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Chemical Detoxification of Small Molecules by *Caenorhabditis elegans*

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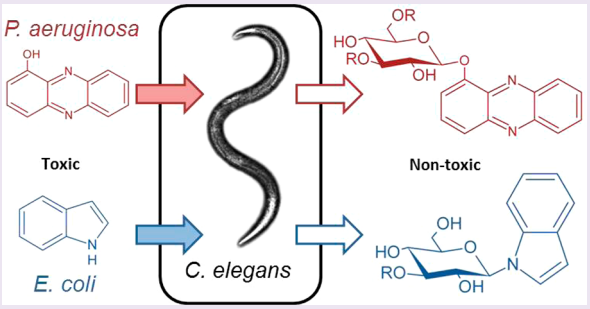
Supporting Information

ABSTRACT: *Caenorhabditis elegans* lives in compost and decaying fruit, eats bacteria and is exposed to pathogenic microbes. We show that *C. elegans* is able to modify diverse microbial small-molecule toxins via both *O*- and *N*-glucosylation as well as unusual 3′-*O*-phosphorylation of the resulting glucosides. The resulting glucosylated derivatives have significantly reduced toxicity to *C. elegans*, suggesting that these chemical modifications represent a general mechanism for worms to detoxify their environments.



Nematodes live in complex microbial environments that present many potential challenges to their health and viability. *Caenorhabditis elegans*, a commonly used model nematode, has an innate immune system that shares many characteristics with mammalian defenses[1,2] and is susceptible to many mammalian pathogens.[3] Its immune system is highly specific: worms can distinguish and mount distinct responses to different pathogens.[4−7] *C. elegans* possesses an array of immune effectors and detoxification enzymes known to be involved in microbial defense and xenobiotic detoxification. Distinct sets of immune effectors, including lysozymes, lipases, and antibacterial peptides, as well as detoxification enzymes such as UDP-glucuronosyl/UDP-glucosyltransferases, cytochrome P450s, and glutathione *S*-transferases, are modulated during these responses.[4−8] Having a large arsenal of detoxification and defense genes is not surprising for an organism that lives in environments rich in decomposing organic material, areas in which potential pathogens are also likely to thrive. In this study we examined two unrelated bacterial toxins, 1-hydroxyphenazine (1-HP, **1**) and indole (**6**), released by *Pseudomonas aeruginosa* and *Escherichia coli*, respectively.[9] Both toxins can kill *C. elegans*, and we found that worms glycosylate both toxins, a modification that significantly lowers their toxicity.

P. aeruginosa is a Gram-negative opportunistic human pathogen that is commonly found in soil and water, although it can survive in a number of environments and is a frequent cause of infection in immunocompromised patients.[10] Several different strains have been used extensively as model pathogens to identify virulence and host defense factors, including PA14, PAK, and PAO1.[3] *P. aeruginosa* PA14 kills *C. elegans* by two distinct modes of action: when grown on low-osmolarity or minimal media agar plates, the pathogen kills over the course of several days,[5] while PA14 grown on high-osmolarity media is able to kill L4 stage *C. elegans* in as little as 6 h through the production of diffusible toxins.[11,12] At least some of these toxins have been identified as phenazines,[11,13] which are redox-active small molecules that are thought to cause damage to cells by producing reactive oxygen species and disrupting normal redox reactions.[14,15] *E. coli* is typically used as a food source for *C. elegans* in the laboratory, but *E. coli* produces indole, a compound that is toxic to many animals and has nematicidal properties.[16] As described in detail below, we have found that *E. coli* bacterial pellets used for feeding *C. elegans* contain indole at concentrations of 3.3 mM, which is sufficient to kill *C. elegans*.

