Chemical Constituents of Habenaria petalodes Lindl. (Orchidaceae)

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Uma nova substância, denominada habenariosídeo \[
[(2R)-2-(2,3,4,6-tetra-O-acetil-β-D-glicopiranosil)oxi]-2-(2-metilpropil)-1,4-dioxo-1,4-butanodiiil]\bis(oximetileno-4,1-fenileno) bis-β-D-glicopiranosídeo, foi isolada das frações polares do extrato etanólico de Habenaria petalodes Lindl. (Orchidaceae), juntamente com duas substâncias conhecidas e estruturalmente relacionadas, a loroglossina e a militarina. Os flavonóides: isoquercitrina, isorhamnetin 3-O-β-D-glicopiranosídeo, e isorhamnetina 3,7-di-O-β-D-glicopiranosídeo, também foram isolados. As estruturas do habenariosídeo e das demais substâncias foram determinadas pela análise dos dados de EM, IV, UV e RMN mono e bidimensional e comparação com os dados publicados na literatura.

Habenarioside, a new natural product identified as \[(2R)-2-(2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl)oxy]-2-(2-methylpropyl)-1,4-dioxo-1,4-butanediyl\bis(oxymethylene-4,1-phenylene) bis-β-D-glucopyranoside, along with two known related metabolites, loroglossin and militarin, were isolated from the ethanol extract of the whole plant Habenaria petalodes Lindl. (Orchidaceae). The flavonoids isoquercitrin, isorhamnetin 3-O-β-D-glucopiranoside and isorhamnetin 3,7-di-O-β-D-glucopiranoside were also isolated. The structures of all compounds were established by analysis of their MS, IR, UV, 1D and 2D NMR spectra, and comparison with published data.

Keywords: orchid, loroglossin, militarin, habenarioside, flavonoids

Introduction

The Brazilian flora is exceedingly rich and comprises more than 56,000 endemic plant species. Among the 22,000 members of the family Orchidaceae that have been described worldwide, approximately 1,800 grow endemically in Brazil.1 In spite of its size and diversity when compared with other plant families, a relatively small number of orchid species have been investigated regarding their chemical composition, with about 300 secondary metabolites reported so far. These included, among others, flavonoids, terpenoids, quinones, phenanthrenes, stilbenes, lignans, and glucosylated benzyl esters of succinic, malic, tartaric and citric acids.2-6 The phytochemical investigations of Brazilian Orchidaceae are also restricted to few published works: a report concerning the presence of methylated C-glycosylflavones as taxonomic markers in fifteen orchids,7 and the investigation of the floral composition of other twenty-one species.8

The genus Habenaria (Orchidaceae) comprises over 600 species of which around 170 occur in Brazil.9 However, to the best of our knowledge only Habenaria repens Nutt. was chemically investigated so far, resulting in the identification of habenariol, a compound with potent feeding deterrent activity.10-12 In the present paper we report the first phytochemical investigation of Habenaria petalodes Lindl., describing the isolation and structure elucidation of six compounds (1-6), including a new 2-malic acid derivative (4) denominated habenarioside.

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Results and Discussion

The whole plant was extracted by maceration with ethanol and the extract was subjected to liquid-liquid partition between CH₂Cl₂ and MeOH:H₂O (1:1) to afford an organic and a hydroalcoholic fraction. The latter was subjected to solid phase extraction (SFE) using RP-18 cartridges followed by semi-preparative RP-HPLC to yield six compounds (Figure 1-2).

