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In Vitro GABA-Transaminase Inhibitory Compounds from the Root of *Angelica dahurica*

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The 80% aqueous MeOH extracts from the root of *Angelica dahurica*, found to inhibit the activities of GABA degradative enzymes GABA transaminase (GABA-T) and succinic semialdehyde dehydrogenase (SSADH), were fractionated using EtOAc, *n*-BuOH and H_2O. Repeated column chromatography for the EtOAc and *n*-BuOH fractions led to the isolation of two new coumarins, oxypeucedanin hydrate-3″-butyl ether and isopraeroside IV along with six known coumarins, isoimperatorin, imperatorin, phellopterin, oxypeucedanin hydrate, nodakenin and 3′-hydroxymarmesinin, and two polyacetylenes, falcarindiol and octadeca-1,9-dien-4,6-diyn-3,8,18-triol. Of the isolated pure compounds, imperatorin and falcarindiol inactivated the GABA-T activities in both time- and concentration-dependent manners. The kinetic studies showed that imperatorin and falcarindiol reacted with the GABA-T with a second-order rate constant of 2.3 ± 0.2 mM^{-1} min^{-1} and 1.5 ± 0.1 mM^{-1} min^{-1}, respectively. It is postulated that imperatorin and falcarindiol are able to elevate the neurotransmitter GABA levels in the central nervous system by an inhibitory action on the GABA degradative enzyme GABA-T. Copyright © 2005 John Wiley & Sons, Ltd.

Keywords: Angelica dahurica; Umbelliferae; oxypeucedanin hydrate-3″-butyl ether; isopraeroside IV; GABA transaminase (GABA-T).

INTRODUCTION

γ-Aminobutyric acid (GABA) is present in many mammalian tissues and is a major inhibitory neurotransmitter in the central nervous system. The concentration of GABA in the brain is controlled by two pyridoxal-5′-phosphate dependent enzymes, i.e. glutamate decarboxylase (GAD; EC 4.1.1.15) and GABA transaminase (GABA-T; EC 2.6.1.19). GAD catalyses the synthesis of GABA from glutamate, which is synthesized through reduction of 2-oxoglutarate catalysed by glutamate dehydrogenase (GDH). Whereas GABA-T catabolizes GABA to succinic semialdehyde in a transamination reaction. Succinic semialdehyde is primarily oxidized to succinate by a succinic semialdehyde dehydrogenase (SSADH; EC 1.2.1.24) and can be also reduced to γ-hydroxybutyrate (GHB) by succinic semialdehyde reductase (SSAR). Succinate then enters the TCA cycle.

When the concentration of GABA in the brain diminishes to below a threshold level, various neurological disorders including seizures, convulsions, Huntington's disease, epilepsy and Parkinsonism may occur (Perry *et al.*, 1973; De Biase *et al.*, 1991). Although it is well known that GAD is the rate controlling enzyme among the GABA shunt enzymes, the observation that the inactivation of GABA-T in brain tissues increases the concentration of neurotransmitter supports the fact that this enzyme exerts a controlling influence on GABA levels (Fletcher and Fowler, 1980). Therefore, the irreversible inhibition of GABA-T by a chemical analog of GABA is the basic mechanism of action of drugs used in the treatment of convulsive disorders (Lippert *et al.*, 1977). The epilepsy drug γ-vinyl GABA (vigabatrin) known as a suicide inhibitor exerts its pharmacological effects by inhibition of GABA-T. It is transformed by GABA-T to an active metabolite, which, thereafter, irreversibly binds to the active site of the enzyme.

Angelica dahurica Bentham et Hooker (Umbelliferae) is a perennial herb distributed in the whole area of Korea, and its root has been most frequently prescribed as a sedative and an analgesic in Chinese medicine (Soka, 1985). Several coumarins that are constituents of *Angelica dahurica* have been extensively studied for their chemical structures (Saiki *et al.*, 1971; Wang *et al.*, 2001) and pharmacological effects (Kimura *et al.*, 1982; Kim *et al.*, 1992; Kwon *et al.*, 1997). Also, although some coumarins that occur in other plants were reported to produce an effect on the central nervous system (Bergendorff *et al.*, 1997), the antispasmodic principal component of *Angelica dahurica* has remained uncharacterized so far.

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In this paper, the principal coumarins and polyacetylenes, isolated from *Angelica dahurica*, were examined for their antispasmodic effects, that is, for their inactivation effect on GABA-T and SSADH. In addition, the chemical structures of the new coumarins were also characterized.

MATERIALS AND METHODS

Materials. The root of *Angelica dahurica* was purchased at a market in Seoul and identified by Dr Hyeung-Kyu Lee, KRIBB, Taejon, Korea. A reference specimen (KHU97246) has been deposited in the laboratory of Natural Products Chemistry, Kyunghee University, Suwon, Korea. Bovine brains were obtained from the Majangdong Packing Company in Seoul, Korea. GABA, α-ketoglutarate and succinic semialdehyde were purchased from Sigma Chemical Co. (St Louis, USA).

