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PRESENCE OF MICROTHECIN IN THE RED ALGA *GRACILARIOPSIS LEMANEIFORMIS* AND ITS FORMATION FROM 1,5-ANHYDRO-D-FRUCTOSE

ANDERS BROBERG,* LENNART KENNE,*‡ and MARIANNE PEDERSÉN†

*Department of Chemistry, Swedish University of Agricultural Sciences, PO Box 7015, S-750 07 Uppsala, Sweden; †Department of Physiological Botany, Uppsala University, Villavägen 6, S-752 36 Uppsala, Sweden

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Key Word Index—*Gracilariopsis lemaneiformis*; Gracilariales; biosynthesis; pyrone; microthecin; 1,5-anhydro-D-fructose.

Abstract—Microthecin, a pyrone isolated from *Gracilariopsis lemaneiformis*, is formed from 1,5-anhydro-D-fructose through two sequential elimination reactions; presumably by the action of a single enzyme.

INTRODUCTION

Starch is known to be the precursor of 1,5-anhydro-D-fructose in morels and red algae [1, 2]. This unusual sugar is produced by the degradation of starch by a novel enzyme, α-1,4-glucan lyase, which has been isolated and characterized from the red alga *Gracilariopsis lemaneiformis* [2]. The metabolic role of anhydrofructose in the studied organisms is not yet understood, but it is a precursor of the pyrone microthecin [2-hydroxy-2-(hydroxymethyl)-2*H*-pyran-3(6*H*)-one] in morels [3]. This compound was initially isolated from bacteria and has been shown to have antibiotic properties [4]. However, the biochemical pathway leading from anhydrofructose to microthecin and the subsequent metabolism of microthecin have not been fully described.

Recently, a biochemical pathway (Scheme 1) explaining the formation of microthecin from anhydrofructose in morels was proposed [5]. This route involves an initial dehydration of anhydrofructose, hydrolysis of the internal ether linkage, ketoenol rearrangement to a 2,3-diketo compound which forms a cyclic hemiketal and finally undergoes a dehydration reaction to produce microthecin. At least two enzymes were suggested to be involved in this mechanism. These findings were principally based on spectrophotometric measurements.

To our knowledge the presence of microthecin has not been reported in any other organism than the fungi investigated by Baute *et al.* [6] and the bacteria from which the compound was originally described [4].

This paper reports the presence of microthecin in the red alga *G. lemaneiformis*, and also indicates that the mechanism leading to the synthesis of microthecin from anhydrofructose in a red alga, is different from the mechanism proposed to occur in morels [5].

