

Contents of 1,4-Benzoxazin-3-ones and 2-Benzoxazolinone from *Stenandrium dulce* (Nees)

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Secondary metabolites, DIBOA, HBOA, 7-OH-HBOA and BOA, were isolated and quantified from *S. dulce* (Nees), a native species in Chile belonging to the Acanthaceae family. The highest DIBOA and HBOA contents were determined in leaves (9.25 mmol kg⁻¹ fr. wt) and root (6.81 mmol kg⁻¹ fr. wt), respectively. Aglycones, 7-OH-HBOA and HBOA, were isolated together from root extracts of Acanthaceae species. Both, HBOA and 7-OH-HBOA should be direct precursors in the biosynthesis of DIBOA and DIMBOA, respectively.

Key words: *Stenandrium dulce* (Nees), Acanthaceae, Hydroxamic Acids

Introduction

1,4-Benzoxazin-3-ones are mainly known from several Gramineae species (Niemeyer, 1988). They occur naturally as 2- β -O-D-glucopyranosides (Fig. 1) which are hydrolyzed by glucosidase released after plant tissue disruption (Hofman and Hofmanova, 1971). 2,4-Dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) is the main hydroxamic acid present in maize and wheat and its demethoxylated analogue (DIBOA) is present in rye (Fig. 1). These aglucones decompose in solution to the respective 2-benzoxazolinones (Fig. 1) (Smitsman *et al.*, 1972; Woodward *et al.*, 1978; Bravo and Niemeyer, 1985; Grambow *et al.*, 1986).

Heterocyclic hydroxamic acids are proposed to participate in various functions in the plant such as disease and insect resistance (Niemeyer, 1988), herbicide tolerance (Hamilton, 1964; Tipton *et al.*, 1971) and mineral metabolisms (Tipton and Buell, 1970). Also they have a broad pharmacological profile, which includes antimicrobial (Bravo and Lazo, 1993, 1996), anticancer (Roberts *et al.*, 1998) and anti-inflammatory activities (Otsuka *et al.*, 1998).

Although knowledge of chemical and biological properties of these hydroxamic acids derives almost entirely from Gramineae; the first natural hydroxamate was isolated from a member of the Acanthaceae family (*Blepharis edulis*) more than 20 years before the discovery of these compounds in cereals (Lal, 1936). However, few studies of 1,4-benzoxazin-3-ones from Acanthaceae species have

been reported. In a screening study of 34 species of Acanthaceae in 21 genera, these compounds were found only in the genera *Acanthus*, *Aphelandra* and *Crossandra* (Pratt *et al.*, 1995). In these genera DIBOA, DIMBOA, N-deoxy-DIMBOA, 7-OH-HBOA, BOA and MBOA were reported. Recently, it has been reported the contents of DIBOA and BOA from leaves, roots and flowers of *Acanthus mollis*, in which the high contents in leaves were viewed as natural factor of resistance against insects (Bravo and Copaja, 2002).

In this work, we isolated and simultaneously quantified by using HPLC method the contents of DIBOA, BOA, N-deoxy-DIBOA (HBOA) and 7-hydroxy-N-deoxy-DIBOA (7-OH-HBOA) from roots and leaves of *Stenandrium dulce* (Nees) the only native Chilean species of the Acanthaceae. Both HBOA and 7-OH-HBOA are considered to be precursors in the biosynthesis of DIBOA and DIMBOA, respectively.

Results and Discussion

Aqueous macerated leaves and roots of *Stenandrium dulce* (Nees) were extracted with ethyl acetate and analyzed by TLC. DIBOA, HBOA and BOA were preliminary identified by using authentic standards (Table I). All three and 7-OH-HBOA were isolated from preparative TLC. Melting points and spectroscopic data were consistent with those reported (Bravo and Copaja, 2002; Woodward *et al.*, 1979; Ozden *et al.*, 1992). This is

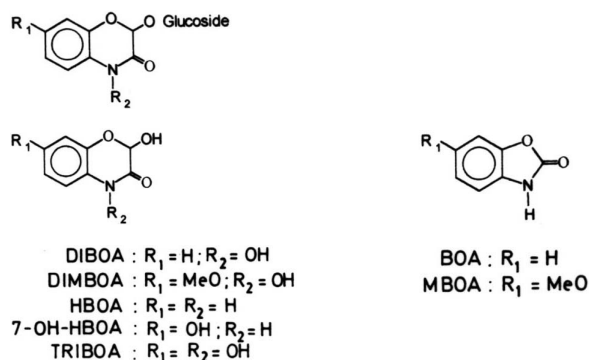


Fig. 1. Structures of 1,4-Benzoxazin-3-ones and 2-Benzoxazolinones.

the first time that the aglycones 7-OH-HBOA and HBOA were isolated and identified together from roots extracts of Acanthaceae species. Hitherto, 7-OH-HBOA has been detected in aqueous extracts of maize seedlings, but it was not isolated, the identification was made only by TMS-MS spectra (Woodward *et al.*, 1979). 7-OH-HBOA-Glc has been isolated from aerial parts of *Acanthus ilicifolius* (Kanchanapoom *et al.*, 2001) and roots of *Coix lacryma-jobi* var. *ma-yven* (Gramineae) (Nagao *et al.*, 1985).

