Anticonvulsant Compounds from the Wood of *Caesalpinia sappan* L.

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80% Aqueous MeOH extracts from the wood of *Caesalpinia sappan*, which showed remarkable anticonvulsant activity, were fractionated using EtOAc, *n*-BuOH, and H_2O. Among them, the EtOAc fraction significantly inhibited the activities of two GABA degradative enzymes, succinic semialdehyde dehydrogenase (SSADH) and succinic semialdehyde reductase (SSAR). Repeated column chromatographies for the fraction guided by activity test led to the isolation of the two active principal components. Their chemical structures were determined to be sappanchalcone and brazilin based on spectral data. The pure compounds, sappanchalcone (**1**) and brazilin (**2**), inactivated the SSAR activities in a dose dependent manner, whereas SSADH was inhibited partially by sappanchalcone and not by brazilin.

Key words: *Caesalpinia sappan*, Anticonvulsant, Brazilin, Sappanchalcone, Succinic semialdehyde reductase, Succinic semialdehyde dehydrogenase

INTRODUCTION

GABA (γ-aminobutyric acid) is present in many tissues of mammalians, also competitive amounts are present in the central nervous system (CNS), where it has been known to be a major inhibitory chemical neurotransmitter (Fletcher and Fowler, 1986). The release of GABA by nerve terminals and its subsequent binding to its receptor must be followed by a rapid inactivation of the neurotransmitter. When the concentration of GABA in brain diminishes to below a threshold level, various neurological disorders including epilepsy, seizures, convulsions, Huntington's disease, and Parkinsonism may occur (Perry *et al.*, 1973; Lloyd *et al.*, 1997; De Biase *et al.*, 1991).

The concentration of GABA in brain is controlled by two pyridoxal-5'-phosphate (PLP) dependent enzymes, i.e., glutamate decarboxylase (GAD) and GABA transaminase (GABA-T). The first enzyme catalyzes the synthesis of GABA, whereas the second enzyme catalyzes the conversion of GABA to succinic semialdehyde in a transamination reaction. Succinic semialdehyde is oxidized to succinate by a succinic semialdehyde dehydrogenase (SSADH) and can also be reduced to γ-hydroxybutyrate (GHB) by succinic semialdehyde reductase (SSAR). The observation that the activation of GAD or the inactivation of the GABA-T, SSADH, and SSAR in brain tissues increases the concentration of neurotransmitter GABA supports the contention that these enzymes exert a controlling influence on GABA levels. It is reported that the irreversible inhibition of GABA-T by chemical analogues of GABA is the basic action mechanism of drugs used in the treatment of convulsive disorders (Lippert *et al.*, 1977).

Caesalpinia sappan L. (Leguminosae) is an indeciduous tree distributed in China and Taiwan, and its heartwood has been traditionally used as an analgesic, a therapy for thrombosis or tumor (Soka, 1985). Even though several neoflavonoid compounds have been isolated from the wood of *C. sappan* (Saitoh *et al.*, 1986; Namikoshi *et al.*, 1987a), and many experiments regarding biological activities such as inhibitory activity in central nervous system (Nagai *et al.*, 1986) and antihypercholesteremic activity (Shimokawa *et al.*, 1985) have been carried out, the principal component of the drugs manifesting anti-spasmodic has not yet been reported so far.

In this paper, the principal compound, isolated from

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the *Caesalpinia sappan*, was examined for sedative or antispasmodic effects, and its inhibitory effect on GABA degrative enzymes (SSADH and SSAR), isolated from bovine brain, was also examined.

MATERIALS AND METHODS

Materials

The wood of *Caesalpinia sappan* was purchased at a market in Seoul and was identified by Dr. Hyeong-Kyu Lee, Korea Research Institute of Bioscience and Biotechnology, Taejon. The voucher specimen are preserved at the Laboratory of Natural Products Chemistry, Kyunghee University, Suwon (KH97124).

Bovine brains were obtained from the Majangdong Packing Company in Seoul, Korea. NAD^+, NADH, ammonium sulfate, succinic semialdehyde, bovine serum albumine, EDTA, 2-mercaptoethanol, NADPH and $NADP^+$ were purchased from Sigma Chemical Co. (St. Louis, U.S.A). CM-Sepharose, Blue-Sepharose, 5'-AMP-Sepharose, Mono-Q and Superose-12 were obtained from Pharmacia/LKB (Uppsala, Sweden).

