New-to-nature sophorose analog: a potent inducer for gene expression in *Trichoderma reesei*

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Running title: New-to-nature sophorose analog

Introduction

25 The filamentous fungus *Trichoderma reesei* is the primary industrial source of cellulases for the development of advanced biofuels and biochemicals from renewable, plant-based materials. *T. reesei* secretes a mixture of endoglucanases, exoglucanases, and beta-glucosidases, under control of the cellobiohydrolase I (cbhI) promoter. The most potent known inducer of cbhI, sophorose (2-O- β -glucopyranosyl-D-glucose) [1], is expensive to manufacture, extremely costly
30 in pure form, and readily metabolized by the host during fermentation. Consequently, industrial cellulase production relies on proprietary chemical or enzymatic glucose reversion reactions, or less potent inducers, such as lactose. In both cases, lack of an effective, economical inducer constrains the ability to optimize the manufacturing process, further limits scaling of the process to serve the many cellulosic ethanol plants envisioned in the future, and makes adaptation to non-
35 glucose feedstocks (such as sucrose) problematic. We report a new, highly efficient, and cost-effective inducer that is only very slowly metabolized by the host. Thus, it does not have to be supplied continuously during the fermentation. Even in impure form, this novel inducer is more potent than previously reported inducers.

Although sophorose seldom is found in its free form in nature, sophorosides are readily
40 available and found in great abundance in plants and microbes. Examples include kaempferol sophoroside from *Sophora japonica* [2], stevioside, a sweetener extracted from *Stevia rebaudiana* [3]; and sophorolipids secreted by *Starmerella bombicola* (formerly *Candida bombicola*) [4]. Natural sophorolipids consist of one sophorose molecule (~50% by weight) linked through C-1' to an hydroxylated fatty acid. With wild-type *S. bombicola* grown on
45 conventional substrates, the secreted sophorolipids are primarily macrolactones, with the 4" position of the sophorose esterified to the carboxylic acid group of the fatty acid [5] (**Figure 1A**),

with a minor fraction in the ring-opened, acid form (**Figure 1B**). As shown in **Figures 1A and 1B**, both types of sophorolipids may contain acetyl groups at 6' and/or 6" positions. *S. bombicola* is capable of cost-effectively producing over 400 g/L sophorolipids at 3.18 g/L/h using glucose
50 and vegetable oil as carbon sources. Both acidic and lactonic sophorolipids have good biosurfactant properties and have been commercialized for applications in the cosmetics, pharmaceutical, and cleaning industries [5].

Previous efforts have failed to find a sophorose analog that induces. Sucrose monopalmitate increases sucrase yields 80 times over sucrose in *T. viride* [6]. Similarly, acetyl cellobioses were
55 more effective than glucose or cellobiose at inducing cellulase in *Penicillium purpurogenum*, with mono-O-acetyl cellobiose being the most active inducer tested [7]. In contrast, modifications of sophorose such as reduction, oxidation, or glycoside formation destroy its activity in *Trichoderma* [1]. Sophoritol, lactitol, lactobionic acid, and cellobionic acid from reduction and oxidation of sophorose, lactose, and cellobiose do not induce. Naturally occurring
60 sophorose-based glycosides do not induce unless hydrolyzed to free sophorose [1].

Natural sophorolipids have been described as protein inducers [8], and co-culture of a natural sophorolipid producer with *T. reesei* Rut-C30 for the production of cellulase has been reported [9]. All these studies were carried out using unmodified sophorolipids which we have observed to have marginal protein induction activity in *Trichoderma* (**Table 1**).

