

SHORT COMMUNICATION



6-Hydroxy-5-nitrobenzo[d]oxazol-2(3H)-one—A degradable derivative of natural 6-Hydroxybenzoxazolin-2(3H)-one produced by *Pantoea ananatis*

Margot Schulz^a, Dieter Sicker^b, Oliver Schackow^b, Lothar Hennig^b, Diana Hofmann^c, Ulrich Disko^c, Marina Ventura^a, and Kateryna Basyuk^a

^aIMBIO Institute of Molecular Physiology and Biotechnology of Plants, University of Bonn, Bonn, Germany; ^bInstitut für Organische Chemie, Universität Leipzig, Leipzig, Germany; ^cIBG-3: Agrosphäre, Forschungszentrum Jülich GmbH, Jülich, Germany

ABSTRACT

Pantoea ananatis is a bacterium associated with other microorganisms on *Abutilon theophrasti* Medik. roots. It converts 6-hydroxybenzoxazolin-2(3H)-one (BOA-6-OH), a hydroxylated derivative of the allelochemical benzoxazolin-2(3H)-one, into 6-hydroxy-5-nitrobenzo[d]oxazol-2(3H)-one. The compound was identified by NMR and mass spectrometric methods. *In vitro* synthesis succeeded with *Pantoea* protein, with isolated proteins from the *Abutilon* root surface or with horseradish peroxidase in the presence of nitrite and H₂O₂. Nitro-BOA-6-OH is completely degraded further by *Pantoea ananatis* and *Abutilon* root surface proteins. Under laboratory conditions, 6-hydroxy-5-nitrobenzo[d]oxazol-2(3H)-one inhibits *Lepidium sativum* seedling growth whereas *Abutilon theophrasti* is much less affected. Although biodegradable, an agricultural use of 6-hydroxy-5-nitrobenzo[d]oxazol-2(3H)-one is undesirable because of the high toxicity of nitro aromatic compounds to mammals.

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6-Hydroxybenzoxazolin-2(3H)-one is produced by many plants as an intermediate for subsequent glycosylation when exposed to the allelochemical benzoxazolin-2(3H)-one.^{1,2} The latter presents the first transformation/degradation product of the bioactive secondary metabolite 2,4-dihydroxy-2H-1,4-benzoxazin-3(4H)-one (DIBOA) occurring in rye and other grasses as glucosides and as an aglycone after root exudation. Bioactive benzoxazinoids are presently investigated for their use as natural insecticides, but the compounds can cause also allelopathic suppression of neighboring plants. However, many plants counter allelopathic suppression by sophisticated detoxification strategies. *Abutilon theophrasti*, a widespread weed, is rather insensitive to benzoxazolin-2(3H)-one. Root-associated microorganisms support the plant in coping with the allelochemical.^{3–5} In this context *Pantoea ananatis*—an Enterobacteriaceae that exists in many aquatic and terrestrial environments as well as in association with plants, insects and humans⁶—was identified as one member of a microbial micro-community that can colonize *Abutilon theophrasti* roots when growing in soils rich in organic matter. Incubation of the bacterium *Pantoea ananatis* with 6-hydroxybenzoxazolin-2(3H)-one (BOA-6-OH) led first to a bright yellow and then to an orange colored medium coincident with the

disappearance of the added BOA-6-OH. To isolate compounds presumably responsible for the colors, the media were immediately used after occurrence of the bright yellow color. TLC of the EtOAc extracts of the media resulted in a major yellow band, traces of an orange and another wine red band which show increasing hydrophilicity. Treatment of the yellow material with KOH or NaOH led to an orange and then to the wine-red colored solution whereas NaCl and KCl addition had no effect. This indicates that the shift to alkaline pH is responsible for the color changes, accompanied by alterations in the solubility. It was therefore concluded, that only one compound is responsible for the different colors. Nevertheless, the bright yellow and the orange preparations were both used for structure identification.

Structure identification

Structural investigation was made using different spectroscopic methods (details and spectra can be found in the supplemental online material). The analyses started with UPLC-ESI-MS measurements, revealing a molecular weight of 196 for the unknown substance. The UPLC-ESI-HR-MS data are coincident with further mass spectrometric analyses. The NMR assignment was

CONTACT Margot Schulz  ulp509@uni-bonn.de  IMBIO Institute of Molecular Physiology and Biotechnology of Plants, Karlrobert Kreiten Strasse 13, 53115, Bonn, Germany.