In this work, the pure aglycone was isolated and the confirmed structure was added to TMS-MS spectra and other spectroscopic data.

Analytical HPLC method was used for the quantitative determination of the compounds. The separation of the natural mixture and standard of these four compounds is shown in Fig. 2.

Contents of DIBOA, HBOA, 7-OH-HBOA and BOA in roots and leaves of *S. dulce* (Nees) are shown in Table II. DIBOA and HBOA were the main aglycones in the leaves and roots, respectively. The BOA contents were lower than DIBOA in both parts of the plants. BOA is the main product of the decomposition of DIBOA in solution. The half-life in phosphate buffers, pH 7.0 and

Table I. TLC R_f values and HPLC retention time (R_t) of DIBOA, HBOA, 7-OH-HBOA and BOA from *S. dulce* (Nees).

Compound	TLC R_f values	Retention time [min]
DIBOA	0.35	12.3 \pm 0.2
HBOA	0.41	11.7 \pm 0.4
7-OH-HBOA	0.23	10.2 \pm 0.3
BOA	0.82	22.0 \pm 0.5

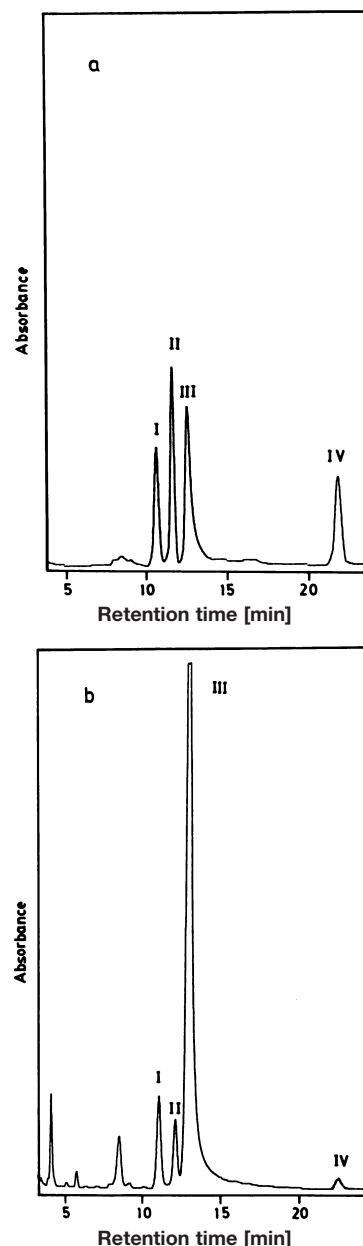


Fig. 2. Chromatograms of an artificial mixture (a) of purified BOA (IV), DIBOA (III), HBOA (II) and 7-OH-HBOA (I) and (b) of an aqueous extract of *S. dulce* (Nees) leaves.

pH 3.0, at room temperature are 15 h and 200 h, respectively (Bravo and Copaja, 2002). Thus, the concentration found should not be produced by decomposition of DIBOA in the extraction process (see experimental). Consequently, BOA may occur naturally in *S. dulce*.

Table II. Contents of DIBOA, HBOA, 7-OH-HBOA and BOA in roots and leaves of *S. dulce* (Nees).

Compound	Roots [mmol kg ⁻¹ fr. wt]	Leaves [mmol kg ⁻¹ fr. wt]
DIBOA	1.8 ± 0.1	9.3 ± 0.3
HBOA	6.8 ± 0.5	1.1 ± 0.1
7-OH-HBOA	1.1 ± 0.2	0.6 ± 0.1
BOA	3.7 ± 0.2	0.4 ± 0.0

Contents of 7-OH-HBOA were smaller than those found for HBOA in roots and leaves, but in both cases, the concentration in roots was always higher than in leaves. These content differences could be related to the biosynthetic pathway of hydroxamic acids DIMBOA and DIBOA.

A proposed biosynthetic route of these compounds (Fig. 3) (Niemeyer, 1988; Glawischning *et al.*, 1997; Desai *et al.*, 1996) suggest that HBOA should be the last precursor of DIBOA. This possibility may be only partially expressed in *S. dulce*. The high contents of HBOA in the roots and the lower content in the leaves suggest that the enzyme producing the N-hydroxylation of HBOA may be less active in the roots tissues.

