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Studies on the Anti-inflammatory Activity of *Aralia continentalis* (I)

Characterization of Continentalic Acid and its Anti-inflammatory Activity

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Abstract □ By tracing albumin stabilizing activity an anti-inflammatory component, continentalic acid was isolated from ether-soluble acidic fraction of *Aralia continentalis*. Continentalic acid in a concentration of 0.115mg/3ml gave 50% inhibition for heat denaturation of albumin. The protein stabilizing potency of it was approximately three and eleven times that of phenylbutazone and that of salicylic acid, respectively. The anti-inflammatory actions of it and its methylester were investigated employing carrageenin-induced edema in rat paw. Continentalic acid administered *s.c.* showed an activity of about three times of hydrocortisone. When administered *p.o.*, it was still active, but its methylester was more active than phenylbutazone, suggesting the poor absorption of it in gastrointestinal tract. Its chemical structure was identified by chemical and spectral studies as (−) pimara-8(14), 15-diene-19-oic acid, which was already isolated from *A. cordata*, but not reported for its biological activity.

Keywords □ Anti-inflammatory action, (−) Pimara-8(14), 15-diene-19-oic acid, (−) Diterpenic acid, *Aralia continentalis*, Albumin stabilizing activity, CMR.

In our previous report[1], 123 species of medicinal plants were screened for their anti-inflammatory actions, based on the stabilizing activity against heat denaturation of bovine serum albumin, which is the simple screening method for determining anti-inflammatory activity of non-steroidal compound in vitro, developed by Mizushima.[2]

One of the strongly effective crude drugs, *Aralia continentalis* K.(Korean name "Dokwhal") which has been used as folk medicine for anti-rheumatic, anti-inflammation and analgesic, was chosen to investigate active principles. By tracing the protein stabilizing activity in a fraction of methanol extracts of the plant roots, we isolated an active component from ether-soluble acidic fraction and designated it as continentalic acid by common name. The protein stabilizing potency of continentalic acid was approximately three-and eleven-fold more than those of phenylbutazone and salicylic acid, when 50% inhibition concentrations for heat denaturation of albumin were compared, respectively.

On rat paw carrageenin edema test, the anti-inflammatory activity of continentalic acid was more potent than that of hydrocortisone and that of phenylbutazone when administered *s.c.* and *p.o.*, respectively.

This paper describes the studies on the isolation and identification of continentalic acid, and on its anti-inflammatory characteristics.

EXPERIMENTAL METHODS

Materials

Roots of *A. continentalis* was purchased from a market, Chongro Street, Seoul. Bovine serum albumin (Cohn fraction V) and carrageenin were obtained from Sigma Chemical Co. Hydrocortisone and phenylbutazone are grade of USP.

