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Isolation of an Antidermatophytic Substance from the Root of *Salvia miltiorrhiza*

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Dihydrotanshinone I was isolated from a Chinese crude drug "Danshen" (the root of *Salvia miltiorrhiza*) as an antidermatophytic substance against *Trichophyton mentagrophytes*. This compound inhibited the mycelial growth of six dermatophytes completely at a concentration as low as 1.56 to 6.25 μg/ml, depending on species, whereas its congener cryptotanshinone was found to be active only against *T. tonsulans* var. *sulfureum* at 100 μg/ml. Cryptotanshinone, however, was as active as dihydrotanshinone I against four species of gram-positive bacteria at 0.195—50 μg/ml.

Keywords——*Salvia miltiorrhiza*; dihydrotanshinone I; cryptotanshinone; antidermatophytic activity; antibacterial activity

"Danshen," the dried roots of *Salvia miltiorrhiza* BUNGE (Labiatae), has been used in Chinese traditional medicine for hemorrhage, menstrual disorder, miscarriage, and swelling.[1)] Fang *et al.*[2)] showed that the orange-red pigments of the root such as cryptotanshinone, dihydrotanshinone I, hydroxytanshinone IIA, methyltanshionate, and tanshinone IIB were inhibitory to the growth of *Staphyloccocus aureus* cultured *in vitro*. Zheng and Ho[3)] also reported that all five varieties of Danshen were active against gram-positive bacteria.

Although an ethanolic extract of Danshen is known to inhibit the growth of various dermatophytes,[1b)] no study has been made on its active principle. The objective of the present work was to determine the antidermatophytic constituents of this crude drug.

Experimental

Isolation of Antidermatophytic Substances——A powdered sample (2.5 kg) of Danshen purchased at a crude drug market in Hong Kong was extracted with MeOH (6 l) four times, and the MeOH was evaporated under reduced pressure to obtain a syrupy extract (413 g). When the extract was distributed between ether and water, the antidermatophytic activity against *Trichopyton mentagrophytes* was found only in the former layer (43 g), which was then subjected to silica gel column (Mallinckrodt, 900 g) chromatography using $CHCl_3$ as the developing solvent. Each fraction (300 ml) eluted was examined by the paper disc method for the antidermatophytic activity against *T. mentagrophytes*. Dihydrotanshinone I[4)] (**1**, 158 mg) and cryptotanshinone[5)] (**2**, 826 mg) were isolated as main active compounds from fr. 13—19 and fr. 44,45, respectively, by preparative TLC using Silica gel G60 (developing solvent: $CHCl_3$). In addition, tanshinone IIA[5)] (**4**, 2653 mg) and tanshinone I[5)] (**3**, 300 mg) were obtained as crystals from fr. 14—19 and fr. 22,23, respectively, but they were found to be inactive against the fungus. The characterization of the above four tanshinones was done by melting point determination, elemental analysis, ultraviolet (UV), infrared (IR), proton nuclear magnetic resonance (^{1}H-NMR), mass spectrum (MS).[4-10)]

Dihydrotanshinone I **1**: Red needles from a mixture of $CHCl_3$ and ethyl acetate, mp 201 °C. $[\alpha]_D^{25}$ −41.3° (c=0.1, $CHCl_3$). *Anal.* Calcd for $C_{18}H_{14}O_3$: C, 77.70; H, 5.04. Found: C, 77.53; H, 4.99. UV λ_{max}^{MeOH} nm (log ε): 240 (4.46), 266 (sh), 290 (4.29). 330 (3.60); VIS λ_{max}^{MeOH}: 410 (3.72). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1670, 1645, 1600. ^{1}H-NMR ($CDCl_3$) δ: 9.18 (1H, d, J=8 Hz), 8.15 (1H, d, J=8 Hz), 7.60 (1H, d, J=8 Hz), 7.40 (2H, m), 4.93 (1H, t, J=9.2 Hz), 4.36 (1H, dd, J=9.2, 6 Hz), 3.60 (1H, m), 2.58 (3H, s), 1.39 (3H, d, J=6.8 Hz). MS m/z: 278 (M^+), 250 (M^+−CO), 235.

