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Aldose Reductase Inhibitory Constituents of the Root of *Salvia miltiorhiza* BUNGE

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The constituents of the MeOH extract of *Salvia miltiorhiza* BUNGE, which showed strong aldose reductase (AR) inhibitory activity, were examined, and two new abietane-type diterpenoids, danshenol A (1) and danshenol B (2), were isolated together with six known ones: dihydrotanshinone I (3), cryptotanshinone (4), tanshinone I (5), tanshinone IIA (6), (−)-danshexinkun A (7), and sugiol (8). Among them, 4, 5, and 8 were weak AR inhibitors with IC_{50} from 4.80 to >10.0 μM, while 1, 2, 3, 6, and 7 were strong inhibitors (IC_{50} from 0.10 to 1.75 μM). Danshenol A (1), the strongest inhibitor, had IC_{50} of 0.10 μM which is comparable to that of epalrestat in clinical use. Moreover, from a consideration of IC_{50} and yield of each compound, it was concluded that tanshinone IIA (6) is the major active constituent of the MeOH extract and danshenol A (1) and (−)-danshexinkun A (7) are the minor ones.

Key words danshenol; abietane-type diterpenoid; aldose reductase inhibitor; tanshinone; *Salvia miltiorhiza*; Lamiaceae

Related complications have become a major problem in the management of diabetes, which is a condition common throughout the world. One possible hypothesis on the development of diabetic complications involves the polyol pathway.[1] The polyol pathway has been found in tissues such as lens, retina, nerve, and kidney, in which diabetic complications appear, and the pathway includes two enzymes, aldose reductase (AR) (alditol: $NADP^+$ oxidoreductase [EC 1.1.1.21]) and sorbitol dehydrogenase (SDH) (L-iditol: NAD^+ 2-oxidoreductase [EC 1.1.1.14]). AR catalyzes the reduction of various aldehydes, including the aldehyde form of D-glucose, to the corresponding sugar alcohol, L-sorbitol, and SDH converts L-sorbitol to D-fructose. AR normally has a low affinity for glucose and therefore requires a relatively high concentration of that sugar for significant activity. In the diabetic condition, however, sufficient glucose may enter the tissues, and the pathway operates to produce both sorbitol and fructose, leading to their intracellular accumulation. This metabolic abnormality results in diabetic complications such as cataracts, retinopathy, neuropathy, and nephropathy. AR is a rate-limiting key enzyme of this pathway, and thus the inhibition of AR may be effective in treating and preventing these complications.

In the course of our search for drugs effective for diabetes[2] and/or diabetic complications,[3] we found a paper reporting that "Danshen (丹参)" strongly inhibits AR.[4] Danshen, dried root and rhizome of *Salvia miltiorhiza* BUNGE (Lamiaceae), is officially listed in the Chinese Pharmacopoeia[5] and is widely used for treatment of hematological abnormalities, heart diseases, menstrual disorder, miscarriage, hepatitis, and swelling.[6,7] Many abietane-type diterpenoids and caffeic acid derivatives have been isolated from this crude drug as pharmacologically active principles,[6] and salvianolic acid A, one of the caffeic acid derivatives, was reported to have AR inhibitory activity.[8] No systematic examination on the AR inhibitory constituents has been done, however, and our preliminary examination indicated that a non-polar fraction, which would contain diterpenoids but not salvianolic acid A, had stronger inhibitory activity than a polar fraction. Thus we began studying the isolation and structure of the AR inhibitory constituents of *S. miltiorhiza* and isolated two new abietane-type diterpenoids, danshenol A (**1**) and danshenol B (**2**), along with six known ones, dihydrotanshinone I (**3**),[9,10] cryptotanshinone (**4**),[9,11] tanshinone I (**5**),[9] tanshinone IIA (**6**),[9,12] (−)-danshexinkun A (**7**),[10] and sugiol (**8**).[13] In this paper, we report the isolation and structure elucidation of these compounds, as well as their AR inhibitory activity.[14]

