Glycoconj J (2009) 26:589–596
DOI 10.1007/s10719-008-9211-z

Antigen 85C-mediated acyl-transfer between synthetic acyl donors and fragments of the arabinan

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Received: 9 July 2008 / Revised: 20 October 2008 / Accepted: 27 October 2008 / Published online: 4 December 2008

Abstract Antigen 85 (ag85) is a complex of acyltransferases (ag85A–C) known to play a role in the mycolation of the D-arabino-D-galactan (AG) component of the mycobacterial cell wall. In order to better understand the chemistry and substrate specificity of ag85, a trehalose monomycolate mimic *p*-nitrophenyl 6-*O*-octanoyl-β-D-glucopyranoside (**1**) containing an octanoyl moiety *in lieu* of a mycolyl moiety was synthesized as an acyl donor. Arabinofuranoside acceptors, methyl α-D-arabinofuranoside (**2**), methyl β-D-arabinofuranoside (**3**), and methyl 2-*O*-β-D-arabinofuranosyl-α-D-arabinofuranoside (**9**) were synthesized to mimic the terminal saccharides found on the AG. The acyl transfer reaction between acyl donor **1** and acceptors **2**, **3**, and **9** in the presence of ag85C from *Mycobacterium tuberculosis (M. tuberculosis)* resulted in the formation of esters, methyl 2, 5-di-*O*-octanoyl-α-D-arabinofuranoside (**10**), methyl 5-*O*-octanoyl-β-D-arabinofuranoside (**11**), and methyl 2-*O*-(5-*O*-octanoyl-β-D-arabinofuranosyl)-5-*O*-octanoyl-α-D-arabinofuranoside (**12**) in 2 h, 2 h and 8 h, respectively. The initial velocities of the reactions were determined with a newly developed assay for acyltransferases. As expected, the regioselectivity corresponds to mycolylation patterns found at the terminus of the AG in *M. tuberculosis*. The study shows that D-arabinose-based derivatives are capable of acting as substrates for ag85C-mediated acyl-transfer and the acyl glycoside **1** can be used *in lieu* of TMM extracted from bacteria to study ag85-mediated acyl-transfer and inhibition leading to the better understanding of the ag85 protein class.

Keywords Mycobacterium tuberculosis · D-Arabino-D-galactan (AG) · Antigen 85 (ag85) complex · TMM · TDM

Abbreviations

My	mycolates
AG	D-arabino-D-galactan
mAGP	mycolyl-arabinogalactan-peptidoglycan
TMM	trehalose mono-mycolate
TDM	trehalose di-mycolate
M. tuberculosis	*Mycobacterium tuberculosis*
AgOTf	silver triflate
NIS	*N*-iodosuccinimide
TBAF	tetrabutylammonium fluoride
EDTA	ethylene diamine tetraacetic acid
DTT	dithiothreitol

Introduction

The cell wall of *M. tuberculosis* is characterized by a highly lipophilic outer layer that is impermeable to many common antibiotics [1]. Identifying strategies to penetrate this structure may lead to the discovery of improved antimycobacterial agents. The main features of this permeation barrier consist of a peptidoglycan matrix covalently connected to a non-repeating polysaccharide called the D-arabino-D-galactan (AG) [2–4]. Mycolates (My), α-branched β-hydroxy fatty acids ranging from 70–90 carbons in length are in turn covalently attached to the

Electronic supplementary material The online version of this article (doi:10.1007/s10719-008-9211-z) contains supplementary material, which is available to authorized users.

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C-5 positions of the terminal and penultimate arabinofuranosides at the non-reducing end of the AG [5–7]. This structure, the mycolyl-arabinogalactan-peptidoglycan (mAGP) interacts with other My-containing glycolipids (trehalose mono- or di-mycolate, TMM and TDM, respectively) to form the outermost layer of the cell envelope.

A potential target for interfering with the synthesis of My-containing glycolipids and the attachment of My to the AG is the antigen 85 (ag85) complex. Ag85 is a homologous series of acyltransferases (ag85A, B and C) expressed in a ratio of 3:2:1 (ag85B:ag85A:ag85C) [8]. The enzymes are responsible for converting TMM to TDM [9–13]. Data that show the involvement of ag85 in the mycolation of the AG (Fig. 1) include gene inactivation for ag85C in *M. tuberculosis* that reduced cell wall mycolation by 40%, and *csp1* gene disruption in *Corynebacterium glutamicum* that reduced cell wall mycolation by 50% [14–16]. Sequential inactivation of the genes encoding for ag85A, B and C and characterization of sites of mycolation on AG in whole cells by chemical derivatization and Smith degradation revealed ag85C to be the most active acyltransferase and show ag85 homologs share identical substrate specificity for the terminal and penultimate C-5 position of the AG [16]. The latter studies are further supported by crystallographic data indicating high similarity between the active sites of the three homologs [17–19]. In our ongoing efforts to identify inhibitors of ag85 we developed a synthetic acyl donor that can be used *in lieu* of TMM, isolated from *M. tuberculosis*, to study ag85-mediated acyl-transfer and to rapidly evaluate ag85 inhibitors [20]. In this communication we demonstrate *p*-nitrophenyl 6-*O*-octanoyl-β-D-glucopyranoside (**1**), Scheme 1, is capable of donating acyl groups in the presence of ag85C to synthetic arabinofuranosides modeling the terminal and penultimate residues of the AG with regiochemistry identical to that seen in the whole cell system.

