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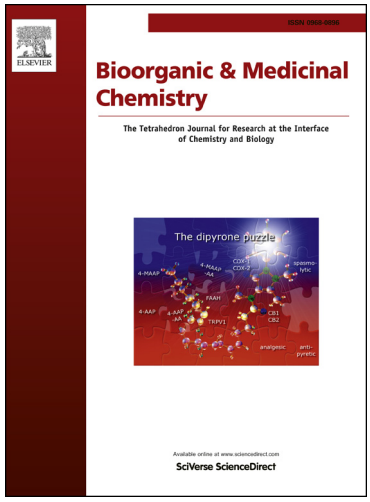

The isolation and synthesis of a novel benzofuran compound from *Tephrosia purpurea*, and the synthesis of several related derivatives, which suppress histamine H_1 receptor gene expression

Manik Chandra Shill, Asish Kumar Das, Tomohiro Itou, Sanmoy Karmakar, Pulok K. Mukherjee, Hiroyuki Mizuguchi, Yoshiki Kashiwada, Hiroyuki Fukui, Hisao Nemoto

PII: S0968-0896(15)30074-2
DOI: http://dx.doi.org/10.1016/j.bmc.2015.09.049
Reference: BMC 12596

To appear in: *Bioorganic & Medicinal Chemistry*

Received Date: 2 September 2015
Revised Date: 29 September 2015
Accepted Date: 30 September 2015

Please cite this article as: Shill, M.C., Das, A.K., Itou, T., Karmakar, S., Mukherjee, P.K., Mizuguchi, H., Kashiwada, Y., Fukui, H., Nemoto, H., The isolation and synthesis of a novel benzofuran compound from *Tephrosia purpurea*, and the synthesis of several related derivatives, which suppress histamine H_1 receptor gene expression, *Bioorganic & Medicinal Chemistry* (2015), doi: http://dx.doi.org/10.1016/j.bmc.2015.09.049

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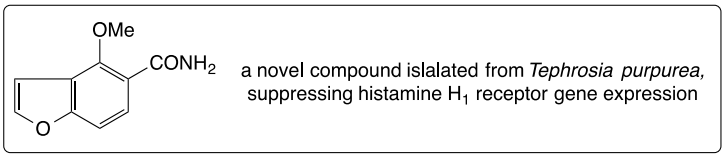

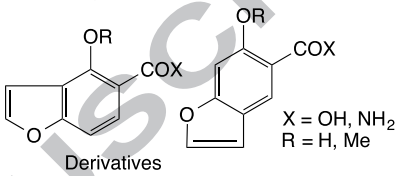

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Bioorganic & Medicinal Chemistry
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The isolation and synthesis of a novel benzofuran compound from *Tephrosia purpurea*, and the synthesis of several related derivatives, which suppress histamine H_1 receptor gene expression

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ARTICLE INFO

Article history:
Received
Received in revised form
Accepted
Available online

Keywords:
Histamin
Allergy
benzofuran

ABSTRACT

A novel naturally occurring compound with a benzofuran skeleton was isolated from a plant, *Tephrosia purpurea* collected in Bangladesh. The chemical synthesis of this compound confirmed its structure, and preliminary biological results showed its suppressive activity towards histamine H_1 gene expression. One isomer and four derivatives were also synthesized, and their suppression activity was investigated. Although only small quantities of this compound can be isolated from its natural source, a 10 g scale synthesis was demonstrated by the newly developed method.

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Introduction

Allergic rhinitis (AR), known as hay fever, affects 5–22% of the population globally,[1] and its causes are gradually being clarified.[2,3] Histamine is a significant key mediator, and acts mainly through histamine H_1 receptor (H1R).[2] Determination of the amino acid sequence of H1R by two of the authors (H. F. and H. M.),[4] has been followed by research into AR therapies,[5] including the survey to identify suitable small molecule drugs.

Advances in biological and chemical technology have led to several modern medicines (mMeds) for palliative treatment of AR.[6] Many mMeds are single compounds. Unfortunately, mMeds often cause clinically negative side effects, and no conclusive therapeutic agent for treating AR has yet been identified. In contrast, several traditionally developed medicines (tMeds) are mildly effective and produce few negative side effects.[7] However, tMeds are generally complicated chemical mixtures and the scientific basis for their action is often unclear. Therefore, many researchers, including us, have been attempting to isolate and determine the structures of key compounds in these complicated mixtures,[8] and examine their therapeutic utility for allergic diseases, including AR.[9,10] tMeds are generally well known in East Asian countries such as China, Japan and Korea.[11] However, Indian subcontinent also directed our attention, because tMeds from Indian subcontinent may exhibit stronger anti-allergic activity than that at East Asian tMeds.[12]

We report the isolation and synthesis of a new compound, 4-methoxybenzofuran-5-carboxamide (**1a**, Scheme 1), extracted from a plant, *Tephrosia purpurea*. *T. purpurea* grows from east India to central Bangladesh. That is the first synthesis of **1a**; this report confirms the chemical structure of **1a** and its suppressive effect on H1R gene expression in allergic model cells.[13] The synthesis of derivatives of **1a** and their suppressive effects are also demonstrated.

