

## Comparative assessment of the anxiolytic-like activities of honokiol and derivatives

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### Abstract

Honokiol has previously been shown to be an effective anxiolytic-like agent in mice when administered for 7 days at 0.2 mg/kg/day prior to evaluation in an elevated plus-maze, while 20 mg/kg is required for efficacy as a single oral dose. The aim of this study was to find analogs of honokiol that are more effective for acute administration. Among the eight analogs evaluated, one partially reduced derivative of honokiol [3'-(2-propenyl)-5-propyl-(1,1'-biphenyl)-2,4'-diol] exhibited significant anxiolytic-like activity at 0.04 mg/kg. Following oral administration of 1 mg/kg of this analog, anxiolytic-like activity was clearly evident at 1 h, peaked at 3 h, and remained significant for longer than 4 h after treatment. Combined administration of the derivative with diazepam led to enhanced anxiolytic-like efficacy. Moreover, as with diazepam, the anxiolytic-like effect of the analog was reduced by flumazenil. In contrast, bicuculline, a GABA<sub>A</sub> antagonist, had no effect on the activity of the derivative. Taken together, these results suggest that this analog of honokiol acts at the benzodiazepine recognition site of the GABA<sub>A</sub>-benzodiazepine receptor complex. © 2000 Elsevier Science Inc. All rights reserved.

**Keywords:** Anxiolytic-like activity; Elevated plus-maze; Honokiol; Honokiol analogs; Hydrogenated honokiols

In our previous studies using an elevated plus-maze test in mice [6,9], honokiol, a neolignan isolated from magnolia bark [1], was shown to be an effective anxiolytic-like agent after administration of 0.2–2 mg/kg, po, for 7 days, but not after acute treatment with the same doses. In order to obviate the need for chronic treatment for development of a clear anxiolytic-like effect, we examined analogs of honokiol to see if any would be effective after acute oral administration of an acceptably low dose.

There are no reports in the literature on the metabolism of honokiol. However, studies with magnolol, an isomer of honokiol, have shown that in vivo, the propenyl groups become reduced, yielding dihydro- and tetrahydromagnolol [2,3]. We, therefore, speculated that reduced forms of honokiol might be accumulating during chronic admini-

nistration, and that these analogs might exhibit enhanced anxiolytic-like activity relative to the parent compound. Described below is our assessment of the anxiolytic-like activity of eight analogs of honokiol and comparison with the results for diazepam, an established benzodiazepine anxiolytic-like agent.

### 1. Materials and methods

#### 1.1. Animals

Male ddY mice, 7 weeks of age, weighing 33–36 g, were obtained from Japan SLC (Hamamatsu, Japan). Randomly chosen mice were housed 10 mice to a polycarbonate cage (20 × 30 × 15 cm with wood chip bedding) with free access to a standard solid diet and tap water. The environment of the animal room was controlled as follows: temperature, 23 ± 1°C; relative humidity, 55 ± 3%; a 12-h light–dark cycle with lights on between 7:00 a.m. and 7:00 p.m. All experimental protocols were approved by The Committee of

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Animal Experiments in Gunma University School of Medicine, and were in accordance with "The Guideline for Animal Experimentation of the Japanese Association of Laboratory Animal Science." For all experiments, except determination of the time course of anxiolytic-like activity, separate mice were utilized for each test. For the time course studies, mice were re-evaluated on the plus-maze up to three different intervals after drug administration in order to minimize the number of mice utilized for this experiment. From pilot studies using ddY mice, we found that a reliable plus-maze result could be obtained if a mouse was re-tested after an interval of more than 5 h from the previous measurement. As such, one group of mice was tested at 1, 6, and 48 h after drug administration, the second group at 2, 8, and 72 h, the third group at 3 and 12 h, and the fourth group at 4 and 24 h.