65 We hypothesized that for an improved inducer to achieve optimal induction, there must be optimal transport, in addition to its being not metabolized or only very slowly metabolized. No protein induction will occur if the inducer molecule is insoluble, if the solubilized portion fails to be taken up by the cells, or if it fails to reach the intracellular induction site intact. On the other hand, too fast an uptake of an active inducer by a cell can decrease the rate of protein induction

70 due to premature metabolization and possible repression. Methods to enhance protein induction
by modifying an active inducer to slow its uptake are known (Mandels, 1960; Sternberg, 1979;
Duff, 1985). Because the uptake and metabolism of sophorose and lactose inducers depend on
permeases and beta-glucosidase (and/or beta-galactosidase) (Kubicek, 1993; Ivanova, 2013), we
further hypothesized that a suitable sophorose analog would retard the action of beta-glucosidase
75 (or beta-galactosidase), resulting in a molecule that is not metabolized or slowly metabolized, but
still efficiently transported by permease, resulting in a more potent inducer. Due to the complex
nature of eukaryotic gene regulation, research into the mechanisms of gene expression in
Trichoderma is on-going (Kubicek, 2009). In this work, we have discovered a novel class of
sophorose analogs that are at least 20-30 times more powerful as inducers than sophorose which
80 was discovered about 53 years ago (Mandels, 1962).

In addition to its use as a potent and cost effective inducer in *Trichoderma* fermentation, the
newly discovered inducer may potentially find use as an effective inducer in other microbial
hosts, it can serve as a powerful research tool to enhance our understanding of fungal gene
expression, and it may also aid in the selection and improvement of host strains. Another area of
85 potential use is consolidated bioProcessing (CBP), where the steps encompassing the production
of enzymes, enzymatic hydrolysis of a pretreated biomass, and fermentation of biomass sugars
are combined in a single reactor using single or multiple microbial hosts (Olson, 2012, Ladisch,
2010). Such a system requires a strong inducer to enhance the limited and unspecific induction
from pretreated biomass. Recently, CBP of pretreated corn stover using cellulytic and enzyme-
90 producing *T. reesei* and a genetically modified *E. coli* capable of producing isobutanol has been
reported (Minty, 2013). Using modified *T. reesei* capable of both secreting enzymes and for
fermenting ethanol has also being proposed for CBP (Xu, 2009).

Materials and Methods

Sophorolipid production

95 To prepare sophorolipids, *S. bombicola* (ATCC 22214) was used. A sample of freeze-dried stock was first transferred into a yeast extract, peptone, dextrose (YPD) culture broth and allowed to grow for 48 hours at 28°C for two days. The culture was then mixed with sterilized glycerol to prepare a final culture containing 20% glycerol (v/v). A cell bank was established using cryovials with 1 mL of seed culture each and stored at -80°C. Sophorolipid was produced
100 using a production medium that included the following components: 100 g/L glucose, 10 g/L yeast extract, 1 g/L urea, and 100 g/L canola oil (CriscoTM, The J.M. Smucker Company, Orrville, OH). Based on the data provided from the manufacturer, the canola oil has 64% monounsaturated fat from mainly oleic fatty acid, and 29% polyunsaturated fat from mainly linoleic and alpha-linolenic fatty acids. In some experiments, sophorolipids were produced with
105 good yields from oleic acid, palm oil, soybean oil, or corn oil feedstocks. To initiate the fermentation, a 1 mL seed of *S. bombicola* was placed into 50 mL of production medium in a 250 mL baffled flask and incubated in a shaking incubator (Innova 4200, New Brunswick Scientific, Edison, NJ) at 28°C and 250 revolutions per minute (RPM). After seven days, sophorolipids appeared in the medium as a viscous brown oily phase, and in some cases, the
110 sophorolipid crystallized into a yellowish residue. Sophorolipids were isolated by heating the culture to 60-80°C at pH 2-3, after which it was allowed to settle in a separation funnel. Crude sophorolipids were collected from the lower phase and washed three times with water to remove impurities. Typical harvested sophorolipid had a moisture content of ~40%. The purity of harvested samples was confirmed by HPLC/MS on a Waters Symmetry C18 column (4.6×250
115 mm, 5 µm) using a Waters 2695 System with a Waters ZQ 2000 mass spectrometer equipped

with electrospray ionization, a Waters 2487 UV detector, and the following gradient: 5% B to 50% B over 10 min., hold 50% B 8 min., 50% B to 85% B over 8 min., and 85% B to 95% B over 2 min. (A: water containing 0.1% formic acid; B: acetonitrile containing 0.1% formic acid). Lactone sophorolipids of >95% purity (Ecover, Malle, Belgium) were used as standards and in
120 some experiments where high-purity starting material was advantageous.

