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Formation of 4-deoxy-*glycero*-hexo-2,3-diulo-furanose from microthecin

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Abstract

4-Deoxy-*glycero*-hexo-2,3-diulose was formed from microthecin [2-hydroxy-2-(hydroxymethyl)-2H-pyran-3(6H)-one] in neutral water solutions by a Michael addition. The compound was determined by NMR spectroscopy, MS and polarimetry to be a racemic mixture of D-and L-forms and existing mainly as two furanosidic C-2 epimers in equilibrium with microthecin. GC–MS analysis showed that 4-deoxy-*glycero*-hexo-2,3-diulose and microthecin were present in extracts of the red alga *Gracilariopsis lemaneiformis*.

Keywords: 1,5-anhydro-D-fructose; Microthecin; 2,3-Diulose; *Gracilariopsis lemaneiformis*; Gracilariales

1. Introduction

In previous papers, we have reported some new aspects on starch metabolism in the red alga *Gracilariopsis lemaneiformis* [1–3]. A new enzyme, α-1,4-glucan lyase, which degrades starch from the non-reducing end to 1,5-anhydro-D-fructose, was isolated and characterised [1]. 1,5-Anhydro-D-fructose has previously been described in some genera of fungi [4,5], and recently also in rat liver [6]. This sugar was found to be further metabolised in *G. lemaneiformis* to the pyrone microthecin [3], also known to occur in several species of fungi, as well as in some bacteria [7,8].

The biological significance of the degradation of floridean starch to 1,5-anhydro-D-fructose and the enzymatic conversion of this unusual sugar to microthecin, remains to be further examined. The Japanese group, which first described microthecin, studied the antibiotic properties of this compound [8]. The antibioticity of microthecin implies a possible role as an antibacterial agent in the studied alga.

We are currently investigating the metabolical fates of 1,5-anhydro-D-fructose and microthecin in the red alga *G. lemaneiformis*. As part of these studies, the present paper reports the transformation of microthecin into 4-deoxy-*glycero*-hexo-2,3-diulose, a compound earlier isolated from the alkaline degradation of maltose and cellobiose [9,10], as well as the presence of this compound in the studied red alga.

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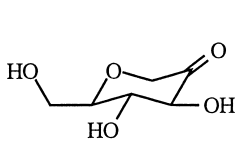
2. Results and discussion

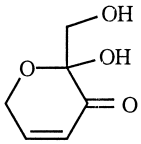
When 1,5-anhydro-D-fructose or microthecin was treated with water extracts from *G. lemaneiformis* containing algal enzymes, a new compound (**1**) was formed. Subsequent experiments showed that microthecin, enzymatically formed from 1,5-anhydro-D-fructose [3], was the precursor of **1**. Furthermore, **1** was shown to be formed from microthecin, also in the absence of algal enzymes. After incubation of microthecin in a citrate buffer of pH 6.6, at 30 °C for 18 h, the relative composition of the mixture was 45% of microthecin and 55% of **1**. Moreover, when lyophilised crude extracts of *G. lemaneiformis* were silylated, and subsequently analysed using GC–MS, compound **1**, as well as microthecin, was shown to be present. This finding shows that **1**, not only is formed in the laboratory, but also in the intact alga.

In a preparative experiment, 1,5-anhydro-D-fructose was incubated in an extract from *G. lemaneiformis* at 30 °C over night. After isolation with flash chromatography on silica gel, it was established, using NMR spectroscopy and MS, that compound **1** was 4-deoxy-*glycero*-hexo-2,3-diulose, a compound known to be formed during alkaline degradation of maltose and cellobiose [9,10]. Furthermore, NMR spectroscopy indicated that **1** existed in water as two furanosidic isomers (> 90%), with a 2:1 ratio between two C-2 epimers. The coupling constants $^{3}J_{H4a,5}$ and $^{3}J_{H4b,5}$ were found to be 9.1 and 6.2 Hz, respectively, for the dominating isomer (in deuterium oxide), and 5.0 and 8.2 Hz, respectively, for the minor form. If the compound was in a pyranosidic form, the difference between $^{3}J_{H4a,5}$ and $^{3}J_{H4b,5}$ would be expected to be larger, since in a pyranosidic ring, these couplings should resemble axial–axial and axial–equatorial couplings, unless the keto-group distorts the overall geometry of the ring to affect the coupling constants.

