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INTERCONVERSIONS OF AGLYCONE AND HOST-SELECTIVE TOXIN FROM *HELMINTHOSPORIUM SACCHARI**

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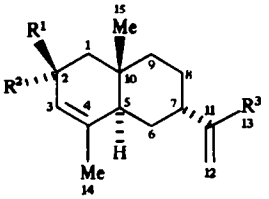
Key Word Index—*Saccharum officinarum*; Gramineae; sugarcane; *Helminthosporium* (*Bipolaris*) *sacchari*; host-selective toxin; biosynthesis of toxin; sesquiterpene (aglycone); disease physiology.

Abstract—*Helminthosporium sacchari*, a fungus that causes disease in sugarcane, produces oligosaccharide-sesquiterpene toxins (HS toxins A, B, and C) that are required for infection and disease development. Two free sesquiterpenes were isolated from mycelium but not from culture fluids of the fungus. One sesquiterpene was identified by HPLC and mass spectrometry as an aglycone of HS toxin C and could be obtained by enzymatic hydrolysis of this toxin. The other sesquiterpene appeared to be the 2-keto form of the first compound. The aglycone from toxin C hydrolysis was labelled with tritium by successive treatments with active manganese dioxide, sodium boro[^{3}H]hydride, and lithium aluminium hydride. The labelled compound was fed to cultures of *H. sacchari*; radioactivity was incorporated into HS toxin C and into lower molecular weight homologues. The results suggest a metabolic route (aglycone → metabolite Y, → HS toxin → metabolite X) for the biosynthesis of HS toxin; metabolites X and Y are lower molecular weight homologues of the toxin.

INTRODUCTION

Helminthosporium sacchari (Van Breda de Haan) Butler, the cause of eyespot disease of sugarcane (*Saccharum officinarum* L.), produces toxins that are host-selective, i.e. they are active against susceptible but not against resistant clones of sugarcane [1]. These toxins are necessary for the pathogenicity of *H. sacchari* to sugarcane, and for disease development in the plant [2]. Macko *et al.* [3] reported that HS toxin is a mixture of three isomers (HS toxins A, B and C), for which there are proposed structures [4]. The toxins are composed of a sesquiterpene linked to two residues of 5-*O*-(β-galactofuranosyl)-β-galactofuranoside (1). The isomers differ only in the positions of a double bond in the sesquiterpene; toxins A, B, and C have the bond at positions C-4/C-5, C-4/C-14, and C-3/C-4, respectively [5]. HS toxin C is the most active form.

Lower molecular weight homologues of the toxins were found in culture filtrates [5–8] and the same homologues were produced by hydrolysis of purified toxins with β-galactofuranosidase [9]. These homologues have fewer galactose units but have the same sesquiterpene moieties as do the toxins. We are following an established system of nomenclature for the homologues [5]. The toxin identification letter (A, B or C) refers to the sesquiterpene moiety of the toxin molecule. Subscript numbers refer to the number of galactose units present and to their position on the core; the first number refers to the galactose attached to position C-2, and the second number refers to the galactose at position C-13 (1). For example, homologue $C_{2,1}$ was produced by removal of one galactose from the original toxin ($C_{2,2}$). The lower molecular weight homologues were found to protect susceptible tissue from the effects of HS toxins [7, 8, 10].



1 R^1 = H, R^2 = OGalGal, R^3 = CH_2OGalGal
2 R^1 = H, R^2 = OH, R^3 = CH_2OH
3 R^1, R^2 = O, R^3 = CHO
4 R^1, R^2 = O, R^3 = CH_2OH

There are no previous reports of free aglycone of HS-toxin in cultures of the fungus or in enzyme-hydrolysed preparations of toxins. Therefore, we attempted to obtain the aglycone from HS toxin by enzymatic hydrolysis and to examine the possible role of the aglycone in HS toxin biosynthesis. We report here: (a) production of the aglycone by enzyme hydrolysis of HS toxin C; (b) the presence of the aglycone in the mycelium of *H. sacchari*; (c) some properties of the aglycone; (d) incorporation of the aglycone into HS toxin by cultures of *H. sacchari* and (e) a proposed scheme for the metabolism of HS toxin by *H. sacchari*.