Instruments

Melting points were determined on a Fisher-John apparatus and uncorrected. ^{1}H- (400 MHz) and ^{13}C-NMR (100 MHz) spectra were measured with JEOL JNM-LA 400 spectometer. EI mass spectra were taken on a JEOL JMS-AX505WA spectrometer. Optical rotations were measured on a JASCO DIP-370 digital polarimeter. IR spectra were taken with a Perkin-Elmer 599B spectrometer. Absorption spectroscopic measurements were carried out in Kontron UVIKON Model 930 double beam spectrophotometer. Fluorescence spectra were recorded in a Kontron SFM 25 spectrofluorometer.

Isolation of sappanchalcone (1) and brazilin (2)

The wood of *C. sappan* (2 kg) was milled and extracted with 80% aqueous MeOH solution (5 L × 2) at room temperature. The filtration of the extracted solution and evaporation under reduced pressure yielded methanolic extracts (153 g). Successive partition of the extracts in H_2O with EtOAc and *n*-BuOH furnished aqueous (CSW, 20 g), EtOAc (CSE, 107 g) and *n*-BuOH (CSB, 23 g) soluble fractions, respectively.

A portion of EtOAc soluble fraction (SME, 30 g) was subjected to silica gel column chromatography (300 g), which was eluted stepwise-gradiently with $CHCl_3$-MeOH (10:1→7:1→5:1→3:1), to be divided into nine subfractions (SME-1~SME-9). SME3 fraction (4.25 g) was successively applied to silica gel column chromatography (125 g, $CHCl_3$-MeOH=20:3 and 110 g, *n*-hexane-EtOAc=1:2) and compound **1** was obtained as a purified one (58 mg).

Compound **1** (sappanchalcone): yellow needles (EtOH), mp 199-200°C, IR_v (KBr, max) 3370, 2965, 1625, 1508, 1445 cm^{-1}, EI/MS *m/z* (%) 286 (M^+, 100), 271 (92), 268 (68), 253 (40), 251 (21), 159 (12), 144 (11), 109 (15). ^{1}H-NMR (400 MHz, CD_3OD, δ) 7.47 (1H, d, *J*=8.5 Hz, H-6'), 7.39 (1H, d, *J*=15.9 Hz, H-β), 7.25 (1H, d, *J*=15.9 Hz, H-α), 7.01 (1H, d, *J*=2.0 Hz, H-2), 6.88 (1H, dd, *J*=2.0, 8.3 Hz, H-6), 6.69 (1H, d, *J*=8.3 Hz, H-5), 6.40 (1H, d, *J*=2.2 Hz, H-3'), 6.35 (1H, dd, *J*=2.2, 8.5 Hz, H-5'), 3.77 (3H, s, $-OCH_3$). ^{13}C-NMR (100MHz, CD_3OD, δ_C) 193.10 (ketone), 164.41 (C-4'), 162.43 (C-2'), 149.44 (C-4), 146.69 (C-3), 144.57 (C-β), 133.70 (C-α), 128.63 (C-1), 125.07 (C-6'), 123.35 (C-6), 121.71 (C-1'), 116.56 (C-5), 115.25 (C-2), 108.92 (C-5'), 100.13 (C-3'), 56.12 ($-OCH_3$).

Repeated silica gel column chromatographies (250 g, *n*-hexane-EtOAc=1:3 and 200 g, *n*-hexane-EtOAc=2:3) of SME-5 (5.18 g) gave a purified compound **2** (1.2 g).

Compound **2** (brazilin): pale-yellow crystals (EtOH-H_2O), mp 249-250°C, $[\alpha]_D$+124°(c=1.12, MeOH), IR_v (KBr, max) 3378, 2915, 2850, 1615, 1510, 1465 cm^{-1}, EI/MS *m/z* (%) 286 (M^+, 100), 268 (69), 267 (62), 229 (43), 213 (11), 110 (16). ^{1}H-NMR (400 MHz, CD_3OD, δ) 7.06 (1H, d, *J*=8.8 Hz, H-1), 6.61 (1H, s, H-8), 6.50 (1H, s, H-11), 6.36 (1H, dd, *J*=2.4, 8.8 Hz, H-2), 6.20 (1H, d, *J*=2.4 Hz, H-4), 3.86 (1H, br. s, H-12), 3.82 (1H, dd, *J*=1.2, 11.2 Hz, H-6), 3.58 (1H, d, *J*=11.2 Hz, H-6), 2.92 (1H, d, *J*=15.6 Hz, H-7), 2.65 (1H, d, *J*=15.6 Hz, H-7). ^{13}C-NMR (100MHz, CD_3OD, δ_C) 157.69 (C-4a), 155.61 (C-3), 145.52 (C-9), 145.20 (C-10), 137.41 (C-11a), 132.21 (C-1), 131.34 (C-7a), 115.52 (C-1a), 112.88 (C-8), 112.42 (C-11), 109.96 (C-2), 104.24 (C-4), 78.05 (C-6a), 70.77 (C-6), 50.95 (C-12), 42.79 (C-7).