Inducer synthesis and structural identification

Hydrolysis of harvested sophorolipids (25 mg/mL) with 0.1 N HCl (100°C 2 hr., sealed glass high-pressure tube, with stirring) resulted in a complex mixture of products, which was resolved
125 by a combination of flash chromatography on silica gel and preparative reverse-phase HPLC. Fractions containing active inducer were submitted for mass spectroscopy and ¹H and ¹³C NMR (Acorn NMR, Livermore, CA). The acid-treated sophorolipid mixture was isolated as a precipitate by centrifugation, and was then purified and fractionated by flash chromatography on a 5 g self-packed silica gel column (230-400 mesh, Grade 60, Fisher Scientific), with a step
130 gradient of methylene chloride/methanol as eluent. Fractions (~5 mL each) were first visualized on thin-layer chromatography (TLC) plates (DC-Kieselgel 60, Merck KGaA, Darmstadt, Germany) according to Asmer et al. [10], and then combined based on R_f values. The combined fractions were tested for cellulase induction following the procedures described below. The combined active fractions were further purified by preparative reverse-phase HPLC (Waters Prep
135 LC 4000 system, Agilent Eclipse XDB C18 column, 9.4×250 mm, 5 μm) using the same HPLC gradient described above.

Cellulase induction studies

Protein induction was assessed in 125 mL baffled shake flasks containing 15 mL of citrate
140 minimal medium (modified from England, et al. [11]) with 2% (w/v) glucose. Glucose with no
added inducer established the baseline level of cellulase activity in the assay. Lactose (0.1%
w/v), cellobiose (0.1% w/v), and sophorose (0.13%, 0.013% w/v) controls were included in each
experiment. To commence fermentation, an aliquot of the standard spore suspension from *T.*
reesei (Rut-C30, ATCC #56765) grown on potato-dextrose-agar plates (Teknova, Hollister, CA,
145 #P0047) was added, and the cultures were then incubated at 28°C and 250 RPM. After 3-4 days,
samples were collected and analyzed for CMC (carboxymethylcellulose) endoglucanase activity
in a 0.3 mL reaction volume [12]. One CMC unit denotes the activity that liberates 1 μ mol of
reducing sugars (expressed as glucose equivalents) in 1 min. at 50°C and pH 4.8.

150 **Results and Discussion**

Due to the size and acetyl and lactonic nature of natural sophorolipids (**Figures 1A, 1B**), it
was unclear to us whether these molecules could be effectively transported into cells in order to
reach an intracellular induction site. Therefore, we investigated various modifications of natural
sophorolipids in order to increase their uptake and, potentially, their protein induction activity.
155 As a result of these experiments, we identified an acid treatment of a natural sophorolipid using
dilute HCl, which produces a complex mixture containing a previously unknown sophorolipid
analog that is highly inductive to *cbh1* gene expression in *T. reesei*.

Partial hydrolysis of natural lactonic sophorolipid with 0.1N HCl (100°C 2hr.) resulted in a
complex mixture of products. Initial attempts to purify the inducer using preparative TLC were
160 unsuccessful due to co-migration of several products with the compound(s) of interest. Using a
combination of flash chromatography on silica gel and preparative reverse-phase HPLC, we

successfully purified two compounds and demonstrated their induction potential (**Table 1**). The acid-treated sophorolipid is highly inductive even as the unpurified mixture. Our analysis and calculations (data not shown) show that only a small amount (<0.5%) of sophorolipid is converted into actual inducer. Purification of this product as Fractions 1 and 2 clearly demonstrated their potent induction (**Table 1**).