1. Results

Isolation and its structural determination of 1a

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As shown in scheme 1, a methanolic extract of *Tephrosia purpurea* was fractionated using various solvents; the suppressive activity of each fraction was examined using phorbol 12-myristate 13-acetate (PMA)-induced up-regulation of H1R gene expression in HeLa cells using our previously established procedure.[10] The chloroform fraction exhibited the highest activity; this fraction was further fractionated by silica gel column chromatography, followed by HPLC, resulting in the isolation of a single molecule. Extensive spectroscopic analysis identified the compound as 4-methoxybenzofuran-5-carboxamide (**1a**).[14,15] Details of the structural determination are described below.

Scheme 1. Extraction and isolation of 1a from *Tephrosia purpurea*

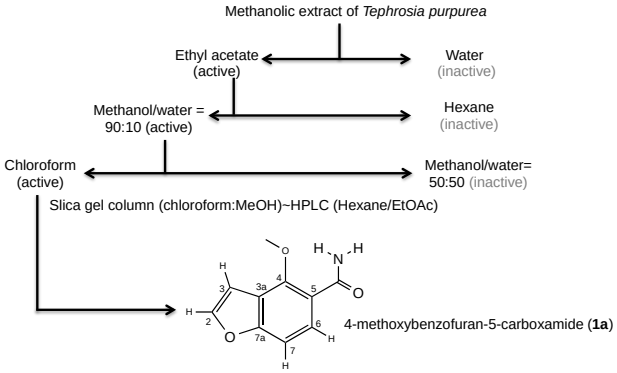


Table 1. ^{1}H (500 MHz) and ^{13}C NMR (125 MHz) data for 1a

Position	δ_H	Multiplicity & Coupling (Hz)	δ_C
2	7.64	d, *J* = 2.2	144.9 (CH)
3	6.98	dd, *J* = 0.8, 2.2	105.2 (CH)
4			152.9 (C)
5			117.5 (C)
6	8.16	d, *J* = 8.8	128.3 (CH)
7	7.32	d, *J* = 0.8, 8.8	107.1 (CH)
7a			158.9 (C)
3a			118.6 (C)
C=O			167.6 (C)
MeO	4.19	s	61.0 (CH_3)
NH	7.78	br	
NH	5.72	br	

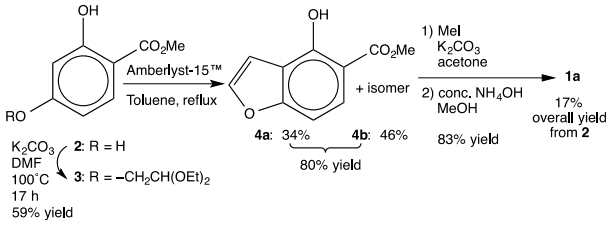
Compound **1a** gave a pseudo-molecular ion peak at *m/z* 214.0498 (calcd for $C_{10}H_9NO_3Na$, 214.0480) on ESI-TOF-HRMS, suggesting the molecular formula is $C_{10}H_9NO_3$. The ^{1}H NMR spectrum showed a pair of *ortho*-coupled aromatic protons [δ_H 8.16 and 7.32 (each 1H, d, *J* = 8.8 Hz)], a pair of conjugated olefinic protons [δ_H 7.64 and 6.98 (each 1H, d, *J* = 2.2 Hz)], and a methoxyl signal (δ_H 4.19) (Table 1). The ^{1}H NMR spectrum also showed two broad D_2O exchangeable peaks at 7.78 and 5.72; these coupled with IR absorption bands at 3453 and 1654 cm^{-1}, implied the presence of a primary carboxamide functionality. The ^{13}C NMR spectrum exhibited ten carbon signals, including a methyl, four sp^2 methines, four sp^2 quaternary carbons, and one carbonyl carbon.

The structure of **1a** was elucidated by 2D-NMR analysis. The olefinic proton signals at δ_H 7.64 (H-2) and 6.98 (H-3) resonated with the carbon signal at δ_C 144.9 and 105.2, respectively, in the HSQC spectrum. These resonances showed the HMBC correlations with the sp^2 quaternary carbon at δ_C 158.9 (C-7a) and 118.6 (C-3a) in each case, suggesting the presence of a furan moiety. In contrast, the following HMBC correlations indicated the presence of 4,5-disubstituted benzofuran structure; the aromatic proton signal at δ_H 7.32 (H-7) with C-3a; the carbon signals at δ_C 117.5 (C-5); the signal at δ_H 8.16 (H-6) with C-7a and the resonances at δ_C 152.9 (C-4). In addition, the positions of the carboxamide and the methoxy group were assigned to be C-5 and C-4, respectively, from the HMBC correlation of H-6 with the carbonyl carbon resonance and of the methoxy signal with C-4. Based on the spectral analysis described above, the structure of **1a** was assigned as 4-methoxybenzofuran-5-carboxamide.