Instrumentation. Melting point: Fisher-John apparatus (uncorrected); NMR: Varian Unity-Inova 400; EI/MS: JEOL JMS-AX505WA; Optical rotations: JASCO DIP-370 digital polarimeter; IR: Perkin-Elmer 599B; Absorption spectroscopic measurements: Kontron UVIKON Model 930 double beam spectrophotometer; Fluorescence spectra: Kontron SFM 25 spectrofluorometer.

Isolation of coumarins and polyacetylenes. Coarsely powdered plant material (1 kg) was extracted with 80% aqueous MeOH (4 L × 2) at room temperature overnight. After removal of the solvent, the residue was poured into water (500 mL) followed by extraction with EtOAc (700 mL × 2) and *n*-BuOH (500 mL × 2) to afford EtOAc (42 g) and *n*-BuOH (55 g) soluble fractions. The EtOAc fraction (36 g) was chromatographed on a silica gel (250 g) column (7 × 30 cm). Stepwise-gradient elution with *n*-hexane–EtOAc with increasing proportions of EtOAc (*n*-hexane–EtOAc = 5:1 → 3:1 → 1:1 → 1:3, each in 2 L) and detection of each eluate with silica gel TLC gave 12 subfractions (ADE-1: between 800–1100 mL, ADE-2: 1101–1400 mL, ADE-3: 1401–1910 mL, ADE-4: 1911–2450 mL, ADE-5: 2451–2960 mL, ADE-6: 2961–3320 mL, ADE-7: 3321–4170 mL, ADE-8: 4171–4890 mL, ADE-9: 4891–5100 mL, ADE-10: 5101–5940 mL, ADE-11: 5941–6990 mL, ADE-12: 6991–7980 mL). Silica gel (100 g) column (4 × 30 cm) chromatography (c.c.) of the subfraction ADE-5 (1.89 g) eluting with *n*-hexane–$CHCl_3$–EtOH (20:20:1, 800 mL) afforded compound **1** (540–620 mL, isoimperatorin, 628 mg, R_f: 0.39 on silica gel TLC in $CHCl_3$–EtOH = 12:1); colorless prisms ($CHCl_3$–EtOH), m.p. 109–110 °C. Lit. (Gu *et al.*, 1990) m.p. 109.5–110.5 °C.

The subfraction ADE-7 (2.75 g) was subjected to a silica gel (85 g) column (3 × 45 cm) eluting with *n*-hexane–$CHCl_3$–EtOH (30:10:1, 1.2 L) to give four subfractions (ADE-7-1: 120–340 mL, ADE-7-2: 341–750 mL, ADE-7-3: 751–920 mL, ADE-7-4: 921–1180 mL) and the third (ADE-7-3, 540 mg) was chromatographed on a silica gel (75 g) column (4 × 25 cm) eluting with *n*-hexane–$CHCl_3$–EtOH (12:12:1, 550 mL) to yield compound **2** (240–460 mL, oxypeucedanin hydrate-3″-butyl ether, 143 mg, R_f: 0.36 on silica gel TLC in $CHCl_3$–EtOH = 10:1).

Oxypeucedanin hydrate-3″-butyl ether (**2**): Pale brownish prisms ($CHCl_3$–EtOH), m.p. 102–103 °C; $[\alpha]^{26}_D$+1.1° ($CHCl_3$, c = 1.1); UV: λ_{max} nm (MeOH, log ε) 252 (3.5), 269 (3.6), 315 (3.9); IR: γ_{max} cm^{-1} ($CHCl_3$) 3465, 3015, 1725, 1620, 1520; EI-MS *m/z*: 360 (M^+), 345, 315, 303, 185, 174; HREI-MS *m/z*: 360.1577 (calcd for $C_{20}H_{24}O_6$, 360.1573); ^{1}H-NMR (400 MHz, $CDCl_3$, δ) 8.14 (1H, d, J = 9.8 Hz, H-4), 7.50 (1H, d, J = 2.2 Hz, H-2′), 7.03 (1H, br. s, H-8), 6.93 (1H, dd, J = 2.2, 0.7 Hz, H-3′), 6.16 (1H, d, J = 9.8 Hz, H-3), 4.53 (1H, dd, J = 10.0, 3.0 Hz, H-1″-a), 4.31 (1H, dd, J = 10.0, 7.8 Hz, H-1″-b), 3.85 (1H, dd, J = 7.8, 3.0 Hz, H-2″), 3.33 (2H, t, J = 6.3 Hz, H-1‴), 1.45 (2H, m, H-2‴), 1.29 (2H, m, H-3‴), 1.19 (3H, s, H-5″), 1.17 (3H, s, H-4″), 0.83 (3H, t, J = 7.3 Hz, H-4‴); ^{13}C-NMR (100 MHz, $CDCl_3$, δ_C) 161.68 (C-2), 158.07 (C-7), 152.50 (C-5), 148.84 (C-8a), 144.88 (C-2′), 139.43 (C-4), 113.85 (C-6), 112.57 (C-3), 107.17 (C-4a), 104.90 (C-3′), 94.22 (C-8), 76.68 (C-2″), 75.59 (C-3″), 74.21 (C-1″), 60.92 (C-1‴), 32.39 (C-2‴), 21.32 (C-4″), 21.15 (C-5″), 19.37 (C-3‴), 13.82 (C-4‴).