Our results seem to indicate that 7-OH-HBOA may be included in the biosynthetic route of DIMBOA. N-Hydroxylation of this precursor could produce TRIBOA, the closer intermediate on the route. Since, DIMBOA was not detected in roots and leaves tissues, it is suggested that the enzymes catalizing the N-hydroxylation and methoxylation of the aromatic hydroxyl group are not expressed in *S. dulce*, leading to the accumulation of 7-OH-HBOA. More experiments will be necessary to clarify fully this possibility.

Experimental

Plant material

Stenandrium dulce (Nees) was collected from VII Región, Chile (35° 04' S; 71° 31' W), in October 2001 (perennial plant). A voucher sample is on deposit at the Talca herbarium under N° 2383 (cod. Ajim).

Chemicals

The DIBOA standard was isolated from extracts of rye shoots (*Secale cereale* cv Tetra-Baer) as previously described (Queirolo *et al.*, 1983; Lyons *et al.*, 1988). The BOA standard was a commercial product (Aldrich Chemical Co). The HBOA stan-

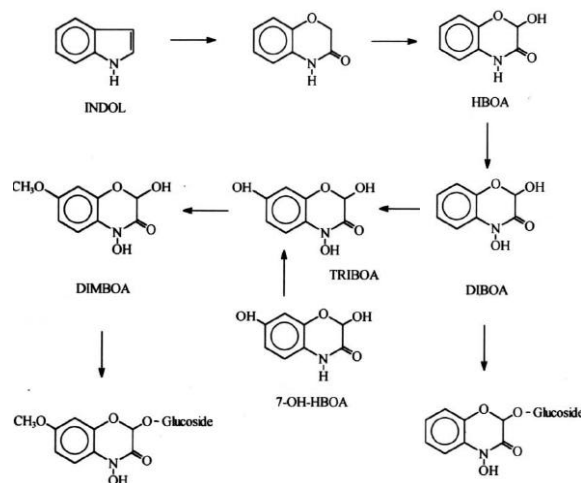


Fig. 3. Hypothetical scheme for biosynthesis of DIMBOA and DIBOA.

dard was synthesized as previously described (Matlin *et al.*, 1979).

Chemical Analysis

0.1 g of plant material, either leaves or roots of *S. dulce* were macerated with 3 × 0.5 ml of deionized water. The aqueous extracts were left at room temperature for 30 min, acidified to pH 3.0 with 0.1 N H₃PO₄ and centrifuged at 7000 × g for 15 min. The volume of supernatant was adjusted to 2.0 ml with deionized water and 50 µl aliquot was directly injected into a HPLC with a Lichrosfer RP-100 C₁₈ column (250 × 4 mm, particle size 5.0 µm). Chromatographic analysis was performed isocratically eluting with 30:70 mixtures of methanol and water, pH 3.0, 0.1 N H₃PO₄. Flow rate was 1.5 ml/min and detection was carried out at 263 nm. The detection limit was 1.0 µmol kg⁻¹ fr. wt. All experiments were done with five replicates. The compound concentrations in test solutions were obtained by linear regression from calibration curves. DIBOA, HBOA and BOA were identified by coincidence of retention time with the standard solutions.

Isolation and thin-layer chromatography

Aqueous macerated leaves and roots (300 g fr. wt) of *S. dulce* were left at room temperature for 24 h and extracted three times with ethyl acetate (300 ml). Solvent was chloroform/methanol (10:1 v/v). The spots were visualized under short-wave-length UV light. Ferric chloride developing was used for the preliminary identification of hydro-

xamic acid. Compounds were isolated using preparative TLC. R_f values of DIBOA, HBOA and BOA (Table I) were identical to the authentic samples. Melting points and spectroscopic data were in agreement with the reported (Woodward *et al.*, 1979; Ozden *et al.*, 1992; Glawischning *et al.*, 1997). The identity of the aglycone 7-OH-HBOA was confirmed by: M.p. 190–195 °C. – UV/vis (MeOH): λ_{\max} (lg ϵ) 247 nm (3.67). – IR (KBr): ν_{\max} = 3310 (O-H), 3190 (N-H), 2900 (C-H), 1680 (C=O), 1620 cm^{-1} . – ^1H NMR (300 MHz, CDCl_3 -

CD_3OD): δ = 5.58 (1H, s, H-2), 6.55 (1H, dd, J = 2.9 Hz, H-6), 6.58 (1H, d, J = 2.2 Hz, H-8), 6.85 (1H, d, J = 7.9 Hz, H-5). – GC-TMS₃-MS (EI, 70 eV): m/z (%) = 397 (69%) [M^+], 296 (22%), 280 (62%), 265 (64%), 237 (35%), 220 (100%), 191 (52%), 147 (58%).

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