Two New Prenylated 3-Benzoxepin Derivatives as Cyclooxygenase Inhibitors from *Perilla frutescens* var. *acuta*†

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Two novel prenyl 3-benzoxepin derivatives, perilloxin (**1**) and dehydroperilloxin (**2**), were isolated from the dichloromethane extract of the stems of *Perilla frutescens* var. *acuta*. Their structures were elucidated as (−)-(*R*)-5-methoxy-2,3-dihydrofuro[2,3-*g*][3]benzoxepin and 5-methoxyfuro[2,3-*g*][3]benzoxepin, respectively, based on UV, MS, ^{1}H and ^{13}C NMR, NOE, ^{1}H−^{13}C COSY, and HMBC spectral data. They were isolated following bioassay-guided fractionation, using an in vitro cyclooxygenase-1 test. Compounds **1** and **2** possess inhibitory activities, with IC_{50} values of 23.2 μM and 30.4 μM, respectively.

Perilla frutescens (L.) Britt. var. *acuta* (Thunb.) Kudo (Lamiaceae) has been used in traditional Chinese medicine for more than a thousand years. The leaves, stems, and fruits are applied for different diseases.[1] *Folium Perillae* is used to induce perspiration, to dispel cold, and to regulate stomach function; *Caulis Perillae*, to regulate the flow of *qi* and the function of the stomach, to alleviate pain, and to prevent abortion; and *Fructus Perillae*, mainly for relieving dyspnea and cough, for eliminating phlegm, and for relaxing the bowels. Some monoterpenes from the leaves have been reported to possess bioactivities, such as promotion of intestinal propulsion,[2] prolongation of hexobarbital-induced sleep in mice,[3,4] and inhibitory effects on xanthine oxidase[5] and aldose reductase.[6] Pan et al. analyzed the constituents of the essential oils from the leaves, stems, and fruits of this plant by GC−MS,[7] and Yuba et al. investigated the genetic variants of the essential oil in *P. frutescens* varieties.[8] Cyanidin, 3-(6-*p*-coumaryl)-β-D-glucoside, arginine, and cumic acid were isolated from the whole plant.[9] However, these reports do not sufficiently explain all the therapeutic functions of the above drug. Because cyclooxygenase (COX) is an important enzyme that catalyzes the formation of mediators involved in the inflammatory process, we tested the dichloromethane extracts of *Perilla*-derived preparations on COX-1 at a concentration of 50 μg/mL. Only *Caulis Perillae* showed significant inhibition on COX-1 (83.0%, indomethacin: 80.2% at a concentration of 5 μM). Using the COX-1 test as a guide for fractionation, two new prenyl 3-benzoxepin derivatives were isolated from the stems of this plant.

Guided by the in vitro assay testing for the inhibition of COX-1-catalyzed prostaglandin biosynthesis from arachidonic acid, a dichloromethane extract of the stems from *P. frutescens* var. *acuta* was fractionated by flash column, Sephadex LH-20, and medium-pressure liquid chromatography (MPLC). This procedure resulted in the isolation of two new prenylated 3-benzoxepin derivatives, perilloxin (**1**) and dehydroperilloxin (**2**), which exhibited inhibitory activities on COX-1 in vitro, with IC_{50} values of 23.2 μM and 30.4 μM, respectively (indomethacin: IC_{50} = 1.5 μM).

Compound **1** is orange-yellow and gives a molecular ion peak at *m*/*z* 274 in the mass spectrum (EIMS). The UV spectrum in EtOH exhibited absorption at 217 (log ϵ 4.2) (sh), 237 (log ϵ 4.3), and 355 (log ϵ 3.6) nm. The presence of a 2-(1-hydroxy-1-methylethyl)-2,3-dihydrobenzofuran moiety was revealed in ^{1}H NMR by a characteristic ABX system at δ 4.50 (1H, t, J = 8.8 Hz), 3.08 (1H, dd, J = 16.4 Hz, 8.8 Hz), and 3.01 (1H, dd, J = 16.4 Hz, 9.5 Hz) and two methyl singlets at δ 1.31, 1.18, which correlated to carbons at δ 89.0, 31.2 and 26.2, 24.2 in the ^{1}H−^{13}C COSY spectrum, and by a quaternary carbon at δ 71.7 in ^{13}C NMR. In addition, a methoxy group linked to the benzene ring was evident from the signal at δ 3.70 in ^{1}H NMR and δ 56.8 in ^{13}C NMR. The remaining 10 carbons in ^{13}C NMR were all unsaturated and, based on the DEPT spectrum, included five quaternary carbons and five methine groups. Four of the methine carbons correlated with the proton signals at δ 5.86, 5.46 (each 1H, d, J = 7.1 Hz) and 5.79, 5.66 (each 1H, d, J = 7.2 Hz), while the fifth correlated with a singlet signal at δ 6.47 in ^{1}H NMR.