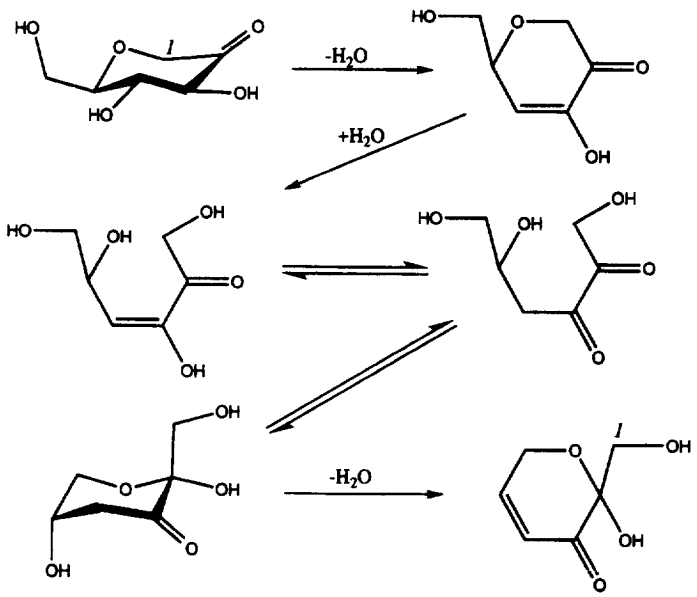
RESULTS AND DISCUSSION

Microthecin was isolated from crude extracts of *G. lemaneiformis* by extraction of algal extracts with ethyl acetate and subsequent flash chromatography on silica gel. The identity of microthecin was confirmed by ^{1}H and ^{13}C NMR spectroscopy and FAB-MS. ^{1}H NMR (D_2O, 70°): H-1a δ3.66 (*d*), H-1b δ3.97 (*d*), H-4 δ6.22 (*ddd*), H-5 δ7.38 (*ddd*), H-6a δ4.53 (*ddd*), H-6b δ4.73 (*ddd*); ^{13}C NMR (D_2O, 25°): C-1 δ63.20, C-2 δ96.03, C-3 δ193.68, C-4 δ124.21, C-5 δ152.11, C-6 δ60.77; FAB-MS (matrix glycerol): *m/z* 167 $[M + Na]^+$, 145 $[M + H]^+$ and 127 $[M + H - H_2O]^+$. The ^{1}H NMR data are in good agreement with earlier published data [6].

The enzymatic synthesis of microthecin was studied by incubating algal extracts with anhydrofructose or ^{13}C-substituted anhydrofructose under various conditions and analysing the microthecin formed.

When anhydro[1-^{13}C]fructose was used as substrate for *in vitro* enzymatic microthecin synthesis, the product was ^{13}C-substituted at C-1, proving that C-1 in anhydrofructose corresponds to C-1 in microthecin. This was found by studying the ^{1}H and ^{13}C NMR spectra of microthecin synthesized from anhydro[1-^{13}C]fructose, which revealed that the doublets corresponding to H-1a and H-1b in microthecin were further split [$^1J_{C,H}$ = 146 Hz] in microthecin produced from anhydro[1-^{13}C]fructose, indicating a coupling to a ^{13}C nucleus. Moreover, the only signal of significance in the ^{13}C NMR spectrum of microthecin produced from

‡Author to whom correspondence should be addressed.



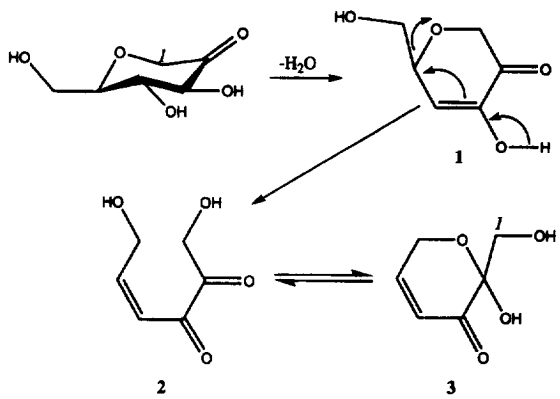
Scheme 1. Proposed route for the biosynthesis of microthecin from anhydrofructose in morels [5].

anhydro[1-^{13}C] fructose was the signal at δ 63.20 corresponding to C-1.

The enzymatic treatment was also performed using D_2O as solvent. When microthecin formed in D_2O was compared to microthecin formed in H_2O, no differences in the corresponding ^{1}H NMR spectra were detectable. This finding was incompatible with the reaction pathway leading from anhydrofructose to microthecin (Scheme 1) proposed by Baute *et al.* [5]. An important step of this suggested mechanism is the conversion of the enol form of the dehydrated anhydrofructose to the corresponding keto form. However, a keto-enol equilibrium in D_2O should incorporate a ^{2}H atom at C-4 in microthecin, which would eliminate the signal in a ^{1}H NMR spectrum corresponding to H-4, and also radically change the coupling patterns of the H-5, H-6a and H-6b signals.

To account for these findings, the pathway from anhydrofructose to microthecin cannot involve hydrolysis of the internal ether linkage, followed by a keto-enol equilibrium. The requirements could, however, be fulfilled by the following tentative pathway (Scheme 2). Upon elimination of H_2O from anhydrofructose, resulting in the intermediate **1**, a β-elimination takes place, resulting in an open-chain form of microthecin [1,6-dihydroxy-(*cis*)-4-hexene-2,3-dione] (**2**). The open-chain form of microthecin is in equilibrium with the cyclic forms of microthecin (**3**) through hemiketal formation. Finally, to explain the lack of deuterium at C-4, the intermediate **1** will not tautomerize to the 2,3-diulose with the concomitant incorporation of ^{2}H from the solvent; instead **2** is directly formed from **1**.