We asked whether *C. elegans* employs specific metabolic transformations to eliminate bacterial toxins such as phenazines and indole. After challenging young adult worms in large-scale liquid culture for 24 h with 200 μM of 1-HP (**1**), HPLC-UV analysis of worm media samples revealed at least three new compounds (**2**, **4**, **5**) present in samples from worms exposed to 1-HP but not present in control samples (Supplementary Figure 1a). In addition, we analyzed whole-body extracts of 1-HP-challenged worms and found at least one novel UV peak (**3**) that was absent in control extracts (Supplementary Figure 1b). These putative 1-HP metabolites were isolated via preparative HPLC and subsequently identified using MS and NMR spectroscopy (Figure 1, Supplementary Figures 2−5,

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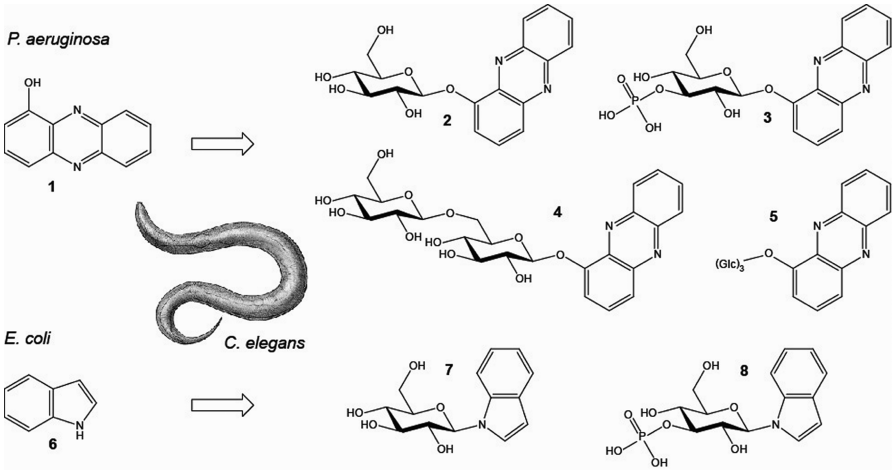

Figure 1. Chemical conversion of 1-hydroxyphenazine (1-HP) (**1**) and indole (**6**) by *C. elegans*. 1-HP is converted into 1-*O*-(β-D-glucopyranosyl)-phenazine (**2**), 1-*O*-(β-D-gentiobiosyl)-phenazine (**4**), and two phenazine-trisaccharides (**5**), which are found in the media and worm pellet. 1-*O*-(3′-*O*-Phospho-β-D-glucopyranosyl)-phenazine (**3**) is found only in the worm pellet. Indole (**6**) is converted into *N*-(β-D-glucopyranosyl)-indole (**7**) and *N*-(3′-*O*-phospho-β-D-glucopyranosyl)-indole (**8**), which are found in the media and pellet.

Supplementary Table 1). We identified 1-*O*-(β-D-glucopyranosyl)-phenazine (**2**) and 1-*O*-(β-D-gentiobiosyl)-phenazine (**4**) as the two most abundant metabolites in the worm media samples, which were accompanied by two compounds (**5**) whose mass and NMR spectra suggested phenazine trisaccharides; however, strong overlap in the NMR spectra of these two compounds prevented their full characterization. In addition, we identified the major phenazine derivative detected in the worm body extracts as 1-*O*-(3′-*O*-phospho-β-D-glucopyranosyl)-phenazine (**3**).

These results show that *C. elegans* converts 1-HP into a series of glycosylated derivatives, including a compound featuring an unusual phosphate substitution. Next we asked whether *C. elegans* also modifies *E. coli*-derived indole (**6**). Notably, HPLC analysis of *C. elegans* large-scale liquid culture extracts revealed only trace amounts of indole 24 h after feeding with indole-rich *E. coli* bacteria. Instead, HPLC analysis revealed two prominent peaks with UV and mass spectra indicative of highly polar indole derivatives. NMR spectroscopic analysis (Figure 1, Supplementary Figure 6, Supplementary Tables 1 and 2) of extract fractions containing these compounds revealed *N*-(β-D-glucopyranosyl)-indole (**7**), of which we prepared an authentic sample via synthesis. In addition, we identified *N*-(3′-*O*-phospho-β-D-glucopyranosyl)-indole (**8**), in direct analogy to the phosphorylated 1-HP derivative (**3**). We quantified the indole glucoside by HPLC-UV and found that more than 80% of indole provided with the OP50 diet appears to be converted into 7 (Supplementary Figure 7a). Analysis of worm body and supernatant extracts further revealed that indole glucoside is predominantly released by *C. elegans* and accumulates in the media (Supplementary Figure 7b). We also studied the time course of the *N*-glucosylation reaction by feeding of [U-D_7]-indole to *C. elegans* liquid cultures and found that the majority of exogenous indole is converted into the glucosides within 6 h (Figure 2c).

