Phenolic Constituents of Liquorice. VII.[1] A New Chalcone with a Potent Radical Scavenging Activity and Accompanying Phenolics from Liquorice

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Twenty-three phenolics including six new compounds were isolated from a commercial liquorice from North-eastern China. Structures 2—7 were assigned for the new compounds, designated as tetrahydroxymethoxychalcone, isolicopyranocoumarin, glycyrrhiza-flavonol A, and glycyrrhiza-isoflavones A, B and C, respectively. Compound 2 showed the most potent scavenging effect on 1,1-diphenyl-2-picrylhydrazyl radical among the tested polyphenols isolated from liquorice. Air-oxidation of 2 in alkaline dimethylsulfoxide solution gave strong ESR signals, indicating the stability of the radical species formed from 2.

Key words liquorice; *Glycyrrhiza*; Leguminosae; phenolic; flavonoid; radical scavenger

Liquorice (licorice), the underground part of the *Glycyrrhiza* species, is one of the most frequently used natural medicines in traditional medicine prescriptions in Asian countries.[2] Recently, the phenolic constituents of liquorice have been found to exhibit a variety of effects such as antibacterial, antiviral and antioxidant activity,[3-5] and inhibition of oxidative enzyme activity.[6] On the other hand, tannins and related polyphenols from medicinal plants are reported to be effective inhibitors of lipid peroxidation in rat liver mitochondria and microsomes,[7] and a radical scavenging mechanism has been shown to be involved in the inhibition.[8] We previously reported that several liquorice polyphenols[9] as well as tannins[8a,10,11] effectively scavenged the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, frequently used as a model compound for free radicals in lipids. Especially, licochalcone B (**1**),[12] which inhibited production of leukotrienes in arachidonate metabolism,[13] showed the most potent scavenging effect among the tested liquorice phenolics.[9] Further investigation of the phenolic constituents of liquorice led us to isolate twenty-three phenolics including six new compounds, one of which exhibited a scavenging effect on the DPPH radical stronger than licochalcone B. This paper deals with the structural elucidation of the new compounds and the radical-scavenging activity of liquorice phenolics.

Results and Discussion

Since a type of commercial liquorice called Tohoku-kanzo in Japan (Tong-pei liquorice, commercial material from North-eastern China; the source plant was assigned to *Glycyrrhiza uralensis* Fisch. *et* DC).[4,14] has been preferably used in traditional medicine in Japan, this type of commercial liquorice was examined in the present study. Dried liquorice roots were homogenized in aqueous acetone, and the concentrated filtrate from the homogenate was extracted with Et_2O. The Et_2O extract was separated by a combination of droplet counter-current chromatography (DCCC), column chromatography on MCI-gel CHP-20P and preparative thin layer chromatography (TLC), to give six new compounds, **2**—**7**, together with licochalcone B (**1**),[9,12] fisetin (**8**),[15] isoglycycoumarin (**9**),[6a] *p*-hydroxybenzoic acid (**10**),[16] glycyrrhisoflavanone (**11**),[9] semilicoisoflavone B (**12**),[17] glycyrrhisoflavone (**13**),[9] glycycoumarin (**14**),[18] isolicoflavonol (**15**),[18] (3*R*)-vestitol (**16**),[19] licoarylcoumarin (**17**),[6a] isoliquiritigenin (**18**),[20] naringenin (**19**),[19] calycosin (**20**),[21] glicoricone (**21**),[6b] licocoumarone (**22**)[5a] and echinatin (**23**).[20,22]

Structures of New Phenolic Compounds Compound **2** showed a UV spectrum [λ_{max} (MeOH): 208 (log ε 4.43), 258 (3.97), 367 nm (4.27)] similar to those of chalcones such as licochalcone B (**1**).[9,12] Its molecular formula $C_{16}H_{14}O_6$, which is 16 mass units larger than that of **1**, was indicated by elemental analysis and by the FAB-MS which showed an $[M+H]^+$ ion peak at *m/z* 303. The ^{1}H-NMR spectrum of **2** (in acetone-d_6) showed signals of a pair of *trans* olefinic protons [δ 7.66 (1H, d, J=15.5 Hz, C_α-H), 7.93 (1H, d, J=15.5 Hz, C_β-H)], ABX protons of a tri-substituted benzene ring [δ 7.60 (d, J=2 Hz, H-2′), 6.93 (d, J=8.5 Hz, H-5′), 7.58 (dd, J=2, 8.5 Hz, H-6′)] and *ortho*-coupled protons of a tetra-substituted benzene ring [δ 6.70 (1H, d, J=8.5 Hz, H-5), 7.28 (1H, d, J=8.5 Hz, H-6)], along with a 3H singlet of a methoxyl group at δ 3.84. Chemical shifts and coupling constants of the olefinic protons and the protons of the tetra-substituted benzene ring showed a good agreement with those of the corresponding protons of **1**, while **2** showed ABX signals for the A-ring protons instead of the A_2B_2 signals of **1**. These data indicate that **2** has an additional hydroxyl group located at the C-2′ or C-3′ of **1**. Since the chemical shifts of the A-ring protons of **2** were obviously different from those of isoliquiritigenin (**18**) [δ 6.33 (H-3′), 6.42 (H-5′), 6.87 (H-6′)][20] which has two hydroxyl groups at C-2′ and C-4′ (see Chart 1), the hydroxyl groups on the A-ring of **2** were assigned at C-3′ and C-4′.

The assignments of the locations of the hydroxyl groups on the A-ring of **2** were verified by 2-dimensional nuclear Overhauser effect spectroscopy (NOESY) of its permethylate **2a** which was obtained by treatment of **2** with dimethyl sulfate and potassium carbonate. The ^{1}H-NMR spectrum of **2a** (in acetone-d_6) showed signals due to five methoxyl groups [δ 3.83 (3H, s), 3.90 (3H, s), 3.91 (6H, s), 3.93 (3H, s)], B-ring protons [δ 6.88, 7.62 (1H each, d,