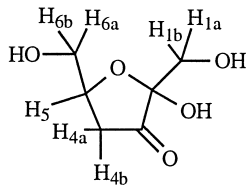
An alternative approach to determine the ring size of compound **1**, is to find a heteronuclear three-bond connectivity derived from the coupling ($^{3}J_{C,H}$) between H-5 and C-2 (for a furanosidic ring) or between either of the protons at C-6 and C-2 (for a pyranosidic ring). This was done by HMBC experiments, in Me_2SO-d_6, with mixing times of 50 and 100 ms, respectively. No cross-peak was observed between H-6a,6b and C-2, but in the latter experiment, a cross-peak between H-5 and C-2 was observed, which strongly supports a furanosidic ring form. This conclusion is further corroborated by studies of the deuterium-induced ^{13}C differential isotope shifts (DIS) of **1** [11]. This technique monitors the upfield shift for signals from carbons with directly bonded or vicinal OH groups, when the protons in the OH groups are exchanged to deuterons. Typically, shifts due to directly bonded OD groups are around 0.15 ppm in size, whereas shifts due to vicinal OD groups normally are 0.03–0.06 ppm [11]. The observed deuterium-induced ^{13}C differential isotope shift of C-6, in the major form of **1**, was 0.11 ppm higher than the DIS of C-5. When compared to the DIS values of C-5 and C-6 in β-D-fructofuranose (0.06 and 0.14 ppm) and in β-D-fructopyranose (0.16 and 0.06 ppm) [11], this finding confirms the conclusion that C-6, and not C-5, is carrying a free hydroxyl group, and thus, that **1** exists as a furanose. DIS studies of the minor form of **1**, resulted in similar results.



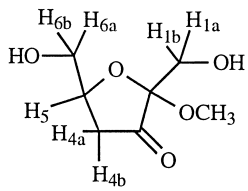
1,5-anhydro-D-fructose



microthecin



1



2

The same conclusion concerning the ring size of compound **1** was drawn from acetylation studies. The largest acetylation shifts will be found for protons attached to carbons carrying acetoxy groups [12]. If **1** was mainly in a pyranosidic form, the H-5 signal would be expected to have a large acetylation shift (1.2–1.5 ppm). However, the acetylation shift for the H-5 signal (+0.37 ppm) was found to be similar to the acetylation shifts for H-4a and H-4b (+0.26 and +0.38 ppm, respectively) whereas the acetylation shifts for H-1a, H-1b, H-6a and H-6b were considerably higher (+0.70, +0.82, +0.58 and +0.59 ppm, respectively), similar in size to those observed for 6-*O*-acetylated hexoses [12].

We did not succeed in determining the identity of the two C-2 isomers. NOE difference ^{1}H NMR ex-

periments on the methyl glycoside (**2**) failed to yield the necessary proofs. To determine the anomeric configuration of the studied isomer, an NOE across the five-membered ring is needed, either between H-1a,1b protons and H-5 or the H-6a,6b, or between the methyl protons and H-5 or H-6a,6b. This was, however, not observed in the performed experiments. Also ROESY and NOESY experiments on compound **1** failed to yield the needed proofs. We also tried to determine the identity of the isomers by studying a 1,2-*O*-isopropylidene derivative of **1**, prepared from **1** and acetone. However, no structurally revealing NOEs were observed.

The molecular mass of trimethylsilylated **1** was determined to be 378, using GC–MS. This corresponds to a molecular mass for **1** of 162, which is consistent with the result from FABMS, in the negative ion mode, which yielded an $[M-H]^-$ ion of *m/z* 161.