Results and discussion

To initiate a study on ag85C-mediated acyl-transfer we are required to synthesize an acyl donor **1**, and three acceptors **2**–**3** [20, 22, 23] and **9** [24]. Acyl donor **1** for enzymatic reaction was synthesized by literature procedure [21]. Synthesis of methyl α-D-arabinofuranoside **2** and its β-anomer **3** was accomplished in high yield employing a kinetically controlled glycosylation to produce products that were separated by flash column chromatography (Scheme 1) [20]. The terminal arabinan fragment **9** was synthesized from glycosyl acceptor **4** [25] and glycosyl donor **5** [26] using a stereoselective protocol developed by Boons *et al.* [27]. Hence, acceptor **4** was glycosylated with **5** in the presence of silver triflate (AgOTf) and *N*-iodosuccinimide (NIS) in toluene–CH_2Cl_2 (1:15) at −35°C to afford **6** and **7** in 25% and 51% yields, respectively. Desilylation of **7** by tetrabutylammonium fluoride (TBAF) followed by debenzylation using Pd-C/H_2 generated disaccharide **9**, via **8**, in 92% yield (in 2 steps).

Acyl-transfer reactions were performed between acyl donor **1** and glycosides **2**, **3**, and **9** in the presence of ag85C at pH=7.4 (sodium phosphate buffer). Reaction of **1** with **2** generated a major 2,5-di-*O*-acylated product **10** [*m/z*= 439 (100), $(M+Na)^+$] with a minor monoacylated product [*m/z*=313 (12), $(M+Na)^+$] in 2 h, whereas **3** produced 5-*O*-acylated product **11** [*m/z*=313 (100), $(M+Na)^+$] with a little starting material left unreacted in 2 h (Scheme 2).

Under the same reaction conditions, reaction of **9** with **1** yielded a major monoacylated product [*m/z*=445 (100), $(M+Na)^+$] accompanied by a minor 5,5′-di-*O*-acylated product **12** [*m/z*=571 (9), $(M+Na)^+$] in 1 h. The latter product [*m/z*=571 (100), $(M+Na)^+$] became predominant product after 8 h (see Supp. Info.). When the reaction was allowed to run for 23 h, a triacylated product [*m/z*=697 (64), $(M+Na)^+$] formed significantly although **12** was still major.

The initial velocity of each reaction was monitored by a newly developed assay-protocol for acyltransferases [21]. The regiochemistry of acylation for products **10** and **11** was determined by ^1H NMR and $^1H-^1H$ gCOSY on $CHCl_3$ extracts of the reaction mixture (see Supp. Info.). As expected, H-2 (4.83 ppm), H-5 (4.24 ppm) and H-5′ (4.31 ppm) of **10**, and H-5 (4.18 ppm) and H-5′ (4.25 ppm) of **11** have shifted downfield, due to acylation. The regiochemistry of acylation for product **12** was more difficult to determine.

To make the assignment authentic **12** was prepared by selective acylation of **9** with *N*-octanoylthiazolidine-2-thione

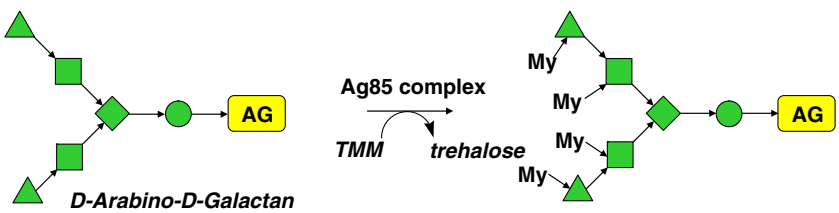
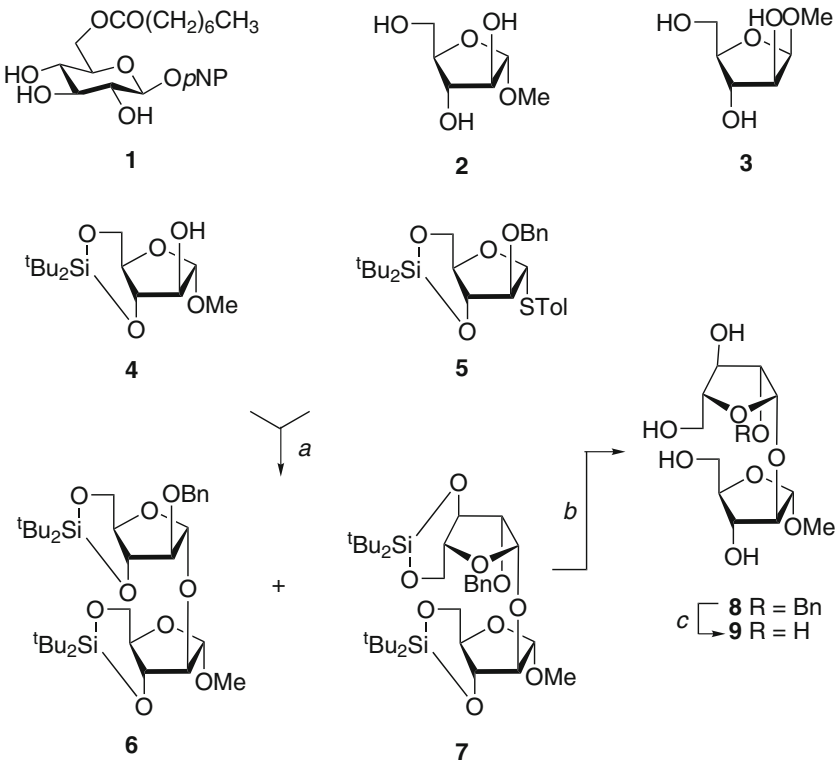