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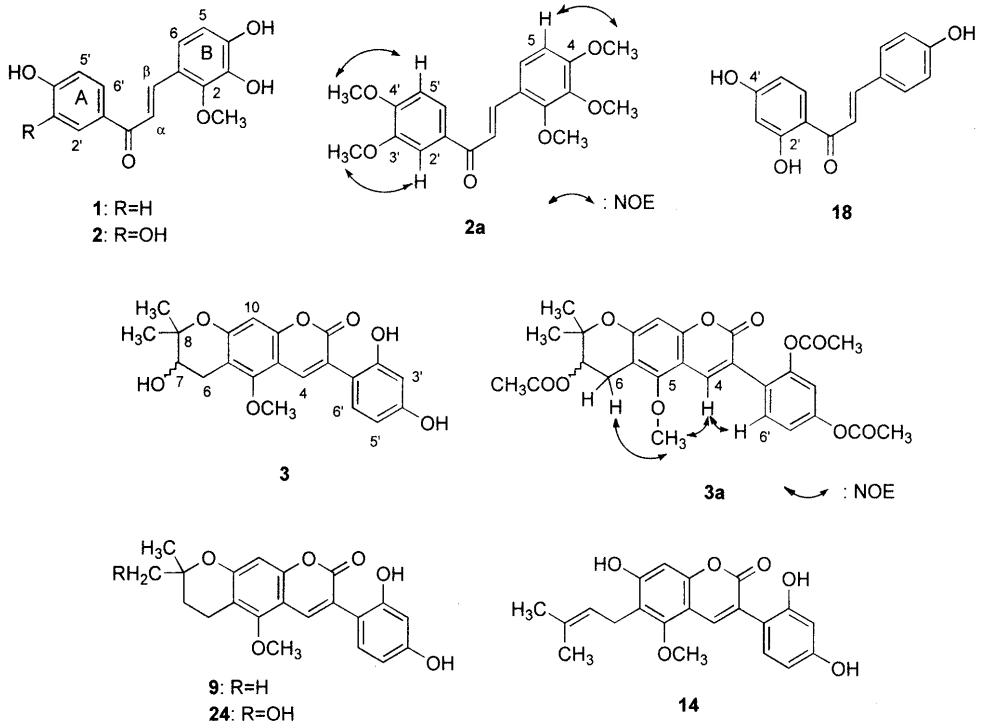


Chart 1

$J=9$ Hz)], A-ring protons [δ 7.07 (d, $J=8$ Hz), 7.64 (d, $J=2$ Hz), 7.80 (dd, $J=2$, 8 Hz)] and *trans*-olefin protons [δ 7.76, 7.98 (1H each, d, $J=16$ Hz)]. Since the 3H singlets at δ 3.83 and 3.93 did not show cross peaks with aromatic protons in the NOESY spectrum, these signals were assigned to the methoxyl groups at C-2 (or C-3) and C-3 (or C-2). The 6H singlet at δ 3.91 was attributed to the methoxyl groups at C-4 and C-4′ based on the cross peaks with an *ortho*-coupled doublet at δ 6.88 (H-5) and with a doublet of $J=8$ Hz at δ 7.07 (H-5′). The remaining 3H singlet at δ 3.90 was ascribed to the methoxyl group at C-3′ based on a cross peak with a doublet of $J=2$ Hz at δ 7.64 (H-2′) (see formula **2a**). If the 2′- and 4′-substituted structure is assumed for the A-ring, this assumption would require NOE between the methoxyl group at C-4′ and H-3′, and that between the methoxyl group and H-5′ which must appear as a double-doublet. However, the methoxyl signal at δ 3.91 showed neither a cross peak due to NOE with the doublet of $J=2$ Hz at δ 7.64 nor that with the double-doublet at δ 7.80 in the NOESY spectrum. The 3′- and 4′-substituted structure was thus assigned for the A-ring of **2**.

The location of the methoxyl group at C-2 in **2** was shown by the electron-impact mass spectrum (EI-MS) of **2**, which had an $[M-31]^+$ ion peak (at *m/z* 271), characteristic of chalcones with a methoxyl group at C-2.[12] Hence, structure **2**, a tetrahydroxymethoxychalcone, was assigned for this compound. The one-bond and long-range ^1H–^{13}C correlation spectroscopy (COSY) measurements showed correlations summarized in Table 1, which satisfied the assigned structure.

Isolicopyranocoumarin (**3**), $[\alpha]_D$ 0°, was obtained as pale-yellow needles. The FAB-MS showed an $[M+H]^+$ ion peak at *m/z* 385 corresponding to the molecular formula $C_{21}H_{20}O_7$, isomeric to licopyranocoumarin

Table 1. One-bond and Long-range ^1H–^{13}C Correlations for **2**[a]

Carbon	δ_C	Proton coupled *via* one bond (δ_H)	Proton coupled *via* 2 or 3 bonds[b]
B-ring			
C-1	121.4		C_α-H, H-5
C-2	149.1		OCH_3, H-6
C-3	139.1		H-5
C-4	149.6		H-6
C-5	112.4	6.70	
C-6	119.9	7.28	
α,β-Unsaturated ketone			
C-α	120.6	7.66	
C-β	138.9	7.93	
C=O	188.2		C_α-H, C_β-H
A-ring			
C-1′	132.1		C_α-H, H-5′
C-2′	116.1	7.60	
C-3′	145.9		H-5′
C-4′	150.7		
C-5′	115.7	6.93	
C-6′	122.8	7.58	H-2′
OCH_3	61.6	3.84	

a) The spectra were recorded at ambient temperature in acetone-d_6. *b*) The average J_{CH} value for long-range couplings was set at 8 Hz.

(**24**).[6a] The UV spectrum of **3** [λ_{max} (MeOH): 210 (log ε 4.48), 252 (3.70), 353 nm (4.12)] was similar to those of 3-arylcoumarins, such as **9**,[6a] **14**[6a,18] and **24**. The ^1H-NMR spectrum of **3** (200 MHz, in acetone-d_6) showed two 1H singlets [δ 7.97 (H-4), 6.52 (H-10)] and three protons forming an ABX system [δ 6.47 (d, $J=2$ Hz, H-3′), 6.43 (dd, $J=2$, 8.5 Hz, H-5′), 7.21 (d, $J=8.5$ Hz, H-6′)]. These signals are due to protons on the 3-arylcoumarin skeleton. The spectrum also showed a methoxyl signal [δ 3.91 (3H, s)], and signals attributable to a C_5 unit forming a hydroxydihydropyrane structure {δ 1.32, 1.38

(3H each, s) ($2\times CH_3$), δ 2.76 (1H, dd, $J=8$, 17 Hz, H-6a), 3.08 (1H, dd, $J=5$, 17 Hz, H-6b), 3.87 (1H, m, H-7), 4.42 (1H, d, $J=5$ Hz, OH at C-7) [$-CH_2-CH(OH)-$]}, which is structurally related to the 3-methyl-2-butenyl (prenyl) residue. The EI-MS, which showed an $[M-71]^+$ ion peak at m/z 313 in the EI-MS, substantiated the presence of the hydroxydihydropyran structure.[23]

Chemical shifts of the protons on the 3-arylcoumarin skeleton and the methoxyl group are closely similar to those of **9**,[6a] indicating that the substitution pattern of the hydroxyl, methoxyl and alkyl (C_5) groups on the 3-arylcoumarin skeleton of **3** is the same as that of **9**. Structure **3** was therefore assigned as isolicopyranocoumarin.

Treatment of **3** with acetic anhydride and pyridine afforded a triacetate (**3a**). The NOESY spectrum of **3a** showed a correlation between the methoxyl signal and H-4 which showed NOE with H-6′, substantiating the location of the methoxyl group at C-5. The NOESY spectrum also showed a correlation between the methoxyl signal and one of the methylene protons, confirming the location (C-7) of the hydroxyl group on the dihydropyran residue (see formula **3a** in Chart 2).

