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Antioxidant and Antimicrobial Constituents of Licorice: Isolation and Structure Elucidation of a New Benzofuran Derivative[1)]

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A new 2-arylbenzofuran derivative named licocoumarone (IIa) was isolated from commercially available xibei licorice (seihoku kanzo) along with a known 3-arylcoumarin derivative, glycycoumarin (Ia), and the structure of IIa was elucidated as 2-(2,4-dihydroxyphenyl)-6-hydroxy-4-methoxy-5-(3-methyl-2-butenyl)coumarone on the basis of spectroscopic and chemical studies. Both Ia and IIa exhibited antimicrobial activities, whereas only IIa had antioxidant activity.

Keywords——2-arylbenzofuran; 3-arylcoumarin; licorice; *Glycyrrhiza* species; antimicrobial activity; antioxidant activity; licocoumarone

Introduction

Several pharmacological activities, such as antiinflammatory, antiallergic, antihepatotoxic and antiviral effects, are shown by the main principle of licorice, glycyrrhizin, and its aglycone, glycyrrhetinic acid,[2)] while the minor principles of licorice, which are mostly flavonoids, have some biological actions supplementing the efficacy of licorice.[3a−c,4a,b,5)] In the present study, the antimicrobial and antioxidant activities of licorice have been examined, and two principles exhibiting such actions have been identified.

Materials and Methods

Apparatus——All melting points were measured on a Yanagimoto melting point apparatus and are uncorrected. Spectral data were obtained using the following apparatus: proton and carbon-13 nuclear magnetic resonance (^{1}H- and ^{13}C-NMR) spectra with a JEOL JMN-GX400 (^{1}H-NMR, 400 MHz, ^{13}C-NMR, 100 MHz) spectrometer with tetramethylsilane as an internal standard; mass spectra (MS) with a JEOL JMS-D300 mass spectrometer; infrared (IR) spectra with a Hitachi 215 grating infrared spectrometer; ultraviolet (UV) spectra with a Hitachi 340 UV spectrometer.

Plant Material——Licorice roots (commercial name: seihoku kanzo in Japanese; xibei ganzao in Chinese) were obtained from Maruzen Kasei Co., Ltd. (Onomichi, Japan).

Assay Procedure for Antioxidant Activity——The antioxidant activity of test samples was evaluated based on the active oxygen method as follows.[6)] Each sample was dissolved in a minimum amount of EtOH, mixed with 18 g of lard at the concentration of 100 ppm and then placed in a fat stability test apparatus (Kuramochi Kagaku Mfg. Co., Ltd., Tokyo). In order to measure the peroxide value (POV), 1 g of mixed lard was taken at each time, and mixed with saturated KI solution to quench the peroxide formed, then generated iodine was titrated with 0.01 N sodium thiosulfate. POV (meq/kg) is expressed as milliequivalents of iodine generated in 1 kg of lard.

Extraction and Isolation of Constituents——The commercially available xibei licorice roots (3.5 kg) were extracted with CH_2Cl_2 under reflux to give the extract (150 g), which was further fractionated into EtOH-soluble (53 g) and insoluble (97 g) parts. The EtOH-soluble fraction was chromatographed over silica gel using $CHCl_3$–