Enzyme purification and assay

The purification of bovine brain SSADH was performed by a method previously described (Lee *et al.*, 1995). For precise kinetic data, the formation of NADH was measured by following the increase in absorbance at 340 nm at which NADH is known to have a molar absorption coefficient of $6.22 \times 10^3 M^{-1}cm^{-1}$. All assays were performed in duplicate and the initial velocity data was correlated with a standard assay mixture containing 10 μM ssucinic semialdehyde and 5 mM NAD^+ in 0.1 μM sodium pyrophosphate (pH 8.4) at 25°C.

SSAR from bovine brain was purified by the method developed in our laboratory (Cho *et al.*, 1993). The method involves four column chromatographic steps: CM-Sepharose, Blue-Sepharose, hydroxyapatite and Mono-Q. To measure the activity of SSAR, the oxidation of NADPH to $NADP^+$ was measured at 340 nm as reported previously (Cho *et al.*, 1993). All assays were performed in duplicates, and initial velocity data was correlated with a standard assay mixture containing succinic semialdehyde (120 μM) and NADPH (50 μM) in 0.01 M potassium phosphate buffer,

with a pH of 7.0 at 25°C. One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 μmol NADPH/min at 25°C. Protein concentration was estimated by the Bradford method with bovine serum albumin as a standard (Bradford, 1976).

Reaction of enzymes with sappanchalcone (1), brasilin (2), sodium valproate and crude extracts

SSADH was preincubated with various concentrations of sappanchalcone (**1**), brazilin (**2**), sodium valproate and 80% aqueous MeOH extracts in 0.05 M potassium phosphate buffer (pH 8.0) at 25°C, respectively. Aliquots withdrawn from each of the incubation mixtures were assayed for enzymatic activity at various times, respectively.

The purified SSAR was preincubated with various concentrations of sappanchalcone (**1**), brazilin (**2**), sodium valproate and 80% aqueous MeOH extracts in 0.01 M potassium phosphate buffer, with a pH of 7.0. After an appropriate time, an aliquot of the incubation mixture was taken and the remaining enzyme activities of an aliquot were measured at 25°C. Protection experiments by coenzyme or substrate were performed in a similar manner, except that the enzyme was preincubated with a substrate or coenzyme for 30 min before modification by sappanchalcone (**1**), brazilin (**2**) and sodium valproate.

RESULTS AND DISCUSSION

Since abnormally low levels of neurotransmitter GABA in brain has been associated with a variety of neurological disorders including epilepsy, seizure and convulsant disorder, a specific inhibitory compound of GABA degradative enzymes (GABA-T, SSADH or SSAR) would be useful in attempts to elevate GABA levels in certain pathological conditions.

Recently, we have screened the effect of MeOH extracts obtained from several plants traditionally known as anticonvulsant drugs on GABA degradative enzymes. Among several plants, the crude extracts of *C. sappan* showed the inhibitory effect on brain SSADH and SSAR by 90% and 70%, respectively. Even though *C. sappan* has been known to have antihypercholesteremic activity (Shimokawa *et al.*, 1985) as well as inhibitory activity in the central nervous system (Nagai *et al.*, 1986), the biological function of *C. sappan* in brain still remains unclear. Therefore we started to isolate the pure active compounds which had inhibitory effect on the GABA degradative enzymes (SSADH and SSAR) by applying fractionation and chromatographic procedures.