### 1.2. Materials

Honokiol was purchased from Nacalai Tesque (Kyoto, Japan), diazepam (Cercine Inj.) from Takeda Chemical Industries (Osaka, Japan), and bicuculline from Sigma (St. Louis, MO, USA). Flumazenil was the generous gift of Hoffman-La Roche (Nutley, NJ, USA). For synthesis of tetrahydrohonokiol (THH), honokiol (20 mg) was hydrogenated over 10% palladium on carbon in 20 ml of methanol under atmospheric pressure of hydrogen for 18 h. After filtration of the insoluble material, the filtrate was concentrated to yield THH as a white solid (20.1 mg, 99%). The seven analogs of dihydrohonokiol (DHH) shown in Fig. 1 were prepared as outlined in Table 1 and Fig. 2, based on the method of Negishi et al. [10]. As an example, for the production of DHH-B3, 1-(methoxymethoxy)-4-propylbenzene (I) was lithiated in tetrahydrofuran and converted to the arylzinc analog by addition of an ethereal solution of zinc chloride. Reaction of the organozinc-I with the aryl bromide-II in the presence of palladium catalyst resulted in formation of the biaryl-III in 27% yield. The methoxymethoxy group was removed by treatment with hydrochloric acid in methanol to produce the biaryl-IV in 100% yield. The identity and purity of each analog was verified by HPLC and mass spectrometry.

### 1.3. Preparation of test solutions

Ethanol (50  $\mu$ l) was added to honokiol and each of the eight analogs, and each mixture was then diluted with distilled water containing 0.1% Tween-80 so that the final ethanol concentration was 0.5%. All test substances were fully soluble under these conditions. The injectable preparation of diazepam was diluted with distilled water. Flumazenil and bicuculline were suspended in Tween-80/0.9% saline. The concentration of each drug preparation was adjusted so that the volume administered would consistently be 0.1 ml/10 g mouse

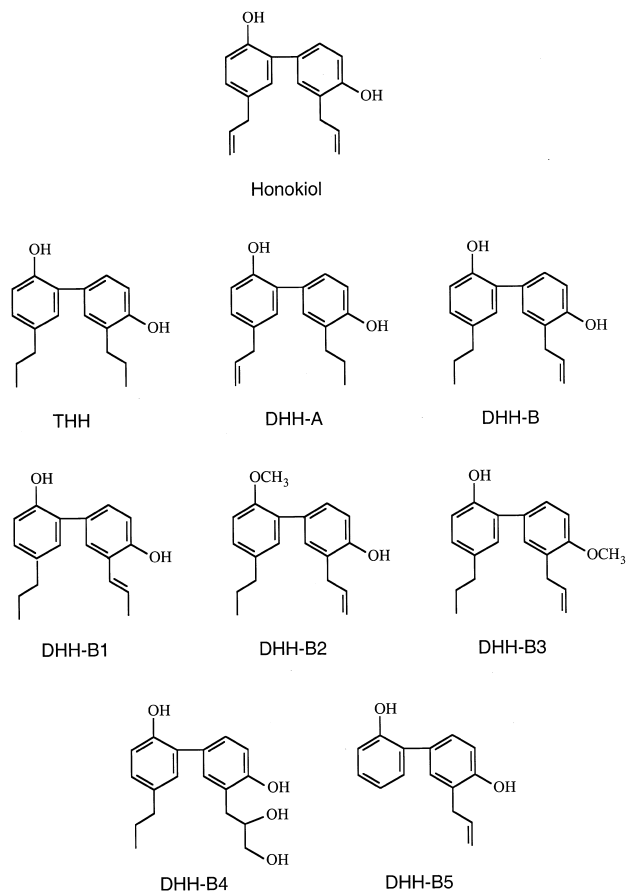


Fig. 1. Structures of honokiol and honokiol analogs.

body weight. Animals in control groups received vehicle alone. In separate experiments, it was confirmed that constituents of the respective vehicles (e.g., ethanol and Tween-80 used for honokiol and analogs, and propylene glycol contained in the diazepam preparation) did not influence plus-maze performance.

