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ISOLATION AND BIOACTIVITY OF NEW TANSHINONES

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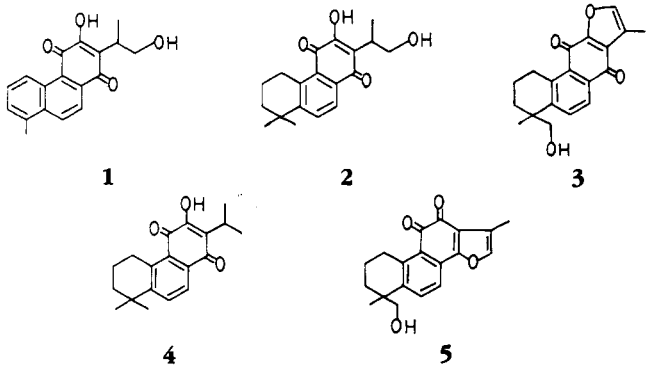
ABSTRACT.—Two new diterpenoids, designated neocryptotanshinone [**2**] and isotanshinone IIB [**3**], have been isolated from "Tan-Shen," the root of *Salvia miltiorrhiza*, together with a known compound, danshexinkun A [**1**]. Their structures are established by spectral and physical data. Isotanshinone IIB exhibits significant inhibitory activity in vitro on ADP- and collagen-induced aggregation.

"Tan-Shen," the root of *Salvia miltiorrhiza* Bunge (Labiatae), is one of the most important herb drugs in use since ancient times in China and is ranked as a "Supergrade" medicine in Shen-Nung's Pen-Ts'ao (Materia Medica) (1).

Phytochemically, the genus *Salvia* is characterized by the fact that it is rich in diterpenoid pigments. More than 20 of them have been isolated and characterized by Japanese, Chinese, and Austrian chemists (2-12). Most of the red crystalline pigments have either a furo-1, 2- or a furo-1,4-naphthoquinone chromophore and are biogentically classified as diterpenoids. This group of secondary metabolites is interesting to organic chemists, pharmacologists, and phytochemists not only for chemical reasons as challenging synthetic targets but also for their remarkable biological properties. The biological activities of "Tan-Shen" span an enormous range including antispasmodic, antiarthritic, tonic, sedative, and astringent. It is highly recommended in cases of hemorrhage, menstrual disorders, and miscarriages (13). Recent studies have shown that "Tan-Shen" exhibited in vitro a significant improvement of the blood flow in the coronary circulatory system and a reduction in myocardial infarction (14,15). Other bioactivities are antipyretic, antimicrobial, and anti-inflammatory (16,17).

In our continuing search for cytotoxic metabolites, we found for the first time that some of the major constituents of "Tan-Shen" showed significant activity in vitro against cells derived from human epidermoid carcinoma of the nasopharynx (KB), and it was speculated that saturation in the ring A of these diterpenoids might be responsible for the antineoplastic activity (18). These purified compounds showed ED_{50} values from 2.0×10^{0} to 2.8×10^{1} μg/ml. However, the crude $CHCl_3$ extract of "Tan-Shen" was found to have a much greater inhibition potency of ED_{50} (1.4×10^{0} μg/ml). This observation prompted us to make a further examination of the roots and led to the isolation and characterization of three minor components named danshexinkun A [**1**], neocryptotanshinone [**2**], and isotanshinone IIB [**3**].

The authors also conducted a preliminary in vitro anti-aggregation test on isotanshinone IIB [**3**] to evaluate its biological activity.



RESULTS AND DISCUSSION

The $CHCl_3$ extract of "Tan-Shen" was prepared as described in the Experimental section and was chromatographed on Si gel with a low pressure column chromatograph to afford three minor components **1-3** in the yield of 3.5, 5.8, 3.0 ppm, respectively.

Compound **1** was identified as a known compound, danshexinkun A, from its uv, ir, nmr, ms spectral data, mp, and elemental analysis.

