Isolation of sophorose during sophorolipid production and studies of its stability in aqueous alkali: epimerisation of sophorose to 2-*O*-β-D-glucopyranosyl-D-mannose

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Abstract

NMR and anion exchange chromatography analysis of the waste streams generated during the commercial production of sophorolipids by the yeast *Candida bombicola* identified the presence of small but significant quantities (1 % w/v) of free sophorose. Sophorose, a valuable disaccharide, was isolated from the aqueous wastes using a simple extraction procedure and was purified by chromatography on a carbon celite column providing easy access to large quantities of the disaccharide. Experiments were undertaken to identify the origin of sophorose and it is likely that acetylated sophorose derivatives were produced by an enzyme catalysed hydrolysis of the glucosyl-lipid bond of sophorolipids; the acetylated sophorose derivatives then undergo hydrolysis to release the parent disaccharide.

Treatment of sophorose with aqueous alkali at elevated temperatures (0.1M NaOH at 50 ^{O}C) resulted in C2-epimerisation of the terminal reducing sugar and its conversion to the corresponding 2-*O*-β-D-glucopyranosyl-D-mannose which was isolated and characterised. In aqueous alkaline solution β-(1,2)-linked glycosidic bonds do not undergo either hydrolysis or peeling reactions.

Key words:

Sophorose, Sophorolipids, β-(1,2)-linked disaccharides, 2-*O*-β-D-glucopyranosyl-D-mannose

1. Introduction

The disaccharide sophorose, 2-*O*-β-D-glucopyranosyl-D-glucose, is an important biologically active molecule. As a free disaccharide, sophorose is a potent inducer of cellulases[1] and is used to generate enzymes for use in bioethanol production. Sophorose is a structural component of the biologically active glycosides saponins[2], including ginsengosides[3]. Sophorose is also the carbohydrate component of sophorolipids[4-6] which are increasingly being manufactured on a large scale for use as surface active agents.

The synthesis and derivatisation of sophorose was first reported in the 1920s by Freudenberg *et al*[7] and subsequently the methods were improved in the 1960s by Coxon and Fletcher[8]. Interest in sophorose grew in the 1950s when it was found to be present in trace amounts in commercial supplies of D-glucose that had been obtained by the mineral acid catalysed hydrolysis of starch[9] and this led to increased efforts to find a more convenient route to sophorose. In the 1950 and 1960s a number of authors reported the isolation of sophorose from naturally occurring sophoryl-glycosides including kaempferol sophoroside isolated from pods of *Sophoar japonica*[10] and from the sweet ester glycoside stevioside from *Stevia rebaudiana*[11]. More recently sophorose and activated sophorose donors have been produced from sophorolipids. Hoffmann *et al*[12] have reported the isolation of per-*O*-acetyl-sophorosyl bromide from the reaction of acetylated sophorolipids with acetic acid and hydrogen bromide. Jourdier and Ben[13] have reported the acid catalysed hydrolysis of sophorolipids generating a mixture of glucolipids, glucose and sophorose from which, after enzyme treatment to remove glucose, they were able to isolate sophorose.

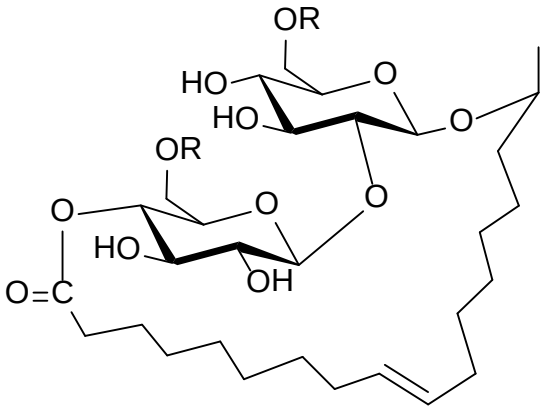
Despite the relatively wide interest in sophorose there is only limited information about its chemical reactivity and that of 1,2-linked glycosides in general; this is partially due to the fact that they are not readily accessible. In this paper we report the isolation of sophorose from a waste stream generated during the commercial production of sophorolipids, provide evidence for its synthesis by a combination of enzyme and base catalysed hydrolysis reactions and we describe the results of our studies of the reaction of sophorose with aqueous alkali.

2. Results and Discussion.

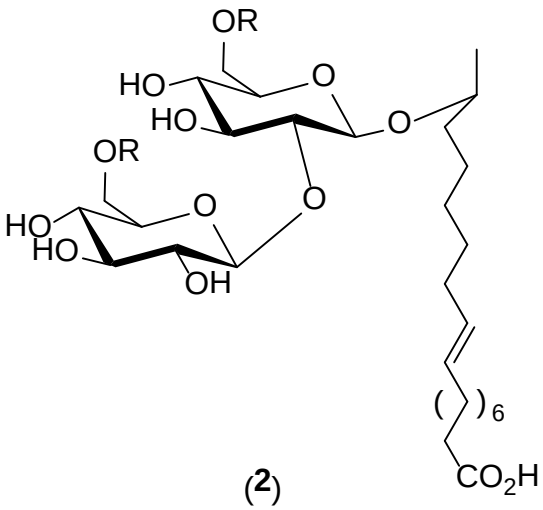
2.1 Isolation of sophorose form sophorolipid process waste streams.