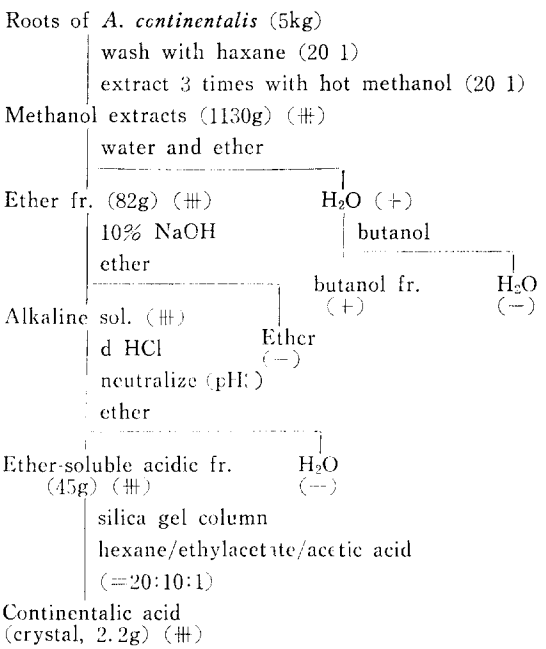
Protein Stabilizing Activity

The simple screening method for determining anti-inflammatry activity developed by Mizushima[2], was modified in order to eliminate the salt effect in test samples. Two ml of 0.75% albumin in 0.1M phosphate-saline solution (pH 5.3) was mixed in a test tube (12mm i.d.) with 1 ml of appropriately diluted test sample solution. Stood at room temperature during 20 min., the reaction mixture was heated at 67°C for 180 sec. in a shaking water bath and then cooled in an ice bath. The degree of heat denaturation of albumin was estimated by the turbidity of colloidal precipitate, of which absorbance was measured at 570nm. An insoluble sample in water was dissolved in 0.3M NaOH and then neutralized pH 7 to 8 with 0.3N HCl. A sodium chloride solution of the same conductivity with that of the test sample solution, was added to a control solution.

One unit of protein stabilizing activity was defined as the amount of sample which gave 50% inhibition rate under the conditions described above. Specific activity was expressed as protein stabilizing activity unit per gram of sample.

Isolation of Continentalic Acid

Roots of *A. continentalis* (5kg) were washed by percolation with 20 l of hexane at room temperature in order to extract oils. The washed roots were extracted 3 times in a boiling water bath with 20 l of methanol and concentrated to dryness in a vacuum. The methanol extract was fractionated by the procedure illustrated in Scheme I, by tracing the protein stabilizing activity.



Scheme I: Separation procedure of an anti-inflammatory component by tracing the albumin stabilizing activity.

The ether-soluble acidic fraction (45g) exhibiting the main activity was chromatographed on a silica gel column, using the elution solvent of hexane/ethylacetate/acetic acid (20:10:1). The fractions with the major activity showed a spot at Rf 0.6 on TLC, were evaporated and recrystallized twice with methanol to give a component (colorless needles) designated as continentalic acid [I] mp 158°, $[\alpha]_D^{25}$ -147.5° ($CHCl_3$).

Methylation of [I]

Methyl ester [II] of [I] was obtained by treatment of diazomethane (in ethylether) and recrystallized with methanol (mp 58°).

Carrageenin Edema Test

Male albino rats (Splague Dawley strain)

weighing 170±20g which had been accomodated for two weeks in our institute, divided into experimental and control groups, each consisting of 4-7 animals. Samples were suspended with 1% CMC solution and administered subcutaneously or orally, twice, six and one hour before edema induction. Edema was induced by subcutaneous injection of 0.05ml of 1% carrageenin saline solution in a hind paw of rat. Edema volume was measured by the method[3] of Harris and Spencer using a plethysmometer. Parallel experiments were conducted with hydrocortisone (s.c.) and phenylbutazone (p.o.) as references. The inhibition per cent of edema induced by each agent was calculated for each animal group with respect to its vehicle-treated control group.

Instrumental Analysis

All melting points were taken on a Mitamura heat block apparatus and given uncorrected values. A UV/visible spectrophotometer, Gilford type 2600 was used for the measurements of UV absorption spectra and the turbidity of heat denaturated albumin solution. Proton NMR spectra were obtained in $CDCl_3$ solution using TMS as an internal standard on a Perkin-Elmer NMR spectrometer (90 MHz). ^{13}C-NMR spectra were obtained in $CDCl_3$ solution using TMS as an internal standard on a 20.1 MHz NMR spectrometer. IR spectra were determined in KBr pellets on a perkin-Elmer type 283 B spectrometer. Gas-liquid chromatograms of II were obtained on a Pye-Unicam type 104 chromatograph under the conditions:column, 3% SE-30, size 4mm ×2m; N_2, flow rate 40ml/min, temperature, column 200°, FID detector 250°. Mass spectra were obtained on a Hewlett Packard GC/Mass spectrometer (type 5985B) using a electron impact method.

RESULTS AND DISCUSSION

Purification of Continentalic Acid

Purification of continentalic acid shown in Scheme I can be divided into two steps; step 1, solvent fractionation of methanol extract; step 2, adsorption chromatography of ether-soluble acidic fraction.