Table 2. One-bond and Long-range $^1H-^{13}C$ Correlations for **3**[a]

Carbon	δ_C	Proton coupled *via* one bond (δ_H)	Proton coupled *via* 2 or 3 bonds[b]
C-2	160.0		
C-3	121.3		
C-4	136.0	7.83	
C-4a	107.1		H-10
C-5	155.3		
C-5a	111.1		H-6
C-6	25.9	—[c]	
C-7	67.1	3.86	CH_3 (δ 1.30)
C-8	78.5		
C-10	99.2	6.56	
C-10a	153.1		H-4
C-1′	113.5		H-4, H-3′, OH at C-2′
C-3′	102.9	6.36	
C-5′	106.4	6.27	H-3′
C-6′	131.7	7.10	
CH_3 at C-8	21.1	1.24	
	25.5	1.30	
OCH_3	62.1	3.82	
C-2′, C-4′, C-9a	156.2, 156.6, 158.6		

a) The spectra were recorded at 30°C. Dimethylsulfoxide-d_6 was used as the solvent because of the low solubility of this compound in acetone. b) The average J_{CH} value for long-range couplings was set at 7 Hz. c) Methylene protons at C-6 (δ 2.62 and δ 2.93) did not show cross peaks with C-6.

Glycyrrhiza-flavonol A (**4**), $[\alpha]_D$ 0°, was obtained as pale-yellow needles. The EI-MS showed a molecular ion peak at m/z 370 corresponding to the molecular formula $C_{20}H_{18}O_7$, and a $[M-71]^+$ ion peak at m/z 299 characteristic of flavonoids with a hydroxydihydropyran moiety. The UV spectrum of **4** [λ_{max} (MeOH): 226 (sh), 250 (log ε 4.34), 267 (4.31), 367 nm (4.41)] suggested a flavonol structure.[24] The ^{1}H-NMR spectrum of **4** (in acetone-d_6+D_2O) showed signals due to the A-ring [δ 6.25 (1H, d, $J=2$ Hz, H-6), 6.52 (1H, d, $J=2$ Hz, H-8)] and B-ring [δ 7.99 (2H, br m, H-2′, H-6′), 6.87 (1H, d, $J=9.5$ Hz, H-5′)] protons of the flavonol skeleton. The spectrum also showed signals of two methyl groups [δ 1.28, 1.37 (3H each, s)] and a $-CH_2-CH(OH)-$ system [δ 2.82 (1H, dd, $J=7.5$, 16.5 Hz, H-1″a), 3.11 (in part overlapped by the HDO signal, H-1″b), 3.84 (1H, dd, $J=5$, 7.5 Hz, H-2″)] of the C_5 unit forming the dihydropyran structure.

Treatment of isolicoflavonol (**15**)[18] with concentrated HCl induced formation of an ether bond between the prenyl group at C-3′ and the hydroxyl group at C-4′, to give the cyclization product, **25**. Chemical shifts of the protons on the flavonol skeleton in the ^{1}H-NMR spectrum of **4** were very similar to the corresponding protons of **25** (see Experimental), in spite of the differences in the signal pattern of the dihydropyran moiety. On the other hand, the ^{13}C-NMR spectrum of **4** showed a marked downfield shift of C-2″ [δ 69.6 (**4**)←δ 32.3 (**25**)], accompanied by downfield shifts of C-1″ [δ 32.2 (**4**)←δ 22.3 (**25**)] and C-3″ [δ 78.8 (**4**)←δ 75.0 (**25**)], relative to the corresponding carbons of **25**. Therefore, structure **4**, where a hydroxyl group was substituted on C-2″ of the dihydropyran moiety of **25**, was assigned as glycyrrhiza-flavonol A.

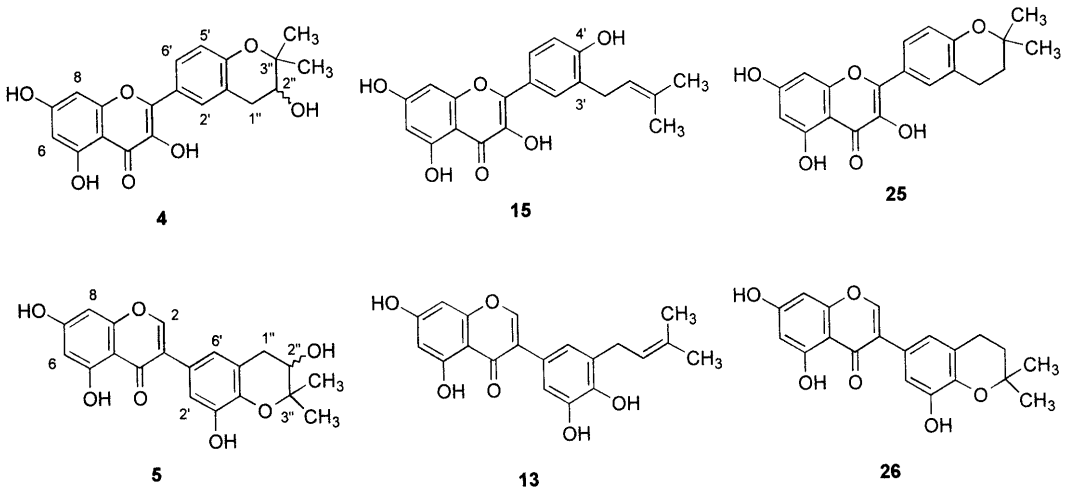


Chart 2

Glycyrrhiza-isoflavone A (**5**), $C_{20}H_{18}O_7$, $[\alpha]_D$ 0°, showed a molecular ion peak at *m/z* 370 in the EI-MS. The spectrum also showed a characteristic $[M-71]^+$ ion peak at *m/z* 299, indicating the presence of a hydroxydihydropyran moiety. The UV spectrum of **5** [λ_{max} (MeOH): 209 (log ε 4.60), 264 (4.32), 286 nm (sh)] was similar to those of isoflavones such as glycyrrhisoflavone (**13**).[9] The ^{1}H-NMR spectrum of **5** (in acetone-d_6 + D_2O) indicated the presence of the isoflavone skeleton [δ 8.14 (1H, s, H-2), 6.27 (1H, d, *J*=2.5 Hz, H-6), 6.40 (1H, d, *J*=2.5 Hz, H-8), 6.92 (d, *J*=2 Hz, H-2′), 6.81 (d, *J*=2 Hz, H-6′)] and a C_5 unit forming the dihydropyran structure {δ 1.21, 1.34 (3H each, s) (2×CH_3), 2.74 (dd, *J*=8, 17 Hz, H-1″a), 3.02 (dd, *J*=5, 17 Hz, H-1″b), 3.83 (dd, *J*=5, 8 Hz, H-2″) [–CH_2–CH(OH)–]}.