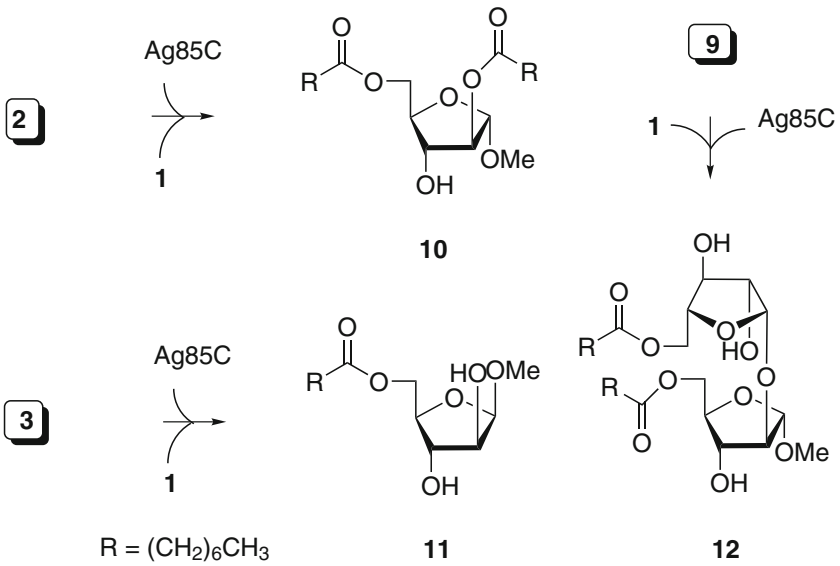
Fig 1 The ag85-mediated mycolyltransfer reaction from TMM to the non-reducing end of AG. *My* mycolate, *filled upright triangle* terminal-β-D-Araf, *filled square* 2-α-D-Araf, *filled diamond* 3,5-α-D-Araf, *filled circle* 5-α-D-Araf, *AG* D-arabino-D-galactan



Scheme 1 Key glycosides and Synthesis of **9**[a]. [a]*Reagents and conditions: a*. AgOTf, NIS, MS 4Å, toluene + CH_2Cl_2 (1:15), −35°C, 1 h: **6**=25%, and **7**= 51% yield; b. TBAF.$3H_2O$, THF, r.t., 24 h; c. Pd-C, H_2, MeOH, 0.5 h, 92% yield (in two steps)

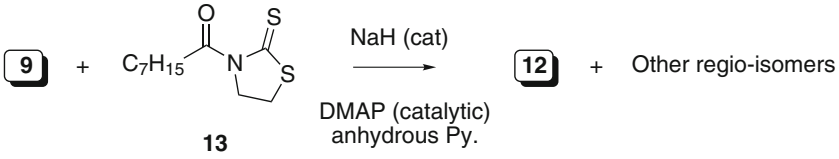
13 [28, 29]; Scheme 3. Comparison of ^{1}H NMR spectra for authentic **12** and $CHCl_3$ extract from the ag85C-mediated acylation confirmed the presence of 5,5′-di-*O*-acylated product in the latter. The characteristic resonances used for comparison were $\delta_{H\text{-}1\beta}$=5.05 ppm (d, *J*=4.8 Hz), $\delta_{H\text{-}1\alpha}$= 4.87 ppm (d, *J*=1.8 Hz), $\delta_{H\text{-}5'\beta}$=4.42 ppm (dd, *J*=7.2, 12.0 Hz), $\delta_{H\text{-}5'\alpha}$=4.32 ppm (dd, *J*=3.0, 11.4 Hz, merged with donor peak), $\delta_{H\text{-}5\alpha}$=4.21 ppm (dd, *J*=6.0, 11.4 Hz), $\delta_{H\text{-}5\beta}$= 4.17 ppm (m, 2 H, merged with H-4α) and δ_{OMe}=3.40 ppm (s) as indicated by arrows a, b, c, d, e, f, and g, respectively, of 5,5′-di-*O*-acylated product (Fig. 2).

MS/MS analysis on $CHCl_3$-extract containing **12** from enzymatic reactions and **12** from chemical synthesis was performed to address whether the ion, *m/z*=445 $(M+Na)^+$, corresponding to monoacylated disaccharide was formed as a result of fragmentation of **12** and to further confirm that synthetic and enzymatic **12** were identical. MS on the *m/z*= 571, $(M+Na)^+$ ion, corresponding to **12**, for both synthetic



Scheme 2 Ag85C-mediated acyl-transfer on D-arabinofuranoside-based compounds[a]. [a]Conditions for substrates: **2** and **3** (100 μL, 100 mM each), **1** (100 μL, 20 mM), ag85C (39 μL, 64 μM), buffer (761 μL) were used. Conditions for substrate: **9** (200 μL, 20 mM), **1** (400 μL, 20 mM), ag85C (156 μL, 64 μM), buffer (1,244 μL) were used. All the reactions were carried out at room temperature. Buffer = sodium phosphate pH=7.4

