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Glycinoeclepins, Natural Hatching Stimuli for the Soybean Cyst Nematode, *Heterodera Glycines*. I. Isolation

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Hatching stimuli, designated as glycinoeclepins, for the soybean cyst nematode (*Heterodera glycines*) have been isolated by repeated chromatography of the aqueous extracts of dried roots of kidney bean (*Phaseolus vulgaris*), one of the host plants of the nematode. One of these compounds, glycinoeclepin A, stimulates the hatching and emergence of larvae in vitro in highly diluted aqueous solutions.

The cyst nematodes (genera *Heterodera, Globodera* and others, ca. 40 species)[1] generally have a limited host range. This specificity arises from the fact that the hatching of dormant second stage larvae from eggs and the emergence of the hatched larvae from cysts occur in response to chemical stimuli in the root diffusates secreted by the host plants.[2-5] This fact was first discovered by Baunacke in 1922 for the potato cyst nematode (*G. rostochiensis*).[6] Since then, much time and effort has been expended in attempts to purify and characterize these stimuli,[2-5] in view of their scientific interest and importance in agriculture. However, success has been limited by the extremely small amounts of active substances available.

The soybean cyst nematode (*H. glycines* Ichinohe) parasitizes a small group of host plants, including soybean (*Glycine max*), kidney bean (*Phaseolus vulgaris*), and adzuki bean (*P. angularis*), and causes "soybean sickness."[7] The life cycle of this nematode is summarized as follows.[7] The female adult, after copulation, is almost completely filled with the ovary and then with the eggs, and dies. Within a few days, the embryo in the egg molts into the first and second stage larva, and enters a domant stage in the dead female called "cyst." Each cyst usually contains about 250 eggs within the body and, without host plants, persists in the soil for many years. In spring, when temperature, moisture, and other factors become desirable, and also when the host plants are cultivated in the soil, the second stage larvae in the eggs hatch, and the hatched larvae emerge out of the cysts. After invading the roots and causing formation of giant cells, the second stage larvae become large and molt into the male and female adults via the third and fourth stage larvae. These larvae feed on the giant cells and hence infect the host plants. This life cycle is completed within 4 weeks, if all the conditions are satisfied. The most interesting stage in this life cycle is hatching and emergence. Various factors affecting the hatching and emergence have been examined in details.[8-13] Of these factors, the most important one is chemical stimuli secreted by the roots of host plants, since these stimuli are connected with the host-parasite specificity. In 1966 Tsutsumi and Sakurai demonstrated[14] that the hatching and the larval emergence of the soybean cyst nematode were stimulated by root diffusates from the host plants. The present investigation was then commenced in 1967 with the purpose of ecological eradication of the nematode, based on these findings, and resulted recently in successful isolation and structure elucidation of the relevant natural hatching stimuli, designated as glycinoeclepins A, B, and C. These results have been reported in preliminary communications.[15-18] In the present article we describe the isolation procedure of these active principles in details.

Bioassay. The method of bioassay for the hatching, required for efficient isolation of the active principles, was carried out as follows. Usually ca. 100 cysts were obtained from ca. 100 g of the nematode-infested soil, which were collected in October at Memuro, Hokkaido, and stored in a refrigerator at 5 °C. The cysts were soaked at 25 °C for 10 d, then dissected in water and sieved through a 325-mesh sieve. The dissected cyst wall fragments remained on the sieve. The eggs and larvae, which had been hatched, were collected on filter papers and transferred to a beaker with water so that 1 ml of the aqueous suspension contained 150—200 eggs. Then 1 ml of this suspension was transferred to a Syracuse watch glass to which 1 ml of the test solution and 8 ml of distilled water were added. The whole suspension was kept at 25 °C, and the number of larvae hatched was counted after 10 d. The per cent hatching rate was expressed as $P=P_t-P_w$, where P_t and P_w denote the per cent hatching observed in the test solution and in distilled water, respectively. The test solution was said to be active, when the hatching rate, P, exceeded 50%. This bioassay is characterized by using the free eggs instead of the cysts, and also by soaking the cysts in water at 25 °C for 10 d before the test. Compared with the bioassays which had been performed with the cyst by most of the previous workers, this bioassay has been found to be simpler and more reproducible.

Preparation of Hatch-Stimulating Concentrates. A large amount of the concentrates, the active raw materials, were prepared from aqueous extracts of the

roots of two subspecies ("Hon-kintoki" and "Beni-kintoki") of kidney bean rather than those from their root diffusates, because the diffusates were highly active, compared with the aqueous extracts, but too unstable for further purification.[19] The two subspecies were cultivated in 1 hectare of the field at Memuro for 3 months (early May to late July). The roots were collected by cutting just before flowering, air-dried for 1—3 months (August to November), and pulverized. The dried and powdered roots (ca. 100 kg) thus obtained were extracted with water (1.5—3 kl) at a temperature below 10 °C for 1 month (November to December). The aqueous suspensions were filtered and concentrated below 30 °C, acidified with concd hydrochloric acid to pH 3, and extracted again with chloroform repeatedly (January to April). The chloroform solution, after concentration, gave the hatch-stimulating concentrates (ca. 100 g) (Chloroform extracts A), which stimulated the hatching and emergence at 10^{-5} g ml^{-1} in water at 25 °C. The dilute (10^{-5} g ml^{-1}) aqueous solution of the concentrates was called "standard solution" and always used for the bioassay for reference.

Isolation. In 1982 we reported[15] the isolation of a natural hatching stimulus, designated as glycinoeclepin A (GEA), for the soybean cyst nematode, as its bis(*p*-bromophenacyl) ester (p-BPE). In 1977, five years before the isolation, we had obtained one fraction [Fraction L (p-BPE)] after repeated chromatography of the hatch-stimulating concentrates according to the procedure shown in Table 1.[20] The fraction had revealed a single peak by analytical high performance liquid chromatography (HPLC) on a variety of columns and showed high activity (active at 10^{-11}—$^{-12}$ g ml^{-1}) for the hatching. However, with a limited amount (ca. 5 μg from ca. 100 g of the concentrates) of the fraction, it had not been feasible in the year of 1977 to obtain any useful MS and ^{1}H NMR spectral data of the fraction, because of unavoidable contamination by impurities of solvent and column origin. Four years later, in 1981, the fraction could be identified as p-BPE of the active principle in question on the basis of the same chromatographic behavior and FD-MS spectrum as those of GEA p-BPE. It implies that the procedure described in Table 1 has resulted in the first isolation

Table 1. Isolation of GEA as its p-BPE

Dried and powdered roots of kidney bean "Beni-kintoki" [135 kg (1 ha)]	
↓ Extraction with water (2 kl) (<10 °C)	
↓ Evaporation (<30 °C) under reduced pressure	
Concentrates	
↓ Continuous extraction with chloroform at pH 2—3	
Chloroform extracts A	(115 g, active at 10^{-5} g ml^{-1})
↓ EtOAc-aq $NaHCO_3$	
Acidic extracts B	(66.5 g, active at 10^{-5}—$^{-6}$ g ml^{-1})
↓ Charcoal-Celite column (aq acetone)	
Fraction C	(5.2 g, active at 10^{-7} g ml^{-1})
↓ Silica-gel column (EtOAc-$CHCl_3$-AcOH, 15:5:1)	
Fraction D	(360 mg, active at 10^{-8} g ml^{-1})
↓ Silica-gel column (Ether-AcOH, 99:1)	
Fraction E	(117 mg, active at 10^{-8} g ml^{-1})
↓ Siliconized Hyflo Super Cel column (MeOH-H_2O-AcOH-$CHCl_3$-$C_8H_{17}OH$, 150:150:2:15:15)	
Fraction F	(25.5 mg, active at 10^{-8}—$^{-9}$ g ml^{-1})
↓ Amberlyst-15 (Ag^+ form) column (EtOH)	
Fraction G	(14.4 mg, active at 10^{-8}—$^{-9}$ g ml^{-1})
↓ Amberlyst XN-1005 (Ag^+ form) column (MeOH)	
Fraction H	(1.53 mg, active at 10^{-9}—$^{-10}$ g ml^{-1})
↓ *p*-Bromophenacylation (*p*-$BrC_6H_4COCH_2Br$, $KHCO_3$, crown ether in MeCN, 80 °C)	
↓ Silica-gel column	
Fraction I (p-BPE)	(0.91 mg, active at 10^{-9}—$^{-10}$ g ml^{-1} after hydrolysis)
↓ Preparative HPLC, MicroPak CN-10 (C_6H_{14}-ether, 45:55)	
Fraction J (p-BPE)	(0.19 mg, active at 10^{-10} g ml^{-1} after hydrolysis)
↓ Preparative HPLC, MicroPak NH_2-10 (C_6H_{14}-CH_2Cl_2-MeCN, 70:18:12)	
Fraction K (p-BPE)	(≈50 μg, active at 10^{-10}—$^{-11}$ g ml^{-1} after hydrolysis)
↓ Preparative HPLC, Hitachi Gel #3011 (MeOH-CH_2Cl_2, 9:1)	
Fraction L (p-BPE) (=GEA p-BPE)	(≈5 μg, active at 10^{-11}—$^{-12}$ g ml^{-1} after hydrolysis)

of GEA.

The isolation procedure summarized in Table 1 will deserve the following comments. a) The hatch-stimulating concentrates were initially fractionated (Fraction A to H) by various types of column chromatography such as adsorption, reversed-phase partition, and ionic exchange chromatography, in the light of the separation procedure of gibberellins[21] and prostaglandins. A number of inactive aliphatic and aromatic acids and abscisic acid-related compounds were isolated and identified during chromatography over Charcoal-Celite (Fig. 1) and silica gel (see Experimental). Usually several active fractions were separated in each of the steps. Fortunately, any special enhancement in the activity was not observed by combination of such active fractions, and only the most active fraction in each step was submitted to the subsequent separation step. The following points are annotated. i) Chromatography over silica gel (Fraction C to E) did not always give satisfactory results in reproducibility; it sometimes resulted in remarkable decrease of the amount without increase or with loss of the activity.[22] ii) Reversed-phase partition chromatography on a siliconized Hyflo Super Cel column (Fraction E to F) led to efficient concentration of active fractions (Fig. 2) under almost the same conditions as those for effective separation of prostaglandins.[23] However, the column was not expected to be suitable for separation of a large amount of the material. iii) Subsequent separation by chromatography over ionic exchange resins (Fraction F to H), Amberlyst-15 (Ag^+ form) and Amberlyst XN-1005 (Ag^+ form) columns, appeared to have been

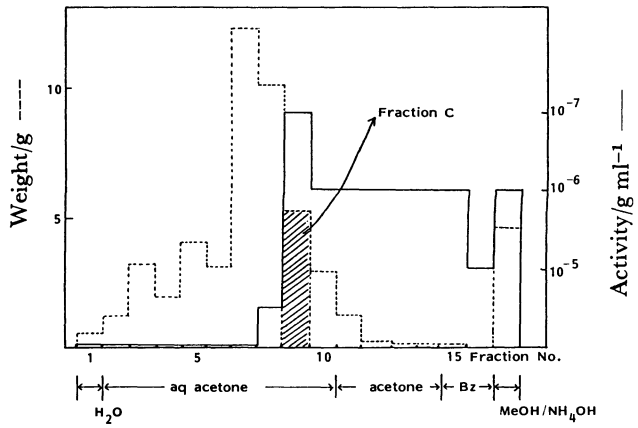


Fig. 1. Column chromatography of Acidic extracts B on the Charcoal-Celite column (Table 1). Eluent: Frs. 1—10, H_2O–acetone (10 : 0, 9 : 1, 8 : 2, 7 : 3, 6 : 4, 3 : 7, 2 : 8, 1 : 9); Frs. 11—14, acetone: Frs. 15—16 (benzene); Fr. 17, (MeOH–concd NH_4OH). The hatching activity was checked with the eggs.

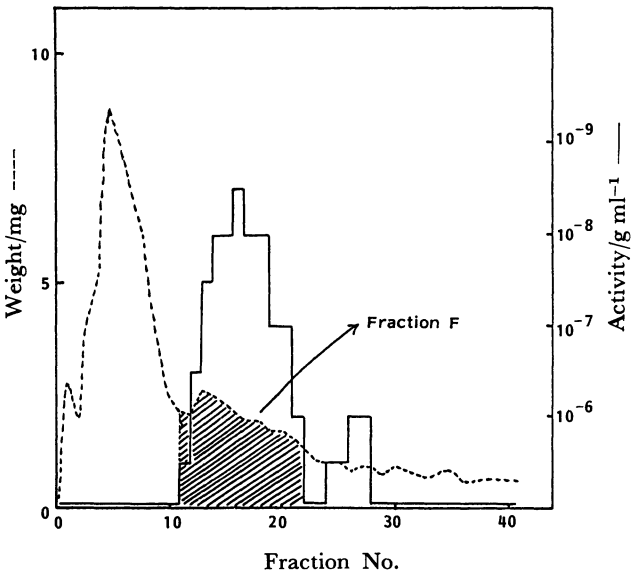


Fig. 2. Reversed-phase partition chromatography of Fraction E on the siliconized Hyflo Super Cel column (Table 1). Moving phase, MeOH–H_2O–AcOH (75 : 75 : 1); stationary phase, $CHCl_3$–2-ethyl-1-hexanol (1 : 1). The hatching activity was checked with the eggs.