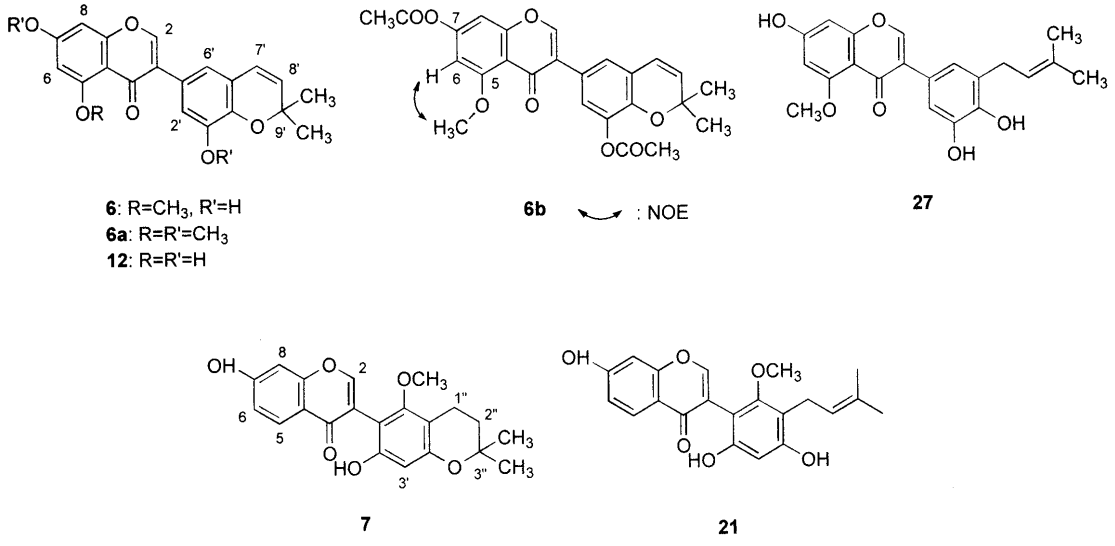
Cyclization of the prenyl group of **13** by treatment with HCl gave cyclo-glycyrrhisoflavone (**26**). Chemical shifts of the protons on the isoflavone skeleton in the ^{1}H-NMR spectrum of **5** were very similar to those of the corresponding protons of **26** (see Experimental), indicating that the substitution patterns on the isoflavone skeletons of the two compounds are the same. On the other hand, the ^{13}C-NMR spectrum of **5** showed a distinctive downfield shift of C-2″ [δ 70.0 (**5**)←δ 32.7 (**26**)], along with downfield shifts of C-1″ [δ 32.1 (**5**)←δ 22.0 (**26**)] and C-3″ [78.7 (**5**)←δ 74.9 (**26**)], relative to the corresponding carbons of **26**, implying location of the hydroxyl group at C-2″. Hence, the structure of glycyrrhiza-isoflavone A was assigned as **5**.

Glycyrrhiza-isoflavone B (**6**) was obtained as colorless needles. The EI-MS showed a molecular ion peak at *m/z* 366, corresponding to the molecular formula $C_{21}H_{18}O_6$. The UV spectrum [λ_{max} (MeOH): 209 (log ε 4.47), 258 (4.52), 282 nm (sh)] suggested an isoflavone skeleton such as that of glisoflavone (**27**).[6a] The ^{1}H-NMR spectrum of **6** (in acetone-d_6) showed signals due to a methoxyl group [δ 3.84 (3H, s)], two methyl groups [δ 1.42 (6H, s)] and a pair of *cis*-olefinic protons [δ 6.40 (1H, d, *J*=10 Hz, H-7′), 5.73 (1H, d, *J*=10 Hz, H-8′)], along with proton signals on the isoflavone skeleton [δ 7.93 (1H, s, H-2), 6.43 (2H, br s, H-6, H-8), 6.95 (1H, d, *J*=2 Hz, H-2′), 6.77 (1H, d, *J*=2 Hz, H-6′)]. Chemical shifts and the coupling pattern of the B-ring protons (H-2′ and H-6′), the olefinic proton signals and the 6H singlet of the two methyl groups are very similar to those of the corresponding protons of semilicoisoflavone B (**12**)[17] where the B-ring moiety of the isoflavone skeleton and the C_5 unit form a benzopyran structure. Treatment of **6** and **12** with diazomethane afforded the same permethyl ether (**6a**).

On the other hand, chemical shifts of the A-ring protons (H-6, H-8) and the methoxyl signal of **6** were comparable with those of the corresponding protons of **27**. The location of the methoxyl group in **6** was therefore assigned to be C-5. Treatment of **6** with acetic anhydride and pyridine gave diacetate **6b**. The methoxyl signal of **6b** in the NOESY spectrum showed only a cross peak with one of the A-ring protons. If the methoxyl group is assumed to be at C-7, the methoxyl protons could show both of the two A-ring protons. The location C-5 of the methoxyl group C-5 was thus confirmed. Based on these data, structure **6** was assigned as glycyrrhiza-isoflavone B.

Glycyrrhiza-isoflavone C (**7**) was obtained as colorless needles. The molecular ion peak at *m/z* 368 in the EI-MS indicated the molecular formula $C_{21}H_{20}O_6$ for this compound. The UV spectrum [λ_{max} (MeOH): 211 (log ε 4.69), 248 (4.45), 288 (4.18), 302 nm (sh)] was similar to that of an isoflavone, glicoricone (**21**).[6b] The ^{1}H-NMR spectrum of **7** (in acetone-d_6 + D_2O) showed signals due to the protons on the isoflavone skeleton [δ 8.06 (1H, s, H-2), 8.05 (1H, d, *J*=8.5 Hz, H-5), 7.02 (1H, br d, *J*=8.5 Hz, H-6), 6.94 (1H, br s, H-8), 6.15 (1H, s, H-3′)], along with those of a methoxyl group [3.48 (3H, s)] and a C_5 unit forming a dihydropyrane structure [δ 2.67 (2H, t, *J*=7 Hz, H-1″), 1.77 (2H, t, *J*=7 Hz, H-2″) (–CH_2–CH_2–), 1.30 (6H, s, 2×CH_3)]. This compound was regarded as being identical with the product obtained by treatment of **21** with concentrated HCl, based on comparison of their ^{1}H-NMR data. Hence, the structure of glycyrrhiza-isoflavone C was formulated as **7**.