The ESI-MS of compounds 1 and 5 exhibited quasi-molecular ion peaks ([M + Na]⁺) at m/z 765 and m/z 749, respectively. Their molecular weight, together with their UV, ¹H and ¹³C NMR data were in accordance with those reported for loroglossin (1) and militarin (5).²,³,¹⁴

Habenarioside (4) was isolated as a resinous material with negative specific rotation ([α]D₂⁵ −30.0°, c 0.16 in MeOH). The positive HRESIMS spectrum showed a quasi-molecular ion peak [M + Na]⁺ at m/z 1079.3578, consistent with the molecular formula C₄₈H₆₄O₂₆ (calc. 1079.3584). The ESI-MS in positive ion mode showed neutral losses of 60 Da, attributed to the presence of acetate groups. Its IR spectrum exhibited absorption bands at 1755 cm⁻¹ (C=O), 1612 and 1512 (C=C), 1234 (C-O) due to ester carbonyl functions and aromatic rings, and strong absorptions at 3433 (OH) and 1072 (C-O) indicating the presence sugar moieties. Acid hydrolysis of 4 followed by reduction and acetylation, and GC-MS analysis of the resulting alditol acetate, confirmed the presence of D-glucose by comparison with an authentic sample. In the UV spectrum of 4, absorptions were similar to those of compounds 1 and 5 (Figure 1), with maxima observed at 224 nm (log ε 3.76) and 271 nm (3.28). NMR spectra (Table 1) showed signals attributed to four acetyl groups at 1755 cm⁻¹ (C=O), 1612 and 1512 (C=C), 1234 (C-O) due to ester carbonyl functions and aromatic rings, and strong absorptions at 3433 (OH) and 1072 (C-O) indicating the presence sugar moieties. Acid hydrolysis of 4 followed by reduction and acetylation, and GC-MS analysis of the resulting alditol acetate, confirmed the presence of D-glucose by comparison with an authentic sample. In the UV spectrum of 4, absorptions were similar to those of compounds 1 and 5 (Figure 1), with maxima observed at 224 nm (log ε 3.76) and 271 nm (3.28).
later form a complete hexose spin system, deducible from the 1H-1H COSY NMR experiment, in which correlations between the following protons pairs could be observed: H-1””” and H-2””” (δ_H 5.21/4.85), H-2””” and H-3””” (δ_H 4.85/5.14), H-3””” and H-4””” (δ_H 5.14/4.95), H-4””” and H-5””” (δ_H 4.95/3.23), H-5””” and H-6_B””” (δ_H 3.23/4.00), and H-5””” and H-6_B””” (δ_H 3.23/3.78). This sugar unit is completely acetylated as can be inferred on the basis of the following HMBC correlations (δ_C/δ_H) between the acete carbonyls and the above mentioned sugar protons: 171.6/4.85 (C=O/H-2”””); 171.8/5.14 (C=O/H-3”””); 171.6/4.95 (C=O/H-4”””); 172.5/3.78 (C=O/H-6_B”””); and, 172.5 /4.00 (C=O/H-6_B”””). Carbons at C-5” and C-5”” (δ_C 159.5) showed long range correlations with the anomeric protons at δ_H 4.93 (H-1”) and δ_H 4.89 (H-1””), indicating the position of the other two sugar units. On the basis of these evidences, compound 4 was identified as [2-[(2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl)oxy]-2-(2-methylpropyl)-1,4-dioxo-1,4-butanediy]bis (oxymethylene-4,1-phenylene) bis-β-D-glucopyranoside, which was given the trivial name habenarioside as it is a mono-glucosylated derivative of habenariol. This compound can also be regarded as a tetra-acetate derivative of dactylorhin A, a natural product previously isolated from Dactylorhiza hatagirea D. Don. and from Gymnadenia conopsea. From the latter species it was also described the presence of acetic and cinnamic esters of dactylorhin A, all at the glucose connected to the C-2 position of the malic acid. These findings indicate that further esters of dactylorhin A may be produced by Orchidaceae species.