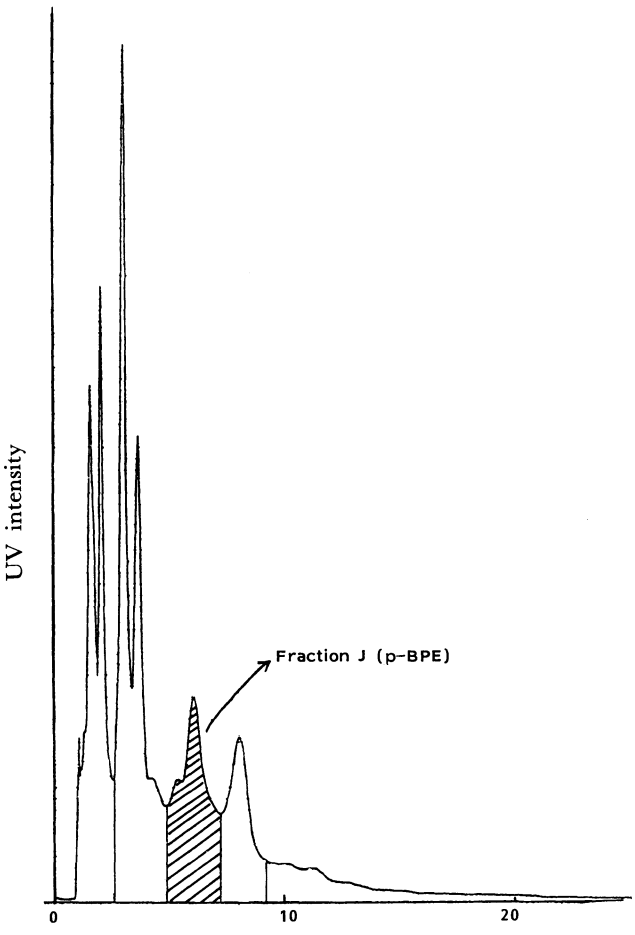


Fig. 3. Preparative HPLC of Fraction I (p-BPE) on the MicroPak CN-10 column (Table 1). Eluent, hexane–ether (45 : 55).

performed efficiently, as had been exemplified by smooth separation of prostaglandins such as PGE_1 and PGE_2.[24,25] However, the silver cations, contaminated in the columns and eluted with solvents, caused serious decrease in the activity of eluates and disturbed the bioassay. It was necessary to wash the columns with distilled water repeatedly (ca. 1 month) in order to remove the silver cations completely and carry out the bioassay in a usual manner. b) *p*-Bromophenacylation of the acid mixture [Fraction H to I (p-BPE)] did not proceed in a satisfactory yield (ca. 30%) by the reported procedure using crown ethers.[26] c) Preparative HPLC on three different types of columns resulted in the effective separation of active fractions, giving only one active fraction in each of the steps without loss of the activity (Figs. 3, 4, and 5). The MicroPak CN-10 column[27] is packed with silica gel (LiChrosorb Si-60) whose surface is bonded chemically with 3-cyanopropylsilyl groups and hence is hydrophobic. The column was found to be superior to silica gel itself (MicroPak Si-10)[20,27] in reproducibility. On the other hand, the MicroPak NH_2-10 column[27] is much polar than the MicroPak CN-10 one, because it is filled up with silica gel (LiChrosorb Si-60) whose surface is modified chemically with 3-aminopropylsilyl groups and interacts with solutes by hydrogen bonding. The Hitachi Gel #3011 column[27b,28] is completely different from these two columns in the separation mode. The packing is a porous polystyrene-divinylbenzene copolymer and the separa-

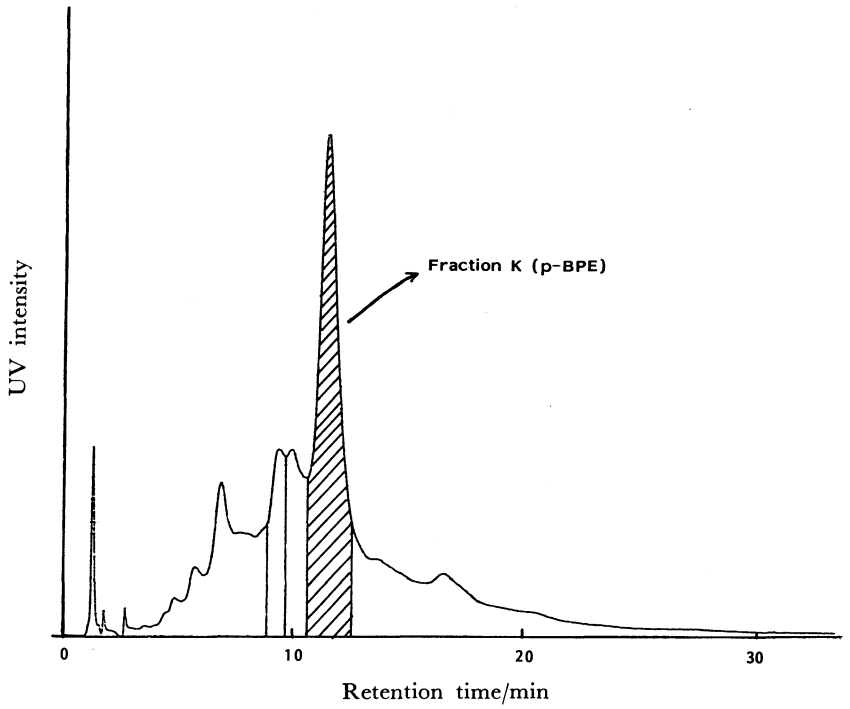


Fig. 4. Preparative HPLC of Fraction J (p-BPE) on the MicroPak NH_2-10 column (Table 1). Eluent, hexane–CH_2Cl_2–MeCN (70 : 18 : 12).

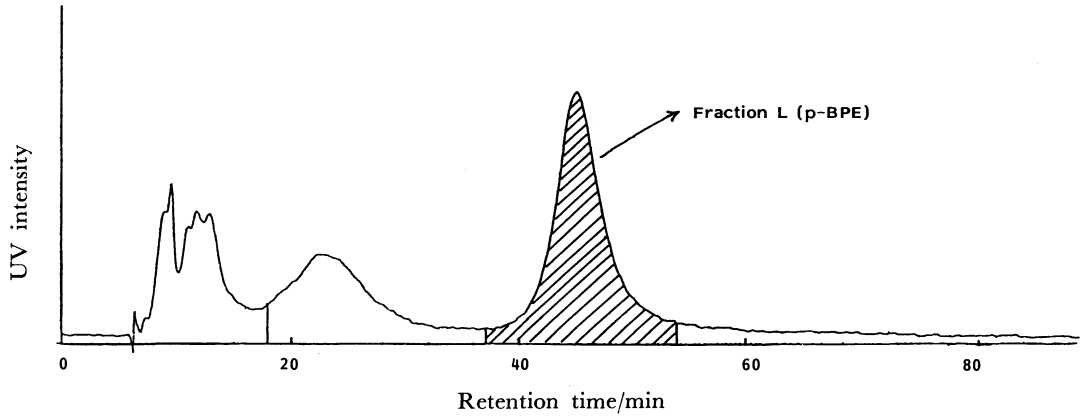


Fig. 5. Preparative HPLC of Fraction K (p-BPE) on the Hitachi Gel #3011 (Table 1). Eluent, MeOH–CH_2Cl_2 (9 : 1).

tion appears to be attributed to a kind of hydrophobic adsorption and/or van der Waals' interaction between the gels and solutes. Examination with p-BPEs of monobasic and dibasic acids suggested that the active principle(s) would probably be dibasic acid(s).[20] All these findings led us to revise the procedure as shown in Table 2 and to obtain a lot of Fraction L (p-BPE) which, if necessary, may be subjected to further purification.

The isolation procedure summarized in Table 2 was improved as follows. a) Initial fractionation of the hatch-stimulating concentrates (Fraction C to F) (Figs. 6 and 7), following the Charcoal–Celite chromatography, proceeded smoothly without loss of the activity and with high recovery of the material by chromatography on columns different from the previous ones; namely, chromatography over silica gel, siliconized Hyflo Super Cel, and ionic exchange resins (Table 1) was replaced by that over Sephadex LH-20 and DEAP-Sephadex LH-20. The Sephadex LH-20 column consists of a kind of methylene-bridged dextran (Sephadex G-25) whose surface (hydroxyl groups) is bonded chemically with 2-hydroxypropyl groups, and is used for gel permeation chromatography as a molecular sieve, including weak interaction (adsorption) between the gels and solutes, and partition chromatography,[29] depending on eluents. The partition chromatography on this column with a 95:5 mixture of dichloromethane and methanol had been used for separation of prostaglandins[29b] as efficiently as that on the siliconized Hyflo Super Cel column. On the other hand, the DEAP-Sephadex LH-20 column[30,31] is packed with Sephadex LH-20 whose surface (hydroxyl groups) is modified chemically by 3-diethylamino-2-hydroxypropyl groups and behaves as a lipophilic anion exchanger. This was effective for group separation of mono-, di-, and polybasic acids as had been shown for separation of fatty acids.[31] b) Efficient *p*-bromophenacylation of the acid mixture [Fraction F to G (p-BPE)] was achieved in the presence of *N,N*-diisopropylethylamine in acetonitrile,[32] giving the corresponding esters in an almost quantitative yield. c) Fractionation of a mixture of the p-BPEs of acids [Fraction G (p-BPE) to I (p-BPE)] was initially performed by preparative HPLC on Hitachi Gel #3019 and then on Bondapak C_{18}/Porasil B columns,[27d] which are recommended for large scale preparative work, because of a large amount (ca. 300 mg) of Fraction G (p-BPE) compared with that (ca. 1 mg) of

Table 2. Isolation of GEA as its p-BPE

Dried and powdered roots of kidney bean "Hon-kintoki" [113 kg (1 ha)]	
↓ Extraction with water (1.5 kl) (<10 °C)	
↓ Evaporation (<30 °C) under reduced pressure	
Concentrates	
↓ Continuous extraction with chloroform at pH 2—3	
Chloroform extracts A	(102 g, active at 10^{-5} g ml^{-1})
↓ EtOAc-aq $NaHCO_3$	
Acidic extracts B	(57 g, active at 10^{-5}–$^{-6}$ g m^{-1})
↓ Charcoal-Celite column (aq acetone)	
Fraction C	(21.5 g, active at 10^{-6} g ml^{-1})
↓ Sephadex LH-20 column (MeOH)	
Fraction D	(10.1 g, active at 10^{-6}–$^{-7}$ g ml^{-1})
↓ DEAP-Sephadex LH-20 column (72% EtOH-AcOH-NH_4OH)	
Fraction E	(2.16 g, active at 10^{-7} g ml^{-1})
↓ Sephadex LH-20 column (CH_2Cl_2-MeOH, 95:5)	
Fraction F	(135 mg, active at 10^{-8} g ml^{-1})
↓ *p*-Bromophenacylation (*p*-$BrC_6H_4COCH_2Br$ and *i*-Pr_2NEt in MeCN, 20 °C)	
↓ Silica-gel column	
Fraction G (p-BPE)	(316 mg, active at 10^{-8} g ml^{-1} after hydrolysis)
↓ Preparative HPLC, Hitachi Gel #3019 (MeOH-CH_2Cl_2, 7:3)	
Fraction H (p-BPE)	(112 mg, active at 10^{-8}–$^{-9}$ g ml^{-1} after hydrolysis)
↓ Preparative HPLC, Bondapak C_{18}/Porasil B (MeOH-H_2O, 8:2)	
Fraction I (p-BPE)	(29 mg, active at 10^{-9} g ml^{-1} after hydrolysis)
↓ Preparative HPLC, μBondapak NH_2 column (C_6H_{14}-CH_2Cl_2-MeCN, 70:18:12)	
Fraction J (p-BPE)	(2.2 mg, active at 10^{-10} g ml^{-1} after hydrolysis)
↓ Preparative HPLC, μBondapak NH_2 column (C_6H_{14}-CH_2Cl_2-MeCN, 80:12:8)	
Fraction K (p-BPE)	(1.2 mg, active at 10^{-10} g ml^{-1} after hydrolysis)
↓ Preparative HPLC, μBondapak NH_2 column (C_6H_{14}-CH_2Cl_2-MeCN, 60:36:4)	
Fraction L (p-BPE) (=GEA p-BPE)	(≈50 μg, active at 10^{-11}–$^{-12}$ g ml^{-1} after hydrolysis)

the corresponding fraction [Fraction I (p-BPE)] in Table 1. These columns, packed with particles of dp ≒50 μm, are similar to Hitachi Gel #3011[27b,28] and μBondapak C_{18}, columns[27b,27c] (dp≒10 μm), respectively, in the chemical constitution and separation selectivity. Of the latter two columns, the μBondapak C_{18} one is packed with silica gel (μPorasil) whose surface is chemically bonded with octadecylsilyl groups and hence hydrophobic, and is useful for high-efficiency reversed-phase partition chromatography, as shown by effective separation of p-BPEs of a variety of fatty acids differing in the number and geometry of double bonds and hydroxyl groups.[33] Thus Fractions G and H (p-BPEs) were separated roughly into several fractions with somewhat loss of active fractions. d) Subsequent preparative HPLC [Fraction I (p-BPE) to L (p-BPE)] on a μBondapak NH_2 column with three different systems of eluents effected smooth separation of active fraction(s), finally giving the relevant fraction, Fraction L (p-BPE). The column[27,34] is packed with silica gel (μPorasil) whose surface is bonded chemically with 3-aminopropylsilyl groups and is used for normal-phase adsorption chromatography. Examination of

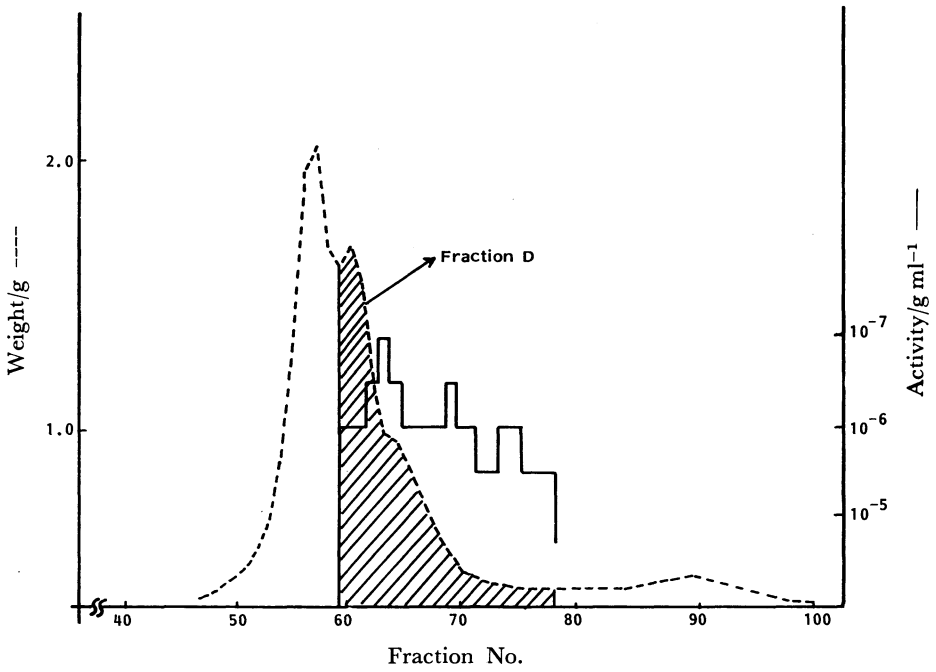


Fig. 6. Column chromatography of Fraction C on the Sephadex LH-20 column (Table 2). Eluent, MeOH. The hatching activity was checked with the eggs.