Synthesis of 1a

Compound **1a** was synthesized as shown in Scheme 2. Ether bond formation between two of the commercially available compounds, 2-bromoacetaldehyde diethyl acetal and methyl 2,4-hydroxybenzoate (**2**), was carried out in anhydrous DMF at 100˚C for 17 h in the presence of anhydrous potassium carbonate afforded **3** in 59% isolated yield. The phenoxide at the 4-position was more reactive than that at the 2-position, because the latter intramolecularly has hydrogen bonds with the neighboring ester functionality. Treatment of **3** in toluene (0.07 mol/L) with Amberlyst-15, a polymer supported sulfonic acid, gave a mixture of benzofuran derivatives **4a** and **4b** in 80% combined yield. In contrast, use of a high concentration of **3** in toluene (0.70 mol/L) provided oligomer-like unidentified mixtures as major products, as expected from a previously reported preparation of benzofuran.[16] It appeared that the concentration of **3** did not strongly influence the reaction rate of consumption of **3**, probably because the furan was produced intramolecularly. On the other hand, over-reactions such as the dimerization of **4** and further oligomerizations are generally strongly inhibited under dilute conditions. Indeed, dilute conditions dramatically improved the chemical yield of **4**. Silica gel column chromatography easily separated **4a**, an important synthetic intermediate for **1a**, from the corresponding isomer **4b**. The isolated yield of **4a** and **4b** was 34% and 46%, respectively.

Scheme 2. Synthesis of 1a



Treatment of **4a** with iodomethane in the presence of potassium carbonate, followed by ammonolysis gave **1a** in 83% overall yield. All the analytical data of ^{1}H and ^{13}C NMR data from synthetic **1a** were identical with that of isolated **1a**, as were the IR and ESI-TOF-HRMS data. The suppressive effect of synthetic **1a** on H1R gene expression (*vide infra*) in allergic model cells was reproducibly as high as that of isolated **1a**.

Synthesis of the isomer and the related derivatives

Treatment of **4a** with concentrated aqueous ammonia gave **5a** in 87% yield, and **4b** was similarly transformed to **5b** (82% yield). Compound **4a** was converted to **7a** via O-methylation and hydrolysis steps[19] in 80% yield, and **4b** was converted to **7b** (89% yield) in the same manner via **6**. Finally, the isomer **1b**, the

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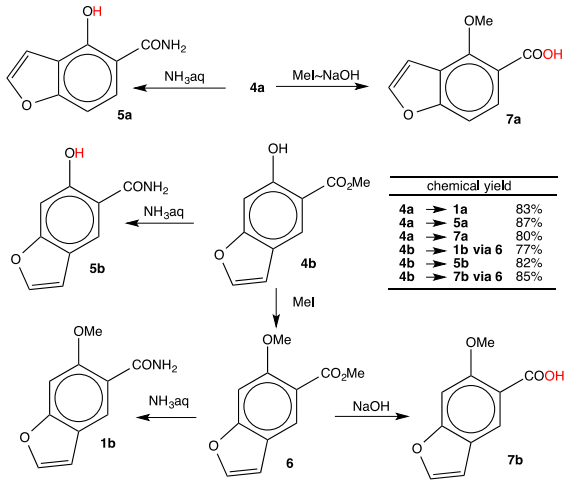
sixth molecule in this small library, was synthesized from **6** in 80% yield via ammonolysis (Scheme 3).

Scheme 3. Synthesis of an isomer 1b and the related derivatives



chemical yield		
4a → 1a		83%
4a → 5a		87%
4a → 7a		80%
4b → 1b via 6		77%
4b → 5b		82%
4b → 7b via 6		85%

Preliminary investigation of anti-allergic activity

The activity of the six synthesized compounds on PMA-induced H1R gene expression in HeLa cells was examined,[5] and the results are summarized in Table 2. Isolated **1a** suppressed H1R mRNA up-regulation in HeLa cells with an IC_{50} of 75.3 μM (entry 1), and synthesized **1a** showed reproducible activity with an IC_{50} 83.7 μM (entry 2). It is noteworthy that *the isomer **1b** suppressed H1R gene expression at a similar level, with an IC_{50} of 49.14 μM* (entry3). Except **5b** (entry 5), the other modified derivatives were inactive.

Table 2. H1R mRNA suppression of the six compounds

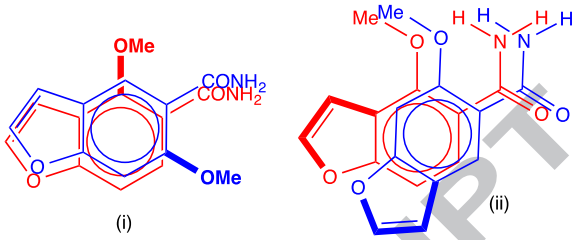
Entry	Compound	Suppression (IC_{50}) (μM)
1	Isolated **1a**	75.3
2	Synthesized **1a**	83.7
3	**1b**	49.1
4	**5a**	–[a)]
5	**5b**	84.6
6	**7a**	–[a)]
7	**7b**	–[a)]

a) No suppression of H1R mRNA expression was observed.

3. Discussion

Our interest is to further the synthesis of library molecules using **1a** as a lead molecule. We believe that the primary carboxamide functionality should be fixed since carboxylic acids **7a** and **7b** were inactive (entries 6 and 7). In contrast, various substituents may be introduced on the phenoxy moiety since demethylated isomer **5b** showed the same level of activity as **1a** (entry 5). Figure 1 provides insights into the observed difference between **1a** and **1b**. Overrapping **1a** and **1b** in the orientation shown in (i) indicates that the relative position of the furan ring and carboxamide of **1a** is exactly the same as that of **1b**, whereas the position of the methoxy group of **1a** is quite different from that of **1b**. In contrast, the orientation shown in (ii) suggests that only the location of the furan plane is different between the two compounds and that benzene ring and all the electronically negative elements overlap well. The interaction of **1a** or **1b** with key proteins/enzymes for allergy is now under investigation.[20)]

Figure 1. Overlapped structures of 1a and 1b.