Results and Discussion

Isolation of Constituents Chopped root of *S. miltiorhiza* was extracted successively with hot water and MeOH to give water and MeOH extracts, both of which showed

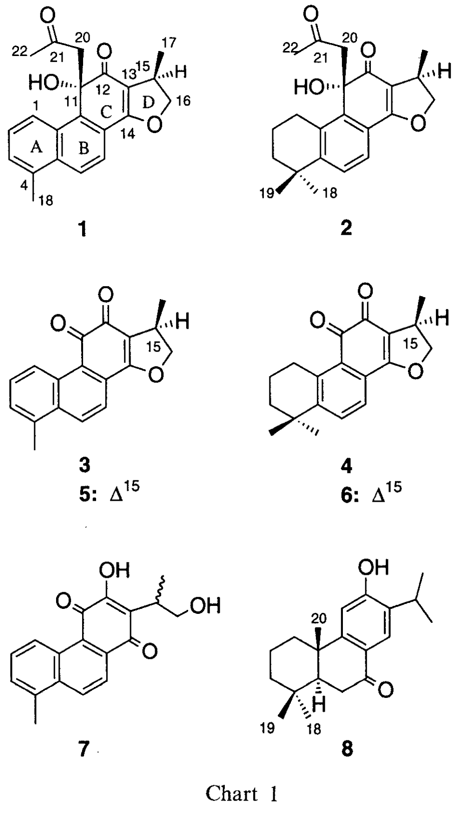

Chart 1

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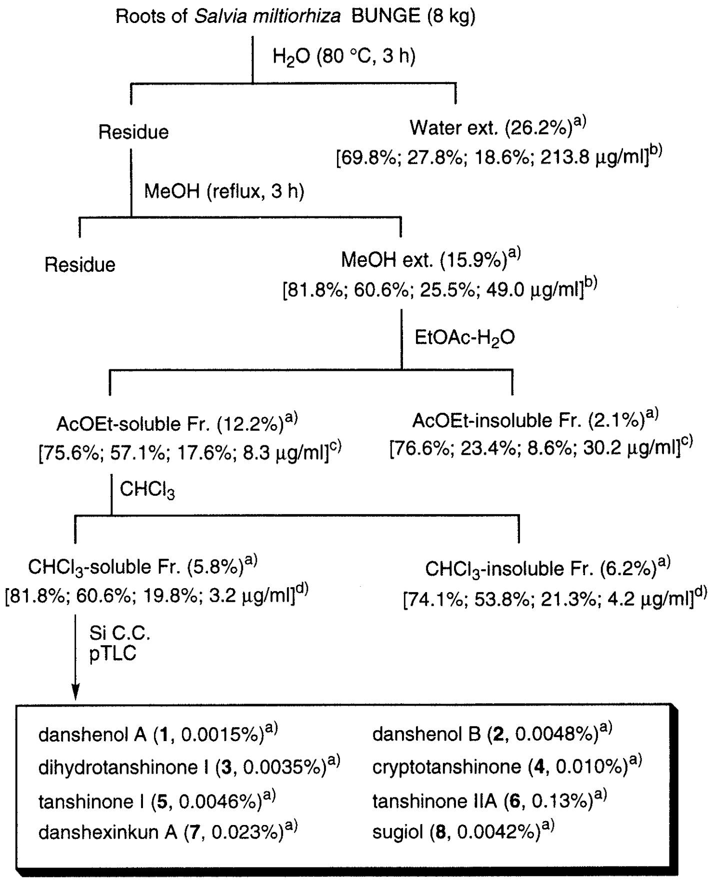


Chart 2. Isolation Procedure and Inhibitory Activity of Each Fraction

a) Yields from the roots (8 kg). b) Inhibitory rate at 500, 100, and 10 μg/ml and IC_{50}. c) Inhibitory rate at 100, 10, and 5 μg/ml and IC_{50}. d) Inhibitory rate at 10, 5, and 1 μg/ml and IC_{50}.