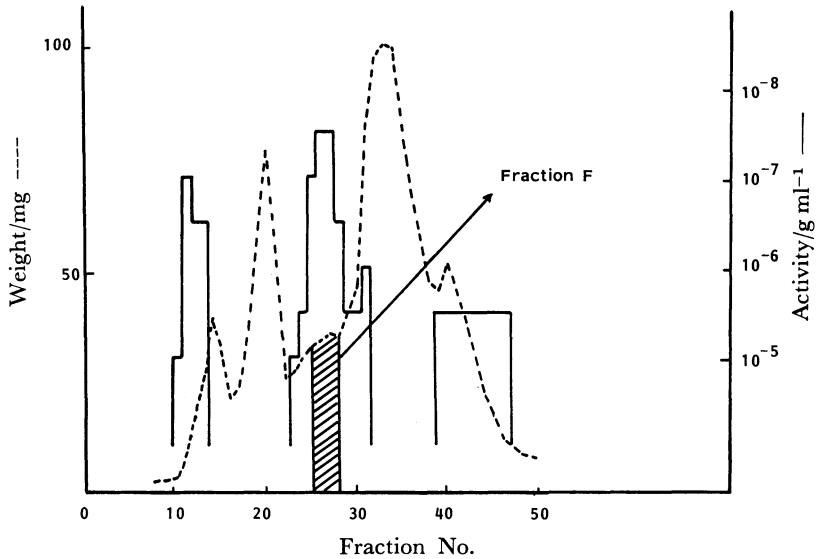


Fig. 7. Column chromatography of Fraction E on the Sephadex LH-20 column (Table 2). Eluent, CH_2Cl_2–MeOH (95 : 5). The hatching activity was checked with the eggs.

model compounds indicated that the separation sequence or selectivity of a series of p-BPEs of fatty acids and their derivatives was influenced remarkably by delicate change of the eluent systems.[20]

In 1981, after the careful chromatographic purification described above, we could achieve the isolation of ca. 50 μg of Fraction L (p-BPE), which showed a single peak by analytical HPLC on μBondapak NH_2, μBondapak C_{18}, and μBondapak/Phenyl columns,[27] and stimulated the hatching at 10^{-11}–$^{-12}$ g ml^{-1} after hydrolysis. With this sample, we could observe a clear triplet, corresponding to the molecular ion peaks, in the FD-MS spectrum[15] and useful signals in the ^1H NMR spectrum.[15] These spectral data led us to assign the molecular formula to GEA and to presume the presence of several oxygen functions.[15] These results, coupled with other evidence discussed later, have led us to believe that Fraction L is the p-BPE of an active principle itself, designated as GEA.[15] However, we could not accomplish the elucidation of the whole structure of GEA, owing to the limited amount (ca. 50 μg) of the isolated GEA p-BPE. It was required to isolate a large amount, at least 500 μg, of GEA p-BPE. Thus the starting hatch-stimulating concentrates (ca. 1 kg) were prepared from dried and powdered roots (ca. 1 ton) of kidney bean, which had been collected in 10 hectares of the field at Memuro, and fractionated, according to the procedure shown in Table 3.

The procedure summarized in Table 3 was practically the same as the previous one (Table 2) in the sequence and efficiency. a) In each of the steps, a dozen or several dozens of chromatographic separations were repeated cautiously and laboriously,

Table 3. Isolation of GEA, GEB, and GEC as their p-BPEs

Dried and powdered roots of kidney bean [1058 kg (10 ha)]
↓ Extraction with water (<10 °C, 15 kl)
↓ Evaporation (<30 °C) under reduced pressure
Concentrates
↓ Continuous extraction with chloroform at pH 2—3
Chloroform extracts A (1011 g, active at 10^{-5} g ml^{-1})
↓ EtOAc-aq $NaHCO_3$
Acidic extracts B (636 g, active at 10^{-5}–$^{-6}$ g ml^{-1})
↓ Charcoal-Celite column (aq acetone)
Fraction C (197.3 g, active at 10^{-6} g ml^{-1})
↓ Sephadex LH-20 column (MeOH)
Fraction D (88.9 g, active at 10^{-6}–$^{-7}$ g ml^{-1})
↓ DEAP-Sephadex LH-20 column (72% EtOH-AcOH-NH_4OH)
Fraction E (18.15 g, active at 10^{-7} g ml^{-1})
↓ Sephadex LH-20 column (CH_2Cl_2-MeOH, 95:5)
Fraction F (3.59 g, active at 10^{-7}–$^{-8}$ g ml^{-1})
↓ Sephadex LH-20 column (CH_2Cl_2-MeOH, 97:3)
Fraction G (951 mg, active at 10^{-8} g ml^{-1})
↓ *p*-Bromophenacylation (*p*-$BrC_6H_4COCH_2Br$ and *i*-Pr_2NEt in MeCN, 20 °C)
↓ Silica-gel column
Fraction H (p-BPE) (2057 mg, active at 10^{-8} g ml^{-1} after hydrolysis)
↓ Hitachi Gel #3019 column (MeOH-CH_2Cl_2, 8:2)
Fraction I (p-BPE) (336 mg, active at 10^{-9} g ml^{-1} after hydrolysis)
↓ Preparative HPLC, μBondapak C_{18} (MeOH-H_2O, 8:2)
Fraction J (p-BPE) (79 mg, active at 10^{-10} g ml^{-1} after hydrolysis)
↓ Preparative HPLC, μBondapak NH_2 (C_6H_{14}-CH_2Cl_2-MeCN, 76:14:10)
→ Fraction K′ (p-BPE) (4.9 mg, inactive ? after hydrolysis)
↓ μBondapak NH_2 (C_6H_{14}-CH_2Cl_2-MeCN, 60:36:4)
Fraction K″ (p-BPE) (1.8 mg, inactive at 10^{-8} g ml^{-1} after hydrolysis)
(=GEC p-BPE)
Fraction K (p-BPE) (8.8 mg, active at 10^{-11} g ml^{-1})
↓ Preparative HPLC, μBondapak NH_2 (C_6H_{14}-CH_2Cl_2-MeCN, 63:33:4)
→ Fraction L′ (p-BPE) (2.6 mg, inactive ? after hydrolysis)
↓ Preparative HPLC, μBondapak NH_2 (C_6H_{14}-CH_2Cl_2-MeCN, 68:13.2:8.8)
Fraction L″ (p-BPE) (1.8 mg, inactive at 10^{-8} g ml^{-1} after hydrolysis)
(=GEB p-BPE)
Fraction L (p-BPE) (1.25 mg, active at 10^{-11}–$^{-12}$ g ml^{-1} after hydrolysis)
(=GEA p-BPE)

because of a large amount of the material to be fractionated. b) Two previous large-scale separation steps (Table 2), which had not always given satisfactory results, were revised as follows. i) Chromatography on the Hitachi Gel #3019 column [Fraction H (p-BPE) to I (p-BPE)] was carried out more easily at atmospheric pressure (Fig. 8) (not at high pressure in Table 2), and ii) preparative HPLC of Fraction I (p-BPE), corresponding to Fraction H (p-BPE) in Table 2, was performed more efficiently on the μBondapak C_{18} column (expensive !) for preparative scale work (Fig. 9) (not on the Bondapak C_{18}/Porasil B one). c) Subsequent fractionation of Fraction J (p-BPE) on the μBondapak NH_2 column afforded Fraction K (p-BPE) as the major fraction, which was active at 10^{-11} g ml^{-1} after hydrolysis, along with the minor one [Fraction K′ (p-BPE)] (Fig. 10). The latter was further purified by preparative HPLC on the same column to give Fraction K″ (p-BPE) [=glycinoeclepin C (GEC) p-BPE] (1.8 mg). The major fraction was further purified by preparative HPLC on the same column with a different eluent

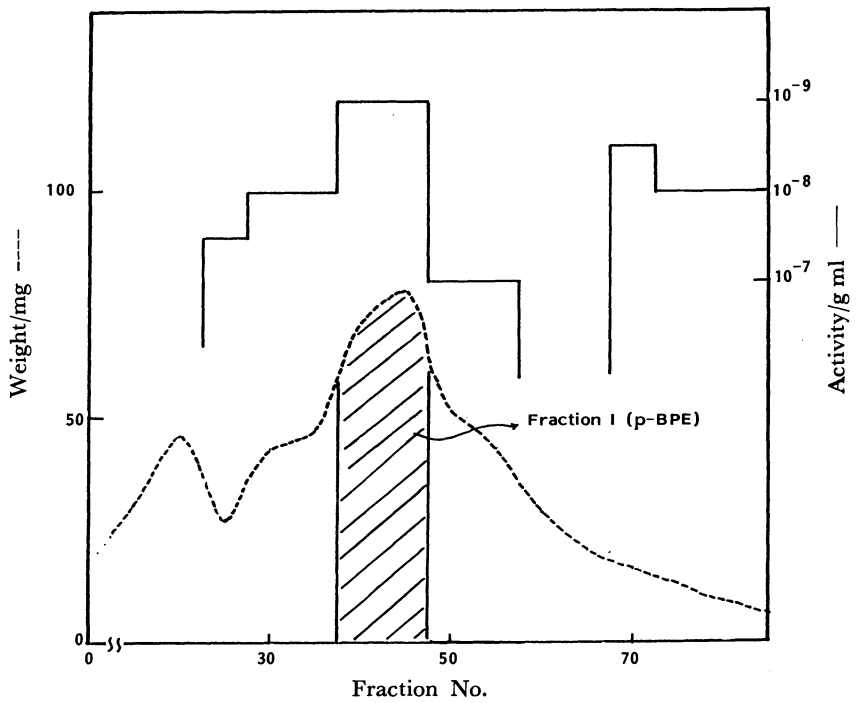


Fig. 8. Column chromatography of Fraction H (p-BPE) on the Hitachi Gel #3019 column (Table 3). Eluent, MeOH–CH_2Cl_2 (8 : 2). The hatching activity was checked with the eggs.

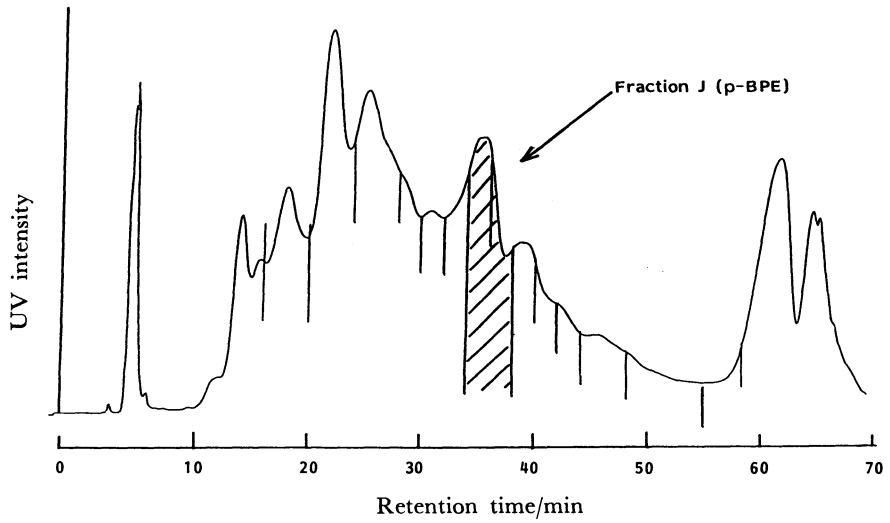


Fig. 9. Preparative HPLC of Fraction I (p-BPE) on the μBondapak C_{18} (Table 3). Eluent: R_t 0—55 min, MeOH–H_2O (8 : 2); R_t 55—70 min, MeOH.

system to give Fraction L (p-BPE) (=GEA p-BPE) (1.25 mg) and Fraction L′ (p-BPE) (Fig. 11). The latter was purified in the same manner to yield Fraction L″ (p-BPE) [=glycinoeclepin B (GEB) p-BPE] (1.8 mg). The p-BPE of GEA was then hydrolyzed with base under mild conditions to give GEA, which, after purification by HPLC on the μBondapak C_{18} column, showed a single peak (Fig. 12) and high activity (active at 10^{-12} g ml^{-1}) for the hatching in water. Likewise, the p-BPEs of GEB and

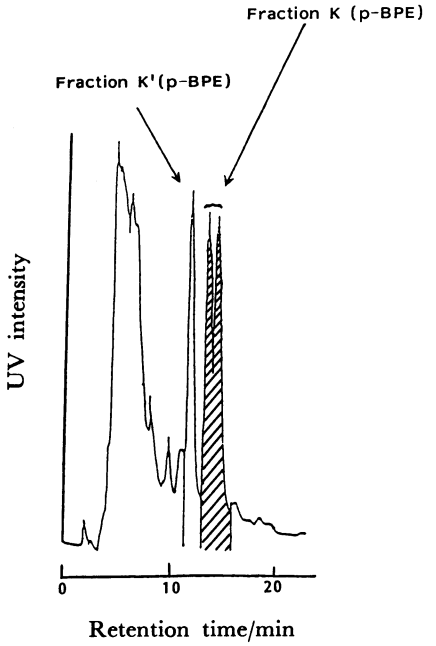


Fig. 10. Preparative HPLC of Fraction J (p-BPE) on the μBondapak NH_2 column (Table 3). Eluent, hexane–CH_2Cl_2–MeCN (76 : 14 : 10).

