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New prenylated benzoic acid derivatives of *Piper hispidum*

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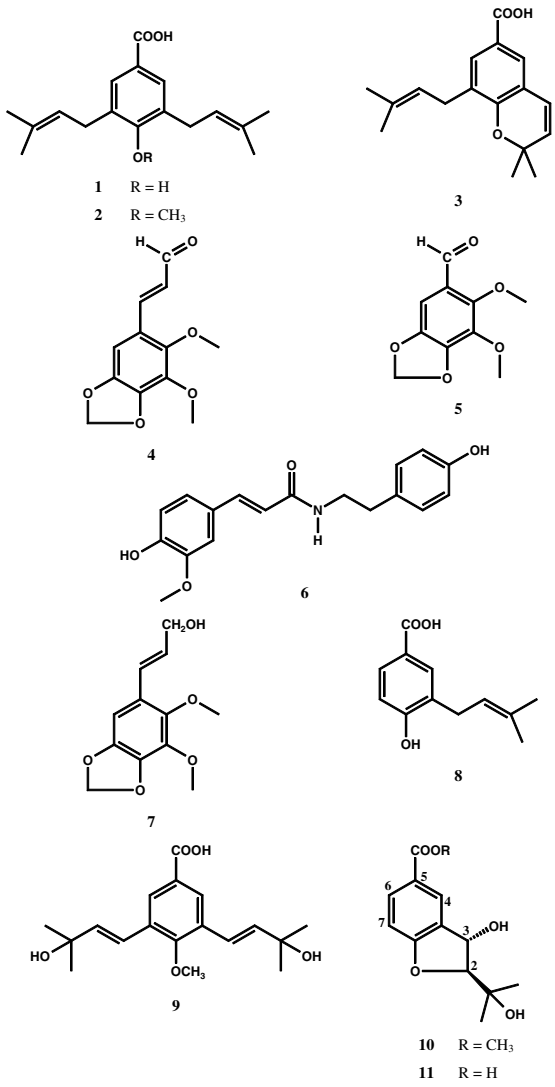
Three new 4-hydroxy-benzoic acid derivatives, 4-methoxy-3,5-bis-(3-hydroxy-3-methyl-1-butenyl)benzoate, 3-hydroxy-2-(1-hydroxy-1-methylethyl)-2,3-dihydrobenzofuran-5-carboxylic acid, and 3-hydroxy-2-(1-hydroxy-1-methylethyl)-2,3-dihydrobenzofuran-5-carboxylic acid methyl ester together with eight known compounds, have been isolated from the stems of *Piper hispidum*. Their structures were elucidated by a detailed spectroscopic analysis. In addition, the cytotoxicity of seven isolated compounds has been evaluated, revealing a moderate activity for three derivatives of dillapiole.

1. Introduction

Piper hispidum Sw., Piperaceae, is an aromatic scenting shrub, which is widely distributed in Central and South America. In Colombia, a leaf decoction is used to treat malaria (Morton 1981). Previous phytochemical investigations led to the isolation of pseudo-dillapiol, nervogenic acid methylether, flavanones and chalcones (Vieira et al. 1980; Burke and Nair 1986). More recently, the occurrence of antifungal amides was reported (Navickiene et al. 2000). A bioactivity-guided fractionation of *P. hispidum* leaves against *Plasmodium falciparum* yielded dillapiol as well as the chalcone derivatives asebogenin and 2′,4′,6′-trihydroxy-dihydrochalcone (Jenett-Siems et al. 1999).
During our ongoing investigations on medicinal plants from Latin America, we analysed the stems of *P. hispidum* and isolated three new benzoic acid derivatives (**9**–**11**) together with eight known compounds (**1**–**8**).