Compounds **3** (imperatorin, 212 mg), **4** (falcarindiol, 502 mg) and **5** (phellopterin, 154 mg) {elution volume: 710–820 mL (**3**), 891–1480 mL (**4**), 1720–2180 mL (**5**); R_f: 0.58 (**3**), 0.51 (**4**), 0.42 (**5**) on silica gel TLC in *n*-hexane–$CHCl_3$–EtOH = 14:14:1} were obtained by silica gel (125 g, 4 × 35 cm) c.c. of ADE-8 (4.0 g), using *n*-hexane–$CHCl_3$–EtOH (25:20:1, 2.4 L) as the eluting solvent. Imperatorin (**3**): Colorless prisms ($CHCl_3$–EtOH), m.p. 101–102 °C. Lit. (Razdan *et al.*, 1982) m.p. 102 °C. Falcarindiol (**4**): Colorless oil, $[\alpha]^{25}_D$+318° (MeOH, c = 1.8). Lit. (Liu *et al.*, 1998) $[\alpha]^{25}_D$+321.4° (CH_3CN). Phellopterin (**5**): Pale yellowish needles (*n*-hexane–EtOAc), m.p. 101–102 °C. Lit. (Sasaki *et al.*, 1982) m.p. 103.5–104.5 °C.

ADE-11 (2.7 g) was applied to a silica gel (125 g) column (4 × 35 cm) eluting with *n*-hexane–EtOAc–acetone (4:6:1, 1.7 L) to give compound **6** (1120–1610 mL, oxypeucedanin hydrate, 517 mg, R_f: 0.58 on silica gel TLC in *n*-hexane–EtOAc–acetone = 2:7:2); colorless needles (*n*-hexane–EtOAc), m.p. 137–138 °C; $[\alpha]^{28}_D$ +18.9° (MeOH, c = 1.2). Lit. (Kozawa *et al.*, 1981) m.p. 136–137 °C; $[\alpha]^{25}_D$+16.9° (EtOH).

ADE-12 (2.76 g) was applied to a silica gel (120 g) column (4 × 30 cm) eluting with *n*-hexane–EtOAc–MeOH (10:3:1, 1.4 L) and *n*-hexane–EtOAc (2:3, 1.2 L), successively, to give compound **7** (910–1120 mL, octadeca-1,9-dien-4,6-diyn-3,8,18-triol, 34 mg, R_f: 0.48 on silica gel TLC in *n*-hexane–EtOAc–MeOH = 7:7:1); colorless oil, $[\alpha]^{26}_D$+308° ($CHCl_3$, c = 1.2). Lit. (Liu *et al.*, 1998) $[\alpha]^{25}_D$+318.2° (CH_3CN).

The *n*-BuOH fraction (40 g) was subjected to a silica gel (250 g) column (7 × 17 cm) chromatography eluting with gradients $CHCl_3$–MeOH–H_2O {10:1:0 (1.0 L) → 7:1:0 (800 mL) → 5:1:0 (800 mL) → 65:35:10 (1.0 L)} to afford eight subfractions (ADB-1: 220–590 mL, ADB-2: 591–920 mL, ADB-3: 921–1420 mL, ADB-4: 1421–1970 mL, ADB-5: 1971–2560 mL, ADB-6: 2561–2950 mL, ADB-6: 2951–3110 mL, ADB-7: 3111–3550 mL). The fifth fraction (ADB-5, 914 mg) was subjected to silica gel (200 g, 7 × 15 cm) c.c. eluting with $CHCl_3$–MeOH (5:1, 1.4 L) to yield three subfractions (ADB-5-1: 280–680 mL, ADB-5-2: 710–840 mL, ADB-5-3: 890–1340 mL). Silica gel (150 g, 5 × 25 cm) c.c. of the ADB-5-1 (572 mg), using *n*-BuOH–EtOAc (2:5,

1.2 L) as the elution solvent, gave three subfractions (ADB-5-1-1: 220–410 mL, ADB-5-1-2: 620–840 mL, ADB-5-1-3: 920–1180 mL). The ADB-5-1-2 (165 mg) was acetylated with pyridine (4 mL) and acetic anhydride (4 mL) at room temperature overnight. The reaction solution was treated with the usual methods and purified with silica gel (75 g, 3 × 24 cm) c.c. eluting with *n*-hexane–EtOAc (1:1, 750 mL) to afford compound **8a** (380–560 mL, isopraeroside IV tetraacetate, 221 mg, R_f: 0.42 on silica gel TLC in *n*-hexane–EtOAc = 1:2).