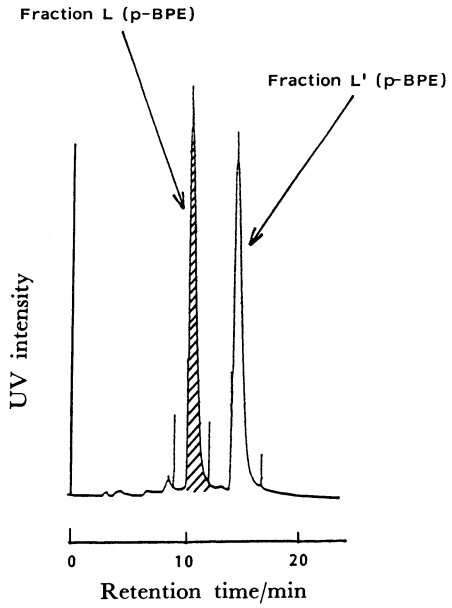


Fig. 11. Preparative HPLC of Fraciton K (p-BPE) on the μBondapak NH_2 column (Table 3). Eluent, hexane–CH_2Cl_2–MeCN (63 : 43 : 3).

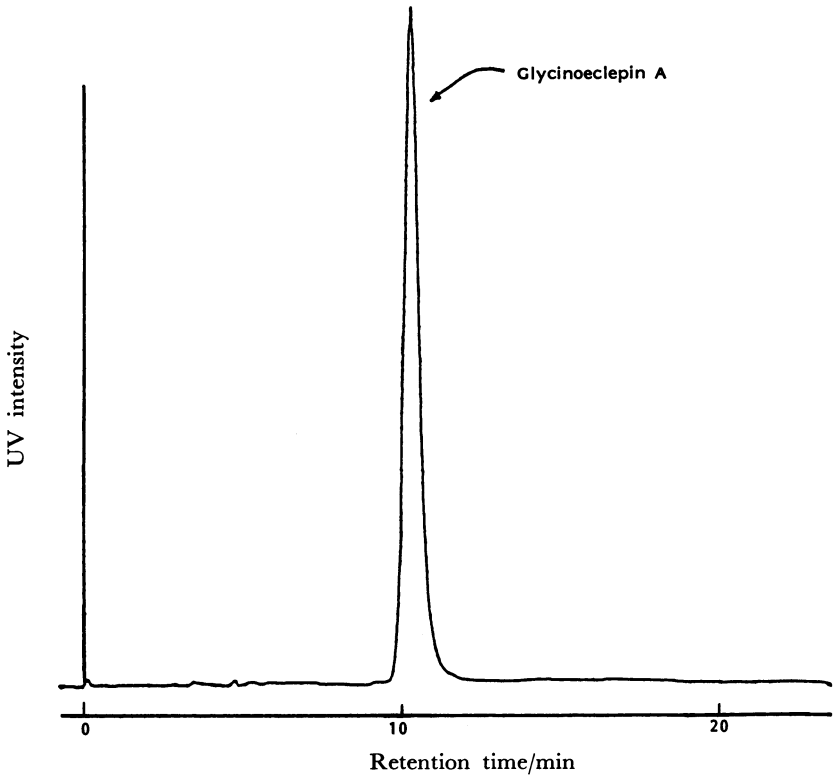


Fig. 12. Analytical HPLC of Glycinoeclepin A on the μBondapak C_{18} column. Eluent, 0.1% AcOH–H_2O and MeOH (35 : 65).

Table 4. Hatching and Larval Emergence of the Soybean Cyst Nematode[a] in an Aqueous Solution of GEA[b]

Test soln	Total eggs	Unhatched eggs	Hatched larvae	
			Unemerged	Emerged
GEA[b]	2158 (8 cysts)	505 (23.4%)[c]	136 (6.3%)	1517 (70.3%)[d]
Water	2875 (9 cysts)	2352 (81.8%)	302 (10.5%)[e]	221 (7.7%)[e]

a) The cysts were soaked at 25 °C and, without dissection, kept in the test soln and in water, respectively, at 25 °C for 10 d. b) A highly dilute soln of GEA (10^{-10} g ml^{-1}) was used. c) Most of the eggs were immature or dead. d) These larvae moved actively. e) These larvae did not move actively, and when kept in the test soln, moved actively.

GEC were converted into the corresponding acids GEB and GEC, which were purified by HPLC on a NOVA-PAK cartridge C_{18}[35] and were not active for the hatching at 10^{-8} g ml^{-1} in water.

Identification of Glycinoeclepin A as the Active Principle. Identification of GEA as the active principle was deduced from the following evidence. i) The fraction isolated always showed a single peak by analytical HPLC over a variety of columns (μBondapak NH_2, MicroPak NH_2-10, μBondapak C_{18}, and μBondapak/Phenyl) with different solvent systems. ii) The fraction was divided into ten fractions by HPLC on a Develosil ODS-5 column. The hatching activity of each divided fraction after hydrolysis paralleled the quantity of the respective fraction, estimated by the UV spectra.[19] iii) The activity (10^{-12} g ml^{-1}) of crystalline GEA p-BPE, obtained by recrystallization from hexane and dichloromethane, was slightly higher than that (10^{-11}—$^{-12}$ g ml^{-1}) of GEA p-BPE contained in the mother liquors after hydrolysis. iv) As described in the preceding section, the fraction was hydrolyzed with base (0.1 M† KOH in MeOH), acidified with acid (0.2 M HCl) to pH 2—3, and extracted with ethyl acetate. The extracts were simply separated by HPLC over μBondapak C_{18} with acidic aqueous methanol to give GEA, which showed a single peak on the column and high activity (10^{-12} g ml^{-1}) (Fig. 12). Surprisingly, the IR spectrum [1572 and 1420 cm^{-1} (KBr)] indicated that GEA was not isolated as the acid itself but as its (potassium) salt. All these facts confirmed that GEA is the active principle itself.

Biological Activity of Glycinoeclepin A. Biological activity of GEA for the hatching and emergence was examined, the result (Table 4) being summarized as follows. The cysts were soaked in water at 25 °C for 10 d, and then kept in a highly dilute solution (10^{-10} g ml^{-1}) of GEA in water at 25 °C for 10 d. In the test solution, ca. 80% of the eggs hatched and most of the hatched larvae emerged out of the cysts, while in water more than 80% of the eggs were not hatched, and less than half (8%) of the hatched larvae emerged out of the cysts. The result indicates that GEA stimulates not only hatching but also emergence of the soybean cyst nematode in a highly dilute solution, and is completely consistent with the Sakurai and Tsutsumi result using the root diffusate of kidney bean.[14] Further examination revealed that (i) most of the unhatched eggs in the test solution were found to be dead or immature under a microscope, and were not hatched by additional addition of the test solution, (ii) the larvae which emerged in the test solution moved actively, while those in water did not move, but moved actively on addition of the test solution, and (iii) naturally, the unhatched eggs in water were hatched when kept in the test solution. These observations imply that the hatching may be only a resultant phenomenon of active motion of the second stage larvae, and GEA may stimulate the motor nervous system of the larvae.

† 1 M=1 mol dm^{-3}.

Experimental

The bioassay was carried out according to the procedure described in the text. The melting points were measured in open capillaries or under a microscope, and uncorrected. Preparative TLC was carried out on silica gel (Wakogel B-5F or Merck Kieselgel 60 F254) and spots were detected by a UV lamp or in iodine vapor unless otherwise stated. The IR and ^{1}H NMR spectra were measured in liquid state and in [^{2}H]chloroform, respectively, unless otherwise stated. Analytical and preparative HPLC were performed at 22 °C with a Varian Aerograph 4200 and a Waters Associates 6000A liquid chromatograph, equipped with a UV monitor (JASCO UVDEC-100), and the chromatograms were recorded on a Unicorder Model-22 M or -225 MB (Nippon Denshi Kagaku Co.). All guranteed solvents (Wako Pure Chemical Ind.) were used as carrier solvents without further purification, unless otherwise stated.

(I) Experimentals Summarized in Table 1. Preparation of Chloroform Extracts A and Acidic Extracts B. Roots of "Beni-kintoki," a subspecies of kidney bean, cultivated in 1 hectare of the field at Memuro, Hokkaido, were collected in late July or in early August just before the flowering time, washed roughly with water, air-dried, and crushed. The powdered roots amounted to 135 kg. A part of the roots (ca. 15 kg) was extracted with water (95 l) below 10 °C for 3 d to be prevented from putrefaction. After being squeezed, the roots were again extracted with water (2×60 l). The combined aqueous (aq) muddy suspension was concentrated

to ca. 10 l below 30 °C by a thin film evaporator (Hitachi Kontro). This process (extraction and concentration) was repeated nine times. All the concentrates (ca. 100 l) were combined and made acidic to pH 2—3 with 6 M hydrochloic acid (HCl). A part of the aq suspension (ca. 40 l) was extracted with chloroform (ca. 60 l) continuously for 50 h by a special Soxhlet apparatus (Shibata Chemical Apparatus Mfg. Co.). This extraction procedure was repeated three times. The combined chloroform solution (soln) was evaporated to leave an oily material (223 g), which was distilled under reduced pressure below 50 °C to remove inactive lower fatty acids, giving Chloroform extracts A (115 g).

The fraction (115 g) was dissolved in ethyl acetate (300 ml) and treated with saturated aq sodium hydrogencarbonate ($NaHCO_3$) (600 ml+4×50 ml). The acetate soln was washed with water (50 ml), dried and evaporated to leave neutral and basic substances, which were not further examined. The combined aq soln (ca. 850 ml) was acidified to pH 2 by addition of 6 M HCl (140 ml) and extracted with ethyl acetate (5×200 ml). The combined acetate soln was washed with water (50 ml) and evaporated to give Acidic extracts B (66.5 g), which was active at 10^{-5}—$^{-6}$g ml^{-1} for the hatching. The acidic aq soln was further extracted with 1-butanol (3×300 ml). The butanol soln was evaporated to leave an acidic oil (7.9 g), which was active at 10^{-5} g ml^{-1} and was not further examined.

Chromatography on a Charcoal–Celite Column (B to C). Charcoal (220 g, Tokusei-Shirasagi Brand, Takeda Chemical Ind.) and Celite-545 (440 g, Johns-Manville) were mixed thoroughly with water and packed in a glass column (7×64 cm). A part (22 g) of Acidic extracts B was dissolved in acetone, mixed with Celite (40 g), evaporated, placed on the top of the column, and then eluted successively with water (2.2 l), water–acetone (9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8 and 1:9, v/v, each 2.2 l), acetone (4×2.2 l), benzene (2×2.2 l), and methanol–concd aqueous ammonia (NH_4OH) (21:1, v/v, 4.4 l). This procedure was repeated three times. Each fraction, other than that eluted with ammoniacal methanol, was evaporated to give an oily or solid eluate. The methanol fraction was dissolved in water (100 ml), acidified and extracted with 1-butanol, and the butanol soln was likewise evaporated. The amount and biological activity of each eluate is described in Fig. 1. The 80% aq acetone eluate (5.2 g) (Fraction C), showing the highest activity (active at 10^{-7} g ml^{-1}), was submitted to further separation. Each eluate, even if inactive, was examined in somewhat detail.

i) Eluates with 40% Aqueous Acetone. The oily eluate (950 mg) was separated into 6 fractions by preparative TLC with benzene–1-butanol–acetic acid (16:3:1): Fraction (Fr.) 1, (R_f=0.7) 125 mg: 2, (0.4) 337; 3, (0.3) 253; 4, (0.2) 40; 5, (0.15) 36; 6, (0.05) 29 mg. To an ethanol soln (10 ml) of Fr. 2 (305 mg), adjusted to pH 6.8 with 0.1 M aq potassium hydroxide (KOH) and one drop of 0.1 M HCl, was added *p*-bromophenacyl bromide (1 g) in ethanol (25 ml). The mixture was heated under reflux for 1.5 h,[36] cooled and extracted with chloroform (4×10 ml). The chloroform soln was worked up as usual to give orange crystals (1048 mg), which were roughly separated by column chromatography over silica gel (Merck 120—230 mesh, 10 g, 1.8×9.5 cm) with chloroform into 3 fractions. The third eluate gave a crystalline mixture of p-BPEs (577 mg), from which 2-hydroxy-4-methylpentanoic acid p-BPE (90 mg), mp 86.5—88 °C ($CHCl_3$–hexane), $[\alpha]_D$ −5° ($CHCl_3$), and 2-hydroxy-3-methylpentanoic acid p-BPE (125 mg), mp 35—39 °C ($CHCl_3$), $[\alpha]_D$ −12° ($CHCl_3$), were isolated by twice preparative TLC with chloroform–benzene (9:1, double development). The IR and ^1H NMR spectra of these esters were identical with those of the p-BPEs of the authentic samples, respectively.[37,38] Fr. 4 was purified by chromatography over silica gel with ethyl acetate to give crude vomifoliol (9 mg), which will be described later.

