# Anticonvulsant Activity of Paeonimetabolin-I Adducts Obtained by Incubation of Paeoniflorin and Thiol Compounds with *Lactobacillus brevis*

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**Seventeen thiopaeonimetabolin-I adducts were obtained as mixtures of diastereoisomers after incubation of paeoniflorin with *Lactobacillus brevis* in the presence of various thiols. The anticonvulsant activity of the adducts was investigated in mice using the maximal subcutaneous pentylenetetrazol seizure test and sodium valproate (1.5 mmol/kg) as a positive control. Thirteen adducts showed dose-dependent prolongation of latencies of clonic and tonic convulsions. Maximal protection against convulsions was effectively demonstrated by 8-(*n*-hexylthio)paeonimetabolin I (8) and 8-benzoylthiopaeonimetabolin I (18) at doses of 0.125 and 0.25 mmol/kg, respectively, while 100% protection was only achieved at 0.5 mmol/kg of 8-cyclopentylthiopaeonimetabolin I and 8-(*p*-tolylthio)paeonimetabolin I. The principal anticonvulsant activity of the diastereoisomers of 8 and 18 was attributed to their 7*S*-isomers [$ED_{50}$ values of 0.09 and 0.12 mmol/kg, and protective indices of 5.0 and 4.0 for 8 (7*S*) and 18 (7*S*), respectively], while the 7*R* counterparts [8 (7*R*) and 18 (7*R*)] showed a muscle relaxation effect.**

**Key words** anticonvulsant; *Lactobacillus brevis*; paeoniflorin; pentylenetetrazole-induced seizure; thiopaeonimetabolin-I adduct

It has been estimated that 0.5—1% of the population suffers from epilepsy, with the highest incidence being found in children and the elderly.[1] Although about 70% of people with epilepsy achieve satisfactory seizure control with the available antiepileptic drugs, about 30% do not respond to these drugs either in monotherapy or in combination.[1] In addition, undesirable side effects from the drugs used clinically often render treatment difficult. This situation has inspired a broad-based search for less toxic, more efficacious and selective agents for the treatment of seizure disorders. One of the most common approaches is the synthesis of derivatives of already known and active agents in order to maximize the activity and duration of action, and to minimize the toxicity.[2] Hattori *et al.* reported that the 7*S*-isomer of paeonimetabolin I (**2**), the major intestinal bacterial metabolite of paeoniflorin (**1**), showed anticonvulsant activity in EI mice, an animal model of heredity epilepsy ($ED_{50}$ value of 41.3 mg/kg, i.v.).[3] When the metabolite was given intraduodenally or intraventricularly to rats, the convulsions induced by pentylenetetrazol (PTZ) were greatly inhibited.[3] Although **2** is one of the most interesting metabolites studied in this laboratory, we did not explore the structure–activity characteristics of this unique compound. Taking advantage of the fact that thiol adducts of **2** can be obtained by the incubation of **1** and thiol compounds (RSH, where R=aliphatic and aromatic groups) with *Lactobacillus brevis*, a human intestinal bacterium,[4] we were able to prepare a series of thiopaeonimetabolin-I derivatives with different RS-residues. In our follow-up work, we report herein the synthesis of 17 thio derivatives (**3**—**19**) and their evaluation as a new class of anticonvulsants.

## MATERIALS AND METHODS

**Instruments** Optical rotations were measured with a Jasco DIP-360 automatic polarimeter. Infrared (IR) spectra were measured with a Jasco FT/IR-230 infrared spectrophotometer. $^1$H- and $^{13}$C-NMR spectra were measured (in $CDCl_3$) with a Varian Gemini 300 ($^1$H, 300 MHz; $^{13}$C, 75 MHz), a Jeol JNM-GX 400 ($^1$H, 400 MHz; $^{13}$C, 100 MHz) or a Varian Unity plus 500 ($^1$H, 500 MHz; $^{13}$C, 125 MHz) spectrometer and chemical shifts are given in $\delta$ ppm relative to tetramethylsilane (TMS). Electron impact (EI) and high resolution (HR) EI-mass spectra were obtained with a Jeol JMS-AX 505 HAD spectrometer at an ionization voltage of 70 eV.

**Chromatography** Thin layer chromatography was carried out on pre-coated silica gel 60 $F_{254}$ plates (0.25 mm thickness, Merck, Darmstadt, Germany) and spots were detected under UV light or after spraying with anisaldehyde–$H_2SO_4$ reagent followed by heating. Column chromatography (CC) was carried out over silica gel 60 (70—230 mesh, Merck) and Diaion HP-20 (Mitsubishi Chemical, Tokyo, Japan). Medium pressure liquid chromatography (MPLC) was performed on a LiChroprep Si 60 column (size A, Merck) or LiChroprep RP-8 column (size A, Merck). Analytical HPLC was carried out on a CCPM-II (Tosoh, Tokyo, Japan) equipped with a Tosoh UV-8020 spectrometer and a Shimadzu C-R 6A chromatopac (Shimadzu, Kyoto, Japan) utilizing a Tosoh TSK-gel ODS-80Ts column [4.6 (i.d.)× 150 mm]. The flow rate was kept at 1.0 ml/min, the peaks were monitored at 254 nm using the following solvent system: A, a buffer solution consisting of 50 mM $KH_2PO_4$ and 0.1% $H_3PO_4$–$CH_3CN$ (95 : 5); B, $H_2O$–$CH_3CN$ (20 : 80) in a gradient mode; the retention time ($t_R$) was recorded in minure. Gas chromatography (GC) spectra were obtained using a GC-17A gas chromatograph (Shimadzu, Kyoto, Japan) fitted with a DB-1 column [0.25 mm (i.d.)×30 m] (J & W Scientific, U.S.A.), coupled to an automass system II benchtop quadrupole mass spectrometer (Jeol, Japan), column temperature: 50 °C, carrier gas: He, flow rate: 15 ml/min.

