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Habenariol, a freshwater feeding deterrent from the aquatic orchid *Habenaria repens* (Orchidaceae)

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Abstract

An uncommon ester, bis-*p*-hydroxybenzyl-2-isobutylmalate, habenariol, has been isolated, by bioassay-guided fractionation, from the organic extract of the freshwater orchid *Habenaria repens*. The structure of habenariol was determined by the interpretation of spectral data. Feeding bioassays showed that habenariol deters feeding by the common freshwater crayfish *Procambarus clarkii*. Habenariol is related to a bis-ester glycoside isolated from the non-aquatic orchid *Galeola faberi*.

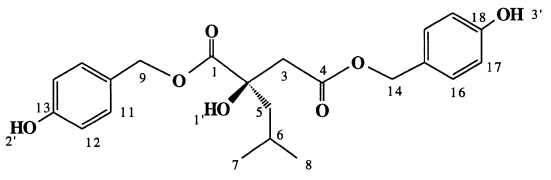
Keywords: Habenaria repens; Orchidaceae; Habenariol; Feeding deterrents

1. Introduction

Traditional studies in freshwater ecology have contended that freshwater herbivores consume fouling diatoms and other small organisms from the surfaces of aquatic macrophytes and avoid grazing directly on the macrophytes (Shelford, 1918; Hutchinson, 1975; Wetzel, 1983; Lamberti & Moore, 1984). The overall consequence of herbivory has thus been predicted to have little direct impact on populations or communities of aquatic macrophytes, resulting in little need for the evolution of chemical defenses against herbivores. Because of this widespread belief, the occurrence and ecological role of secondary metabolites produced by freshwater plants have rarely been investigated (Ostrovsky & Zettler, 1986). Only one example is currently known of a specific metabolite from a freshwater macrophyte acting as a feeding deterrent against herbivores (Newman, Kerfoot, & Hanscom III, 1996). However, a recent ecological investigation suggested that chemical defenses against herbivory could be common among freshwater macrophytes and demonstrated that the aquatic orchid *Habenaria repens* (Orchidaceae) was a low preference food for an omnivorous crayfish, and that the orchid's low susceptibility to the crayfish was chemically mediated (Bolser, Hay, Lindquist, Fenical, & Wilson, submitted). Bioassay-guided fractionation of the CH_2Cl_2 extract of this orchid yielded habenariol (**1**) as the only lipophilic metabolite to significantly reduce crayfish feeding. In this paper, we report the elucidation of the structure of this interesting new bioactive aquatic plant component.

2. Results and discussion

Habenariol (**1**), was isolated from the dichloromethane extract of *Habenaria repens* by bioassay-guided, gradient elution flash chromatography on silica gel using 60–80% ether/hexane mixtures. Fractions demonstrating feeding deterrence against the common crayfish *Procambarus clarkii* were further purified by silica HPLC to yield habenariol as a viscous oil.



1

Habenariol (**1**), showed $[\alpha]_D + 12^\circ$ and was analyzed for $C_{22}H_{26}O_7$ by both low resolution EI and high resolution FAB mass spectral analysis (observed: 401.1612, for $[M-H]^-$, calculated: 401.1600, $\Delta = 3.0$ ppm for

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$C_{22}H_{26}O_7$). This formula indicated habenariol possessed ten degrees of unsaturation. IR absorption bands indicated the presence of hydroxyl (3382 cm^{-1}) and ester (1731 cm^{-1}) functionalities and suggested the presence of a *para*-disubstituted benzene ring (1614, 1517, 829 cm^{-1}). Analysis of 1D proton and carbon NMR data indicated a nearly symmetrical structure possessing two very similar aromatic rings and two ester carbonyls, consistent with the extent of unsaturation.

