Pergamon

PII: S0031-9422(97)01012-1

Phytochemistry, Vol. 49, No. 3, pp. 719–722, 1998

Printed in Great Britain
0031-9422/98/$—see front matter

3-β-D-GLUCOPYRANOSYL-BENZOXAZOLIN-2(3*H*)-ONE—A DETOXIFICATION PRODUCT OF BENZOXAZOLIN-2(3*H*)-ONE IN OAT ROOTS¶

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(*Received* 31 *July* 1997)

Key Word Index—*Avena sativa*; Poaceae; oat; allelopathy; detoxification; 3-β-D-glucopyranosyl-benzoxazolin-2(3*H*)-one; benzoxazolin-2(3*H*)-one.

Abstract—Methanolic extracts of *Avena sativa* roots previously incubated with the natural phytotoxin benzoxazolin-2(3*H*)-one contain, in addition to two already described detoxification products, a third one of hitherto unknown structure. The compound has now been identified by NMR and mass spectral methods as 3-β-D-glucopyranosyl-benzoxazolin-2(3*H*)-one. This structure was confirmed by an independent synthesis. The ability of oat to produce the N-glucoside is supposed to be one reason for a less pronounced sensitivity to the phytotoxin.

INTRODUCTION

Cyclic hydroxamic acids are secondary constituents of a variety of Poaceae and some dicotyledonous species [1–4]. They can be released into the environment passively by rotting plant material or actively by root exudation [5, 6]. Once released into the soil, 2,4-dihydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one (DIBOA) is converted to benzoxazolin-2(3*H*)-one (BOA), which accumulates prior further metabolism by microbial organisms [7–10]. Cyclic hydroxamic acids, as well as BOA, are bioactive compounds as they have inhibitory effects on seedling growth and development. However, the negative effects were found to be dose and species dependent: For instance, monocotyledons are often less sensitive than most dicotyledons and very low concentrations of the phytotoxins can stimulate radicle growth and activate plasma membrane ATPase activity [11].

The species-dependent differences in sensitivity to BOA were assumed to be due to a less or more pronounced detoxification capacity of the species tested and, indeed, *Avena sativa*, *Triticum aestivum* and *Secale cereale* are able to detoxify the phytotoxin BOA by two different routes. By contrast, *Vicia faba* has only one possibility with a lower efficiency of detoxification. The way found in all species tested starts with a hydroxylation in position C-6, resulting in BOA-6-OH, which is subsequently glucosylated to form BOA-6-O-β-D-glucoside. A third product, representing the second route of detoxification, is synthesized by the cereals tested. It accumulates in the roots, but was also found to be exuded by wheat roots [12].

This compound with a slightly more hydrophobic behaviour in HPLC runs than BOA-6-OH, and with UV spectrometric characteristics very similar to those of BOA, represents a yet undescribed natural detoxification product. The isolation, structural analysis and synthesis of this material is reported here.

RESULTS AND DISCUSSION

Methanolic extracts from oat roots that were incubated in presence of 500μM BOA for 75 h contain as a major detoxification product BOA-6-O-β-D-glucoside, a small amount of the intermediate BOA-6-OH and a third compound assumed to be another BOA derivative. In most experiments, 5–10% of the total BOA absorbed was converted into this product. However, in some cases where detoxification via BOA-6-OH did not appear for unknown reasons, it was the only product found, but, hitherto, it could not be assigned to a definite structure. Due to its retention time the equipment of BOA with a hydrophilic unit

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¶ Dedicated to Professor Gerhard Werner on the occasion of his 65th birthday.

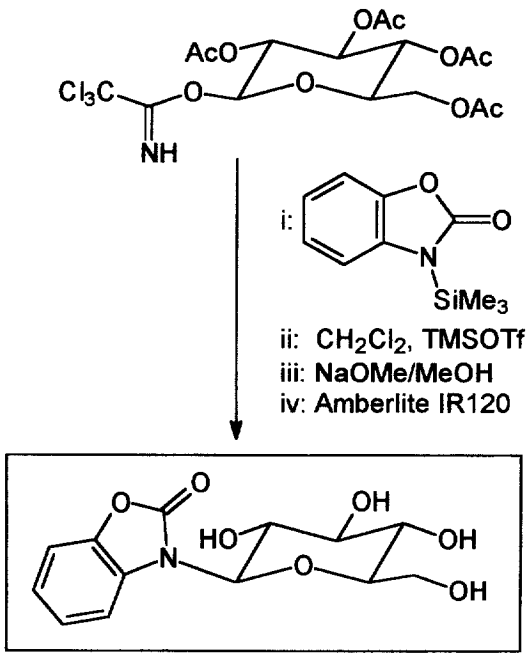

Fig. 1. Synthesis of 3-β-D-glucopyranosyl-benzoxazolin-2(3*H*)-one isolated from *Avena sativa*.