HMBC was used to establish the connection between the fragments. The linkages C-6/C-5a and C-10/C-10a were established by the correlation of H-6 with C-5 and C-10a and of H-10 with C-5a and C-10b, which, together with correlations between positions 7 and 9, indicated the connection of the two double bonds through an oxygen. Thus, the structural skeleton of a dihydrofuro[3]benzoxepin with a methoxy group attached to the aromatic ring was proposed for this compound. Its color and UV and NMR spectral data were in agreement with this inference.[10−12] Moreover, the position of OMe was determined by the correlation of methoxy protons with C-5, and the direction, as well as position, of the dihydrofuran ring was fixed by correlation of the aromatic proton with C-3, C-5a, and C-10b. Its absolute configuration was determined as (−)-(*R*) based on the negative optical rotation, in comparison with the positive rotation of the related compound, (+)-(*S*)-fomannoxin and with (−)-(*R*)-anodenroic acid.[13,14] Therefore, the structure of **1** was determined as (−)-(*R*)-5-methoxy-2,3-dihydrofuro[2,3-*g*][3]benzoxepin, named perilloxin.

Further confirmation of the proposed structure was provided by NOEDS. Irradiation of the OMe gave an NOE effect on the signal of the aromatic proton H-4 at 6.47 (12.6%) and of the oxepin proton H-6 at δ 5.66 (1.3%). A sizable NOE was observed for the aromatic proton (4.4%)

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Table 1. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR Data of **1** and **2** (TMS, $CDCl_3$, δ ppm)

	1				**2**	
no.	^{1}H (J, Hz)	^{13}C	DEPT	HMBC	^{1}H (J, Hz)	^{13}C
2	4.50 (t, 8.8)	89.0	CH			163.9
3	3.08 (dd, 16.4, 8.8)	31.2	CH_2	C2, C3a, C10b,	6.43 (s)	100.8
	3.01 (dd, 16.4, 9.5)			$C(CH_3)_2OH$		
3a		127.2	C			120.6[a]
4	6.47 (s)	108.5	CH	C3, C5a, C10b	6.72 (s)	101.3
5		150.8	C			152.6[b]
5a		123.0	C			120.9[a]
6	5.66 (d,7.2)	108.2	CH	C5, C7, C10a	5.92 (d, 6.9)	110.3[c]
7	5.79 (d, 7.2)	145.3	CH	C5a, C6, C9	5.89 (d. 6.9)	145.0
9	5.86 (d,7.1)	146.8	CH	C7, C10, C10a	6.06 (d, 6.8)	148.3
10	5.46 (d, 7.1)	107.2	CH	C5a, C9, C10b	5.92 (d, 6.9)	107.5[c]
10a		118.2	C			127.7[a]
10b		150.7	C			146.6[b]
$-C(CH_3)_2OH$		71.7	C			69.4
$-C(CH_3)_2OH$	1.31 (s)	26.2	CH_3	C2, CH_3, CMe_2OH	1.64 (s)	28.7
	1.18 (s)	24.2	CH_3	C2, CH_3, CMe_2OH		
OCH_3	3.70 (s)	56.8	CH_3	C5	3.80 (s)	56.3

[a–c] Assignments may be interchanged.

by the irradiation of the methylene group in the dihydrofuran ring. Conversely, irradiation of the aromatic proton H-4 gave NOEs both with the OMe (3%) and the methylene group (1.2%).