2. Investigations, results and discussion

A methanolic extract of the stems of *P. hispidum* was successively extracted with petrol ether, CH_2Cl_2, and EtOAc. Further separation of the petrol ether-soluble fraction by cc on RP-18 material and preparative TLC afforded nervogenic acid (**1**) and its derivatives nervogenic acid methylether (**2**) (Orjala et al. 1993a), and 2,2-dimethyl-8-(3-methyl-2-butenyl)-2*H*-chromene-6-carboxylic acid (**3**) (Orjala et al. 1993b), as well as the dillapiole derivatives dillapional (**4**) (Tomar and Mukerjee 1981) and dillapiole aldehyde (**5**) (Walia et al. 1985). The CH_2Cl_2-soluble fraction was again subjected to cc on RP-18 material and further purified by preparative TLC to give *N-trans*-feruloyltyramine (**6**) (Rahman et al. 1992), ϖ-hydroxyisodillapiole (**7**) (Falkiner et al. 1972), and 4-hydroxy-3-(3-methyl-2-butenyl)benzoate (**8**) (Abraham and Arfmann 1990) together with the new compounds **9** and **10**. Separation of the EtOAc-soluble fraction yielded **11**.



Compound **9** showed a molecular ion peak at m/z 320, corresponding to a molecular formula of $C_{18}H_{24}O_5$ (HRMS) which is two oxygen atoms more than nervogenic acid methylether (**2**). In addition to the aromatic signal at δ 8.07 and the methoxy group, the ^{1}H NMR spectrum displayed signals for two isolated *trans*-double bonds at δ 6.55 (2 H, d, J = 16.0 Hz) and 6.91 (2 H, d, J = 16.0 Hz). The four methyl groups of the prenyl moieties are shifted highfield compared to **2**, indicating that the double bond was shifted from the 2,3-position to the 1,2-position upon introduction of hydroxyl groups in the C-3 position. Thus, **9** had to be 4-methoxy-3,5-bis-(3-hydroxy-3-methyl-1-butenyl)benzoate.

Compound **10** displayed a molecular ion peak at m/z 252, corresponding to a molecular formula of $C_{13}H_{16}O_5$ (HRMS). A fragment at m/z 194 with a molecular formula of $C_{10}H_{10}O_4$ pointed to the loss of a hydroxy-isopropyl residue. The ^{1}H NMR spectrum exhibited characteristic signals for a 1,3,4-trisubstituted aromatic system and a methyl ester. Furthermore, two hydroxymethine protons at δ 4.37 and 5.46, respectively, and two methyl groups were observed. These findings hinted to a dihydrobenzofuran derivative similar to anodendroic acid methyl ester which was already isolated from *P. aduncum* (Orjala et al. 1994) but with an additional hydroxy group in position 3. The relative stereochemistry of the positions 2 and 3 could be deduced from the coupling constants, which should be 7.0 Hz in case of a *cis* configuration and 4.0–5.0 Hz in *trans*-derivatives (Kawasaki et al. 1984). As a coupling constant of $J_{2,3} = 4.0$ Hz was observed in **10**, the dihydrofuran ring had to be 2,3-*trans*-configurated.

Compound **11** showed a $[M\text{-}H]^-$ peak at m/z 237 in the (−)-FABMS, corresponding to a molecular formula of $C_{12}H_{14}O_5$. Characteristic features in the ^{1}H NMR spectrum were again the 1,3,4-trisubstituted aromatic system displaying nearly the same chemical shifts as in **10** and a singlet for two methyl groups. Thus, **11** had to be another prenylated derivative of 4-hydroxy-benzoic acid. The remaining two signals at δ 4.29 (1 H, d, J = 4.5 Hz) and 5.32 (1 H, d, J = 4.5 Hz) were also quite similar to the hydroxymethine protons in **10** but no methyl ester signal was observed. From this data, **11** was identified as 3-hydroxy-2-(1-hydroxy-1-methylethyl)-2,3-dihydrobenzofuran-5-carboxylic acid.