**Materials** Paeoniflorin (**1**) was isolated from the dried

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roots of *Paeoina lactiflora* Pall. according to the method of Kaneda *et al.*[5]; its purity was determined by HPLC to be >95%. General anaerobic medium (GAM) broth was purchased from Nissui Seiyaku Co., Ltd. (Tokyo, Japan). Sodium valproate (VPA) was obtained from Sigma (St. Louis, MO, U.S.A.) and PTZ was from Aldrich Chemical Co. (Milwaukee, U.S.A.). Mercaptans were of analytical grade and *s*-butylthiol was used as an enantiomeric mixture. Thiopaeonimetabolin-I derivatives (**3**—**19**) were prepared according to the method of Akao *et al.*[4] Briefly, a precultured bacterial suspension (600 ml) of *Lactbacillus brevis* was added to GAM broth (6 l) and anaerobically incubated for 18 h at 37 °C. The culture was centrifuged at 8000×*g* for 10 min. The pellets were washed with saline solution and suspended in 50 mM K-phosphate buffer (pH 7.3, 900 ml). Alkyl-, cycloalkyl- or arylthiol (5 mmol in 5 ml $H_2O$ or MeOH) and **1** (1.2 g, 2.5 mmol in 10 ml buffer solution) were added to the bacterial suspension. The mixture was anaerobically incubated for 6 h at 37 °C with occasional shaking. The reaction mixture was then applied to a column of Diaion HP-20. The column was washed with $H_2O$ (3 l) and then with MeOH (2 l). The MeOH eluate was evaporated *in vacuo* to give a residue (0.3—0.6 g). Repeated CC of this residue over silica gel [50 g; eluted with benzene–acetone (9 : 1)] afforded an oily residue of the desired adduct (**3**—**19**) as diastereoisomers. The ratio of the diastereoisomers (7*S* and 7*R*) in **9** and **12**—**19** was determined by HPLC or by GC/MS (for **3**—**8**, **10** and **11**). MPLC [column, LiChroprep Si-60; mobile phase, benzene–acetone (9 : 1)] of **8** (50 mg) afforded (7*S*)-8-(*n*-hexylthio)paeonimetabolin I [**8** (7*S*), 19 mg] and (7*R*)-8-(*n*-hexylthio)paeonimetabolin I [**8** (7*R*), 10 mg]. Likewise, MPLC [column, LiChroprep RP-8; mobile phase, methanol–water (7 : 3)] of **18** (50 mg) gave (7*S*)-8-benzoylthiopaeonimetabolin I [**18** (7*S*), 20 mg] and (7*R*)-8-benzoylthiopaeonimetabolin I [**18** (7*R*), 10 mg].[4]

**8-(*n*-Propylthio)paeonimetabolin I (3)** Colorless oil (185 mg, 27%). The 7*S*- and 7*R*-isomers are in a ratio of 2.0 : 1.0. IR $\nu_{max}$ (film) $cm^{-1}$: 3400 (br, OH), 1720 (C=O). $^1$H-NMR: two singlets at $\delta$ 5.44 and 5.50 [each 1H, s, H-9 (7*S* and 7*R*)], and signals for a *n*-propylthiomoiety. EI-MS *m/z* (rel. int.): 272 $[M]^+$ (98), 197 (96), 151 (99), 123 (50), 89 (71), 69 (95). HR-EIMS: Found 272.1039, Calcd for $C_{13}H_{20}O_4S$ $[M]^+$: 272.1083.

**8-(*iso*-Propylthio)paeonimetabolin I (4)** Colorless oil (165 mg, 24%). The 7*S*- and 7*R*-isomers are in a ratio of 2.2 : 1.0. IR $\nu_{max}$ (film) $cm^{-1}$: 3400 (br, OH), 1720 (C=O). $^1$H-NMR: two singlets at $\delta$ 5.44 and 5.50 [each 1H, s, H-9 (7*S* and 7*R*)], and signals for an *iso*-propylthio moiety. EI-MS *m/z* (rel. int.): 272 $[M]^+$ (40), 197 (25), 151 (100), 83 (69), 69 (63). HR-EIMS: Found 272.1009, Calcd for $C_{13}H_{20}O_4S$ $[M]^+$: 272.1083.

**8-(*n*-Butylthio)paeonimetabolin I (5)** Colorless oil (150 mg, 21%). The 7*S*- and 7*R*-isomers are in a ratio of 2.3 : 1.0. IR $\nu_{max}$ (film) $cm^{-1}$: 3400 (br, OH), 1720 (C=O). $^1$H-NMR: two singlets at $\delta$ 5.45 and 5.51 [each 1H, s, H-9 (7*S* and 7*R*)], and signals for a *n*-butylthio moiety. EI-MS *m/z* (rel. int.): 286 $[M]^+$ (56), 197 (37), 151 (100), 69 (52). HR-EIMS: Found 286.1219, Calcd for $C_{14}H_{22}O_4S$ $[M]^+$: 286.1239.

**8-(*sec*-Butylthio)paeonimetabolin I (6)** Colorless oil (180 mg, 25%). The (7*S*,*S*)-, (7*S*,*R*)-, (7*R*,*S*)- and (7*R*,*R*)-isomers are in a ratio of 1.2 : 1.2 : 1.1 : 1.0. IR $\nu_{max}$ (film) $cm^{-1}$: 3400 (br, OH), 1720 (C=O). $^1$H-NMR: four singlets at $\delta$ 5.43 and 5.46, and $\delta$ 5.50 and 5.51 [each 1H, s, H-9 (7*S* and 7*R*)], and signals for (+)- and (−)-*s*-butylthio moieties. EI-MS *m/z* (rel. int.): 286 $[M]^+$ (89), 197 (53), 151 (100), 69 (98). HR-EIMS: Found 286.1210, Calcd for $C_{14}H_{22}O_4S$ $[M]^+$: 286.1239.

**8-(*iso*-Butylthio)paeonimetabolin I (7)** Colorless oil (180 mg, 25%). The 7*S*- and 7*R*-isomers are in a ratio of 2.3 : 1.0. IR $\nu_{max}$ (film) $cm^{-1}$: 3400 (br, OH), 1720 (C=O). $^1$H-NMR: two singlet at $\delta$ 5.45 and 5.51 [each 1H, s, H-9 (7*S* and 7*R*)], and signals for an *iso*-butylthio moiety. EI-MS *m/z* (rel. int.): 286 $[M]^+$ (99), 197 (97), 151 (97), 123 (60), 69 (100), 57 (81). HR-EIMS: Found 286.1235, Calcd for $C_{14}H_{22}O_4S$ $[M]^+$: 286.1239.

**8-(*n*-Hexylthio)paeonimetabolin I (8)** Colorless oil (160 mg, 20%). The 7*S*- and 7*R*-isomers are in a ratio of 1.7 : 1.0. IR $\nu_{max}$ (film) $cm^{-1}$: 3400 (br, OH), 1720 (C=O). $^1$H-NMR: two singlets at $\delta$ 5.44 and 5.50 [each 1H, s, H-9 (7*S* and 7*R*)], and signals for a *n*-hexylthio moiety. EI-MS *m/z* (rel. int.): 314 $[M]^+$ (86), 197 (70), 151 (100), 69 (96). HR-EIMS: Found 314.1507, Calcd for $C_{16}H_{26}O_4S$ $[M]^+$: 314.1552.