MeOH (40 : 1) as an eluent to give six fractions, fr. 1 (3.7 g), fr. 2 (12.5 g), fr. 3 (3.5 g), fr. 4 (3.5 g), fr. 5 (2.7 g) and fr. 6 (12.2 g). Fraction 5 was rechromatographed on a reverse-phase silica gel column (μ-Bondapak C-18, 4.6 cm × 30 cm, Waters) with MeCN–H_2O (1 : 2) to give nine fractions. Recrystallization of fr. 5-4 and 5-5 from benzene–acetone afforded 170 mg of glycycoumarin (Ia) in a pure form. Yellow plates, mp 243.5—244.5 °C. UV λ_{max}^{MeOH} nm (log ε): 354 (4.29), 250 (4.01). IR ν_{max}^{KBr} cm^{-1}: 3375, 3250, 1700, 1605. HRMS: Calcd for $C_{21}H_{20}O_6$: 368.1260. Found: 368.1276. MS *m/z*: 368 (M^+), 313 ($M^+ - (CH_3)_2C=CH$). ^{1}H-NMR (d_6-DMSO) δ: 1.64 (3H, s, CH_3–C=C), 1.73 (3H, s, CH_3–C=C), 3.26 (2H, d, J=6.7 Hz, –CH_2–), 3.76 (3H, s, OMe), 5.16 (1H, t, J=6.7 Hz, –CH=), 6.26 (1H, dd, J=8.4, 2.1 Hz, 5′-H), 6.36 (1H, d, J=2.1 Hz, 3′-H), 6.59 (1H, s, 8-H), 7.10 (1H, d, J=8.4 Hz, 6′-H), 7.80 (1H, s, 4-H). ^{13}C-NMR (d_6-DMSO) δ: 17.6 (4″-C), 22.2 (1″-C), 25.4 (5″-C), 62.7 (OMe), 97.8 (8-C), 102.6 (3′-C), 105.9 (1′-C), 106.2 (5′-C), 113.4, 118.3, 120.1, 122.6 (2″-C), 130.6 (3″-C), 131.4 (6′-C), 136.4 (4-C), 152.9, 155.2, 155.9, 158.3, 159.5, 160.0 (C=O). Fractions 5-7 and 5-8 were combined (324 mg) and subjected to Sephadex LH-20 column chromatography (eluted with MeOH) to give seven fractions. Fraction 8 (130 mg) was recrystallized from EtOH–H_2O to afford 65 mg of pure licocoumarone (IIa). Colorless needles, mp 183—185 °C. UV λ_{max}^{MeOH} nm (log ε): 336 (4.51), 320 (4.57). IR ν_{max}^{KBr} cm^{-1}: 3500, 3350, 1620, 1600. HRMS: Calcd for $C_{20}H_{20}O_5$: 340.1311. Found: 340.1313. MS *m/z*: 340 (M^+), 285 ($M^+ - (CH_3)_2C=CH$), 269. ^{1}H-NMR (d_6-acetone) δ: 1.62 (3H, s, CH_3–C=C), 1.73 (3H, s, CH_3–C=C), 3.27 (2H, d, J=7 Hz, –CH_2–), 3.95 (3H, s, 4-OMe), 5.17 (1H, t, J=7 Hz, –CH=), 6.35 (1H, dd, J=8.8, 2.1 Hz, 5′-H), 6.56 (1H, d, J=2.1 Hz, 3′-H), 6.77 (1H, s, 7-H), 7.15 (1H, s, 3-H), 7.68 (1H, d, J=8.8 Hz, 6′-H). ^{13}C-NMR (d_6-DMSO) δ: 17.6 (4″-C), 22.3 (1″-C), 25.4 (5″-C), 59.7 (OMe), 92.2 (7-C), 100.3 (3′-C), 102.9 (3-C), 107.0 (5′-C), 108.9 (1′-C), 112.6, 114.1, 123.9 (2″-C), 126.5 (6′-C), 129.3 (3″-C), 150.1, 150.4, 153.0, 153.1, 155.2, 158.1.

Glycycoumarin Trimethylether (Ic)——Glycycoumarin (Ia) was methylated with diazomethane and the reaction mixture was worked up in the usual manner to give a trimethylether (Ic). Recrystallization from MeOH–H_2O afforded colorless needles, mp 77—79 °C. Lit.[4a] mp 78—80 °C. ^{1}H-NMR ($CDCl_3$) δ: 1.68 (3H, s, CH_3–C=C), 1.78 (3H, s, CH_3–C=C), 3.37 (2H, t, J=7 Hz, –CH_2–), 3.81, 3.83, 3.85, 3.89 (3H, each, s, OMe), 5.15 (1H, t, J=7 Hz, –CH=), 6.56 (1H, dd, J=2.5, 1 Hz, 3′-H), 6.57 (1H, dd, J=8.5, 2.5 Hz, 5′-H), 6.65 (1H, d, J=1 Hz, 8-H), 7.34 (1H, ddd, J=8.5, 1, 1 Hz, 6′-H), 7.89 (1H, dd, J=1 Hz, 4-H).