Our results demonstrate that *C. elegans* modifies two chemically distinct bacterial toxins, 1-HP and indole, by glycosylation and additional 3′-*O*-phosphorylation, suggesting that these modifications may be part of a general detoxification pathway in the nematode. Therefore, we tested the modified glycosides for their toxicity to *C. elegans*. For this purpose we used synthetic *N*-(β-D-glucopyranosyl)-indole (**6**) and isolated samples of the 1-HP glycosides (**2**–**5**). Using a plate-based assay, we found that whereas 1-HP at concentrations of 200 μM kills about 80% of L4 worms after 6 h, exposure to the same concentrations of 1-HP glycosides results in less than 5% mortality (Figure 2a,b). Next we compared the toxicity of indole and *N*-(β-D-glucopyranosyl)-indole in plate and liquid-culture assays. We found that indole is more toxic in liquid media assays in comparison to plate-based assays (not shown). In liquid media indole kills *C. elegans* at concentrations of 4 mM, whereas lower concentrations of 2 and 3 mM result in reversible paralysis. In contrast, indole glucoside did not kill or elicit paralysis at concentrations as high as 5 mM (Figure 2d–f). Although our toxicity assays differ significantly from the large-scale liquid culture conditions employed for isolation of glucosylation products, our results demonstrate that the bacterial toxins, 1-HP and indole, kill *C. elegans*, whereas their glucosylation products are much less toxic.

C. elegans possesses at least 60 ABC transporter genes, 15 of which are p-glycoproteins (pgp), a subfamily of ABC transporters that is presumed, by homology to mammalian genes, to be involved in drug resistance.[17] Several *C. elegans* pgp genes have been shown to be involved in xenobiotic transport.[18] A previous study showed that a *C. elegans pgp-1;pgp-3* double mutant was much more susceptible to fast killing by PA14 than wild type worms (N2) and that this susceptibility was dependent on production of phenazines by PA14.[11] Although we confirmed increased susceptibility of the *pgp-1;pgp-3* mutants to PA14, there was no increase in lethality of the double mutant compared to wild-type when exposed to 1-HP (Supplementary Figure 8). We tested a selection of *pgp* knockout strains and identified a second strain (a *pgp-12* knockout, VC26) that is more susceptible than N2 worms to PA14. Furthermore, VC26 worms are more susceptible to 1-HP than wild type, suggesting that *pgp-12* is involved in detoxification of 1-HP (Supplementary Figure 8). However, *pgp-12* mutant worms are able to produce and release 1-HP glycosides (Supplementary Figure 9), suggesting that other transporters contribute to detoxification in addition to *pgp-12*. These results show that the *C. elegans* detoxification machinery is based, at least in part, on components that are homologues of