AR inhibitory activity dose-dependently (Chart 2). The MeOH extract, which showed the stronger AR inhibitory activity, was further separated as shown in Chart 2 to give two new diterpenoids, danshenol A (**1**, 0.0015% yield from the dried material) and danshenol B (**2**, 0.0048%), along with dihydrotanshinone I (**3**, 0.0035%), cryptotanshinone (**4**, 0.010%), tanshinone I (**5**, 0.0046%), tanshinone IIA (**6**, 0.13%), (−)-danshexinkun A (**7**, 0.023%), and sugiol (**8**, 0.0042%). The structures of the known diterpenoids **3**—**8** were determined by analyses of their spectral data and comparisons with the literature data.[9−13)]

Structures of Danshenol A (1) and Danshenol B (2) Danshenol A (**1**) was obtained as dark yellow needles, mp 182 °C (MeOH), and showed $[\alpha]_D$ −136.4° ($CHCl_3$). Its molecular formula was determined by high-resolution MS (HR-MS) measurement to be $C_{21}H_{20}O_4$ (*m/z* 336), which was C_3H_6O more than that of dihydrotanshinone I (**3**). The ^1H- and ^{13}C-NMR spectra of **1** were partially similar to those of **3** (Tables 1 and 2), and analyses of the ^1H–^1H and ^1H–^{13}C correlation spectroscopy (COSY) spectra of **1** indicated the presence of the same A-, B-, and D-rings as **3**. In addition, they showed signals due to a methylene (C20-H_2) and a methyl (C22-H_3) group, and the ^{13}C-NMR spectrum of **1** revealed the presence of an oxygen-bearing quaternary carbon (δ_C 79.3) and a low-field shift of two carbonyl carbons (**1**: δ_C 204.8, 196.1; **3**: δ_C 185.3, 175.8). These data suggested that danshenol A was an abietane-type diterpenoid with an additional C3 unit (CH_3–CO–CH_2 grouping) on the C-ring, which was supported by the fact that tris(dipivaloylmethanato)europium $Eu(DPM)_3$[15)] caused a *retro*-aldol reaction to give **3**.

The location of the additional C3 unit was determined by the heteronuclear multiple bond correlation (HMBC) spectrum (Fig. 1, left). The long-range correlations observed in the spectrum, especially that between C-9 and the methylene protons (20-H_2) on the additional C3 unit, indicated that the location of this unit should be at C-11. Thus, the planar structure of danshenol A was concluded to be **1**.

In the difference nuclear Overhauser effect (NOE) experiments, irradiation of 17-H_3 caused the NOE increase at 22-H_3 and *vice versa*, indicating that these methyls were in *cis* relation. On the other hand, dihydrotanshinone I (**3**) prepared through the *retro*-aldol reaction by $Eu(DPM)_3$ showed $[\alpha]_D$ −146.6° ($CHCl_3$, $c=0.09$) (natural **3**, $[\alpha]_D$ −332.8°), indicating that the configuration at C-15 is *R*. Thus the structure of danshenol A, including the absolute stereochemistry, was determined to be **1**.

Danshenol B (**2**), yellow needles, mp 176 °C (MeOH), $[\alpha]_D$ −131.6° ($CHCl_3$), showed the molecular ion at *m/z* 354 and its molecular formula was determined as $C_{22}H_{26}O_4$ by HR-MS measurement. The ^1H-NMR spectrum of **2** was partially similar to that of cryptotanshinone (**4**) (Table 1) and showed the signals of three coupled methylenes (1-H_2, 2-H_2, 3-H_2), two coupled aromatic protons (6-H, 7-H), a CH_3–CH–CH_2–O grouping

Table 1. ^{1}H-NMR Data (δ, J in Hz) for Compounds **1**—**7** (400 MHz) in $CDCl_3$