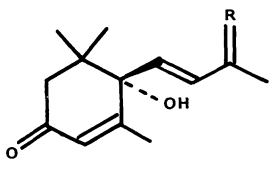
ii) Eluates with 50% Aqueous Acetone. A part (2.05 g) of the solid eluate was separated into 6 fractions by preparative TLC with a 1:4 mixture of ethyl acetate and an upper phase of benzene–propionic acid–water (8:3:5): Fr. 1, (R_f=0.8) 600 mg; 2, (0.6) 327; 3, (0.4) 216; 4, (0.25) 130; 5, (0.2) 161; 6, (0.1) 128. Fr. 1 (25 mg) gave 3- furoic acid (10 mg), mp 120—120.5 °C, on recrystallization from water. Fr. 2 (100 mg) was separated into 3 fractions by preparative TLC with an upper phase of benzene–acetic acid–water (3:3:1). The lst fraction (R_f=0.6) gave a crystalline substance (25 mg), which on recrystallization from water afforded suberic acid (3 mg), mp 135—137°C. The mother liquor (18 mg), after sublimation, gave 2-furoic acid (4 mg), mp 128—129 °C. The 2nd fraction (R_f=0.5) gave a crystalline substance (18 mg), which on recrystallization from water yielded maltol (3 mg), mp 158—159 °C.

Fr. 5 (150 mg) was purified by preparative TLC with ethyl acetate to give a crystalline substance (55 mg), which on recrystallization from benzene gave vomifoliol (i) (24 mg), mp 112—113°C; $[\alpha]_D$ +220° ($CHCl_3$); CD, λ_{max}^{MeOH} ($\Delta\varepsilon$) 318 nm (−0.65) and 240 (+11.9); UV (MeOH), 237 nm (ε 13900); IR (Nujol), 3400, 1662, 1617, and 973 cm^{-1}; ^1H NMR, δ=1.01 and 1.07 (each 3H, s), 1.28 (3H, d, J=6 Hz), 1.88 (3H, d, J=1.5 Hz), 2.07 (2H, 2OH, D_2O-exchangeable), 2.23 and 2.42 (each 1H, ABq, J=17 Hz), 4.38 (1H, dq, J=6 and 3.5 Hz), 5.75 (1H, d, J=16 Hz), 5.83 (1H, dd, J=16 and 3.5 Hz), and 5.88 (1H, q, J=1.5 Hz); MS m/z 206 (M^+ −18), 168, and 124 (base). This was identified by direct comparison with (+)-vomifoliol[39–43] (blumenol A) from *Podocarpus blumei* Endl.[41]

Fr. 3 (130 mg) was purified by preparative TLC with ether–benzene (9:1) and then with ethyl acetate to give dehydrovomifoliol (ii) (11 mg), oil; $[\alpha]_D$ +159°($CHCl_3$); CD, λ_{max}^{EtOH} ($\Delta\varepsilon$) 318 nm (−1.95), 240 (+27.6); UV (EtOH), 237 nm (ε 20000); IR ($CHCl_3$), 3400, 1666, 1626, and 982 cm^{-1}; ^1H NMR, δ=1.01 and 1.09 (each 3H, s), 1.87 (3H, d, J=1.5 Hz), 2.29 (3H, s), 2.34 and 2.48 (each 1H, ABq, J=17 Hz), 5.95 (1H, q, J=1.5 Hz), and 6.45 and 6.83 (each 1H, ABq, J=16 Hz); MS, m/z 166 (M^+ −56) and 124 (base). These spectral data indicated the compound (**ii**) to be a dehydro derivative of vomifoliol (**i**). This was confirmed by conversion of **i** into **ii** as follows. To a soln of **i** (23 mg) in acetone (0.5 ml) was added Jones reagent [CrO_3 (10 mg), H_2SO_4 (0.01 ml), and H_2O (0.03 ml)]. The mixture was stirred for 3 min, diluted with water (2 ml), and extracted with ethyl acetate (3×1 ml). The combined acetate soln was evaporated to leave an oil (22 mg), which was purified by preparative TLC with benzene–ethyl acetate (1:1) to yield **ii**. The compound (**ii**), named (+)-dehydrovomifoliol,[42–46] appeared to be a new compound isolated from natural sources, although its racemate had been prepared by two groups.[39,47,48] (+)-Dehydrovomifoliol (**ii**) was converted

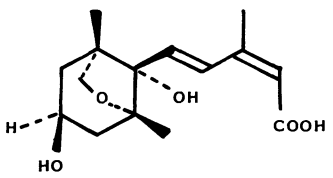
into (+)-abscisic acid in the same manner[47] as the synthetic racemate[47,48] as follows. A mixture of **ii** (18 mg) and (ethoxycarbonylmethylene)triphenylphosphorane (29 mg) in toluene (1 ml) was heated under reflux for 4 h and distilled to remove the toluene. The residue was heated at 150—170 °C for 40 min, cooled, and separated by preparative TLC with benzene–ethyl acetate (1:1) to give a mixture (9 mg) of esters. The mixture (8 mg) was hydrolyzed with 2M methanolic KOH (6 ml) at room temperature (temp) for 23 h. The reaction mixture was evaporated, acidified with 1.5 M HCl (4 ml), and extracted with ether (3×2 ml). The ether soln gave a 1:1 mixture[39] of (+)-abscisic acid[49] and its 2-*trans*-isomer:[47,48] CD, $\lambda_{max}^{0.006\,M\,HCl\,in\,EtOH}$ ($\Delta\varepsilon$) 318 nm (−0.92), 265 (+28.2), and 230 (−21.0); UV (0.006 M HCl in EtOH), 262 nm (ε 21000). These spectral values indicated that (+)-dehydrovomifoliol (**ii**) possessed the same absolute configuration at the carbon atoms corresponding to C-6 of abscisic acid.[39]

Fr. 6 (80 mg) was purified by preparative TLC with benzene–1-butanol–acetic acid (14:5:1) to give dihydrophaseic acid (**iii**) (34 mg), amorphous; CD, λ_{max}^{MeOH} ($\Delta\varepsilon$) 265 nm (−2.8); UV (MeOH), 259 nm (ε 15500); ^{1}H NMR (CD_3COCD_3), δ=0.90 and 1.06 (each 3H, s), 1.80 (4H, br), 1.95 (3H, d, J=1.5 Hz), 3.60 (1H, d, J=7.5 Hz), 3.73 (1H, dd, J=7.5 and 1.5 Hz), 4.10 (1H, br), 5.66 (1H, br s), and 6.54 and 8.06 (each 1H, d, J=16 Hz); MS, m/z 282 (M^+) and 264. The acid (**iii**) was converted with ethereal diazomethane into its methyl ester, amorphous; IR ($CHCl_3$), 3420, 1710, 1674, and 1610 cm^{-1}; ^{1}H NMR, δ=0.95 and 1.17 (each 3H, s), 1.80 (4H, br), 2.05 (3H, d, J=1.5 Hz), 3.72 (3H, s), 3.77 (2H, br), 4.26 (1H, m, W_H=20 Hz), 5.73 (1H, q, J=1.5 Hz), 6.40 and 7.98 (each 1H, d, J=16 Hz); MS, m/z 296 (M^+), 278, 264, 247, 246, 154, 125, 122, 94, and 43 (base). The ester was oxidized with Jones reagent as follows. To a soln of the ester (6 mg) in acetone (0.05 ml) was added Jones reagent [CrO_3 (5 mg), H_2SO_4 (0.005 ml), and H_2O (0.02 ml)] for 3 min. The mixture was diluted with water (0.3 ml) and extracted with ethyl acetate (3×0.3 ml). The acetate soln was dried and evaporated to leave an oil (3 mg), which was separated by preparative TLC with benzene–ethyl acetate (1:1) to give the dehydro ester (1 mg), mp 152—155 °C (benzene); CD, λ_{max}^{MeOH} ($\Delta\varepsilon$) 314 nm (−0.55), 262 (+5.2), and 230 (−4.2); ^{1}H NMR, δ=2.50 and 2.64 (each 2H, br s), 3.73 (1H, d, J=8 Hz), and 3.95 (1H, dd, J=8 and 1.5 Hz); MS, m/z 294 (M^+), 276, 121, and 43 (base). The ester was identified as methyl phaseate[49-51] by comparison of the spectral data (MS, IR, and ^{1}H NMR), and hence compound **iii** was named dihydrophaseic acid.[52]



i R=—H, ---OH

ii R==O



iii

iii) Eluates with 60% Aqueous Acetone. The solid eluate (453 mg) was separated into 6 fractions by preparative TLC with an upper phase of benzene–acetic acid–water (8:3:5): Fr. 1, (R_f=0.4) 178 mg; 2, (0.3) 35; 3, (0.25) 97; 4, (0.15) 28; 5, (0.1) 37; 6, (0.0) 16. Frs. 1 (167 mg) and 2 (30 mg) gave benzoic acid (160 mg) and vanillic acid (19 mg) after sublimation, respectively. Fr. 3 (90 mg) was purified by preparative TLC with ethyl acetate–chloroform–acetic acid (15:5:1) and recrystallized from water to give azelaic acid (46 mg). Fr. 5 (30 mg) was crystallized and recrystallized from chloroform to give *p*-hydroxybenzoic acid (2 mg).

iv) Eluates with 70% Aqueous Acetone. Benzoic, vanillic, and *p*-hydroxybenzoic acids were detected, when the eluate was developed on a silica-gel plate with an upper phase of benzene–acetic acid–water (8:3:5).

v) Eluates with 90% Aqueous Acetone. The solid eluate (2.9 g) was separated by chromatography over silica gel (Merck 120—230 mesh, 300 g, 5×42.5 cm) with ethyl acetate–chloroform–acetic acid (15:5:1). The eluates (each 15 ml) were collected, evaporated and assayed: Frs. 1—36, (537 mg) inactive; 37—41, (506) active at 10^{-6} g ml^{-1}; 42—68, (1033) active at 10^{-7}—$^{-8}$; 69—88, (285) active at 10^{-6}; 89—200, (257), inactive. Fr. 33 (90 mg) was separated into 5 fractions by preparative TLC with an upper phase of benzene–acetic acid–water (8:3:5, developed twice): Fr. 1′, (R_f=0.50) 34 mg; 2′, (0.45) 12; 3′, (0.35), 8; 4′, (0.25) 12; 5′, (0.10) 18. Fr. 1′ (30 mg) gave salicylic acid (8 mg) after sublimation in twice and recrystallization from benzene. Fr. 2′ (10 mg) afforded dodecanedioic acid (4 mg) after recrystallization from chloroform.

vi) Eluates with Acetone, Benzene, and Ammoniacal Methanol. Salicylic acid was detected on a silica-gel plate with ethyl acetate–chloroform–acetic acid (15:5:1).

Chromatography on a Silica-Gel Column (C to D).[53] Fraction C (5.2 g) was separated by chromatography over silica gel (Merck 70—325 mesh, 520 g, 5×66.5 cm) with ethyl acetate–chloroform–acetic acid (15:5:1, v/v/v). The eluates (each 19.5 ml) were collected, evaporated and assayed. The amount and activity of each or collected eluates are summarized as follows: Frs.31—39, (55 mg) inactive; 40—55, (2468) inactive; 56—57, (203) active at 10^{-6} g ml^{-1}; 58—60, (241) active at 10^{-7}; 61—67, (360) active at 10^{-8}; 68—83, (402) active at 10^{-7}; 84—100, (231) active at 10^{-6}; 101—140, (384) active at 10^{-6}; 141—154, (97) inactive; 155—226, (307) inactive. Frs. 61—67 (360 mg) (Fraction D) was the most active and submitted to further separation. Other inactive or less-active fractions were examined in somewhat detail.

i) Fraction 45. The fraction (194 mg) was separated into 5 fractions by preparative TLC with an upper phase of benzene–acetic acid–water (8:3:5): Fr. 1, (R_f=0.45) 53 mg; 2, (0.35) 46; 3, (0.25) 29; 4, (0.10) 28; 5, (0.03) 20. Fr. 2 was treated with ethereal diazomethane and analyzed by GC-MS; column, 5% diethylene glycol adipate polyester on Anakrom Q. Sebacic, undecanedioic acid (ca. 90%), and dodecanedioic acids were identified.

ii) Fraction 53. The fraction (145 mg) was separated into

8 fractions by preparative TLC (twice development) in the same manner as Fr. 45: Fr. 1, (R_f=0.50) 2 mg; 2, (0.45) 2; 3, (0.35) 11; 4, (0.30) 10; 5, (0.25) 35; 6, (0.15) 29; 7, (0.05) 19; 8, (0.00) 19. Fr. 1 gave veratric acid, and Fr. 3 was found to consist of sebacic (trace), undecanedioic (ca. 90%), and dodecanedioic acids by GLC of the trimethylsilyl derivatives (2% OV-17 on Anakrom Q). Fr. 4 gave syringic acid (3 mg) after chromatography over silica gel. Fr. 5, when recrystallized from diisopropyl ether–hexane, gave 4-hydroxydodecanedioic acid γ-lactone (13 mg), mp 48.5—50 °C, which was converted with diazomethane into its methyl ester, mp 20—22 °C: acid, IR ($CHCl_3$), 1760, 1705, and 1170 cm^{-1}: ester, ^1H NMR, δ=1.2—1.9 (14H, br), 2.26 (2H, t, J=7 Hz), 2.5 (2H, m), 3.60 (3H, s), and 4.46 (1H, m, W_H=18 Hz); MS, m/z 242 (M^+), 224, 211, 192, 151, and 85 (base). Frs. 6—8 were found to consist of a mixture of long-chain fatty acids on the basis of their NMR spectra.

iii) Fractions 101—154. The fractions (480 mg) were crystallized from methanol–ethyl acetate and recrystallized from acetic acid to give 9,10,13-trihydroxyoctadecanoic acid (16 mg), mp 139—139.5 °C, which formed its methyl ester, (with diazomethane), mp 117.5—119.5 °C, and trimethylsilyl derivative [with trimethylsilyl *N*-(trimethylsilyl)acetimidate]. The planar structure was elucidated on the basis of the spectral data and chemical reactions.[54] The filtrate (465 mg) was dissolved in ethanol (2 ml). To the soln, adjusted to pH 6.6 with 0.1 M aq KOH and a few drops of 0.1 M HCl, was added *p*-bromophenacyl bromide (607 mg) in ethanol (2 ml). The mixture was heated under reflux for 2.5 h,[36] cooled, concentrated, diluted with water (15 ml), and then extracted with chloroform (2×10 ml). The chloroform soln was worked up as usual to leave an oily residue (1.0 g), which was roughly separated by chromatography over silica gel (Merck 120—230 mesh, 10 g, 2×6.4 cm), with benzene (950 ml), benzene–chloroform (1:1, 600 ml), chloroform (200 ml), and ethyl acetate (200 ml). A fraction eluted with ethyl acetate afforded a mixture of p-BPEs (563 mg), which was further separated into more than 215 fractions [Frs. 1′—137′ (each eluent 9.1 g) and Frs. 138′—215′, (each eluent 14.7 g)] by chromatography over silica gel containing 10% silver nitrate (Merck 120—230 mesh, 70 g, 2×40 cm) with a mixture of chloroform, ethyl acetate, and methanol as eluents.