Isopraeroside IV tetraacetate (**8a**): Pale yellowish oil, $[\alpha]^{28}_D$+54° (MeOH, *c* = 1.2); IR: γ_{max} cm^{-1} ($CHCl_3$) 1715, 1610, 1515; ^{1}H-NMR (400 MHz, $CDCl_3$, δ) 7.62 (1H, d, *J* = 9.3 Hz, H-4), 7.25 (1H, d, *J* = 8.8 Hz, H-5), 6.71 (1H, d, *J* = 8.8 Hz, H-6), 6.20 (1H, d, *J* = 9.3 Hz, H-3), 5.13 (1H, dd, *J* = 9.5, 9.5 Hz, glc-4), 4.94 (1H, dd, *J* = 9.7, 8.5 Hz, glc-2), 4.84 (1H, dd, *J* = 9.7, 9.5 Hz, glc-3), 4.75 (1H, d, *J* = 8.5 Hz, glc-1), 4.69 (1H, dd, *J* = 2.6, 5.6 Hz, H-2′), 3.93 (1H, dd, *J* = 5.3, 12.2 Hz, glc-6a), 3.56 (1H, dd, *J* = 2.4, 12.2 Hz, glc-6b), 3.48 (1H, m, glc-5), 3.25 (1H, dd, *J* = 2.6, 12.0 Hz, H-1′a), 3.23 (1H, dd, *J* = 5.6, 12.0 Hz, H-1′b), 1.96, 1.95, 1.92, 1.91 (all 3H, each s, acetyl-methyl), 1.29 (3H, s, H-4′), 1.22 (3H, s, H-5′); ^{13}C-NMR (100 MHz, $CDCl_3$, δ_C) 171.14, 170.52, 170.34, 169.32 (acetyl-carbonyl), 163.75 (C-2), 160.95 (C-7), 151.31 (C-8a), 143.87 (C-4), 128.65 (C-5), 113.96 (C-8), 113.12 (C-4a), 112.32 (C-3), 106.43 (C-6), 95.62 (glc-1), 90.40 (C-2′), 78.62 (C-3′), 72.90 (glc-5), 71.52 (glc-3), 71.49 (glc-2), 69.46 (glc-4), 61.68 (glc-6), 60.38 (C-2′), 27.70 (C-1′), 22.75 (C-4′), 22.65 (C-5′), 20.59 (x 2), 20.56, 20.53 (acetyl-methyl).

Compound **8a** (50 mg) was dissolved in 5 mL of 2% KOH solution (H_2O–MeOH = 1:3) and stirred at room temperature for 30 min. The reaction mixture was neutralized by the addition of Dowex 50w × 8 (H^+ form), filtered and evaporated to dryness, followed by silica gel (50 g, 3 × 12 cm) c.c. (*n*-hexane–EtOAc = 8:5, 450 mL) to yield the compound **8** (210–320 mL, isopraeroside IV, 12.2 mg, R_f: 0.61 on silica gel TLC in $CHCl_3$–MeOH–H_2O = 65:35:10).

Isopraeroside IV (**8**): White powder (*n*-hexane–EtOAc–MeOH), m.p. 117–118 °C; $[\alpha]^{26}_D$+66° (MeOH, *c* = 1.2); IR: γ_{max} cm^{-1} ($CHCl_3$) 3382, 1710, 1604, 1490; EI-MS *m*/*z*: 408 (M^+), 275, 246, 228, 187, 175; HREI-MS: Found: 408.1422, Calcd. For $C_{20}H_{24}O_9$: 408.1420; ^{1}H-NMR (400 MHz, CD_3OD, δ) 7.77 (1H, d, *J* = 9.5 Hz, H-4), 7.30 (1H, d, *J* = 8.6 Hz, H-5), 6.67 (1H, d, *J* = 8.6 Hz, H-6), 6.09 (1H, d, *J* = 9.5 Hz, H-3), 4.49 (1H, d, *J* = 7.8 Hz, glc-1), 4.12 (1H, dd, *J* = 2.1, 5.5 Hz, H-2′), 3.05 (1H, dd, *J* = 2.1, 12.0 Hz, H-1′a), 3.01 (1H, dd, *J* = 5.5, 12.0 Hz, H-1′b), 1.28, 1.26 (both 3H, each s, H-4′, 5′); ^{13}C-NMR (100 MHz, CD_3OD, δ_C) 165.60 (C-2), 163.25 (C-7), 152.54 (C-8a), 146.33 (C-4), 130.27 (C-5), 115.53 (C-8), 114.46 (C-4a), 112.36 (C-3), 107.86 (C-6), 98.91 (glc-1), 91.90 (C-2′), 79.16 (C-3′), 78.13 (glc-5), 77.50 (glc-3), 75.12 (glc-2), 71.39 (glc-4), 62.35 (glc-6), 28.27 (C-1′), 23.79, 22.47 (C-4′, 5′).