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RESULTS

Aglycone prepared by enzymatic hydrolysis of HS toxin C

The toxin was hydrolysed with a concentrated solution of β-galactofuranosidase from *Penicillium charlesii*, using vigorous stirring of the reaction mixture. This procedure gave a good yield of aglycone (2) with 30–45% hydrolysis of HS toxin C. ^{1}H NMR and mass spectral data on the aglycone and its derivatives indicated a complete removal by sugars from the HS toxin C molecule. Previously, Livingston and Scheffer [9] detected only a small amount of an apparent aglycone when HS toxin was hydrolysed with β-galactofuranosidase from *H. sacchari*. The enzyme preparation used in the experiment described here was much more concentrated.

Preparation of ^{3}H-*aglycone*

The aglycone (2) was easily converted to the keto aldehyde (3) by use of active manganese dioxide. The signals at δ4.12 (2H) and 4.30 (1H) observed in the ^{1}H NMR spectrum of the aglycone disappeared and a new signal at δ9.50 (1H) appeared in the spectrum of the keto aldehyde. Some signals in the ^{1}H NMR spectrum of the aglycone were shifted down-field in the spectrum of the keto aldehyde, because of the ketone and aldehyde groups.

Attempts to convert the keto aldehyde directly into the aglycone with sodium borohydride, using various conditions, were not successful. The hydroxy ketone (4) was produced when sodium borohydride was added to the solution of the keto aldehyde; longer reaction times and higher temperatures resulted in mixtures of many products. Reduction of the keto aldehyde or the hydroxyketone with lithium aluminium hydride gave a mixture of the aglycone and its epimer at position C-2.

Labelled aglycone was prepared by use of sodium boro[^{3}H]hydride plus lithium aluminium hydride, or by use of lithium aluminium [^{3}H]hydride alone although the sodium boro[^{3}H]hydride method has several advantages. Extensive purification of the products by HPLC showed that the keto aldehyde was reduced by sodium boro[^{3}H]hydride and lithium aluminium hydride, with a 34% yield of 13-[^{3}H]aglycone (11.2 mCi/mmol).

Presence of the aglycone in the mycelium of H. sacchari

Mycelial mats of fungus were extracted and the extracts were purified. Each purification step was monitored by analytical TLC, along with a known sample of the aglycone. An aliquot of the fraction obtained after final purification with preparative TLC gave two prominent peaks with retention times of 10.4 and 13.1 min on HPLC. The compound with retention time of 13.1 min was the aglycone (2) as shown by the mass spectrum of the trimethylsilyl derivatives. The spectrum showed prominent ions at *m/z* 380, 365, 290, 275, 219, 200, 185, 170, 157, 145, 143, 141 and 129 and it was indistinguishable from the spectrum of the aglycone prepared by the hydrolysis of HS toxin C. The yield of the aglycone from the mycelium of 3-week-old cultures was *ca* 1 mg/l. of medium (0.001% fr. wt mycelium). The compound giving the peak at 10.4 min was identified tentatively as the hydroxyketone (4) on the basis of the retention time and the mass spectrum (70 eV) using a direct insertion probe. There were prominent ions at *m/z* 234, 219, 216, 201, 173, 161, 159, 147, 135 and 95. This compound was not studied further. Analysis of the culture filtrate by the same procedures as used with mycelial extracts gave no prominent peak at 13.1 min on HPLC (data not shown). Presence of the aglycone in the fungus suggested that the aglycone might be a precursor in toxin biosynthesis.

Conversion of the aglycone into HS toxin and other metabolites by H. sacchari

The [3H]aglycone was added to the medium of 2-week-old cultures of *H. sacchari* and incubated for 24, 48 and 96 hr. After incubation, the labelled aglycone and its conversion products were removed from the culture filtrate by solvent extraction. The amount of the [^{3}H]aglycone in the medium decreased gradually during incubation and concomitantly, there was an increase in ^{3}H-labelled products (Table 1). Sephadex LH-20 column chromatography gave three radioactive peaks for each incubation time (Fig. 1); the first peak appeared to be HS toxin C, as indicated by elution volume. The fractions for 96 hr cultures were also analysed by HPLC. The first peak of radioactivity eluted from the HPLC column (19.8 min) correspond to that for HS toxin C. The radioactivity which was incorporated into HS toxin C by 96 hr cultures was *ca* 6.2% of the radioactivity of the labelled aglycone initially added to the medium and 12.0% of the radioactivity in the *n*-butanol extract.