4. Conclusions

A novel compound, comprising a primary carboxamide on a benzofuran skeleton was isolated from a plant found in west India to central Bangladesh. The structure was determined by IR, ESI-TOF-HRMS, and NMR measurements. Preliminary examination of H1R mRNA suppression activity confirmed this compound to hold promise for the treatment of allergic diseases, including rhinitis. The chemical structure and the activity of the compound were confirmed by its total synthesis and two novel molecules designed from the isolated compound exhibited potential therapeutic utility. Although little of the lead compound can be isolated from natural source, the synthetic route described here will allow its synthesis on a scale of 10 g or more with high reproducibility. Our chemical synthesis and biological research efforts will continue in order to identify optimal drugs for treating allergies.

5. Exprimental Section

IR spectra were recorded on a FT-IR 6200 spectrophotometer. ^{1}H NMR spectra were measured in chloroform-d_1 ($CDCl_3$) and referenced to tetramethylsilane using 400 MHz and 500 MHz spectrometers unless otherwise noted. ^{13}C NMR were measured in $CDCl_3$ and referenced to $CDCl_3$ (δ = 77.0) using 125 MHz spectrometers unless otherwise noted. Column chromatography was performed on silica gel (N-60). Thin layer chromatography as performed on pre-coated plates (0.25 mm, silica gel Kieselgel 60_{F254}). All the extract procedures were performed with commercially available organic solvents without further purification. All the chemical reactions were performed in oven-dried glassware equipped with calcium chloride tube. Reaction mixtures were stirred magnetically. Methanol (MeOH) was distilled over magnesium. N,N-dimethylformamide (DMF) was distilled over calcium hydride under reduced pressure. Toluene was distilled under normal pressure. Acetone was distilled over anhydrous potassium carbonate (K_2CO_3).

Phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma-Aldrich. The pre-developed TaqMan Assay Reagent of human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was from Applied Biosystems (Foster City, CA, USA). Minimal essential medium (MEM)-α was from Invitrogen (Carlsbad, CA, USA). RNAiso Plus and the PrimeScript RT reagent Kit were from Takara Bio Inc. (Kyoto, Japan). Methyl 2, 4-dihydroxy benzoate and bromoacetaldehyde diethyl acetal were purchased from Sigma-Aldrich and Tokyo Chemical Company (USA) respectively. All other chemicals and solvents were of analytical grade.

Plant Material

Aerial parts of *Tephrosia purpurea* Pers. were collected in Bangladesh. The plant was identified by Dr. Sarder Nasir Uddin, National Herbarium Mirpur, Dhaka, Bangladesh (Accession No.

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41236). The plant was then shed dried, grounded to obtained coarse powder material and stored until the present investigation.

Extraction and isolation of 1a

The powdered plant (4.0 kg) was extracted by maceration at room temperature using MeOH. The extract was concentrated in vacuo to afford a residue (118.8 g), which was dissolved in ethyl acetate (EtOAc), and washed with water. The organic layer was concentrated in vacuo to afford a residue (65.34 g), which was dissolved in MeOH:H_2O (90:10) and washed with hexane. The aqueous methanolic layer was concentrated in vacuo to afford a residue (35.0 g), which was dissolved in chloroform and washed with MeOH:H_2O=50:50. The chloroform layer was concentrated in vacuo to afford a residue (30 .0 g). Afterward, 16.0 g was subjected to silica gel column chromatography eluted with chloroform:MeOH (98.5:1.5, 97:3, 94:6, 90:10, 80:20, 1:1, and 0:1) to afford 24 fractions based on TLC examination. A part (30.0 mg) of fraction 9 (500.0 mg) was purified by HPLC (Cosmosil 5 SL, 4.6 mm id × 250 mm length) eluted with EtOAc:hexane (6:4, flow rate = 1.0 mL/min) in an isocratic condition to afford **1a** (6.0 mg). FT-IR (KBr): 3453, 2360, 1654, 1458, 1420, 1387, 1256, 1066, 978 cm^{-1}, ^1H NMR (500 MHz, $CDCl_3$) see Table 1; ^{13}C NMR (125 MHz, $CDCl_3$) see Table 1; HRESIMS m/z calcd for $C_{10}H_9NNaO_3$ $[M + Na]^+$ 214.0480, found: 214.0498.