seemed possible as a hypothesis. Therefore, in a series of HPLC runs, a first isolate of 150 μg was isolated which was subjected to a HPLC-coupled 500 MHz NMR spectrometer. This isolate was highly enriched in the unknown component of interest, but was not pure. Thus, using a direct coupling of HPLC with NMR the method of choice was to obtain an insight into the structure of the unknown metabolite. By means of several HPLC-NMR runs it was shown, that the compound consisted of an aromatic part and most likely, a saccharide moiety. Furthermore, the characteristic pattern of the protons at positions C-4, C-5, C-6, and C-7 of the aromatic portion of the starting benzoxaxolinone unit proved to be unchanged. Hence, a hydrophilic unit must have been attached to the oxazolinone ring. Clearly, in this unit, only the NH function was able to undergo such a transformation. In principle, a glycosylation should be possible either directly at the NH-unit or after N-hydroxylation.

At this point, it was decided to collect a new isolate from our solution containing the BOA-metabolites by analytical HPLC. In a series of 40 runs a sample of 300 μg was collected, which was analysed conventionally by NMR spectroscopy to determine the structure of the unknown compound as 3-β-D-glucopyranosyl-benzoxazolin-2-(3*H*)-one (Fig. 1).

Analysis of the spectroscopic data obtained from HMBC and HMQC experiments proved that the aromatic part of the compound had no modifications at positions C-4, C-5, C-6, and C-7 and allowed the assignment of all C and H atoms of the monosaccharide unit. The combination of these findings with a $[M]^+$ of *m/z* 297 obtained from the EI-mass spectrum led to the recognition of a glycosylated form of BOA. The crucial question to clarify concerned the elucidation of the correct regio- and stereochemistry.

The classification of the β-configuration at the anomeric center (C-1′) was possible by examining the coupling constant of $^3J_{1',2'} = 9.3$ Hz of the anomeric proton H-1′ at δ 5.28 with its vicinal proton H-2′. This coupling constant is rather large for a β-glucoside, but can be explained by the unusual structure. However, an α-glucosylation was excluded, as in this case the coupling constant should not be larger than 4 Hz. Proton H-2′ coupled with the vicinal protons H-1′ and H-3′, which resulted in a coupling constant of about 9 Hz, for each, indicating a 1,2-*trans*-configuration. The same configuration at C-1′, C-2′, and C-3′ is a feature of β-D-glucose. The multiplet at δ 3.5, originating from C-4′ and C-5′, showed signals with even spacings within it which were all larger than 8 Hz. This indicated a *trans*-configuration of C-4′ and C-5′, also as expected for glucose. The spectrophotometrical characteristics of both C-6′ protons point to an equatorial configuration, which is again only possible in case of a glucose-configuration of H-5′.

The ^{1}HNMR spectral data obtained left the position of the bond to D-glucose unclear, since unequivocal identification of either a N- or an O-glucosidic link is impossible by this means. Therefore, HMBC spectrometry was used to ascertain the mode of the link to glucose. In the case of an O-glucoside, the anomeric proton was expected to show long range couplings to C-2′, C-3′, and C-5′, but not to the carbonyl carbon of the oxazolinone ring (C-2) and C-3a, respectively. By contrast, the HMBC spectral data obtained exhibited long-range couplings between H-1′ and C-2′, C-3′, C-5′, C-2, and C-3a representing characteristic features only explainable for the N-glucoside. Surprisingly, the proton H-2′ at δ 4.04 was also considerably deshielded. This indicated the presence of an electronegative atom close to H-2′ which was in agreement with the direct C-1′-N bond in 3-β-D-glucopyranosyl-benzoxazolin-2-(3*H*)-one.