The yeast *Candida bombicola* produces sophorolipids in a biosynthetic pathway[14,15] involving the hydroxylation of a fatty acid (normally C18) to generate a (ω-1)-hydroxyfatty acid which is then glucosylated twice, initially at the (ω-1)-hydroxyl-group and then at the 2'-position of the newly added glucose residue to generate a sophorolipid. The pathway is concluded with the partial acetylation of the sugar primary hydroxyls (6' and 6'') and by partial lactonisation of the 4''-OH with the lipid fatty acid to give a mixture of lactone (**1, R=H or OAc**) and acid (**2, R=H or OAc**) forms of the sophorolipids (Scheme 1).

Scheme 1 here.



(**1**)



(**2**)

During a typical fermentation, sophorolipid production is allowed to proceed until the pH of the fermentation mixture falls to approximately 3.5 and the mixture settles into two phases: a lipid rich bottom layer containing the sophorolipids[16] and an upper aqueous phase (spent liquors). To isolate the deacetylated acid form of the sophorolipid (**2 R=H**) it is normal practice to recover the pure lactone and then to convert it to the acid. The lactone is recovered from the lipid rich layer by extraction of acidic impurities by adjustment of the pH to above the pKa of the acid group using cold aqueous alkali (pH 5.5). After separation of the layers, the lipid rich layer contains predominately the lactone form and the aqueous phase (lactone waste) which is brown in colour contains a number of impurities. To isolate the acid form the lactone is converted into the acid in a

hydrolysis step involving heating with aqueous alkali[17]. In an attempt to determine the composition of the different phases and to understand the extraction and hydrolysis reaction the different phases were analysed by NMR and HPAEC. HPAEC analysis of the lactone waste identified the presence of free sugars, interestingly the HPAEC chromatograph (Fig 1) contained peaks consistent with the presence of both glucose and sophorose (**3**) (ratio 1: 10 w/w). NMR analysis confirmed the presence of sugars and acid sophorolipids.

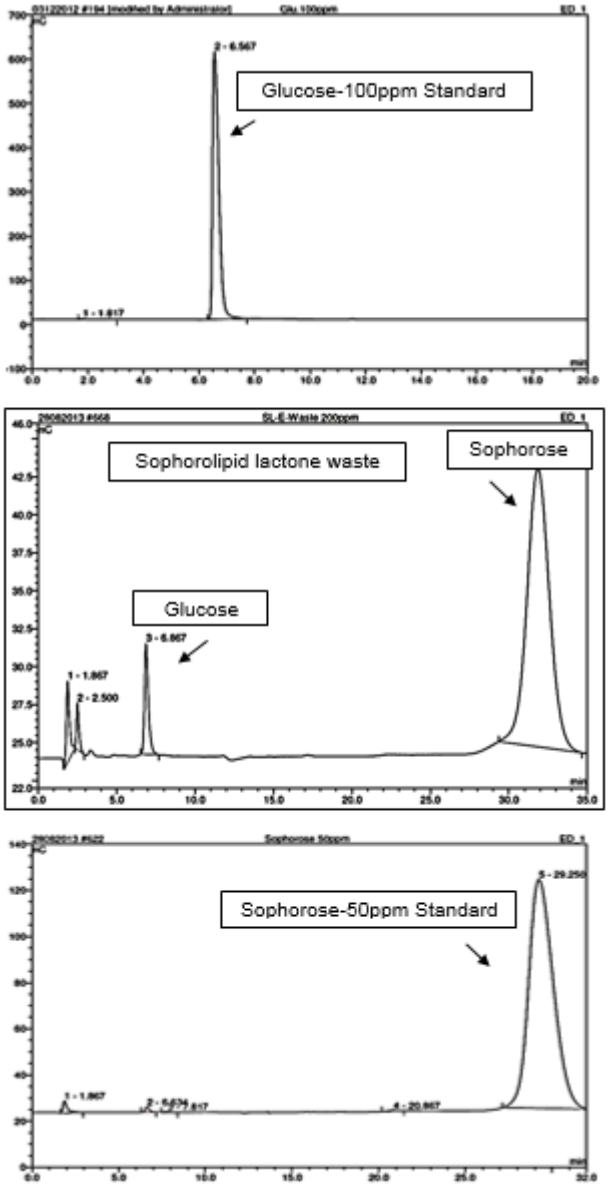


Figure 1: High Performance Anion Exchange Chromatographs (HPAEC) chromatographs recorded using a Dionex ICS3000 and employing a Carbopac PA20 column. Analytes (50-200 ppm) were eluted with a mobile phase composed of 20 mM KOH with a flow rate of 0.3 ml.min^{-1}. Top: A 100 ppm glucose standard; Middle: A sample of the lactone waste; Bottom: A 50 ppm sophorose standard.

As the commercial process generates large volumes of lactone waste (1000s of litres) an attempt was made to isolate the sophorose. Initially, the waste (98g, 100mL) was diluted with an equal volume of ultrapure water and the sugars were extracted into the aqueous phase leaving the lipid components as a separate organic phase. After separation of the two layers, the aqueous phase was evaporated to leave a brown solid residue which contained a range of salts as well as the desired sugars (5.5g, 5.6% w/w of the original waste). NMR analysis of the solid (Fig 2a) confirmed that the organic component of the material was a mixture of glucose and sophorose and that no lipids were present. Comparison of the integrals for the anomeric protons (α plus β-anomers) for the two sugars indicated that the extracted mixture contained a 1: 2.3 (w/w) ratio of glucose to sophorose and HPAEC analysis suggested a slightly higher ratio of 1:2.9 (w/w). The solid was dissolved in ultra pure water and applied to a carbon-celite column and the column was eluted initially with water to remove salts and then with an aqueous mobile phase containing increasing concentrations of ethanol. Sophorose eluted as a single peak when the eluent was composed of 20% ethanol; after evaporation sophorose was recovered as a crystalline white solid (1.0g). NMR analysis (Fig 2b) indicated that sophorose with a purity of greater than 95% had been recovered and this corresponds to approximately one percent of the original waste. Given the large volume of lactone waste available this represents a potentially large source of sophorose.