**8-Allylthiopaeonimetabolin I (9)** Colorless oil (200 mg, 30%). The 7*S*- and 7*R*-isomers are in a ratio of 2.1 : 1.0. IR $\nu_{max}$ (film) $cm^{-1}$: 3400 (br, OH), 1725 (C=O). $^1$H-NMR: two singlets at $\delta$ 5.40 and 5.47 [each 1H, s, H-9 (7*S* and 7*R*)], and signals for an allylthio moiety. EI-MS *m/z* (rel. int.): 270 $[M]^+$ (99), 197 (61), 151 (98), 69 (98). HR-EIMS: Found 270.0921, Calcd for $C_{13}H_{18}O_4S$ $[M]^+$: 270.0926.

**8-Cyclopentylthiopaeonimetabolin I (10)** Colorless oil (150 mg, 20%). The 7*S*- and 7*R*-isomers are in a ratio of 1.7 : 1.0. IR $\nu_{max}$ (film) $cm^{-1}$: 3400 (br, OH), 1720 (C=O). $^1$H-NMR: two singlets at $\delta$ 5.45 and 5.51 [each 1H, s, H-9 (7*S* and 7*R*)], and signals for a cyclopentylthio moiety. EI-MS *m/z* (rel. int.): 298 $[M]^+$ (47), 197 (27), 151 (100), 69 (41). HR-EIMS : Found 298.1242, Calcd for $C_{15}H_{22}O_4S$ $[M]^+$: 298.1239.

**8-Cyclohexylthiopaeonimetabolin I (11)** Colorless oil (155 mg, 20%). The 7*S*- and 7*R*-isomers are in a ratio of 2.0 : 1.0. IR $\nu_{max}$ (film) $cm^{-1}$: 3400 (br, OH), 1720 (C=O). $^1$H-NMR: two singlets at $\delta$ 5.45 and 5.50 [each 1H, s, H-9 (7*S* and 7*R*)], and signals for a cyclohexylthio moiety. EI-MS *m/z* (rel. int.): 312 $[M]^+$ (30), 197 (21), 151 (74), 83 (25), 69 (33). HR-EIMS: Found 312.1336, Calcd for $C_{16}H_{24}O_4S$ $[M]^+$: 312.1396.

**8-Phenylthiopaeonimetabolin I (12)** Colorless oil (230 mg, 30%). The 7*S*- and 7*R*-isomers are in a ratio of 1.6 : 1.0. IR $\nu_{max}$ (film) $cm^{-1}$: 3400 (br, OH), 1720 (C=O). $^1$H-NMR: two singlets at $\delta$ 5.43 and 5.53 [each 1H, s, H-9 (7*S* and 7*R*)], and signals for a phenylthio moiety. EI-MS *m/z* (rel. int.): 306 $[M]^+$, (55), 197 (6), 151 (50), 110 (100), 69 (50). HR-EIMS: Found 306.0901, Calcd for $C_{16}H_{18}O_4S$ $[M]^+$: 306.0926.

**8-(*o*-Tolylthio)paeonimetabolin I (13)** Colorless oil (185 mg, 23%). The 7*S*- and 7*R*-isomers are in a ratio of 1.6 : 1.0. IR $\nu_{max}$ (film) $cm^{-1}$: 3400 (br, OH), 1725 (C=O). $^1$H-NMR: two singlets at $\delta$ 5.43 and 5.55 [each 1H, s, H-9 (7*S* and 7*R*)], and signals for an *o*-tolylthio moiety. EI-MS *m/z* (rel. int.): 320 $[M]^+$ (99), 197 (4), 151 (99), 124 (100), 91 (36), 69 (98). HR-EIMS: Found 320.1039, Calcd for $C_{17}H_{20}O_4S$ $[M]^+$: 320.1083.

**8-(*m*-Tolylthio)paeonimetabolin I (14)** Colorless oil

(180 mg, 22.5%). The 7*S*- and 7*R*-isomers are in a ratio of 1.0 : 1.0. IR $\nu_{max}$ (film) $cm^{-1}$: 3400 (br, OH), 1725 (C=O). $^1$H-NMR: two singlets at $\delta$ 5.43 and 5.53 [each 1H, s, H-9 (7*S* and 7*R*)], and signals for a *m*-tolylthio moiety. EI-MS *m/z* (rel. int.): 320 $[M]^+$ (100), 197 (2), 151 (41), 124 (98), 91 (10), 69 (36). HR-EIMS: Found 320.1078, Calcd for $C_{17}H_{20}O_4S$ $[M]^+$: 320.1083.

**8-(*p*-Tolylthio)paeonimetabolin I (15)** Colorless oil (190 mg, 24%). The 7*S*- and 7*R*-isomers are in a ratio of 1.2 : 1.0. IR $\nu_{max}$ (film) $cm^{-1}$: 3400 (br, OH), 1720 (C=O). $^1$H-NMR: two singlets at $\delta$ 5.41 and 5.52 [each 1H, s, H-9 (7*S* and 7*R*)], and signals for a *p*-tolylthio moiety. EI-MS *m/z* (rel. int.): 320 $[M]^+$ (99), 197 (18), 151 (48), 137 (57), 124 (100), 91 (26), 69 (59). HR-EIMS: Found 320.1089, Calcd for $C_{17}H_{20}O_4S$ $[M]^+$: 320.1083.

**8-(2-Naphthylthio)paeonimetabolin I (16)** Colorless oil (205 mg, 23%). The 7*S*- and 7*R*-isomers are in a ratio of 1.0 : 1.2. IR $\nu_{max}$ (film) $cm^{-1}$: 3400 (br, OH), 1725 (C=O). $^1$H-NMR: two singlets at $\delta$ 5.44 and 5.56 [each 1H, s, H-9 (7*S* and 7*R*)], and signals for a 2-naphthylthio moiety. EI-MS *m/z* (rel. int.): 356 $[M]^+$ (98), 197 (15), 160 (100), 151 (34), 128 (42), 115 (72), 69 (38). HR-EIMS: Found 356.1084, Calcd for $C_{20}H_{20}O_4S$ $[M]^+$: 356.1083.