Biosynthetic interconversions of aglycone, toxin and putative intermediates

When [^{3}H]aglycone was incubated with cultures of *H. sacchari*, the three new ^{3}H-peaks increased with time, as shown by Sephadex LH-20 chromatography (Fig. 1). To establish the relationship of these conversion products to HS toxin metabolism, each conversion product (Fig. 1) from 96 hr cultures was purified with HPLC and each was incubated for 72 hr with 2-week-old cultures of *H. sacchari*. The fungus converted HS toxin C (first peak) to the metabolite contained in the second peak (metabolite X) with a 10.4% yield (Table 2). However, the fungus did not convert metabolite X to other products. The metabolite in the third peak (metabolite Y) was converted to HS toxin (10.3% yield) and to metabolite X (5.9% yield). In control experiments, labelled HS toxin, product X and product Y were each added to individual samples of culture filtrates (rather than to living cultures). There were no conversions, indicating that the conversion products were not artifacts formed during purification nor were they contaminants of the other metabolites.

Table 1. Conversion of aglycone of HS toxin to other metabolites by *Helminthosporium sacchari**

Fraction†	Incubation time (hr) 24	48	96
Dichloromethane	1 120 000	920 000	735 000
n-Butanol	296 000	436 000	966 000

*[^{3}H]Aglycone (1 772 000 dpm) was added at 0-time to 14-day-old cultures of *H. sacchari*.

†After incubation, the cultures were extracted and the material divided into two fractions by solvent partition. The aglycone is soluble in dichloromethane, not in *n*-BuOH.

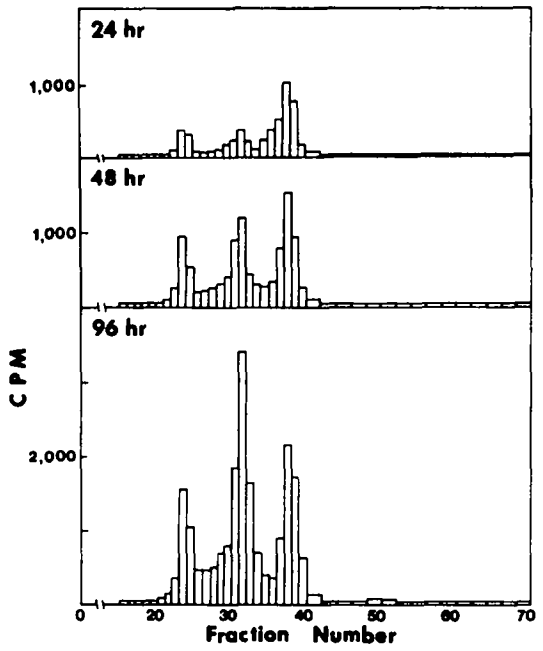

Fig. 1. Sephadex LH-20 column chromatogram of the metabolites produced from the aglycone by cultures of *H. sacchari*. The labelled aglycone was incubated with 2-week-old cultures of *H. sacchari* for 24, 48 and 96 hr. The column (1.8 × 82 cm) was developed with 50% methanol. Fractions (4 ml each) were collected and an aliquot (400 μl) of each fraction was assayed using a liquid scintillation counter.

Table 2. Biosynthetic interconversions of toxin and related metabolites

Compound fed*	HPLC Fractions†		
	8–11	19–21	22–26
	dpm × 10^{-3}		
HS toxin	4.3	0.5	0
Control‡	3.6	0	0
Metabolite X	0	5.1	0
Control‡	0	4.0	0
Metabolite Y	0.7	0.4	5.7
Control‡	0	0	6.4

*Labelled toxin and labelled metabolites X and Y were recovered from cultures that were fed [^{3}H]aglycone. These products were purified by LH-20 chromatography (Fig. 1) and by HPLC and fed to new cultures.

†After incubation for 96 hr, the ^{3}H-conversion products were analysed by HPLC, using an ODS column developed with 25% acetonitrile in H_2O at a flow rate of 2 ml/min. Two ml fractions were collected and counted by liquid scintillation.