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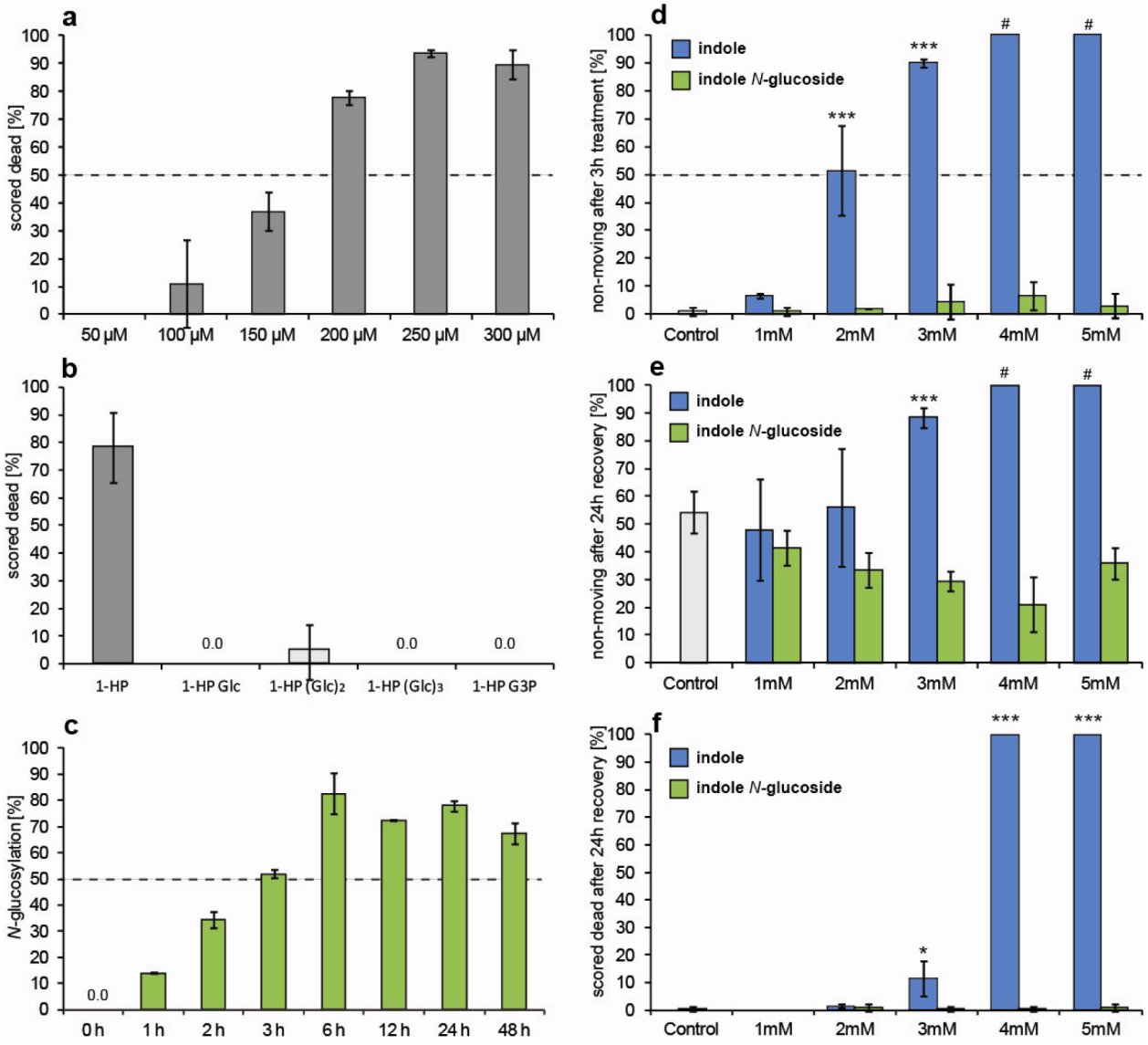

Figure 2. Toxicity assays. Error bars represent mean ± SD. (a) Killing of L4 stage worms on M9 agar plates containing increasing concentrations of 1-HP after 6 h (N = 2 or 3 with 20−40 worms per trial). (b) Killing of L4 stage worms on M9 agar plates containing 200 μM of each phenazine glucoside after 6 h (N = 3 with 40−50 worms). (c) Time course for N-glucosylation of indole by *C. elegans* determined by application of 0.5 mg/100 mL [U-D_7]-indole. (d) Paralysis of L4 worms after treatment with increasing amounts of indole or indole-N-glucoside in M9 media for 3 h (#: worms scored dead after recovery: see panel f). (e) Paralysis of L4 worms after treatment with indole or indole-N-glucoside for 3 h and 24 h recovery in M9 (#: worms scored dead after recovery: see panel f). (f) Killing of L4 worms after treatment with indole or indole-N-glucoside for 3 h and 24 h recovery in M9 (N = 4 with 50 worms per trial).

genes involved in mammalian small-molecule detoxification. Because transporters and glycosyltransferases often have broad specificity and thus their substrate ranges may overlap, more detailed characterization of this pathway will require systematic study of deletion mutants of different combinations of transporter and glycosyltransferase genes.