**8-Benzylthiopaeonimetabolin I (17)** Colorless oil (160 mg, 20%). The 7*S*- and 7*R*-isomers are in a ratio of 3.0 : 1.0. IR $\nu_{max}$ (film) $cm^{-1}$: 3400 (br, OH), 1720 (C=O). $^1$H-NMR: two singlets at $\delta$ 5.35 and 5.43 [each 1H, s, H-9 (7*S* and 7*R*)], and signals for a benzylthio moiety. EI-MS *m/z* (rel. int.): 320 $[M]^+$ (48), 229 (62), 197 (19), 183 (29), 151 (64), 124 (57), 91 (100), 69 (42). HR-EIMS: Found 320.1096, Calcd for $C_{17}H_{20}O_4S$ $[M]^+$: 320.1183.

**8-Benzoylthiopaeonimetabolin I (18)**[4] Colorless oil (170 mg, 20%). The 7*S*- and 7*R*-isomers are in a ratio of 2.7 : 1.0. IR $\nu_{max}$ (film) $cm^{-1}$: 3400 (br, OH), 1720 (C=O), 1690 (thioether C=O). $^1$H-NMR: two singlets at $\delta$ 5.39 and 5.41 [each 1H, s, H-9 (7*S* and 7*R*)], and signals for a benzylthio moiety. EI-MS *m/z* (rel. int.): 272 $[M]^+$ (2), 197 (99), 151 (99), 69 (100). HR-EIMS: Found 334.0885, Calcd for $C_{17}H_{18}O_5S$ $[M]^+$: 334.0875.

**8-Acetylthiopaeonimetabolin I (19)** Colorless oil (185 mg, 27%). The 7*S*- and 7*R*-isomers are in a ratio of 2.2 : 1.0. IR $\nu_{max}$ (film) $cm^{-1}$: 3400 (br, OH), 1720 (C=O), 1660 (thioether C=O). $^1$H-NMR: two singlets at $\delta$ 5.31 and 5.15 [each 1H, s, H-9 (7*S* and 7*R*)], and signals for an acethylthio moiety. EI-MS *m/z* (rel. int.): 334 $[M]^+$ (8), 197 (67), 151 (99), 138 (60), 105 (100), 77 (98), 69 (91). HR-EIMS: Found 272.0683, Calcd for $C_{12}H_{16}O_5S$ $[M]^+$: 272.0718.

**(7*S*)-8-(*n*-Hexylthio)paeonimetabolin I [8 (7*S*)]** Colorless oil. $[\alpha]_D = -2.8°$ (0.25, $CHCl_3$). $^1$H-NMR: $\delta$ 2.69—2.73 (2H, H-2), 2.81 (1H, m, H-4), 2.06 (1H, dd, *J*=13.7, 2.2 Hz, H-5), 2.34 (1H, dd, *J*=13.7, 3.3 Hz, H-5), 1.87 (1H, br t, *J* =7.7 Hz, H-7), 2.53 (1H, dd, *J*=13.7, 7.7 Hz, H-8), 2.74 (1H, dd, *J*=13.7, 7.7 Hz, H-8), 5.44 (1H, br s, H-9), 1.30 (3H, s, H-10), *n*-hexyl residue: 2.50 (2H, t, *J*=7.4 Hz, H-1); 1.56 (2H, quin, *J*=7.4 Hz, H-2). 1.37 (2H, quin, *J*=7.4 Hz, H-3), 1.24—1.35 (4H, H-4 and H-5), 0.89 (3H, t, *J*=7.4 Hz, H-6). $^{13}$C-NMR: $\delta$ 78.0 (C-1), 47.7 (C-2), 210.4 (C-3), 47.9 (C-4), 30.9 (C-5), 102.0 (C-6), 42.8 (C-7), 31.7 (C-8), 101.4 (C-9), 21.3 (C-10), *n*-hexyl residue: 32.5 (C-1), 29.7 (C-2), 28.8 (C-3) 31.7 (C-4), 22.8 (C-5), 14.3 (C-6). HR-EIMS: Found 314.1524, Calcd for $C_{16}H_{26}O_4S$ $[M]^+$: 314.1552.

**(7*R*)-8-(*n*-Hexylthio)paeonimetabolin I [8 (7*R*)]** Colorless oil. $[\alpha]_D = +3.5°$ (0.20, $CHCl_3$). $^1$H-NMR: $\delta$ 2.63 (2H, s, H-2), 2.82 (1H, m, H-4), 2.17 (1H, dd, *J*=13.7, 2.2 Hz, H-5), 2.35 (1H, dd, *J*=13.7, 3.3 Hz H-5), 2.06 (1H, m, H-7), 2.27 (1H, dd, *J*=13.2, 9.9 Hz, H-8), 2.54 (1H, dd, *J*=13.2, 4.9 Hz, H-8), 5.50 (1H, br s, H-9), 1.30 (3H, s, H-10), *n*-hexyl residue: 2.49 (2H, t, *J*=7.4 Hz, H-1), 1.55 (2H, quint, *J*=7.4 Hz, H-2), 1.36 (2H, quint, *J*=7.4 Hz, H-3), 1.24—1.32 (4H, H-4 and H-5), 0.88 (3H, t, *J*=7.0 Hz, H-6). $^{13}$C-NMR: $\delta$ 78.5 (C-1), 47.1 (C-2), 209.5 (C-3), 48.6 (C-4), 34.5 (C-5), 101.7 (C-6), 43.9 (C-7), 30.9 (C-8), 101.0 (C-9), 21.4 (C-10), *n*-hexyl residue: 33.1 (C-1), 29.8 (C-2), 28.8 (C-3), 31.7 (C-4), 22.8 (C-5), 14.3 (C-6). HR-EIMS: Found 314.1544, Calcd for $C_{16}H_{26}O_4S$ $(M)^+$: 314.1552.