Synthesis

Methyl 4-hydroxy benzoate 2-acetaldehyde diethyl acetal (**3**): To a solution of methyl 2,4-dihhydroxy benzoate (**2**) (1.00 g, 5.95 mmol) in DMF (10 mL), suspended with anhydrous potassium carbobate (K_2CO_3) (1.23 g, 8.93 mmol, 1.5 equiv.) with vigorously stirring, was added bromoacetaldehyde diethyl acetal (5.87 g, 29.8 mmol) dropwise over 5 min. The resulting suspension was heated at 100 ºC with stirring for 17 h. After being cooled, the suspension was poured into 5% of aqueous solution of potassium hydrogen sulfate ($KHSO_4$aq, 100 mL) at 0˚C, and extracted with EtOAc (120 mL × 3). The combined organic layers were washed with brine, dried over anhydrous sodium sulfate (Na_2SO_4) and concentrated in vacuo. The residue was purified by silica gel column chromatography using hexane and EtOAc (8:2) to afford **3** as a colorless oil (994.0 mg, 3.50 mmol, 59% yield). FT-IR (neat): 3145, 2977, 2884, 1917, 1672, 1624, 1584, 1506, 1442, 1374, 1350, 1257, 1225, 1195, 1178, 1136, 1072, 980, 958, 886, 855, 780, 733, 697, 655 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 10.94 (s, 1H, OH), 7.73 (d, J = 9.2 Hz, 1H), 6.48–6.45 (m, 2H), 4.83 (t, J = 5.2 Hz, 1H), 4.02 (d, J = 5.2 Hz, 2H), 3.91 (s, 3H), 3.76 (dq, J = 6.8, 9.4 Hz, 2H), 3.63 (dq, J = 6.8, 9.4 Hz, 2H), 1.25 (t, J = 6.8 Hz, CH_3 × 2); ^{13}C NMR (125 MHz, $CDCl_3$): δ 170.4 (C), 164.5 (C), 163.7 (C), 131.3 (CH), 107.8 (CH), 105.8 (C), 101.5 (CH), 100.2 (CH), 68.6 (CH_2), 62.8 (CH_2), 52.0 (CH_3), 15.4 (CH_3); HRESIMS m/z calcd for $C_{14}H_{19}O_6$ $[M – H]^+$ 283.1182, found: 283.1172.

Methyl 4-hydroxybenzofuran-5-carboxylate (**4a**) **and methyl 6-hydroxybenzofuran -5-carboxylate** (**4b**): A solution of **3** (389.5 mg, 1.37 mmol) in toluene (20 mL) suspended with Amberlyst-15® (51.0 mg) was stirred at reflux for 4 h, equipped with dean-stark apparatus to remove water. After being cooled, the resulting suspension was filtered, and the residue was washed with toluene (80 mL). The combined filtrate was concentrated in vacuo. The residue was purified by silica gel column chromatography eluted with EtOAc/hexane (1:9) to afford **4a** (89.5 mg, 0.47 mmol; 34% yield) and **4b** (121.1 mg, 0.63 mmol, 46% yield), respectively.

4a: FT-IR (KBr): 3414, 3149, 3125, 3029, 2971, 2859, 1868, 1678, 1629, 1471, 1445, 1357, 1284, 1235, 1195, 1170, 1133, 1050, 982, 748 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 11.47 (s, 1H, OH), 7.78 (d, J= 8.8 Hz, 1H), 7.57 (d, J=2.0, 1H), 7.04 (d, J=8.8 Hz, 1H), 6.98 (d, J=2.0, 1H), 3.97 (s, 3H); ^{13}C NMR (125 MHz $CDCl_3$) δ 171.2 (C), 159.6 (C), 157.5 (C), 144.4 (CH), 126.0 (CH), 117.1 (C), 106.0 (C), 104.8 (CH), 103.9 (CH), 52.3 (CH_3); HRESIMS m/z calcd for $C_{10}H_8NaO_4$ $[M + Na]^+$ 215.0320, found: 215.0322.

4b: FT-IR (KBr): 3413, 3120, 1663, 1557, 1443, 1414, 1369, 1286, 1258, 1212, 1186, 1143, 1072, 1013, 948, 845, 790 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 10.92 (s, 1H, OH), 8.13 (s, 1H), 7.54 (d, J=2.4 Hz, 1H), 7.07 (s, 1H), 6.70 (d, J = 2.4 Hz, 1H), 3.98 (s, 3H); ^{13}C (125 MHz, $CDCl_3$): δ 170.9 (C), 159.8 (C), 159.4 (C), 145.5 (CH), 123.1 (CH), 120.3 (C), 109.2 (C), 106.6 (CH), 99.4 (CH), 52.4 (CH_3); HRESIMS m/z calcd for $C_{10}H_7O_4$ $[M – H]^+$ 191.0350, found: 191.0351.

4-Hydroxybenzofuran-5-carboxamide (**5a**): To a solution of **4a** (90.0 mg, 0.468 mmol) in MeOH (2.0 mL) was added an aqueous solution of conc. Ammonia (NH_3aq) (28%, 10 mL). The resulting mixture was stirred at 36º C for 24 h, and concentrated in vacuo. The residue was poured into HClaq (1N, 5.0 mL) and extracted with EtOAc (50mL × 3). The combined organic layers were washed with brine, dried over Na_2SO_4 and concentrated in vacuo. The residue was purified by silica gel column chromatography eluted with chloroform/MeOH (9:1) to afford **5a** (72.0 mg, 0.41 mmol, 87% yield). FT-IR (KBr): 3552, 3407, 3179, 1611, 1531, 1471, 1423, 1390, 1309, 1265, 1226, 1201, 1131, 1090, 1049, 1018, 941, 878, 754 cm^{-1}; ^1H NMR (500MHz, DMSO-d_6) δ 14.28 (s, 1H, OH), 8.45 (brs, 1H, NH), 7.96 (d, J = 2.0 Hz, 1H), 7.93 (brs, 1H, NH), 7.81 (d, J = 8.5 Hz, 1H), 7.13 (d, J = 8.5 Hz, 1H), 7.03 (d, J = 2.0 Hz, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 173.1 (C), 157.8 (C), 157.3 (C), 145.3 (CH), 124.2 (CH), 116.8 (C), 107.4 (C), 104.6 (CH), 102.6 (CH); HRESIMS m/z calcd for $C_9H_7NO_3$ $[M]^+$: 177.0426, found: 177.0424.