Scheme 3 Chemical Synthesis of **12** from **9** and **13**



and enzymatic products produced identical fragmentation patterns. The major fragments resulting from MS on *m/z*= 571, $(M+Na)^+$, were *m/z*=313 $(M+Na)^+$, which corresponds to fragmentation at the glycosidic linkage and *m/z*=439 $(M+Na)^+$, which corresponds to a diacylated monosaccharide. No fragmentation corresponding to monoacylated disaccharide product having *m/z*=445 $(M+Na)^+$ (see Supp. Info.) was observed. Thus, NMR and MS/MS data together led us to conclude the following: (a) the minor monoacylated disaccharide product having *m/z*=445 $(M+Na)^+$ was formed independently as detected by ESI-MS described earlier and was not due to the fragmentation of diacylated disaccharide product **12** and (b) the regiochemistry of compound **12** in the $CHCl_3$-extract is same as the authentic **12**.

To further characterize the range of substrates available to ag85C, steady-state kinetic experiments were performed with three acyl-acceptors (Fig. 3). While, the catalytic parameters of the two β-anomers are very similar, the α-anomer produces a pronounced difference in the V_{max} of the reaction. This may be a reflection of the two possible binding orientations of the α-anomer within the enzyme active site as seen from the NMR and MS experiments.

Conclusion

In summary, ag85C-mediated reactions on substrates **3** and **9** afforded **11** and **12**, respectively, with acyl functions at C-5 positions. The results are consistent with that found in mycobacterial cell wall, where the arabinan is mycolylated at the C-5 positions of the terminal and penultimate saccharides. Ag85C could acylate an unnatural glycoside **2** with acylations at C-2 and C-5. The results agree with previous findings [14] that ag85C is not specific for a given type of acyl chain. The study shows clearly that D-arabinose-based derivatives are capable of acting as substrate for ag85C-mediated acyl-transfer and model acyl donor can be used *in lieu* of difficult to obtain TMM for studying the ag85 protein class.

Experimental section

General Methods All fine chemicals such as D-arabinose, thiocresol, acetic anhydride, and benzyl bromide, and anhydrous solvents such as anhydrous methanol, and

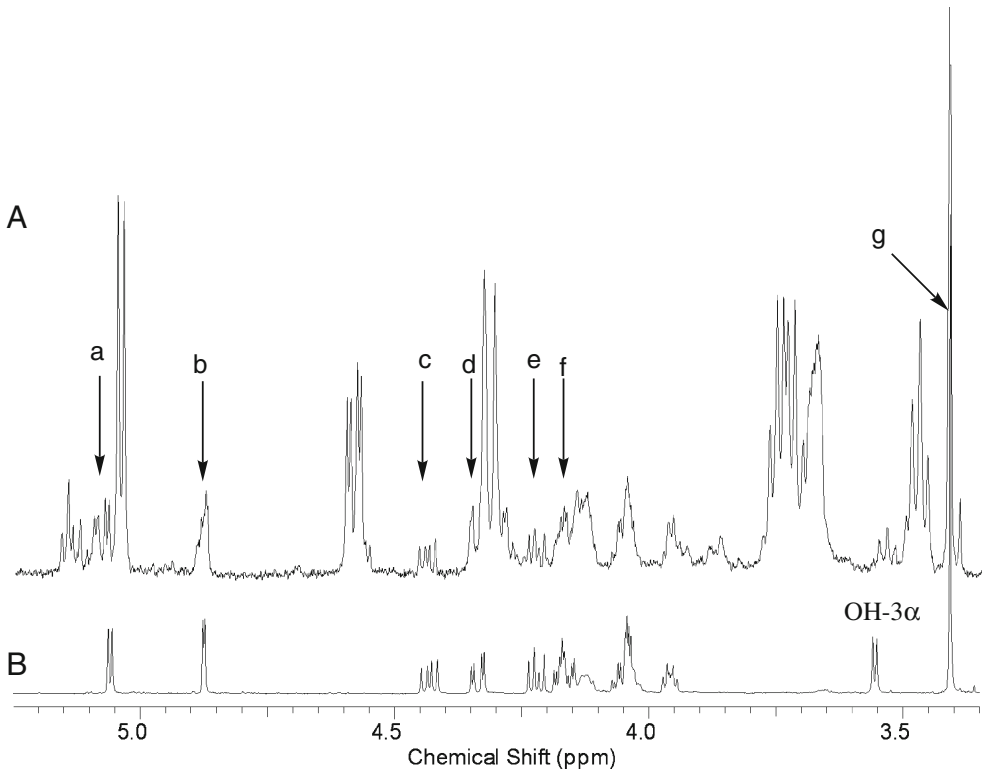


Fig 2 Comparison of characteristic region of ^1H NMR. **A** Spectrum of crude **12** from the enzymatic reaction. **B** Spectrum of pure **12** obtained by chemical synthesis (Scheme 3)