Frs. 111′—135′ (81 mg), eluted with chloroform–ethyl acetate (2:3), was crystallized and recrystallized from ethyl acetate containing a trace of methanol to give (11*E*)-9,10,13-trihydroxy-11-octadecenoic acid p-BPE (20 mg), mp 127—128 °C. This planar structure was deduced from the following experiment. The ester (7.6 mg) was hydrolyzed with 0.1 M KOH in methanol (2 ml) at room temp for 24 h. The mixture was evaporated, acidified with 3M HCl and extracted with 1-butanol (2×2 ml). The butanol extracts were treated with ethereal diazomethane in methanol to give a mixture of its methyl ester and methyl *p*-bromobenzoate, which was submitted to chromatography over silica gel (Merck 120—230 mesh, 2 g, 1×7 cm) with chloroform and/or ethyl acetate. Eluates with ethyl acetate was evaporated and recrystallized from ethyl acetate to give its methyl ester (3.1 mg), mp 88.5—90.5 °C; IR (KBr), 3270, 1735, and 968 cm^{-1}; ^1H NMR, δ=0.90 (3H, br t, J=7 Hz), 1.2—1.8 (23H, br, 20H on addition of D_2O), 2.30 (2H, t, J=7 Hz), 3.60 (1H, br), 3.66 (3H, s), 4.12 (2H, br), and 5.77 (2H, m, W_H=9 Hz). The methyl ester (0.5 mg) was treated with trimethylsilyl *N*-(trimethylsilyl)acetimidate (40 μl) at 55 °C for 5 min. The mixture was mixed with water (100 μl) and extracted with hexane (2×100 μl). The combined hexane soln was washed with water (30 μl) and evaporated to leave its tris(trimethylsilyl ether), oil, which showed the following MS spectrum: m/z 545 (M^+−15), 460 [M^+−$C_6H_{12}O$ (hexanal)], 455, 439, 399, 387, 301, 297, 259, 227, 173 (base), 155 [$^+O{\equiv}C(CH_2)_7CHO$], 147, 129, 103, 83, 75, and 73.[54] On the other hand, the methyl ester was oxidized with potassium periodate[55] as follows. A soln of the ester (not of the ether) (0.23 mg) in acetic acid (0.5 ml) was combined with 0.02 M aq potassium periodate (0.05 ml) and stirred at room temp for 30 min. To the soln was added ethylene glycol (0.005 ml) in water (0.02 ml) and then 0.4% 2,4-dinitrophenylhydrazine in 4 M HCl (0.5 ml). The mixture was kept at 2 °C for 3 d, and the precipitates (ppts) were collected by filtration, washed with water and dried. The hydrazone mixture was proved to consist of methyl 9-oxononanoate 2,4-dinitrophenylhydrazones by HPLC (TSK-G1000H8) and TLC by comparison of the authentic samples,[56] which were prepared from 9-oxononanoic acid[57] by the known procedure.

Frs. 181′-200′ (65 mg), eluted with chloroform–ethyl acetate (1:4), were crystallized and recrystallized from ethyl acetate to give (15*Z*)-9,10,13-trihydroxy-15-octadecenoic acid p-BPE (21 mg), mp 112—113 °C. ^1H NMR, δ=0.97 (3H, t, J=7 Hz), 1.35—1.64 (16H, br), 2.07 and 2.28 (each 2H, m), 2.48 (2H, t, J=7 Hz), 2.61 (3H, br s, D_2O-exchangeable), 3.62 (3H, br), 5.27 (2H, s), 5.48 (2H, m), and 7.60 and 7.76 (each 2H, d, J=9 Hz). This p-BPE was converted into the corresponding methyl ester, which was further derived into the tris(trimethylsilyl ether): The methyl ester, oil; IR (neat), 3300 and 1735 cm^{-1}: the ether, oil; MS, m/z 529 (M^+−31), 491, 455, 439, 401, 389, 311, 301, 299, 259, 227, 171, 155, 147, 129, 103, 81, 75, and 73 (base).[54]

Fr. 216′ (6 mg), eluted with ethyl acetate, and Fr. 217′ (19 mg), eluted with ethyl acetate and methanol (9:1), were combined, crystallized and recrystallized from ethyl acetate to give (11*E*, 15*Z*)-9,10,13-trihydroxy-11,15-octadecadienoic acid p-BPE (3 mg), mp 114—115 °C; ^1H NMR, δ=0.96 (3H, t, J=7 Hz), 1.35—1.62 (12H, br), 2.07 and 2.28 (each 2H, m), 2.47 (2H, t, J=7 Hz), 2.60 (3H, br, s, D_2O-exchangeable), 3.60 (1H, br), 4.12 (2H, br), 5.27 (2H, s), 5.47 (2H, br), 5.78 (2H, m, W_H=9Hz), and 7.60 and 7.76 (each 2H, d, J=9 Hz). The p-BPE was converted into the corresponding methyl ester, which was further derived into the tris(trimethylsilyl ether): The methyl ester, oil; IR (neat), 3360, 1735, and 973 cm^{-1}; the ether, oil; MS, m/z 543 (M^+−15), 460 [M^+−$C_6H_{10}O$ (hexenal)], 453, 437, 399, 387, 299, 297, 259, 171, 155, 147, 129, 103, 81, 75, and 73 (base).[54]

Chromatography on a Silica-Gel Column (D to E). Fraction D (360 mg) was separated by chromatography over silica gel (Merck 70—325 mesh, 55 g, 2×28.5 cm) with ethyl ether–acetic acid (99:1, v/v). The eluates (each 6 ml) were collected, evaporated, and assayed: Frs. 1—33, (3 mg) inactive; 34—44, (127) inactive; 45—62, (117) active at 10^{-8} g ml^{-1}; 63—74, (28) active at 10^{-7}; 75—120, (12) active at 10^{-6}—$^{-7}$. The fractions 45—62 (Fraction E) was the most active and submitted to further separation.

Reversed-Phase Partition Chromatography on a Siliconized Hyflo Super Cel Column (E to F). Hyflo Super Cel

(Johns-Manville; Nakarai Chemicals) was washed successively with 6 M HCl, distilled water and methanol, dried at 110 °C for 46 h, cooled, and kept in a desiccator containing dimethyldichlorosilane for 10 d. The siliconized Hyflo Super Cel was washed with methanol until the washings became neutral, and dried at 110 °C for 3 h.[58] The hydrophobic Hyflo Super Cel (18 g) thus obtained was mixed with a lower (stationary) phase (16 ml) of methanol–water–acetic acid–chloroform–(2-ethyl-1-hexanol) (150:150:2:15:15, by vol.) for 3 min. To the mixture was added the upper (mobile) phase (50 ml).[59] The slurry mixture was poured into a glass tube (12 mm in diameter), homogenized by rapid up and down movement of a stainless plunger. The resulting fine suspension was then packed in the tube to give a column of 33 cm in length.[58] Fraction E (115 mg) dissolved in the mobile phase (1 ml) was separated by chromatography on the column with the same solvent mixture. The eluates (each 20 ml) were collected, evaporated in vacuo, and assayed. The amount and activity of each or collected eluates are summarized in Fig. 2. As shown in Fig. 2, a large amount of inactive substances were eluted in early fractions (Frs. 1—10), and two groups of active fraction [Frs. 11—22 (23.9 mg) and Frs. 24—28 (4.6 mg)] were then eluted separately. The former active fractions were combined (Fraction F, active at 10^{-8}—$^{-9}$ g ml^{-1}, 23.9 mg) and submitted to further separation. The inactive fractions were examined in somewhat detail.

Fractions 5 and 6. The fractions (16.7 mg) gave a crystalline mixture, which was again purified by chromatography on the hydrophobic Hyflo Super Cel (18 g) under the same conditions described above to yield hydroxy dibasic acid (11.8 mg), mp 149—153 °C. This acid was converted easily into its dimethyl ester, oil; IR ($CHCl_3$), 1726 cm^{-1}; ^{1}H NMR, δ=1.2—1.7 (14H, br), 1.9 (1H, br s, D_2O-exchangeable), 2.32 (2H, t, J=7 Hz), 2.45 (2H, m), 3.69 and 3.73 (each 3H, s), and 3.9 (1H, br); MS, m/z 225 (M^+−31−18), 211 (M^+−31−32), 210 [(OH)CH$(CH_2)_8$COOCH$_3$], 193 (201—18), 172 [$(CH_2)_8$COOCH$_3$+H], 169 (201—32), 151 (193—42), 143, 129, 103 [base, (OH)CHCH$_2$COOCH$_3$], 87, and 74. The acid was identified as 3-hydroxydodecanedioic acid by comparison with a racemic, synthetic sample,[60] mp 105—107 °C.

Chromatography on an Amberlyst-15 (Ag^+ form) Column (F to G). Amberlyst-15 (Rohm & Haas; Organo Ltd., 9.0 g) was washed with ethanol in a Soxhlet apparatus for 20 h, packed into a glass tube (1.0 cm in diameter), and washed with distilled water. The resin was then converted into the silver form by passing 5% aq silver nitrate ($AgNO_3$), until silver cations were detected in the effluent (ca. 130 ml). The resin was then washed with distilled water thoroughly, when silver ions had not been detected, and then with ethanol.[23] Fraction F (23.1 mg) in ethanol was placed on the column, and eluted with ethanol (total 18 ml) and then with 5% cyclohexene in ethanol (total 50 ml). All eluates were collected in 2 ml portions. The odor of cyclohexene was detected in the fractions after Fr. 19. Frs. 4—10, 11—18, and 19—34 were combined, evaporated and assayed, respectively: Frs. 4—10, (14.4 mg) active at $10^{-8\sim-9}$ g ml^{-1}; 11—18, (0.5) active at 10^{-6}; 19—34, (3.0) active at 10^{-6}. The second fraction (Frs. 4—10) (Fraction G) was the most active and submitted to further separation. Less active fractions were examined in somewhat detail.

The Fourth Fraction (Frs. 19—34) gave a crystalline mixture (0.3 mg), which was treated with diazomethane in ether, evaporated, and then treated with trimethylsilyl *N*-(trimethylsilyl)acetimidate (25 μl) for 24 h. The soln was mixed with water (50 μl) and extracted with hexane (2×50 μl). The hexane soln was washed with water (20 μl), evaporated to leave an oily residue, whose MS spectrum [m/z 455 and 453 (M^+−15), 439 and 437 (M^+−31)] indicated that the fraction would contain dihydroxyoctadecadienoic and dihydroxyoctadecatrienoic acids.

Chromatography on an Amberlyst XN-1005 (Ag^+ form) Column (G to H). Amberlyst XN-1005 (Rohm & Haas; Organo Ltd., 100 g) placed in a large column was washed successively with 1 M aq sodium hydroxide (NaOH) (600 ml), distilled water (600 ml), 1 M HCl (1200 ml), distilled water (600 ml), 95% ethanol (600 ml), and distilled water (600 ml). This procedure was repeated three times. The purified resin was converted into the silver form by addition of 5% aq $AgNO_3$ (1200 ml) and washed with distilled water, until silver ions were not detected in the effluent, and then with methanol. The resin thus treated was packed into a long glass column (1.0 cm in diameter) to form a bed of 216 cm in length.[25] Fraction G (14.4 mg) in methanol was placed on the column and eluted with methanol to give 46 fractions (each 5 ml). An aliquot (2 μl) of each fraction was assayed. Active fractions [Frs. 28—33, (0.90 mg) active at 10^{-9}—$^{-10}$ g ml^{-1}, and Frs. 34—39, (0.63) active at 10^{-9}] were combined to amount to 1.53 mg (Fraction H) and submitted to further purification.