It is possible that this mechanism is catalysed by the activity of a single enzyme, which is responsible for the H_2O elimination step and the initiation of the β-elimination resulting in **2**. This could explain why no deuterium is incorporated at C-4 in microthecin, since intermediate



Scheme 2. Revised pathway for the biosynthesis of microthecin from anhydrofructose.

1 would only be transiently present at the active site of the enzyme, which would minimize the proton exchange process otherwise normally detectable. However, the presence of **1** has not been detected in this investigation. When 1,5-anhydro-D-fructose was incubated with an algal extract of *G. lemaneiformis*, no other compound was detected prior to the formation of microthecin. There are two possible explanations for this observation; either the intermediate **1** is a very short lived species occurring transiently at the active site of the enzyme, or the two elimination steps occur simultaneously at the active site of the enzyme.

The single enzyme hypothesis does not correlate with the mechanism proposed by the French group, since in that mechanism two enzymes responsible for the different dehydration steps are needed plus an additional enzyme to break the internal ether linkage, a hydrolysis reaction

unlikely to be induced merely by increased ring tension as suggested by Baute *et al.* [5].

When a H_2O solution of anhydrofructose is kept at neutral pH in the absence of algal extracts, no apparent reaction occurs. However, anhydrofructose is not stable in alkaline H_2O solutions [7]. The quantitative formation of the enolate form of **1** is reported as the first intermediate when anhydrofructose is treated with aqueous alkali. In that reaction no microthecin is detected. Instead, **1** tautomerizes to a 2,3-diulose which undergoes a benzylic acid rearrangement to 3*R*,5*S*-3-hydroxy-5-(hydroxymethyl)oxolane-3-carboxylic acid [7]. Thus, the enzymatic reaction is different from the reactions occurring in aqueous alkali, and furthermore, the dehydration of anhydrofructose resulting in **1** and the subsequent reactions in the biosynthesis of microthecin have to be under enzymatic control.

EXPERIMENTAL

Algal material. The red alga *Gracilariopsis lemaneiformis* [(Bory) Dawson, Acleto, et Foldvik] was cultivated as previously described [8, 9]. Freshly harvested alga was ground in liquid N_2, using a mortar and pestle, and then 2 ml buffer (10 mM citrate–NaOH, pH 6.6)/g fr.wt of alga was added. Following thawing, the resulting suspension was filtered through two layers of cloth and centrifuged at 36 600 × *g* for 30 min at 4°. The supernatant (also used as a crude extract) was transferred to dialysis tubing (24 Å cut-off) and dialysed twice against 50 vol buffer. Lyophilization yielded the enzyme mixture used in this study. Before use, the lyophilized algal extract was dissolved in H_2O (0.27 ml mg^{-1} freeze-dried algal powder). The enzyme extract to be used in the ^2H labelling studies was lyophilized twice from D_2O.

Presence of microthecin in G. lemaneiformis. To a crude extract (see above) of *G. lemaneiformis* was added $(NH_4)_2SO_4$ to a final concentration of 40% (w/w) and the precipitated proteins were removed by centrifugation of 36 600 × g for 30 min at 4°. The resulting supernatant was extracted with 5 vol EtOAc. Following concn of the organic phase under red. pres., the resulting substance was dissolved in H_2O and lyophilized. The presence of microthecin was shown as described below.