Licocoumarone Trimethylether (IIb)——Licocoumarone trimethylether was prepared as stated above. Colorless needles, mp 113—115 °C (MeOH–H_2O). Lit.[4a] mp 114—115 °C. ^{1}H-NMR ($CDCl_3$) δ: 1.67 (3H, s, CH_3–C=C), 1.80 (3H, s, CH_3–C=C), 3.41 (2H, d, J=7.3 Hz, –CH_2–), 3.86, 3.87, 3.97, 4.04 (3H, each, s, OMe), 5.22 (1H, t, J=7.3 Hz, –CH=), 6.56 (1H, d, J=2.5 Hz, 3′-H), 6.60 (1H, dd, J=8.8, 2.5 Hz, 5′-H), 6.80 (1H, s, 7-H), 7.23 (1H, s, 3-H), 7.89 (1H, d, J=8.8 Hz, 6′-H).

Test Organisms Used for Antimicrobial Activity Assay——All microorganisms used were obtained from the Institute for Fermentation (Osaka, Japan). The following strains were used in this experiment: *Streptococcus mutans* IFO 13955, *Staphylococcus aureus* IFO 3060, *Bacillus subtilis* IFO 3007, *Escherichia coli* IFO 3366, *Saccharomyces cerevisiae* IFO 0306, *Candida utilis* IFO 1086, *Pichia nakazawae* IFO 1668, *Rhizopus formosaensis* IFO 4756 and *Aspergillus niger* IFO 4407. Bacteria were cultured in the standard agar medium (Nissui, Tokyo) which contains 2.5 g of yeast extract, 5 g of peptons, 1 g of glucose and 15 g of agar per 1 l. Yeasts and fungi were cultured in the potato dextrose standard agar medium (Nissui, Tokyo) which contains 4 g of potato extract, 20 g of glucose and 15 g of agar per 1 l.

Determination of Minimal Inhibitory Concentration (MIC)——The agar dilution method was used to assess the antimicrobial activity of each sample. Test compounds were dissolved in a minimum amount of EtOH and a series of dilutions was made with the culture medium. The maximum concentration of EtOH was 5%, which did not inhibit the growth of microorganisms. Each culture medium containing a test sample was poured into a Petri dish, onto which cells of each microorganism were streaked. The culture medium was incubated at 30 °C for 24 h for bacteria, and 72 h for fungi and yeasts. The MIC (g/ml) was the lowest concentration giving complete suppression of growth on the dish after incubation.

Results and Discussion

The separation of the CH_2Cl_2 extract of commercially available licorice roots, which exhibited significant antioxidant (Fig. 1) and antimicrobial activity, was pursued according to the scheme shown in Chart 1. Since the activity was detected mainly in the 50% EtOH-soluble part of the extract, it was subjected to silica gel column chromatography, and each fraction eluted with $CHCl_3$–MeOH (40 : 1) was monitored by means of the antioxidant and antimicrobial assays. The active fraction (fr. 5) was further chromatographed over reverse-phase SiO_2 (MeCN : H_2O = 1 : 2) to give fr. 5-4 and fr. 5-5 which exhibited only antimicrobial activity, and fr. 5-7 and fr. 5-8 which exhibited both antimicrobial and antioxidant activities. The latter fractions were separated on Sephadex LH-20 (MeOH) to give compound B as an active principle. The former fractions were combined and recrystallized from benzene–