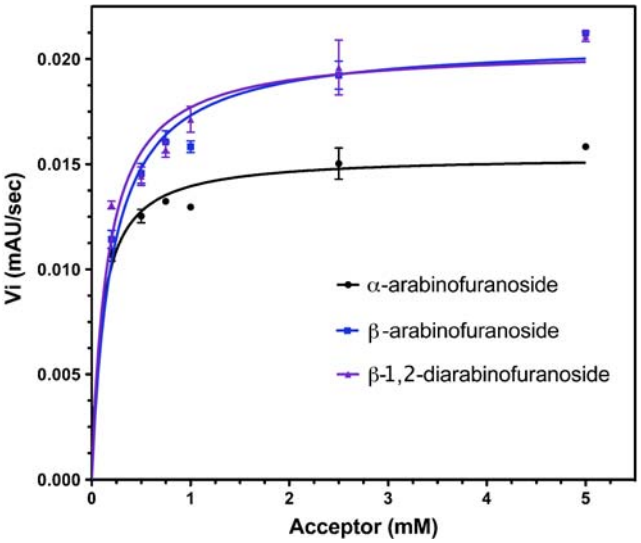


Fig 3 Kinetic consequence of using different acyl-acceptors

N,*N*-dimethylformamide were purchased from Acros, Di-*tert*-butylsilylbis(trifluoromethanesulfonate) was from Aldrich and was used without further purification. All solvents were obtained from Fisher and used as received except pyridine, which was dried and distilled following the standard procedures [30]. Silica (230–400 mesh) for flash column chromatography was obtained from Sorbent Technologies; thin-layer chromatography (TLC) precoated plates were from EMD. TLCs (silica gel 60, f_{254}) were visualized under UV light or by charring (5% H_2SO_4–MeOH). Flash column chromatography was performed on silica gel (230–400 mesh) using solvents as received. ^{1}H NMR were recorded either on a Varian VXRS 400 MHz or an INOVA 600 MHz spectrometer in $CDCl_3$ or CD_3OD using residual $CHCl_3$ and CH_3OH as internal references, respectively. ^{13}C NMR were recorded on a Varian INOVA 150 MHz in $CDCl_3$ using the triplet centered at δ 77.273 or CD_3OD using the septet centered at δ 49.0 as internal reference. The mass spectra were recorded on a Bruker Daltonics Esquire LC/MS mass spectrometer in MeOH. High resolution mass spectrometry (HRMS) was performed on a Micromass Q-TOF2 instrument.

*Methyl 2-O-(2-O-benzyl-3,5-O-(di-tert-butylsilylene)-α-D-arabinofuranosyl)-3,5-O-(di-tert-butylsilylene)-α-D-arabinofuranoside (**6**) and methyl 2-O-(2-O-benzyl-3,5-O-(di-tert-butylsilylene)-β-D-arabinofuranosyl)-3,5-O-(di-tert-butylsilylene)-α-D-arabinofuranoside (7)* Acceptor **4** (0.5 g, 1.642 mmol), donor **5** (0.96 g, 1.97 mmol) and activated powdered MS 4Å (1.5 g) were taken in a 250 mL two-neck round-bottom flask and dried under vacuum for 0.5 h. Anhydrous CH_2Cl_2 (30 mL) was added to the flask. The resulting suspension was stirred at room temperature for 1 h under N_2 atmosphere. Temperature was lowered to −35°C using a dry ice-acetone cooling bath. *N*-Iodosuccinimide (0.66 g, 2.95 mmol) was quickly added and stirring was continued for 5 min. A solution of AgOTf (0.25 g, 0.98 mmol) in anhydrous toluene (2 mL) was added dropwise and the resulting reddish colored suspension was stirred further for 1 h at −35°C. Reaction mixture was allowed to attain 0°C and TEA (2 mL) was added. Resulting suspension was filtered through a Celite® bed. Filtrate thus obtained was washed with saturated $Na_2S_2O_3$ and brine. Aqueous phases were back extracted with CH_2Cl_2 and the combined organic phase was dried (anhydrous Na_2SO_4) and filtered, and the filtrate was concentrated under reduced pressure to get the crude residue. The crude material was purified by silica gel flash column chromatography (10×4 cm). Elution with 0.1:0.1:0.2:9.6 MeOH–acetone–$CHCl_3$–hexanes generated **6** as colorless fluffy mass: yield 0.28 g (25%); R_f=0.78 (1:4 EtOAc-hexanes); ^{1}H NMR (600 MHz, $CDCl_3$): δ 0.99 (s, 9 H, ^{t}Bu), 1.01 (s, 9 H, ^{t}Bu), 1.04 (s, 9 H, ^{t}Bu), 1.07 (s, 9 H, ^{t}Bu), 3.39 (s, 3 H, OMe), 3.92 (m, 2 H, H-4α & H-5α), 3.95 (m, 2 H, H-4β & H-5β), 3.99 (dd, 1 H, *J*=3.0, 7.2 Hz, H-2β), 4.05 (m, 2 H, H-2α & H-3α), 4.11 (m, 1 H, H-3β), 4.33 (dd, 1 H, *J*=3.0, 7.2 Hz, H-5′β), 4.35 (dd, 1 H, *J*=4.2, 7.8 Hz, H-5′α), 4.75 (dd, 2 H, *J*=12.0, 46.2 Hz, C*H*$_2$Ph), 4.83 (d, 1 H, *J*=2.4 Hz, H-1α), 5.28 (d, 1 H, *J*=3.0 Hz, H-1β), 7.30 (m, 1 H, aromatic), 7.35 (t, 2 H, *J*=7.2 Hz, aromatic), 7.38 (d, 2 H, *J*=7.2 Hz, aromatic); ^{13}C NMR (150 MHz, $CDCl_3$): δ 20.33, 20.34, 22.86, 22.88, 27.34 (3× Me of ^{t}Bu), 27.37 (3× Me of ^{t}Bu), 27.71 (3× Me of ^{t}Bu), 27.75 (3× Me of ^{t}Bu), 56.26 (OMe), 67.75, 67.84, 72.06 (*C*H$_2$Ph), 73.49, 74.21, 81.39, 81.88, 87.14, 87.95, 106.98 (C-1β), 108.22 (C-1α), 127.87, 127.90, 128.55, 138.08; mass spectrum (HRMS), *m/z*=689.3492 $(M+Na)^+$ ($C_{34}H_{58}NaO_9Si_2$ requires 689.3517) and **7** as colorless fluffy mass: yield 0.56 g (51%); R_f=0.65 (1:4 EtOAc-hexanes); ^{1}H NMR (600 MHz, $CDCl_3$): δ 0.98 (s, 9 H, ^{t}Bu), 1.00 (s, 9 H, ^{t}Bu), 1.04 (s, 9 H, ^{t}Bu), 1.08 (s, 9 H, ^{t}Bu), 3.37 (s, 3 H, OMe), 3.86 (m, 1 H, H-4β), 3.89 (m, 2 H, H-4α & H-5α), 3.94 (m, 2 H, H-2β & H-5β), 4.02 (dd, 1 H, *J*=3.0, 7.2 Hz, H-2α), 4.08 (m, 1 H, H-3α), 4.27 (dd, 1 H, *J*=5.4, 9.0 Hz, H-5′β), 4.32 (m, 1 H, H-5′α), 4.43 (t, 1 H, *J*=9.0 Hz, H-3β), 4.80 (dd, 2 H, *J*=12.0, 39.6 Hz, C*H*$_2$Ph), 4.87 (d, 1 H, *J*=2.4 Hz, H-1α), 5.02 (d, 1 H, *J*=4.8 Hz, H-1β), 7.31 (t, 1 H, *J*=7.2 Hz, aromatic), 7.36 (t, 2 H, *J*=7.2 Hz, aromatic), 7.42 (d, 2 H, *J*=7.2 Hz, aromatic); ^{13}C NMR (150 MHz, $CDCl_3$): δ 20.30, 20.34, 22.79, 22.86, 22.35 (3× Me of ^{t}Bu), 27.41 (3× Me of ^{t}Bu), 27.61 (3× Me of ^{t}Bu), 27.79 (3× Me of ^{t}Bu), 55.98 (OMe), 67.80, 68.83, 72.15 (*C*H$_2$Ph), 74.15, 74.24, 78.38, 80.46, 80.95, 87.36, 100.05 (C-1β), 108.23 (C-1α), 127.99, 128.30, 128.57, 138.08; mass spectrum (HRMS), *m/z*=689.3469 $(M+Na)^+$ ($C_{34}H_{58}NaO_9Si_2$ requires 689.3517).

