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Structure of three isomeric host-specific toxins from *Helminthosporium sacchari*

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Summary. Extended spectroscopic analysis has led to the derivation of complete structures for the 3 isomeric host-specific toxins $C_{39}H_{64}O_{22}$ previously isolated from *H. sacchari*; these compounds represent bis-5-O-(β-galactofuranosyl)-β-galactofuranosides differing in the position of 1 double bond in their sesquiterpenoid aglycone component.

In a previous communication[4] on the host specific toxin complex from *H. sacchari*, the causal organism of eyespot disease of sugar cane, we have described the isolation of three isomeric toxins $C_{39}H_{64}O_{22}$, each of which produces characteristic symptoms on susceptible sugar cane at levels as low as 2×10^{-11} moles, and it was shown that these compounds consist of 4 galactose units linked to aglycone components with the molecular formula $C_{15}H_{24}O_2$. A detailed spectroscopic investigation has now enabled us to deduce complete structures for these 3 isomers[5], henceforth referred to as HS-toxin A, B, and C, respectively, according to their order of elution from a reverse phase HPLC column.

Confirmatory evidence for the composition of the toxins was obtained by Chemical Ionisation/Desorption Mass Spectroscopy (CI/D-MS)[6] of the mixture of the 3 isomers with ammonia as the reagent gas; next to the expected molecular ion at 884 daltons the spectrum displays fragments with m/e 722, 560, 398 and 236 daltons, which correspond to the sequential loss of 4 hexose units, and intense peaks at m/e 219 and 201 daltons resulting from sequential loss of water molecules from the protonated aglycones of mol.wt 236. In addition, fast atom bombardment mass spectroscopy (FAB-MS) of the pure isomers gave essentially identical spectra displaying intense ions at 907($M+Na^+$) and 885($M+H^+$) (positive mode), and at 883($M-H^+$) (negative mode), corresponding to a mol.wt of 884.