6-Hydroxybenzofuran-5-carboxamide (5b): To a solution of **4b** (97.6 mg, 0.508 mmol) in MeOH (2.0 mL) was added NH_3aq (28%, 10 mL). The resulting mixture was stirred at 36º C for 5 h, and concentrated in vacuo. The residue was poured into HClaq (1N, 5.0 mL) and extracted with EtOAc (50 mL × 3). The combined organic layers were washed with brine, dried over Na_2SO_4 and concentrated in vacuo. The residue was purified by silica gel column chromatography eluted with chloroform/MeOH (9:1) to afford **5b** (74.0 mg, 0.418 mmol, 82% yield). FR-IR (KBr): 3547, 3422, 3195, 1666, 1638, 1605, 1543, 1466, 1400, 1284, 1236, 1189, 1150, 1114, 1060, 1016, 832 cm^{-1}; ^1H NMR (400 MHz, DMDO-d_6) δ 13.26 (s, 1H, OH), 8.48 (brs, 1H, NH), 8.18 (s, 1H), 7.90 (brs, 1H, NH), 7.90 (d, J = 2.0 Hz, 1H), 7.04 (s, 1H,), 6.90 (d, J = 2.0 Hz, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 172.6 (C), 159.6 (C), 157.5 (C), 145.9 (CH), 120.9 (CH), 119.0 (C), 111.2 (C), 106.7 (CH), 98.8 (CH); HRESIMS m/z calcd for $C_9H_7NNaO_3$ $[M + Na]^+$: 200.0324, found: 200.0336.

Methyl 6-methoxybenzofuran-5-carboxylate (6): A mixture of **4b** (486mg, 2.529 mmol), K_2CO_3 (1.818g, 13.15 mmol), iodomethane (2.59g, 25.29 mmol) in acetone (25 mL) was refluxed for 5 h. After being cooled, the resulting suspension was filtered. The filtrate was concentrated in vacuo. The residue was diluted with ether (50 mL), and the resulting suspension was filtered. The filtrate was concentrated in vacuo. The residue was purified by silica gel column chromatography (Hexane/EtOAc, 7:3) to afford **6**, a pale yellow oil (500 mg, 2.425mmol, 96% yield). FT-IR (neat): 3107, 2951, 2840, 1720, 1590, 1538, 1475, 1433, 1353, 1298, 1253, 1199, 1177, 1137, 1070, 1008, 899, 834

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cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 8.07 (s, 1H), 7.57 (d, J = 2.4 Hz, 1H), 7.10 (s, 1H), 6.73 (d, J = 2.4 Hz), 3.95 (s, 3H), 3.91 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 166.8 (C), 158.0 (C), 157.8 (C), 145.2 (CH), 124.8 (CH), 120.0 (C), 116.6 (C), 106.7 (CH), 95.4 (CH), 56.4 (CH_3), 52.1 (CH_3); HRESIMS m/z calcd for $C_{11}H_{10}NaO_4$ $[M + Na]^+$: 229.0477, found: 229.0473.

4-Methoxybenzofuran-5-carboxylic acid (7a): A solution of **4a** (200.0 mg, 1.04 mmol) in acetone (11 mL) were added K_2CO_3 (747.0 mg, 5.41 mmol) and iodomethane (1.47 g, 10.4 mmol), and the resulting suspension was stirred at reflux for 3 h. After being cooled to room temperature, the resulting suspension was filtered. The filtrate was concentrated in vacuo, and diluted with ether (30 mL). The resulting suspension was again filtered, and the filtrate was concentrated in vacuo. The residue was used in the next step without further purification. To a solution of the residue in MeOH (4.0 mL) was added an aqueous solution of sodium hydroxide (NaOHaq) (3N, 3.0 mL). The mixture was stirred for 7 h at room temperature, poured into HClaq (2N, 15 mL), extracted with EtOAc (40 mL × 3). The combined organic layers were washed with brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by silica gel column chromatography eluted with chloroform/methanol (9:1) to afford **7a** (160mg, 0.833 mmol, 80% yield); FT-IR (KBr): 3413, 3138, 2993, 1693, 1617, 1550, 1472, 1427, 1417, 1366, 1339, 1285, 1261, 1239, 1176, 1076, 987, 924, 750 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 10.93 (s, 1H), 8.15 (d, J = 8.8 Hz, 1H), 7.69 (d, J = 2.4 Hz, 1H), 7.36 (d, J = 8.8 Hz, 1H), 7.02 (d, J = 2.4 Hz, 1H), 4.34 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 166.0 (C), 159.8 (C), 153.4 (C), 145.6 (CH), 129.4 (CH), 117.8 (C), 113.9 (C), 107.9 (CH) 105.2 (CH), 61.6 (CH_3); HRESIMS m/z calcd for $C_{10}H_8NaO_4$ $[M + Na]^+$: 215.0320, found: 215.0321.