Gitterman and co-workers demonstrated that in Cephalotaxus (Cephalotaxaceae) 2-isobutyl-malic acid is biosynthesized from L-leucine, and have R configuration at C-2. Assuming that this is also the case in Orchidaceae, and based on structures of compounds already isolated from this family, a biosynthetic route is suggested for habernarioside (Scheme S1 - Supplementary Material). Accordingly, 2R-isobutyl-malic acid could be subjected to a sequence of esterification and glucosylation reactions to afford habenariol, militarin, dactylorhin A, and gymnoside III, as intermediates in the biosynthesis of habernarioside. This hypothetical sequence assumes that all intermediates have 2R configuration in order to avoid the unlikely inversion/reversion of the C-2 configuration. However, habenariol was reported to have [α]_D ^25 + 12° and 2S configuration. On the other hand, catalytic hydrogenation of militarin, and comparison of the resulting 2-isobutyl malic acid with a synthetic standard, established its absolute stereochemistry as 2R. This data rises doubt about the published configuration for habenariol. Even though, it is possible to devise a pathway bypassing this compound, and use only 2R intermediates. In such pathway, militarin would be produced via esterification of 2R-isobutyl-malic with gastrodin (Scheme S1) keeping the R configuration at C-2. Based on the above discussion we propose that habernarioside have 2R configuration.

Malic acid derivatives 1 and 5 were previously isolated from Orchis militaris L., Dactylorhiza hatagirea D. Don. and Coeloglossum viride (L.) Hartm. var. bracteatum (Willd.). whilst 5, together with Gymnoside III (Scheme S1), were recently found in tubers of Gymnadenia conopsea R. Br. Zhang et al. reported that a fraction from the rhizomes of Coeloglossum viride rich in 1 and 5 significantly improved the memory of mice treated with scopolamine, cycloheximide or alcohol. These authors also demonstrated that this fraction protected neurons against injury by β-amyloid or H_2O_2, and suggested that these components could be of value in the treatment of Alzheimer’s disease and other dementia.

Compounds 2, 3 and 6 were isolated as yellow powders, and their UV spectra in MeOH exhibited absorptions consistent with those of flavonoids. The ESI-MS in the negative ion mode of compound 2 showed a [M - H]^- quasi-molecular ion peak at m/z 463, while the ESI-MS in the positive ion mode of compounds 3 and 6 showed [M + Na]^+ quasi-molecular ion peaks at m/z 501 and 663, respectively. Their 1H NMR spectra showed signals characteristic of glycosylated flavonoids, and comparison with published data allowed 2 to be identified as isoquercitrin, 3 as isorhamnetin 3-O-β-D-glucopyranoside, and 6 as isorhamnetin 3,7-di-O-β-D-glucopyranoside. Although isoquercitrin and isorhamnetin 3-O-β-D-glucoside have been previously isolated from some Orchidaceae species, this is the first report on the occurrence of isorhamnetin 3,7-di-O-β-D-glucopyranoside in this family.

The present study contributes to the phytochemical characterization of Orchidaceae, disclosing the presence of a new compound and the occurrence of two known compounds reported for the first time in this family.

Experimental

General experimental procedures

TLC analysis were conducted on pre-coated silica gel G-60/F_254 (0.25 mm, MERCK) eluted with mixtures of CHCl_3:MeOH:H_2O (65:50:1) and EtOAc:HOAc:HCOOH (100:11:1:26). Spots were visualized after spraying the plates with vanillin-H_2SO_4 or NP/PEG (Natural products-polyethylene glycol) reagents. Optical rotations were determined on a Perkin-Elmer 341 polarimeter. UV spectra were recorded in methanol on
a Beckman DU® Series 600 spectrophotometer, whilst infrared spectra were measured in KBr pellets on a Shimadzu FTIR-8400 instrument. 1H NMR (400 MHz), 13C NMR (100 MHz), DEPT, HMQC and HMBC experiments were carried out using a Bruker DRX 400 spectrometer. ESI-MS were recorded on a Thermo Finnigan LCQ-Advantage spectrometer, whilst high resolution mass measurements were obtained using a Micromass Q-TOF Micro™ instrument equipped with an ESI source operated in the positive ion mode. Extract, fractions and compounds were analyzed by HPLC using a Shim-pack® C18 column (5 µm, 250 mm × 4.6 mm i.d.) in a Shimadzu
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**Plant material**