### 1.4. Measurement of anxiolytic-like activity

The elevated plus-maze used in this study was constructed in this laboratory using a modification of the original apparatus for rats [11] and mice [8], as described in our earlier reports [4,5,9]. Briefly, the plus-maze, which was maintained at a 40-cm elevation, consisted of four arms (6  $\times$  30 cm each) that extended from a central platform (8  $\times$  8 cm). Two of the arms had 10-cm high sidewalls. Like the central platform, both the floor and sidewalls of these arms were nontransparent and painted gray. The other two arms had no sidewalls, and the floor was constructed of transparent acrylic. For testing, each mouse was placed on the central platform, randomly facing one of the walled arms. The cumulative time spent in one of the open-sided arms during the ensuing 5-min period was recorded by a

Table 1  
Synthesis of DHH analogs

DHH analog	Starting material <sup>a</sup>		Intermediate <sup>a</sup>		Product <sup>a</sup>	
	I	II	III	Yield (%)	IV	Yield (%)
A	R <sup>1</sup> = Br R <sup>2</sup> = allyl R <sup>3</sup> = MOM	R <sup>4</sup> = MOM R <sup>5</sup> = 1-propyl	R <sup>2</sup> = allyl R <sup>3</sup> = MOM R <sup>4</sup> = MOM R <sup>5</sup> = 1-propyl	41	R <sup>2</sup> = allyl R <sup>3</sup> = H R <sup>4</sup> = H R <sup>5</sup> = 1-propyl	90
B	R <sup>1</sup> = Br R <sup>2</sup> = propyl R <sup>3</sup> = Me	R <sup>4</sup> = MOM R <sup>5</sup> = 2-allyl	R <sup>2</sup> = propyl R <sup>3</sup> = Me R <sup>4</sup> = MOM R <sup>5</sup> = allyl	53	R <sup>2</sup> = propyl R <sup>3</sup> = H R <sup>4</sup> = H R <sup>5</sup> = 2-allyl	66
B1	R <sup>1</sup> = H R <sup>2</sup> = propyl R <sup>3</sup> = MOM	R <sup>4</sup> = MOM R <sup>5</sup> = 1-propenyl	R <sup>2</sup> = propyl R <sup>3</sup> = MOM R <sup>4</sup> = MOM R <sup>5</sup> = 1-propenyl	30	R <sup>2</sup> = propyl R <sup>3</sup> = H R <sup>4</sup> = H R <sup>5</sup> = 1-propenyl	66
B2	R <sup>1</sup> = H R <sup>2</sup> = propyl R <sup>3</sup> = Me	R <sup>4</sup> = MOM R <sup>5</sup> = 2-propenyl	R <sup>2</sup> = propyl R <sup>3</sup> = Me R <sup>4</sup> = MOM R <sup>5</sup> = 2-propenyl	11	R <sup>2</sup> = propyl R <sup>3</sup> = Me R <sup>4</sup> = H R <sup>5</sup> = 2-propenyl	94
B3	R <sup>1</sup> = H R <sup>2</sup> = propyl R <sup>3</sup> = MOM	R <sup>4</sup> = Me R <sup>5</sup> = 2-propenyl	R <sup>2</sup> = propyl R <sup>3</sup> = MOM R <sup>4</sup> = Me R <sup>5</sup> = 2-propenyl	27	R <sup>2</sup> = propyl R <sup>3</sup> = H R <sup>4</sup> = Me R <sup>5</sup> = 2-propenyl	100
B4	R <sup>1</sup> = H R <sup>2</sup> = propyl R <sup>3</sup> = MOM	R <sup>4</sup> = MOM R <sup>5</sup> = 2-propenyl	R <sup>2</sup> = propyl R <sup>3</sup> = MOM R <sup>4</sup> = MOM R <sup>5</sup> = 2-propenyl	20	R <sup>2</sup> = propyl R <sup>3</sup> = H R <sup>4</sup> = H R <sup>5</sup> = 2,3-diOHpr <sup>b</sup>	18
B5	R <sup>1</sup> = H R <sup>2</sup> = H R <sup>3</sup> = MOM	R <sup>4</sup> = MOM R <sup>5</sup> = 2-propenyl	R <sup>2</sup> = H R <sup>3</sup> = MOM R <sup>4</sup> = MOM R <sup>5</sup> = 2-propenyl	29	R <sup>2</sup> = H R <sup>3</sup> = H R <sup>4</sup> = H R <sup>5</sup> = 2-propenyl	44