A summary of the procedures for purification of continentalic acid is illustrated in Table I. The yield of continentalic acid recrystallized was about 2.2g from 5kg of roots and about 177 fold purification over the protein stabilizing activity at methanol extract was achieved.

Table I: Summary of purification of continentalic acid.

Step	Total amounts (g)	Total* activity (units)	Specific** activity (units/g)	Yield (%)
A. continentalis	5,000	—	—	—
Methanol extract	1,130	5.5×10^4	48.7	100
1. Solvent fractionation				
ether-soluble acidic fr.	45	3.73×10^4	828.9	67.8
butanol fr.	28	5.25×10^3	187.5	9.5
2. Silica gel chromatography				
continentalic acid (recrystallized)	2.2	1.91×10^4	8,636.4	34.7

* One unit of protein stabilizing activity was defined as the amount of sample which gave 50% inhibition rate.

** Protein stabilizing activity units per gram of sample.

The Purity of Continentalic Acid and its Content

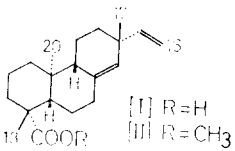
Continentalic acid and its methylester were chromatographically pure on silica gel plates. Gas chromatogram of methyl ester gave a single peak (Rt 5.3). The content of continentalic acid was 0.39% in the plant roots when determined by GLC technique after methylation of ether-soluble acidic fraction.

The Chemical Structure of Continentalic Acid

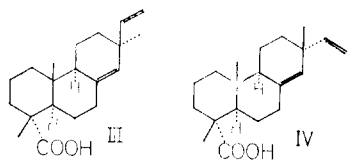
Continentalic acid [I] was positive in Libermann-Buchard reaction and negative in nitrogen test and Zimmermann test. The UV spectrum (in hexane) of [I] gave only an end absorption at 212.5 nm ($E_1\%$—258.3), revealing the absence of conjugated double bonds. The IR spectrum of [I] showed absorption bands at 3000-2500, 1685 cm^{-1} (COOH), 996, 912 cm^{-1} (monosubstituted double bond), 860, 846cm^{-1} (trisubstituted double bond) [I] afforded methyl ester (colorless needles from methanol) [II] mp 58°. The IR spectrum of [II] showed absorption band at 1725 cm^{-1} (ester) and did not at 4000-3000 cm^{-1}, revealing the absence of ketone and hydroxyl groups.

The mass spectrum of [I] showed the molecular ion at m/e 302, giving a possible molecular formular $C_{20}H_{30}O_2$. Proton NMR of [I] gave $\delta^{CDCl_3}_{ppm}$; 10(1H, diffused, COOH); 0.64, 0.99 and 1.25 (3×3H, s, three tertiary methyls); 4.66 to 5.94 (3H, ABC type coupling, J_{AB}=10 Hz, J_{AC}=17Hz, J_{BC}=2Hz, three protons of a vinyl group); 5.07 (1H, d, allylic coupling, a proton of trisubstituted double bond); one of the doublet peaks was observed to be partially overlapped with a peak of the vinyl protons. Proton NMR of [II] showed the presence of a carboxy methyl ester group (3H, s, 3.61) and some up-field shifts of three methyl signals (0.50, 0.95 and 1.15).

The patterns of proton NMR of [I] and [II], and other spectral data mentioned above are identical with those of (−) pimara-8(14), 15-dien-19-oic acid, which was already isolated from Aralia cordata.[4]



Additional confirmation of the structure of [I] was obtained by considering the fragmentation pattern as shown in Scheme II, whose mass spectrum (Fig. 1) is quite similar to the spectra of pimaric acid [III] and sandaracopimaric acid [IV].[5]