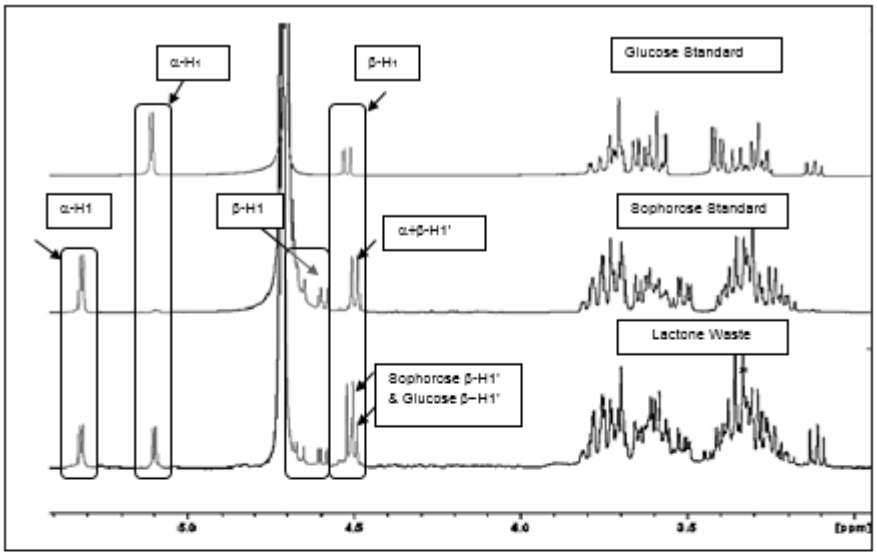
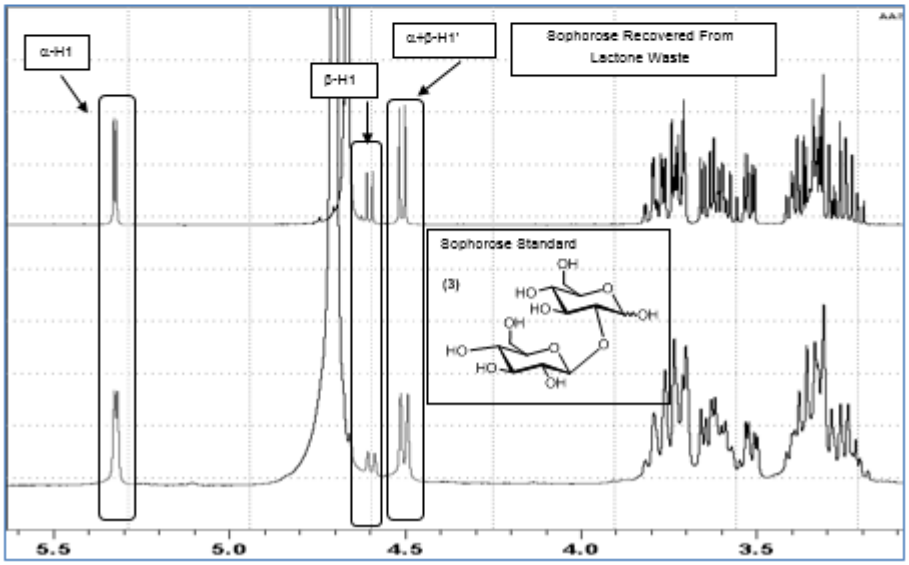


Figure 2b



129

130 **Figure 2:** ^{1}H-NMR spectra recorded at 70 ^{o}C in D_2O on a Bruker 500MHz Avance Spectrometer (a)
131 comparison of NMRS od a glucose standard, a sophorose standard and a sample of the lactone
132 waste: a) top: glucose standard; middle: sophorose standard; bottom: sugar sample extracted from
133 lactone waste (b) top: NMR of the sophorose isolated from a carbon-celite column; bottom: NMR of
134 a sophorose standard.

135 As the biosynthetic pathway involves sequential addition of glucose residues to the lipid chain[15],

136 free sophorose was not expected to be a product of the fermentation process.

137 *2.2 Attempt to observe base catalysed hydrolysis of sophorolipids.*

138 In an attempt to determine if sophorose was being produced by a base catalysed hydrolysis of the

139 sophorolipid during the processing of the crude fermentation products, an experiment was

140 undertaken to see if sophorose was released from the acid form of the sophorolipid on heating with

aqueous alkali. The acid form of the sophorolipid was chosen because of its solubility in aqueous alkali and because of the absence of acetyl-ester groups, the hydrolysis of which would consume base. The reaction of the acid sophorolipid with aqueous alkali (pH 12, 50 °C) was monitored by HPAEC and NMR over a period of 24 h. No reaction was observed by NMR, however, analysis of the HPAEC chromatographs (Fig 3) identified the rapid production of very small quantities of glucose, small quantities of sophorose and a second disaccharide which was identified as the C2-epimer 2-*O*-β-D-glucopyranosyl-D-mannose (see Section 2.4). These additional sugars were produced in the first three hours after which time no further reaction was observed and the combined peak area of the new sugars suggested that less than 0.5% of the starting material had been converted to sophorose. Under the relatively mild alkaline conditions employed, sophorolipids were not expected to undergo glycosidic bond hydrolysis or to participate in either substitution or elimination processes centred at the lipid ω-1 carbon; it is more likely that the very small amount of sophorose that is produced is generated through the rapid hydrolysis of small quantities of the corresponding acetylated-sugars that are present as minor impurities in the starting acid. This result suggests that under moderately basic conditions hydrolysis of sophorolipids does not lead to the production of sophorose.