Further evidence for the identity of **1** was achieved by comparing ^{1}H NMR spectra of the compound originating from microthecin with spectra of 4-deoxy-D-*glycero*-hexo-2,3-diulose prepared by alkaline degradation of maltose [9]. The spectra were found to be identical, both concerning the chemical shifts and coupling constants, as well as the relative intensities of the signals from the α- and β-forms. The alkaline degradation of maltose also resulted in the formation of a small amount of microthecin, isolated by flash chromatography on silica gel and identified by ^{1}H NMR spectroscopy. This is most likely the result of dehydration of 4-deoxy-D-*glycero*-hexo-2,3-diulose. The same reaction was observed when 4-deoxy-*glycero*-hexo-2,3-diulose, originating from microthecin, was kept in neutral water solutions at room temperature over night. The observed equilibrium between compound **1** and microthecin should result in racemisation of 4-deoxy-D-*glycero*-hexo-2, 3-diulose isolated from maltose or cellobiose, making it difficult to determine the optical rotation for the isolated compound [9,10].

The equilibrium between microthecin and compound **1**, at neutral pH, appears to be positioned towards **1**, as illustrated by the ratio 9:11 for microthecin:**1** after 18 h at 30 °C in citrate buffer of pH 6.6. The mechanism by which **1** is formed, is likely to be a Michael addition of water to the double bond in microthecin. This addition can result in two C-5 epimers of **1**, the D- or L-form, and since no optical activity has been found for **1** formed from microthecin, it most likely exists as a racemic mixture.

If one of the two C-5 epimers of **1** could be transformed to another compound by the action of an enzyme, an optically active compound would be formed, and furthermore, the action of this enzyme would indirectly facilitate the formation of **1** from microthecin. This means that **1** can be able to function as a precursor for other compounds, even though its formation is not enzymatically catalysed.

A series of preliminary experiments implies a possibility that compound **1**, or microthecin, indeed is a precursor for other metabolites. Microthecin was incubated in extracts of *G. lemaneiformis* at room temperature for 24 h, without preceding dialysis of the extracts. When samples were analysed after different incubation durations, microthecin was found to decrease rapidly in concentration. The concentration of compound **1** was increasing with time, but this increase was much too low to explain the decrease in concentration of microthecin [unpublished data]. If, however, the extracts were dialysed prior to the incubation, the total concentration of microthecin and compound **1**, present at the end of the incubation period, were roughly equal to the concentration of microthecin at the beginning of the experiment [unpublished data].

These analyses were performed using GC–MS after silylation of lyophilised crude incubation mixtures.

A possible explanation to these observations, is that a small solute, e.g. UTP, necessary for activating microthecin or compound **1** for further reactions, or a cofactor of an enzyme, is removed during the dialysis of the algal extracts, and thus making the corresponding enzymatic elaboration of microthecin or compound **1** impossible. A second possibility is that the enzyme, responsible for the disappearance of microthecin and compound **1**, is unstable, and is rendered inactive during the dialysis.

3. Experimental

General methods.—NMR-experiments were performed on Varian VXR 400 and Bruker DRX 400 NMR spectrometers at 400 MHz (^{1}H) and 100 MHz (^{13}C).

GC–MS was run on a HP 5890 gas chromatograph (DB-5, 30 m × 0.25 mm, carrier gas He) connected to a HP 5970 quadrupole mass spectrometer (70 eV). The temperature program employed was 120–260 °C at 5°/min, after 3 min at 120 °C. The injector was held at 240 °C and the GC–MS interface at 260 °C.

FABMS spectra were recorded on a JEOL JMS-

SX/SX102A four-sector tandem mass spectrometer (matrix glycerol).

Optical rotation was measured on a Perkin-Elmer model 141 polarimeter.

Preparation of extracts from G. lemaneiformis.—*G. lemaneiformis*, [(Bory) Dawson, Acleto, et Foldvik], cultivated in the laboratory, was ground in liquid N_2, using a mortar and pestle [13,14]. To the resulting powder, 50 mM citrate buffer of pH 6.6 was added (3 mL/g fresh weight alga). Following thawing, the suspension was filtered through a two-layer cloth and the resulting extract was centrifuged for 10 min at 15,000 rpm (4 °C, Sorvall RC-5B, SS-34 rotor). The supernatant was drawn off and dialysed (cut off 6–8000 Da) twice against 50 mM citrate buffer of pH 6.6, or used directly without dialysis.