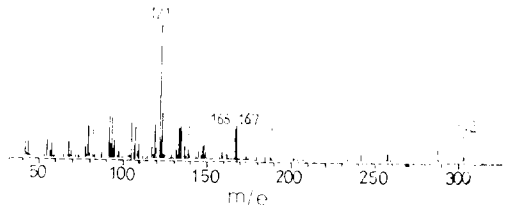
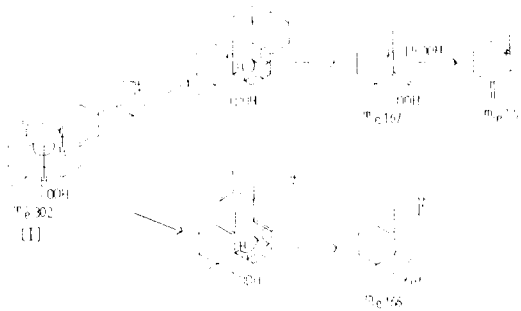


Fig. 1: Mass spectrum of continentalic acid.



Scheme II: Possible mass fragmentation of continentalic acid.

The ^{13}C-NMR spectra provided conclusive assignment for the structure of [I]. Application

Table II: CMR chemical shifts of continentalic acid.

	I[a]	III[6]	IV[6]		I	III	IV
C-1	38.1(t*, —**)	38.6	38.4	C-11	19.6(t, —)	19.5	18.8
C-2	19.3(t, —)	18.5	15.3	C-12	29.7(t, —)	36.0	34.6
C-3	36.5(t, —)	37.5	37.1	C-13	39.3(s, —)	39.0	37.4
C-4	44.1(s, —)	47.6	47.2	C-14	128.2(d, +)	128.2	129.3
C-5	50.7(d, +)	49.1	48.7	C-15	147.3(d, +)	147.8	149.0
C-6	24.2(t, —)	25.5	24.9	C-16	113.0(t, —)	113.2	110.5
C-7	35.9(t, —)	35.8	35.5	C-17	29.2(q, +)	29.9	26.2
C-8	138.1(s, —)	138.5	136.2	C-18	29.4(q, +)	17.6	16.8
C-9	56.3(d, +)	51.9	50.7	C-19	184.4(s, —)	185.7	185.3
C-10	38.5(s, —)	38.1	37.8	C-20	13.8(q, +)	15.4	15.3

a) Spectra taken at 20.1 MHz on a Fourier transform spectrometer; chemical shifts in parts per million downfield from TMS. In $CDCl_3$ solution; $\delta_{TMS} = \delta_{CDCl_3} + 77.1$ppm.

* Off-resonance decoupled spectra; abbreviation: s=singlet, d=doublet, t=triplet, q=quartet

** Gated spin echo spectra

of chemical shift theory to noise decoupled, off-resonance decoupled and gated spin echo spectra of [I] yielded the the data in Table II, which was collated with those of [III] and [IV].[6]

The Protein Stabilizing Activity of Continentalic Acid

To determining the protein stabilizing activity of continentalic acid, dose-response curves were drawn by plotting values for the inhibition rate of different amounts of continentalis acid together with phenylbutazone and salicylic acid as non-steroidal anti-inflammatory references.

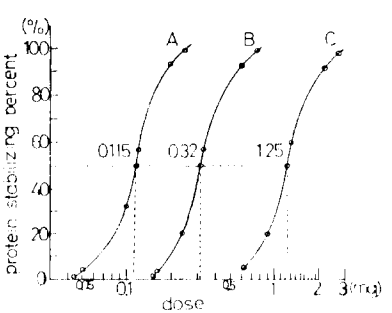


Fig. 2: Protein stabilizing potency of continentalic acid.
A : continentalic acid
B : phenylbutazone
C : salicylic acid

The curves (Fig. 2) show that the protein stabilizing activity of continentalic acid in the concentration of 0.115mg/3ml matchs to that of phenylbutazone 0.32mg/3ml and to that of salicylic acid 1.25mg/3ml, when 50% inhibition concentrations for heat denaturation of albumin are compared. The results indicate that continentalic acid is three and eleven times as effective as phenylbutazone and salicylic acid, respectively.