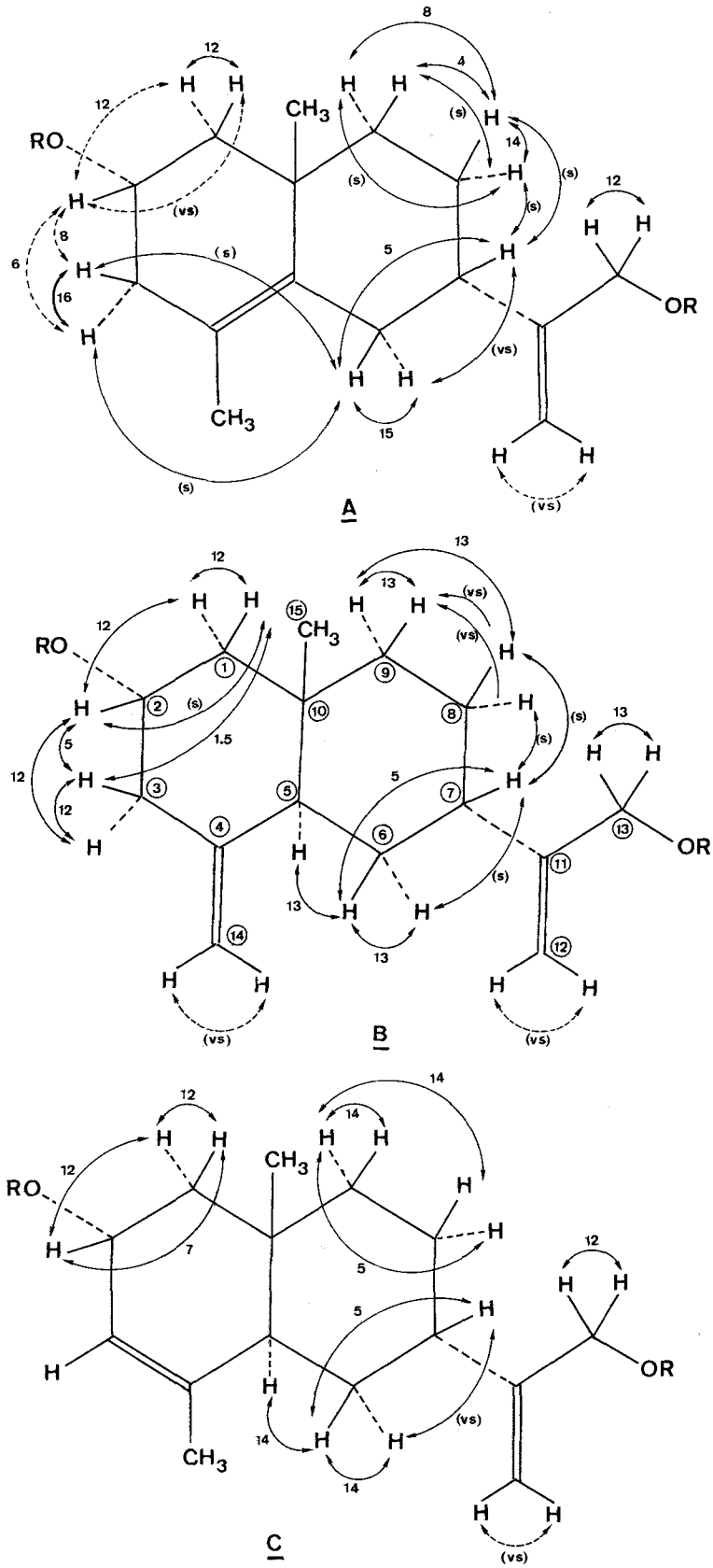
The most valuable structural information came from high-resolution NMR investigations of the pure toxins. The ^{13}C-NMR spectrum of each isomer, measured in D_2O at 75 MHz, is consistent with the expected presence of 39 carbon atoms; chemical shifts and signal multiplicities for the aglycone and sugar moieties are listed in tables 1 and 2, respectively. The ^{1}H-NMR spectra were recorded in D_2O at 300 and 600 MHz and the relevant data, including the outcome of double resonance experiments, are summarized in tables 3 and 4 and in figures 1-3.

The ^{13}C-NMR spectra of each aglycone component (table 1) reveal the presence of 4 sp^2- and of 2 oxygen carrying sp^3-carbon atoms; since according to the IR-spectrum the toxins do not contain carbonyl groups, it follows that all 3 aglycones must possess a

Table 1. ^{13}C-NMR data[a] for aglycone moieties

Carbon No.	Isomer A	Isomer B	Isomer C
1	46.8 t	49.0 t	46.7 t
2[b]	72.4 d	75.4 d	75.1 d
3	39.2 t	43.0 t	121.2 d
4	123.0 s	147.5 s	142.4 s
5	134.6 s	44.0 d	42.1 d
6[b]	23.7 t	22.7 t	22.6 t
7	37.1 d	35.8 d	36.1 d
8[b]	28.3 t	25.7 t	24.9 t
9	37.7 t	36.5 t	35.9 t
10	36.9 s	36.0 s	36.2 s
11	149.0 s	149.3 s	147.7 s
12	114.3 t	114.9 t	114.8 t
13	71.2 t	71.5 t	71.6 t
14	25.4 q	108.6 t	21.0 q
15	19.3 q	16.9 q	16.0 q

[a] δ-Values relative to internal dioxane = 67.4 ppm.
[b] Intensity increased upon feeding of [5-^{13}C]-mevalonic acid.