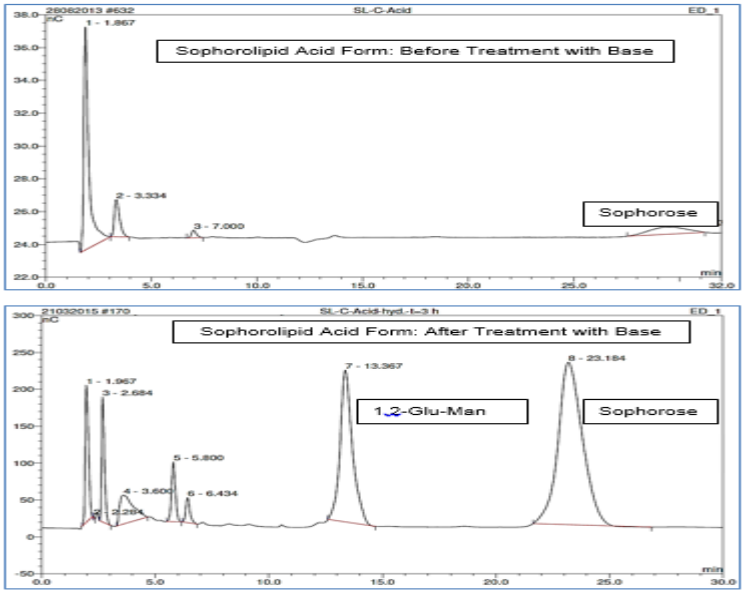


Figure 3: High performance anion exchange chromatographs recorded using a Carbopac PA20 column and eluting analytes (50-200 ppm) with a mobile phase composed of 20 mM KOH with a flow rate of 0.3 ml.min^{-1}. Top: Acid form of the sophorolipid; bottom: reaction mixture obtained after treatment of the acid sophorolipid with aqueous alkali (10 mM, 3 h, 50 °C).

2.3 Observation of an enzyme catalysed hydrolysis of sophorolipids. One of the reasons why the yeast *C. bombicola* is thought to synthesise sophorolipids is to allow it to sequester available fatty acids. It has previously been suggested that under conditions of starvation the yeast is able to metabolise sophorolipids[14]. In order to investigate the possibility that the yeast releases an enzyme which is able to catalyse the hydrolysis of the gluco-lipid bond of the acid or lactone forms of the sophorolipids, two experiments were undertaken in which the spent liquors recovered at the end of a large scale fermentation were filtered to remove any biomass and then immediately added to a solution containing either the diacetylated-lactone sophorolipid (**1, R=Ac**) or the deacetylated acid form (**2, R=H**) of the sophorolipid.

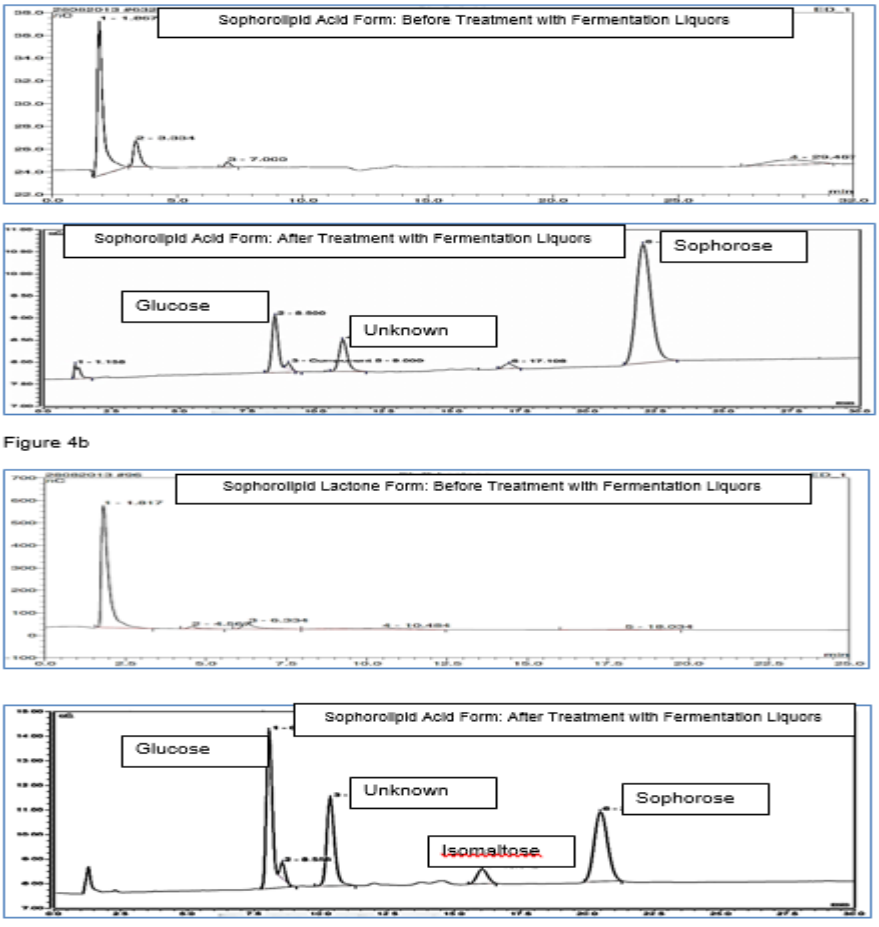


Figure 4b

Figure 4: Use of high performance anion exchange chromatography to monitor the enzyme catalysed hydrolysis of sophorolipids[1]