‡Controls: toxin, metabolite X, or metabolite Y was added to culture filtrates (rather than to live cultures); products were then purified.

Metabolites X and Y, isolated from *H. sacchari* cultures that had been incubated with [^{3}H]aglycone, were compared with known samples (homologues of HS toxin), using Sephadex LH-20 chromatography, HPLC and mass spectrometry. For HPLC, a Bondapak C_{18} column was developed for 5 min with acetonitrile–H_2O (20:80); the acetonitrile was then gradually increased for 10 min to 27%, where it was maintained for 20 min. The elution patterns from the column indicated that metabolite X was a 2-galactose homologue ($C_{2,0}$), of toxin and that metabolite Y contained two radioactive compounds, a 2-galactose and a 1-galactose form ($C_{0,2}$ and $C_{0,1}$) [8]. The elution patterns from the Sephadex LH-20 column suggested that X was the 2-galactose form ($C_{2,0}$) and that Y was a 1-galactose form. FAB-MS data indicated that X was the 2-galactose form and that Y was a mixture of a 1-galactose and a 2-galactose homologue.

DISCUSSION

The HS toxin C molecule is composed of two 5-*O*-(β-galactofuranosyl)-β-galactofuranoside units and an aglycone. The feeding experiments by Macko *et al.* [4] suggested that the aglycone is synthesized from mevalonic acid; the unique structure of HS toxin suggested that the aglycone is the biosynthetic precursor of HS toxin. If so, the aglycone should exist as the free form in the mycelium or in the culture fluids of *H. sacchari*. Our results showed that the aglycone in fact is present in the mycelium of *H. sacchari*, but is not in the culture fluids. This finding fits the idea that the aglycone is a biosynthetic precursor of HS toxin, and that toxin is excreted into the culture medium after galactose is attached to the aglycone. The hypothesis was tested by adding [^{3}H]aglycone to the medium of growing cultures of *H. sacchari*. Radioactivity was incorporated into the HS toxin molecule, and [3H]toxin was recovered from the culture filtrates. There is an earlier suggestion that HS toxin may be formed by α-glucosidase hydrolysis of higher molecular weight homologues which have α-glucopyranosyl units attached to position C-2 of the external galactofuranose units [5]. We have not examined this possibility.

Two labelled products in addition to toxin were obtained, as shown by chromatography with a Sephadex LH-20 column, from cultures incubated with [^{3}H] aglycone. This finding indicated that HS toxin and the metabolites in these two peaks have the aglycone as a common precursor. Further feeding experiments indicated the following facts: (a) HS toxin was converted to metabolite X (second peak in the eluate, Fig. 1), suggesting that HS toxin is not the end product; (b) metabolite X was not converted by *H. sacchari* and (c) metabolite Y (third peak, Fig. 1) was converted to HS toxin and to metabolite X. These facts suggest the following metabolic pathway: aglycone → metabolite Y → HS toxin → metabolite X. The scheme could explain changes with time in the three peaks (Fig. 1). However, it is possible that some of metabolite Y was degraded by *H. sacchari* to the aglycone and that the aglycone was then converted to HS toxin and metabolite X.

Metabolites X and Y were examined by use of HPLC, LH-20 chromatography and FAB-MS, using known standards for comparison [8]. Results of these analyses indicated that metabolite X is a 2-galactose homologue of toxin ($C_{2,0}$) and that metabolite Y probably is a mixture of 1-galactose ($C_{0,1}$) and 2-galactose ($C_{0,2}$) homologues.

Therefore, it is possible that a 5-*O*-(β-galactofuranosyl)-β-galactofuranoside unit is attached to one of the two hydroxyl groups of the aglycone to give metabolite Y, and that an additional unit is attached to the other hydroxyl group to give HS toxin. Metabolite X could be formed when one β-galactofuranoside unit is removed from HS toxin by galactofuranosidase [9].

The action of HS toxin is still an open question. There must be a specific receptor or sensitive site because lower homologues competitively inhibit the effects of HS toxin on sugarcane tissues [7, 8, 10]. Conclusive data on receptor sites will require use of toxin with much greater radioactivity than has been used in the past [2]. Adequately labelled toxin can be prepared by the methods outlined above. Another possible procedure is to attach the 5-*O*-β-galactofuranosyl-β-galactofuranoside units to the labelled aglycone by the method of Kochetkov *et al.* [11]. This approach seems possible and should be reliable because the synthesis of 5-*O*-β-galactofuranosyl-β-galactofuranoside has been reported [12]. The dilution of radioactivity *in vivo* will not occur if the Kochetkov method is used.