	1	**3**	**5**	**2**	**4**	**6**	**7**[a)]
1	9.02 d (8.5)	9.28 d (8.5)	9.21 d (9)	3.12 dt (17.5, 5) 3.41 ddd (17.5, 9, 5)	3.20 t (6.5) (2H)	3.18 t (6.5)	9.44 d (8.5)
2	7.51 dd (8.5, 6.5)	7.56 dd (8.5, 7)	7.53 dd (9, 6.5)	1.70 m 1.87 m	1.78 m (2H)	1.80 m	8.43 d (8.5)
3	7.42 d (6.5)	7.39 d (7)	7.33 d (6.5)	1.69 m (2H)	1.65 m (2H)	1.66 m	8.23 d (8.5)
5							
6	8.07 d (8.5)	8.28 d (8.5)	8.27 d (8.5)	7.41 d (8.5)	7.63 d (8.5)	7.63 d (8)	7.62 d (7)
7	7.78 d (8.5)	7.73 d (8.5)	7.30 d (8.5)	7.49 d (8.5)	7.48 d (8.5)	7.53 d (8)	7.48 d (7)
11							
14							
15	3.65 tq (9, 6.5)	3.65 dqd (9.5, 7, 6.5)		3.58 tq (8.5, 7)	3.59 dqd (9.5, 7, 6)		3.49 sextet (6.5)
16	4.39 t (9) 4.98 t (9)	4.43 dd (9.5, 6.5) 4.97 t (9.5)	7.24 s	4.30 t (8.5) 4.90 t (8.5)	4.37 dd (9.5, 6) 4.90 t (9.5)	7.21 q (1.5)	3.84 dd (10.5, 6.5) 3.95 dd (10.5, 6.5)
17	1.45 d (6.5)	1.42 d (7)	2.25 s	1.39 d (7)	1.36 d (7)	2.26 d (1.5)	1.32 dd (6.5, 1.5)
18	2.72 s	2.69 s	2.64 s	1.34 s	1.32 s	1.31 s	2.75 s
19				1.30 s	1.31 s	1.31 s	
20	3.06 d (12.5) 3.28 d (12.5)			2.83 d (12.5) 3.10 d (12.5)			
22	1.98 s			2.06 s			
11-OH	4.68 br s			4.54 br s			

a) Measured in $CDCl_3$–CD_3OD (1 : 1).

Table 2. ^{13}C-NMR Data (δ) for Compounds **1**—**7** (100 MHz) in $CDCl_3$

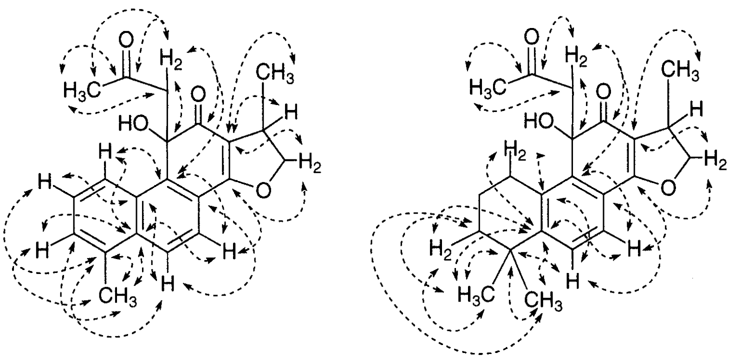
	1	**3**	**5**	**7**[a)]	**2**	**4**	**6**
1	125.8	125.1	124.7	125.0	29.2	29.6	29.9
2	126.7	130.5	130.6	129.5	19.9	19.3	19.1
3	128.5	128.9	128.3	128.6	38.7	37.7	37.8
4	135.2	135.0	135.2	134.8	35.3	34.8	34.7
5	134.9	134.8	132.9	133.0	151.6	143.6	144.1
6	125.5	131.9	133.0	131.3	127.3	132.5	133.3
7	120.1	120.4	118.7	121.6	122.2	122.5	120.8
8	120.4	128.2	118.7	129.8	120.3	128.8	127.4
9	141.3	126.2	153.1	124.4	140.9	126.1	126.5
10	131.0	132.2	129.6	134.6	137.3	152.3	150.1
11	79.3	185.3	183.3	183.5	79.6	184.0	183.6
12	196.1	175.8	207.2	155.4	196.0	175.5	175.7
13	113.6	118.4	120.3	121.7	112.5	118.2	119.9
14	171.3	170.4	162.5	185.5	171.6	170.8	171.7
15	34.5	34.7	122.2	32.0	34.4	34.6	121.4
16	81.4	81.7	142.1	64.3	81.1	81.5	141.1
17	18.0	18.8	8.3	13.9	17.9	18.7	17.9
18	20.1	19.9	19.8	18.9	32.4	31.7	31.2
19					32.6	31.8	31.2
20	57.3				54.8		
21	204.8				205.1		
22	32.1				32.1		