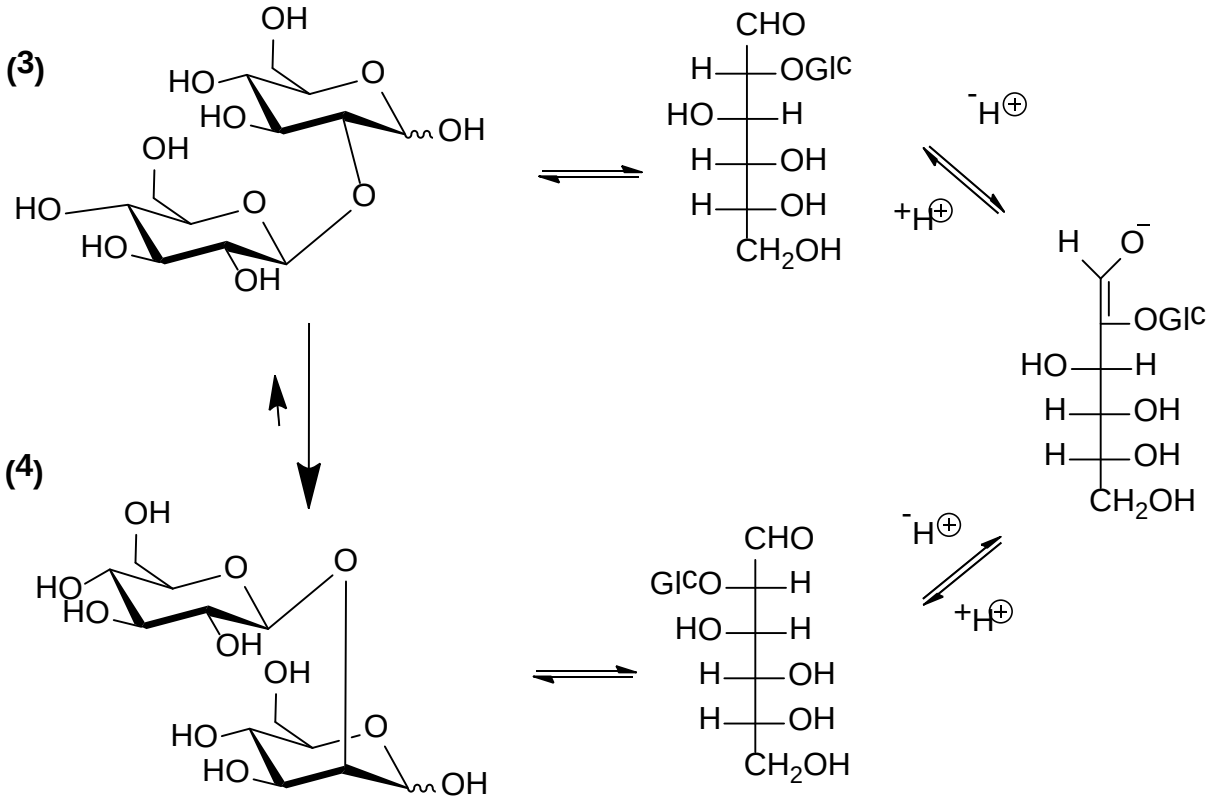
(a) Acid form of the sophorolipid; top: before addition of fermentation liquor and bottom: after treatment with spent fermentation liquor

(b) Lactone form of the sophorolipid; top: before addition of fermentation liquor and bottom: after treatment with spent fermentation liquors.

Chromatographs generated during HPAEC analysis of the products (Fig 4a Acid after treatment and 4b Lactone after treatment) showed the presence of sophorose and glucose along with a small amount of isomaltose and an unknown product (potentially glucolipid). The amount of sophorose was significantly higher when the acid form of the sophorolipid was used as a substrate. The results are consistent with an enzyme catalysed hydrolysis of the sophorolipids with the acid form having generated sophorose, in comparison, the product formed from the lactone would be expected to be diacetylated-sophorose. The latter result, whilst not being conclusive evidence, is consistent with the presence of enzyme/s that are able to cleave the 1,2-glycosidic bond and the lipid-glycosidic bond. An enzyme catalysed cleavage of the 1,2-glycosidic link would, in the first instance, generate a gluco-lipid and a glucosyl-enzyme intermediate. Attack by water on the glucosyl-enzyme intermediate would generate glucose and release the enzyme. Attack by a primary hydroxyl of a second glucose molecule on the glucosyl-enzyme intermediate would lead to production of isomaltose and release the enzyme. Production of isomaltose requires the transferase activity of an inverting enzyme.

2.4 Studies of the chemical reactivity of sophorose and isolation of 2-O-β-D-glucopyranosyl-D-mannose. Very little is known about the chemical reactivity of 1,2-linked glycosides. Treatment of 1-3, 1-4 and 1-6-linked glycosides with aqueous alkali results in the observation of peeling reactions and the generation of multiple small chain fatty acids including saccharinic acids[18,19]. As peeling proceeds through enolisation and migration of the carbonyl carbon to C2 and beyond (a process that is not possible in C2-linked glycosides) it was of interest to study the stability of sophorose in aqueous alkali. After treatment of sophorose with sodium hydroxide solution using conditions which would promote peeling of glycosides, the HPAEC chromatographs contained signals identifying the presence of two carbohydrates: a late eluting peak for sophorose and a second disaccharide peak. NMR analysis of the reaction mixture indicated that deuterium exchange was occurring at the C2 position of the starting material and, as was the case in the HPAEC analysis, a second disaccharide was present. These results were consistent with the epimerisation of the reducing sugar to form the corresponding 2-*O*-β-D-glucopyranosyl-D-mannose (4, Scheme 2).