The Anti-inflammatory Activity of Continentalic Acid

The anti-inflammatory effects of continentalic acid, 3, 10 and 30mg/kg, *s.c.*, and 100mg/kg, *p.o.*, and its methyl ester, 33 and 100mg/kg, *p.o.*, on carrageenin-induced edema in rat hind paw were investigated and the results were compared with that of hydrocortisone, 30mg/kg, *s.c.* (Table III) and that of phenylbutazone, 100mg/kg, *p.o.* (Table IV).

After the s.c. administration, continentalic acid, 3, 10 and 30mg/kg, and hydrocortisone, 30mg/kg, suppressed significantly the edema

Table III: Anti-inflammatory activity of continentalic acid administered *s.c.*

Compound	Dose mg/kg s.c.	No. of animals	Edema increaser per cent(Inhibition per cent) 0.5hr	1.5hr	2.5hr	3.5hr	4.5hr
Control	CMC	18	43.32±11.52	82.12±24.06	108.40±19.00	81.02±30.25	64.86±28.06
Hydrocortisone	30	20	31.21±14.46 (27.95)*	38.08±16.92 (53.62)	37.98±19.97 (64.96)	32.91±19.30 (59.38)	20.00±12.85 (69.16)
[I]	30	7	25.04± 8.02 (42.20)*	14.68±10.72 (82.12)	34.93±10.99 (67.78)	17.35±16.36 (78.59)	15.00±12.37 (76.87)
[I]	10	15	29.53±17.47 (31.83)**	35.18±21.50 (57.16)	36.87±21.81 (65.99)	37.18±21.67 (54.11)	32.37±18.51 (50.09)
[I]	3	5	39.12± 9.55 (9.70)****	58.81±10.67 (28.39)***	53.03±15.22 (51.08)	39.11± 6.70 (51.73)*	25.34± 7.90 (60.93)*

* $P<0.01$ ** $P<0.02$ *** $P<0.1$ **** $P>0.1$
Other values are highly significant ($P<0.001$).

Table IV: Anti-inflammatory activity of continentalic acid administered *p.o.*

Compound	Dose mg/kg p.o.	No. of animals	Edema increaser per cent(Inhibition per cent) 0.5hr	1.5hr	2.5hr	3.5hr	4.5hr
Control	CMC	5	15.49±10.10	22.58± 7.01	40.46± 9.82	49.55±14.40	26.79±20.88
Phenylbutazone	100	4	7.36± 2.92	14.50± 2.80	19.90± 4.56 (50.82)**	24.71± 3.23 (50.13)**	14.50± 6.40
[I]	100	5	9.74± 4.47	16.78± 3.96	25.95± 4.23 (35.86)**	22.83± 5.77 (53.93)*	16.45± 6.64
[II]	100	4	3.78± 3.78	8.18± 5.75	13.20± 4.16 (67.38)*	16.16± 3.52 (67.39)*	5.72± 5.16
[II]	33	5	13.38± 4.20	24.82± 1.78	31.91± 4.55 (21.13)	21.37± 4.17 (56.87)*	16.20± 6.23

* $P<0.01$ **$P<0.05$
Other values are non-significantly different from control.

volumes by 51, 65, 68 and 65% on average 2.5 hour after the treatment with carrageenin, respectively. By a rough estimation, continentalic acid shows an anti-inflammatory activity of about three times of hydrocortisone.

In contrast, when administered *p.o.*, continentalic acid, its methyl ester and phenylbutazone, each of the doses of 100mg/kg cause significant inhibitions of 54, 67 and 50% on average 3.5 hour after the treatment with carrageenin, respectivley. The results administered *p.o.* indicate that continentalic acid exhibits an anti-inflammatory activity almost equipotent to that of phenylbutazone, but its methyl ester is more active, suggesting the poor absorption of continentalic acid in gastrointestinal tract of rat.

In conclusion, it will be summarized that continentalic acid is a nonsteriodal anti-inflammatory agent isolated as an active principle of A. continentalis. It is exceptional that an anti-inflammatory component isolated from natural products shows an action more potent than phenylbutazone and hydrocortisone used clinically now.

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