*Methyl 2-O-(2-O-benzyl-β-D-arabinofuranosyl)-α-D-arabinofuranoside (**8**) and methyl 2-O-β-D-arabinofuranosyl-α-D-arabinofuranoside (**9**)* A solution of **7** (0.06 g, 0.089 mmol) and TBAF.$3H_2O$ (0.116 g, 0.368 mmol) in anhydrous THF (10 mL) was stirred at ambient temperature under N_2 atmosphere. The reaction was monitored by TLC and appeared complete after 24 h. Excess solvent was removed under reduced pressure and crude material was purified by silica gel flash column chromatography (9× 3 cm). Elution with 3:3:14 MeOH–acetone–$CHCl_3$ produced **8** as a yellow amorphous solid: yield 0.033 g (95%); R_f=0.22 (1:9 MeOH–$CHCl_3$); ^{1}H NMR (600 MHz, CD_3OD): δ 3.33 (s, 3 H, OMe), 3.58 (dd, 1 H, *J*=5.4, 12.0 Hz, H-5β), 3.61 (dd, 1 H, *J*=6.6, 12.0 Hz, H-5α), 3.68-3.76 (m, 3 H, H-5′α, H-5′β, & H-4β), 3.84 (m, 1 H, H-4α), 3.89 (dd, 1 H, *J*=4.2, 7.8 Hz, H-2β), 3.98 (m, 2 H, H-2α & H-3α), 4.13 (t, 1 H, *J*=7.8 Hz, H-3β), 4.61 (dd, 2 H, *J*=12.0, 24.0 Hz, CH_2Ph), 4.75 (d, 1 H, *J*=2.4 Hz, H-1α), 4.97 (d, 1 H, *J*=4.2 Hz, H-1β), 7.27 (t, 1 H, *J*=7.2 Hz, aromatic), 7.32 (t, 2 H, *J*=7.2 Hz, aromatic), 7.37 (d, 2 H, *J*=7.2 Hz, aromatic); ^{13}C NMR (150 MHz, CD_3OD): δ 55.62 (OMe), 62.75, 64.57, 73.81 (*C*H_2Ph), 74.77, 76.42, 83.97, 84.18, 85.78, 89.26, 101.23 (C-1β), 108.33 (C-1α), 129.11, 129.47, 129.58, 129.59, 139.42; mass spectrum (HRMS), *m/z*=409.1447 $(M+Na)^+$ ($C_{18}H_{26}NaO_9$ requires 409.1475). On a 500 mg scale, compound **7** was desilylated following the procedure as described above and the product **8** was purified by a silica gel flash column (10×4 cm) using 1.2:1.2:7.6 MeOH–acetone–$CHCl_3$. Purified product **8** (503 mg) having an impurity of TBAF was dissolved in anhydrous MeOH (25 mL) and Pd-C (300 mg) was added into it. The resulting suspension was stirred in the presence of a balloon of H_2 and monitored by TLC. The reaction appeared complete within 0.5 h. Suspended solids were filtered off by passing the reaction mixture through a pad of Celite® bed. The Celite® bed was washed with MeOH (two bed volumes). Combined filtrate was concentrated under reduced pressure to afford a crude material, which was purified by flash column chromatography (8×3 cm) on silica gel with 1:4 MeOH–$CHCl_3$ to afford **9** as a light yellow gum. Trituration with $CHCl_3$ provided **9** (Scheme 1) as a white amorphous powder: yield 0.204 g (92% in 2 steps); R_f=0.21 (1:4 MeOH–$CHCl_3$).