Scheme 2



The partial epimerisation of sophorose has already been reported but in this earlier study the product 2-*O*-β-D-glucopyranosyl-D-mannose was not isolated and was not fully characterised[20]. The NMR spectra recorded here suggested that under the conditions of the current experiments 75% of the starting sophorose was converted to 2-*O*-β-D-glucopyranosyl-D-mannose (**4**). The mechanism for such epimerisation reactions was first proposed by Lobry de Bruyn and Alberda van Ekenstein[21] and involves ring-opening of the reducing glucose and base catalysed reversible enolate formation between C2 and C1. Reprotonation of the enolate anion on C2 can take place on either the top Re-face to regenerate glucose or from the bottom Si-face to generate mannose. At equilibrium, and under the conditions employed in the current experiments, the thermodynamically more favourable mannose epimer dominates (see scheme 2).

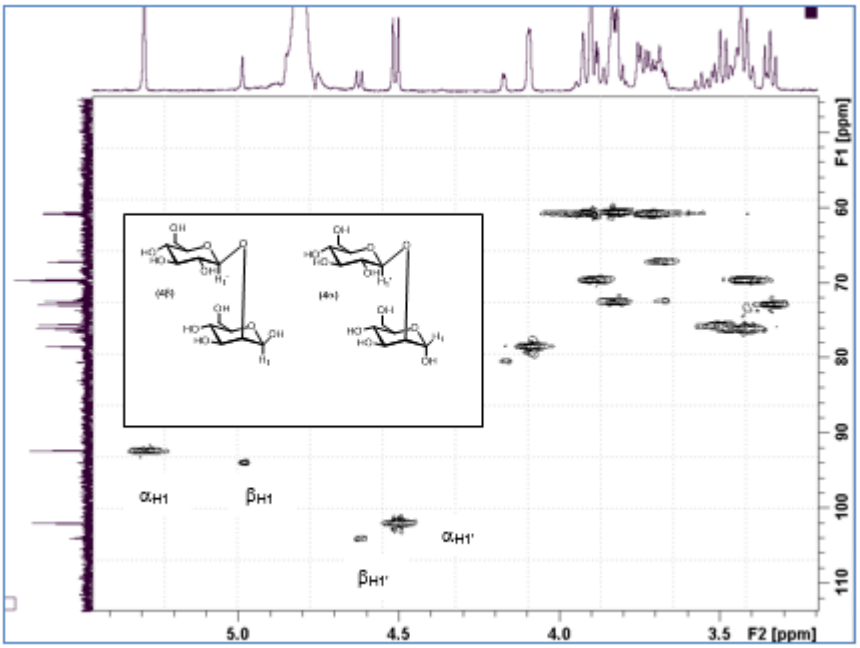


Figure 5. Selected region of the HSQC NMR spectrum of *2*-O-*β-D-glucopyranosyl-α/β-D-mannose.* The F1-axis provides the ^{13}C -spectrum and the F2-axis the ^{1}H -spectrum. The location of the individual resonances for the different protons and carbons are listed in the table. The spectrum was recorded at 70 °C in D_2O.

In an attempt to isolate the 2-*O*-β-D-glucopyranosyl-D-mannose a small quantity of sophorose was treated with aqueous alkali and after HPAEC analysis confirmed that the majority of the starting material had been converted to 2-*O*-β-D-glucopyranosyl-D-mannose the reaction was quenched by acidification with acetic acid. The crude products were then applied to a carbon-celite column and the new disaccharide was isolated by elution with aqueous ethanol. The product eluted as a single peak in 20% ethanol and, after evaporation of the solvent, 2-*O*-β-D-glucopyranosyl-D-mannose was recovered as a white powder. The proton NMR (Fig 5 F2 (x)-axis) of an aqueous solution of 2-*O*-β-D-glucopyranosyl-D-mannose indicated that the product was present as a pair of anomers. At 30 °C and in an aqueous solution of D_2O 2-*O*-β-D-glucopyranosyl-D-mannose exists as a mixture containing 77% of the alpha-anomer and 23% of the beta-anomer. A number of 1D and 2D-NMRs were recorded (^{1}H, ^{13}C and HSQC spectra are shown in Fig 5.) and a full assignment for the proton and carbons of the alpha anomer and for the protons of the beta-anomer (for the beta-anomer carbon signals were weak and only the anomeric signals were clearly visible above the spectral noise); the chemical shifts for both the protons and carbons are listed in the table. It should be

noted that the proton assignment for the anomeric glucose (H1')-protons are different to those in the literature[20] (the values in the literature for the H1' proton for alpha and beta have been inverted). Even after prolonged exposure to high pHs there was no evidence for hydrolysis of the glycosidic bond and there was no evidence for decomposition via peeling reactions.

In summary, the valuable β-(1,2)-linked disaccharide sophorose was isolated from waste streams generated during the commercial manufacture of sophorolipids and we have provided evidence which indicates that sophorose is generated by an enzyme catalysed hydrolysis of the parent sophorolipid. When treated with aqueous alkali, sophorose did not undergo either hydrolysis or a peeling reaction and instead underwent epimerisation at C2 to generate 2-*O*-β-D-glucopyranosyl-D-mannose which was isolated and characterised by NMR.