***p*-Bromophenacylation [H to I (p-BPE)].** A mixture of Fraction H (1.53 mg), potassium hydrogencarbonate ($KHCO_3$) 2.7 mg, 0.027 mmol), *p*-bromophenacyl bromide (9.4 mg, 0.034 mmol), and dicyclohexano-18-crown-6 (1.31 mg, 0.0035 mmol) in acetonitrile (60 μl) was heated in a sealed tube at 75 °C for 30 min,[26] cooled, and evaporated to remove the acetonitrile. The residue was dissolved in a small volume of benzene, placed on a silica-gel column (Merck 120—230 mesh, 3 g, 1.3×5.5 cm), and then eluted successively with benzene (Fr. 1), chloroform (Fr. 2), chloroform–ethyl acetate (9:1 and 1:1) (Frs. 3 and 4), ethyl acetate (Fr. 5), and ethyl acetate–acetic acid (9:1) (Fr. 6) to give 6 fractions (each 15 ml). An aliquot (3 μg) of each of Frs. 1—5 was hydrolyzed with 0.1 M KOH in methanol (25 μl) at room temp for 15 h, respectively. After removal of the methanol, the reaction mixture was acidified with 0.2 M HCl (25 μl) and extracted with ethyl acetate (3×50 μl). The combined ethyl acetate soln was washed with distilled water (15 μl) and evaporated in vacuo or under flashing argon. The residue and a part (2 μg) of Fr. 6 were dissolved in distilled water (2 ml) and assayed, respectively: Fr. 1, (8.7 mg) inactive; 2, (0.10) active at 10^{-8}—$^{-9}$ g ml^{-1}; 3, (0.91) active at 10^{-10}; 4, (0.55) active at 10^{-9}; 5, (0.11) active at 10^{-8}—$^{-9}$; 6, (0.47) active at 10^{-8}—$^{-9}$. Fr. 3 (0.91 mg) was the most active, named Fraction I (p-BPE), and submitted to further separation.

Preparative HPLC on a MicroPak CN-10 Column [I (p-BPE) to J (p-BPE)]. Fraction I (p-BPE) (0.90 mg) was dissolved in chloroform (100 μl), and the soln was injected in 20 μl portions onto the column (Varian, MicroPak CN-10, 2.2 mm×25 cm)[27c] and separated into 5 fractions, a 45:55 mixture of hexane and diethyl ether being used as an eluent at flow rate of 0.83 ml min^{-1} (Fig. 3). An aliquot (3 μg) of each fraction was hydrolyzed as described above and

assayed, respectively: Fr. 1, (0.225 mg) active at 10^{-8} g ml^{-1}; 2, (0.225), active at 10^{-8}—$^{-9}$; 3, (0.190) active at 10^{-10}—$^{-11}$; 4, (0.170) active at 10^{-7}—$^{-8}$; 5, (0.110) active at 10^{-7}—$^{-8}$. Fr. 3 (0.190 mg) was the most active, named Fraction J (p-BPE), and submitted to further purification.

Preparative HPLC on a MicroPak NH_2-10 Column [J (p-BPE) to K (p-BPE)]. Fraction J (p-BPE) (0.180 mg) was dissolved in chloroform, and the soln was injected onto the column (Varian, MicroPak NH_2-10, 2.0 mm×25 cm),[27c] and separated into 5 fractions, a 70:18:12 mixture of hexane, dichloromethane and acetonitrile being used as an eluent at flow rate of 0.5 ml min^{-1} (Fig. 4). An aliquot (ca. 0.3 μg) of each fraction was hydrolyzed as described above, and then assayed, respectively: Fr. 1, (≈45 μg) inactive; 2, (≈15) inactive; 3, (≈15) inactive; 4, (≈50) active at 10^{-11} g ml^{-1}; 5, (≈55) inactive. Fr. 4 [Fraction K (p-BPE)] (≈50μ g) was the most active and submitted to further purification.

Preparative HPLC on a Hitachi Gel #3011 Column [K (p-BPE) to L (p-BPE) (=GEAp-BPE)]. Fraction K (p-BPE) (≈50 μg) was dissolved in chloroform (5 μl) and separated into 4 fractions by preparative HPLC on a Hitachi Gel #3011 column [Hitachi Ltd., 4 mm×50 cm; eluent, methanol–dichloromethane (9:1); flow rate, 1 ml min^{-1}] (Fig. 5). One-two hundredth of each separated fraction was hydrolyzed as described above, dissolved in water, and assayed. Only Fr. 3 [Fraction L (p-BPE) =GEA p-BPE] 5—10 μg, estimated by the UV intensity of the chromatogram) was active after hydrolysis, and was purified by simple chromatography on the MicroPak NH_2-10 column under the same conditions to remove impurities contaminated from the gels and/or solvents.

The homogeneity of Fraction L (p-BPE) was further checked by analytical HPLC on a MicroPak CH-10 column [Varian, 2.2 mm×25 cm, methanol–water (8:2); flow rate, 0.67 ml min^{-1}], when a single peak (R_t 9 min) was observed on the chromatogram.

(II) Experimentals Summarized in Table 2. Preparation of Chloroform Extracts A and Acidic Extracts B. Roots of "Hon-kintoki," a subspecies of kidney bean, cultivated in 1 hectare of the field at Memuro, were collected in early August, washed roughly with water, dried in the shade and crushed. The dried and powdered roots amounted to 113 kg. A part (41 kg) of the roots was extracted with water (300 l) at 2—8 °C for 4 d. After being squeezed, the roots were further extracted with water (2×200 l). The combined aq muddy suspension (ca. 650 l) was concentrated to 13 l below 30 °C by a thin-film evaporator. This process was repeated three times. All the concentrates (38 l) were combined, made acidic to pH 2—3 with 6 M HCl, and extracted with chloroform (ca. 60 l) continuously for 50 h by a special Soxhlet apparatus. The chloroform soln was evaporated to give an oily material (Chloroform extracts A) (102 g), which was active at 10^{-5} g ml^{-1} for the hatching in water at 25 °C.

The extracts (102 g) was dissolved in ethyl acetate (400 ml) and treated with saturated aq $NaHCO_3$ (500+6×100 ml). The combined aq soln was acidified to pH 2 by addition of 6 M HCl (250 ml) and extracted with ethyl acetate (300+5×200 ml). The combined acetate soln was washed with water (2×50 ml), dried over sodium sulfate, and evaporated to give an acidic oily material (Acidic extracts B) (56.9 g), which was active at 10^{-5}—$^{-6}$ g ml^{-1}.

Chromatography on a Charcoal–Celite Column (B to C). A mixture of Charcoal (500 g) and Celite-545 (1 kg) in water was packed in a glass column (8.4×88 cm). Acidic extracts B (56.5 g) was dissolved in acetone, mixed with Celite (60 g), evaporated, placed on the top of the column, and eluted successively with water (5 l) (Fr. 1), water–acetone (9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, and 1:9, v/v, each 5 l) (Frs. 2—10), and methanol–concd NH_4OH (22:1, v/v, 12 l) (Fr. 11). All the fractions, except the last one, were simply evaporated and assayed, while that (Fr. 11) eluted with ammoniacal methanol, after being evaporated, was dissolved in water (100 ml), acidified with 3 M HCl, and extracted with ethyl acetate (3×100 ml). The acetate solution was then dried, evaporated, and assayed. The hatching activity of each fraction is summarized as follows: Fr. 1, (0.01 g) inactive; 2, (0.07) inactive; 3, (0.53) active at 10^{-6} g ml^{-1}; 4, (0.68) active at 10^{-5}; 5, (2.08) inactive; 6, (5.17) inactive; 7, (7.49) inactive; 8, (8.25) active at 10^{-6}—$^{-7}$ g ml^{-1}; 9, (5.66) active at 10^{-6}; 10, (3.18) active at 10^{-5}; 11, (4.41) active at 10^{-6}. Frs. 8—11 were combined (Fraction C) (21.5 g) and submitted to further separation.

Chromatography on a Sephadex LH-20 Column (C to D). Fraction C (21.5 g) in methanol (50 ml) was separated by chromatography on a Sephadex LH-20 column (Pharmacia, 1013 g) (8.4×74 cm). The column was eluted with methanol (6.5 l), 130 fractions (each 50 ml) being collected. The amount and biological activity of each eluate is summarized in Fig. 6. Fractions 59—78 were active nearly at 10^{-6}—$^{-7}$ g ml^{-1}, combined (Fraction D) (10.1 g), and submitted to further separation.

Chromatography on a DEAP-Sephadex LH-20 Column (D to E). A suspension of DEAP-Sephadex LH-20 (120 g), prepared according to the reported procedure,[30] in 72% aq ethanol was packed in a glass column (3.1×63 cm) and washed successively with 0.5 M aq potassium acetate in 72% aq ethanol (6 l), which had been prepared and adjusted to pH 10 by addition of 1 M aq KOH, 72% aq ethanol (4 l), 0.1 M acetic acid in 72% aq ethanol (4 l), and 72% aq ethanol (4 l), when the packing was converted into the acetate form (3.1×81.5 cm). Fraction D (10.1 g) in 72% aq ethanol (90 ml) was placed on the top of the column and then eluted successively with 72% aq ethanol (4 l) (Fr. 1), 0.1 M acetic acid in 72% aq ethanol (4 l) (Fr. 2), 0.3 M acetic acid in 72% aq ethanol (4 l), which had been adjusted to pH 6.0 by addition of concd NH_4OH (Fr. 3), and 0.3 M acetic acid in 72% aq ethanol (4 l), which had been adjusted to pH 9.0 by addition of concd NH_4OH (Fr. 4).[30,31] Frs. 1 and 2 were evaporated thoroughly to dryness to give inactive oily mixtures (3.18 and 4.87 g) of neutral compounds and monobasic acids, respectively. Fr. 3 was evaporated, dissolved in water (150 ml), acidified with 3 M HCl (130 ml), and extracted with ethyl acetate (4×100 ml). The combined acetate solution was washed with water (3×50 ml), dried, and evaporated to leave an oily mixture of dibasic acids (1.99 g), which was active at 10^{-7} g ml^{-1}. Fr. 4 was treated in the same manner to give an amorphous mixture of polybasic acids (0.17 g) (active at 10^{-6} g ml^{-1}). The mixtures of dibasic and polybasic acid were combined (Fraction E) (2.16 g) and submitted to further separation.

Chromatography on a Sephadex LH-20 Column (E to F). Fraction E (2.12 g) was separated by partition chromatography on a Sephadex LH-20 column (106 g) (2.5×90 cm). The column was eluted with dichloro-

methane–methanol (95:5), 50 fractions (each 30 ml) being collected. The amount and biological activity of each eluate is summarized in Fig. 7. Frs. 25—28 were combined (Fraction F) (135 mg) and submitted to *p*-bromophenacylation.

***p*-Bromophenacylation [F to G (p-BPE)].** A mixture of Fraction F (135 mg), *p*-bromophenacyl bromide (1.70 g), and *N,N*-diisopropylethylamine (0.5 ml) was stirred in dry acetonitrile (15 ml) at room temp for 19 h and then evaporated to leave an oily material. The residue was dissolved in benzene (5 ml) and separated simply by chromatography on a silica-gel column (Wakogel C-200, 20 g) (2.1×21.5 cm) with benzene (Fr. 1), chloroform (Fr. 2), chloroform–ethyl acetate (9:1 and 1:1) (Frs. 3 and 4), ethyl acetate (Fr. 5), and ethyl acetate–acetic acid (99:1) (Fr. 6) (each eluent 100 ml): Fr. 1, (1364 mg); 2, (20.9); 3, (315.7); 4, (27.3); 5, (5.3); 6, (7.7). A part (0.20—0.40 mg) of each eluate was treated with 0.1 M KOH in methanol (50 μl) at room temp for 20 h. The reaction mixture was evaporated, acidified with 0.2 M HCl (50 μl), and extracted with ethyl acetate (3×100μl). The combined acetate solution was washed with water (50 μl) and evaporated under a stream of nitrogen. The residue was dissolved in water (1 ml for 0.02 mg of the initial weight) and assayed: Frs. 1 and 2, both inactive; 3, active at 10^{-8} g ml^{-1}; 4 and 5, both inactive; 6, active at 10^{-6}–$^{-7}$ g ml^{-1}. Fr. 3 [Fraction G (p-BPE)] (315.7 mg) was submitted to further separation.

Preparative HPLC on a Hitachi Gel #3019 Column [G (p-BPE) to H (p-BPE)]. A soln of Fraction G (p-BPE) (316 mg) in chloroform (1.4 ml) was injected in portions of 60—100 μl onto a Hitachi Gel #3019 column (8 mm×50 cm) and separated roughly into 4 fractions, methanol–dichloromethane (7:3) being used as an eluent at flow rate of 4 ml min^{-1}. Each fraction (40 μg) was hydrolyzed as described above and then assayed: Fr. 1, (59.5 mg) inactive; 2, (57.0) active at 10^{-6}–$^{-7}$ g ml^{-1}; 3, (112) active at 10^{-8}–$^{-9}$; 4, (67.7) active at 10^{-8}. Fr. 3 [Fraction H (p-BPE)] (112 mg) was submitted to further separation.

Preparative HPLC on a Bondapak C_{18}/Porasil B Column [H (p-BPE) to I (p-BPE)]. A soln of Fraction H (p-BPE) (112 mg) in a 1:1 mixture (1 ml) of chloroform and methanol was injected onto a Bondapak C_{18}/Porasil B column (7.8 mm×61 cm) and separated into 6 fractions, methanol–water (8:2) being used as an eluent at flow rate of 6 ml min^{-1}. Each fraction (40 μg) was hydrolyzed as described above and assayed: Fr. 1, (9.1 mg) active at 10^{-8} g ml^{-1}; 2, (14.9) active at 10^{-8}; 3, (7.7) active at 10^{-9}; 4, (21.6) active at 10^{-9}; 5, (7.1) active at 10^{-8}; 6, (30.7) active at 10^{-7}–$^{-8}$. Frs. 3 and 4 were combined [Fraction I p-BPE)] (29 mg) and submitted to further separation.

Preparative HPLC on a μBondapak NH_2 Column [I (p-BPE) to J (p-BPE)]. A soln of Fraction I (p-BPE) (29 mg) in chloroform (1.3 ml) was injected in portions of 100—150 μl onto a μBondapak NH_2 column (3.9 mm×30 cm) and separated into 4 fractions, hexane–dichloromethane–acetonitrile (70:18:12) being used as an eluent at flow rate of 1.5 ml min^{-1}. Each fraction (4 μg) was hydrolyzed as described above and then assayed: Fr. 1, (18.18 mg) active at 10^{-6}–$^{-7}$ g ml^{-1}; 2, (0.93) active at 10^{-8}–$^{-9}$; 3, (2.17) active at 10^{-10}; 4, (1.90) active at 10^{-8} g ml^{-1}. Fr. 3 [Fraction J (p-BPE)] (2.17 mg) was submitted to further separation.