 Supplemental data for this article can be accessed on the [publisher's website](#).

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performed using standard 2D techniques (HSQC, HMBC). Characteristic shift differences in ^1H and ^{13}C were found between a neutral and ionic form of the compound. Additionally, line broadening of C-5, C-6 and C-7 was observed in the carbon spectrum of the salt. A bathochromic shift in the spectrum from 383 nm to 418 nm occurred as a result of salt formation. According to these data, the colored compound produced by *Pantoea ananatis* was identified as 6-hydroxy-5-nitrobenzo[d]oxazol-2(3H)-one. It exists in the medium first in a yellow neutral and then in an orange anionic form due to the increasing pH during continued culturing (Fig. 1).

In vitro synthesis of 6-hydroxy-5-nitrobenzo[d]oxazol-2(3H)-one

BOA-6-OH was converted to 6-hydroxy-5-nitrobenzo[d]oxazol-2(3H)-one by proteins isolated from *P. ananatis*

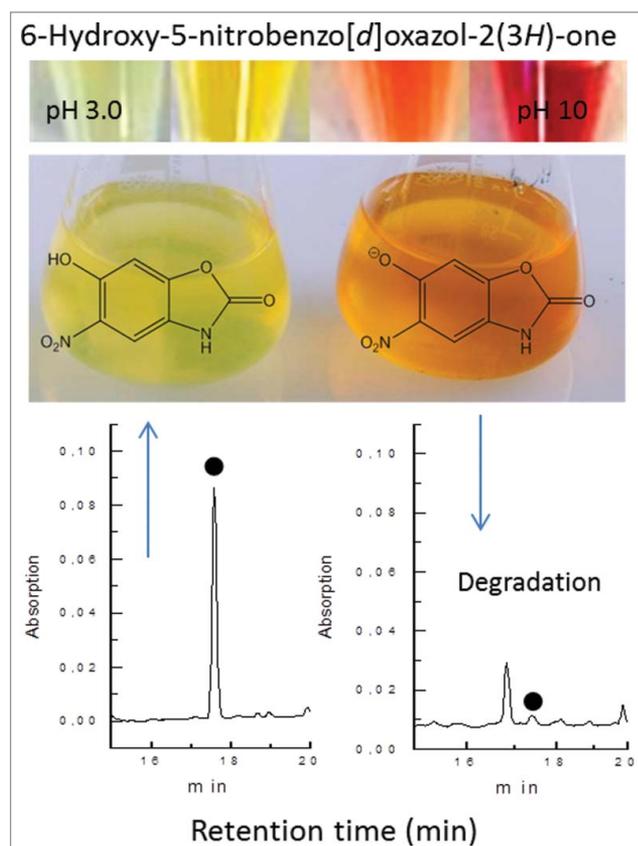


Figure 1. The color of 6-hydroxy-5-nitrobenzo[d]oxazol-2(3H)-one depends on pH: pH 3 greenish; pH 7 yellow; pH 8 orange; pH 9–10 wine-red. *Pantoea ananatis* cultures, producing the compound from BOA-6-OH, change the color from yellow to orange due to the pH value which increases over time. The anionic, orange form of the compound is degraded, as shown by the HPLC chromatograms (left: analysis of the yellow medium immediately after adding synthetic nitro-BOA-6-OH (black points) before the medium turned to orange. After 3 h (right), only traces of the compound are left.

in presence of nitrite and H_2O_2 . Replacing nitrite with nitrate decreased product formation (Fig. 2). Thus, we concluded that nitrate present in the Czapek medium is reduced by bacterial nitrate reductase and the resulting nitrite is used for the nitration of BOA-6-OH by a peroxidase. Secretion of peroxidase and periplasmic nitrate reductase by certain bacteria is known.^{7,8} *Pantoea ananatis* possesses genes for nitrate reductase (UniProtKB-A0A0H3KVM0 (A0A0H3KVM0_PA-NAA) and catalase/hydroperoxidase (NCBI Reference Sequence: WP_014592998.1).^{9,10} Nitrate reductase may be located within the periplasmic space of the Gram-negative *Pantoea ananatis*.