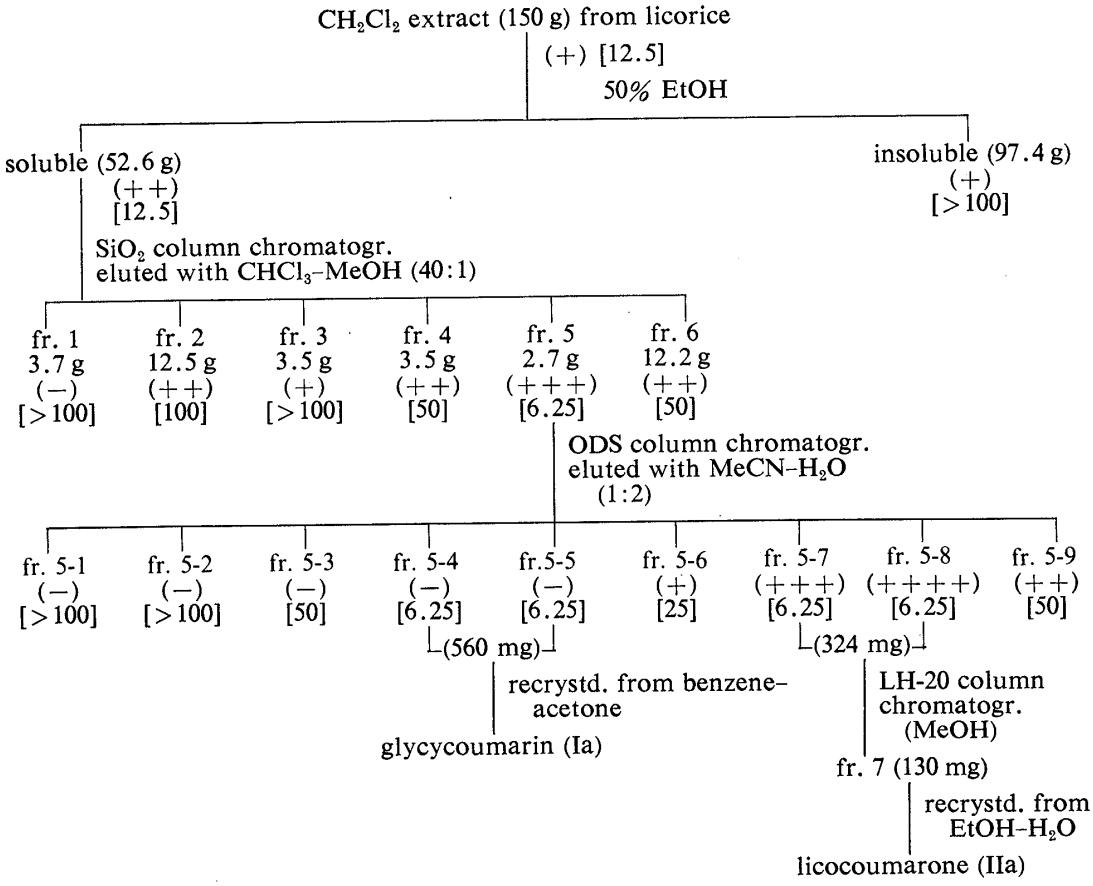
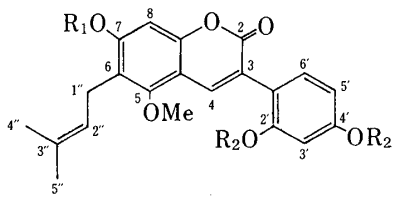
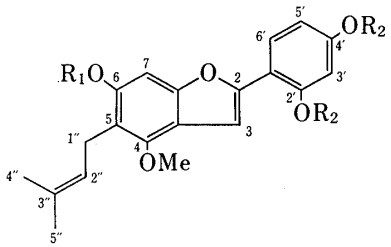


Chart 1. Procedure for Separation of Glycycoumarin and Licocoumarone

The antioxidant activity was evaluated by the active oxygen method. The relative activity of each fraction was expressed as the span of time (h) required for POV in lard to exceed 20 meq/kg in the presence of samples: − (0—5); + (5—10); ++ (10—15); +++ (15—20); ++++ (>20). The antimicrobial activity was determined by the agar dilution method against *Staphylococcus aureus*. Figures in parentheses indicate MIC (μg/ml).