Compound **2**, orange-red needles, $[\alpha]^{23}D=+29.8$ (0.84, $CHCl_3$), analyzed for $C_{19}H_{22}O_4$ (M^+ 314) by mass spectral and elemental analysis. The uv spectrum is almost identical to that of **4**, a synthetic compound previously reported (8). It shows characteristic absorption maxima at 248 (4.24), 256 (4.26), 282 (4.08), 348 (3.56), and 460 (3.48) nm in 95% EtOH and their absorptions at 3340, 1665, 1645 cm^{-1}. These spectral properties suggest the presence of a 2-hydroxyl-1, 4-naphthoquinone moiety in this pigment (8). The ^1H-nmr spectrum of **2** indicates the presence of a geminal dimethyl group at δ 1.30 (s, 6H), a methyl (δ 1.24, d, 3H, J=7.8 Hz) bonded to a methine group (δ 3.50, m, 1H). Signals for two protons with an ABX type splitting are found at δ 3.88 with coupling constants of 10.0, 6.0, and 4.0 Hz, respectively, which are clearly indicative of diastereotopic methylene protons attached to a chiral center and an electron withdrawing group (-OH) (19). Signals for three more methylene groups are seen in δ 1.75 (br m, 4H) and 3.25 (br t, 2H). The latter is assigned to two benzylic methylene protons. The signals for an AB-quartet at lower field (ABq centered at δ 7.85, Δδ=0.23 ppm, J=8.0 Hz) are assigned to two adjacent aromatic protons. The fairly low field of these signals is apparently attributed to the anisotropic effect of a *peri*-carbonyl in the 1,4-quinone moiety. A molecular ion peak at *m/z* 314 and prominent peaks at *m/z* 299 (M^+-CH_3·), 296 (M^+-H_2O), 284 (M^+-CH_2O·), 281 (296-CH_3·), 253 (281-CO), and 225 (253-CO) are in excellent agreement with those of the expected fragmentation patterns. Hence, based on the spectroscopic evidence and on analogy with the structure of known tanshinones, the structure **2** was assigned to neocryptotanshinone.

Compound **3**, red platelets, analyzed for $C_{19}H_{18}O_4$ (M^+ 310) and has the same molecular formula with that of tanshinone IIB [**5**]. The ir spectrum of this red pigment shows the presence of a furan ring (ν 3150, 1540, 1025, 845 cm^{-1}) (20). The ^1H-nmr spectrum strikingly resembles that of tanshinone IIB [**5**] (21) and shows signals for a methyl group (δ 1.46, s, 3H), a methyl (δ 2.28, d, 3H, J=2.0 Hz) bonded to a furan ring which is unsubstituted in the α-position (the α-proton appears at δ 7.30, d, 1H, J=2.0 Hz), and four methylene groups at 1.87-2.10 (m, 4H), 3.36 (br t, 2H), and 3.80-3.98 (m, 2H). However, there is a conspicuous difference between the two spectra, whereas an AB-quartet of aromatic protons is centered at δ 7.64 (Δδ=0.24 ppm, J=9.0 Hz) in the spectrum of tanshinone IIB (5), the corresponding AB-quartet is shifted to lower field (δ 7.75, Δδ=0.31 ppm, J=8.0 Hz) in that of isotanshinone IIB [**3**]. The shift effect is once again due to the anisotropic effect of a *peri*-carbonyl group. A major difference is seen in the uv-vis spectra of these two pigments, tanshinone IIB [**5**] shows absorption maxima at 226, 252, 269, 276 (sh), 348, and 465 nm (21), but isotanshinone IIB [**3**] shows absorption maxima at 260 (4.40), 2.84 (4.47), 346 (3.78), and 467 nm (3.47). The ms spectrum of **3** shows a molecular peak at *m/z* 310 and prominent peaks at 292 (M^+-H_2O), 279 (M^+-CH_2OH·), 251 (279-CO), 223 (251-CO). Therefore, based on the spectroscopic evidence and on analogy with the structure of known tanshinones, the structure **3** was assigned to isotanshinone IIB.

The anti-aggregation activity of isotanshinone IIB [**3**] for the ADP- and collagen-induced platelet aggregation is shown in Table 1. For comparison, the data for aspirin are given. Compound **3** displays a remarkable inhibitory activity on platelet aggregation. This inhibition is concentration-dependent on **3**. Owing to the extreme scarcity

of **1-3** in natural sources, an experiment for a large-scale extraction of these compounds for further investigations on the mechanisms and the median inhibition concentration (IC_{50}) of platelet aggregation and other bioactivities is to be conducted.

TABLE 1. Anti-aggregation Activity of Isotanshinone IIB [**3**]

Compound[a]	Dose (μg/ml)	Inhibition of Platelet Aggregation[b]	
		ADP-induced	Collagen-induced
3	10	40	81
	5	—	4
Aspirin	50	74	72

[a]Saline was used as solvent.
[b]Inhibitions exceeding 50% are effective.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Melting points were determined using a Thomas-Hoover melting point apparatus and are uncorrected. Uv spectra were obtained with a Perkin-Elmer model 555 double beam spectrophotometer using 95% EtOH as the solvent, and ir spectra were obtained on a Perkin-Elmer model 983G spectrophotometer with polystyrene calibration at 1601 cm^{-1}. ^{1}H-nmr spectra were recorded on a JEOL FX-90Q instrument at 100 MHz using TMS as an internal standard. Low resolution mass spectra were run on a Hewlett-Packard 5985-B instrument at 70 eV. Optical activity was determined with a Perkin-Elmer model 241 polarimeter using a 0.1 cm microcell and $CHCl_3$ as the solvent. Low pressure column chromatography was performed on a preparative scale with an Eyela flash chromatograph model EF-10 using E. Merck Lichroprep Si 60 (25-40 mesh) as the stationary phase.