a) Measured in $CDCl_3$–CD_3OD (1 : 1).

($17\text{-}H_3$, $16\text{-}H_2$, 15-H), and two singlet methyls ($18\text{-}H_3$, $19\text{-}H_3$). The signals due to the CH_3–CO–CH_2 grouping in the ^{1}H-NMR spectrum of **1** were also observed. Thus **2** was assumed to have the same modified C-ring as **1**, which was confirmed by the ^{1}H–^{1}H COSY, ^{1}H–^{13}C COSY, and HMBC spectra (Fig. 1, right). The stereochemistry of danshenol B, including the absolute one, was determined to be **2**, based on the facts that in the difference NOE experiment NOEs were observed between $17\text{-}H_3$ and $22\text{-}H_3$ and that $Eu(DPM)_3$ caused a *retro*-aldol reaction to give **4** ($[\alpha]_D$ $-56.8°$, $CHCl_3$, $c=0.09$) (natural **4**, $[\alpha]_D$ $-76.5°$).

Danshenols A (**1**) and B (**2**) have structures believed to be derived from aldol condensation between dihydrotanshinone I (**3**) or cryptotanshinone (**4**) and an acetone molecule. However, they are considered to be natural products even though possible precursors (**3**, **4**) are present in the extract, because we did not use acetone in the isolation procedure, the TLC analysis (AcOEt–benzene = 13 : 87) of the MeOH extract indicated the presence of **1** (*Rf*, 0.24) and **2** (*Rf*, 0.33), and the treatment of dihydrotanshinone I (**3**) with silica gel in refluxing acetone resulted in recovery of **3**. These are the first examples of C_{21} and C_{22} abietane-type diterpenoids having the modified C-ring. Although many abietane-type diterpenoids have been isolated from the root of *S. miltiorhiza*,[7)] they are C_{18} (*e.g.*, **3**, **5**, **7**) or C_{19} (*e.g.*, **2**, **4**, **6**) diterpenoids, except for salvilenone (C_{20}),[16)] tanshinlactone (C_{17}),[17)] and danshexinkun D (C_{21}) (**9**, Fig. 2).[18)] However, the structure of danshexinkun D is very doubtful, because the ^{13}C-NMR data reported for danshexinkun D contains the signals ascribable to a ketone carbonyl (δ_C 204.1, s) and an oxygen-bearing quaternary carbon (δ_C 78.9, s), which are not in accord with the structure formula **9**. These and other ^{13}C-NMR data, and also other spectral data, are very similar to danshenol A (**1**), which would suggest danshexinkun D is the same as danshenol A (**1**), although direct comparison has not been done.