*Methyl 2-O-(5-O-octanoyl-β-D-arabinofuranosyl)-5-O-octanoyl-α-D-arabinofuranoside (**12**)* To a solution of compound **9** (0.26 g, 0.877 mmol) in anhydrous pyridine (6 mL) was added catalytic amounts of NaH and DMAP and the resulting solution was stirred for 15 min under N_2 atmosphere. *N*-octanoylthiazolidine-2-thione **13** (0.43 g, 1.75 mmol) in anhydrous pyridine (9 mL) was added dropwise. The reaction was stirred at ambient temperature and monitored by TLC for 2 h during which time, a number of spots were found to form on TLC. Crude material, obtained after removing excess solvent under reduced pressure, was absorbed on silica gel and purified twice by silica gel flash column chromatography (6×4.5 cm). First, elution with 0.4:0.4:9.2 and then 1:1:8 MeOH–acetone–$CHCl_3$ generated three monoacylated products 85 mg (H-5α-monoacylated), 26 mg (H-5β-monoacylated), and 26 mg with R_f=0.07, 0.15, and 0.27 in 0.4:0.4:9.2 MeOH-acetone–$CHCl_3$, respectively and a mixture of three spots higher in R_f than the pure fractions above. Purification of the mixture obtained from the first stage of purification, with 0.2:0.2:9.6 MeOH–acetone–$CHCl_3$ afforded the desired 5,5′-diacylated product **12** with R_f=0.33 in 0.4:0.4:9.2 MeOH–acetone–$CHCl_3$ (Scheme 3) and two other diacylated products having R_f=0.39 and R_f=0.43. The diacylated product with R_f=0.43 possessed diacylation in β-ring. Regiochemistry of acylations for all the assigned products described above were established by ^{1}H NMR and ^{1}H−^{1}H gCOSY experiments. For **12**: yield 0.011 g (2%); ^{1}H NMR (600 MHz, $CDCl_3$): δ 0.88 (t, 6 H, *J*=6.6 Hz, 2×Me), 1.29 (m, 16 H), 1.65 (m, 4 H), 2.35 (dd, 4 H, *J*=7.2, 14.4 Hz), 2.63 (d, 1 H, *J*=9.0 Hz, OH-2β), 2.78 (d, 1 H, *J*=2.4 Hz, OH-3β), 3.40 (s, 3 H, OMe), 3.55 (d, 1 H, *J*=4.8 Hz, OH-3α), 3.96 (m, 1 H, H-3α), 4.04 (m, 3 H, H-3β, H-4β & H-2α), 4.12 (m, 1 H, H-2β), 4.17 (m, 2 H, H-4α & H-5β), 4.21 (dd, 1 H, *J*=6.0, 11.4 Hz, H-5α), 4.32 (dd, 1 H, *J*=3.0, 11.4 Hz, H-5′α), 4.42 (dd, 1 H, *J*=7.2, 12.0 Hz, H-5′β), 4.87 (d, 1 H, *J*=1.8 Hz, H-1α), 5.05 (d, 1 H, *J*=4.8 Hz, H-1β);^{13}C NMR (150 MHz, $CDCl_3$): δ 14.33 (Me), 14.34 (Me), 22.85, 22.86, 25.08, 25.13, 29.17, 29.20, 29.30, 29.31, 29.95, 31.89, 31.91, 34.36, 34.37, 55.72 (OMe), 63.71, 65.19, 76.52, 76.78, 77.92, 80.25, 80.33, 89.12, 101.70 (C-1β), 106.78 (C-1α), 174.11 (C=O), 174.37 (C=O); mass spectrum (HRMS), *m/z*=571.3082 $(M+Na)^+$ ($C_{27}H_{48}NaO_{11}$ requires 571.3094).