Ia : $R_1=R_2=H$
Ib : $R_1=Me$, $R_2=H$
Ic : $R_1=R_2=Me$



IIa : $R_1=R_2=H$
IIb : $R_1=R_2=Me$

acetone to give compound A in a pure form.

The UV spectrum of compound A showed an absorption maximum at 354 nm and the fluorescent nature of compound A suggested that it might be a 3-arylcoumarin derivative. The ^{1}H-NMR spectrum revealed the presence of one isopentenyl group, one methoxy group and 5 aromatic protons. Since its permethylether (Ic) was identical with that of glycyrin (Ib)[4a] (mixed melting point and ^{1}H-NMR), compound A should have the same functional group disposition as glycyrin. The location of the only methoxyl group in compound A was assigned as the 5-position based on an nuclear Overhauser effect (NOE) experiment in which irradiation of a methoxyl signal at δ 3.76 induced a 20% increase in the intensity of 4-H at δ 7.80. The spectral data of this compound were in good agreement with those of

glycycoumarin (Ia), which was also isolated from Chinese licorice roots independently by three groups.[3a,5,7] Since glycyrin had been correlated chemically to the known isoflavone licoricone, whose structure was established by X-ray crystallography,[8] the structure of glycycoumarin was confirmed as Ia by the above findings.

Compound B, named licocoumarone was obtained as colorless needles of mp 183—185 °C, whose molecular formula was determined by high-resolution mass spectroscopy as $C_{20}H_{20}O_5$, one carbon and one oxygen less than that of glycycoumarin. The IR spectrum showed no band in the carbonyl region. The UV spectrum had absorption maxima at 336 and 320 nm, closely resembling that of a 2-arylbenzofuran derivative.[9] The ^{1}H-NMR spectrum revealed the presence of one methoxyl group and one isopentenyl group in addition to five resonances (ABX and two singlets) in the aromatic region. The singlet at δ 7.29 was assignable to the furanic proton at the 3-position. Methylation of compound B with diazomethane gave a tetramethylether, suggesting the presence of three phenolic hydroxyls. The 2-arylbenzofuran skeleton of compound B was finally confirmed by the finding that its permethylether was identical (mixed melting point, ^{1}H-NMR and IR) with 2-(2,4-dimethoxyphenyl)-4,6-dimethoxy-5-(3-methyl-2-butenyl)coumarone (IIb) derived from glycyrin permethylether (Ic).[4a] The location of a methoxyl group was determined to be at the 4-position by the observation of substantial NOE (14%) between 4-OMe (δ 4.02) and 3-H (δ 7.29). Thus, the above results unambiguously elucidated the structure of licocoumarone as IIa.

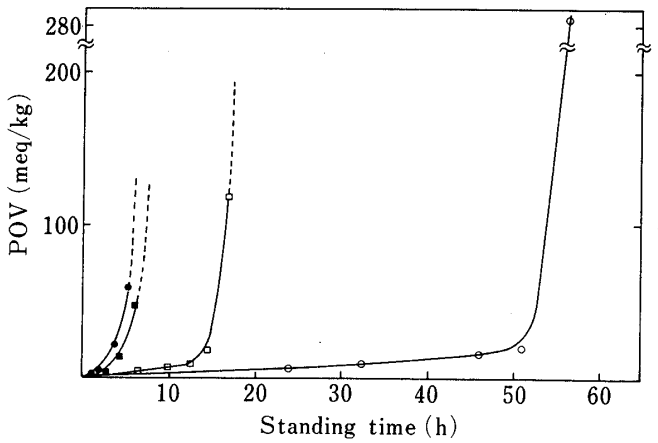


Fig. 1. Antioxidant Activity of Glycycoumarin (Ia) and Licocoumarone (IIa)

●, blank; □, CH_2Cl_2 extract; ■, glycycoumarin; ○, licocoumarone.