Radical Scavenging Effect of Liquorice Phenolics The scavenging effects of the liquorice phenolics on the DPPH



6: R=CH_3, R'=H
6a: R=R'=CH_3
12: R=R'=H

6b : NOE

27

7

21

Chart 3

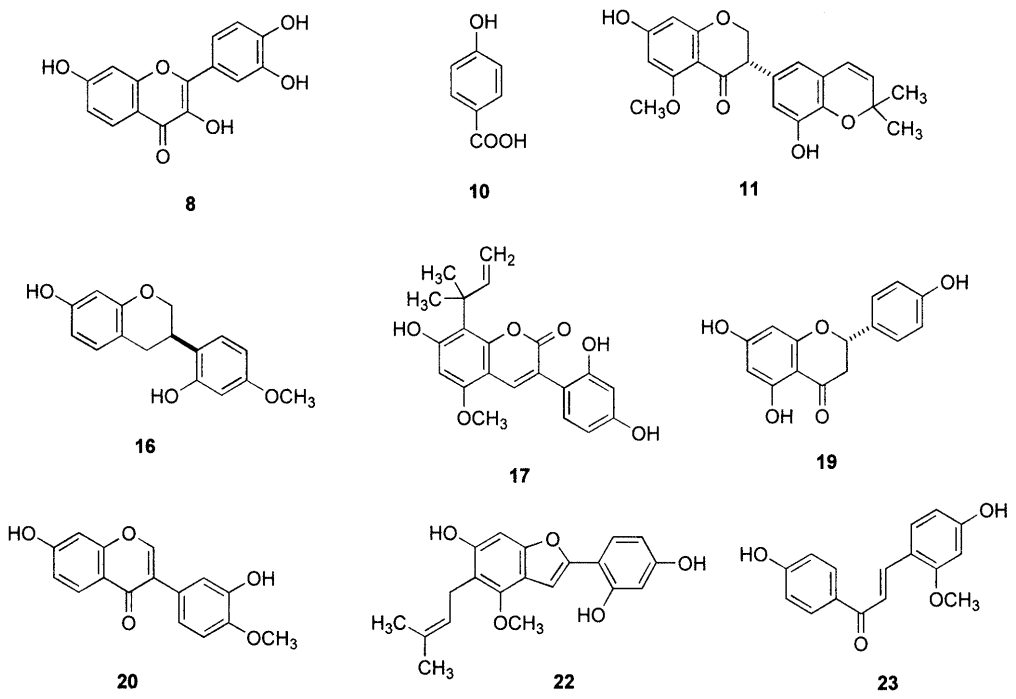


Chart 4

Table 3. Scavenging Effects of Liquorice Phenolics on the 1,1-Diphenyl-2-picrylhydrazyl Radical

Compounds	EC_{50} (M)[a]	Number of phenolic hydroxyl groups
Chalcone		
Tetrahydroxymethoxy-chalcone (**2**)	1.4×10^{-5}	4
3-Arylcoumarin		
Isolicopyranocoumarin (**3**)	$> 1.0 \times 10^{-3}$	2
Licoarylcoumarin (**17**)	6.1×10^{-5}	3
2-Arylcoumarone		
Licocoumarone (**22**)	4.1×10^{-5}	3
Flavonol		
Glycyrrhiza-flavonol A (**4**)	8.7×10^{-5}	2
	3.7×10^{-5}	3
Isoflavone		
Glycyrrhiza-isoflavone A (**6**)	$> 1.0 \times 10^{-3}$	3
Glicoricone (**21**)	$> 1.0 \times 10^{-3}$	3

a) Concentration required for a 50% reduction in the absorbance of the diphenylpicrylhydrazyl radical at 520 nm in MeOH.

radical were estimated colorimetrically,[8a,9,10] and the results are summarized in Table 3. Tetrahydroxymethoxychalcone **2** showed the most potent activity among the liquorice polyphenols examined (including those tested previously).[9]

Previously, we reported that the radical-scavenging activity of tannins depends largely on the number of hydroxyl groups they possess.[11] Liquorice polyphenols which have four [tetrahydroxymethoxychalcone (**2**), 1.4×10^{-5} M; glycyrrhisoflavone (**13**),[9] 3.8×10^{-5} M] and three phenolic hydroxyl groups [licochalcone B (**1**),[9] 2.2×10^{-5} M; glycycoumarin (**14**),[9] 4.1×10^{-5} M; licoarylcoumarin (**17**), 6.1×10^{-5} M; licocoumarone (**22**), 4.1×10^{-5} M; fisetin (**8**), 3.7×10^{-5} M; isolicoflavonol (**15**),[9] 4.0×10^{-5} M] generally showed more potent scavenging effects than the other compounds. However, glicoricone (**21**), glycyrrhiza-isoflavone A (**5**) and isoliquiritigenin (**18**),[9] all of which have three phenolic hydroxyl groups, showed practically no scavenging activity. Participation of one of the hydroxyl groups in a hydrogen bond (**5** and **18**), and steric hindrance (for **21**) of the reaction with DPPH may affect the activity of these compounds.

The strong activity of **2** was also attributable to the formation of stable radicals when **2** donates a hydrogen of the phenolic hydroxyl groups to the radical species, owing to the presence of two *ortho*-diphenol structures and delocalization of electrons in **2**.

Since **1** and **2** showed strong scavenging activity, electron spin resonance (ESR) spectra of their solutions in aqueous alkaline dimethylsulfoxide (DMSO) were recorded, in order to clarify whether these compounds can form stable radicals.[8,25] Air-oxidation of the two compounds in alkaline solution gave strong ESR signals, indicating the stability of the radical species formed.

When aqueous KOH (0.05 M) was added to the solution of licochalcone B (**1**) in DMSO [0.05 M KOH (20%)–DMSO (80%)], an intense ESR signal due to a radical from **1** was observed (Fig. 1a). The hyperfine splitting (hfs) constants of the signal, 0.29, 0.18, 0.10 and 0.08 mT, were respectively ascribed to four protons on the B-ring and the α,β-unsaturated ketone. The radical on this compound was therefore regarded as being delocalized in this region.

Compound **2** also showed an ESR signal in the presence of 10% aqueous alkali, whose hyperfine splitting pattern (Fig. 1b) was similar to that due to the radical from **1**. However, in the presence of higher concentrations of alkali (30—40% 0.05 M KOH), the spectra were dominated by another ESR signal with higher intensity (Fig. 1c). The hfs constants of the latter signal, 0.44, 0.08, 0.03 and 0.03 mT, were obviously different from those for the radical from **1**, although this hfs pattern was also ascribable to