ADB-5-3 (29 mg) was acetylated by similar procedures to those of ADB-5-1-2 and applied to a silica gel (30 g) column (3 × 9 cm) eluting with *n*-hexane–EtOAc (5:6, 240 mL) to afford compound **9a** (140–190 mL). The same treatment of compound **9a** as that of compound **8a** and silica gel (30 g, 3 × 9 cm) c.c. ($CHCl_3$-MeOH = 4:1, 320 mL) gave a purified compound **9** (180–270 mL, nodakenin, 17 mg, R_f: 0.52 on silica gel TLC in $CHCl_3$–MeOH–H_2O = 65:35:10); white powder (*n*-hexane–EtOAc), m.p. 224–225 °C; $[\alpha]^{26}_D$+28° (MeOH, *c* = 0.7). Lit. (Gu *et al.*, 1990) m.p. 217–219 °C; $[\alpha]^{25}_D$+24° (EtOH–H_2O = 1:1, *c* = 0.9).

ADB-7 (1.5 g) was subjected to silica gel (150 g, 5 × 25 cm) c.c. using $CHCl_3$–EtOH (3:1, 1.6 L) as eluents to obtain five subfractions (ADB-7-1: 210–320 mL, ADB-7-2: 340–460 mL, ADB-7-3: 520–680 mL, ADB-7-4: 780–1120 mL, ADB-7-5: 1210–1480 mL), and the fourth one (ADB-7-4, 44 mg) was chromatographed on a silica gel (50 g) column (3 × 14 cm, *n*-hexane–EtOAc–EtOH = 2:10:3, 720 mL) to afford compound **10** (460–580 mL, 3′-hydroxymarmesinin, 22 mg, R_f: 0.33 on silica gel TLC in $CHCl_3$–MeOH–H_2O = 65:35:10); colorless needles (*n*-hexane–EtOAc–EtOH), m.p. 262–263 °C; $[\alpha]^{26}_D$–24° (MeOH, *c* = 0.7). Lit. (Kim *et al.*, 1992) m.p. 217–218 °C; $[\alpha]^{25}_D$–11° (pyridine, *c* = 0.1).

Enzyme purification and assays. The purification of bovine brain GABA transaminase was performed by a method developed in our laboratory (Choi *et al.*, 1993). Succinic semialdehyde dehydrogenase was prepared from bovine brain by a combination of CM-Sepharose, Blue-Sepharose and hydroxyapatite chromatographic methods (Lee *et al.*, 1995). The protein concentration was determined with a protein assay kit from Bio-Rad using bovine serum albumin as a standard.

A coupled assay system consisting of two purified enzymes, i.e. GABA transaminase and succinic semialdehyde dehydrogenase was used to study the catalytic conversion of GABA into succinic semialdehyde.

$$\text{GABA} + \alpha\text{-ketoglutarate} \xrightarrow{GABA\text{-}T} \text{succinic semialdehyde (SSA)} + \text{glutamic acid} \quad (1)$$

$$\text{SSA} + NAD^+ + H_2O \xrightarrow{SSADH} \text{succinate} + NADH \quad (2)$$

Enzymatic assays were performed in 0.1 M sodium pyrophosphate buffer, pH 8.4, containing 1 mM 2-mercaptoethanol, 5 mM NAD^+, 30 mM GABA, and 10 mM α-ketoglutarate. The progress of the reaction was monitored by measuring the absorbance changes at 340 nm due to the reduction of NAD^+. The coupled assay system is appropriate to measure the rate of transamination when the concentration of SSADH is at least five-fold higher than that of GABA transaminase. A unit of enzyme activity is defined as the amount of enzyme that produces 1 μmol · min^{-1} of succinic semialdehyde at 25 °C.

To measure the catalytic activity of SSADH, the formation of NADH was measured by the increase in absorbance at 340 nm. All assays were performed in duplicate and the initial velocity data were correlated with a standard assay mixture containing 100 μM succinic semialdehyde and 1 mM NAD^+ in 0.1 M sodium pyrophosphate (pH 8.4) at 25 °C.

Inactivation of enzymes with pure compounds isolated from the root of *Angelica dahurica*. Purified GABA-T (10 μM) was treated with various concentrations of the sample. Changes in catalytic activity were measured using the method described above. An antiepileptic drug vigabatrin was used as a positive control. The reaction was initiated by the addition of the sample at 25 °C in 0.1 M of potassium phosphate buffer (pH 7.4). At intervals after the initiation of the inactivation, aliquots were withdrawn for the activity assay. Protection experiments

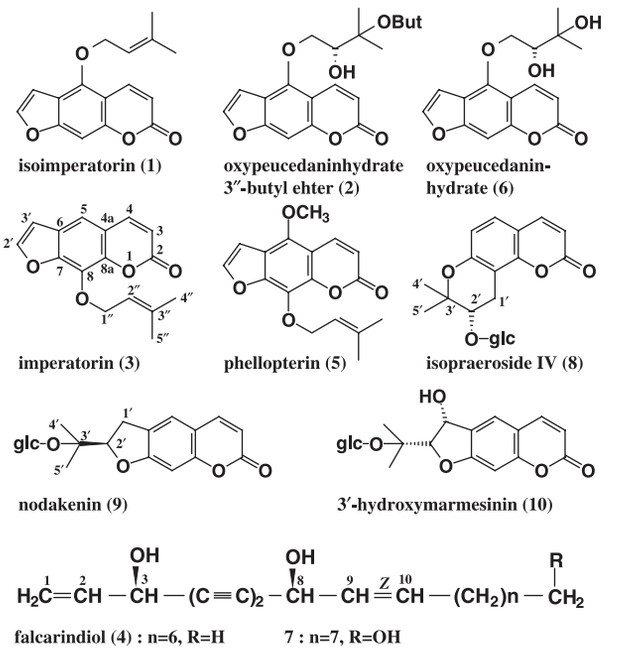

Figure 1. Chemical structures of coumarins and polyacetylenes from the root of *Angelica dahurica.*