To determine whether peroxidase activity is able to perform the nitration step, commercial horseradish peroxidase (HRP) was investigated. This enzyme was successfully used by Sakihama et al.¹¹ for nitration of p-coumaric acid and cinnamic acids. Similar to the observations of Sakihama et al.¹¹ with other acceptor molecules, nitration of BOA-6-OH in position 5 was almost immediately accomplished after application of horseradish peroxidase (Fig. 2) resulting in a product identical to the previously identified compound. The OH group at C-6 might increase electron density at C-5 and C-7 due to its own donor effect, which facilitates nitration. For steric reasons, C-5 should be preferred for electrophilic attacks and is therefore favored for nitration of BOA-6-OH. Since benzoxazolin-2(3H)-one (BOA) lacks the OH group, nitration with horseradish peroxidase failed when BOA was used as a substrate in the assay. Aromatic nitro compounds are rare in nature¹² and natural nitrated benzoxazolinones have not been previously

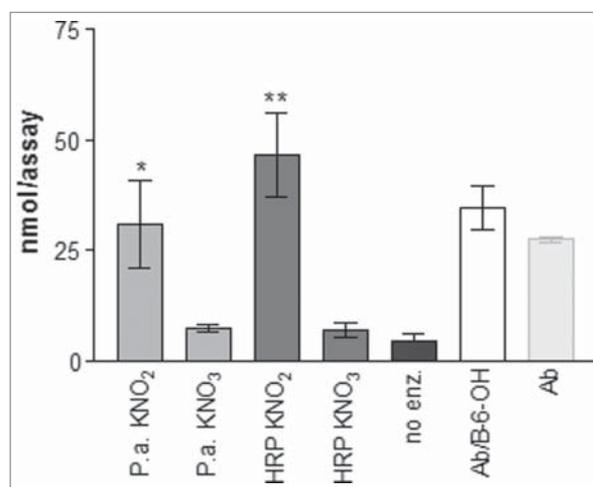


Figure 2. Enzymatic nitration by *Pantoea ananatis* (P.a.) exuded protein, horseradish peroxidase (HRP) and *Abutilon* (Ab) root surface proteins in presence of nitrite; P.a. and HRP also in the presence of nitrate. No enz. = no enzyme was added to the assay. Ab/B-6-OH: root surface proteins from seedlings pre-incubated with BOA-6-OH. Means \pm SD are shown; asterisk(s) indicate significant differences (t-test, * = $p < 0.05$; *** = $p < 0.0001$).