In addition, the cytotoxicity of the known dillapiol derivatives **4**, **5** and **7**, as well as the benzoic acid derivatives **1**–**3**, and **8** was determined using the human bladder carcinoma cell line ECV-304. In this assay, the dillapiol derivatives turned out to be moderately toxic (IC_{50} values: **4** = 31.7 μg/ml, **5** = 31.1 μg/ml, **7** = 31.8 μg/ml), whereas the benzoic acid derivatives were not cytotoxic (IC_{50} values > 50 μg/ml).

3. Experimental

3.1. Equipment

^{1}H NMR spectra were run on a Bruker AVANCE DPX 400 (400 MHz, TMS as internal standard). EIMS were recorded on a Varian MAT CH7A (70 eV), HRMS on a Varian MAT 711 (80 eV), and FABMS on a Varian MAT CH_5DF. Preparative cc was performed on LiChroprep® RP-18 material (40–63 μm). Preparative HPLC separation was performed on a Knauer pumping system with a Knauer variable wavelength detector (225 nm) equipped with a Knauer Nucleosil 300 C-18 column (10 μm, 22 × 250 mm). Preparative TCL was performed on silica gel 60 plates (F_{254}, 20 × 20 cm, Merck, Germany).

3.2. Plant material

Aerial parts of *Piper hispidum* were collected at the road from El Llano to Carti, Panama. The species was identified by Prof. M. A. Correa D., Herbarium of the Universidad de Panama, Panama-City, Panama, where voucher specimens (Florpan 2761, PMA) are deposited.

3.3. Extraction and isolation

Ground, dried stems of *P. hispidum* (350 g) were extracted with MeOH. After solvent evaporation, the residue was resuspended in H_2O and successively extracted with petrol ether, CH_2Cl_2, and EtOAc. Further separation of the petrol ether-soluble fraction (2.4 g) by cc on RP-18 material with H_2O/MeOH mixtures (60 : 40; 50 : 50; 40 : 60, 30 : 70, 20 : 80) and MeOH yielded 11 fractions. Fraction 7 which was eluted with H_2O/MeOH 40 : 60 was purified by preparative TLC with $CHCl_3$/EtOAc/HCOOH 90 : 10 : 1 to yield **1** (R_f = 0.43, 2 mg) and **5** (R_f = 0.57, 1 mg). Fraction 8, which eluted with H_2O/MeOH 30 : 70 contained pure **4** (6 mg). Fraction 9, which was obtained with the same solvent mixture, was further purified by preparative HPLC (H_2O/MeOH 40 : 60 → 10 : 90 in 40 min) and yielded 5 mg of **3** (R_t = 26 min). Fraction 10, which eluted with H_2O/MeOH 20 : 80 was further separated by prep. HPLC (H_2O/MeOH 40 : 60 → 100% MeOH in 60 min) to give 3 mg of **2** (R_t = 34 min). The CH_2Cl_2-soluble fraction (2.0 g) was fractionated by column chromatography on RP-18 material with H_2O/MeOH mixtures (60 : 40; 50 : 50; 40 : 60, 30 : 70, 20 : 80) and MeOH to yield 11 fractions. Fraction 4, which was eluted with H_2O/MeOH 50 : 50 was further purified by prep. TLC ($CHCl_3$/MeOH 90 : 10) to afford **9** (R_f = 0.12, 3 mg) and **10** (R_f = 0.35, 1 mg). Fraction 5, which was obtained with the same solvent mixture, was purified by prep. TLC using $CHCl_3$/MeOH 80 : 20 to yield **6** (R_f = 0.57, 2 mg). Fraction 6, which eluted with H_2O/MeOH 40 : 60 afforded **7** (R_f = 0.62, 3 mg) and **8** (R_f = 0.49, 4 mg) upon preparative TLC with $CHCl_3$/MeOH 80 : 20 as solvent system. The EtOAc-soluble fraction (1.8 g) was again fractionated by cc on RP-18 material with H_2O/MeOH mixtures (80 : 20; 70 : 30; 60 : 40, 40 : 60) and MeOH to yield 10 fractions. Fraction 4, which was eluted with H_2O/MeOH 70 : 30 was further purified by prep. TLC (EtOAc/HCOOH/H_2O 82 : 9 : 9) to afford **11** (R_f = 0.73, 1 mg).