Preparation of **1** *from 1,5-anhydro-*D*-fructose/microthecin.*—1,5-Anhydro-D-fructose (900 mg) was dissolved in dialysed algal extract (200 mL) in a round bottomed flask. The solution was incubated at 30 °C for 48 h, and finally lyophilised. During the incubation, the formation of microthecin and **1** was followed by TLC [17:3 $CHCl_3$–MeOH, Rf 0.5 and 0.35, respectively, visualisation with anisaldehyde/H_2SO_4 (2.5%/2.5% in EtOH)]. For comparison, pure microthecin (3.7 mg/mL) was incubated at 30 °C over night in 50 mM citrate buffer of pH 6.6 and in dialysed algal extracts under the same conditions. At the end of the incubations, the reaction solutions were lyophilised. The control reactions were analysed with ^{1}H NMR spectroscopy by measuring the relative areas of the signals corresponding to H-1b of microthecin and H-6b of **1** (major form).

Isolation of **1**.—By treating the freeze-dried powder (from the large scale enzymatic treatment) with MeOH (3×50 mL), **1** was extracted together with 1,5-anhydro-D-fructose and microthecin. Following filtration, silica gel (5 g) was added to the pooled MeOH extracts, and the suspension was concentrated under reduced pressure (30 °C). Finally, the resulting dry powder was loaded onto a column (5×17 cm) of silica gel and chromatographed in 17:3 $CHCl_3$–MeOH. The fractions containing **1**, according to TLC (as above), were collected and concentrated at 30 °C under reduced pressure to yield 80 mg of **1** after lyophilisation from water.

*Preparation of 4-deoxy-*D*-glycero-hexo-2,3-diulose from maltose.*—Maltose (5.15 g) was dissolved in degassed aqueous 50 mM KOH (250 mL) under N_2 and stirred at room temperature for 24 h. After neutralisation with Dowex 50 (H^+) ion-exchanger and subsequent filtration, the solution was concentrated at 40 °C under reduced pressure and lyophilised. The resulting material was dissolved in MeOH (100 mL) followed by concentration in the presence of silica gel (5 g). The dry sample was chromatographed as described above to yield 60 mg of 4-deoxy-D-*glycero*-hexo-2,3-diulose.

Identification and characterisation of **1**.—Compound **1** was identified with NMR spectroscopy (^{1}H, ^{13}C, HMQC, HMBC), GC–MS and FABMS.

^{1}H NMR data (D_2O, 20°C): Major form: δ 3.68 (^2J 12.1 Hz, H-1a), 3.75 (H-1b), 2.54 (^2J 19.0 Hz, $^3J_{4a,5}$ 9.1 Hz, H-4a), 2.79 ($^3J_{4b,5}$ 6.2 Hz, H-4b), 4.65 (m, H-5), 3.76 (^2J 12.5 Hz, $^3J_{5,6a}$ 5.0 Hz, H-6a), 3.93 ($^3J_{5,6b}$ 3.0 Hz, H-6b). Minor form: δ 3.66 (^2J 11.9 Hz, H-1a), 3.77 (H-1b), 2.65 (^2J 19.2 Hz, $^3J_{4a,5}$ 5.0 Hz, H-4a), 2.84 ($^3J_{4b,5}$ 8.2 Hz, H-4b), 4.65 (m, H-5), 3.72 (^2J 12.2 Hz, $^3J_{5,6a}$ 5 Hz, H-6a), 3.83 ($^3J_{5,6b}$ 3.3 Hz, H-6b). ^{13}C NMR data (7:1 H_2O–D_2O, 20 °C). Major form: (C-1) δ 62.42, (C-2) 99.63, (C-3) 214.13, (C-4) 37.29, (C-5) 75.23, (C-6) 63.13. Minor form: (C-1) δ 63.21, (C-2) 99.30, (C-3) 215.15, (C-4) 37.21, (C-5) 75.89, (C-6) 64.33. The presented NMR data are given relative to sodium 3-trimethylsilylpropionate (δ_H 0.00) and acetone (δ_C 30.7). ^{1}H and ^{13}C NMR signals were assigned with HMQC and HMBC experiments. HMBC experiments were also run in Me_2SO-d_6, at 25 °C, with mixing times of 50 and 100 ms, respectively, to study the ring size of compound **1**.