AR Inhibitory Activity The inhibitory activity of **1**—**8** against AR isolated from the eye lens of rats was compared with those of quercetin, a natural AR inhibitor,[19)] and epalrestat, a strong AR inhibitor in clinical use[20)] (Table 3). Plasma comprises about 4% albumin, and in most cases any drug administered in the animal body is bound to albumin or other proteins present in the blood, if they are not selective, and hence their activity is remarkably decreased. Thus we also examined the AR inhibitory activity in the presence of 1% bovine serum albumin (BSA). As can be seen in Table 3, three compounds (**4**, **5**, **8**) inhibit AR (IC_{50} from 4.80 to >10.0 μM) more weakly than quercetin (IC_{50}, 5.60 μM), while five



1 **2**

Fig. 1. Significant Long-Range Correlations Observed in the HMBC Spectra of **1** and **2**

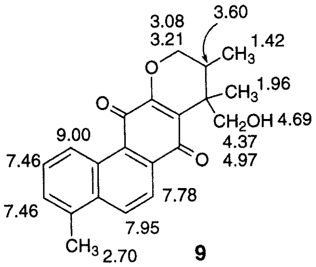


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Fig. 2. Structure and ^{1}H-NMR Data (δ) of Danshexinkun D (**9**)[18] in $CDCl_3$

Table 3. Aldose Reductase Inhibitory Activity (IC_{50}, μM) of Compounds **1**—**8**, Quercetin, and Epalrestat

	Experimental IC_{50} (μM)		Calculated IC_{50}[a] (μM)
	without BSA	with BSA	
1	0.10	29.1	0.014
2	1.75	32.3	0.042
3	1.19	58.1	0.039
4	10.0	67.3	0.10
5	4.80	39.6	0.051
6	1.14	37.8	1.39
7	0.87	38.2	0.24
8	>10[b]	>100[c]	0.043
Quercetin	5.60	39.8	
Epalrestat	0.038	0.86	

a) Calculated from the yield of each compound and the IC_{50} of the MeOH extract (49.0 μg/ml). *b*) Inhibitory rate at 10 μM was 30.2%. *c*) Inhibitory rate at 100 μM was 20.7%.

compounds (**1**, **2**, **3**, **6**, **7**) are stronger inhibitors (IC_{50} from 0.10 to 1.75 μM) than quercetin. Danshenol A (**1**) is the strongest inhibitor with IC_{50} of 0.10 μM which is comparable to that of epalrestat (IC_{50}, 0.038 μM).

Compounds **1**—**7** have varied AR inhibitory activity and yield; for example, danshenol A (**1**) is the strongest inhibitor (IC_{50}, 0.10 μM) but its yield is low (0.0015%), while tanshinone IIA (**6**) is the main compound (0.13%) but its activity is slightly weak (IC_{50}, 1.14 μM). Thus in order to determine which compound contributes to the activity of the MeOH extract, we compared the experimental IC_{50} of each compound with the calculated one (Table 3). The experimental IC_{50} of **6** (1.14 μM) was comparable to the calculated one (1.39 μM), while the experimental ones of **1** (0.10 μM) and **7** (0.87 μM) are several times more than the calculated ones (**1**, 0.014 μM; **7**, 0.24 μM). The experimental and calculated IC_{50} of the other compounds differed by one to two orders of magnitude. It was thus concluded that tanshinone IIA (**6**) is the major active constituent in the MeOH extract and danshenol A (**1**) and (−)-danshexinkun A (**7**) are the minor ones.

Many synthetic and natural AR inhibitors have been reported,[21] and they can be roughly classified into four groups: those containing a carboxylic acid moiety, those containing rigid spirohydantoins or related ring systems, flavonoids, and others.[1] Despite the apparent structural diversity of AR inhibitors, certain common electronic and steric features have become apparent through computer modeling, molecular orbital calculations, and known structure–activity relationships; *i.e.* a generally planar structure with hydrophobic (aromatic) region(s) and a common region susceptible to charge–transfer interaction are required.[22,23] Seven inhibitors obtained from the root of *S. miltiorhiza* contain a naphthalene (**1**, **3**, **5**, **7**) or a tetrahydronaphthalene (**2**, **4**, **6**) ring as the planar hydrophobic aromatic ring(s). They also have ketone carbonyl (**1**, **2**), *ortho*-quinone (**3**, **4**, **5**, **6**), or *para*-quinone (**7**). These groups may be responsible for the region susceptible to charge–transfer interaction.