3.4. 4-Methoxy-3,5-bis-(3-hydroxy-3-methyl-1-butenyl)benzoate (9)

Colourless oil. EIMS (80 eV, m/z, %): 320 $[M]^+$ (9), 302 (6), 287 (17), 269 (14), 232 (26), 217 (25); HREIMS (m/z): 320.16214 (calc. for $C_{18}H_{24}O_5$, 320.16238); ^{1}H NMR (400 MHz, CD_3COCD_3): δ 1.39 (12 H, s, H-4′/H-4″/H-5′/H-5″), 3.74 (3 H, s, OCH_3), 6.55 (2 H, d, J = 16.0 Hz, H-1′/H-1″), 6.91 (2 H, d, J = 16.0 Hz, H-2′/H-2″), 8.07 (2 H, s, H-2/H-6).

3.5. 3-Hydroxy-2-(1-hydroxy-1-methylethyl)-2,3-dihydrobenzofuran-5-carboxylic acid methyl ester (10)

Colourless oil. EIMS (80 eV, m/z, %): 252 $[M]^+$ (27), 219 (69), 194 (4), 177 (100), 59 (72); HREIMS (m/z): 252.09955 (calc. for $C_{13}H_{16}O_5$, 252.09978); 194.05780 (calc. for $C_{10}H_{10}O_4$, 194.05791); ^{1}H NMR (400 MHz, CD_3OD): δ 1.26 (3 H, s, H-2′/H-3′), 1.28 (3 H, s, H-2′/H-3′), 3.84 (3 H, s, $COOCH_3$), 4.37 (1 H, d, J = 4.0 Hz, H-3), 5.46 (1 H, d, J = 4.0 Hz, H-2), 6.85 (1 H, d, J = 8.5 Hz, H-7), 7.91 (1 H, dd, J = 1.5; 8.5 Hz, H-6), 8.01 (1 H, d, J = 1.5 Hz, H-4).

3.6. 3-Hydroxy-2-(1-hydroxy-1-methylethyl)-2,3-dihydrobenzofuran-5-carboxylic acid (11)

Colourless oil. (−)-FABMS (m/z): 237 $[M\text{-}H]^-$; ^{1}H NMR (400 MHz, CD_3OD): δ 1.25 (6 H, s, H-2′/H-3′), 4.29 (1 H, d, J = 4.5 Hz, H-3), 5.32 (1 H, d, J = 4.5 Hz, H-2), 6.81 (1 H, d, J = 8.0 Hz, H-7), 7.91 (1 H, dd, J = 1.0; 8.0 Hz, H-6), 8.01 (1 H, d, J = 1.0 Hz, H-4).

3.7. In vitro cytotoxicity assay

The cytotoxicity of the substances was estimated by a proliferation assay using the MTT-assay (Mosmann 1983). Test substances were dissolved in DMSO and diluted with medium to the desired concentrations. Human bladder carcinoma cells (ECV-304) were cultivated in Eagle Medium 199 supplemented with 10% foetal calf serum in 96-well plates in an atmosphere of 5% CO_2 at 37 °C in a humidified environment. Endothelial cells were seeded at a density of approximately 1000 cells per well. After 24 h they were supplemented with 100 μl test substance in medium and cultivated for further 4 days. The cell viability was measured by the MTT-assay using DMSO to dissolve the formed purple formazan. The absorbance was quantified at 580 nm with an ELISA plate reader.

Data are presented as the mean of 8 parallel samples for each concentration. The IC_{50} values were calculated by linear regression.

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