The ^{13}C NMR spectrum of **1**, with DEPT sequence analysis, suggested that the molecule possessed two aliphatic methyl groups (δ 23.4, 24.3), four methylenes (δ 44.5, 47.6, 66.6, 67.7), one methine carbon (δ 23.9), seven quaternary carbons (δ 75.4, 127.0, 127.4, 156.0, 156.0, 170.8, 175.4) and two sets of four aromatic methines as overlapping signals centered at δ 115.4 and δ 130.4, respectively. These aromatic carbon chemical shifts, as well as the UV absorption data [$\lambda_{max} = 224$ nm (14000), 278 nm (2800)], were consistent with a *p*-hydroxy alkylbenzene fragment. The observed extinction coefficients provided support for the presence of two aromatic chromophores in the molecule (Pretsch, Clerc, Seibl, & Simon, 1989). The EI mass spectral fragmentation pattern for **1**, which illustrated an intense fragment (the base peak) at $m/z = 107$ indicative of a *p*-hydroxybenzyl cation, suggested that the ester functionalities were *p*-hydroxybenzyl esters.

COSY ^1H NMR data, specifically two methyl doublets (C-7 and C-8) at δ 0.77 and 0.89, observed coupled to a methine multiplet (C-6) at δ 1.66 and a methylene doublet (C-5) at δ 1.55 coupled to the C-6 methine proton, indicated the presence of an isolated isobutyl functionality. Five additional isolated spin systems, three methylene pairs and two *p*-disubstituted aromatic rings, were also observed. The three methylenes were characterized by AB double doublets at δ 2.67 and 2.88, at δ 4.93 and 4.98 and at δ 5.00 and 5.06, while the aromatic protons were composed of four overlapping multiplets centered at δ 6.76 and 7.13, each integrating for four protons. Finally, D_2O-exchangeable resonances (one proton singlet at δ 3.72, and a two proton singlet at δ 5.22) were assigned to three hydroxyl protons.

Analysis of 2D HMQC and HMBC heterocorrelation NMR data permitted complete assignment of all protons and carbons, thus leading to the structure assignment of habenariol. HMBC correlations from the protons at C-3, C-5, C-6, C-9, and C-16 were particularly diagnostic, as indicated in Table 1. The observed positive optical rotation indicated habenariol possessed the C-2 *S* absolute configuration. This configuration is assigned by anal-

Table 1
NMR spectral data for habenariol (**1**)[a]

Carbon #	^1H[b]	^{13}C[c]	COSY	HMQC	HMBC
1		175.4			
2		75.4			
3	2.67, 2.88				
	(AB, 16.2)[c]	44.5	2.88, 2.67	44.5	75.4, 170.8, 175.4
4		170.8			
5	1.55 (d, 6.3)	47.6	1.66	47.6	23.4, 23.9, 24.2, 44.5, 75.4, 175.4
6	1.66 (m)	23.9	0.77, 0.89, 1.55	23.9	23.4, 24.2, 47.6, 75.4
7	0.77 (d, 6.6)	23.4	1.66	23.4	23.9, 47.6
8	0.89 (d, 6.6)	24.2	1.66	24.2	23.4
9	5.00, 5.06				
	(AB, 11.8)	67.7	5.06, 5.00	67.7	127.0, 130.4, 175.4
10		127.0			
11	7.13 (m)	130.4	6.76	130.4	66.6, 67.7, 130.4, 156.0
12	6.76 (m)	115.4	7.13	115.4	115.4, 127.0, 156.0
13		156.0			
14	4.93, 4.98				
	(AB, 12.0)	66.6	4.98, 4.93	66.6	127.4, 130.4, 170.8
15		127.4			
16	7.13 (m)	130.4	6.76	130.4	66.6, 67.7, 130.4, 156.0
17	6.76 (m)	115.4	7.13	115.4	115.4, 127.4, 156.0
18		156.0			
1′	3.72 (s)				
2′	5.22 (br s)				
3′	5.22 (br s)				

[a] Spectra recorded at 400 MHz in $CDCl_3$.
[b] Coupling constants (*J*) in Hz.
[c] The number of attached protons in the carbon NMR experiment was determined using DEPT experiments.

ogy to numerous synthetic 2-alkylmalic acids found in the literature (Eck & Simon, 1994). This assignment is, however, inconsistent with an assignment suggested for a related analog of **1** (Li, Zhou, & Hong, 1993), which adds to the confusion, already noted in the literature, regarding the absolute stereochemistry of 2-alklymalate derivatives [*Dictionary of Organic Compounds*, 1996).