<sup>a</sup> Abbreviations: MOM, methoxymethoxy; Me, methyl; 2,3-diOHpr, 2,3-dihydroxypropyl.

<sup>b</sup> Produced from 2-propenyl by: (1) oxidation; (2) base hydrolysis; (3) acid hydrolysis.

trained observer who was not apprised of the treatment code for the individual groups. A mouse was considered to have entered an open-sided arm if all four paws crossed the border between the central platform and the open arm.

### 1.5. Activity test

Immediately following the plus-maze test, the motor activity of each mouse was measured for 5 min with a tilting ambulometer, which had a bucket-like Plexiglas activity cage (20 cm in diameter; SMA-1; O'Hara, Tokyo, Japan). This apparatus could selectively detect horizontal movement (ambulation) of the mouse.

### 1.6. Statistical analysis

Statistical significance was assessed by a one-way ANOVA followed by Student–Newman–Keuls test. Values of  $P < .05$  were considered significant.

## 2. Results

As shown in Table 2, honokiol (20 mg/kg, po), DHH-B (1 mg/kg, po), and diazepam (1 mg/kg, po) significantly prolonged the time spent in the open-sided arms without causing a change in motor activity. It is important to note that a dose of 0.04 mg/kg of DHH-B also exhibited a

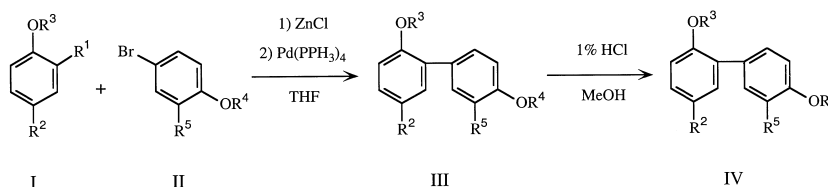


Fig. 2. Reaction sequence for preparation of honokiol analogs.

Table 2  
Anxiolytic-like activity of honokiol and analogs after acute oral administration

Treatment				
Drug <sup>a</sup>	Dose (mg/kg)	N	Time in open-sided arms (s) <sup>b</sup>	Activity (counts/5 min) <sup>b</sup>
Vehicle		20	5.8±2.0	32.4±1.7
Honokiol	20	10	16.9±3.5 *	37.0±7.2
DHH-A	1	10	10.1±3.9	35.3±4.7
DHH-B	1	10	21.5±5.2 *	33.0±2.4
THH	10	10	5.3±2.3	31.2±1.8
Vehicle		10	1.3±0.8	31.0±3.2
DHH-B1	2	10	6.1±2.3	40.8±3.8
DHH-B2	2	10	5.3±2.7	36.9±3.3
DHH-B3	2	10	9.6±3.8 *	39.1±4.9
DHH-B4	2	10	2.9±1.8	33.6±4.1
DHH-B5	2	10	1.3±0.7	36.6±4.6
Vehicle		10	3.2±1.3	28.6±3.0
Diazepam	1	10	22.1±5.8 *	33.6±3.1

<sup>a</sup> All drugs were administered orally 3 h before evaluation on the elevated plus-maze except for diazepam, which was given orally 10 min prior to testing; N, number of mice per test group.