The glycosylation and release of a common anthelminthic drug, albendazole,[19] and of several small molecules from a synthetic library[20] have been described, but the function of glycosylation in *C. elegans* has not previously been explored. In this paper, we demonstrate that N- and O-glycosylation serve to detoxify two chemically very different xenobiotics, indole and 1-HP, indicating that glycosylation and subsequent phosphorylation represent general mechanisms to convert and remove environmental toxins. This pathway may involve specific glycosylation enzymes and transporter proteins, whose identification will enable comparing the detoxification systems of nematodes with those of other metazoans. Furthermore, existing treatments of parasitic nematode infections in humans, livestock, and plants could be augmented by inhibiting small molecule glycosyltransferases and/or transporters to increase drug efficacy by slowing down or preventing release into the environment.[19,21]

METHODS

Toxicity Assays (Phenazine). *C. elegans* N2 eggs were arrested at the L1 stage for 24 h in M9 buffer and then grown for 42−48 h at 20 °C on NGM plates to L4 stage as verified by observation with a stereoscope. Worms were washed off plates, allowed to settle, and dispensed onto test plates made with M9 buffer and 2% agar (a small lawn of OP50 was placed on the plate to discourage worms from

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crawling off). After 6 h, worms that failed to respond to physical touch with a platinum pick were scored as dead.

Toxicity Assays (Indole). Toxicity was determined in 12-well plates using 50 worms per well, which were placed into 0.5 mL of M9 buffer (control), or buffer containing 1, 2, 3, 4, or 5 mM indole or indole glucoside; 0.1% methanol was present in all experiments. Worms were inspected for spontaneous movement after 3 h, the solution was removed, and the worms were washed 2 times with 2 mL of fresh M9 buffer. After 24 h recovery, worms were inspected for spontaneous movement and scored for survival by physical touch with a platinum pick.

Quantification of Indole in *E. coli*. *E. coli* OP50 from a 1 L culture was centrifuged at 5251*g* for 45 min, and the resulting bacterial pellet (3.1 mL) was lyophilized. The resulting material (637 mg) was sonicated in 5 mL methanol (10 min) and extracted with methanol (3 × 15 mL). The filtered extract was concentrated *in vacuo*, and the residue was taken up in 3 mL methanol and analyzed by HPLC using a DAD-detector. Indole was quantified by comparison of the UV absorbance at 230 and 260 nm with those of a synthetic standard of known concentration.

Glucoside Collection and Analysis (Phenazine). Adult *C. elegans* were synchronized with a bleach solution. The eggs were hatched overnight into L1s, which were then grown at a worm density of 10,000 worms/mL at 22 °C at 250 rpm in S-complete medium supplemented with 2% *E. coli* (strain HB101) for 43 h until they reached young adult stage verified by observation with a stereoscope. The worms were concentrated to 30,000 worms/mL, washed once with M9, and fed 1% *E. coli* HB101. 1-HP from a DMSO stock or the appropriate amount of DMSO was added to the cultures, which were incubated at 22 °C at 250 rpm for 24 h. The supernatant was removed by centrifugation (2,000 rpm, 2 min), filtered through a 0.22 μm nitrocellulose filter, lyophilized, extracted with 5 mL methanol, filtered, and concentrated. The residue was resuspended in 1 mL of methanol and used for HPLC analysis. The worm pellet was washed 4 times in M9 buffer, resuspended in 1 vol of 80% methanol and homogenized with a BioSpec MiniBeadbeater-8 (3 cycles of 30 s, with 1 min on ice). The lysate was spun at 14,000 rpm for 10 min, and the supernatant was dried under N_2 gas. The residue was resuspended in 1 mL of methanol and used for HPLC analysis.

Worm water was analyzed on an Agilent 1100 Series HPLC system equipped with a diode array detector and an automated fraction collector. Absorbance was monitored at 254 nm. For worm media separation, an aqueous methanol gradient was used from 5% to 95% on a Zorbax SB C-18 column (4.6 cm × 150 mm, 5 μm particle diameter). For worm pellet separation, 5% methanol (A) and 95% 5 mM Phosphate buffer pH 7.2 (B) was held isocratically for 4 min, increasing to 95% A and 5% B over 30 min and then held for 5 min, followed by re-equilibriation of the column, at a flow rate of 2 mL min^{-1} in a Zorbax SB C-18 column (9.4 cm × 250 mm, 5 μm). Fractions were collected automatically by peak detection. Aliquots of each fraction were analyzed by high-resolution mass spectrometry by the University of Florida Spectroscopy Service in the Chemistry Department or in the Biomedical Mass Spectrometry Core at the Clinical and Translational Science Institute at the University of Florida.