As shown by a total ion current (TIC) scan from HPLC/MS of the natural sophorolipid (**Figure 2A**) and the acid-modified material (**Figure 2B**), both were complex mixtures. We fractionated the mixture by column chromatography and found that most fractions of the mixture are inactive, while a select few are highly inductive. After further purification, we have isolated two relatively pure fractions (**Figures 2C, 2D**), determined their molecular weights, identified their structures through NMR, and most importantly, found their induction power to be at least 28 times (by mass) that of sophorose under the conditions tested. As shown by a cellulase assay (**Table 1**), the acid-modified sophorolipid mixture, even though it contains only a small percent of active inducer, is already very powerful and highly efficient. At 1.1 g/L, it easily surpasses the induction power of lactose and cellobiose at 1.0 g/L. Its specific induction power (expressed as units CMC / mg inducer) is an order of magnitude higher than lactose and cellobiose. More importantly, when the sophorose loading is increased 10-fold to 1.3 g/L, the specific induction power is lowered by half, indicative of a loss of induction efficiency at high sophorose concentration where most of sophorose might be prematurely metabolized. In contrast, increasing the concentration of the acid-treated sophorolipid mixture increases the specific induction.

Even though the impure, modified sophorolipid is highly inductive, an equivalent mass of the purified component isolated from the mixture of modified sophorolipid is even more powerful.

185 The specific induction activity of Fraction 1 (**Figure 2C**) at 0.033 g/L and a molar concentration of 0.05 mM, measured at day 3, is 1,000 and 2,000 times more powerful than the traditional inducers, lactose and cellobiose, respectively, and approximately 28 times that of sophorose (the current gold standard) on a mass basis and 56 times on a molar basis. On the other hand, the specific induction power of Fraction 2 (**Figure 2D**) is approximately 10 times that of the
190 sophorose on a mass basis and approximately 20 times on a molar basis. In an important contrast, we found the induction ability of the raw, unmodified sophorolipids to be minimal in comparison with sophorose (**Table 1**). In batch culture, it is probable that high levels of cellulase expression quickly exhausted the carbon and nutrient source, thus reaching a maximum induction. In contrast, industrial enzyme production relies on continuous-feed processes, in which the carbon
195 source is continually supplied for protein synthesis with its feed rate optimized to minimize growth and repression. The actual induction potential of these inducers might be $\gg 28$ under industrially relevant fed-batch conditions. We recently demonstrated the potential of this newly discovered inducer under fed-batch conditions (data not shown).

Analysis by HPLC/MS of the culture medium from the 0.05 mM culture of Fraction 1 shows
200 that undegraded inducer is present after 3 days of culture (data not shown). In our experience, 0.05 mM sophorose (applied only at the beginning of the fermentation) would not be detectable after 3 days. While these data are preliminary, the result is consistent with a highly potent, slow-metabolizing, and long-acting inducer. The state of the art employs inducer to turn on gene expression when cell density and other process parameters are optimal for rapid, high-yield
205 protein production. Induction shifts the host's genetic and translational machinery from growth and metabolism to secretion of one or a few proteins of interest. Unlike lactose which serves both as inducer and substrate for *Trichoderma* fermentation, a more powerful inducer allows

induction and cell metabolism to be decoupled: a major advantage for industrial enzyme production, allowing more rigorous process optimization. This novel inducer has the potential to improve yield and productivity in enzyme manufacturing plants by providing an efficient and constant induction to the fermentation culture, while not requiring the inducer to be continually supplied to the fermentation.

Crude sophorolipids were found to be primarily (typically ~85%) lactonic sophorolipids containing hydroxy oleate with two acetyl groups at C-6' and C-6'' (688 Da, 711 Da $[M+Na]^+$, **Figure 1A**), and minor fractions were monoacetylated lactonic sophorolipids and acidic sophorolipids (**Figure 1B**). Following dilute acid hydrolysis, products were analyzed by HPLC/MS (**Figure 2**), and purified fractions containing the active inducers were analyzed by HPLC/MS mass spectroscopy (**Figures 3, 4**). Fractions 1 and 2 were observed to contain compounds of molar mass 664 Da, 665 Da $[M+H]^+$, **Figure 3A**) and 622 Da, 623 Da $[M+H]^+$, **Figure 4A**), respectively. The structures of the sophorose esters in these two fractions were further confirmed by 1H and ^{13}C nuclear magnetic resonance (NMR, Varian 500 MHz) and in-source fragmentation studies using HPLC/MS with electrospray ionization cone voltage increased from 10 V to 60 V. Based on the 1H NMR, ^{13}C -NMR, and two dimensional ^{13}C - 1H HSQC NMR, Fraction 1 was confirmed to be a 6'' monoacetylated sophorose ester, esterified at C-4'' with an 18-carbon monounsaturated fatty alcohol hydroxylated at C-17 (**Figure 1C**). The assignments of the protons and carbons of Fraction 1 are summarized in **Table 2**. The results from the 1H NMR spectrum of Fraction 2 were similar to the results from the 1H NMR spectrum of Fraction 1, except that a singlet peak at 2.04 ppm was not observed. This observation suggests the presence of a deacetylated sophorose ester (**Figure 1D**), esterified at C-4'' with an 18-carbon monounsaturated fatty alcohol hydroxylated at C-17.