EXPERIMENTAL

Analyses. ^{1}H NMR spectra were obtained on a Brucker WH-250 instrument using $CDCl_3$ as the solvent. Mass spectra (70 eV) were obtained with a Hewlett Packard 5985A or a Hitachi RMU 6MG instrument using the direct insertion probe. TMS derivatives were obtained by treating the sample with *N,O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA)–pyridine (1:2) at 80° for 2 hr. Optical rotation was measured with a Perkin–Elmer Model 141 polarimeter. HPLC was with a Varian 5000 chromatograph connected to an ODS column (Waters, μBondapak C_{18}, 0.78 × 30 cm) or a silica gel column (Whatman, Partisil 10, 0.40 × 25 cm); absorbance was monitored at 215 nm. Precoated TLC plates of silica gel 60F-254 (Merck) were used for TLC. Evaporation of solvents or concn of solns was at 40° under red. pres. (10 mm Hg). Radioactivity of each sample was determined by scintillation counting.

Isolation of HS toxin C. Cultures of *H. sacchari* (from Jack L. Dean of the U.S.D.A. Sugarcane Research Station, Canal Point, FL) were maintained and grown for toxin production as described previously [6, 9]. Culture filtrates (10 l.) from 21-day-old cultures were concentrated to 1.0 l. An equal vol. of MeOH was added to the concentrate and the soln was stored for 20 hr at 4°. The filtrate was concd to 300 ml and the aq. soln was purified by countercurrent distribution (10 fractions) using H_2O and *n*-BuOH. Fractions 4 (most polar) to 8 were combined and concd to dryness. The residue was dissolved in 50 ml of H_2O and passed through a column of Amberite XAD-2 (100 ml bed vol.). The column was washed with 250 ml of H_2O and then with 500 ml of 10% MeOH in H_2O. The fraction that eluted with 500 ml of 60% MeOH in H_2O was evapd to dryness and the residue was purified with silica gel flash chromatography [8]. A 30 mg sample of the dried toxin preparation from flash chromatography was dissolved in a small amount of H_2O and was passed through an ODS preparative cartridge (Sep-Pak C_{18}, Waters Associates), which was then washed with 5 ml of 5% CH_3CN in H_2O. The fraction that eluted from the cartridge with 10 ml of 30% CH_3CN in H_2O was evapd to dryness. Final purification was by repeated HPLC using an ODS column and 20% CH_3CN in H_2O [8]. Yields varied from 10 to 20 mg of HS toxin C per l. of culture fluids.

Purified HS toxin C gave a single, symmetrical peak on HPLC; the retention time was the same as that of the toxin isolated previously [8]. In addition, a bioassay based on induced loss of electrolytes from leaf discs gave biological activity comparable to that reported previously [8]. The ^{1}H NMR spectrum in D_2O (data not shown) was indistinguishable from that of HS toxin C reported by Macko *et al.* [4].

Enzyme hydrolysis of HS toxin C. β-Galactofuranosidase was prepared from *Penicillium charlesii* NRRL 1887 which was grown as described elsewhere [13]. Culture filtrates (300 ml) were concd to 50 ml. Ammonium sulphate (23 g) was added and the ppts collected by centrifugation, resuspended in 10 ml of 2.5 mM sodium acetate/acetic acid (pH 4.6), and dialysed against the same buffer.

HS toxin C (100 mg) was dissolved in 6 ml of the enzyme soln and incubated at 36° for 15–20 hr with stirring. The soln was then extracted (5 × CH_2Cl_2) and the extracts washed, (sat. NaCl) dried and filtered. The residue from evapn of solvent was purified with repeated HPLC, using an ODS column with 65% aq. MeOH at a flow rate of 2 ml/min; retention time was 13.1 min. Yield was 8–12 mg of the pure aglycone of HS toxin C (**2**) which was a colourless solid.