6-Methoxybenzofuran-5-carboxylic acid (7b): To a solution of **6** (360.5 mg, 1.75 mmol) in MeOH (5.0 mL) was added NaOHaq (3N, 3.0 mL). The mixture was stirred for 5 h at room temperature, poured into HClaq (2N, 15 mL), and extracted with EtOAc (40 mL × 3). The combined organic layers were washed with brine (60 mL), dried over Na_2SO_4 and concentrated in vacuo. The residue was purified by silica gel column chromatography eluted with chloroform/methanol (9:1) to afford **7b** (300.0 mg, 1.56 mmol, 89% yield). FT-IR (KBr): 3413, 3235, 1690, 1638, 1618, 1581, 1540, 1475, 1427, 1406, 1356, 1270, 1209, 1188, 1146, 1066, 1003, 901, 828 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 10.85 (s, 1H, COOH), 8.49 (s, 1H), 7.64 (d, J = 2.4 Hz, 1H), 7.19 (s, 1H, 6.81 (d, J = 2.4 Hz, 1H), 4.13 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 165.7 (C), 158.4 (C), 156.3 (C), 146.2 (CH), 127.3 (CH), 122.0 (C), 113.9 (C), 107.0 (CH), 95.3 (CH), 57.1 (CH_3); HRESIMS m/z calcd for $C_{10}H_8NaO_4$ $[M + Na]^+$: 215.0320, found: 215.0312.

4-Methoxybenzofuran 5-carboxamide (synthetic **1a**): A solution of **4a** (200 mg, 1.04 mmol) in acetone (11 mL) were added K_2CO_3 (747.4 mg, 5.41 mmol) and iodomethane (1.48 g, 10.43 mol), and the resulting suspension was stirred at reflux for 3 h. After being cooled to room temperature, the resulting suspension was filtered. The filtrate was concentrated in vacuo, and diluted with ether (30 mL). The resulting suspension was again filtered, and the filtrate was concentrated in vacuo. The residue was used in the next step without further purification. To a solution of the residue in MeOH (5.0 mL) was added NH_3aq (40 mL, 28%), and the mixture was stirred at 35 ºC for 27 h. The resulting solution was concentrated in vacuo. To the residue was added HClaq (1N, 10 mL), extratected with EtOAc (60 mL × 3). The combined organic layers were washed with a saturated aqueous solution of sodium hydrogen carbonate (50 mL), brine (20 mL), dried over Na_2SO_4 and concentrated in vacuo. The residue was purified by silica gel chromatography eluted with chloroform/methanol (9:1) to afford **1a** (165.0 mg, 0.863 mmol, 83% yield). FT-IR (KBr): 3459, 3162, 1668, 1605, 1579, 1475, 1461, 1419, 1387, 1348, 1322, 1251, 1233, 1067, 975, 742 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 8.16 (d, J = 8.8 Hz, 1H), 7.77 (brs, 1H, NH), 7.63 (d, J = 2.2 Hz, 1H), 7.31 (d, J = 8.8 Hz, 1H), 6.98 (d, J = 2.2 Hz, 1H), 5.76 (brs, 1H, NH), 4.19 (s, 3H); ^{13}C (125 MHz, $CDCl_3$) δ 167.3 (C), 158.9 (C), 152.8 (C), 145.0 (CH), 128.4 (CH), 118.6 (C), 117.6 (C), 107.2 (CH), 105.3 (CH), 61.2 (CH_3); HRESIMS m/z calcd for $C_{10}H_9NNaO_3$ $[M + Na]^+$ 214.0480, found: 214.0485.

Synthesis of 4-Methoxybenzofuran 5-carboxamide (1a) on a large scale from 2: Two times of the large-scale examinations of the three steps (from **2** to **3**, from **3** to **4a**, and from **4a** to **1a**) confirmed that the overall yield from **2** to **1a** was reproducible (17% ± 5%, 0.59 × 0.34 × 0.83 = 0.17). The following results were the one of the two examinations. Compound **2** (56.7 g, 336.9 mmol) was converted to **3** (56.5 g, 198.8 mmol) in 59% yield. Compound **4a** (13.0 g, 37.6 mmol) was obtained from **3** (198.8 mmol) in 34% yield along with **4b**. Compound **1a** (10.72 g, 56.1 mmol) was afforded from **4a** (37.6 mmol) in 83% yield.

6-Methoxybenzofuran-5-carboxamide (1b): To a solution of **6** (502 mg, 2.435 mmol) in MeOH (7.0 mL) was added NH_3aq (28%, 30 mL). The mixture was stirred at 35ºC for 12 h, concentrated in vacuo, poured into HClaq (1N, 15 mL) at 0˚C, and extracted with EtOAc (100 mL × 3). The combined organic layers were washed with NaOHaq (1N, 100 mL), brine (150 mL), dried over Na_2SO_4 and concentrated in vacuo. The residue was purified by silica gel chromatography eluted with chloroform/MeOH (9:1) to afford **1b** (373.0 mg, 1.95 mmol, 80% yield). FR-IR (KBr): 3404, 3179, 1643, 1603, 1538, 1475, 1435, 1376, 1288, 1201, 1154, 1067, 1003, 905, 826 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 8.50 (s, 1H), 7.59 (d, J = 2.4 Hz , 1H), 7.77 (brs, 1H, NH), 5.79 (brs, 1H, NH), 7.12 (s, 1H), 6.78 (d, J = 2.4 Hz, 1H), 4.02 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 167.4 (C), 157.7 (C), 156.4 (C), 145.3 (CH), 125.7 (CH), 121.0 (C), 117.5 (C), 107.0 (CH), 94.8 (CH), 56.4 (CH_3); HRESIMS m/z calcd for $C_{10}H_9NNaO_3$ $[M + Na]^+$: 214.0480, found: 214.0512.