Figures 1-3. Full double pointed arrows indicate the results of double resonance experiments conducted at 300 MHz in both ways with the corresponding J-values given in Hz. (s) = small ($J \sim 1$-3 Hz); (vs) = very small ($J < 1$ Hz). Single pointed arrows indicate that the decoupling has only been conducted in one way. Arrows with broken line are used where the indicated connectivities and J-values have not been corroborated by irradiation experiments. Multiplicities for the individual signals and J-values for the H-H interactions were obtained mostly from the 600 MHz spectra.

doubly unsaturated bicarbocyclic framework. Comparison of the data in tables 1-4 and in figures 1-3 makes it abundantly clear that the 3 isomers differ only in the position of 1 double bond. Exploitation of this kinship simplified the interpretation of the ^{1}H-NMR results which led eventually to the derivation of the connectivities indicated in figures 1-3. Whereas most signals for the aglycone protons can be easily recognized as such, 2 of them are overlapping in the region between 3.6 and 4.1 ppm with the many resonances from the sugar residues; their positions were located, whenever possible, by double resonance experiments. One of these signals represents the B-component of an AB-system assigned to the (C-13)-H_2OR group. The complex multiplet displayed by (C-2)-H at δ 3,88 in isomer B is shifted downfield in isomer C in a manner which is characteristic for allylic protons, and this serves as a basis for assigning the 2nd oxygen of the aglycon to position 2.

The relative configurations assigned to the 3 isomers in the figures rest on the following evidence. The equatorial orientation of the oxygen substituent at C-2 follows from the large axial-axial coupling ($J \sim 12$ Hz) caused by interaction of the corresponding geminal proton with the axially oriented protons in the adjacent position(s). The protons at the ring junctions in isomers B and C must be axial with respect to the side chain bearing ring, as indicated by their large couplings with (C-6)-H_a, and also with respect to the other ring, since the characteristic downfield shift expected for an equatorial, syn-allylic proton is not observed in the spectrum of isomer B; hence the ring junction must be trans. These conclusions received independent support from the detection of nuclear Overhauser effects experienced by the 3 axially oriented protons at C-2, C-6 and C-8 upon irradiation of the angular methyl group in isomer C. Finally, the characteristic shape of the signal for (C-7)-H (broad signal, containing 4 small couplings) indicates an axial orientation for the side chain appended to the same carbon atom. The choice of absolute configurations indicated for the 3 aglycones in the figures needs experimental verification, as it rests solely on extrapolations from data showing a predominance of the same steric arrangement at C-7 in a variety of related metabolites from lower organisms in general and specifically from *Helminthosporium* species[7].

The ^{13}C-resonances for the galactose residues (table 2) show that the 4 units must be arranged in 2 groups. The 1st group displays identical or nearly identical chemical shifts that match very closely those of a terminal β-galactofuranosyl unit[8], whereas signals from the 2nd group differ slightly among themselves as well as from the previous ones and form a pattern which is characteristic for 5-O-substituted β-galactofuranosides[8]. A sample of benzyl 5-O-(β-galactofuranosyl)-β-galactofuranoside[9] was synthesized as a reference and the close correspondence of the relevant part of its ^{13}C-NMR spectrum (table 2) with those of the 3 toxins leaves little doubt that the latter must possess the general structure gal-gal-X-gal-gal (where X stands for the aglycone). This proposal is supported by the pattern of resonances in the anomeric region of

Table 2. ^{13}C-NMR data[a] for sugar moieties

Atom No.	Type	Isomer A	Isomer B	Isomer C	Ref. comp.[b]
1	Terminal	108.0 (2)d	107.9 (2)d	107.9 (2)d	108.0 d
	Internal	106.9 d	107.2 d	107.3 d	107.4 d
		106.2 d	106.1 d	106.9 d	
2	Terminal	82.0 (2)d	82.0 (2)d	82.0 (2)d	82.0 d
	Internal	82.0 (2)d	82.0 (2)d	82.0 d	82.0 d
				81.9 d	
3	Terminal	77.4 (2)d	77.4 d	77.4 d	77.4 d
			77.3 d	77.3 d	
	Internal	77.0 (2)d	77.3 d	77.2 d	77.3 d
			77.0 d	77.1 d	
4	Terminal	83.7 (2)d	83.6 (2)d	83.6 d	83.4 d
				83.5 d	
	Internal	82.5 (2)d	82.4 d	82.3 d	82.4 d
			82.0 d	82.2 d	
5	Terminal	71.4 (2)d	71.3 (2)d	71.3 (2)d	71.3 d
	Internal	76.8 (2)d	76.7 d	76.7 d	76.7 d
			76.9 d	76.9 d	
6	Terminal	63.7 (2)t	63.6 t	63.6 (2)t	63.6 t
			63.7 t		
	Internal	62.0 (2)t	62.0 t	62.0 (2)t	62.0 t