TABLE I. Antimicrobial Activity of Glycycoumarin and Licocoumarone

Microorganisms	MIC (μg/ml) Glycycoumarin (Ia)	Licocoumarone (IIa)	Streptomycin sulfate
Bacteria			
Streptococcus mutans[a]	12.5	12.5	3.13
Staphylococcus aureus[a]	3.13	6.25	3.13
Bacillus subtilis[a]	6.25	6.25	3.13
Escherichia coli[b]	>100	>100	50
Yeasts			
Saccharomyces cerevisiae	25	25	>100
Candida utilis	50	25	>100
Pichia nakazawae	25	25	>100
Fungi			
Rhizopus formosaensis	>100	50	>100
Aspergillus niger	>100	>100	>100

a) Gram positive. *b*) Gram negative.

A number of 2-arylbenzofuran derivatives occur in nature, and those isolated from leguminous plants are considered to be of isoflavonoid origin.[10,12] Biosynthetic experiments[10] showed that some 2-arylbenzofurans are derived from the corresponding isoflavonoid by loss of one carbon atom, which has not yet been identified. The co-occurrence of 2-arylbenzofuran and the corresponding 3-arylcoumarin in licorice represents evidence for this hypothesis.

The antioxidant activity of licocoumarone is shown in Fig. 1. It is of interest to note that glycycoumarin has no significant activity, though it is of the same isoflavonoid origin as licocoumarone. The antioxidant activities of 2-arylbenzofurans are being investigated. The isolation of a potent antioxidant component from licorice is of particular interest from the pharmacological point of view, since it quenches active oxygen, which is toxic and damaging to normal biological systems.

Namba *et al.* have investigated licorice roots (seihoku kanzo) for antibacterial activity against *Streptococcus mutans* (also shown in Table I for reference), one of the bacterial species in the oral flora, and isolated glycycoumarin as one of the active principles.[3a] We also investigated the growth-inhibitory activity of glycycoumarin and licocoumarone against various types of microorganisms including bacteria, yeasts and fungi. As shown in Table I, both glycycoumarin and licocoumarone inhibited the growth of gram-positive bacteria as strongly as streptomycin, whereas they were inactive against gram-negative bacteria. Both compounds also exhibited significant activities against yeasts (against which streptomycin is inactive), while licocoumarone showed inhibitory activity against fungi such as *Rhizopus formosaensis*. The biological activities of the minor principles of licorice root other than the main principle, glycyrrhizin, are noteworthy, since licorice extracts are widely used not only medicines, but also as food additives.

Seihoku kanzo (xibei licorice) is one of the main licorices imported from China. Though extensive chemical investigations have been made into this licorice, [3a,4a,7] little information is available on its taxonomic origin. The name of seihoku kanzo is given to the dried roots of licorice produced in the northwestern part of China (excluding Xinjiang Province). Namba *et al.*[3a] reported the occurrence of four coumarin derivatives, glycycoumarin, glycyrin, glycyrol and isoglycyrol, in the extracts of seihoku kanzo and tohoku kanzo (dongbei ganzao; licorice produced in the northeastern region of China), and showed that the high performance liquid chromatography (HPLC) profiles of the two extracts closely resembled each other, as was also observed by us.[11] Since these characteristic coumarin derivatives are known to occur only in seihoku and tohoku kanzo,[12] it is reasonable to presume that both licorices consist of the same *Glycyrrhiza* species from the chemotaxonomical point of view. According to the detailed phytogeographical studies by Lin and Tung on the distribution of *Glycyrrhiza* species of commercial value in China,[13] *Glycyrrhiza uralensis* FISCHER *et* DC. is the only species occurring in the northeastern region, and is predominant in the northwestern region excluding Xinjiang Province. Consequently it seems to be more reasonable to assign seihoku kanzo to *G. uralensis* FISCHER *et* DC. than to *G. glabra* L. var. *glandulifera* REG. *et* HERD., to which seihoku kanzo has been assigned, whithout clear evidence.[3a] However, it should be noted that there have been no scientific reports to confirm this identification based on an on-the-spot survey in the regions where the licorice roots are actually collected.

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