Sugar\Position	H1	H2	H3	H4	H5	H6s	C1	C2	C3	C4	C5	C6s
Reducing	5.29	4.17	3.88	3.81	3.69	3.93 & 3.72	92.3	78.6	69.6	72.4	67.2	60.7
Non-reducing	5.51	3.34	3.49	3.43	3.41	3.83	101.9	72.9	75.6	76.0	69.6	60.4

β-anomer

Sugar\Position	H1	H2	H3	H4	H5	H6s	C1
Reducing	4.98	4.09	3.68	3.55	3.41	ND	93.9
Non-reducing	4.62	3.41	3.48	ND	ND	ND	104.1

Table: Individual chemical shifts for protons and carbons for *2*-O-*β-D-glucopyranosyl-α-D-mannose* (top) and specific resonances for *2*-O-*β-D-glucopyranosyl-α-D-mannose* (bottom) (recorded in D_2O at 70 °C on a Bruker Avance 500 MHz spectrometer).

Experimental

3.1 Materials and Chemicals

Monosaccharides and disaccharide standards were purchased from Carbosynth (Berkshire, UK). All other reagents were purchased from Aldrich (Poole, UK) unless otherwise stated and were used as supplied.

3.1.1 Sophorolipid and sophorolipid process wastes. Samples of sophorolipids and sophorolipid process wastes were provided by Croda Biotechnology Group (Widnes, UK) and were generated from a large scale fermentation employing an appropriate lipid and glucose as feeds. Fermentations (>10,000 L) were initiated by inoculation of the media with the yeast strain *Candida*

bombicola and at the end of the fermentation the fermentation broth was allowed to settle generating two phases: a lipid-rich phase containing sophorolipids and an aqueous phase. Adjustment of the pH of the sophorolipid layer by addition of aqueous alkali to 5.5 provided a sophorolipid lactone and generated an aqueous phase containing sophorose and which will be subsequently referred to as 'lactone-waste'. Pure samples of the lactone form (**1**) and the acid form of the sophorolipids (**2**) were also supplied by Croda.

3.2 General Analytical Procedures.

Analysis of monosaccharides and disaccharides was performed using high pressure anion exchange chromatography coupled to a pulsed amperometric detector (HPAEC-PAD). Standards and unknowns were separated on a Dionex ICS3000 HPAEC system incorporating a Carbopac PA 20 column operating at 30 ^{o}C and using a mobile phase containing 20mM KOH running at a flow rate of 0.3 ml.min^{-1}. Standards of glucose, isomaltose and sophorose were prepared in ultra-pure water (50-200 ppm).

NMR analyses of sophorolipids, sophorose, isomaltose and 2-*O*-β-D-glucopyranosyl-D-mannose were performed on a Bruker DPX500 NMR spectrometer, a series of 1D and 2D-NMR spectra were recorded in D_2O at a probe temperature of 70 ^{o}C and employing acetone as either an internal or external standard. The 2D-spectra recorded included homonuclear ^{1}H-^{1}H-COSY and TOCSY (80 ms mixing time) and heteronuclear ^{1}H-^{13}C HSQC, ^{1}H-^{13}C- HMBC, ^{1}H-^{13}C- HSQC-TOCSY and finally a NOESY spectrum was recorded for *2*-O-*β-D-glucopyranosyl-α/β-D-mannose* .

3.3 Isolation of disaccharides.

3.3.1. Isolation of sophorose (2-O-β-D-glucopyranosyl-α/β-D-glucose). Lactone waste (100 ml, 98 g) was added to ultra-pure water (100 ml) and was left to stir at 35-40 °C for 1h. The resulting brown suspension was centrifuged (4200 rpm for 10 mins) and the supernatant was collected. After completion of the separation process, the aqueous phase was rotary evaporated at reduced pressure (10 mmHg) and at a temperature of 45°C to give a crude mixture of salts and sugars as a brown syrup (5.5g). The salt and sugar mix (5.5 g) was redissolved in UPW (3 ml) and the sample was sonicated for 5 mins before being applied to the top a carbon-celite column (5 x 50 cm

prepared from Darco G 60, 100 mesh carbon, 200 g, and Celite 535, 200 g). Elution of sugars was carried out with an increasing concentration of aqueous ethanol (from 0 to 20% ethanol in UPW in 5% incremental steps of 2 L), HPAEC analysis of the different fractions identified salt plus glucose in the fractions eluting with 5% ethanol (4.0 g) isomaltose in the fractions eluting with 10% ethanol (0.5g) and sophorose from the fractions eluting with 20% ethanol (1.0g).

3.3.2 Isolation of 2-O-β-D-glucopyranosyl-α/β-D-mannose.

Sophorose (50 mg) was treated with aqueous sodium hydroxide (100 mM, 20 ml at pH 13) in a pressure tube and was held at 50 ^{o}C for 24 h. The progress of the reaction was monitored using HPAEC; samples (1 ml) were removed every 3 h and the pH of the sample was adjusted to 6 using glacial acetic acid. Samples were then transferred to small glass vials and injected into the HPAEC using sodium hydroxide (50 mM) as the mobile phase. HPAEC analysis suggested that the reaction had reached equilibrium after 24 h at which point the remaining solution was neutralised with glacial acetic acid and the solvent removed under vacuum to give a crude mixture of sugars and sodium acetate as a yellow solid. The sample was redissolved in ultrapure water (1 ml) and was applied to the top of a carbon-celite column (2.5 x 20 cm). Elution of sugars was carried out with an increasing concentration of aqueous ethanol (from 0 to 20% ethanol in UPW in 5% incremental steps each of 200 ml). The desired product eluted with 20% aqueous ethanol and the combined fractions were rotary evaporated to give a pure sample of 2-*O*-β-D-glucopyranosyl-α/β-D-mannose as a white solid (10 mg, 40% overall yield based on the volume of solution remaining at the end of the reaction). The identity and anomeric purity of the product was determined by 1D-and 2D-NMR (see Section 2).

