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Islation of Long-Chain Phenols from *Ginkgo biloba* L. and Their Bio-Activer Principles *1

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要 約

은행나무 추출물의 생리활성에 대한 실험결과, 그 추출물이 GAG release에 의한 IL-1의 억제효과(87%)를 나타냄을 관찰하였다. 그 추출물을 chromatography로 분리한후 분광학적 분석을 이용하여 그 함유물질들이 anarchardic acid 및 그와 유사한 페놀성 물질임을 밝혔다. 각 성분에 대하여 활성효과를 다시 실험한 결과, 주성분인 Ⅲb와 Ⅲc는 활성효과를 보이지 않았으며, 단지, 소량물질인 Va와 Vb만이 중간정도의 효과(각각 43과 55%)를 나타내었다. 이 결과에 의하면, 은행나무의 추출성분이 IL-1의 억제효과를 분명히 나타내나, 그 효과가 주성분에 기인하는 것이 아님을 보여 주었다.

Keywords : Long-chain phenol, *Ginkgo biloba* L., bio-activer, column, HPLC, NMR spectra

1. INTRODUCTION

Ginkgo biloba L. is the only surviving species of the Giinkgoaceae, which is recognized as a popular street and park tree in Far East Asia such as Japan, Korea and China. It has many exceptional botanical and taxonomical characteristics, e. g. dioecism, flagellated pollen, ovule dissemination as well as significant tracheid compositions in the xylem[1]. Its leaves and seeds have long been used as ingredients of Chinese medicine in the treatment of many disease[2]. The therapeutic and pharmacological properties for treating thrombosis, inflammation, allergy, platelet aggregation, antimicrobial and cytotoxic activity, etc, have been demonstrated in the literatures[3~6]. These activities are mainly based on the content of flavonoids and terpenoids[4]. However, the actions of this drug are not fully charaterized because of the large number of substances which are found in the extract. The ginkgolides(I) and bilobalide(II) are well known components in the extract of the tree(Figure 1).

Moreover, Itokawa and coworkers reported that long-chain phenols (IIIa-Vb) from the sarcotesta of the tree have antitumour activity and structure-activity relationship of long-chain phenols has also been demonstrated based on various chain length for antitumour activity[7,8].

As part of an ongoing program for the development of anti-inflammatory agents from the natural sources[9], the study of *G. biloba* ex-

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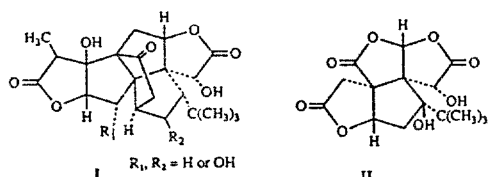

	R_1	R_2	R_3	R_4
IIIa	OH	COOH	$(CH_2)_{12}CH_3$	H
IIIb	OH	COOH	$(CH_2)_7$	H
IIIc	OH	COOH	$(CH_2)_9$	H
IVa	OH	H	$(CH_2)_7$	OH
IVb	OH	H	$(CH_2)_9$	OH
Va	OH	H	$(CH_2)_7$	H
Vb	OH	H	$(CH_2)_9$	H

Fig. 1. The structures of I－Vb

tract appears to be of great interest. The present paper describes the isolation, purification, structure elucidation and biological evaluation of long-chain phenols isolated from the *G. biloba* ract.

2. EXPERIMENTAL

2. 1 General

G. For flash chromatography Merck silica gel 60(0.032－0.063mm) was used. All solvents were of high purity at purchase and were redistilled before use. High performance liquid chromatography(HPLC) was carried out on Dynamax-60A octadecylsilyl(ODS) column (21.4mmID ×250mmL, 8 μm pore size) with MeOH and silica gel(10.0mmID×250mmL, 8 m pore size) column using TMP /EtOAc(10 : 1) as eluent. Mass spectra(MS) were determined on a Hewlett-Packard model HP 5988 GC /MS system. Samples were introduced by means of a direct in sertion probe(DIP) and vaporized with electron impact ionization(50 eV) method. Nuclear magnetic resonance (NMR) spectra were obtained using Bruker-200 (^{1}H : 200.13 MHz, ^{13}C : 50.32MHz) and Varian-MHz spectrometers.

2. 2 Separation of long-chain phenols.

G. biloba extract was subjected to flash column chromatography using TMP /EtOAc solvent system. The active fraction was further separated with HPLC with ODS counm shown in Figure 2. The major fractions were further purified using HPLC with normal phase silica gel column to give compounds IIIb, IIIc, Va and Vb.