Conventional production of sophorolipids, for use as biosurfactants and for other applications, converts the lactone form into the acidic form through ring-opening of the ester linkage at C-4" to regenerate the free carboxyl group of the hydroxy fatty acid, which remains linked to the sugar through a glycosidic linkage at C-1' (**Figure 1B**). In contrast, the inducer is produced when ring-opening occurs through breakage of the ether-like linkage at C-1' (the anomeric carbon), regenerating the hydroxyl group of the hydroxy fatty acid, which remains linked by an ester bond to C-4" (**Figure 1C**). We refer to this family of novel sophorolipid analogs as sophorose ester alcohols (SophoroSeal™). Mass spectra (**Figures 3, 4**) and NMR (**Table 1**) were consistent with the SophoroSeal™ structure (**Figure 1C, 1D**). For in-source fragmentation studies, increasing ionization voltage from 10 V to 60 V (**Figure 3**) resulted in the fragmentation of the parent molecule (**Figure 1C**, 664 Da, 665 Da $[M+H]^+$) into fragments of 647 Da $[M+H-H_2O]^+$, 485 Da $[M+H-H_2O-C_6H_{10}O_5]^+$, 467 Da $[M+H-2H_2O-C_6H_{10}O_5]^+$, 263 Da ($C_{18}H_{31}O$), and other associated fragments ($C_6H_{10}O_5$ and $C_{18}H_{31}O$ denote glucose and oleic acid residues, respectively). The fragmentation pattern of deacetylated SophoroSeal™ (**Figure 4B**) is similar to the monoacetylated SophoroSeal™ (**Figure 3B**) with a difference of an acetyl group (42 Da). We observed close correspondence between the fragmentation pattern of our compounds with those of natural monoacetylated and deacetylated lactonic sophorolipids as reported by de Koster et al. [13] (structure B2, Scheme 1; note that these authors described a hypothetical ionized structure to explain an observed fragmentation pattern, rather than an isolated compound; the compound we have discovered has never been described in the literature.) These sophorose analogs are not present at detectable levels in the fermentation products of *S. bombicola*. Nor are they produced during conventional alkaline hydrolysis of the lactone, under which conditions the ester linkage is preferentially cleaved to generate acidic sophorolipids. A patent has been filed on these

compounds and their use as novel inducers [14].

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Conclusion

We have identified a new-to-nature sophorose analog with greatly enhanced induction efficiency for cellulase production in *T. reesei* Rut-C30. This novel 4"-sophorose hydroxy fatty acid ester resists degradation by the host and remains effective for at least seven days following a single addition to the fermentation. With further improvements to the synthesis, combined with economies of scale, the new inducer could be as enabling for *Trichoderma* cellulase, as well as heterologous protein, expression as isopropyl β -D-1-thiogalactopyranoside (IPTG), the standard inducer for *E. coli*, which is used daily in molecular biology and industry. Furthermore, the ready availability of a potent inducer can enable rapid deployment of cellulase production, using a variety of feedstocks including sucrose, in proximity to cellulosic sugar facilities, without the need for lactose or the requirement to run a glucose reversion process in parallel with fermentation to produce inducer.