against inactivation were performed in a similar manner except that the enzyme was pre-incubated with a substrate (GABA or α-ketoglutarate) before the modification was initiated by the addition of samples. Inhibition kinetic constants were calculated by using a least-squares fit or the data points on a Kitz & Wilson plot (Kitz and Wilson, 1962). All kinetic data presented here are mean values obtained from more than two experimental points (Fig. 1).

RESULTS AND DISCUSSION

The MeOH crude extracts obtained from the root of *Angelica dahurica* show the inhibitory activities on GABA degradation enzymes GABA-T and SSADH. They were fractionated with EtOAc, *n*-BuOH and H_2O, successively, and the treatment of EtOAc and *n*-BuOH fractions with the concentration of 100 μg/mL significantly inhibited the activity of GABA-T by 76% and 62% and that of SSADH by 62% and 58%, respectively.

The major components of the fractions, which have been shown as coumarins and polyacetylenes, were isolated by repeated silica gel column chromatography. (Yield – **1**: 0.063%; **2**: 0.017%; **3**: 0.025%; **4**: 0.059%; **5**: 0.018%; **6**: 0.060%; **7**: 0.004%; **8**: 0.007%; **9**: 0.002%; **10**: 0.003%.) Compounds **1**, **3**, **5** and **6** showed the typical characteristics of linear furanocoumarin in the several spectral data including ^{1}H and ^{13}C-NMR. Their physical or spectral data were compared with those reported in the literature leading to their identifications as isoimperatorin (**1**) (Bergendorff *et al.*, 1997; Gu *et al.*, 1990; Kozawa *et al.*, 1981), imperatorin (**3**) (Bergendorff *et al.*, 1997; Razdan *et al.*, 1982; Kozawa *et al.*, 1981), phellopterin (**5**) (Kwon *et al.*, 1997; Kozawa *et al.*, 1981) and oxypeucedanin hydrate (**6**) (Kozawa *et al.*, 1981; Ishihara *et al.*, 2001). Also, ^{1}H- and ^{13}C-NMR data of compounds **9** and **10** indicated the presence of a linear hydrofurancoumarin skeleton and a glucopyranosyl moiety. Also, compounds **9** and **10** were identified to be nodakenin (Kim *et al.*, 1992; Gu *et al.*, 1990; Matano *et al.*, 1986) and 3′-hydroxymarmesinin (Kim *et al.*, 1992), respectively, through the comparison of several physical and spectral data with those reported in the literature.

The ^{1}H- and ^{13}C-NMR spectra of compound **2** exhibited signals that resembled those of oxypeucedanin hydrate (**6**) with the exception of the additional presence of a *n*-butyloxy unit {δ_H: 3.33 (2H, t, J = 6.3 Hz), 1.45 (2H, m), 1.29 (2H, m), 0.83 (3H, t, J = 7.3 Hz); δ_C: 60.92 (t), 32.39 (t), 19.37 (t), 13.82 (q)}. The comparison of several physical and spectral data with those of the literature (Kozawa *et al.*, 1981; Ishihara *et al.*, 2001) led to the structure determination of compound **2** as oxypeucedanin hydrate 3″-butyl ether. From Umbelliferae plants, derivatives of oxypeucedanin hydrate such as 3″-methoxy (Chi and Kim, 1981) or 3″-ethoxy (Harkar *et al.*, 1982) have been isolated, but 3″-butoxy derivative has not been reported so far. To confirm that compound **2** was not an artefact during extraction or isolation procedures, the root of *Angelica dahurica* was extracted in acetone and partitioned between EtOAc and H_2O. The presence of compound **2** in the EtOAc layer was confirmed by direct comparison of the TLC for the EtOAc layer and the authentic compound.

The ^{1}H- and ^{13}C-NMR data of compound **8** were nearly the same as praeroside IV (Takata *et al.*, 1988) with the exception of the marked shift of the C-2′ signal toward the low magnetic field, and the smaller coupling constants of H-2′, which was commonly observed in the C-2′ stereoisomer of praeroside derivatives (Takata *et al.*, 1988). Finally, the careful inspection of several spectral data including ^{1}H-^{1}H COSY, gHSQC and gHMBC of compound **8a**, along with the comparison with those of the literature (Takata *et al.*, 1988), revealed the chemical structure to be 2′S-praeroside IV, named isopraeroside IV.