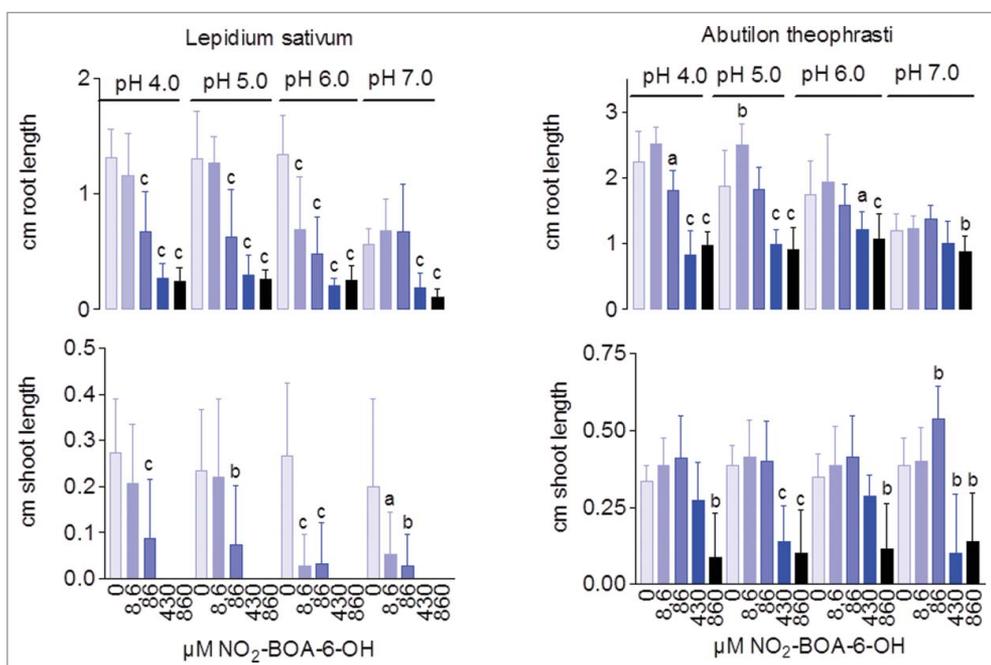


Figure 3. 6-Hydroxy-5-nitrobenzo[*d*]oxazol-2(3*H*)-one (NO₂-BOA-6-OH) inhibits the growth of *Lepidium sativum* (I50 values: lowest at pH 6.0 with 20 μ M for root growth and 10 μ M for the shoot growth). As expected, *A. theophrasti* is rather insensitive. The I50 values for nitrated BOA-6-OH increased with pH from averaged 300 μ M at pH 4.0 and 5.0 to 1.7 mM at pH 6.0 and 2 mM at pH 7.0 for root growth. The averaged I50 value of 0.7 mM for *Abutilon* shoot growth was approximately the same for all tested pH values. Means \pm SD are shown; different letters indicate significant differences (t-test, a = $p < 0.05$; b = < 0.005 ; c = $p < 0.0001$).

reported. Zikmundova et al.¹³ who identified N-(2-hydroxy-5-nitrophenyl)acetamide and N-(2-hydroxy-3-nitrophenyl)acetamide as fungal detoxification products of benzoxazolinone-derived 2-aminophenol, assumed an enzymatic reduction of nitrate, a constituent of the culture medium, and use of the resulting nitrite for nitration. This mechanism was also suggested by Rousseau et al.¹⁴ for tocopherol nitration by *Streptomyces catenulae*.

A question was whether the compound would be formed at the root surface. Therefore, the root surface proteins were collected and assayed for peroxidase-dependent synthesis in the presence of nitrite. After 3 min, approximately 30 nmol of nitro compound could be extracted from the assay mixture; when seedlings were pre-incubated with 2 mM BOA-6-OH (Fig. 2) more product was formed.

Degradation of 6-Hydroxy-5-nitrobenzo[*d*]oxazol-2(3*H*)-one

Since the compound disappeared during prolonged cultivation of *P. ananatis*, biological degradation was considered because the isolated, purified compound did not show any indication of instability. To elucidate whether *P. ananatis* can metabolize 6-hydroxy-5-nitrobenzo[*d*]oxazol-2(3*H*)-one when existing in the anionic form, the bacterium was taken from a previous culture grown in the presence of BOA-6-OH and incubated with the purified, *in vitro*

synthesized compound. Aliquots of the media revealed that anionic 6-hydroxy-5-nitrobenzo[*d*]oxazol-2(3*H*)-one is almost completely metabolized within 3 h (Fig. 1). The compound is also degraded by proteins collected from the *Abutilon* root surface colonized by the microbial community (shown in the supplemental online material). Thus, 6-hydroxy-5-nitrobenzo[*d*]oxazol-2(3*H*)-one presents an intermediate of a catabolic sequence.

The nitration of 6-hydroxybenzoxazolin-2(3*H*)-one and its subsequent degradation presents a hitherto unknown possibility for the elimination of benzoxazolinone by a plant-bacterium cooperation. For BOA-6-OH, nitration seems to be a necessary step to initiate degradation. Electron-withdrawing NO₂-group substitution at the aromatic ring position 5 may facilitate enzymatic attacks by destabilizing the heterocyclic molecule and starting the decomposition. It is known that nitro aromatic compounds can be completely degraded by several bacteria such as *Pseudomonas*, *Ralstonia* and *Comamonas* species resulting in small biooxidizable molecules that can enter the TCA cycle.¹⁵

Bioassays

The purified compound was tested for its effects on germinating seeds of *Abutilon theophrasti* and *Lepidium sativum* using the bioassay procedure of Macias and

coworkers.¹⁶ Since the water solubility of the compound changes with pH, the bioassays were performed at pH 4, 5, 6 and 7.0. *Abutilon* root growth was, however, reduced with increasing pH, the growth of *Lepidium* roots only at pH 7.0. *L. sativum* exhibits a higher sensitivity to nitrated-BOA-6-OH than *Abutilon theophrasti* at all pH values (Fig. 3). Exposure to low amounts of nitro-BOA-6-OH results in shoot growth stimulation of *Abutilon* (significant with 86 μM at pH 7.0.). Taken together, nitro-BOA-6-OH is less toxic for *Abutilon* than for *Lepidium*. The results of the bioassays obtained under laboratory conditions indicate that only sensitive plant species such as *L. sativum* may be considerably inhibited, but it is questionable whether the compound has any phytotoxic effects under field conditions when bacteria able to degrade the compound are present. An agricultural use of nitrated BOA-6-OH is undesirable because of the known toxicity of nitro aromatic compounds against animals due to their mutagenic, carcinogenic and teratogenic properties.^{17,18} The results are in line with the formerly observed insensitivity of *A. theophrasti* against benzoxazinoids.³⁻⁵ It is assumed that root associated microorganisms can trigger effects of benzoxazinone derived allelochemicals by molecule modifications and by shortening the time of exposure due to degradation processes.