Cryptotanshinone **2**: Brownish red needles from a mixture of $CHCl_3$ and ethyl acetate. mp 191 °C. $[\alpha]_D^{25}$ −87.8° (c=0.1, $CHCl_3$). *Anal.* Calcd for $C_{19}H_{20}O_3$: C, 77.03; H, 6.76. Found: C, 76.63; H, 6.78. UV λ_{max}^{MeOH} nm (log ε): 263

(4.45), 272 (4.41), 293 (3.89), 355 (3.25); VIS λ_{max}^{MeOH}: 445 (3.26). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1680, 1650, 1610, ^{1}H-NMR ($CDCl_3$) δ: 7.42 (2H, ABq, J=8 Hz), 4.83 (1H, t, J=9.2 Hz), 4.31 (1H, dd, J=9.2, 6 Hz), 3.55 (1H, m), 3.17 (2H, br t), 1.65 (4H, m), 1.40 (3H, d, J=6.8 Hz), 1.28 (6H, s). MS m/z: 296 (M^+), 268 ($M^+ - CO$), 253.

Tanshinone I **3**: Brownish orange needles from a mixture of $CHCl_3$ and ethyl acetate, mp 241 °C. *Anal.* Calcd for $C_{18}H_{12}O_3$: C, 78.25; H, 4.38. Found: C, 78.04; H, 4.31. UV λ_{max}^{MeOH} nm (log ε): 244 (4.40), 267 (sh), 320 (3.55); VIS λ_{max}^{MeOH} 415 (3.60). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1660, 1590, 1550. ^{1}H-NMR ($CDCl_3$) δ: 9.17 (1H, d, J=8 Hz), 8.13 (1H, d, J=8 Hz), 7.60 (1H, d, J=8 Hz), 7.20—7.30 (3H, m), 2.60 (3H, s), 2.25 (3H, s). MS m/z: 276 (M^+), 248 ($M^+ - CO$), 191.

Tanshinone IIA **4**: Reddish orange needles from a mixture of $CHCl_3$ and ethyl acetate, mp 216 °C. *Anal.* Calcd for $C_{19}H_{18}O_3$: C, 77.53; H, 6.16. Found: C, 77.68; H, 6.09. UV λ_{max}^{MeOH} nm (log ε): 252 (4.35), 267 (4.50), 350 (3.31); VIS λ_{max}^{MeOH} 460 (3.53). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1665, 1579, 1535. ^{1}H-NMR ($CDCl_3$) δ: 7.42 (2H, ABq, J=8 Hz), 7.10 (1H, q, J=2 Hz), 3.15 (2H, br t), 2.25 (3H, d, J=2 Hz), 1.70 (4H, m), 1.30 (6H, s). MS m/z: 294 (M^+), 279 ($M^+ - CH_3$), 261.

Assay of Antimicrobial Activity——The bacteria and fungi used in the present experiments were cultured on the heart infusion agar medium and Sabouraud's agar medium, respectively, at 37 °C (for bacteria and yeasts) and 27 °C (for dermatophytic fungi) in the dark.[12] During the isolation and purification processes of antidermatophytic substances, the samples were tested against *T. mentagrophytes* by the paper disc method as described elsewhere.[12] For the estimation of the minimum inhibitory concentration (MIC), the agar streak method was employed using the heart infusion medium and Sabouraud's medium (9.5 ml), to which the MeOH solution (0.5 ml) of a test sample or a reference drug was added.[11]

In order to examine fungicidal concentrations, a MeOH solution (0.25 ml) of **1** (3.9—2000 μg/ml) was mixed with Sabouraud's liquid medium (4.75 ml) and a piece of filamentous mycelia of *T. mentagrophytes* was put on the test medium. After incubation for 6 d at 27 °C, the mycelia were transferred to fresh Sabouraud's agar medium and incubated for another week at 27 °C to check the survival of the mycelia.

Results and Discussion

As shown in Table I, dihydrotanshinone I (**1**) proved to be highly active against all six species of fungi tested at low concentrations (1.56—6.25 μg/ml). However, the growth of mycelia of *T. mentagrophytes* recovered when the mycelia were transferred to **1**-free medium, indicating that the effect of this compound is not fungicidal but fungistatic. It was also demonstrated that **1** is strongly antibacterial against five strains of gram-positive bacteria, inhibiting their growth at concentrations ranging from 0.78 to 12.5 μg/ml.

