Journal of Natural Products
Vol. 48, No. 1, pp. 59-63, Jan-Feb 1985

BENZOXAZOLINONE, 2,4-DIHYDROXY-1,4-BENZOXAZIN-3-ONE, AND ITS GLUCOSIDE FROM *ACANTHUS MOLLIS* SEEDS INHIBIT VELVETLEAF GERMINATION AND GROWTH

REBECCA B. WOLF, GAYLAND F. SPENCER, and RONALD D. PLATTNER

Northern Regional Research Center, Agricultural Research Service, US Department of Agriculture,[1] *Peoria, Illinois 61604*

ABSTRACT.—Seeds of *Acanthus mollis* contain (4% by dry weight) the glucoside **1** of 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA). Enzymatic hydrolysis of this glucoside yields DIBOA which easily degrades to benzoxazolinone (BOA). DIBOA completely inhibits velvetleaf (*Abutilon theophrasti*) germination at a concentration of 2 mM; BOA is less active. The glucoside has no effect on germination but causes unusual growth of velvetleaf seedlings. This compound is known to occur in the seedlings (but not the seeds) of several grasses (e.g., corn, rye, and wheat). The phytotoxicity of BOA and DIBOA suggests that they might be involved in the allelopathic activity attributed to rye.

Benzoxazolinone (BOA) was first discovered in nature by Virtanen and Hietala (1) as an anti-*Fusarium* factor in rye seedlings. In further work, the same group found that BOA is formed by heating the hydroxamic acid 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA), and that DIBOA is actually the autolysis product of the glucoside **1** (2-4). Since then, this family of compounds or their methoxy derivatives have also been found in corn, wheat, wild rye (*Elymus gayanus* L.), giant reed (*Arundo donax* L.), Job's tears (*Coix lacryma-jobi* L.), and a bamboo (*Chusquea cumingii* Kunth.) (5-7), all members of the grass (Gramineae) family. It appears that only the parent glucosides of DIBOA and its methoxy derivative (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one, DIMBOA) occur in significant amounts in uninjured plants, although traces of DIMBOA have been found in corn (8). However, most measurements of these compounds have been made on the autolysis products or on BOA itself after heating the aglucone. As indicated by such measurements, the glucoside of DIMBOA usually occurs in greater amounts in plants than the corresponding nonmethoxylated compound. For example, corn shoots have up to 61.9 mmoles/kg dry weight methoxy-BOA (9,10), whereas only 10.3 mmoles BOA/kg dry weight was measured in the same seedlings (9). However, the amount of these compounds present varies greatly depending upon species, age, and plant part analyzed (6-14). Before our discovery in bear's breech (*Acanthus mollis* L.), they had not been detected in seeds (1,14,15), although they have been found in seedlings as early as two or three days after germination (9,14).

The biological activities of these compounds have also been investigated. In particular, both BOA and DIBOA inhibit the mycelial growth of *Fusarium* and *Helminthosporum* (1,16,17); DIBOA is also lethal to the aphid *Schizaphis graminum* and has been implicated in insect antifeeding studies (7,9). BOA has demonstrated anticonvulsant activity in small animals (18), but more germane to this study is the single report of its plant growth inhibitory activity. Aiupova and coworkers (19) demonstrated that the radicle elongation of cucumbers, oats, radishes, and cabbage was sensitive to BOA and that doses required for 50% inhibition of elongation varied from 1.5 to 8.2 kg/ha. Unfortunately, with the information provided, it is not possible to determine the molar concentrations used. However, germination of these seeds was not affected at the doses given.

To the best of our knowledge, this is the first report of the presence of this family of

[1]The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

compounds in seeds or in a dicotyledonous plant. Additionally, the antigermination activity of the three compounds is now reported.

MATERIALS AND METHODS

GENERAL EXPERIMENTAL PROCEDURES.—Nmr, ms, hplc, and bioassay equipment and procedures are described in previous reports (20,21). Bioassays were conducted in petri dishes with treated filter paper. Each dish contained 20 velvetleaf seeds, and two dishes comprised one treatment. For every two treatments, a blank control was added. Germination of controls varied from 85 to 100%. Each experiment was repeated at least once and the data was averaged. For ^{1}H-nmr spectra, field strength was 300 MHz, and samples were dissolved in D_2O. Ir spectra were determined on a Perkin-Elmer 337 in $CHCl_3$. A Bausch and Lomb Spectronic 2000 or a Beckman DK-2A was used for uv spectra, with samples in EtOH. Silica tlc plates (0.25 mm, Brinkmann) were developed in $CHCl_3$-MeOH-H_2O (29:10:1), and spots were visualized under shortwave uv light. Authentic BOA was purchased from Aldrich Chemical Co., Milwaukee, WI. All chromatographic procedures were followed by tlc and/or bioassay.