Based on our experience in the synthesis of natural 1,4-benzoxazin-3(4*H*)-one glucosides [13] we were interested to prove the structure of the new product by an independent chemical synthesis. In the two first approaches, the glucoside should be formed by the reaction of benzoxazolin-2(3*H*)-one with 1,2,3,4,6-penta-*O*-acetyl-β-D-glucopyranose or 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranose trichloroacetimidate [14] in the presence of excess trimethylsilyltriflate. Unfortunately, in both cases due to the low nucleophilicity of the amide function no reaction could be observed after 24 h. In a third approach, we tried to make use of the Vorbrüggen protocol [15], which is well known as a standard procedure in nucleoside synthesis. Therefore, BOA was silylated with N,O-bistrimethylsilyl-trifluoroacetamide and reacted with 1,2,3,4,6-penta-*O*-acetyl-β-D-glucopyranose in the presence of trimethylsilyltriflate. Unexpectedly, under

these conditions no glucosylation could be achieved. Eventually, by changing the glucosyl donor to an excess of the more reactive 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranose trichloroacetimidate we obtained 3-(2,3,4,6-tetra-*O*-acetylglucopyranosyl)-benzoxazolin-2(3*H*)-one (Fig. 1). Finally, separation and deprotection of the tetraacetate gave 3-β-D-glucopyranosyl-benzoxazolin-2(3*H*)-one, though in moderate yield only. The synthetic compound proved to be identical in its properties to the detoxification product.

The only N-glycosylated oxazolinone compound reported yet is, to the best of our knowledge, 5-amino-3-β-D-glucopyranosyl-7-methyl-3*H*-oxazolo[4,5-d] pyrimidin-2-one [16]. Thus, the identified compound is the first natural N-glycosylated oxazolinone described. N-glucoside formation is known from herbicide (3-amino-2,5-dichlorobenzoic acid, Chloramben, Amiben) detoxification [17, 18], or from nicotinic acid and cytokinin metabolisms [19–21]. Amiben-tolerant species, e.g., soybean, tomato, and sugar beet, converted the herbicide to N-(3-carboxy-2,5-dichlorophenyl)glucosamine. In contrast to hydroxylation and subsequent (reversible) glycosylation or ester glycoside formation, which was found to give rise in susceptible species to the major detoxification products, N-glycosylation is supposed to present an irreversible detoxification of the herbicide. Coincidently, the transformation of BOA to 3-β-D-glucopyranosyl-benzoxazolin-2-(3*H*)-one in oat roots may be a reason for the relatively low sensitivity to BOA. On the other hand, N-glucosylation of natural substances can probably not be regared as an irreversible conjugation in general, since metabolism of at least nicotinate-N-glucoside has been described [19]. Ongoing research will clarify, whether BOA-N-glucosylation leads in general to a lower sensitivity of plants to this phytotoxin and whether the N-glucoside is indeed a stable end product of BOA detoxification.

EXPERIMENTAL

Plant material

Oat seedlings were grown, the roots harvested and incubated as described in Ref. [12].

Extraction

After 3 days the roots were rinsed with distilled water, dried and homogenized by freezing in liquid nitrogen and grinding with quartz sand using a mortol and pestle, then extracted with 10% MeOH (3 ml g^{-1} fr. wt). The mixture was shaken at room temp. for 30 min, squeezed through Miracloth, and the filtrate centrifuged for 15 min at 1000 *g*. The supernatant was evaporated to dryness, the residue dissolved in MeOH (100 μl g^{-1} fr. wt). The soln was subjected to HPLC (Beckman model 126 chromatograph equipped with a diode array detector 168 and an ultrasphere ODS PR 18 column). Eluents were 0.1% TFA in H_2O (eluent A) and MeOH (eluent B). The following gradient was used: 0–1 min 100% A, 1–20 min 20% B, 21–23 min 100% B, detection wavelengths were 280 and 227 nm, the flow rate was 1 ml min^{-1}). The substance of interest eluting with a retention time of 20.8 min was collected, concentrated and purified by a second HPLC procedure, using 0.1% TFA as eluent A and MeCN as eluent B and the following gradient: 0–4 min 0–10% B; 4–8 min 90% B; 8–18 min 90% B; 18–20 min 100% B. The flow rate was 0.8 ml min^{-1}, the detection wavelength was 227 nm. The compound eluted with a retention time of 11.3 min was collected, evapd to dryness and used for measuring ^{1}HNMR and ^{13}CNMR and mass spectra as reported below.