Cryptotanshinone (**2**), which was considered as an antibacterial constituent of Danshen in the screening test by the paper disc method, failed to inhibit the growth of the dermatophytes except for *T. tonsulans* var. *sulfureum* even at the highest concentration

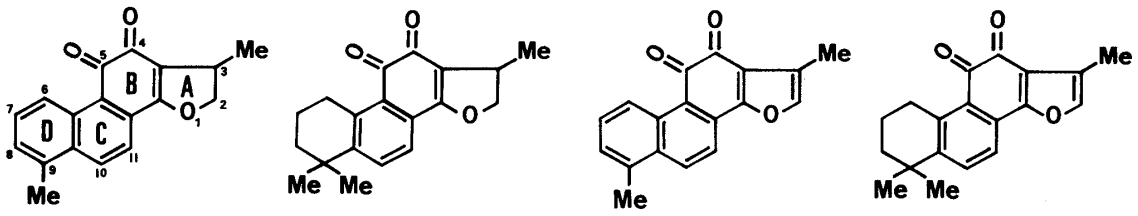
TABLE I. Antimicrobial Activities of the Methanol Extract and Active Substances from "Danshen"

Microorganism	MIC (μg/ml)			Reference drugs	
	MeOH extract	Dihydro-transhinone I	Crypto-tanshinone	Chloram-phenicol	Griseo-fulvin
Gram-positive bacteria					
Staphylococcus aureus 209P	800	12.5	50	6.25	—[a]
Sarcina lutea ATCC 381	50	3.125	0.195	1.56	—
S. lutea ATCC 382	50	0.78	0.78	3.125	—
Bacillus polymyxa IAM 1189	100	1.56	0.78	3.125	—
B. subtilis PCI 219	200	1.56	1.56	3.125	—
Dermatophytic fungi					
Trichophyton rubrum IFO 5808	200	3.125	>100	—	6.25
T. mentagrophytes IFO 5809	1600	6.25	>100	—	1.56
T. tonsulans var. *sulfureum* IFO 5945	200	3.125	100	—	6.25
Microsporum gypseum IFO 8307	1600	1.56	>100	—	6.25
Sabourandites canis IFO 9182	100	6.25	>100	—	1.56
Epidermophyton floccosum IFO 9045	400	1.56	>100	—	1.56

a) Not tested.

(100 μg/ml) tested by the agar streak method. Interestingly, however, **2** was highly active (MIC: 0.195—50 μg/ml) against the gram-positive bacteria (Table I).

Neither **1** nor **2** showed any antimicrobial activity against the following gram-negative bacteria and yeasts even at the highest concentration tested (100 μg/ml): *Escherichia coli, Pseudomonas aeruginosa, Citrobacter freundii, Serratia marcescens, Candida albicans, C. krusei, C. mycoderma, C. tropicalis, C. utilis,* and *Saccharomyces sake.* As for the compounds **3** and **4**, no significant activity was detected against any of the tested microorganisms, including gram-positive bacteria, at the concentration of 100 μg/ml.



1 2 3 4

Chart 1. Structure of Tanshinones Isolated from "Danshen"

1, dihydrotanshinone I; **2**, cryptotanshinone; **3**, tanshinone I; **4**, tanshinone IIA.

The four compounds (**1**—**4**) isolated from Danshen have mutually similar chemical structures (Chart 1); **1** and **2** have a dihydrofuran ring (A), whereas **3** and **4** possess a furan ring (A) in common. Furthermore, **1** and **3** have a conjugated benzene ring (D) with a methyl group at the 9 position in contrast to **2** and **4**, which have a cyclohexane ring D with two methyl groups. The existence of a dihydrofuran ring seems to be essential to the antimicrobial activity, since both **3** and **4** are inactive. Also, the aromatization of the ring D and/or the presence of a single methyl group at the 9 position must be important for the antifungal activity, as **1** shows a much stronger activity than **2**.

As far as the *in vitro* tests in the present work are concerned, the antidermatophytic activity of **1** is comparable to that of griseofulvin, an antibiotic used orally for serious cases of athlete's foot. From our own experience, we have found that **1** is more active than other antidermatophytic compounds such as tryptanthrin (MIC: 3.1—6.3 μg/ml),[11] maackiain (MIC: 12.5—25 μg/ml),[12] perillaldehyde (MIC: 100—200 μg/ml), citral (MIC: 25—100 μg/ml),[13] osthol (MIC: 6.25—12.5 μg/ml), and isopimpinellin (MIC: 6.25—>200 μg/ml),[14] which we have isolated from various medicinal plants. The compound **1** also shows a stronger activity than such antidermatophytic substances isolated from other higher plants as cinnamic aldehyde (MIC: 8—32 μg/ml),[15] hinokitiol (MIC: 8—16 μg/ml),[16] ethyl *p*-methoxycinnamate (MIC: 2—50 μg/ml),[17] and *o*-methoxycinnamaldehyde (MIC: 3.12—6.25 μg/ml).[18]

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