As shown in Fig. 1, we identified pure compounds to be sappanchalcone and brazilin which are known as major biological components in *C. sappan*. Compound **1** showed the absorbance band due to hydroxyl and conjugated double bond at 3370, 1625, 1508 cm^{-1} in the IR

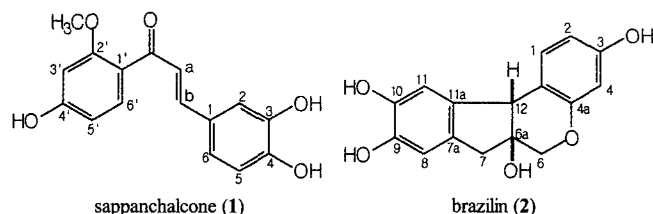

Fig. 1. Chemical structure of sappanchalcone (**1**) and brazilin (**2**)

spectrum (KBr). In the ^{1}H-NMR spectrum (400 MHz, CD_3OD) of **1**, two 1,2,4-trisubstituted benzene {(δ 7.47, d, *J*=8.5 Hz), (δ 6.40, d, *J*=2.2 Hz), (δ 6.35, dd, *J*=2.2, 8.5 Hz)}, {(δ 7.01, d, *J*=2.0 Hz), (δ 6.88, dd, *J*=2.0, 8.3 Hz), (δ 6.69, d, *J*=8.3 Hz)}, one pair of olefinic methine with trans-vicinal-coupling in with each other (δ 7.39, 7.25, d, *J*=15.9 Hz) and one methoxyl (δ 3.77, 3H, s) proton signals were observed indicating compound **1** to have a flavonoid skeleton. In the ^{13}C-NMR (100MHz, CD_3OD) spectrum, sixteen carbon signals including one methoxy carbon were observed. The following signals derived from one ketone (δ 193.10, s) and two olefinic methine carbons neighboring each other (δ 144.57, 133.70, d) revealed compound **1** to be a chalcone. Among twelve carbons composing A and B rings, four oxygenated quaternary (δ 164.41, 162.43, 149.44, 146.69, s), two quaternary (δ 128.63, 121.71, s), six methine carbon (δ 125.07, 123.35, 116.56, 115.25, 108.92, 100.13, d) of benzene ring and one methoxyl (δ 56.12, q) signals were observed. When the above results were compared with data of reference (Nagai et *al.*, 1984), the chemical structure of compound **1** was found to be 3',4',7-hydroxy-9-methoxy chalcone which is sappanchalcone.

Compound **2** showed IR absorbance of hydroxyl (3378 cm^{-1}) and conjugated dienes (1615, 1510 cm^{-1}). ^{1}H-NMR data (400 MHz, CD_3OD) of compound **2** showed the presence of one 1,2,4,5-tetrasubstituted (δ 6.61, 6.50, s) and one 1,2,4-trisubstituted {(δ 7.06, d, *J*=8.8 Hz), (δ 6.36, dd, *J*=2.4, 8.8 Hz), (δ 6.20, d, *J*=2.4 Hz)} benzen ring. One methylene (δ 2.92, δ 2.65, each d, both *J*=15.6 Hz), another oxygenated methylene (δ 3.82, δ 3.58, each d, both *J*=11.2 Hz) showing germinal coupling, and one methine (δ 3.86) signal were observed. The above results indicate that compound **2** is a flavonoid. In addition, the absence of carbonyl and the presence of sixteen carbons in the molecule confirmed from ^{13}C-NMR spectrum (100 MHz, CD_3OD) indicate that compound **2** is a neoflavonoid. Four oxygenated olefinic quaternary carbons (δ 157.69, δ 155.61, δ 145.52, δ 145.20, all s), three olefinic quaternary (δ 137.41, δ 131.34, δ 115.52, all s), and one oxygenated quaternary (δ 78.05, s) carbon resonances were observed. This indicates that compound **2** is a brazilin (Kim *et al.*, 1997), the major component of *C. sappan*. The stereo configurations of both C-6a and C-12

were determined to be *S* by optical specific rotation value ($[\alpha]_D$+124°) (Namikoshi et *al.*, 1987b; Kim *et al.*, 1997) of compound **2**.

Both purified compounds, sappanchalcone (**1**) and brazilin (**2**) inactivated the SSAR in a dose dependent manner (Fig. 2A and 2B), whereas sappanchalcone only has a partial inhibitory effect on the SSADH. Even though the crude extracts of *C. sappan* inhibited the SSADH completely (Table I), the major pure compounds purified from *C. sappan* showed very low inhibitory effects. Therefore, the pure compounds of *C. sappan* which are effective on SSADH still remains to be isolated and identified.