**(7*S*)-8-Benzoylthiopaeonimetabolin I [18 (7*S*)]**[4] Colorless oil. $[\alpha]_D = -49°$ (0.15, $CHCl_3$). $^1$H-NMR: $\delta$ 2.69—2.73 (2H, H-2), 2.75 (1H, m, H-4), 2.11 (1H, dd, *J*=13.7, 2.2 Hz, H-5), 2.56 (1H, dd, *J*=13.7, 3.3 Hz, H-5), 1.96 (1H, t, *J*=7.7 Hz, H-7), 3.23 (2H, d, *J*=7.7 Hz, H-8), 5.39 (1H, br s, H-9), 1.31 (3H, s, H-10), benzoyl residue: 7.45 (2H, br t, *J*=7.7 Hz, H-3 and H-5), 7.59 (1H, br t, *J*=7.7 Hz, H-4), 7.95 (2H, br d, *J*=7.7 Hz, H-2 and H-6). $^{13}$C-NMR: 78.2 (C-1), 47.7 (C-2), 209.9 (C-3), 47.7 (C-4), 31.1 (C-5), 102.0 (C-6), 43.8 (C-7), 28.0 (C-8), 101.5 (C-9), 21.3 (C10), benzoyl residue: 136.5 (C-1), 128.8 (C-2 and C-6), 127.5 (C-3 and C-5), 133.8 (C-4), 190.9 (C-7).

**(7*R*)-8-Benzoylthiopaeonimetabolin I [18 (7*R*)]**[4] Colorless oil. $[\alpha]_D = -49$ (*ca.* 0.13, $CHCl_3$). $^1$H-NMR: 2.68 (2H, s, H-2), 2.97 (1H, m, H-4), 2.21 (1H, dd, *J*=13.2, 2.7 Hz, H-5), 2.34 (1H, dd, *J*=13.2, 3.3 Hz, H-5), 2.22 (1H, t, *J*=7.0 Hz, H-7), 2.95 (2H, d, *J*=7.0 Hz, H-8), 5.41 (1H, br s, H-9), 1.32 (3H, s, H-10), benzoyl residue: 7.45 (2H, br t, *J*=7.7 Hz, H-3 and H-5), 7.58 (1H, br t, *J*=7.7 Hz, H-4), 7.96 (2H, br d, *J*=7.7 Hz, H-2 and H-6). $^{13}$C-NMR: 78.7 (C-1), 47.1 (C-2), 209.6 (C-3), 48.1 (C-4), 34.4 (C-5), 101.7 (C-6), 44.5 (C-7), 27.6 (C-8), 101.2 (C-9), 21.4 (C-10), benzoyl residue: 136.7 (C-1), 128.8 (C-2 and C-6), 127.4 (C-3 and C-5), 133.7 (C-4), 191.3 (C-7).

**Animals** Male ddY mice (Japan SCL, Shizuoka) weighing 30—35 g were maintained under controlled conditions of temperature (23±1 °C) with 60% humidity, in a 12 h light/dark cycle at the animal house one week before the start of the experiments. All experiments were performed between 13:00—17:00 hrs.

**Anticonvulsant Testing in Mice [Subcutaneous PTZ Seizure Test]**[6] Each compound at doses of 0.125, 0.25 and 0.50 mmol/kg (in 0.5% Tween 80 in saline) was injected intraperitoneally (i.p.) in groups of 6 animals. After 30 min, PTZ was injected subcutaneously (s.c.) at a dose of 100 mg/kg dissolved in 0.9% NaCl. The animals were then observed for 1 h for clonic and tonic convulsions and for death. Protection was defined as the failure to detect even a single episode of clonic spasms of at least 5 seconds duration. The median anticonvulsant potency was determined by the graphic presentation method.

**Rotorod Toxicity Test** The sedative effect of **3**—**19** was assessed in mice by evaluating rolling roller performance (RRP) according to the procedure of Dunham and Miya.[7] Briefly, each treated animal, previously trained to balance on a rod rotating at 6 rpm, was allowed three trials to remain on the rod for 1 min. Failure to maintain equilibrium was noted

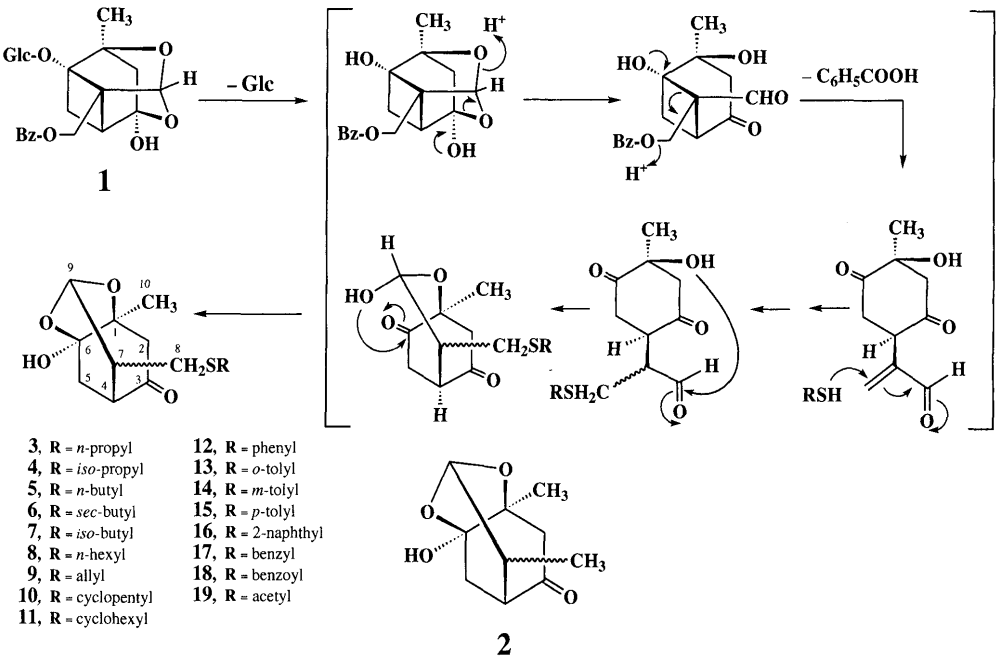


Chart 1. Possible Pathway for the Formation of Thiopaeonimetabolin-I Derivatives after Incubation of **1** and Thiol Compounds with *Lactobacillus brevis*

as a lack of RRP. After i.p. administration of the compound, each animal was tested in this manner every 15 min for a total duration of 2 h. The median neurotoxic dose ($TD_{50}$) of each compound was calculated by the up and down method.[8]

**Statistical Analysis** The latencies of clonic and tonic convulsions were expressed as the mean±S.E.M. The $ED_{50}$ values were determined by using the graphic presentation method, and the $TD_{50}$ values were determined from the rotorod test. The protective indices (PI) were defined as $TD_{50}/ED_{50}$. The data were analyzed by Fisher's protected LSD super ANOVA analysis of variance.