EXTRACTION OF *ACANTHUS MOLLIS* SEEDS.—Ground *A. mollis* seed was defatted with hexane and extracted with Me_2CO in a Soxhlet apparatus for 17 h. The Me_2CO extract was dried on a rotary evaporator, taken up in H_2O, and extracted 3 times with $CHCl_3$. The aqueous residue was freeze-dried, taken up in a small volume of H_2O, mixed with an equal volume of silica, placed on a silica column (silica gel grade 12, 28-200 mesh) and eluted successively with 5% and 50% MeOH in $CHCl_3$ (v:v), resulting in fractions A and B, respectively.

Fraction A was reduced on a rotary evaporator and separated by reverse-phase (C8 column) hplc with H_2O-MeOH (40:60). This procedure yielded pure BOA; DIBOA collected at this time was further purified by the same procedure. Isolated BOA was compared to authentic standard by gc-ms, ir, and nmr.

Fraction B was separated by reverse-phase hplc with H_2O-MeOH-HOAc (50:50:1); like DIBOA, two chromatographic separations were required to yield pure glucoside.

EXTRACTION OF GLUCOSIDE FROM RYE SEEDLINGS.—Since rye is known to contain **1** (2), it was used as a source for standard material. Eight-day-old rye (*Secale cereale* L.) seedlings were immersed in boiling H_2O for 20 min, ground in a Waring Blendor, and boiled again for 45 min with stirring. The slurry was centrifuged, and the supernatant was decanted and extracted 3 times with $CHCl_3$. The aqueous residue was then extracted 3 times with *n*-BuOH, and the BuOH fraction was dried on a rotary evaporator. The residue was redissolved in H_2O and extracted 3 times with Et_2O. The aqueous fraction was separated by hplc on a carbohydrate column (0.5×25 cm, Waters) with CH_3CN-H_2O-HOAc (87:13:1). The fraction containing the glucoside was further purified by repetition of this hplc procedure, and finally by anion exchange (Bio-Rad AG-1-X_2, 50-100 mesh, OH^- form). The uv spectrum of the resulting glucoside was identical to that published by Virtanen and Hietala (2,3).

QUANTITATION OF THE GLUCOSIDE IN *A. MOLLIS* SEEDS.—*A. mollis* seeds (26.1 g) were immersed in boiling MeOH for 20 min, removed from the solvent, allowed to surface dry for a few minutes, and ground in a coffee grinder to a fine powder. The meal was returned immediately to boiling MeOH and stirred for 15 min. The slurry was then filtered and reextracted 4 times with boiling MeOH-H_2O (70:30). No glucoside was detected in the final extract by tlc. All other extracts (including the original MeOH extract) were combined and reduced on a rotary evaporator. Neither DIBOA nor BOA were detectable by tlc in this fraction. The residue was redissolved in H_2O and extracted 3 times with $CHCl_3$. The aqueous fraction was diluted to a known volume and analyzed by hplc with the addition of coumarin (ICN Pharmaceuticals, Plainview, NY) as an internal standard. Hplc conditions were: 9.4×250 mm Zorbax C8 (DuPont, Wilmington, DE) column; MeOH-H_2O (60:40) solvent system; flow rate 5 ml/min; Hitachi Model 100-10 (modified for hplc use) uv detector at 255 nm. (The extract alone showed no peak at the same elution time as coumarin under these conditions.) A calibration curve was made using five different mixtures of pure glucoside (obtained from procedures described above) and coumarin.

All samples including the unknown were analyzed twice and the results averaged.

RESULTS AND DISCUSSION

CHARACTERIZATION AND QUANTITATION.—The ir spectra for isolated and authentic BOA are identical. The gc-ms spectra are also the same and are consistent with data published previously (22). Nmr spectra were essentially identical; however, resolution of signals for aromatic protons of the isolated sample was not as sharp as of the authentic BOA.