Specimens of *Habenaria petalodes* Lindl. were collected in Belo Horizonte-MG, Brazil, in April 2004. The plant material was identified by Prof. João Renato Stehmann of the Universidade Federal de Minas Gerais, Belo Horizonte-MG, Brazil. A voucher specimen was deposited in the Herbarium BHCB (Instituto de Ciências Biológicas, Belo Horizonte-MG, Brazil) under the code BHCB-88339.

**Extraction and isolation**

Whole fresh plants (185 g) were chopped into small pieces and macerated in ethanol for 3 weeks at room temperature. After filtration and solvent evaporation, 8 g of the extract were obtained. An aliquot (6.2 g) was suspended in 100 mL MeOH:H₂O (1:1), and extracted with CH₂Cl₂ (3 × 100 mL) to afford an organic fraction (610 mg) and a hydroalcoholic fraction (4.7 g). The latter fraction (23.9 mg) was purified by semi-preparative HPLC in a RP-18 column eluted with MeCN:H₂O at a flow rate of 10 mL min⁻¹ and detection at 230 nm and 254 nm.

**Characterization of the sugar moieties**

Compound 4 (2 mg) was hydrolyzed in 0.5 TFA at 100 °C for 4 h. To the reaction mixture was added methanol (0.5 mL) and the solution evaporated to dryness under a stream of nitrogen. This operation was repeated to completely remove the TFA. To the resulting solid were added 0.3 mL of a solution of 1 mg/ml aqueous NaBH₄ and the reaction kept at 25 °C. After 30 min the reaction was stopped by addition of 0.5 mL 10% AcOH in MeOH, and the reaction mixture evaporated to dryness. The later steps, that is, addition of acidified methanol and evaporation, were repeated twice. The residue was acetylated by adding 0.1 mL of Ac₂O and heating at 100 °C for 30 min. Toluene (0.5 mL) was added to the reaction mixture and the solution evaporated under a stream of nitrogen at 90 °C. This operation was repeated three to four times to completely remove the acetic anhydrate. The residue was then partitioned (3×) between H₂O-EtOAc (1:1) and the organic phase was pooled, concentrated and dissolved in 0.2 mL of EtOAc. Control experiments with sugar standards were run in parallel. The alditol acetate solutions thus obtained were analyzed in a Shimadzu GC-17A gas chromatograph in line with a QP5050A mass spectrometer. One microliter of each solution was injected in a capillary column DB-5 (30 m × 0.25 mm i.d.), using helium as carrier gas (1.5 mL min⁻¹) and the following temperature programming: 100 to 200 °C in 20 min, 200-300 °C in 5 min and keeping at 300 °C for 5 min. The injector and detector were kept at 230 °C and 250 °C, respectively. Under these conditions the retention times of the alditol acetates obtained from the standard sugars were: ribose (17.77 min), arabinose (17.95 min), xylose (18.34 min), mannose (21.78 min), glucose (21.88 min), and galactose (21.96 min).