## RESULTS AND DISCUSSION

**1. Synthesis of Thiopaeonimetabolin-I Derivatives** Seventeen thiopaeonimetabolin-I derivatives (**3**—**19**) were obtained following the procedure reported by Akao *et al.* (Chart 1).[4] Assignment of the chemical structures of these compounds (obtained in yields of 20—30%) relied on the structure of **2**, established by a single crystal X-ray analysis.[9] The structures of these compounds (obtained in yields of 20—30%) were determined by careful inspection of their spectroscopic data. The IR spectra of **3**—**19** exhibited diagnostic absorption bands at 3400 and 1720—1730 $cm^{-1}$ characteristic for hydroxyl and carbonyl groups, respectively. The EI-MS spectra of **3**—**19**, with a common fragment ion peak at *m/z* 197 $[M-SR]^+$ accounted for by the removal of R-thio residues placed at C-8, together with the HR-EIMS, are indicative of their molecular formula. The $^1$H-NMR spectra of **3**—**19** showed paired singlets at $\delta_H$ 5.15—5.55 (in **2**, at $\delta_H$ 5.14—5.17) characteristic for H-9, with the preference of 7*S* isomers in general, as determined by GC/MS and HPLC. In the $^{13}$C-NMR spectra, the downfield shift of C-8 resonance (observed at $\delta$ 28.7—34.6) relative to the corresponding chemical shift of **2** (at $\delta$ 13.5 or 15.0) established the presence of thio residues at C-8. Other signals assigned for the cage-like skeleton of **3**—**19** remained essentially unaffected. From these findings, it was apparent that **3**—**19** are the C-8 thiol adducts of **2**.

**2. Anticonvulsant Activities of Thiopaeonimetabolin-I Derivatives** Table 1 shows the latencies of clonic and tonic convulsions, the % inhibition of both convulsions and the % mortality values for all compounds (**3**—**19**). When compared with **2**, **3**—**19** were considered to be potent anticonvulsant agents. In a dose range of 0.125—0.5 mmol/kg, a dose-dependent prolongation of the latencies of clonic and tonic convulsions was observed for thirteen compounds (**3**—**8**, **10**, **12**—**16** and **18**). In the case of **9**, **17** and **19**, the latency of clonic convulsion (for **9**) and of both clonic and tonic convulsions (for **17** and **19**) gradually increased and then decreased at a dose of 0.5 mmol/kg (Table 1). However, **11** showed a prolongation of both latencies at a dose of 0.125 mmol/kg, which then gradually decreased at higher doses. The mechanisms underlying the bell-shaped effects of these compounds remain unclear. These compounds may be able to interact with a variety of sites of action. Complete protection against convulsion was effectively demonstrated by **8** (at a dose of 0.125 mmol/kg) and **18** (at 0.25 mmol/kg), while 100% protection by **10** and **15** was only achieved at 0.5 mmol/kg.

Next, we decided to follow up the anticonvulsant activity of the individual diastereoisomers of the active compounds, and the most active ones, **8** and **18**, were selected. It was interesting to note that the 7*S*-isomers of both compounds were more potent than their counter parts, whereas the 7*R*-isomers showed muscle relaxation effects at higher doses of 1.0 and 0.25 mmol/kg, respectively.

The toxicity of these compounds was preliminarily investigated using an acute toxicity test. Since acute toxicity from anticonvulsant drugs in experimental animals is exhibited by some types of neurological abnormalities, the positional sense test, righting test, gait and stance test, muscle tone test, equilibrium test and grip test were performed.[10] None of the tested compounds [**3**—**19**, **8** (7*S*) and **18** (7*S*)] showed any appreciable toxicity within the tested doses (Table 2).