3.4 Monitoring the base catalysed hydrolysis of sophorolipids

Acid sophorolipid (200 mg, 0.322 mM) was dissolved in aqueous sodium hydroxide (10 mM, 20 ml at pH 12) and stirred in a pressure tube at a temperature of 50 °C for 270 min. Samples (1 ml) were taken at 30 min intervals and the pH of the samples was adjusted to pH 6

using glacial acetic acid. Samples were then transferred into small glass vials and injected into the HPAEC.

3.5 *Monitoring the stability of sophorose in aqueous alkaline solution.*

Sophorose (5 mg, 0.015 mM) was treated with aqueous sodium hydroxide (100 mM, 20 ml at pH 13) in a pressure tube at room temperature for 32 h. Samples (1 ml) were taken out every 1 h and the pH of the sample was adjusted to 6 using glacial acetic acid. Samples were then transferred into small glass vials and injected into the HPAEC using sodium hydroxide (50 mM) as the mobile phase.

The same experiment was carried out on sophorose (8 mg), but in an alkaline solution of NaOD (0.1M, 600 μL in D_2O) at room temperature. The progress of the reaction was followed by running ^{1}H-NMR spectra after 1, 12 and 24 h.

3.6. Hydrolysis of sophorolipids by spent fermentation broth

A sample of a fermentation broth (100 ml) recovered at the end of the fermentation process was supplied by Croda Biotechnology and was stored in a cold-room for 24 h before being used to test for the presence of enzyme activity that may hydrolyse sophorolipids (pH 3.5). In separate experiments, both lactone and acid sophorolipids (0.5 g) were added to a 250 ml clean and autoclaved conical flask and a third flask without any sophorolipid was used as a control. The newly prepared mixtures were kept in a shake–flask incubator. The incubator conditions were set as follows; 30◦C, under agitation at 120 rev/min for 3 days (Stuart Orbital Incubator, SI500). After 24 h intervals, samples (1 ml) were taken and filtered through (0.45 μl disk filter) and the supernatant was diluted with UPW (2 ml) then transferred into small vials. The sugar composition of the flasks was assayed by HPAEC-PAD.

References:

1. Mandels, M.; Parrish, F.W.;. Reese, E.T. *J. Bacteriol*, **1962**, *83*, 400-408.
2. Francis, G.; Kerem, Z.; Makkar, H.P.S.; Becker K. *Br. J.of Nutr*., **2002,** *88*, 587-605.
3. Attele, A.S.; Wu, J.A.; Yuan, C.S. *Biochem. Pharmacol*., **1999**, *58*, 1685-1693.
4. Asmer, H.J.; Lang, S.; Wagner, F.; Wray, V. *J. Am. Oil Chem. Soc.,* **1988**, *65*, 1460-1466.

5. Mulligan, C.N. *Environ.Pollut. (Amsterdam, Neth.),* **2005**,*133,* 183-198.

6. Van Bogaert, I.N.A.; Saerens, K.; De Muynck, C.; Develter, D.; Soetaert, W.; Vandamme, E.J. Appl. Microbiol. *Biotechnol.,* **2007**, *76*, 23-34.

7. Freudenberg, K.; Toepffer, H.; Andersen, C.C. *Ber.,* **1928**, *61*, 1750.

8. Coxon, B.; Fletcher Jr, H.G. *J.Org. Chem*., **1961**, *26,* 2892-2894.

9. Thompson, A.; Anno, K.; Wolfrom, M.L.; Inatome, M. *J. Am. Chem. Soc*., **1954,** *76,* 1309-1311.

10. Clancy, M.J. *J.Chem. Soc. (Resumed),* **1960,** 4213-4217.

11. Wood Jr, H.B.; Allerton, R.; Diehl, H.W.; Fletcher Jr, H.G. *J.Org.Chem*., **1955**, *20*, 875-883.

12. Hoffmann, N.; Pietruszka, J.; Söffing, C.; *Adv. Synth. & Catal*., **2012**, *354*, 959-963.

13. Jourdier, E.; Ben, C.F. World patent WO201468208 A1, **2014**.

14. Van Bogaert, I.N.A.; Holvoet, K.; Roelants, S.L.K.W.; Li, B.; Lin, Y.C.; Van de Peer, Y.; Soetaert, W. *Mol. Microbiol.* **2013**, *88*, 501-509.

15. Van Bogaert, I.N.A.; Zhang, J.; Soetaert, W. *Process Biochem*., **2011**, *46*, 821-833.

16. Weber, A.; May, A.; Zeiner, T.; Górak, A. in: *Chem.Eng. Trans.(*DOI: 10.3303/CET1227020, **2012,** 115-120.

17. Rau, U.; Heckmann, R.; Wray, V.; Lang S., *Biotechnol. Lett*., **1999**, *21,* 973-977.

18. Gakhokidze, R. *Russ. Chem. Rev.,* **1980**, *49*, 222-236.

19. Knill, C.J.; Kennedy, J.F. *Carbohydr. Polym*., **2003**, *51*, 281-300.

20. Kobayashi, H.; Shibata, N.; Konno, S.; Hisamicha, K.; Suzuki, S. *Carbohydr. Res.,* **1992**, *229* 369-375.

21. Lobry de Bruyn, C.A.; Alberda van Ekenstein, W. *Recl. Trav. Chim. Pays-Bas*, **1895,** *14*, 156.