Table 3. ^{1}H-NMR data[a] for aglycone moieties

Carbon No.	Isomer A		Isomer B		Isomer C	
	H_a	H_β	H_a	H_β	H_a	H_β
C-1	1.40	1.80	1.28	1.8	1.88	1.88
C-2	-	?	-	3.88	4.20	4.20
C-3	2.04	2.40	1.97	2.77	5.50	
C-5	-	-	1.92	-	-	-
C-6	2.73	2.24	1.8	1.63	1.52	1.52
C-7	-	2.61	-	2.62	2.64	2.64
C-8	1.64	2.00	1.8	1.8	1.93	1.93
C-9	1.37	1.37	1.43	1.32	1.29	1.29
C-12	5.11; 5.20		5.19; 5.30		5.24; 5.33	
C-13	4.03; 4.23		~4.07; 4.22		4.05; 4.23	
C-14	1.70		4.64; 4.95		1.72	
C-15	1.15		0.78		0.92	

[a] δ-Values in ppm relative to internal DSS = 0.

Table 4. ^{1}H-NMR data[a] for anomeric protons

Isomer	Terminal units		Internal units (C-2)-O-C*H*		(C-13)-O-C-*H*	
	δ	J	δ	J	δ	J
A	5.23 (2 H)	2.0	5.17	2.75	4.99	2.2
B	5.25 (2 H)	2.2; 1.9	5.14	2.9	5.02	2.3

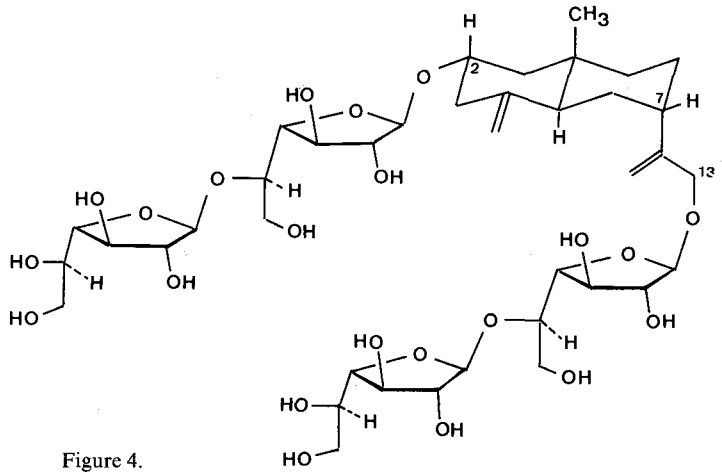
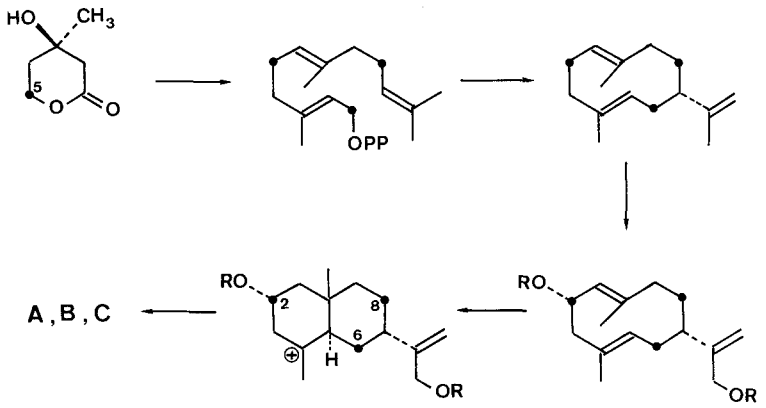


Figure 4.