Compound IIIb : Colorless powder, mp 38－40℃ : IR(KBr) cm^{-1} 3565, 3560, 3470, 3108, 1672, 1662, 1438, 1300, 1238, 1167 ; ^{1}H NMR ($CDCl_3$) δ0.88 (3H, t, J=7.0 Hz), 1.28 (16H, m), 1.59 (2H, quintet, 7.6), 2.01 (4H, q, 6.0), 2.98 (2H, t, 7.6), 5.34 (2H, m), 6.77 (1H, dd, 1.0, 7.4), 6.87 (1H, dd, 1.0, 7.4), 7.36 (1H, t, 7.4), 11.0 (1H, broad s) ; ^{13}C NMR ($CDCl_3$, multiplicity) 14.12 (t), 22.67 (t), 27.20 (t), 28. 88 (t), 29.20 (t), 29.24 (t), 29.38 (t), 29.46 (t), 29.60 (t), 29.75 (t), 31.78 (t), 32.01 (t), 36.49 (t), 110.33 (s), 115.88 (d), 122.78 (d), 129.83 (d), 129.96 (d), 135.48 (d), 147.80 (s), 163.65 (s), 176.02 (s), ; MS m/z (rel int) : 302 (20. M^+-CO_2), 276(15), 147(14), 133(8), 121(12), 120(35), 108(100), 107(68).

Compound IIIc : Colorless powder, mp 46～48 ℃ ; IR (KBr) cm^{-1} 3565, 3563, 3475, 3115, 2840, 1612, 1610, 1598, 1488, 1480, 1294, 1248, 1148 ; ^{1}H NMR ($CDCl_3$) δ0.88 (3H, t, J=7.0Hz), 1.28(20H,m), 1.61 (2H, quintet, 7.6), 2.01 94H, q, 6.0), 2.98 (2H, t, 7.6), 5.34 (2H, m), 6.77 (1H, dd, 1.0, 7.4), 6.87 (1H, dd, 1.0, 4), 7.36 (1H, t, 7.4), 10.55 (1H, broad s) ; ^{13}C NMR ($CDClz_3$, multiplicity) 14.12(t), 22.67(t), 27.20 (t), 28.99 (t), 29.27 (t), 110.34 (s), 115.85 (d), 122.76 (d), 129.89 (d), 129.90 (d), 135.43 (d), 147.77 (s), 163.62 (s), 176.76 (s) ; MS m /z (rel int) : 330 (52, M^+-CO_2), 304 (35), 234 (10), 175 (8), 149 (14), 133 (8), 121 (18), 120 (36), 108 (100), 107 (62).

Compound Va : Colorless oil : IR(KBr) cm^{-1} 3640, 3460, 3070, 1618, 1598, 1470, 1464, 1302, 1270, 1165, 1146 ; ^{1}H NMR ($CDCl_3$) 30.88 (3H, t, J=7.0Hz), 1.28 (16H, m), 1.57 (2H, quintet, 8.0), 2.01 (4H, q, 6.0), 2.53 (2H, t, 7.8), 4.62 (1H, broad s), 5.34 (2H, m), 6.64 (1H,

quintet, 7.5), 6.65 (1H, s), 6.74 (1H, d, 7.6), 7.11 (1H, t, 7.6), ; ^{13}C NMR ($CDCl_3$, multiplicity) 14.10 (q), 22.67 (t), 27.21 (t), 27.23 (t), 29.00 (t), 29.23 (t), 29.30 (t), 29.43 (t), 29.67 (t), 29.75 (t), 31.28 (t), 31.88 (t), 35.79 (t), 112.38 (s), 115.30 (d), 120.68 (d), 129.32 (d), 129.96 (d), 144.82 (s), 155.47 (d) ; MS m/z (rel int) : 302 (38, m^+), 276 (12), 206 (6), 175 (10), 161 (8), 149 (6), 147 (10), 133 (20), 120 (52), 108 (100), 107 (56).

Compound Vb : Colorless oil : IR (KBr)cm^{-1} 3620, 3458, 3056, 2938, 1616, 1586, 1476, 1453, 1451, 1273, 1186, 1154, 1146 ; ^{1}H NMR ($CDCl_3$) δ0.88 (3H, t, J=7.0Hz), 1.29 (20H, m), 1.57 (2H, quintet, 8.0), 2.01 (4H, 1, 6.0), 2.53 (2H, t, 8.0), 4.64 (1H, broad s), 5.34 (2H, m), 6.64 (1H, d, 7.5), 6.65 (1H, s), 6.74 (1H, d, 7.6), 7.11 (1H, t, 7.6) ; ^{13}C NMR ($CDCl_3$, multiplicity) 14.10 (q), 22.67(t), 27.21(t), 27.23(t), 29.00 (t), 29.23(t), 29.30(t), 29.43(t), 29.67(t), 29.75(t), 31.28(t), 31.88(t), 35.79(t), 112.38(s), 115.30(d), 120.68(d), 129.32 (d), 129.76 (d), 129.96 (d), 144.82 (s), 155.47 (d) ; MS m/z (rel int) : 302 (38, M^+), 276 (12), 206 (6), 175 (10), 161 (8), 149 (6), 147 (10), 133 (20), 120 (52), 108 (100), 107 (56).