Table 1. Preliminary Screening of the Anticonvulsant Activity of **3**—**19**

| Compound | Dose (mmol/kg) | Latency (min, mean±S.E.M.) | | % Inhibition[a] | | % Mortality[b] |
|---|---|---|---|---|---|---|
| | | Clonic convulsion | Tonic convulsion | Clonic convulsion | Tonic convulsion | |
| **2** | 0 | 8.2±0.5 | 12.8±1.9 | 0 | 0 | 100 |
| | 0.125 | 8.5±1.0 | 10.2±0.9 | 0 | 0 | 100 |
| | 0.25 | 7.8±0.8 | 9.3±2.0 | 0 | 0 | 100 |
| | 0.5 | 17.2±2.4*** | 21.3±2.7** | 0 | 0 | 100 |
| **3** | 0 | 7.7±0.8 | 12.3±1.2 | 0 | 0 | 100 |
| | 0.125 | 8.0±1.4 | 13.7±1.5 | 0 | 0 | 100 |
| | 0.25 | 9.0±1.3 | 17.5±2.3 | 0 | 0 | 100 |
| | 0.5 | 19.0±1.2*** | 31.8±6.0** | 0 | 33 | 100 |
| **4** | 0 | 7.7±0.5 | 12.3±1.2 | 0 | 0 | 100 |
| | 0.125 | 9.8±1.4 | 15.0±2.1 | 0 | 0 | 100 |
| | 0.25 | 15.0±3.3 | 20.7±3.5 | 0 | 0 | 100 |
| | 0.5 | 26.5±6.2 | 35.8±6.1 | 17 | 17 | 83 |
| **5** | 0 | 7.5±1.0 | 16.0±1.5 | 0 | 0 | 100 |
| | 0.125 | 7.5±1.0 | 17.7±1.7 | 0 | 0 | 100 |
| | 0.25 | 8.5±1.6 | 18.3±1.9 | 0 | 0 | 100 |
| | 0.5 | 28.5±6.5*** | 34.8±6.0*** | 17 | 17 | 67 |
| **6** | 0 | 6.2±0.8 | 9.7±1.7 | 0 | 0 | 100 |
| | 0.125 | 9.5±1.9 | 10.3±1.6 | 0 | 0 | 100 |
| | 0.25 | 10.7±2.1 | 13.3±1.9 | 0 | 0 | 100 |
| | 0.5 | 21.0±8.2*** | 32.0±6.0*** | 17 | 17 | 83 |
| **7** | 0 | 6.2±0.8 | 9.7±1.7 | 0 | 0 | 100 |
| | 0.125 | 11.5±1.5 | 15.7±1.9 | 0 | 0 | 100 |
| | 0.25 | 17.7±2.6 | 21.7±3.0 | 0 | 0 | 100 |
| | 0.5 | 30.3±9.9** | 41.5±6.2*** | 33 | 33 | 67 |
| **8** | 0 | 5.0±0.7 | 16.0±1.5 | 0 | 0 | 100 |
| | 0.125 | >60.0*** | >60.0*** | 100 | 100 | 0 |
| | 0.25 | >60.0*** | >60.0*** | 100 | 100 | 0 |
| | 0.5 | >60.0*** | >60.0*** | 100 | 100 | 0 |
| **9** | 0 | 5.4±0.4 | 19.7±1.3 | 0 | 0 | 100 |
| | 0.125 | 17.8±8.9 | 10.3±1.6 | 17 | 33 | 67 |
| | 0.25 | 28.3±10.1* | 13.3±1.9 | 33 | 50 | 50 |
| | 0.5 | 11.3±1.6 | 32.0±6.0*** | 0 | 33 | 50 |
| **10** | 0 | 6.5±0.8 | 9.7±1.7 | 0 | 0 | 100 |
| | 0.125 | 10.2±1.1 | 16.0±1.3 | 0 | 0 | 100 |
| | 0.25 | 31.8±9.0** | 38.0 ±7.5*** | 33 | 33 | 50 |
| | 0.5 | >60.0*** | >60.0*** | 100 | 100 | 0 |
| **11** | 0 | 9.2±2.0 | 17.7±3.3 | 0 | 0 | 100 |
| | 0.125 | 37.8±7.0*** | 45.2±5.0*** | 33 | 33 | 67 |
| | 0.25 | 29.8±6.2* | 32.0±5.7* | 17 | 17 | 83 |
| | 0.5 | 18.8±2.5 | 19.1±2.6 | 0 | 0 | 100 |
| **12** | 0 | 10.3±1.3 | 17.5±2.6 | 0 | 0 | 100 |
| | 0.125 | 9.5±1.6 | 14.5±1.9 | 0 | 0 | 100 |
| | 0.25 | 13.0±2.3 | 18.0±2.1 | 0 | 0 | 100 |
| | 0.5 | 29.0±7.1** | 29.5±4.6** | 17 | 0 | 83 |
| **13** | 0 | 10.3±1.3 | 17.5±2.6 | 0 | 0 | 100 |
| | 0.125 | 12.5±1.6 | 15.5±1.6 | 0 | 0 | 100 |
| | 0.25 | 18.7±2.3* | 27.8±2.9* | 0 | 0 | 67 |
| | 0.5 | 50.3±4.5*** | 47.2±4.7*** | 50 | 67 | 50 |
| **14** | 0 | 6.2±0.6 | 9.8±1.5 | 0 | 0 | 100 |
| | 0.125 | 32.2±8.8** | 36.3±8.1* | 33 | 33 | 67 |
| | 0.25 | 43.2±7.6*** | 43.2±6.5*** | 50 | 33 | 50 |
| | 0.5 | 55.8±4.2*** | 57.0±3.0*** | 83 | 83 | 17 |
| **15** | 0 | 9.2±2.0 | 17.7±3.3 | 0 | 0 | 100 |
| | 0.125 | 28.2±10.2 | 31.0±9.2 | 33 | 33 | 67 |
| | 0.25 | 45.0±9.5* | 49.5±6.9** | 67 | 67 | 33 |
| | 0.5 | >60.0*** | >60.0*** | 100 | 100 | 0 |
| **16** | 0 | 8.2±0.5 | 12.8±1.9 | 0 | 0 | 100 |
| | 0.125 | 9.5±0.8 | 14.3±1.9 | 0 | 0 | 100 |
| | 0.25 | 39.8±7.2*** | 36.0±5.7*** | 0 | 17 | 67 |
| | 0.5 | 41.3±8.8*** | 43.8±4.4*** | 17 | 17 | 67 |
| **17** | 0 | 4.5±0.4 | 19.7±1.3 | 0 | 0 | 100 |
| | 0.125 | 49.8±6.4*** | 50.5±6.0*** | 67 | 67 | 33 |
| | 0.25 | >60.0*** | >60.0*** | 100 | 100 | 0 |
| | 0.5 | 23.3±1.1*** | 23.3±1.1 | 0 | 0 | 100 |
| **18** | 0 | 5.7±0.9 | 20.0±1.9 | 0 | 0 | 100 |
| | 0.125 | 46.0±8.9*** | 48.7±5.4*** | 67 | 50 | 50 |
| | 0.25 | >60.0*** | >60.0*** | 100 | 100 | 0 |
| | 0.5 | >60.0*** | >60.0*** | 100 | 100 | 0 |

Table 1. (continued)

| Compound | Dose (mmol/kg) | Latency (min, mean±S.E.M.) | | % Inhibition[a] | | % Mortality[b] |
|---|---|---|---|---|---|---|
| | | Clonic convulsion | Tonic convulsion | Clonic convulsion | Tonic convulsion | |
| **19** | 0 | 5.7±0.8 | 20.0±1.9 | 0 | 0 | 100 |
| | 0.125 | 34.5±1.4* | 39.5±6.6** | 50 | 33 | 33 |
| | 0.25 | 51.3±5.5*** | 57.5±2.5*** | 67 | 83 | 17 |
| | 0.5 | 33.2±8.7* | 44.7±5.2*** | 33 | 33 | 67 |
| **8** (7*S*) | 0 | 9.3±1.9 | 16.0±1.3 | 0 | 0 | 100 |
| | 0.06 | 7.8±1.4 | 11.8±1.0 | 0 | 0 | 100 |
| | 0.08 | 21.0±7.9 | 25.8±7.0 | 17 | 17 | 83 |
| | 0.09 | 49.5±6.7*** | 41.7±6.1** | 33 | 33 | 67 |
| | 0.10 | 35.5±8.1* | 51.7±5.4*** | 67 | 67 | 33 |
| | 0.125 | >60.0*** | >60.0*** | 100 | 100 | 0 |
| **8** (7*R*) | 0 | 9.8±0.9 | 16.0±2.0 | 0 | 0 | 100 |
| | 0.25 | 12.0±1.6 | 17.3±2.9 | 0 | 0 | 100 |
| | 0.5 | 10.7±1.8 | 16.3±1.9 | 0 | 0 | 100 |
| | 1.0[c] | >60.0*** | >60.0*** | 100 | 100 | 0 |
| **18** (7*S*) | 0 | 9.7±1.8 | 17.0±3.1 | 0 | 0 | 100 |
| | 0.06 | 10.2±2.0 | 19.0±2.6 | 0 | 0 | 100 |
| | 0.08 | 19.7±8.2 | 26.7±7.5 | 17 | 17 | 83 |
| | 0.10 | 43.8±5.8*** | 44.7±5.8** | 33 | 33 | 67 |
| | 0.125 | 49.2±7.1*** | 49.2±7.1** | 67 | 67 | 33 |
| | 0.25 | >60.0*** | >60.0*** | 100 | 100 | 0 |
| **18** (7*R*) | 0 | 9.2±1.1 | 15.8±2.2 | 0 | 0 | 100 |
| | 0.25 | —[c] | —[c] | —[c] | —[c] | —[c] |