In the diterpenoids having aromatized A-ring (**1**, **3**, **5**, **7**), the order of inhibitory intensity is **1**, **7**, **3**, and **5**, while in the diterpenoids having saturated A-ring (**2**, **4**, **6**) the order is **6**, **2**, and **4**. The former diterpenoids may have adequate hydrophobicity by the presence of the aromatized A-ring, and thus the electron density at the carbonyl carbon susceptible to charge–transfer interaction may correspond to the strength of the AR inhibition. In the latter diterpenoids, on the other hand, the hydrophobicity may be insufficient, and thus their inhibitory activity is weaker than the corresponding A-ring aromatized compound and may be affected by presence or absence of a hydrophobic group (aromatized D-ring in **6**, 2-oxopropyl group in **2**).

Conclusion

We isolated eight abietane-type diterpenoids, including two new ones named danshenol A (**1**) and danshenol B (**2**), from the root of *S. miltiorhiza*. They showed the AR inhibitory activity dose-dependently and with different intensity, and the activity of **1** was comparable to that of epalrestat, a strong AR inhibitor in clinical use. Though more detailed examinations are needed, "Danshen (丹参)"

or its extract is expected to be effective in the treatment or prevention of diabetic complications such as cataracts, retinopathy, neuropathy, and nephropathy.

Experimental

All melting points were determined with a Kofler-type apparatus and are uncorrected. Optical rotations were measured on a JASCO DIP-4 automatic polarimeter at 25 °C. IR spectra were taken on a Hitachi 260-10 IR spectrophotometer in a KBr disk and UV spectra on a Shimadzu UV 2200 UV-visible spectrophotometer in $CHCl_3$ solutions. NMR spectra were taken on a JEOL GX-400 spectrometer with tetramethylsilane (TMS) as an internal standard, and MS and HR-MS were taken on a JEOL JMS-SX 102A mass spectrometer using a direct inlet system (ionization voltage, 70 eV; accelerating voltage, 5.0 kV).

The root of *S. miltiorhiza* Bunge was supplied by Alps Pharmaceutical Co., Ltd., Furukawa, Japan in 1994. The plant was identified by an expert and a voucher sample was preserved in the Museum for Materia and Medica, Analytical Research Center of Ethnopharmacology in our institute (No. 15481).

Extraction and Isolation Chopped root (8.00 kg) of *S. miltiorhiza* was extracted successively with water (5 l, 80 °C, 3 h, ×4) and MeOH (2.5 l, reflux, 3 h, ×4), and the extract was concentrated to give a water extract (2.10 kg) and a MeOH extract (1.28 kg), respectively. A part of the MeOH extract (600 g) was suspended in water and partitioned with EtOAc to give an EtOAc-soluble (460 g) and an EtOAc-insoluble (78.7 g) fraction, and a part of the EtOAc-soluble fraction (58.7 g) was then separated into a $CHCl_3$-soluble (27.8 g) and a $CHCl_3$-insoluble (29.6 g) fraction. The former fraction was subjected to silica gel column chromatography with a $CHCl_3$–hexane solvent system to give six fractions [fr. A, 10% $CHCl_3$–hexane eluate, 1.65 g; fr. B, 20—30% $CHCl_3$–hexane eluate, 4.76 g; fr. C, 40—50% $CHCl_3$–hexane eluate, 2.80 g; fr. D, 60—70% $CHCl_3$–hexane eluate, 3.32 g; fr. E, 80—90% $CHCl_3$–hexane eluate, 3.12 g; fr. F, $CHCl_3$ eluate, 6.92 g].