Compound **2** showed the same color as **1** and a similar UV spectrum with absorptions at 208 (log ϵ 4.1) (sh), 223 (log ϵ 4.2) (sh), 247 (log ϵ 4.3), 336 (log ϵ 3.5) nm. The MS gave a molecular ion peak at *m*/*z* 272. Besides the four protons of an oxepin ring at δ 6.06, 5.92 (each 1H, d, J = 6.8 Hz) and 5.92, 5.89 (each 1H, d, J = 6.9 Hz) and an aromatic proton at δ 6.72, a singlet proton signal appeared at δ 6.43 instead of the ABX system observed in the ^{1}H NMR of compound **1**. This indicated that **2** is a dehydrogenated derivative of **1**. The downfield shifts of the benzene and oxepin protons of **2** relative to **1** are similar to those observed in the ^{1}H NMR spectra of 2-methylnaphtho[1,2-*b*]furan and its 2,3-dihydro derivative.[15,16]

NOEDS experiments on **2** supported the proposed structure. Irradiation of the aromatic proton H-4 caused NOEs for both the methoxyl group (3.3%) and the furan proton (2%), whereas irradiation of the furan proton enhanced the aromatic proton H-4 (1.9%) and, only to a slight extent (0.3%), the proton of the geminal methyl groups. Irradiation of the protons of the geminal methyls yielded 6.5% enhancement for the furan proton. A large NOE (16.9%) was observed for the aromatic proton H-4 on irradiation of the methoxyl group, which resulted also in a small enhancement (1.3%) of the oxepin proton H-6. Due to the roof effect of the latter proton signal it was possible to assign all oxepin protons.

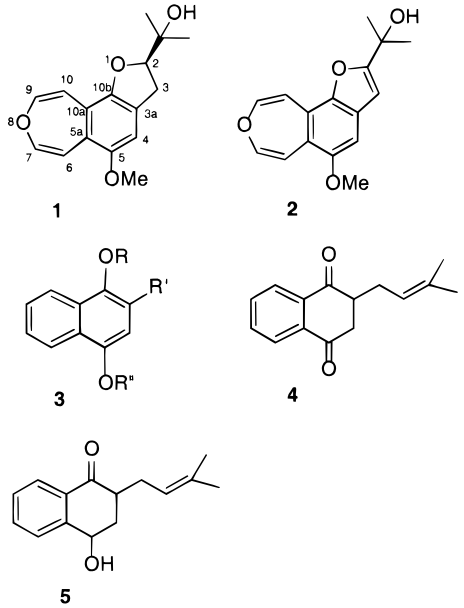
Finally, ^{13}C signals of the protonated carbon atoms could be assigned by the ^{1}H−^{13}C COSY spectrum of compound **2** (see Table 1), thus providing a confirmation of the structure of **2** as 5-methoxyfuro[2,3-*g*][3]benzoxepin, named dehydroperilloxin.

To our knowledge, 3-benzoxepin derivatives unsubstituted in the oxepin ring have not been reported as plant constituents. Recently, however, two plant products were claimed to possess a 3-benzoxepin ring system carrying three substituents in the seven-membered ring.[17]

In the case of our isolated compounds, the positions of the furan and methoxy oxygens may provide a clue to a common biosynthetic pathway with the well-studied prenylnaphthoquinones occurring in other families such as Tubiflorae.[18,19] Thus, enzymatic epoxidation[20,21] of a naphthalene precursor of type **3** followed by valence isomerization[22,23] could lead directly to the observed 3-benzoxepin ring system.

Enzymatic, as well as chemical, epoxidation of the 2,3-position of naphthalene has not been previously observed, in contrast to the well-known 1,2-epoxidation.[18,19] The chemical synthesis of the parent 3-benzoxepin was performed by Wittig reaction of phthalaldehyde.[11,24]

As an alternative mechanism of formation of **1** and **2**, the enzymatic epoxidation could also occur at an earlier stage of the presumed biosynthetic pathway, for instance by oxidation of compounds **4** or **5**.[18,19] In this case, however, the presumably unstable arene oxide would have to endure the steps of aromatization of the second ring until reaching the more stable[10,11] 3-benzoxepin ring system.