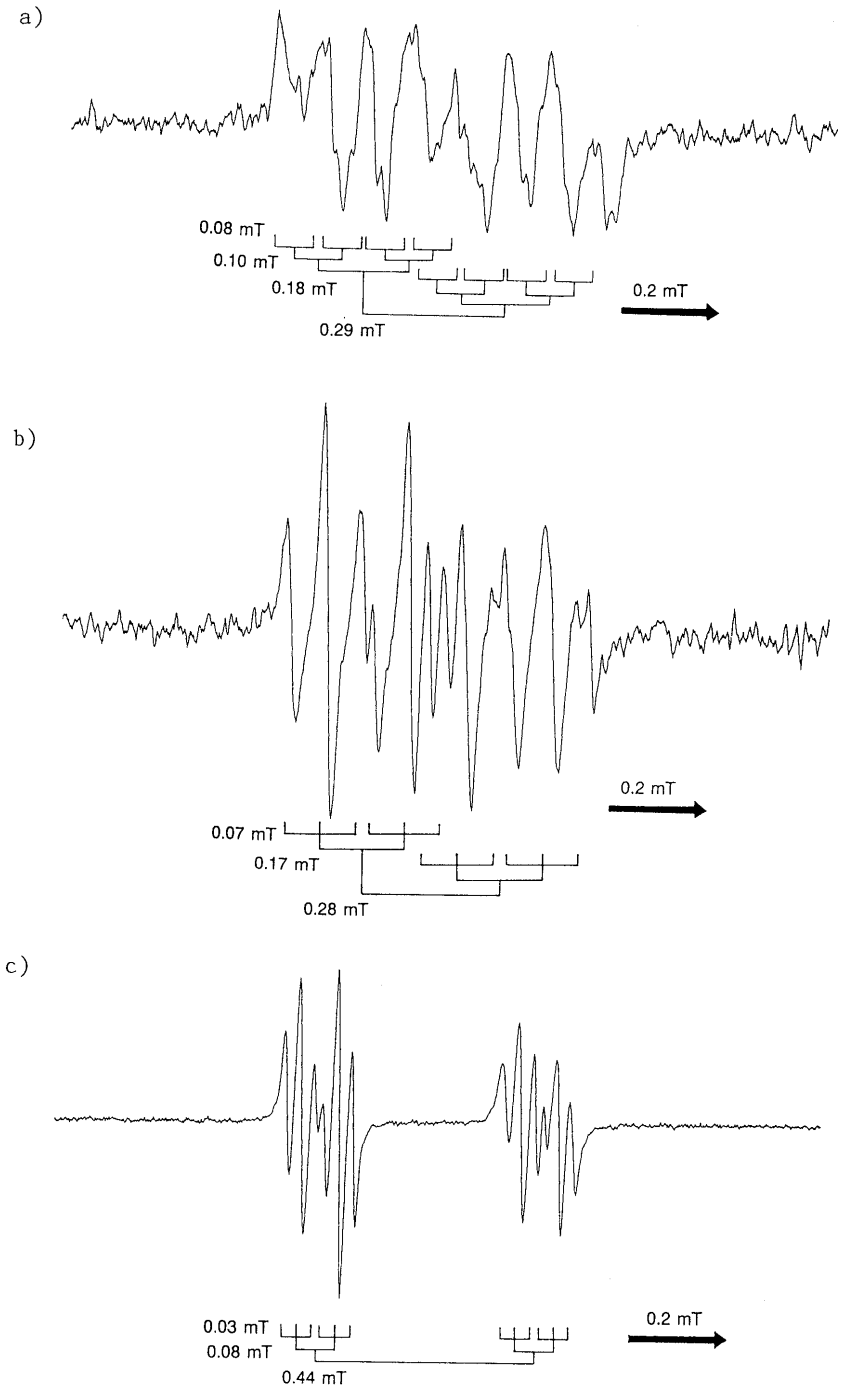


Fig. 1. ESR Signals of Solutions of Liquorice Phenolics in Aq. Alkaline DMSO

a) Licochalcone B (**1**) in 0.05 M KOH–DMSO (2:8). Modulation width, 0.005 mT; time constant, 0.03 s; spectrum accumulation, 8 times; amplitude, 400. b) Tetrahydroxymethoxychalcone (**2**) in 0.05 M KOH–DMSO (1:9). Modulation width, 0.005 mT; time constant, 0.03 s; spectrum accumulation, 8 times; amplitude, 500. c) Compound **2** in 0.05 M KOH–DMSO (4:6). Modulation width, 0.0025 mT; time constant, 0.01 s; spectrum accumulation, 4 times; amplitude, 50.

the protons on the B-ring and the α,β-unsaturated ketone moiety. On the other hand, licochalcone B (**1**) did not show signals due to a different splitting pattern with higher intensity in the presence of higher concentrations of alkali (30%). Therefore, the A-ring with the two phenolic hydroxyl groups in **2** was considered to help stabilize the radical shown in Fig. 1c. The change in the hfs pattern observed for **2**, caused by changes in the concentration of alkali, is ascribable to a difference in the conformation of the radical species involving rotation around the bonds[25] between the two aromatic rings, accompanied by changes in the conjugation system in the molecule.

Experimental

UV spectra were recorded on a Shimadzu UV-180 spectrometer, and optical rotations on a JASCO DIP-1000 polarimeter. ^{1}H- and ^{13}C-NMR spectra were recorded on a Varian VXR 500 instrument (500 MHz for ^{1}H, and 126 MHz for ^{13}C) in acetone-d_6 or acetone-d_6 containing D_2O (*ca.* 3%), unless mentioned otherwise. Chemical shifts are given in δ values (ppm), based on the signals of the solvents [δ_H 2.04 and δ_C 29.8 (acetone-d_6); δ_H 2.49 and δ_C 39.7 (DMSO-d_6)]. FAB-MS and EI-MS were recorded on a VG-70SE mass spectrometer. 3-Nitrobenzyl alcohol was used as the matrix agent for the FAB-MS. Electrospray ioniza-

tion mass spectra (ESI-MS) were recorded on a Micromass Autospec OA-Tof mass spectrometer, with 50% aq. MeOH containing 0.1% ammonium acetate as a solvent. Droplet countercurrent chromatography (DCCC) was performed on an apparatus consisting of 95 glass tubes (3.2 mm i.d. × 1.2 m). $CHCl_3$–MeOH–*n*-PrOH–H_2O (45 : 60 : 10 : 40) was used as the solvent system. Kieselgel 60 PF_{254} (Merck) was used for preparative TLC. Reversed-phase HPLC was performed on a YMC PACK ODS-A304 column (4 mm i.d. × 25 cm) with solvent systems, MeCN–H_2O–AcOH (40 : 5 : 55), MeCN–H_2O–AcOH (25 : 5 : 70) or MeOH–H_2O (2 : 1) at 40 °C in an oven. Detection was by UV absorption at 254 nm or at 280 nm, and the flow rate was set at 1.0 ml/min.

Isolation of Phenolic Constituents from Liquorice Tohoku kanzo (liquorice from North-eastern China) (500 g), purchased from Tochimoto-tenkai-do, Osaka, Japan, was homogenized in aqueous acetone (2.8 l × 3), and the concentrated filtrate from the homogenate (1.8 l) was extracted with Et_2O (1.2 l × 3), EtOAc (1.2 l × 3) and *n*-BuOH (1.2 l × 3), successively. The Et_2O extract (15 g) was subjected to DCCC (descending method). Fractions were further purified by DCCC, column chromatography on MCI-gel CHP-20P with aq. MeOH, preparative TLC with $CHCl_3$–acetone–HCOOH (85 : 12 : 3) and/or preparative HPLC with MeCN–H_2O–AcOH (40 : 5 : 55 or 25 : 5 : 70), to give licochalcone B (**1**, 39.2 mg), fisetin (**8**, 7.0 mg), isoglycycoumarin (**9**, 0.8 mg), *p*-hydroxybenzoic acid (**10**, 3.9 mg), tetrahydroxymethoxychalcone (**2**, 37.1 mg), isolicopyranocoumarin (**3**, 79.4 mg), glycyrrhiza-isoflavone A (**4**, 9.1 mg), glycyrrhisoflavanone (**11**, 1.3 mg), glycyrrhiza-isoflavone B (**5**, 4.0 mg), glycyrrhiza-flavonol A (**6**, 10.0 mg), semilicoisoflavone B (**12**, 18.1 mg), glycyrrhisoflavone (**13**, 4.1 mg), glycycoumarin (**14**, 118.5 mg), isolicoflavonol (**15**, 8.4 mg), (3*R*)-vestitol (**16**, 7.8 mg), licoarylcoumarin (**17**, 13.1 mg), isoliquiritigenin (**18**, 0.8 mg), glycyrrhiza-isoflavone C (**7**, 6.3 mg), naringenin (**19**, 4.0 mg), calycosin (**20**, 2.6 mg), glicoricone (**21**, 5.0 mg), licocoumarone (**22**, 18.2 mg) and echinatin (**23**, 2.1 mg).