Two subsequent ^{13}C NMR experiments, with deuterium oxide and 9:1 water–deuterium oxide, respectively, as solvents, were used to calculate the deuterium-induced ^{13}C differential isotope shifts. In each experiment, 11 mg of **1** was dissolved in 500 μL solvent, and 1% 1, 4-dioxan (δ_C 67.40) was used as reference compound.

Compound **1** (1 mg) was treated with 0.1 mL Sigma-Sil-A (6:5:1 pyridine–hexamethyldisilazane–chlorotrimethylsilane) at room temperature for 2 h to yield the corresponding trimethylsilyl ether. The resulting suspension was filtered through glass wool and centrifuged (13,000 rpm for 5 min). The resulting solution was analysed with GC–MS. The same method of derivatisation was employed when crude incubation mixtures, as well as algal extracts, were analysed with GC–MS.

FABMS analysis of compound **1** was run in the negative ion mode with glycerol as matrix.

Acetylation of **1**.—Compound **1** (1 mg) was treated over night with 0.5 mL dry pyridine and 0.5 mL Ac_2O at room temperature. Following coevaporation

with MeOH, the product in Me_2SO-d_6 was analysed with ^1H NMR spectroscopy at 30 °C. The acetylation shifts were calculated by comparing these NMR data with NMR data for **1** (data not shown).

Preparation of **2** *from* **1**.—Compound **1** (30 mg) was treated with MeOH (100 mL) and Dowex 50 (H^+) ion-exchanger at room temperature for 24 h, under N_2-atmosphere. Following filtration, the solvent was evaporated and the residues chromatographed on a column of silica gel (1.5 × 10 cm) in 20:1 $CHCl_3$–MeOH. The fractions containing the methyl glycosides of **1**, as indicated by TLC [the same eluent, visualisation with anisaldehyde/H_2SO_4 (2.5%/2.5% in EtOH)], were examined by GC–MS (following silylation, as above). A mixture of the C-2 epimers was collected and concentrated at 30 °C under reduced pressure and finally separated on semi-preparative C-18 reverse phase HPLC using 1:9 acetonitrile–water as eluent. The separation was followed by UV detection at 205 nm. The fractions containing the dominating isomer of **2** (> 90% pure), corresponding to the major form of **1**, were collected and evaporated to dryness at 40 °C under reduced pressure. ^1H NMR data (25°C, $CDCl_3$): δ 3.74 (^2J 11.3 Hz, H-1a), 3.86 (H-1b), 2.63 (^2J 18.9 Hz, $^3J_{4a,5}$ 7.3 Hz, H-4a), 2.75 ($^3J_{4b,5}$ 7.6 Hz, H-4b), 4.65 (m, H-5), 3.66 (^2J 12.1 Hz, $^3J_{6a,5}$ 2.8 Hz, H-6a), 4.08 ($^3J_{6b,5}$ 2.3 Hz, H-6b), 3.32 (OCH_3). The ^1H NMR data are given relative to $CHCl_3$ (δ 7.26). The ^{13}C chemical shifts of the methyl glycoside (from HMQC and HMBC experiments, data not shown) were found to be very similar to the ^{13}C chemical shifts of **1**, which strongly indicated that the studied isomer of **2** was in the same ring form as **1**. The NOE difference ^1H NMR spectra were obtained using Bruker pulse sequence Noemul, with irradiation of all signals within the different multiplets (total irradiating time 2.5 s).

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