Experimental Section

General Experimental Procedures. ^{1}H (500 MHz), ^{13}C (125 MHz), DEPT, ^{1}H−^{13}C COSY, and HMBC spectra were recorded on a Bruker DRX 500 spectrometer with TMS as internal standard in $CDCl_3$. NOE difference spectra were measured on a Varian VXR-300 spectrometer (300 MHz). EIMS was carried out at 70 eV using a Finnigan 1020 GC−EIMS. UV spectra were obtained on-line by a diode array detector of a liquid chromatograph HP 1050 (Hewlett-Pack-

ard). Optical rotation was measured with a Perkin-Elmer polarimeter 241 C (in EtOH). Column chromatography was carried out on Si gel (230−400 mesh, Merck) and over Sephadex LH-20 (Pharmacia). MPLC columns were filled with RP_{18} Si gel (25−40 μm, Merck).

Plant Material. The material was supplied by Sinomed, Kötzting. It was imported from China and identified as the stems of *Perilla frutescens* (L.) Britt. var. *acuta* (Thunb.) Kudo on the basis of macroscope and microscope description. A voucher specimen is deposited in the Institute of Pharmaceutical Biology, University of Düsseldorf.

Extraction and Isolation. The stems (950 g) were powdered and extracted with dichloromethane in a Soxhlet apparatus for 8 h. The solvent was evaporated to give 5.8 g of extract. The extract was separated by flash chromatography (Si gel, 150 g) with a solvent gradient of *n*-hexanes−EtOAc (from 100:0 to 1:1), the elution volume of each gradient was 500 mL. Further isolation was guided by the COX-1 assay. Active fractions 3 (0.13 g) and 4 (0.12 g) were rechromatographed on Sephadex LH-20 (each with 30 g) with *c*-hexane−dichloromethane−MeOH (7:4:1), then purified on MPLC with H_2O−MeCN (6:4) to yield compounds **1** (7.4 mg) and **2** (1.2 mg).

(−)-(*R*)-5-Methoxy-2,3-dihydrofuro[2,3-*g*][3]benzoxepin (1): orange-yellow, viscous oil, $C_{16}H_{18}O_4$; $[\alpha]^{25}_D$ −278° (*c* 0.05, EtOH); UV (EtOH) λ_{max} (log ϵ) 355 (3.6), 237 (4.3), 217 (4.2) (sh) nm; ^{1}H NMR and ^{13}C NMR ($CDCl_3$), see Table 1; EIMS *m*/*z* 274 $[M]^+$, 259, 241, 201, 187, 173, 159, 145, 128, 115, 91, 77, 59, 43.

5-Methoxyfuro[2,3-*g*][3]benzoxepin (2): orange-yellow viscous oil, $C_{16}H_{16}O_4$; UV λ_{max} (log ϵ) 336 (3.5), 247 (4.3), 223 (4.2) (sh), 208 (4.1) (sh) nm; ^{1}H NMR and ^{13}C NMR ($CDCl_3$), see Table 1; EIMS *m*/*z* 272 $[M]^+$, 254, 254, 239, 226, 211, 183, 165, 152, 145, 139, 127, 115, 91, 77, 63, 59, 43.

Cyclooxygenase-1 Assay. The assay was performed in a microtiter scale with a preparation of microsomal cyclooxygenase from ram seminal vesicles as described in the literature.[25] The incubation mixture contained 1.0 μg of an enzyme preparation in 170 μL of 0.1 M Tris buffer (pH 8.0), 1 nM reduced glutathione, 1 mM epinephrine-hydrogentartrate, and 0.05 mM Na_2EDTA. Of each extract, dissolved in EtOH p.a. (1 mg/mL), 10 μL were added and preincubated for 5 min at room temperature. The reaction was started by adding 10 μL of 4.5 μM 1-[^{14}C]-arachidonic acid (0.05 μCi abs) and incubated for 25 min at 37 °C. Arachidonic acid and its radiolabeled metabolites were separated and determined by reversed-phase HPLC using a Berthold radioactivity monitor. Inhibition refers to reduction of PGE_2 formation, in comparison to a blank run without inhibitor. The results are means of three single experiments. Indomethacin was used as a positive control. IC_{50} values have been determined from three concentrations via graphical evaluation of the log regression.

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