*a*) % Inhibition=(number of non-protected animals/number of total animals used)×100. *b*) % Mortality=(number of animals died/number of total animals used)×100. *c*) Complete muscle relaxation of the animals as detected by muscle tone test, equilibrium test, grip test and general behavior of the animals. VPA showed 100% protection at 1.5 mmol/kg. Statistical significance: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Table 2. Anticonvulsant Potency and Neurotoxicity of **8** (7*S*) and **18** (7*S*)

| Compound | $ED_{50}$ (mmol/kg) | $TD_{50}$ (mmol/kg) | PI[a] |
|---|---|---|---|
| **8** (7*S*) | 0.09 | 0.47 | 5.0 |
| **18** (7*S*) | 0.12 | 0.47 | 4.0 |
| VPA | 1.05 | 1.90 | 1.8 |

*a*) PI, protective index calculated as $TD_{50}/ED_{50}$. The doses of compound required to produce 100% protection in 50% of the animals ($ED_{50}$) or minimal neurological toxicity in 50% of the animals ($TD_{50}$) were calculated by the graphic presentation method and up- and down-method, respectively.

In the present study, the anticonvulsant activity of the compounds was preliminarily evaluated in mice using PTZ-induced generalized tonic-clonic seizures at a dose of 100 mg/kg, s.c. It has been reported that mice given a fixed dose of PTZ (100 mg/kg, s.c.) characteristically exhibit a variable number of clonic episodes followed by a maximal tonic-extensor seizure and death.[6] By using this model, we evaluated the compounds which affect both seizure spread and seizure threshold.

Although no clear correlation between the structure and the anticonvulsant activity emerged from the present result, it was obvious that the introduction of an R-thio residue at C-8 of **2** effectively enhanced the anticonvulsant activity of **2**. On the other hand, the number of carbon atoms and the nature (branching, unsaturation, alicyclic or aromatic) of the RS-residue at C-8 possibly affected the anticonvulsant potency of these compounds. Of the same series, alkyl substituents with 6 carbon atoms demonstrated maximal potency (**8**>**5**), while branching of the alkyl group showed variable potencies, as in **4**, **6** and **7**, and their unsaturation was accompanied by a decrease in the latency of clonic convulsion, as in **9**. In the case of alicyclic (**10** and **11**) and aromatic substitutions with the same number of carbon atoms, alicyclic substitution with 5 carbon atoms was more potent than that with 6 carbon atoms (**10**>**11**) or that of aromatic analogs with the same number of carbon atoms (**12**). As for compounds with a substituted aryl thio (**13**—**15**, **17** and **18**), the introduction of a carbonyl group, a methyl or a methylene group into the aryl substituent significantly affected the latency for both clonic and tonic convulsions (Table 1). The highest potency was observed for **18** (with a benzoyl), followed by the tolyl substituents (**15**>**14**>**13**). The effect of carbonyl and methylene was variable since complete blockade of the convulsion seizure was observed for **17** (with benzyl) at a dose of 0.25 mmol/kg, but at a dose of 0.5 mmol/kg, the activity was significantly decreased. The observation that only the *p*-tolyl derivative (**15**) completely suppressed the clonic-tonic convulsions at a dose of 0.5 mmol/kg can be explained by possible steric effects of the substituted aromatic analogs **13** and **14**.

Since different stereoisomers interact differentially in a chiral environment, the stereoisomeric ratio and the anticonvulsant profiles of the individual stereoisomers of **8** and **18** were evaluated. As it was clear from the initial screening, marked stereospecificity was noticed for both compounds, where the 7*S*-isomers displayed anticonvulsant activity while the diastereoisomers (7*R*—ones) showed skeletal muscle relaxation effects. The observation that the anticonvulsant effects of the 7*S*-isomers was not twice as potent as their respective diastereomates suggested that the muscle relaxation effect of the *R* form in the mixture enhanced the anticonvulsant effect of the *S* one, and that the results of the diastereomate were a combination of both effects.

When the $ED_{50}$ values of the 7*S*-isomers of **8** and **18** were compared with that of VPA, **8** (7*S*) and **18** (7*S*) were approximately 11.7 and 8.8-times as active as VPA, respectively.

Furthermore, the higher protective indices ($TD_{50}/ED_{50}$) (4.0 and 5.0, respectively) for **8** (7*S*) and **18** (7*S*), when compared with that of VPA (PI=1.8), suggested that the maximal anticonvulsant effect of these compounds was achieved at non-toxic doses.

To date, a wide variety of chemicals exhibit anticonvulsant activity, many of which are cyclic amides such as imides, carboximides, sulfonamides, carbamates, hydantoins and ureas. However, **2** and its R-thio derivatives (**3**—**19**) are structurally distinct monoterpenes obtained from **1** by human intestinal bacteria; they exhibited significant anticonvulsant activity. It is interesting to note that paeoniflorigenone (PFG), a monoterpene isolated from peony roots,[11] and **18** (7*S*) share most structural elements except that a sulfur atom was introduced at C-8 in **18**, relative to an oxygen atom at C-8 of PFG, and the other difference is that PFG is the 7*R*-isomer. Interestingly, PFG was found to have a blocking effect on the neuromuscular junction in phrenic nerve diagram preparations of mice.[12]

The present findings provided more information about the role of human intestinal bacteria in introducing R-thio residues at C-8 of **2**, raising the possibility of producing other analogs *in vivo* after the oral administration of **1** or crude drugs containing related glycosides. From kinetic studies of **1** or **2** after *p.o.* or i.v. administration to rats, **2** was found at a higher concentration in blood and remained longer than **1**.[13] As the adducts of **2** with various thiols (RSH) seem to be more lipophilic, their absorption and distribution to the site of action in both brain and muscles are expected to be very fast.

Although substantial evidence has been initially obtained from the present experiment where R-thio analogies of **2** with potent anticonvulsant activities were synthesized, pharmacological and pharmacokinetic evaluation of these compounds are necessary to clarify their mechanisms of actions.

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