	R_3	R_4	R_5
1/2	α-OH	OH	OH	H	β-CH_3 / α-CH_3
3/4	ΔO	OH	OH	H	β-CH_3 / α-CH_3
5/6	β-OH	H	OH	H	β-CH_3 / α-CH_3
7/8	ΔO	H	OH	H	β-CH_3 / α-CH_3
9/10	α-OH	H	ΔO	OH	α-CH_3 / β-CH_3
11/12	ΔO	H	ΔO	H	β-CH_3 / α-CH_3
13/14	ΔO	H	H	H	β-CH_3 / α-CH_3

Fig. 1. Chemical structures of ergostanes isolated from *Antrodia camphorata*.

In this paper, we report SFC separation of seven pairs of 25*R*/*S*-ergostane epimers isolated from *A. camphorata*. The separation selectivity of a chiral 4-methylbenzoate cellulose (Chiralcel OJ-H) column and an achiral 2-ethylpyridine (2-EP) column was studied. The optimized method was employed to purify the *R*/*S* forms of antcin A, and to analyze an ergostane-enriched extract of *A. camphorata*.

2. Experimental

2.1. Chemicals and reagents

Methanol, isopropanol, trifluoroacetic acid (DikmaPure, CA, USA), acetonitrile (J.T. Baker, Phillipsburg, NJ, USA), and formic acid (Sigma-Aldrich, MO, USA) were of HPLC grade. De-ionized water was prepared using a Milli-*Q* water purification system (Millipore, MA, USA). High-purity carbon dioxide (CO_2, 99.999%) was from AP BAIF Gases (Beijing, China). All ergostane reference compounds were isolated from *A. camphorata* by the authors. Their structures are given in Fig. 1. These compounds included (25*S*)-antcin K (**1**), (25*R*)-antcin K (**2**), antcamphin E (**3**), antcamphin F (**4**), antcamphin K (**5**), antcamphin L (**6**), (25*S*)-antcin C (**7**), (25*R*)-antcin C (**8**), (25*R*)-antcin H (**9**), (25*S*)-antcin H (**10**), (25*S*)-antcin B (**11**), (25*R*)-antcin B (**12**), (25*S*)-antcin A (**13**), and (25*R*)-antcin A (**14**). Among them, **3**–**6** were reported as new compounds in our recent publication [2]. Compounds **13** and **14** were purified in this study.

2.2. Crude drug materials

The fruiting bodies of *A. camphorata* were cultivated and identified by Professor Yew-Min Tzeng at Chaoyang University of Technology, Taiwan. A voucher specimen (YMT 1307) was deposited at the Herbarium of School of Pharmaceutical Sciences, Peking University, Beijing, China. The mixture of **13** and **14** was isolated from the fruiting bodies of *A. camphorata* [2].

2.3. Sample preparation

Preparation of reference standard solutions: Reference compounds were, respectively, dissolved in methanol, and then mixed to produce a mixed standard solution (containing all the 14 ergostanes), a mixed (*S*)-form standard solution (containing **1**, **3**, **5**, **7**, **10**, **11** and **13**), and a mixed (*R*)-form standard solution (containing **2**, **4**, **6**, **8**, **9**, **12** and **14**) (80 μg/mL for each compound). Each 25*R*/*S*-ergostane epimeric pair was separately dissolved in methanol to produce epimer standard solutions (30–50 μg/mL for each compound). The above Solutions were filtered through 0.22 μm membranes before use.

Preparation of ergostane-enriched extract: The crude drug of *A. camphorata* was pulverized into fine powder. An aliquot of 200 mg was extracted with 6 mL of 50% methanol in an ultrasonic water bath for 30 min, and then filtered. Two milliliter of the filtrate was evaporated to dryness, suspended in water (5 mL) and then extracted with dichloromethane (5 mL). The organic layer was dried and dissolved in 1.5 mL of methanol to obtain the ergostane-enriched extract. The sample was filtered through 0.22 μm membranes before use.

2.4. Analytical columns

The following analytical columns were used (or tested) in this study: Chiralcel OJ-H (4.6 × 250 mm, 5 μm, Daicel Industries, Tokyo, Japan); Chiralpak IC (4.6 × 250 mm, 5 μm, Daicel); Princeton 2-ethylpyridine (4.6 × 250 mm, 3 μm, Princeton Chromatography, NJ, USA); Princeton SFC Diol (4.6 × 250 mm, 3 μm); Agilent Zorbax RX-SIL (4.6 × 250 mm, 5 μm, Agilent Technologies, Waldbronn, Germany); YMC-Pack ODS-A (4.6 × 250 mm, 5 μm, YMC Co. Ltd, Kyoto, Japan) protected by an XTerra MS C_{18} guard column (3.9 × 20 mm, 5 μm, Waters, MA, USA).

2.5. Analytical supercritical-fluid chromatography

An Agilent 1260 Infinity analytical SFC system was used. The system consists of an SFC Fusion™ A5 module, a modified 1260 Infinity LC system, a degasser, an SFC binary pump, an SFC autosampler, a thermostated column compartment, and a diode array detector with high pressure SFC flow cell. Unless otherwise stated, the flow rate was 2 mL/min, the back pressure regulation (BPR) pressure was 120 bar, and the column temperature was 40/37 °C (inlet/outlet). The UV detection wavelength was set at 250 and 270 nm. An aliquot of 5 μL sample was injected for analysis. The 25*R*/*S*-ergostane solutions were separated on the Chiralcel OJ-H column by isocratic elutions. The mixed standard solutions and the ergostane-enriched extract were separated on Chiralcel OJ-H column (0–10 min, 5–18%; 10–15 min, 18–20% MeOH in CO_2, v/v) and Princeton 2-ethylpyridine column (0–15 min, 10–30%; 15–20 min, 30% MeOH in CO_2, v/v). Chiralpak IC, Princeton SFC Diol and Zorbax RX-SIL columns were tested using the same conditions as the OJ-H column, with slight modifications on the elution program. Data were processed by Openlab CDS Chemstation C.01.03 software.

2.6. Preparative scale SFC

The 25*R*/*S* epimeric mixture of antcin A (96.1 mg) was purified by a customized preparative SFC system (TOYO KOATSU Co. Ltd, Hiroshima, Japan). The sample (6.5 mg/mL in methanol) was separated on a preparative Chiralcel OJ-H column (30 × 250 mm, 10 μm, Daicel Industries), and was detected at 250 nm. The flow rate was 70 mL/min and the injection volume was 0.3 mL. The back pressure regulation (BPR) pressure was 120 bar, and the column temperature was 35 °C. An isocratic elution (CO_2/MeOH = 90/10, v/v) was used to obtain (25*S*)-antcin A (**13**, t_R = 1.07 min, 45.4 mg) and (25*R*)-antcin A (**14**, t_R = 1.80 min, 19.9 mg). The absolute configurations for C-25 of **13** and **14** were determined by the modified Mosher's method, according to previous reports (see Supporting information) [2,4].

(25*S*)-antcin A (**13**): white amorphous powder; $[\alpha]^{25}_D$ + 144 (ca. 0.1, MeOH); IR (KBr) ν_{max} 2962, 2925, 1736, 1619, 1456, 1172 cm^{-1};