<sup>b</sup> Values represent mean ± S.E.

significant anxiolytic-like effect (12.3±3.4 s in the open-sided arms) without impairing motor function. In contrast, neither DHH-A nor THH was effective.

Since DHH-B was substantially more active than DHH-A, only analogs of DHH-B were subsequently examined. As can be seen in Table 2, of the five analogs tested, only DHH-B3 exhibited weak anxiolytic-like activity. During the course of these studies, it was observed that 1 mg/kg of diazepam produced muscle relaxation and mild ataxia. However, neither honokiol nor any of the analogs caused any motor dysfunction (data not shown).

Table 3  
Time course for anxiolytic-like activity of DHH-B

Time after treatment (h)	DHH-B <sup>a</sup>		Control <sup>a</sup>	
	Plus-maze (s)	Activity (counts/5 min)	Plus-maze (s)	Activity (counts/5 min)
1	7.4±2.5	39.2±2.9	4.2±2.2	28.6±4.5
2	13.5±2.8 *	33.6±2.0	6.4±2.3	30.7±3.7
3	18.8±3.6 **	42.9±4.8	3.6±2.1	33.9±3.7
4	17.1±3.3 **	22.9±2.5	4.2±2.6	18.3±1.9
6	10.8±2.8	22.0±1.6	5.5±1.6	23.0±3.4
8	7.9±3.3	25.0±2.6	1.5±1.3	22.5±3.8
12	8.3±3.1	23.8±4.4	0.4±0.4	18.3±2.1
24	6.5±4.7	18.0±2.0	0.3±0.3	27.9±4.1
48	3.7±2.4	27.2±4.2	2.7±1.2	29.6±2.5
72	7.8±2.2	30.3±2.0	2.6±4.1	30.0±4.1

<sup>a</sup> Treatment with 1 mg/kg, po, DHH-B or vehicle (control); 10 mice were tested at each time point.

\*  $P < .05$  compared to the corresponding vehicle-treated group.

\*\*  $P < .01$  compared to the corresponding vehicle-treated group.

Table 4  
Anxiolytic-like activity of DHH-B in combination with other agents

Drug <sup>a</sup>	Time in open-sided arms (s) <sup>b</sup>	Activity (counts/5 min) <sup>b</sup>
Vehicle	2.1±1.0	27.7±2.1
Flumazenil	12.8±4.6*	29.0±2.6
Vehicle	3.9±0.9	28.7±2.5
Diazepam	17.8±3.7*	32.5±4.2
DHH-B	14.9±5.6*	32.4±4.4
DHH-B + diazepam	34.4±10.7*†**	36.8±8.0
Vehicle	4.9±1.5	33.0±3.9
DHH-B	22.8±4.3*	33.8±2.1
DHH-B + flumazenil	9.0±1.9†	29.2±4.6
Diazepam	19.4±2.8*	31.4±4.1
Diazepam + flumazenil	7.4±3.0**	31.7±5.0
Vehicle	3.2±1.3	28.6±3.0
Bicuculline	4.0±1.3	35.9±4.3
DHH-B	16.6±6.1*	36.2±2.6
DHH-B + bicuculline	13.8±3.2	33.0±4.1
Diazepam	25.4±7.1*	25.4±2.8
Diazepam + bicuculline	8.4±1.8**	48.3±5.9**

<sup>a</sup> Drug administration was as follows: flumazenil, 0.3 mg/kg ip, 10 min before plus-maze test; diazepam, 1 mg/kg po, 10 min; DHH-B, 1 mg/kg po, 3 h; bicuculline, 0.1 mg/kg sc, 10 min; 10 mice were tested in each group.

<sup>b</sup> Values represent mean ± S.E.

\*  $P < .05$  compared to vehicle-treated group.

\*\*  $P < .05$  compared to diazepam.