The aglycone was characterized as follows: specific optical rotation was $[\alpha]_D^{22} - 53.6$ (*c* 0.028, MeOH); ^{1}H NMR characteristic signals were observed at δ0.85 (3H), 1.63 (3H), 2.08 (1H), 2.64 (1H), 4.12 (2H), 4.30 (1H), 5.05 (1H), 5.22 (1H), and 5.38 (1H). MS *m/z* (rel. int.): 221 (9), 218 (58), 203 (38), 200 (17), 185 (71), 161 (35), 160 (38), 159 (30), 157 (100), 149 (25), 147 (28), 145 (52), and 143 (85). MS of TMS derivative *m/z* (rel. int.): 380 (1.6), 365 (6.8), 290 (7.6), 275 (39), 219 (8), 200 (20), 185 (15), 170 (15), 157 (14), 145 (9), 143 (10), 141 (12), 129 (8), 105 (12), 91 (13), 75 (57), and 73 (100). A molecular ion was not observed in the EI–MS; however, the TMS derivative of the aglycone gave a molecular ion at *m/z* 380, along with characteristic ions at *m/z* 365, 290, and 275. These data and data on the derivatives of the aglycone indicated that complete removal of sugars from the HS toxin C molecule occurred during enzymatic hydrolysis.

Preparation of radioactive aglycone. Aglycone (**2**, 30 mg) was shaken with a suspension of 100 mg of active MnO_2 in 1.0 ml $CHCl_3$ for 10 hr at 21–22°. The MnO_2 was removed, the filtrate evapd, and the residue purified with TLC using 25% EtOAc in C_6H_6 to give the keto aldehyde (**3**) (R_f 0.4); yield was 22.3 mg. ^{1}H NMR: δ0.93 (3H, *s*), 1.22–2.42 (9H, *m*), 1.80 (3H, *d*, *J* = 1.4 Hz), 3.10 (1H, *m*), 5.86 (1H, *q*, *J* = 1.4 Hz), 6.18 (1H, *d*, *J* = 1.4 Hz), 6.50 (1H, *d*, *J* = 1.4 Hz), and 9.50 (1H, *s*). MS *m/z* (rel. int.): 232 (77), 217 (45), 199 (66), 189 (17), 171 (13), 161 (13), 159 (34), 147 (25), 135 (100), 121 (26), 109 (17), 107 (17) and 105 (25).

The dry keto aldehyde (**3**) (10 mg) in 1 ml absolute EtOH was stirred at 0° and $NaBH_4$ (480 μg: 100 mCi/mmol) in 50 μl of 0.1 M NaOH was added. The soln was held for 1 min at 0°, a few drops of HOAc were added and the reaction mixture poured into 10 ml 0.01 N HCl. The soln was extracted 5 × EtOAc; the combined extracts were washed with sat. NaCl, dried, filtered and the EtOAc removed by evapn to give the hydroxyketone (**4**) as an oil. ^{1}H NMR: δ0.90 (3H, *s*), 1.25–2.27 (8H, *m*), 1.87 (3H, *br s*), 2.49 (1H, *br d*, *J* = 12 Hz), 2.70 (9H, *m*), 4.15 (2H, *br s*), 5.07 (1H, *br s*), 5.28 (1H, *br s*) and 5.85 (1H, *br s*). MS *m/z* (rel. int.): 234 (73), 219 (17), 216 (32), 201 (71), 176 (20), 175 (20), 174 (24), 173 (45), 161 (32), 159 (52), 147 (28), 145 (32), 135 (93), 121 (36), 119 (32), 109 (43), 108 (44), 107 (46), 105 (46), 95 (100), 93 (63), and 91 (73).

$LiAlH_4$ (2 mg) in dry Et_2O (500 μl) was added to the hydroxyketone (**4**) in 1 ml dry Et_2O, under dry N_2 at 0°. The mixture was allowed to stand for 15 min at 0°. After addition of 500 μl of EtOAc, the reaction mixture was poured into 10 ml of 0.01 N HCl. The soln was extracted 3 × with EtOAc, washed, dried and the residue purified by repeated HPLC using an ODS column developed with 60% MeOH in H_2O at a flow rate of 2 ml/min. Retention time was 17.3 min; retention time of the

epimer was 14.4 min. The next step was HPLC with a silica gel column developed with 5% EtOH in *n*-hexane, at a flow rate of 2 ml/min; retention time was 13.0 min. Final yield was 3.1 mg of the aglycone (2). ^{3}H-Labelled aglycone (3.5 mg, 11.2 mCi/mmol) was stored in 2 ml of 5% EtOH in C_6H_6 at 4° until use.