The uv spectrum of the glucoside isolated from *A. mollis* seeds was identical to that

published by Virtanen and Hietala (2,3) and to that obtained from the rye glucoside. Maxima were 254 (ϵ=8300) and 281 (ϵ=6380). A comparison of nmr data showed slight inexplicable differences in the aromatic protons between the two glucosides, perhaps due to a difference in concentration. The presence of α-D-glucose was authenticated by comparison with methyl-α-D-glucose and with published data (23). Cims with a direct exposure probe of the *A. mollis* sample showed the expected protonated molecular ion at *m/z* 344 (rel. % 31), and an intense ion at 182 (83) representing the aglucone DIBOA. The peaks at 328 (44) and 166 (100) probably represent MH^+-oxygen and 182-oxygen, respectively. Other ions produced were *m/z* 180 (19), 165 (14), 164 (33), 163 (38), 145 (24), and 136 (21). The spectrum of the glucoside isolated from rye seedlings is the same.

The identity of DIBOA was established by cims and by its transformation to BOA (as detected by tlc) upon heating. The mass spectrum of DIBOA is as expected, with the protonated molecular ion at *m/z* 182 (rel. % 42), and peaks at 166 (100), 148 (18) MH^+-H_2O_2, and 136 (95) MH^+-NO_2. Other ions were 167 (12), 165 (12), 164 (23), 138 (16), and 137 (11).

A. mollis proved to be a very good source of the glucoside: based on dry weight this compound comprises almost 4% of the seed. This value converts to 281 mmoles per kg dry weight. It is difficult to compare this measurement with many others in the literature for two reasons: first, most reported values have been based on fresh weights; and second, most measurements were made on the products of the glucoside, either DIBOA or BOA (and, in other cases, their methoxy derivatives). These methods presume quantitative conversion of the parent glucoside. In the present case, we preferred to measure the intact glucoside to avoid potential error due to incomplete hydrolysis or formation of alternate products.

A. mollis seeds are by far the richest known source of compound **1**. The next highest value reported is 10.3 mmoles per kg dry weight in corn seedling tissue, based on the measurement of BOA present (10). It is also interesting to note that this family of compounds is not found in any seeds of the grass species when present in the same seedlings (1,14,15).

BIOLOGICAL ACTIVITY.—Of the three compounds, DIBOA is the most active against velvetleaf (*Abutilon theophrasti* Medic.) seed germination (Table 1). A dose of 2

TABLE 1. Effect of BOA, DIBOA, and Glucoside **1** on Velvetleaf (*Abutilon theophrasti*) Seed Germination[a]

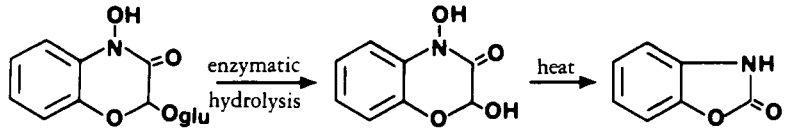
Compound	Dose (mM)	% Germination[b]	
		2 days	4 days
DIBOA	2	0[c]	0[c]
	1	6[c]	72[c]
	0.8	40[c]	82
	0.6	59[c]	97
BOA	5	14[c]	75
	1	111	116
	0.5	118	125[c]
Glucoside **1**	5	89	

[a]For complete assay procedure see ref. 21.

[b]Relative to controls.

[c]Significantly different than controls at 95% level or better by Chi-square 1-tailed test.

mM is lethal: none of the seeds germinated after two more weeks. At lower concentrations, germination was delayed, and seedling growth was slower than that of controls. BOA had no lasting effect at 5 mM, but at one-tenth that concentration actually promoted germination. Antigermination activity was not shown by the glucoside at 5 mM; however, it did have a pronounced effect on growth at this dosage.



glucoside **1** DIBOA BOA

These concentrations are within the range of those reported for other sorts of biological activities caused by BOA or DIBOA. Wahlroos and Virtanen (16) found that 3.7 mM BOA inhibits mycelial growth of *Fusarium nivale*; lethal doses are higher. *Helminthosporum maydis* is more sensitive: 0.1 mM BOA or DIBOA is growth inhibiting (17). Two to 8 mM DIBOA is effective against aphids (7). And, as mentioned earlier, BOA has been shown to inhibit radicle elongation of several species (19). It is interesting to note that rye plants have been implicated in allelopathy, and much effort has been made toward the isolation of the compounds responsible (24-31). Perhaps upon further investigation, the three compounds discussed in this report will be found to contribute to the phytotoxic effects of rye plants.