Materials and methods

Pantoea ananatis was isolated from a microbial micro-community colonizing *Abutilon theophrasti* roots. The identity of the bacterium was confirmed by the DMSZ (Leibniz-Institut - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig). The bacterium was pre-cultured in LB medium for 2 weeks. One ml of the cultures was added to 50 ml flasks with 25 ml liquid Czapek medium supplemented with 1–5 mg BOA-6-OH (stocks from Dieter Sicker, University of Leipzig). Subsequent culturing was without shaking at 23 °C. The culture media were filtered as soon as the color of the medium had turned luminous yellow and the filtrate was extracted with ethyl acetate. The organic phase was evaporated to dryness in vacuo and the yellow residue was dissolved in ethyl acetate. The fraction was purified on TLC Silgur-25 sheets (Macherey-Nagel) with water-saturated chloroform:EtOAc 3:2 as the liquid phase. The yellow band was extracted with EtOAc, centrifuged and evaporated to dryness. The remaining solid was washed with water. Yellow crystals precipitated during washing. They were collected, and the residual liquid was evaporated. During the first extraction, a portion of the yellow compound turned to orange accompanied by a higher hydrophilicity. The orange portion could be separated by further TLC runs using the liquid phase

mentioned above. The orange band was extracted with 50% methanol. The solution was centrifuged and evaporated to dryness.

Identification of 6-hydroxy-5-nitrobenzo[*d*]oxazol-2(3*H*)-one based on the following data:

6-hydroxy-5-nitrobenzo[*d*]oxazol-2(3*H*)-one: yellow solid; UV-Vis (MeOH) λ_{max} 223, 250, 291, 380 nm. IR (KBr): $\sim \nu$ [cm^{-1}] = 3443, 3079, 1816, 1622, 1540, 1482. ¹H-NMR (DMSO-*d*₆, 300 MHz) δ 7.54 (1 H, s, H-4), 7.04 (1H, s, H-7). ¹³C-NMR (DMSO-*d*₆, 75 MHz) δ 154.6 (C, C-2), 150.9 (C, C-6), 149.1 (C, C-7a), 132.2 (C, C-5), 123.7 (C, C-3a), 105.6 (CH, C-4), 100.7 (CH, C-7). HR-MS (ESI negative): 195.00497; Calc. C₇H₄N₂O₅: 196.01202. EI-MS *m/z* 196 [M]⁺ (100), 180 (1), 179 (2), 166 (10), 150 (4), 149 (2), 138 (5), 122 (4), 121 (4), 106 (7), 93 (5), 80 (10), 69 (21), 53 (17), 52 (18), 39 (10). Salt of 6-hydroxy-5-nitrobenzo[*d*]oxazol-2(3*H*)-one: orange, amorphous; UV-Vis (MeOH): λ_{max} 231, 291, 418 nm; IR (KBr): $\sim \nu$ [cm^{-1}] = 3647, 3432, 1699, 1532, 1471, 1439; ¹H-NMR (DMSO-*d*₆, 300 MHz) δ 7.20 (1 H, s, H-4), 6.40 (1H, s, H-7). ¹³C-NMR (DMSO-*d*₆, 75 MHz) δ 160.4 (C, C-2), 158.7 (C, C-6), 155.0 (C, C-7a), 131.7 (C, C-5), 129.3 (C, C-3a), 103.7 (CH, C-4), 99.8 (CH, C-7). The nature of the cation could not be detected by spectroscopy. However, the Czapek medium contains mainly potassium salts, and the orange compound is likely a K⁺ salt of the 6-hydroxy-5-nitrobenzo[*d*]oxazol-2(3*H*)-one