PLANT MATERIAL.—"Tan-Shen" was supplied from Chien-Yuan Co., Taipei, and was identified by Prof. W. L. Wu of the National Defense Medical Center, where a voucher specimen is deposited.

EXTRACTION AND FRACTIONATION.—The air dried (40°) and powdered "Tan-Shen" (11.27 kg) was extracted with $CHCl_3$ at room temperature for 3 days. The $CHCl_3$ extract (325 g) was partitioned between $CHCl_3$-EtOH (80:20) and 5% Na_2CO_3. The aqueous layer was neutralized with HCl, and then extracted repeatedly with $CHCl_3$. After concentration in vacuo, the $CHCl_3$ extract (25 g) was chromatographed on Si gel (70-230 mesh) by elution with $CHCl_3$ to give danshexinkun A [**1**] (40.32 mg) and neocryptotanshinone [**2**] (65.25 mg), followed by Me_2CO and MeOH. The Me_2CO and MeOH eluates were combined and concentrated under reduced pressure. The mixture was then purified on Si gel (24-40 mesh) by low pressure flash chromatography by eluting with H_2O-MeCN (60:40) to afford isotanshinone IIB [**3**] (34.56 mg).

IDENTIFICATION OF **1**.—Orange-red needles, mp 184-186° [lit. (22) mp 183-185°]. Identification was established by comparison (uv, ir ^{1}H-nmr, ms) with an authentic sample of danshexinkun A.

CHARACTERIZATION OF NEOCRYPTOTANSHINONE [**2**].—Orange-red needles, mp 165-167°; $[\alpha]^{23}D = +29.8$ (0.84, $CHCl_3$); uv λ max (95% EtOH) (log ε) 248 (4.24), 256 (4.26), 282 (4.08), 348 (3.56), 460 (3.48) nm; ir ν max (KBr) 3340, 1665, 1645, 1200, 1020, 750 cm^{-1}; ^{1}H nmr ($CDCl_3$) δ 1.24 (d, 3H, J=7.8 Hz), 1.30 (s, 6H, Me×2), 1.75 (br m, 4H, CH_2×2), 3.25 (br t, 2H, CH_2-Ar), 3.50 (m, 1H, CH), 3.88 (ddd, 2H, J= 10.0, 6.0 and 4.0 Hz, CH_2-O), 7.20 (br s, quinoid OH), and 7.73, 7.96 ppm (ABq, 2H, J=8.0 Hz, ArH); ms *m/z* (rel. int.) 314 (M^+, 58.4), 299 (M^+-CH_3·, 5.6), 296 (M^+-H_2O, 25.6), 284 (M^+-CH_2O·, 100), 283 (M^+-CH_2OH·, 7.4), 281 (M^+-H_2O-CH_3·, 17.9), 253 (181-CO, 22.0), 225 (253-CO, 13.0), 197 (16.5), 171 (23.4), 165 (33.6). Calcd for $C_{19}H_{22}O_4$: C, 72.59; H, 7.05. Found: C, 72.71; H, 7.32.

CHARACTERIZATION OF ISOTANSHINONE IIB [**3**].—Red platelets, mp 206-209°; uv λ max (95% EtOH) (log ε), 260 (4.50), 284 (4.47), 346 (3.78), 467 (3.47) nm; ir ν max (KBr 3520, 3150, 1670, 1580, 1540, 1390, 1025, 845, 795 cm^{-1}; ^{1}H nmr ($CDCl_3$) 1.46 (s, 3H, Me), 1.87-2.10 (m, 4H, CH_2×2), 2.28 (d, 3H, J=2.0 Hz, Me-furan), 3.36 (br t, 2H, -CH_2-Ar), 3.80-3.98 (m, 2H, CH_2-O), 7.30 (d, 1H, J=2.0 Hz, H-furan), and 7.59, 7.90 (ABq, 2H, J=8.0 Hz, ArH) ppm; ms *m/z* (rel. int.) 310 (M^+, 10.0), 292 (M^+-H_2O, 14.0), 279 (M^+-CH_2OH·, 100), 251 (279-CO, 23.6), 223 (251-CO, 21.7), 165 (14.7), 152 (10.0). Calcd for $C_{19}H_{18}O_4$: C, 73.53; H, 5.85. Found: C, 73.49; H, 5.99.

ANTI-AGGREGATION ACTIVITY.—The in vitro assessment of anti-aggregation was performed on ADP- and collagen-induced platelet-rich plasma by the turbidimetric method (23). If the percentage inhibition is greater than 50, the compound is considered effective. In this test aspirin was used as a reference.

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