Glucoside Collection and Analysis (Indole). *C. elegans* worms from 10 cm NGM plates were washed using M9 medium into a 100 mL S-medium preculture where they were grown for 4 days at 22 °C on a rotary shaker at 220 rpm. Concentrated OP50 derived from 1 L of bacterial culture (grown for 16 h in LB media) was added as food at days 1 and 3. Subsequently, the preculture was divided equally into four 1 L Erlenmeyer flasks containing 400 mL of S-medium for a combined volume of 425 mL of S-medium, which was then grown for an additional 10 d at 22 °C on a rotary shaker. Concentrated OP50 derived from 1 L of bacterial culture was added as food every day from days 1 to 9. Subsequently, the cultures were centrifuged, and the supernatant media and worm pellet were lyophilized separately. The lyophilized materials were extracted with 95% ethanol (250 mL 2 times) at RT for 12 h. The resulting yellow suspensions were filtered, and the filtrate was evaporated *in vacuo* at RT, producing media and worm pellet metabolite extracts. The media metabolite extract from two cultures was adsorbed on 6 g of octadecyl-functionalized silica gel and dry loaded into an empty 25 g RediSep *Rf* sample loading cartridge. The adsorbed material was then fractionated via a reversed-phase RediSep *Rf* GOLD 30 g HP C18 column using a water–methanol solvent system, starting with 100% water for 4 min, followed by a linear increase of methanol content up to 100% methanol at 42 min, which was continued up until 55 min. The fractions generated from this fractionation were evaporated *in vacuo*, and the residue was analyzed by HPLC-MS and 2D-NMR spectroscopy.

Nuclear Magnetic Resonance. Pooled fractions were dried, resuspended in 150 μL of 99.95% methanol-d_4 with 0.111 mM TSP as an internal standard, and transferred into 2.5 mm NMR tubes. 1D ^1H and 2D COSY spectra along with $^1H-^{13}C$ HSQC and $^1H-^1H$ NOESY where appropriate were collected on a Bruker Avance II 600 MHz spectrometer using a 5 mm TXI cryoprobe or an Agilent 600 MHz spectrometer using a 5 mm cryoprobe in the AMRIS facility at the University of Florida or Varian INOVA 600, INOVA 500, and INOVA 400 spectrometers at Cornell's NMR facility. Spectra were processed and analyzed with MestReNova 7.0 (Mestrelab Research) or Varian VNMR.

Feeding Experiment with [U-D_7]-Indole. *C. elegans* were cultivated in 100 mL S-complete medium by providing *E. coli* OP50 as a food source. After a worm density of 88,000 worms/mL was reached, 0.6 mg [U-D_7]-indole in 100 μL methanol was added to the culture. Aliquots of 6 mL were taken after 0, 1, 2, 3, 6, 12, and 24 h and centrifuged at 5251*g* for 10 min, and 5 mL of supernatant was lyophilized, extracted with 2 mL methanol, filtered, and concentrated *in vacuo*. The residues were taken up in 200 μL methanol and analyzed by HPLC with UV and ESI-MS detection.

Synthesis of *N*-(β-D-Glucopyranosyl)indole (7). A solution of indoline (1.0 g, 8.4 mmol) in ethanol (60 mL) was treated with β-D-glucose (0.7 g, 3.9 mmol) in water (2 mL) and stirred at 90 °C. Additional water (0.8 mL) was added after 7 and 14 h. After 28 h the solution was concentrated *in vacuo*, and the residue was fractionated on silica using a gradient of 0–10% methanol in dichloromethane to get *N*-(β-D-glucopyranosyl)indoline (1.05 g, 3.7 mmol, 96% yield) as a yellowish solid. A solution of *N*-(β-D-glucopyranosyl)indoline (85 mg, 300 μmol) in 1,4-dioxane (15 mL) was treated with 2,3-dichloro-5,6-dicyano-1,4-benzochinone (82 mg, 360 μmol). After stirring for 14 h the mixture was concentrated. Flash column chromatography on silica gel using 20% methanol in dichloromethane afforded *N*-(β-D-glucopyranosyl)indole (70 mg, 251 μmol, 83% yield). Reverse phase HPLC on a C_{18} column afforded a pure sample for toxicity testing.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

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Notes

The authors declare no competing financial interests.

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