The synthesis of the BOA-6-OH derivative succeeded with *Pantoea ananatis* protein, horseradish peroxidase (HRP) and *Abutilon* root surface protein. *Pantoea ananatis*, cultured as described above with BOA-6-OH, was pelleted at 4000 g for 15 min from cultures that started to turn yellow. The proteins in the supernatant were precipitated on ice with 80% (NH₄)₂SO₄. After centrifugation at 10,000 g, the precipitate was dissolved in 50 mM phosphate buffer pH 5.8 and desalted with PD-columns using the phosphate buffer for elution. Fractions with the highest protein content according to the Bradford method were combined and assayed for peroxidase activity. To determine the class of enzyme responsible for introduction of the NO₂-group, additional assays were run with horseradish peroxidase (HRP; Sigma). The assays contained 50 μl 50 mM phosphate buffer pH 5.8, 50 μl of the protein preparation, 160 μM NaNO₂, 80 μM BOA-6-OH, 140 μM H₂O₂, 8 μM FeSO₄ and 40 μM catalase inhibitor 3-amino-1,2,4-triazole (Sigma). Assays with HRP were performed with 1 μl enzyme solution (100 mU), 98 μl 50 mM phosphate buffer pH 5.8, 160 μM NaNO₂, 80 μM BOA-6-OH, and 140 μM H₂O₂. After rigorous vortexing with 200 μl EtOAc, the phases were separated by centrifugation for 2 min at 10,000 g, and the organic phase was analyzed for products by HPLC-DAD (Shimadzu) using a 0–100% methanol gradient as described.⁵

Abutilon theophrasti seeds were purchased from Herbiseed (UK). They were germinated and grown hydroponically under greenhouse conditions for 10 d on cheesecloth-covered containers filled with tap water. The seedlings were transferred successively with their roots to 5 ml 50 mM phosphate buffer pH 5.8 supplemented with protease inhibitor cocktail (Sigma) and sonicated for 5 sec. The resulting protein solution was centrifuged for 10 min at 10000 g and 4 °C. The supernatant was assayed for 6-hydroxy-5-nitrobenzo[d]oxazol-2(3H)-one synthesis using the assay conditions described for the *P. ananatis* protein for 3 min. All EtOAc phases of the assays were analyzed by HPLC. Calculations of the product amounts based on standard curves established with the purified 6-hydroxy-5-nitrobenzo[d]oxazol-2(3H)-one.

For the degradation of 6-hydroxy-5-nitrobenzo[d]oxazol-2(3H)-one, BOA-6-OH primed *Pantoea ananatis* cultures were supplemented with 500 μ l 40 μ M 6-hydroxy-5-nitrobenzo[d]oxazol-2(3H)-one and incubated at 25 °C. Aliquots of the culture medium were taken directly after addition of the nitro-compound and then every hour over a period of 3 h and analyzed by HPLC. The *Abutilon* root surface protein fraction was assayed for compound degradation by adding 5 μ l of 10 mM 6-hydroxy-5-nitrobenzo[d]oxazol-2(3H)-one to 50 μ l phosphate buffer (pH 6.5).

The inhibitory activity of the nitro compound was bioassayed with *Abutilon theophrasti* and *Lepidium sativum*. *Lepidium sativum* seeds were purchased from a local garden supplier. *Abutilon* and *Lepidium* seedlings (*Abutilon* 8; *Lepidium* 15 germinating seeds/ dish) with emerging radicles were transferred on filter paper placed in Petri dishes. The bioassays were performed as described by Macias and coworkers,¹⁶ at pH 4.0, 5.0, 6.0 (all 100 mM Mes buffers) and 7.0 (100 mM Hepes buffer) with each 0, 8.6, 86, 430 and 860 μ M 6-hydroxy-5-nitrobenzo[d]oxazol-2(3H)-one. Root and shoot lengths were measured after 1.5 d. The bioassays were repeated 5 times. IC₅₀ values were determined as described by Baerson and coworkers.¹⁹ The t-test was used for statistical analysis of all data. The error bars are based on the SD. In figures, the data are presented as the mean \pm standard deviation. Each data point is based on at least 3 biologic replicates from 3 independent experiments, if not otherwise noted.

Abbreviations

6-Hydroxy-5-nitrobenzo NO₂-BOA-6-OH
[d]oxazol-2(3H)-one

BOA-6-OH 6-hydroxybenzoxazolin-2
(3H)-one
UPLC-ESI-MS Ultra Performance Liquid
Chromatography- Electrospray
Ionisation Mass Spectrometry

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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