Scheme

the ^{1}H-NMR spectrum (table 4); in each case 2 identical signals can be observed for the terminal units together with 2 slightly different signals at higher field for the internal units, the difference arising from the diversity in the point of attachment to the aglycone. That the low field signal at 5.14 ppm in the spectrum of isomer C belongs to the sugar attached to the (C-2)-oxygen of the aglycone was proved by detecting an increase of its intensity upon irradiation of the (C-2)-proton. The point of attachment of the 2nd chain is defined independently by the AB-patterns observed for the CH_2-OR groups, in contrast to the A_2-pattern displayed by the -CH_2OH groups of relevant model compounds[10]. Thus, the structural formulae of the three toxins can be completed as indicated in figure 4 for isomer B. Similar conclusions concerning the nature and arrangement of the 4 sugar units have been reached by Beier et al.[11], using a toxin preparation which must, however, represent an unresolved mixture of the 3 isomers.

Supporting evidence for the expected sesquiterpenoid nature of the 3 aglycones and for the correctness of the structures assigned to them was obtained in feeding experiments with [5-^{13}C]-mevalonic acid, which resulted in each case in a specific increase of the three signals due to carbon atoms 2, 6 and 8 in accordance with the operation of the appended scheme.

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Modulation of protein synthesis in primary myogenic cells from chicken by cultivation in the serum-free, hormonally defined medium 'DMN'[1]

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Summary. Chicken muscle cells secrete characteristic proteins when grown in the serum-free and hormonally defined culture medium 'DMN'. The most prominent band detected by gel electrophoresis represents a protein of mol.wt 22,000. Fibroblasts released a mol.wt 16,000 protein and fibronectin (mol.wt 220,000) into the medium. The mol.wt 22,000 protein band resolved in 2 dimensional gels into 2 spots which migrated to the same positions as small heat shock proteins as well as butyrate-inducible proteins (BIP) which can be demonstrated in whole cell extracts after butyrate treatment in the presence of serum. The synthesis and release of the mol.wt 22,000 protein is repressed by supplementing the culture medium with serum but not with chick embryo extract.

Introduction

The induction of a characteristic protein synthesis pattern as a response to environmental stress was originally detected in invertebrate cells ('heat shock response'). This characteristic cellular response can be demonstrated in a wide variety of species from plants to mammals[4–7]. Apart from elevated temperature, several toxic agents can induce an identical or a closely related response[8–10], which suggests a broad cellular stress response of which heat shock is only one of the possible initiating factors. The function of the 'stress proteins' is still unclear, but there is evidence that some of these proteins are components of the cytoskeleton and of myofibrils[11], whereas others are reported to migrate into the cell nucleus[12,13].

We report in this paper evidence that myogenic cells in a defined medium synthesize one of these stress proteins and release it into the culture medium.

Materials and methods

a) Materials: (^{35}S) methionine and En(3H)ance were from New England Nuclear, sodium dodecylsulfate (SDS) was from BDH, acrylamide from Serva and bisacrylamide from Eastman. The cell culture medium MCDB 201 was purchased from Seromed, Munich. The suppliers of the additional medium components have been listed previously[14].

b) Methods: Chicken cell cultures were prepared as described previously[14]. Sample preparation and SDS-polyacrylamide gel electrophoresis (SDS-PAGE): 30-µl aliquots of conditioned medium, labelled with 15 µCi (^{35}S)methionine/ml medium for the times indicated in the legends were boiled for 5 min in 20 µl sample buffer (in the presence of beta-mercaptoethanol) and then subjected to a 3–15% gradient SDS-polyacrylamide gel electrophoresis according to Laemmli[15]. The fixed gels were impregnated with En(3H)ance for 1 h, washed in running water for 30 min and, after drying, subjected to fluorography at −70 °C for 3–7 days. Two-dimensional gel electrophoresis and sample preparation was performed according to O'Farrell[16]. Extracts of cell cultures were essentially prepared as described by Caravatti et al.[17]. Cells were scraped from plates, sonicated and extracted for 1 h at 4 °C.