Preparative HPLC on a μBondapak NH_2 Column [J (p-BPE) to K (p-BPE)]. A soln of Fraction J (p-BPE) (2.17 mg) in chloroform (0.5 ml) was injected in portions of 100, 200, and 200 μl onto the same column as described above and separated into 4 fractions, hexane–dichloromethane–acetonitrile (80:12:8) being used as an eluent at flow rate of 2.5 ml min^{-1}. Each fraction (0.4 μg) was hydrolyzed and assayed as described before: Fr. 1, (0.28 mg) active at 10^{-8} g ml^{-1}; 2, (0.07) active at 10^{-8}; 3, (0.44) active at 10^{-10}; 4, (0.76) active at 10^{-10}. Frs. 3 and 4 [Fraction K (p-BPE)] (1.20 mg) were submitted to further separation.

Preparative HPLC on a μBondapak NH_2 [K (p-BPE) to L (p-BPE) (=GEA p-BPE)]. A soln of Fraction K (p-BPE) (1.20 mg) in chloroform (120 μl) was injected in portions of 10—25 μl (6 times) onto the aforementioned column and separated into 3 fractions, a 60:36:4 mixture of hexane–dichloromethane–acetonitrile being used as an eluent at flow rate of 1.5 ml min^{-1}. Each fraction (0.4 μg) was hydrolyzed as described above and then assayed: Fr. 1, (0.77 mg) active at 10^{-8}–$^{-9}$ g ml^{-1}; 2, (0.05) active at 10^{-11}–$^{-12}$; 3, (0.07) active at 10^{-10}–$^{-11}$ g ml^{-1}. The second fraction [Fraction L (p-BPE)] showed a single peak by analytical HPLC on the following columns and the highest activity for the hatching after hydrolysis. Analytical HPLC: column, solvent (flow rate), retention time; μBondapak NH_2 (3.9 mm×30 cm), hexane–dichloromethane–acetonitrile (60:36:4) (1.5 ml min^{-1}), 9.5 min; μBondapak C_{18} (3.9 mm×30 cm), methanol–water (8:2) (2.0 ml min^{-1}), 8.5 min; μBondapak/Phenyl (3.9 mm×30 cm), methanol–water (8:2) (2.0 ml min^{-1}), 8.5 min.

(III) Experiments Summarized in Table 3. Preparation of Chloroform Extracts A and Acidic Extracts B. Dried and powdered roots (1058 kg) of "Beni-kintoki" and "Hon-kintoki," subspecies of kidney bean, which had been collected in late July during several years at Memuro (10 hectares) were extracted with water, concentrated and then extracted with chloroform continuously as described above. The total Chloroform extracts A, prepared from all the roots, amounted to 1011 g which stimulated the hatching and emergence at 10^{-5} g ml^{-1} in water at 25 °C.

A part (120 g) of Chloroform extracts A was dissolved in ethyl acetate (500 ml) and treated with saturated aq $NaHCO_3$ (6×200 ml) repeatedly. The aq solns were combined, acidified with 6 M HCl (250 ml) to pH 2—3, and extracted again with ethyl acetate (5×200 ml) and then with chloroform continuously for 18 h. The neutral acetate extracts were dried over anhydrous sodium sulfate and evaporated to leave a dark gum (27.3 g) (inactive), while the acidic acetate extracts were treated in the same manner to give a viscous oil (Acidic extracts B) (84 g) (636 g from total Chloroform extracts A), which was active at 10^{-5}–$^{-6}$ g ml^{-1}. The chloroform extracts also afforded an active oil (4.2 g) (active at 10^{-5}–$^{-6}$ g ml^{-1}).

Chromatography on a Charcoal-Celite Column (B to C). A part (88.2 g) of Acidic extracts B was dissolved in acetone (200 ml) and mixed with Celite-545 (70 g) under vigorous stirring. The mixture, after being evaporated, was placed on the top of a column (11.4×92 cm) prepared from Charcoal (1 kg) and Celite-545 (2 kg) in water, and eluted with water (10 l) (Fr. 1), water–acetone (9:1 to 1:9, each 10 l) (Frs. 2—10), acetone (10 l) (Fr. 11), and methanol–concd NH_4OH (19:1, 20 l) (Fr. 12). The final fraction (Fr. 12) was evaporated, acidified and extracted with ethyl acetate (3×

100 ml), and the acetate extracts were combined with Fr. 11. Frs. 9—11, eluted with 80 and 90% aq acetone and acetone, were more active than other fractions and combined to give a resinous oil (Fraction C) (26.6 g) (197.3 g from total Acidic extracts B), which was active at 10^{-6} g ml^{-1}.

Chromatography on a Sephadex LH-20 Column (C to D). A part (17.57 g) of Fraction C in methanol (40 ml) was separated by chromatography on a Sephadex LH-20 column (1 kg) (8.4×72 cm) in methanol. The column was eluted with methanol (5 l), 180 fractions (each 25—30 ml) being collected. Frs. 76—100 were more active (10^{-6}—$^{-7}$ g ml^{-1}) than those 130—160 (10^{-6} g ml^{-1}), and were combined to give a viscous oil (Fraction D) (7.40 g) (88.9 g from total Fraction C).

Chromatography on a DEAP-Sephadex LH-20 Column (D to E). A part (38.4 g) of Fraction D in 72% aq ethanol (390 ml) was submitted to chromatography on a DEAP-Sephadex LH-20 column[30] (128 g) (3.1×82 cm) packed in 72% aq ethanol (v/v). The column was eluted with 72% aq ethanol (3.9 l) (Fr. 1), 0.1 M acetic acid in 72% aq ethanol (4 l) (pH 4.0) (Fr. 2), and then with 0.3 M acetic acid in 72% aq ethanol (4 l) and concd NH_4OH (127 ml) (pH 9.0) (Fr. 3). Frs. 1 and 2 were evaporated to dryness to give inactive oily residues (20.57 and 9.35 g), respectively. Fr. 3 was evaporated, dissolved in water (100 ml), acidified with 6 M HCl (195 ml) to pH 2, and extracted with ethyl acetate (5×100 ml). The acetate extracts were washed with saturated brine (3×50 ml), dried and evaporated to leave a slightly dark oil (Fraction E) (7.75 g) (18.15 g from total Fraction D), which was active at 10^{-7} g ml^{-1}.

Chromatography on a Sephadex LH-20 Column (E to F and to G). A part (3.21 g) of Fraction E in dichloromethane–methanol (95:5, v/v) (7 ml) was separated by chromatography on a column (2.7×75 cm), which had been packed by Sephadex LH-20 (120 g) with methanol and then with dichloromethane–methanol (95:5). The column was eluted with the same solvent mixture (total 2 l), 60 fractions (each 35 ml) being collected. Most of the fractions were active to some extent. Frs. 19—31 were more active than other fractions and combined to give an oil (Fraction F) (636 mg) (3.59 g from total Fraction E), which was active at 10^{-7}—$^{-8}$ g ml^{-1}.

Fraction F (3.59 g) in dichloromethane–methanol (95:5, v/v) was separated by rechromatography on the same column (2.7×75 cm) packed with Sephadex LH-20 (140 g). The column was eluted with dichloromethane–methanol (97:3) (total 2.5 l), 18 fractions (each ca. 150 ml) being collected. Frs. 9—13 were more active than others and combined to give an oil (Fraction G) (0.95 g), which was active at 10^{-8} g ml^{-1}.

***p*-Bromophenacylation [G to H (p-BPE)].** To a soln of Fraction G (0.95 g) and *p*-bromophenacyl bromide (4.01 g) in acetonitrile (10 ml) was added *N,N*-diisopropylethylamine (2 ml), and the mixture was stirred vigorously at room temp for 19 h, when white ppts appeared. The whole suspension was evaporated to leave a semisolid residue (6.42 g), which was simply purified by chromatography (5.5×26 cm) over silica gel (Wakogel C-200) (250 g). The column was eluted with benzene (1.5 l), chloroform (1 l), chloroform–ethyl acetate (9:1 and 1:1) (each 1 l), ethyl acetate (1 l), and ethyl acetate–methanol (1:1) (1 l). Fracitons eluted with chloroform–ethyl acetate (9:1 and 1:1) were combined to give a gum [Fraction H (p-BPE)] (2.21 g), which was active at 10^{-8} g ml^{-1} after hydrolysis as described below.

A part (0.1 mg) of Fraction H (p-BPE) was treated with 0.1 M KOH in methanol (0.2 ml) at room temp for 4 h. The reaction mixture was evaporated, acidified with 0.2 M HCl (0.4 ml), and extracted with ethyl acetate (3×0.1 ml). The extracts were washed with water (0.1 ml), dried and evaporated to leave a residue, which was dissolved in acetone (one drop) and diluted with water for the bioassay.

Chromatography on a Hitachi Gel #3019 Column [H (p-BPE) to I (p-BPE)]. A part (944 mg) of Fraction H (p-BPE) in dichloromethane–methanol (3:7) (2 ml) was separated by chromatography on a column (2.5×64 cm) packed with Hitachi Gel #3019 (100 g) in dichloromethane–methanol (2:8). The column was eluted with dichloromethane–methanol (2:8) (total 1.6 l), 100 fractions (each 16 ml) being collected (Fig. 8). Frs. 36—50 were more active than others and combined to give a brown oil [Fraction I (p-BPE)] (149 mg) [336 mg from total Fraction H (p-BPE)], which was active at 10^{-9} g ml^{-1} after hydrolysis.

Preparative HPLC on a μBondapak C_{18} Column [I (p-BPE) to J (p-BPE)]. A part (5 mg) of Fraction I (p-BPE) in acetone (0.2 ml) was submitted to preparative HPLC on a μBondapak C_{18} column (7.8 mm×30 cm) and separated into 15 fractions, 80% aq methanol (v/v) (Frs. 1—13) and methanol (Frs. 14 and 15) being used as eluents at flow rate of 2 ml min^{-1} (Fig. 9). The eluents were monitored by the UV spectra and checked by the hatching activity after hydrolysis. Frs. 8 and 9 were more active than others after hydrolysis and combined to give an oil [Fraction J (p-BPE)] (ca. 1.2 mg), which was active at 10^{-9}—$^{-10}$ g ml^{-1} after hydrolysis. This procedure was repeated 70 times to give 79 mg of the Fraction J (p-BPE).

Preparative HPLC on a μBondapak NH_2 Column [J (p-BPE) to K (p-BPE) and K″ (p-BPE)(=GEC p-BPE)]. A part (1.5 mg) of Fraction J (p-BPE) in dichloromethane (0.24 ml) was separated into 6 fractions by preparative HPLC on a μBondapak NH_2 column (7.8 mm×30 cm), hexane–dichloromethane–acetonitrile (76:14:10, v/v/v) being used as an eluent at flow rate of 1.5 ml min^{-1} (Fig. 10). Only Fr. 5 (R_t 13—16 min) was highly active after hydrolysis. This fraction was collected by repeated HPLC (55 times) to amount to 8.8 mg of an oily substance [Fraction K (p-BPE)], which was active at 10^{-10}—$^{-11}$ g ml^{-1} after hydrolysis. On the other hand, Fr. 4 (R_t 11 min) [Fraction K′ (p-BPE)] [4.9 mg from total Fraction J (p-BPE)], showing a practically single peak, was further purified by preparative HPLC on the same column with a mixture of hexane, dichloromethane, and acetonitrile (60:36:4, v/v/v) to give Fraction K″ (p-BPE) (=GEC p-BPE) [1.8 mg from total Fraction J (p-BPE)].

Preparative HPLC on a μBondapak NH_2 Column [K (p-BPE) to L (p-BPE) (=GEA p-BPE) and L″(p-BPE) (=GEB p-BPE). A part (0.50 mg) of Fraction K (p-BPE) in dichloromethane (0.1 ml) was purified by preparative HPLC on a μBondapak NH_2 column (7.8 mm×30 cm), a 63:33:14 (v/v/v) mixture of hexane, dichloromethane, and acetonitrile being used as an eluent at flow rate of 1.5 ml min^{-1}. The eluents were monitored by the UV spectra, when two major fractions with R_t 10.5 and 14.5 min were separated, as shown in Fig. 11. This procedure was repeated 20 times. The former, which was active at 10^{-11}—$^{-12}$

g ml^{-1} in water after hydrolysis, was crystallized on trituration with hexane and dichloromethane to give GEA p-BPE (1.25 mg). On the other hand, the latter [Fraction K' (p-BPE)] [2.6 mg from total Fraction K (p-BPE)] was much less active after hydrolysis and was further purified by preparative HPLC on the same column with a 68:13.2:8.8 (v/v/v) mixture of hexane, dichloromethane, and acetonitrile (flow rate, 3 ml min^{-1}) to give an oil [Fraction L'' (p-BPE)] (=GEB p-BPE)] [1.80 mg from total Fraction K (p-BPE)].

GEA p-BPE (0.1 mg) was treated with 0.1 M KOH in methanol (0.2 ml) at room temp for 4 h. After being evaporated, the soln was acidified with 0.2 M HCl (0.4 ml) to pH 2—3 and extracted with ethyl acetate (3×0.1 ml). The acetate extracts were washed with water (0.1 ml) and purified by preparative HPLC on a μBondapak C_{18} column (7.8 mm×30 cm), 0.1% aq acetic acid–methanol (35:65, v/v) being used as a solvent at flow rate of 2 ml min^{-1} (Fig. 12). The eluent was monitored by the UV spectrum to give an amorphous substance with R_t 10.1 min, GEA (as its potassium salt), which was active at 10^{-12} g ml^{-1}.

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