Compounds **4** and **7** exhibited typical characteristics of polyacetylenes in several spectral data including ^{1}H and ^{13}C-NMR. Their physical or spectral data were compared with those of literature (Liu *et al.*, 1998) leading to their identification as falcarindiol and octadeca-1,9-dien-4,6-diyn-3,8,18-triol. The simultaneous occurrence of C_{17}- and C_{18}-polyacetylenes in the same plant is a very rare case.

To our knowledge, compounds **2** and **8** have never been reported for the isolation from natural sources so far, and compounds **4** and **7** were first isolated from *Angelica dahurica*. However, some major coumarins of *Angelica dahurica* including byak-angelicin or scopoletin were not detected in this experiment.

All the isolated compounds were evaluated for their inhibitory effects on the activity of SSADH and GABA-T. Most compounds did not show any marked effects on the activity of SSADH. On the other hand, imperatorin (**3**) and falcarindiol (**4**) significantly inhibited the activity of GABA-T, while octadeca-1,9-dien-4,6-diyn-3,8,18-triol (**7**) did a little (Table 1). Although the compounds were structurally very similar to each other, their inhibitory effects were different. In other studies referring to the principal coumarins' regulation of the central nervous system, phellopterin was reported to inhibit strongly the binding of diazepam to the

Table 1. The remaining activity of GABA-T and SSADH after treatment of coumarins and polyacetylenes isolated from the root of *Angelica dahurica*

Compound No	1	2	3	4	5	6	7	8	9	10	Vigabatrin
GABA-T	87	84	24	35	78	98	62	100	82	93	63
SSADH	92	90	93	88	78	95	87	99	107	100	–

Either GABA-T (10 μM) or SSADH (10 μM) was treated with 7 mM of each isolated compound for 10 min prior to performing enzyme assays as described under Materials and Methods. Reaction of purified GABA-T with 1 mM of vigabatrin is included as a positive control. The percent values shown are averages of duplicate determinations that agreed within 5%.

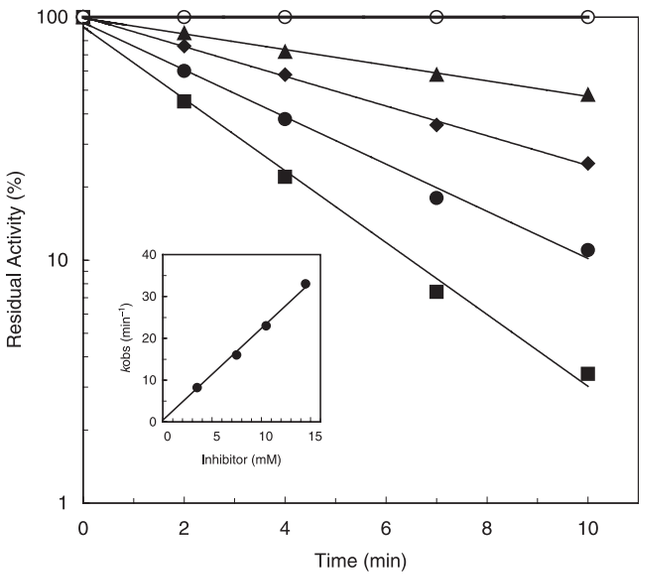

Figure 2. Time-dependent inactivation of GABA-T by imperatorin (**3**). GABA-T (10 μM) was incubated with no (○), 3.5 mM (▲), 7 mM (□), 10.5 mM (●), and 14 mM (■) of imperatorin (**3**) at 25 °C in 0.1 M of potassium phosphate buffer (pH 7.4). At given time intervals, aliquots were removed and assayed for remaining enzyme activities. Pseudo first-order rate constants of inactivation k_{inact} were obtained from the slopes of straight lines fitted to data points by least square linear regression. Each point is the mean of a triplicate determination. Ranges of individual values were less 5% in all cases. Error bars are omitted for clarity. Insets, determination of the second-order rate constant of inactivation.

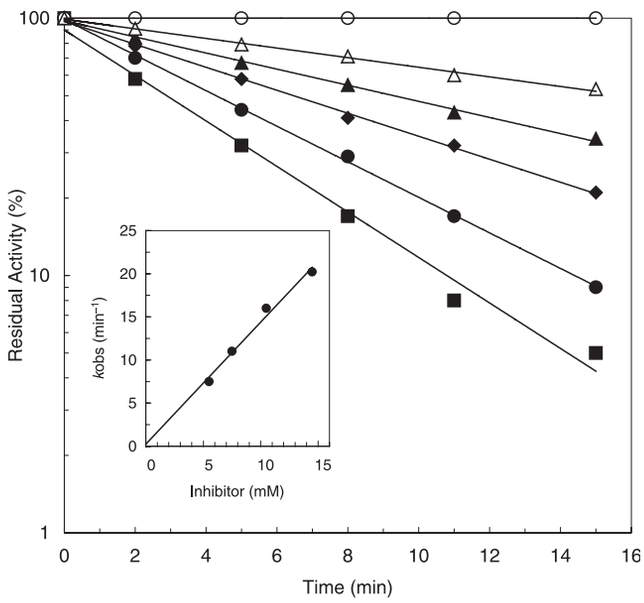

Figure 3. Time-dependent inactivation of GABA-T by falcarindiol (**4**). GABA-T (10 μM) was incubated with no (○), 5.7 mM (▲), 7 mM (□), 10.5 mM (●), and 14 mM (■) of falcarindiol at 25 °C in 0.1 M of potassium phosphate buffer (pH 7.4). At given time intervals, aliquots were removed and assayed for remaining enzyme activities. Reaction of purified GABA-T with 1 mM of vigabatrin is included as a positive control (□). See Fig. 2 legend for detail.