Synthesis of 3-β-D-*glucopyranosyl-benzoxazolin*-2-(3H)-*one*

Benzoxazolin-2(3*H*)-one (270 mg, 2 mmol) was silylated with N,O-bistrimethylsilyl-trifluoroacetamide (970 mg, 3.8 mmol, 1 ml) at 110° for 1 h under exclusion of moisture. Excess silylating agent was removed *in vacuo* and the remaining oily residue was dissolved in dry CH_2Cl_2 (20 ml). 2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranose trichloroacetimidate (1.97 g, 4 mmol) and trimethylsilyl-triflate (250 μl) were added and the soln was stirred for 24 h at room temp. in a sealed flask. To the soln were added CH_2Cl_2 (50 ml), H_2O (30 ml) and $NaHCO_3$ (1 g). After shaking for 2 min, the organic layer was sepd, washed with H_2O (3 × 20 ml), dried ($MgSO_4$) and evapd *in vacuo*. The remaining residue was chromatographed (silica gel 60, 60–200 μm, Merck, CH_2Cl_2) to yield a colourless oil, which was dissolved in dry MeOH (20 ml). NaOMe (10 mg) was added and the soln was stirred for 2 h at room temp. Ion exchange resin (Amberlite IR 120, 1 g) was added and the soln was stirred for 30 min. The resin was filtered off, washed with MeOH (2 × 20 ml) and the combined organic phases were evapd *in vacuo*. The remaining residue was chromatographed [silica gel 60, 60–200 μm, Merck, $CHCl_3$–MeOH (5:1)] to yield 207 mg (35%) of pure 3-β-D-glucopyranosyl-benzoxazolin-2(3*H*)-one (mp 125–128°) as colourless crystals. ^{1}HNMR (199.975 MHz, CD_3OD): δ 3.47–3.58 (*m*, 3H, H-3′, H-4′, H-5′), 3.73 (*dd*, 1H, $^2J_{6b',6a'}$ = 12.1 Hz, $^3J_{6b',5'}$ = 5.4 Hz, H-6b′), 3.91 (*d*, 1H, $^2J_{6a',6b'}$ = 12.1 Hz, H-6a′), 4.04 (*dd*, 1H, $^3J_{2',1'}$ = 9.3 Hz, $^3J_{2',3'}$ = 8.9 Hz, H-2′), 5.28 (*d*, 1H, $^3J_{1',2'}$ = 9.3 Hz, H-1′), 7.17 (*dd*, 1H, $^3J_{6,5}$ = 7.5 Hz, $^3J_{6,7}$ = 7.6 Hz, H-6), 7.21 (*dd* 1H, $^3J_{5,6}$ = 7.5 Hz, $^3J_{5,4}$ = 7.8 Hz, H-5), 7.26 (*d*, 1H, $^3J_{4,5}$ = 7.8 Hz, H-4), 7.35 (*d*, 1H, $^3J_{7,5}$ = 7.6 Hz, H-7). ^{13}C-NMR (50.289 MHz, CD_3OD): δ 62.7 (C-6′), 71.1 (C-2′), 71.3 (C-4′), 78.7 (C-3′), 81.2 (C-5′), 85.7 (C-1′), 111.0 (C-4), 112.5 (C-7), 124.1 (C-6), 125.2 (C-5), 130.5 (C-3a), 144.1 (C-7a), 155.5 (C-2). EI-MS *m/z* (rel. int.): 297 (M^+, 8), 281 (4), 234 (4), 206 (5), 178 (6), 135 (100). IR cm^{-1}: 1759, 1342, 1073, 1032. $[\alpha]_{589}^{26}$ = +30° (MeOH; *c* 1.0). CD $\Delta\varepsilon_{270}$ +7.2 (H_2O; *c* 1.945). Found: C, 52.4; H, 5.0; N, 4.5%. $C_{13}H_{15}NO_7$ requires: C, 52.5; H, 5.1; N, 4.7%.

Acknowledgements—Financial support by the Deutsche Forschungsgemeinschaft (grant Schu 1012/3-1-764/95) is gratefully acknowledged. B.S. wants to express his gratitude for the personal loan of a HPLC-coupled Bruker NMR-spectrometer DRX-500 to the Deutsche Forschungsgemeinschaft. D.S. thanks the Fonds der Chemischen Industrie for generous support of this work.

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