†  $P < .05$  compared to DHH-B.

The time course for efficacy of DHH-B is shown in Table 3, where it can be seen that significant activity was apparent by 2 h after oral administration of 1 mg/kg, with peak effectiveness at 3 h. Furthermore, treatment with DHH-B did not cause any significant changes in motor activity at any time period.

As illustrated in Table 4, combined administration of DHH-B and diazepam led to an enhancement in the anxiolytic-like effect. Flumazenil inhibited the anxiolytic-like efficacy of both DHH-B and diazepam. However, bicuculline was not able to reduce the anxiolytic-like activity of DHH-B at a dose that significantly inhibited the action of diazepam.

### 3. Discussion

We have previously shown using BALB/c [6] and ddY [9] mice that honokiol is an effective anxiolytic-like agent when administered daily at 0.2 mg/kg for 7 days prior to evaluation in an elevated plus-maze, while 20 mg/kg is required for efficacy as a single oral dose. The half-life of honokiol in rat plasma has been reported to be 49–56 min, indicating no accumulation of honokiol following daily administration [13]. Hattori et al. [2,3] found that

daily administration of magnolol, an isomer of honokiol, resulted in increased plasma levels of partially hydrogenated magnolol derivatives. Based on these findings, we hypothesized that repeated administration of honokiol might be leading to metabolite accumulation, and that the anxiolytic-like effect observed after chronic administration of honokiol might be induced by active metabolite(s) rather than the parent compound. We, therefore, generated hydrogenated derivatives of honokiol as well as other analogs, and evaluated the anxiolytic-like activities of these compounds.

The present study demonstrated that of the eight analogs tested, DHH-B was the most potent anxiolytic-like agent with significant efficacy after a single dose of 0.04 mg/kg. Following administration of 1 mg/kg of DHH-B, anxiolytic-like activity was observed at 1 h after administration and persisted for up to 4 h, peaking at 3 h. Furthermore, DHH-B did not cause any significant change in motor activity or muscle relaxation/ataxia. It has previously been shown in our laboratory [7] and by Watanabe et al. [14–16] that there is a comparatively lower likelihood of motor dysfunction after treatment with honokiol, the precursor of DHH-B, in contrast to benzodiazepine anxiolytics, which have well-known motor function side effects [12].

In earlier studies [4,6], we showed that the benzodiazepine receptor antagonist, flumazenil, inhibited the anxiolytic-like effect of both diazepam and honokiol, even though flumazenil alone slightly prolonged the time spent in the open-sided arms. Here, we demonstrated that the anxiolytic-like activity of DHH-B could be completely inhibited by flumazenil, strongly suggesting that the benzodiazepine binding sites within the GABA<sub>A</sub> receptor are involved in development of the anxiolytic-like effect of DHH-B. However, in the present investigation, it was also found that unlike diazepam and honokiol [6], the anxiolytic-like activity of DHH-B could not be reduced by bicuculline, a GABA<sub>A</sub> receptor antagonist that interacts with the GABA binding site. In preliminary in vitro studies of muscimol-stimulated <sup>36</sup>Cl<sup>-</sup> uptake into mouse cerebral synaptosomes, we recently ascertained that DHH-B significantly enhanced chloride ion influx in a dose-dependent manner (data not shown). These results suggest that the anxiolytic-like activity of DHH-B may be mediated by a receptor-gated chloride ion channel, such as the GABA<sub>A</sub> or GABA<sub>C</sub> receptor complex. However, the bicuculline experiments reported here indicate that although DHH-B may act as a benzodiazepine receptor agonist, GABA binding sites on the GABA<sub>A</sub> receptor are less directly involved in the anxiolytic-like activity of DHH-B than for the benzodiazepine anxiolytics. Further studies are required to elucidate the binding sites (or receptors) for DHH-B, and to discover the mechanisms of pharmacological action of this partially hydrogenated analog of honokiol.

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