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Acknowledgements

Dr. Huang owns shares in Sophoro Biotechnologies, LLC. This research was supported by Small Business Innovative Research (SBIR) Phase I (award #2012-33610-19542) and Phase II (award #2013-33610-21420) grants from the US Department of Agriculture (USDA) to Panorama Research. We acknowledge the kind assistance of Dr. Hanxiao Jiang with the interpretation of the NMR results.

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List of Figures (with legends separate from the images)

Table 1. Activity of novel inducer (SophoroSeal™) greatly exceeds activity of conventional inducers.

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Table 2. NMR peak assignment of ^{13}C and ^1H signals for SophoroSeal™ (Figure 1C).

Figure 1. Chemical structures: (A) lactone, (B) acidic sophorolipid, (Where $\text{R}^a = \text{H}$ or $\text{C}(\text{O})\text{CH}_3$ and $\text{R}^b = \text{H}$ or $\text{C}(\text{O})\text{CH}_3$), (C) hydroxy fatty acid ester of 6'' acetylated alpha-sophorose (SophoroSeal™), and (D) hydroxy fatty acid ester of alpha-sophorose (SophoroSeal™). Oleic acid forms are shown here for the lipid portion of the molecule.

Figure 2. HPLC/MS total ion current scan of (A) the mixture of natural, unmodified sophorolipids from *S. bombicola*, (B) acid-treated sophorolipid mixture that is highly inductive, and two purified fractions: Fraction 1 (C) and Fraction 2 (D) which are super-inductive (10-28x sophorose, Table 1).

Figure 3. MS of purified product (Fraction 1, Figure 1C, SophoroSeal™) from dilute acid hydrolysis where the cone voltage of electrospray ionization is at (A) 10 V and (B) 60 V.

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Figure 4. MS of purified product (Fraction 2, Figure 1D, SophoroSeal™) from dilute acid hydrolysis where the cone voltage of electrospray ionization is at (A) 10 V and (B) 60 V.

Figure 1

Figure 2

Figure 3

Figure 4

Table 1. Activity of novel inducer (SophoroSeal™) greatly exceeds activity of conventional inducers.

Induction media	[Inducer]		Cellulase activity (day 3)	Specific induction
	g/L	mM	CMC U/mL	CMC U/mg inducer
glucose control	20	0	0.28 ± 0.17	NA
lactose	1	2.92	0.64	0.64
cellobiose	1	2.92	0.34	0.34
sophorose	0.13	0.38	3.58	26.9
sophorose	1.3	3.90	15.0	11.2
natural and unmodified sophorolipids *	1.1	NA	2.90	2.72
acid-treated (0.1N HCl, 2 hr.) sophorolipid mixture *	0.15	NA	1.50	10.2
acid-treated (0.1N HCl, 2 hr.) sophorolipid mixture *	1.1	NA	14.9 ± 2.6	13.9 ± 2.4
Fraction 1 of acid-treated sophorolipid	0.033	0.05	24.7	741.0
Fraction 2 of acid-treated sophorolipid	0.073	0.12	18.8	256.4
* All sophorolipid mixtures were precipitated and washed with water to remove residual sophorose.				

Table 2. NMR peak assignment of ^{13}C and ^1H signals for SophoroSeal™ (Figure 1C).

Functional groups	^{13}C -NMR $\delta(\text{ppm})$	^1H -NMR $\delta(\text{ppm})$
Sophorose		
1'	92.4	5.31
1''	104.7	4.53
2'	82.1	3.42
2''	74.1	3.40
3'	74.1	3.56
3''	72.3	3.85
4'	70.2	3.36
4''	70.6	4.82
5'	71.9	3.66
5''	67.3	3.68
6' a, b	61.4	3.68, 3.78
6'' a, b	62.6	4.08, 4.14
Acetyl group		
-C=O	171.2	
CH3	19.5	2.04
Acyl group		
1	172.3	
2	33.7	2.36
3	24.6	1.62
4-7, 12-15	25.6 – 29.6	1.27-1.46
8, 11	26.9	2.00-2.05
9, 10	129.6	5.34
16	39.0	1.40
17	71.5	3.78
18	22.2	1.14