central nervous system benzodiazepine receptors *in vitro* (Bergendorff *et al.*, 1997), and osthol and isoimperatorin were reported to show significant analgesic effects (Kosuge *et al.*, 1985).

Incubation of GABA-T with varying concentrations of imperatorin (**3**) and falcarindiol (**4**) resulted in a time-dependent loss of enzyme activity. The imperatorin (**3**) inactivates the enzyme in both a time- and concentration-dependent manner (Fig. 2). Inactivation follows a pseudo-first order kinetic behavior at each fixed concentration of sample. A plot of the pseudo-first order constant at each inhibitor concentration, taken from the slopes of the lines in Fig. 2, gives a straight line (inset of Fig. 2), demonstrating that the inactivation process is the results of a simple bimolecular reaction. Likewise, the incubation of GABA-T with varying concentrations of falcarindiol (**4**) resulted in a time-dependent loss of enzyme activity (Fig. 3) and exhibited a bimolecular kinetics (inset of Fig. 3). The second-order rate constants (k_{inact}) obtained for the inactivation was equal to $2.3 \pm 0.2\ mM^{-1}\ min^{-1}$ for imperatorin (**3**), a value higher than that calculated for falcarindiol (**4**) ($1.5 \pm 0.1\ mM^{-1}\ min^{-1}$).

Since the coupled assay system contains two enzymes, GABA-T and SSADH, the inhibitory effect of the sample on SSADH was independently investigated in a similar manner. Pre-incubating purified SSADH with increasing concentrations of samples up to 14 mM did not lead to any significant loss of the catalytic activity. This result indicated that the inhibitory effect was due solely to the inactivation of GABA-T activity.

To demonstrate that imperatorin (**3**) and falcarindiol (**4**) act as an active-site-directed irreversible inhibitor of GABA-T activity, the ability of the substrates to protect against the inactivation was investigated. GABA-T (10 μM) in 0.1 M KH_2PO_4, pH 7.4 (25 °C), was inactivated with 7 mM of each sample, and the residual activity was determined after 10 min of incubation. Protection was achieved by incubating for 20 min with substrate, 10 mM α-ketoglutarate or 20 mM GABA before the start of the inactivation. As shown in Table 2, the inactivation by imperatorin (**3**) was completely protected when the enzyme was pre-incubated with α-ketoglutarate, whereas only a partial effect was observed with GABA. Similar results for substrate protection were obtained for falcarindiol (**4**) inactivation, except that little or no effect was obtained for the protection by GABA. These results indicated that the

Table 2. Substrate protection from inactivation of GABA-T by imperatorin (3) and falcarindiol (4)

Reaction mixture	Residual activity (%)
GABA-T (10 μM)	100
GABA-T + imperatorin (14 mM)	14
GABA-T with α-ketoglutarate (10 mM) + imperatorin	98
GABA-T with GABA (20 mM) + imperatorin	74
GABA-T + falcarindiol (14 mM)	23
GABA-T with α-ketoglutarate (10 mM) + falcarindiol	95
GABA-T with GABA (20 mM) + falcarindiol	98

Residual activity was determined after 5 min of incubation with imperatorin or falcarindiol. Protections were achieved by pre-incubating with substrates for 30 min before the start of the inactivation by imperatorin or falcarindiol.

inactivation resulted from selective modification of a functional group of the enzyme active center rather than from a nonspecific reaction mechanism.

Time-dependency of the inhibitory process could be used to show that the type of inhibition is irreversible. To further evaluate the irreversibility of the interaction between enzyme and inhibitor, the ability to recover GABA-T activity by dilution was examined. GABA-T was pre-incubated for 20 min at 25 °C with 7 mM of imperatorin (**3**) or falcarindiol (**4**) and then diluted by up to 10-fold prior to measurement of enzyme activity. Under these conditions, inhibition by a reversible inhibitor would be reduced in parallel to the degree of dilution, whereas inhibition by an irreversible inactivator would remain unchanged. The resulting inhibitory effects were not significantly changed by dilution (data not shown). Taken together, these observations indicate that both imperatorin (**3**) and falcarindiol (**4**) act as irreversible inactivators of GABA-T.

In conclusion, this study reports for the first time that the naturally occurring compounds imperatorin (**3**) and falcarindiol (**4**) bind to the active site of GABA-T, and behave as effective irreversible inhibitors of the enzyme. Further studies to elucidate the alteration of GABA levels by this compound in brain may provide insights into a potent therapeutic anticonvulsant.

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