Enzymatic production of microthecin. Algal extract (1 ml) was incubated at 30° for 1 hr with anhydrofructose (2 mg), prepared enzymatically from amylose by the use of α-1,4-glucan lyase isolated from *G. lemaneiformis* [2, 10]. The reaction mixture was filtered in Centricon-10 tubes (10 kDa cut-off) and the filtrate extracted with EtOAc (6 × 3 ml). After concentration of the organic phase under red. pres., the residue was dissolved in $CHCl_3$–MeOH (17:3; 1 ml) and flash chromatographed on a column of silica gel. Frs were collected and those containing microthecin, as indicated by TLC (the same eluent, visualization with UV_{254} and anisaldehyde–H_2SO_4, a blue spot at R_f 0.5), were pooled and concd under red. pres. The resulting substance was dissolved in H_2O and lyophilized. For comparison, the same experiment was carried out with only algal extract present or with only anhydrofructose present in the reaction mixts. These experiments were analysed by TLC and ^1H NMR spectroscopy. The formation of microthecin was also followed directly by ^1H NMR spectroscopy by incubating 1,5-anhydro-D-fructose (4 mg) and algal extract (20 μl) in 0.7 ml D_2O at 30° in an NMR-tube.

Identification of microthecin. Microthecin was identified by ^1H and ^{13}C NMR spectroscopy and FAB-MS. ^1H NMR (400 MHz, D_2O, 70°): H-1a δ3.66 [$^2J_{H\text{-}1a,H\text{-}1b}$ = 11.6 Hz], H-1b δ3.97, H-4 δ6.22 [$^2J_{H\text{-}4,H\text{-}5}$ = 10.4 Hz, $^4J_{H\text{-}4,\ H\text{-}6a}$ = 2 Hz, $^4J_{H\text{-}4,H\text{-}6b}$ = 2 Hz], H-5 δ7.38 [$^3J_{H\text{-}5,H\text{-}6a}$ = 4 Hz, $^3J_{H\text{-}5,H\text{-}6b}$ = 2 Hz], H-6a δ4.53 [$^2J_{H\text{-}6a,H\text{-}6b}$ = 19.8 Hz], H-6b δ4.73; ^{13}C NMR (100 MHz, D_2O, 25°): C-6 δ60.77, C-1 δ63.20, C-2 δ96.03, C-4 δ124.21, C-5 δ152.11, C-3 δ193.68. ^1H and ^{13}C NMR data are given relative to TSP (sodium 3-trimethylsilylpropionate, δ_H0.00) and acetone-d_6 (δ_C 28.9), respectively. Assignments of the ^{13}C NMR signals were achieved by a HMQC experiment. FAB-MS (matrix glycerol): *m/z* 167 $[M + Na]^+$, 145 $[M + H]^+$ and 127 $[M + H - H_2O]^+$.

Studies of the reaction mechanism. [1-^{13}C]amylose (12 mg) prepared from [1-^{13}C]glucose [10], was dissolved in aq. 1 M KOH (1 ml). The pH was adjusted to 7.0 with aq. 1 M HCl. After dilution with H_2O (2 ml), α-1,4-glucan lyase (10 U) was added to the soln. The reaction mixture was transferred to a dialysis tube (24 Å cut off) placed in 100 ml distilled H_2O, and incubated over night at 30°. After lyophilization, the dialysate was extracted with $CHCl_3$–MeOH (7:3; 5 × 1 ml). The organic phase was evaporated and the resulting mixture, containing mainly anhydro[1-^{13}C]fructose, was dissolved in H_2O and lyophilized. ^{13}C-substituted microthecin was synthesised by incubating anhydro[1-^{13}C]fructose (2 mg) with 1 ml algal extract at 30° for 1 hr. [1-^{13}C]microthecin was isolated as described above. The incorporation and location of the ^{13}C nucleus in microthecin was studied using ^1H and ^{13}C NMR spectroscopy (solvent D_2O).

Algal extract (1 ml, prepared in D_2O) was incubated at 30° for 1 hr with anhydrofructose (2 mg). At the end of the incubation the mixture was filtered in Centricon-10 tubes (10 kDa cut-off) and extracted with EtOAc as previously described. The organic phase was evaporated under red. pres. and the residues dissolved in H_2O and lyophilized. The incorporation of ^2H in microthecin was studied by comparison of signal intensities in the ^1H NMR spectrum (solvent D_2O).

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