The finding that habenariol is a potent feeding deterrent provides substantive evidence that some freshwater plants are indeed chemically defended against aquatic herbivores. The structure of habenariol is uncommon as well, apparently being derived from amino acids and (degradation) products of phenylalanine. Habenariol is precedented, however, by a single report in the orchid phytochemical literature of a related 2-[1-methylpropyl]malate ester isolated from *Galeola faberi*, a non-aquatic climbing orchid (Li et al., 1993). Given the bioactivity of habenariol, it is conceivable that this latter metabolite also contributes toward the defense of *Galeola faberi*. Two pyrrolizidine alkaloids, with incorporated 2-isobutylmalic acid esters, have been reported from the unrelated plants *Parsonsia heterophylla* and *P. spiralis* (Apocynaceae) (Edgar, Eggers, Jones, & Russell, 1980).

3. Experimental

3.1. General experimental procedures

NMR spectra are reported as δ values in parts per million relative to internal chloroform (δ 7.24 for ^{1}H, 77.00 for ^{13}C). IR spectra were obtained as films on NaCl plates, with strong (s), medium (m) and weak (w) used to denote peak intensities. UV spectra were recorded in MeOH in 1 cm quartz cells.

3.2. Plant material

The orchid, *Habenaria repens*, was collected from a small, freshwater pond located in the coastal plain of North Carolina, U.S.A. *H. repens* is a perennial, aquatic herb which occurs infrequently from North Carolina, U.S.A., to northern South America (Godfrey & Wooten, 1979). Upon collection, plants for chemical analysis were wet-frozen and stored at $-70^{\circ}C$. A voucher specimen, identified by James R. Massey, has been deposited in the Herbarium of the University of North Carolina at Chapel Hill.

3.3. Bioassay-guided isolation of habenariol (*1*)

The frozen plants (165 ml, 15 g dry mass) were thawed and then exhaustively extracted with a CH_2Cl_2:MeOH (2:1) mixture. The combined extracts were reduced to an aqueous methanolic residue *in vacuo* and the residue was then partitioned between CH_2Cl_2 and H_2O. The CH_2Cl_2-soluble material was initially fractionated by silica gel gradient elution vacuum flash chromatography eluting with 100% hexanes to 100% diethyl ether mixtures in 10% increments (11 fractions collected). Fractions shown by TLC to contain identical metabolites were combined. Combined fractions were tested for feeding deterrence activity against *Procambarus clarkii* (Decapoda, Cambaridae), a freshwater crayfish, using concentrations (mg/g) calculated to occur naturally in plant tissues. Fraction No. 4 (60–80% ether:hexanes) reduced crayfish feeding by 44.2% ($N=22$, $P_{1\text{-tailed}}=0.044$). No other fractions were significant feeding deterrents, although the fractions eluted immediately before and after fraction No. 4 exhibited non-significant trends toward deterrence, possibly suggesting that there was some overlap of deterrent metabolite between these fractions. The individual compounds in fraction No. 4 were then purified by silica HPLC [6:4 ethyl acetate:isooctane delivered at 3 ml/min; compound detection was determined by refractive index and photo-diode array detection]. Habenariol eluted as a single peak comprising $2.0\pm0.27\%$ (mean $\pm$ 1 S.E., $N=4$) of leaf dry mass. When tested across a range of concentrations (10, 15 and 20 mg/g), habenariol (**1**) significantly reduced crayfish feeding by 54 and 66% at the two higher concentrations ($N=25$, $P_{1\text{-tailed}}=0.002$ and $N=26$, $P_{1\text{-tailed}}<0.0001$, respectively).

3.4. Habenariol (*1*)

Habenariol (**1**) was obtained as a pale green oil (2.0% dry mass) which decomposed upon standing at room temperature. The complete, assigned NMR spectra data for **1** are given in Table 1. Habenariol showed the following physical and spectral features: $[\alpha]^{23}_{D}+12^{\circ}$ (c 0.05, CH_2Cl_2); IR (film): 3382, 2957, 1731, 1614, 1518, 1450, 1379, 1224, 1170, 829 cm^{-1}; UV $\lambda^{(MeOH)}_{max}$ (ε): 224 nm (14000) and 278 nm (2800); HRMS (negative FAB): 401.1612 amu (calcd: 401.1600 for MH^{-}, $\Delta=3.0$ ppm); EIMS (70 eV): m/z 402 (0.38%), 296 (3.1), 145 (15), 123 (19), 107 (100).

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