Isolation of the aglycone from the mycelium. The mycelium from 5 cultures (200 ml each, 21 days old) of *H. sacchari* was dried and extracted 3 × Me_2CO. After removal of Me_2CO by evapn, the aq. residue was washed with *n*-hexane and extracted 3 × with CH_2Cl_2. The extract was washed (sat. NaCl), dried, filtered and evapd to dryness. The residue was dissolved in ETOAc and applied to a silica gel column (60–200 mesh, 2.2 × 15 cm). The column was developed successively with 200 ml each of 0, 10, 20, 40 and 60% EtOAc in *n*-hexane; 40 ml fractions were collected. Fractions 18–23 were combined, concd, and purified by prep. TLC (EtOAc–*n*-hexane, 60:40). The R_f 0.2–0.4 zone was eluted with EtOAc and subjected to HPLC, using an ODS column developed with 65% MeOH in H_2O at a flow rate of 2 ml/min.

Feeding experiment. An aliquot of [^{3}H]aglycone was diluted 50 times with non-labelled aglycone. The diluted ^{3}H aglycone (400 μg, 886 000 dpm) in 200 μl of Me_2CO was added aseptically to the medium of each 2-week-old culture of *H. sacchari* in a Roux bottle. The cultures were incubated with the [^{3}H]aglycone at 21–22° for 24, 48 and 96 hr. Filtrate (400 ml) from two cultures was passed through an Amberite XAD-2 column (bed vol. 60 ml), which was washed with 100 ml H_2O and 100 ml 5% MeOH in H_2O. The fraction that eluted with 300 ml of MeOH was evapd to dryness. The residue was dissolved in H_2O and extracted 3 × with CH_2Cl_2. The aq. residue was extracted 5 × with 20 ml H_2O-saturated *n*-BuOH; the BuOH extracts were evapd and the residue dissolved in 2 ml of 50% aq. MeOH and placed on a Sephadex LH-20 column (1.8 × 82 cm) which was developed with 50% aq. MeOH; 4 ml fractions were collected.

The significant fractions (Fig. 1) from the Sephadex LH-20 column (for 96 hr cultures) were processed further. Fractions 24 and 25 were combined, evapd, analysed and purified with HPLC, using an ODS column developed with 20% CH_3CN in H_2O at a flow rate of 2 ml/min. The HPLC fractions that eluted at 19–20.5 min contained HS toxin C, as shown by spectral data and bioassay. Fractions 31–33 were purified the same as were fractions 24–25, except that the ODS column was developed with 25% CH_3CN in H_2O. The HPLC fractions that eluted at 18–20 min contained metabolite X. Fractions 37–40 were processed the same as were fractions 31–33; the HPLC fractions which eluted at 21–25 min contained metabolite Y.

Each ^{3}H-metabolite was fed to individual cultures of *H. sacchari*, to determine conversion relationships. Labelled HS toxin C was dissolved to 500 μl of MeOH; 100 μl of the soln were added aseptically to the medium of each 2-week-old culture, incubated at 21–22° for 72 hr and harvested. The culture filtrate obtained from two cultures (200 ml each) was purified as described above to the *n*-BuOH step. The BuOH extract was evapd to dryness and dissolved in a small amount of H_2O. The sample was passed through a Sep Pak C_{18} cartridge, which was washed with 5 ml of 5% CH_3CN in H_2O. The fraction that was eluted with 10 ml of 30% CH_3CN in H_2O was evaporated to dryness. An aliquot of the residue was analysed with HPLC, using an ODS column developed with 25% acetonitrile in H_2O at a flow rate of 2 ml/min. Fractions (2 ml) were collected and assayed by liquid scintillation counting. As a control, 200 μl of MeOH containing [^{3}H]HS toxin C was added to the filtrate (400 ml) from two cultures (17 days old) of *H. sacchari*. After incubation, the preparation was purified as described for the *H. sacchari* cultures incubated with the same radioactive metabolites. Metabolites X and Y also were examined by this whole procedure, to determine their fate in cultures of *H. sacchari*.

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