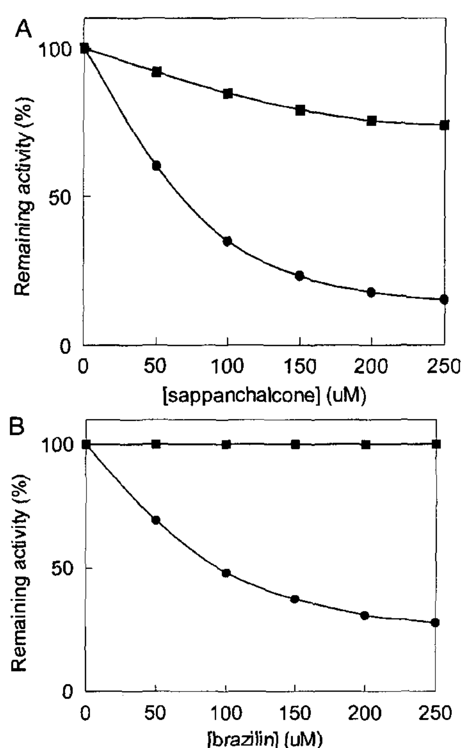

Fig. 2. A. Inhibition of succinic semialdehyde dehydrogenase (■) and reductase (●) by sappanchalcone. The enzyme (5 μM) was preincubated with various concentrations of sappanchalcone (50, 100, 150, 200, 250 μM) in 50 mM potassium phosphate buffer (pH 7.0) at 25°C. B. Inhibition of succinic semialdehyde dehydrogenase (■) and reductase (●) by brazilin. The enzyme (5 μM) was preincubated with various concentrations of brazilin (50, 100, 150, 200, 250 μM) in 50 mM potassium phosphate buffer (pH 7.0) at 25°C.

Table I. Effect of crude extracts from *Caesalpinia sappan* on GABA degradative enzymes

Reaction Mixture	Remaining Activity (%)
SSADH (5 μM)	100
SSADH (5 μM)+crude extracts (0.1 %)	10
SSAR (5 μM)	100
SSAR (5 μM)+crude extracts (0.1%)	30

Complete protections from the inactivation of SSAR by the two compounds were afforded by the coenzyme NADPH (Table II and Table III). In marked contrast to NADPH, succinic semialdehyde, substrates of SSAR, did not protect against the inactivation by inhibitors.

According to the inhibitory effect of sappanchalcone (**1**) and brazilin (**2**) against SSAR, we can assume that the inhibition of this enzyme is important in the elevation of neurotransmitter GABA levels in CNS. It has been known that sodium valproate (Epilim) is an effective anticonvulsant drug both in clinical and experimental epilepsies (Simler *et al.*, 1973; Simon and Penry, 1975; Anlezark *et al.*, 1976). Administration of this compound has been shown to raise the cerebral GABA level (Godin *et al.*, 1969). Our previous studies of sodium valproate for the elucidation of the possible action mechanism have suggested that it inhibits the SSAR effectively (Choi *et al.*, 1993). The sappanchalcone (**1**) and brazilin (**2**) isolated from *C. sappan* showed higher inhibition effect than valproate (Table IV) and may be an effective anticonvulsant and antiepileptic therapeutic drug.

Table II. Inactivation of succinic semialdehyde reductase by sappanchalcone

Reaction Mixture	Remaining Activity (%)
SSAR (5 μM)	100
SSAR (5 μM)+sappanchalcone (250 μM)	15
SSAR (5 μM)+SSA (3 mM)+sappanchalcone (250 μM)	20
SSAR (5 μM)+NADPH (3 mM)+sappanchalcone (250 μM)	95

Table III. Inactivation of succinic semialdehyde reductase by brazilin

Reaction Mixture	Remaining Activity (%)
SSAR (5 μM)	100
SSAR (5 μM)+brazilin (250 μM)	35
SSAR (5 μM)+SSA (3 mM)+brazilin (250 μM)	40
SSAR (5 μM)+NADPH (3 mM)+brazilin (250 μM)	92

Table IV. Inactivation of succinic semialdehyde reductase by sodium valproate

Reaction Mixture	Remaining Activity (%)
SSAR (5 μM)	100
SSAR (5 μM)+sodium valproate (250 μM)	38
SSAR (5 μM)+SSA (3 mM)+sodium valproate (250 μM)	45
SSAR (5 μM)+NADPH (3 mM)+sodium valproate (250 μM)	96

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