LITERATURE CITED

1. A.I. Virtanen and P.K. Hietala, *Acta Chem. Scand.*, **9**, 1543 (1955).
2. A.I. Virtanen and P.K. Hietala, *Acta Chem. Scand.*, **14**, 499 (1960).
3. P.K. Hietala and A.I. Virtanen, *Acta Chem. Scand.*, **14**, 502 (1960).
4. E. Honkanen and A.I. Virtanen, *Acta Chem. Scand.*, **14**, 504 (1960).
5. C.L. Tipton, J.A. Klun, R.R. Husted, and M.D. Pierson, *Biochemistry*, **6**, 2866 (1967).
6. C.-S. Tang, S.H. Chang, D. Hoo, and K.H. Yanagihara, *Phytochemistry*, **14**, 2077 (1975).
7. G.E. Zuniga, V.H. Argandona, H.M. Niemeyer, and L.J. Corcuera, *Phytochemistry*, **22**, 2665 (1983).
8. O. Wahlroos and A.I. Virtanen, *J. Pharm. Sci.*, **53**, 844 (1964).
9. J.A. Klun and J.F. Robinson, *J. Econ. Entomol.*, **62**, 214 (1969).
10. J.A. Klun, C.L. Tipton, J.F. Róbinson, D.L. Ostrem, and M. Beroza, *J. Agric. Food Chem.*, **18**, 663 (1970).
11. P.K. Hietala and A.I. Virtanen, *Acta Chem. Scand.*, **12**, 119 (1958).
12. A.J. Scism, J.N. BeMiller, and A.L. Caskey, *Anal. Biochem.*, **58**, 1 (1974).
13. M.D. Woodward, L.J. Corcuera, J.P. Hegelson, A. Kelman, and C.D. Upper, *Plant Physiol.*, **63**, 14 (1979).
14. V.H. Argandona, H.M. Niemeyer, and L.J. Corcuera, *Phytochemistry*, **20**, 673 (1981).
15. O. Wahlroos and A.I. Virtanen, *Acta Chem. Scand.*, **13**, 1906 (1959).
16. O. Wahlroos and A.I. Virtanen, *Acta Chem. Scand.*, **12**, 124 (1958).
17. D.H. Dawe, *Diss. Abstr. Int.*, B **34**, 3031 (1973).
18. J.A. Orcutt, J.P. Prytherch, M. Konicov, and S.M. Michaelson, *Arch Int. Pharmacodyn.*, **152**, 121 (1964).
19. A.T. Aiupova, L.V. Molchanov, C.S. Kadryov, N.A. Aliev, K. Giasov, N.P. Loi, Z. Tsoi, and A.A. Umarov, *Agrokhimiia*, **10**, 107 (1979).
20. G.F. Spencer, R.B. Wolf, and D. Weisleder, *J. Nat. Prod.*, **47**, 730 (1984).
21. R.B. Wolf, G.F. Spencer, and W.F. Kwolek, *Weed Sci.*, **32**, 612 (1984).
22. M.L. Thomson and D.C. DeJongh, *Can. J. Chem.*, **51**, 3313 (1973).
23. K. Bock and H. Thogersen, *Annu. Rep. NMR Spectrosc.*, **13**, 38 (1983).

24. Z.A. Patrick and L.W. Koch, *Can. J. Bot.*, **36**, 621 (1958).
25. H. Börner, *Bot. Rev.*, **26**, 393 (1960).
26. Z.A. Patrick, T.A. Toussoun, and W.C. Snyder, *Phytopathology*, **53**, 152 (1963).
27. D.H. Chou and Z.A. Patrick, *J. Chem. Ecol.*, **2**, 369 (1976).
28. D.G. Shilling and A.D. Worsham, *Weed Sci.*, Abstract #145 (1984).
29. D.G. Shilling and A.D. Worsham, *Weed Sci.*, Abstract #195 (1984).
30. J.P. Barnes and A.R. Putnam, *J. Chem. Ecol.*, **9**, 1045 (1983).
31. J.P. Barnes and A.R. Putnam, *Weed Sci.*, Abstract #146 (1984).

Received 18 June 1984