HeLa cell experiments:

HeLa cells were cultured at 37 °C under a humidified (5% CO_2, 95% air) atmosphere in MEM-α medium containing 8% FCS (Sigma-Aldrich) supplemented with 100 IU/ml penicillin (Sigma-Aldrich) and 50 μg/ml streptomycin (Sigma-Aldrich). HeLa cells were cultured to 70% confluence in 35-mm dishes and were serum starved for 24 h at 37 °C before treatment with 100 nM of phorbol-12-myristate-13-acetate (PMA) for 3 h. Cells were pretreated with TP extract, its different fractions, isolated compound (**1a**: 50, 100, 150, 200, and 250 μM) as well as synthesized compounds (**1a**: 50, 100, 150, 200, and 250 μM; **1b**: 25, 50, 75, 100, and 150 μM; **5a**: 50, 100, 200, and 300 μM; **5b**: 50, 100, 200, and 300 μM; **7a**: 50, 100, 200, and 300 μM; **7a**: 50, 100, 200, and 300 μM) for 1 h prior to PMA stimulation.

***Real-time quantitative RT-PCR*:**

After a 3-h treatment with PMA, the cells were harvested with 700 μl of RNAiso Plus (Takara Bio Inc.), mixed with 140 μL of chloroform, and centrifuged at 17,400 × *g* for 15 min at 4 °C. The aqueous phase was collected, and RNA was precipitated by the addition of isopropyl alcohol. After centrifugation at 17,400 × *g* for 15 min at 4 °C, the resulting RNA pellet was washed with ice-cold 75% ethanol. Total RNA pellet was resolved in 10 μl of diethylpyrocarbonate-treated water, and 1 μg of each RNA samples was used for the reverse transcription reaction. RNA samples were reverse-transcribed to cDNA using a PrimeScript

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RT reagent Kit. TaqMan primers and the probe were designed using Primer Express software (Applied Biosystems). Real-time PCR was conducted using a GeneAmp 7300 sequence detection system (Applied Biosystems). The sequences of the primers and TaqMan probe were as follows: forward primer for human H1R, 5′-CAGAGGATCAGATGTTAGGTGATAGC-3′; reverse primer for human H1R, 5′-AGCGGAGCCTCTTCCAAGTAA-3′; TaqMan probe, FAM-CTTCTCTCGAACGGACTCAGATACCACC-TAMRA. To standardize the starting material, the human GAPDH gene (Applied Biosystems) was used, and data were expressed as the ratio of H1R mRNA to GAPDH mRNA.

Acknowledgments

This work was financially supported in part by Grants-in-Aid for Scientific Research (B) from the Japan Society for the Promotion of Science (26305004 to H.F.), and by a grant from the Osaka Medical Research Foundation for Incurable Diseases.

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13. Two of the authors (H. F. and H. M.) have recently established the standard cell model examination procedure, where H1R gene is up-regulated in patients with allergic rhinitis and its expression level strongly correlates with the severity of symptoms.[5g] We also have reported that some of the alternative potential molecules suppressing H1R gene up-regulation alleviate allergic symptoms in allergy model rats.[5c,5e,5h,5i]
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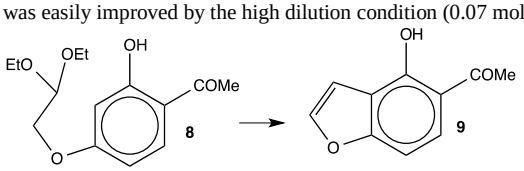
16. At the beginning, we chose 0.7 mol/L as concentration of the reaction of **3**, due to the value reported in the following paper,[17] where the reaction from ketone **8** to **9** was described. In the next related paper,[18] same authors reported the dimerization reaction of **9**. Those results described both in references 17 and 18 confused us because the condition (including the concentration value) from **8** to **9** was almost same as the condition for dimerization of **9**. Therefore, we assumed that the value of the concentration in reference 17 was wrong. Accordingly, the step from **3** to **4a**/**4b** was easily improved by the high dilution condition (0.07 mol/L).



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19. Synthesis of **7a** was reported via a different route. Foster, R. T.; Robertson, *J. Chem. Soc.* **1948**, 115–116.
20. A manuscript entitled "A novel benzofuran, 4-methoxybenzofuran-5-carboxamide, from Tephrosia purpurea suppressed histamine H1 receptor gene expression through a

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protein kinase C-δ-dependent signaling pathway" has been submitted. Shill, M. C.; Mizuguchi, H.; Karmakar, S.; Kadota, T.; Mukherjee, P. K.; Kitamura, Y.; Kashiwada, Y.; Nemoto, H.; Takeda, N.; Fukui. *Int. Immunopharmacol*.

Supplementary Material

Supplementary material for 2D NMR of isolated **1a,** for ^{1}H and ^{13}C NMR of compounds **1a** (isolated and synthesized), **1b**, **3**, **4a**, **4b**, **5a**, **5b**, **6**, **7a**, and **7b** are available.

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