Tetrahydroxymethoxychalcone (2) Yellow needles, mp 196 °C. *Anal.* Calcd for $C_{16}H_{14}O_6 \cdot H_2O$: C, 60.00; H, 5.00. Found: C, 60.43; H, 5.24. EI-MS *m/z*: 302 ($[M]^+$), 271 ($[M-OCH_3]^+$), 137, 109. FAB-MS *m/z*: 303 ($[M+H]^+$). ^{1}H-NMR: see text. ^{13}C-NMR: see Table 1.

Methylation of 2 A mixture of **2** (2 mg), dimethyl sulfate (5 μl) and potassium carbonate (5 mg) in acetone (1 ml) was stirred at room temperature overnight, and then refluxed for 2 h. After removal of insoluble material by centrifugation, the supernatant was evaporated and the residue was subjected to preparative HPLC with MeOH–H_2O (3 : 2) to give **2a** (0.2 mg), high-resolution EI-MS *m/z*: 358.1464 ($[M]^+$; Calcd for $C_{20}H_{22}O_6$, 358.1416). ^{1}H-NMR δ: 3.83 (3H, s, OCH_3), 3.90 (3H, s, OCH_3 at C-3′), 3.91 (6H, s, OCH_3 at C-4, C-4′), 3.93 (3H, s, OCH_3 at C-3), 6.88 (1H, d, *J* = 9 Hz, H-5), 7.07 (1H, d, *J* = 8 Hz, H-5′), 7.62 (1H, d, *J* = 9 Hz, H-6), 7.64 (1H, d, *J* = 2 Hz, H-2′), 7.76 (1H, d, *J* = 16 Hz, C_α-H), 7.80 (1H, dd, *J* = 2, 8 Hz, H-6′), 7.98 (1H, d, *J* = 16 Hz, C_β-H).

Isolicopyranocoumarin (3) Pale-yellow needles, mp 251 °C. $[\alpha]_D$ 0° (*c* = 1, MeOH). *Anal.* Calcd for $C_{21}H_{20}O_7$: C, 65.62; H, 5.24. Found: C, 65.25; H, 5.54. EI-MS *m/z*: 384 ($[M]^+$), 313 ($[M-71]^+$). FAB-MS *m/z*: 385 ($[M+H]^+$). ^{1}H-NMR: see text. ^{13}C-NMR: see Table 2.

Compound **3** was treated with acetic anhydride and pyridine in the usual way to give a triacetate (**3a**), colorless needles, mp 162 °C. ^{1}H-NMR δ: 1.38, 1.39 (3H each, s, 2 × CH_3), 2.02, 2.13, 2.80 (3H each, s, 3 × $OCOCH_3$), 2.91 (1H, dd, *J* = 5, 17.5 Hz, H-6a), 3.18 (1H, dd, *J* = 5, 17.5 Hz, H-6b), 3.92 (3H, s, OCH_3), 5.11 (1H, t, *J* = 5 Hz, H-7), 6.59 (1H, s; H-10), 7.08 (1H, d, *J* = 2 Hz, H-3′), 7.11 (1H, dd, *J* = 2, 8 Hz, H-5′), 7.55 (1H, d, *J* = 8 Hz, H-6′), 7.94 (1H, br s, H-4).

Glycyrrhiza-flavonol A (4) Yellow needles, mp 163 °C. $[\alpha]_D$ 0° (*c* = 1, MeOH). High-resolution EI-MS *m/z*: 370.1004 ($[M]^+$; Calcd for $C_{20}H_{18}O_7$, 370.1052). EI-MS *m/z*: 370 ($[M]^+$), 299 ($[M-71]^+$). ^{1}H-NMR: see text. ^{13}C-NMR δ: 21.1, 26.1 (CH_3), 32.2 (C-1″), 69.6 (C-2″), 78.8 (C-3″), 94.6 (C-8), 99.2 (C-6), 104.1 (C-4a), 117.8 (C-5′), 121.5 (C-3′), 123.8 (C-1′), 128.1 (C-6′), 130.6 (C-2′), 136.8 (C-3), 146.9 (C-2), 156.1 (C-8a), 157.9 (C-4′), 162.3 (C-5), 165.2 (C-7), 174.6 (C-4).

Treatment of Isolicoflavonol (15) with HCl Isolicoflavonol (**15**) (10.3 mg) in MeOH (0.70 ml) was treated with concentrated HCl (0.70 ml) at room temperature for 36 h to give **25** (4.0 mg), yellow needles, mp 143 °C. UV λ_{max} (MeOH) nm (log ε): 206 (4.44), 228 (sh), 251 (4.10), 267 (4.11), 371 (4.09). ^{1}H-NMR δ: 1.35 (6H, s, 2 × CH_3), 1.88 (2H, t, *J* = 7 Hz, H-2″), 2.88 (2H, t, *J* = 7 Hz, H-1″), 6.26 (1H, d, *J* = 2 Hz, H-6), 6.53 (1H, d, *J* = 2 Hz, H-8), 6.86 (1H, d, *J* = 8 Hz, H-5′), 8.02 (2H, br m, H-2′, H-6′). ^{13}C-NMR δ: 22.3 (C-1″), 26.3 (CH_3), 32.3 (C-2″), 75.0 (C-3″), 93.7 (C-8), 98.3 (C-6), 103.4 (C-4a), 117.3 (C-5′), 121.3 (C-3′), 122.5 (C-1′), 127.2 (C-6′), 129.5 (C-2′), 135.9 (C-3), 146.2 (C-2), 156.1 (C-8a), 157.0 (C-4′), 161.5 (C-5), 164.1 (C-7), 175.7 (C-4).

Glycyrrhiza-isoflavone A (5) Colorless needles, mp 141 °C. $[\alpha]_D$ 0° (*c* = 1, MeOH). High-resolution EI-MS *m/z*: 370.1036 ($[M]^+$; Calcd for $C_{20}H_{18}O_7$, 370.1052). EI-MS *m/z*: 370 ($[M]^+$), 299 ($[M-71]^+$). ^{1}H-NMR: see text. ^{13}C-NMR δ: 20.7, 25.9 (2 × CH_3), 32.1 (C-1″), 70.0 (C-2″), 78.7 (C-3″), 94.5 (C-8), 99.8 (C-6), 106.2 (C-4a), 114.6 (C-2′), 121.4 (C-5′), 121.6 (C-6′), 123.7 (C-1′), 124.2 (C-3), 141.9, 146.5 (C-3′, C-4′), 154.4 (C-2), 159.0 (C-8a), 164.0, 165.0 (C-5, C-7), 181.6 (C-4).