2. 3 Chondrocyte Matrix Breakdown Assay

This bioassay has been conducted by Osteo Arthritis Sciences, Inc. as part of a drug screening project for anti-inflammatory actibity. Primary bovine chondrocytes were isolated from the calf cartilage. The chondrocytes were plated at 8×2cm_2 per well with 0.5ml of 1 : 1(v/v) DMEM/F12 supplemented with 10% fetal bovine serum in 24 well plates and incubated for 4 days. The cultures were thenrefed on days 4, 7, 11, 14, 18 and 21 with 0.5ml/well of DMEM/F12 plus 10%fetal bovine serum. On day 22, the wells were rinsed 2×1 ml with Delbuccos phosphate buffered saline (D-PBS) and incubated for 30min in 0.5ml of DMEM/F12 per well This starve media is removed, replaced with 0.5ml/well of DMEM/F12 plus 10 uCi sulfur-35 per well and incubated for 48hr at 37℃. On day 24, the labeling media is removed and disposed and this is then refed with 0.5ml of DMEM/F12 plus 10% fetal bovine serum. The cultures are "chased"with cold sulfate (in the tissue culture media) for two more days and on day 26 are refed with 0.5ml of fresh DMEM/F12 plus 10% fetal bovine serum. On day 27, the wells are rinsed 2×1ml with D-PBS and incubated for 22~24 hr with 0.5ml/well of serum free DMEM/F12 plus the experimental additions. On the day 28, the 0.5ml of media is removed and counted in a mini-vial with 4 ml of scintillation fluids. The cell layer is rinsed 1×1ml with D-PBS and harvested with 0.5ml of trypsin-EDTA for scintillation counting. The data is expressed as % radiolabel released in the media of the total according to the formula :

#Chondrocytes Per Matrix (CPM) in media
/#CPM in media+#CPM in cell layer
=% reoleased

3. RESULTS & DISCUSSION

The crude extract was subjected to flash column chromatography with one major fraction showing significant activity(87%) in the screening test. The test of inhibitory activity for interlukin-1(IL-1) induced glucuruno amino gylcan(GAG) release was conducted by means of the chondrocyte matrix breakdown assay(CMB) (see experimental for details). The active fraction was further separated by HPLC which provided fractions 1, 2, 3 and 4 (Figure 2).

Further chromatography of fraction 3 yielded the major compound IIIb and minor compound Va. Fraction 4 was also purified to yield compound IIIc and Vb. The isolation of pure compounds from fractions 1 and 2 was not attempted since relatively small amounts are present. Therefore, the CMB inhibition assay was tested on fractions 1, 2, and compounds IIIb, IIIc, Va and Vb. As a result of the test, it turns out that the major compounds IIIb and IIIc showed almost no activity(5 and 6%,

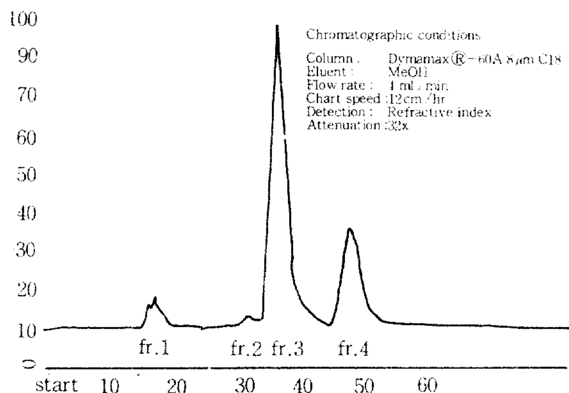

Fig. 2. HPLC chromatogram for the active fraction of *G. biloba* extract.