Fraction B was subjected to silica gel column chromatography [EtOAc–hexane 20 : 80] to give tanshinone IIA (**6**, 360 mg) and cryptotanshinone (**4**, 18 mg), and frs. C and D gave **6** (280 mg), **4** (13 mg), and danshexinkun A (**7**, 109 mg) and **4** (17 mg), danshenol A (**1**, 7 mg), and dihydrotanshinone I (**3**, 10 mg), respectively, by silica gel column chromatography [EtOAc–hexane 30 : 70] followed by preparative TLC [EtOAc–benzene 5 : 95]. Fractions E and F were separated by a combination of silica gel column chromatography [EtOAc–hexane 40 : 60] and preparative TLC [EtOAc–benzene 25 : 75], and tanshinone I (**5**, 22 mg) and danshenol B (**2**, 23 mg) were obtained from fr. E and **3** (7 mg) and sugiol (**8**, 20 mg) from fr. F.

Danshenol A (1) Dark yellow needles, mp 182 °C (MeOH). $[\alpha]_D$ $-136.4°$ ($CHCl_3$, $c=0.07$). EI-MS m/z: 336 (M^+), 296, 279, 268, 253. HR-MS: 336.1395 ($C_{21}H_{20}O_4$ requires 336.1361). UV λ_{max} nm (log ε): 231 (4.32), 265 (sh), 275 (4.19), 287 (4.04), 299 (3.90), 343 (sh), 356 (3.42), 372 (sh), 403 (3.12). IR ν_{max} cm^{-1}: 3350, 1600, 1380, 1170, 1160, 910. ^1H- and ^{13}C-NMR: Tables 1 and 2.

Danshenol B (2) Yellow needles, mp 176 °C (MeOH). $[\alpha]_D$ $-131.6°$ ($CHCl_3$, $c=0.1$). EI-MS m/z: 354 (M^+), 311, 296, 268, 253. HR-MS: 354.1866 ($C_{22}H_{26}O_4$ requires 354.1831). UV λ_{max} nm (log ε): 214 (4.18), 230 (4.10), 275 (4.06), 287 (3.84), 299 (3.90), 343 (sh), 356 (3.42), 372 (sh), 403 (3.12). IR ν_{max} cm^{-1}: 3350, 1620, 1460, 1260, 1175, 1085, 1020, 790. ^1H- and ^{13}C-NMR: Tables 1 and 2.

$Eu(DPM)_3$ Treatment of Danshenol A (1) and Danshenol B (2) After measurement of ^1H-NMR spectrum, $Eu(DPM)_3$ (10 mg) was added to the $CDCl_3$ solution (0.6 ml) of **1** or **2** (2 mg), and the solution was set at room temperature for overnight. After elimination of the $Eu(DPM)_3$ reagent with short silica gel column, the eluate was evaporated, and the residue was subjected to ^1H-NMR and $[\alpha]_D$ measurements without further purification.

Isolation of Crude Enzyme The crude AR was isolated from the eye lens of ether anesthetized male Wistar rats (eight-week-old, 260 g) by the procedure of Shimizu *et al.*[24] The lenses were collected in a chilled phosphate buffer (pH 6.2) containing NADP (1 mM) and mercaptoethanol (1 mM). The lens was homogenized and centrifuged at 10000 × ***g*** for 15 min. The supernatant was considered to be a crude AR and was preserved at −80 °C until used.

Measurement of AR Inhibitory Activity The frozen enzyme was melted in ice-cold water 2 h before starting the experiment. The assay was performed by the method of Kador and Sharpless,[23] with slight modification. The reaction mixture (1 ml) consisted of a phosphate buffer (970 μl) containing D,L-glyceraldehyde (10 mM) and NADPH (0.1 mM), DMSO or the sample in DMSO (10 μl), and the crude enzyme (20 μl). The reaction was carried out at 25 °C and a time course of 200 s was observed at 340 nm. The experiment was repeated until two concurrent readings were observed.

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References and Notes

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