**Loroglossin (1)**

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[\alpha]_{D}^{25} = 28.0^\circ (\text{MeOH}, c 0.23) \quad \{\text{Lit.} 14 [\alpha]_{D}^{25} = 34.0 (\text{MeOH}, c = 0.36)\} \quad \text{UV} \lambda_{\text{max}}^{\text{MeOH/} \mu \text{m}} (\log e): 201 (4.22), 221 (4.18), 268 (3.42). \quad \text{H NMR (CD}_3\text{OD, 400 MHz):} \quad \delta^1_H 0.80 (3H, d, J 6.5 Hz, H-7), 0.94 (3H, d, J 6.5 Hz, H-8), 1.68 (1H, m, H-6), 1.91 (2H, m, H-5), 4.36 (1H, s, H-3), 4.97 (d, J 7.4 Hz...
Hz, J₁' = J₂' = 11.8 Hz, H-1' and H-1''), 5.09, 4.98 (2H, d, J₂' = J₃' = 12.0 Hz, H-1''' and H-1''''), 7.30 (2H, d, J 8.6 Hz, H-3',7'), 7.18 (2H, d, J 8.8 Hz, H-3'',7''), 7.12 (2H, d, J 8.6 Hz, H-4',6''), 7.07 (2H, d, J 8.8 Hz, H-4'',6''), 3.45 (m, H-2''/H-3', H-3'''/H-5', H-5''), 3.40 (m, H-4', H-4''), 3.71 (m, H-6', H-6'''), 3.89 (m, H-6'', H-6'''); 13C NMR (CD₃OD, 100 MHz): δC 22.5 (C-7), 23.3 (C-8), 23.7 (C-6), 44.8 (C-3), 47.7 (C-5), 56.1 (C-6', C-6'''), 65.8 (C-1''), 66.6 (C-1'), 70.0 (C-4', C-4''), 73.5 (C-2', C-2'''), 75.3 (C-2), 76.6/76.7 (C-3''/C-5', C-5'''), 100.9 (C-1'', C-1''''), 116.4 (C-4', C-4'''), 129.4 (C-2', C-2''), 129.6/129.8 (C-3', C-3''), 157.8 (C-5', C-5''), 170.2 (C-4), 174.6 (C-1). ESI-MS (positive mode) [M + Na]+ (m/z 765).

Habenarioside (4)

Resinous solid; [α]D25 = -30.0 (c 0.16, MeOH); UV (MeOH) λmax/nm (log ε): 203 (4.19), 219 (4.10), 269 (3.47), IR (KBr) νmax/cm⁻¹: 3433 (OH), 2959 (aliphatic), 2874 (aliphatic), 1612 (aromatic), 1512 (aromatic), 1736 (carbonyl), 1458 (aromatic), 1234 (C-O), 1076 (C-O), 1045 (C-O); 1H NMR (CD₃OD, 400 MHz): δH 1.61 (2H, m, H-5), 1.72 (1H, m, H-6), 2.65, 2.95 (1H, d, J₁' = J₂' = 15.6 Hz, H-3 and H-3'), 5.01 (4H, s, H-1' and H-1''), 7.30-7.25 (4H, m, H-3'',7'''), 7.11-7.07 (4H, m, H-4'',6'', H-4'',6'''), 4.91 (d, J 7.4 Hz, H-1'', H-1''''), 3.45 (m, H-2'', H-2'''/H-3', H-3''''/H-5', H-5'''), 3.39 (m, H-4'', H-4'''), 3.70 (m, H-6', H-6'''), 3.87 (m, H-6'', H-6'''''); 13C NMR (CD₃OD, 100 MHz): δC 22.5 (C-7), 23.3 (C-8), 23.7 (C-6), 44.8 (C-3), 47.7 (C-5), 61.1 (C-6', C-6'''), 65.8 (C-1'''), 66.6 (C-1'), 70.0 (C-4', C-4''), 73.5 (C-2', C-2'''), 75.3 (C-2), 76.6/76.7 (C-3''/C-5', C-5'''), 100.9 (C-1'', C-1''''), 116.4 (C-4', C-4'''), 129.4 (C-2', C-2''), 129.6/129.8 (C-3', C-3''), 157.8 (C-5', C-5''), 170.2 (C-4), 174.6 (C-1).

Supplementary Information

Supplementary data including IR, LC-DAD, 1H and 13C NMR and HRMS spectra are available free of charge at http://jbcs.sbq.org.br as PDF file.

Acknowledgments

The authors wish to thank CAPES for a fellowship (B. B. Cota) and FIOCRUZ for financial support.

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Received: December 17, 2007
Web Release Date: July 11, 2008