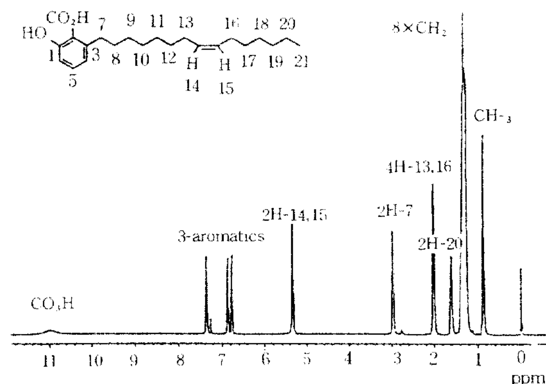

Fig. 3. The structure of compound IIIb and its ^{1}H NMR spectrum (500MHz, $CDCl_3$).

respectively). On the other hand, compounds IIIb, IIIc, Va and Vb. As a result of the test, it turns out that the major compounds IIIb and IIIc showed almost no activity(5 and 6%, respectively). On the other hand, compounds Va and Vb exhibited moderate activity (43 and 55%, respectively)in this assay. These results demonstrate that *Ginkgo biloba* extract is able to inhibit IL−1 induced GAG release, but the substance responsible for this effect is not the major long-chain phenols. The chemical stuctures of these compounds found in active fraction are determined as follows :

Compound IIIb was isolated as a major component from fraction 3. In the proton nuclear magnetic resonance (^{1}H-NMR) spectrum(Figure 3) of IIIb, three peaks at δ6.77(1H, dd, J=1.0, 7.4 Hz), 6.87(1H, dd, 1.0, 7.4), and 7.36 (1 H, t, 7.4) ppm indicated that it is a *trisubstituted* aromatic compound.

A huge peak at δ1.28(16 H, m)ppm in the ^{1}H NMR spectrum in dicated aliphatic long-chain in the molecule. A broad singlet (1 H) appeared at δ11.0 ppm suggested the presence of a free carboxyl group. The overall spectral data led to conclude that this is one of the anacardic acids IIIb[7]. The configuration of the double bond was determined to be *cis* based on the observation of a peak at δ27.20 ppm in the carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectum[10]. The chain-length and the ^{1}H-NMR spectrum, molecular ion peak in the mass spectrum as well as other spectral data reported in the literatures[7]. The ^{1}H-NMR spectrum of compounds IIIc appeared to be superimposable to that of compound IIIb suggesting compound IIIc is closely related to IIIb. The only spectal difference between two compounds was that the integration of the peak at δ1.28 ppm for IIIc (equivalent to twenty protons)is longer than that of compound IIIb(equivalent to sixteen protons). The overall spectral data and mass fragmentational pattern reported in the literature[7] led to the structure of compound IIIc.

Compounds Va and b were determined to be cardanols by procedures similar to those used to identify anacardic acids. The side chain lengths of Va and Vb were determined to be $C_{15}H_{27}$ and $C_{17}H_{33}$ by direct comparison to those of anacardic acids and the literature data[7]. The aromatic proton signals appeared

at δ6.64(1 H, d, J=7.5Hz), 6.65(1 H, s), 6.74 (1 H, d, 7.6) and 7.11(1 H, t, 7.6)ppm which are differ from those of anacardic acids in dicating the absence of carboxyl group in the ring system (Figure 4).

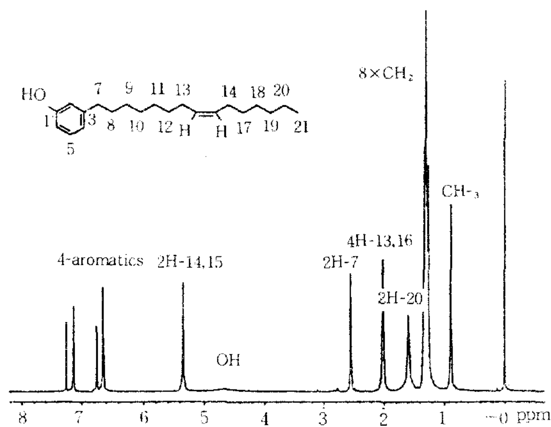

Fig. 4. The structure of compound Va and its ^{1}H NMR spectrum (500MHz, $CDCl_3$).

The non-active mixtures in fractions 1 and 2 were not intended for further purification. The ^{1}H-NMR and MS spectral data of the mixture were compared with the data reported in the literature[7] and suggested that the mixtures contain other long-chain phenols.

4. CONCLUSION

Ginkgo viloba L. extract exhibited significant inhibitory activity (87%) for IL-1 induced GAG release in chondrocyte matrix breakdown assay. Chromatographic separation followed vy spectroscopic analysis of the extract yielded long-chain phenolic compounds, andacardec acids(Ⅲb and c), cardanols(Va and b). The major long-chain phenols(Ⅲb and c)had no signifecant effect in this assay, although the minor long-chain phenols(Va and b) exhibited the moderate activity(43 and 55%, respectively). These results demonstrate that *Ginkgo biloba* extract is able to inhibit IL-1 induced GAG release, but the substance responsible for this effect is not the major long-chain phenols.

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