Treatment of Glycyrrhisoflavone (13) with HCl Concentrated HCl (0.7 ml) was added to a solution (0.70 ml) of **13** (8.5 mg) in MeOH, and the mixture was left to stand for 36 h. The solvent was evaporated, and the residue was purified by preparative TLC with $CHCl_3$–acetone–HCOOH (16 : 2 : 1), to give cyclo-glycyrrhisoflavone (**26**) (5.6 mg), colorless needles, mp 140 °C. UV λ_{max} (MeOH) nm (log ε): 210 (4.54), 264 (4.49). ^{1}H-NMR δ: 1.35 (6H, s, 2 × CH_3), 1.86 (2H, t, *J* = 7 Hz, H-2″), 2.81 (2H, t, *J* = 7 Hz, H-1″), 6.28 (1H, d, *J* = 2 Hz, H-6), 6.41 (1H, d, *J* = 2 Hz, H-8), 6.83 (1H, d, *J* = 2 Hz, H-2′), 6.93 (1H, d, *J* = 2 Hz, H-6′), 8.15 (1H, s, H-2). ^{13}C-NMR δ: 22.0 (C-1″), 26.1 (CH_3), 32.7 (C-2″), 74.9 (C-3″), 93.6 (C-8), 99.0 (C-6), 105.4 (C-4a), 113.5 (C-2′), 120.5 (C-6′), 121.0 (C-5′), 122.3 (C-1′), 123.4 (C-3), 141.9, 145.8 (C-3′, C-4′), 153.6 (C-2), 158.2 (C-8a), 163.1, 164.1 (C-5, C-7), 180.8 (C-4).

Glycyrrhiza-isoflavone B (6) Colorless needles, mp 197 °C. High-resolution ESI-MS *m/z*: 367.1153 ($[M+H]^+$; Calcd for $C_{21}H_{18}O_6$ + H, 367.1182). EI-MS *m/z*: 366 ($[M]^+$), 351 ($[M-CH_3]^+$). UV λ_{max} (MeOH) nm (log ε): 209 (4.47), 258 (4.52). ^{1}H-NMR: see text. ^{13}C-NMR δ: 27.8 (CH_3), 56.3 (OCH_3), 77.1 (C-9′), 95.7 (C-8), 97.1 (C-6), 107.6 (C-4a), 117.6 (C-7′), 118.9 (C-6′), 122.1 (C-5′), 123.1 (C-2′), 125.9 (C-3), 126.1 (C-1′), 131.7 (C-8′), 140.5 (C-4′), 146.5 (C-3′), 151.1 (C-2), 160.5 (C-8a), 162.6 (C-5), 163.3 (C-7), 174.8 (C-4).

Diacetate (**6b**). ^{1}H-NMR δ: 1.40 (6H, s, 2 × CH_3), 2.25, 2.30 (3H each, s, 2 × $OCOCH_3$), 3.90 (3H, s, OCH_3), 5.79 (1H, d, *J* = 10 Hz, H-8′), 6.47 (1H, d, *J* = 10 Hz, H-7′), 6.78 (1H, d, *J* = 2 Hz, H-6), 6.91 (1H, d, *J* = 2 Hz, H-8), 7.18 (2H, br m, H-2′, H-6′), 8.14 (1H, s, H-2).

Methylation of 6 Compound **6** (1.2 mg) in EtOH (0.2 ml) was treated with ethereal diazomethane at room temperature overnight. After removal of the solvent, the residue was subjected to preparative TLC with $CHCl_3$–MeOH (49 : 1), to give **6a** (0.3 mg). ^{1}H-NMR δ: 1.42 (6H, s, 2 × CH_3), 3.82, 3.88, 3.93 (3H each, s, 3 × OCH_3), 5.74 (1H, d, *J* = 10 Hz, H-8′), 6.40 (1H, d, *J* = 10 Hz, H-7′), 6.49 (1H, d, *J* = 2 Hz, H-6), 6.58 (1H, d, *J* = 2 Hz, H-8), 6.87 (1H, d, *J* = 2 Hz, H-2′), 7.10 (1H, d, *J* = 2 Hz, H-6′), 8.04 (1H, s, H-2). Methylation of semilicoisoflavone B (**12**) also gave **6a**, which was identified by comparison of the ^{1}H-NMR data.

Glycyrrhiza-isoflavone C (7) Colorless needles, mp 159 °C. High-resolution ESI-MS *m/z*: 369.1307 ($[M+H]^+$; Calcd for $C_{21}H_{20}O_6$ + H, 369.1338). ^{1}H-NMR: see text. ^{13}C-NMR δ: 17.1 (C-1″), 26.2 (CH_3), 32.3 (C-2″), 59.5 (OCH_3), 74.0 (C-3″), 100.8 (C-3′), 102.4 (C-8), 105.8 (C-5′), 106.4 (C-1′), 115.1 (C-6), 117.4 (C-4a), 118.7 (C-3), 127.6 (C-5), 155.7 (C-2′), 155.8 (C-2), 156.1 (C-4′), 157.8 (C-8a), 158.1 (C-6′), 162.7 (C-7), 176.5 (C-4). This compound was identified by ^{1}H-NMR spectral comparison with the product obtained by acid-catalyzed cyclization of the prenyl group of glicoricone (**21**) with HCl.[6b]

Estimation of the Effects of Liquorice Phenolics on the DPPH Radical A solution (4 ml) of a test compound in MeOH was added to a solution (1 ml) of DPPH in MeOH (final concentration of DPPH: 2.0×10^{-4} M). After mixing for 10 s on a vortex mixer, the solution was left to stand for 30 min, and the absorbance of the resulting solution at 520 nm was measured. The scavenging activity on the DPPH radical was expressed as EC_{50}, the concentration of the test compound required to give a 50% reduction in the absorbance from that of 2.0×10^{-4} M DPPH in MeOH.

ESR Measurements of Radicals Formed from Liquorice Phenolics ESR spectra were recorded on a JEOL JES-RE1X/HR spectrometer equipped with a Hewlett Packard 382 Controller (X-band; field modulation frequency, 100 kHz), at ambient temperature (24 °C). Microwave power was set at 1 mW. To a solution of test compound in dimethylsulfoxide (2 mg/ml), 0.05 M aq. KOH was added, and the mixture was agitated for 10 s on a vortex mixer. The resulting solution (*ca.* 25 μl) was transferred to a micro-tube (0.7 mm i.d.) to record the spectrum. The modulation width was set at 0.0025—0.005 mT. Each spectrum was recorded after 4 or 8 times accumulation.

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