Procedures for enzymatic reactions and enzymatic assays

Part A: general methods for enzymatic reactions Solutions of synthetic compounds **2**, **3** and **9** of appropriate concentrations were prepared by using the respective synthetic compound in sodium phosphate buffer of pH=7.4. Acyl donor **1** was similarly prepared in DMSO. Recombinant ag85C was expressed in *E-coli* and purified by using nickel affinity and size exclusion chromatography. The concentration (64 μM) in crystallization buffer (10 mM tris pH=7.5, 2 mM EDTA and 1 mM DTT) was accurately determined by reading the absorbance at 280 nm using the theoretical extinction co-efficient of 84,340 M^{-1} cm^{-1}. All the reactions were carried out at room temperature. Reactions of **2** and **3** separately with **1** in the presence of ag85C were

performed without additional shaking whereas reaction of **9** with **1** required shaking on a shaker.

Typically, appropriate substrate and acyl donor were mixed in a sample vial of 5 mL capacity in sodium phosphate buffer (pH=7.4) and into it ag85C was added. The resulting solution or suspension was either left at room temperature or placed on a shaker as required. After the reaction was complete (confirmed by mass spectrometry analysis), MeOH (ca. 0.5 mL) was added to stop the reaction. The aqueous layer was extracted with $CHCl_3$ (3× 30 mL). Combined $CHCl_3$ layer was dried (anhydrous Na_2SO_4), filtered and the filtrate was concentrated to dryness under reduced pressure to produce the crude material.

Part B: general methods for enzymatic assay The enzymatic assay was run between each acyl-acceptor **2**, **3**, or **9**, and a single octanoyl donor **1**. The concentration of **1** was kept constant at 175 μM while the acceptor concentration was varied between 0 and 5 mM. A master mix was prepared by combining 76 μL of a 0.05 M sodium phosphate buffer (pH= 7.4), 2 μL of 1,000 U/mL β-glucosidase and 10.55 μL of ag85C enzyme (0.948 μM). In each well, 2 μL of **1** (8.75 mM in DMSO), the appropriate amount of acyl-acceptor (0–5 μL of a 0.1 M stock) and the volume of buffer needed to bring the volume to 11.45 μL. To this was added 88.55 μL of the master mix to start the reaction. Reactions at each acceptor concentration were run in triplicate.

Methyl 2,5-di-O-octanoyl-α-D-arabinofuranoside (10) Compound **2** (100 μL, 100 mM) was reacted with **1** (100 μL, 20 mM) in the presence of ag85C (39 μL, 64 μM) in sodium phosphate buffer (761 μL) at ambient temperature as per the general procedure described in part A to afford **10** (2 mg) as a crude material (Scheme 2). The latter was a 1:1 mixture of **10** and **1** (unreacted donor); Scheme 1. ^{1}H NMR and ^{1}H−^{1}H gCOSY of the crude material were recorded and characteristic peaks in ^{1}H NMR of **10** are as follows: ^{1}H NMR (600 MHz, $CDCl_3$): δ 3.42 (s, 3 H, OMe), 3.10 (d, 1 H, J=5.4 Hz, OH-3), 3.93 (m, 1 H, H-3), 4.19-4.24 (m, 2 H, H-4 & H-5), 4.31 (m, 1 H, H-5′), 4.83 (d, 1 H, J= 1.8 Hz, H-2), 4.99 (s, 1 H, H-1). Characteristic peaks due to the alkyl chain have been merged with those present in **1**; mass spectrum (ESI-MS), m/z=439.5 $(M+Na)^+$ ($C_{22}H_{40}NaO_7$ requires 439.26).

Methyl 5-O-octanoyl-β-D-arabinofuranoside (11) Compound **3** (100 μL, 100 mM) was reacted with **1** (100 μL, 20 mM) in the presence of ag85C (39 μL, 64 μM) in sodium phosphate buffer (761 μL) as per the general procedure described in part A to generate **11** (2 mg) as a crude material (Scheme 2). The latter was a 1.5:1 mixture of **11** and **1** (unreacted donor); Scheme 1. ^{1}H NMR and ^{1}H−^{1}H gCOSY of the crude material were recorded and characteristic peaks in ^{1}H NMR of **11** are as follows: ^{1}H NMR (600 MHz, $CDCl_3$): δ 3.44 (s, 3 H, OMe), 4.01 (m, 1 H, H-4), 4.07 (m, 2 H, H-2 & H-3), 4.18 (dd, 1 H, J=5.4, 11.4 Hz, H-5), 4.25 (dd, 1 H, J=4.2, 12.0 Hz, H-5′), 4.83 (d, 1 H, J=4.8 Hz, H-1). Characteristic peaks due to the alkyl chain have been merged with those present in **1**; mass spectrum (ESI-MS), m/z=313.3 $(M+Na)^+$ ($C_{14}H_{26}NaO_6$ requires 313.16).

Methyl 2-O-(5-O-octanoyl-β-D-arabinofuranosyl)-5-O-octanoyl-α-D-arabinofuranoside (12) Compound **9** (200 μL, 20 mM) was reacted with **1** (400 μL, 20 mM) in the presence of ag85C (156 μL, 64 μM) in sodium phosphate buffer (1244 μL) as per general procedure described in part A to yield **12** (2 mg) as a crude material (Scheme 2). The latter is a mixture of **12** (minor) and **1** (unreacted donor); Scheme 1. ^{1}H NMR and ^{1}H−^{1}H gCOSY of the crude material were recorded and characteristic peaks in ^{1}H NMR of **12** are as follows: δ 3.41 (s, 3 H, OMe), 4.22 (dd, 1 H, J=6.0, 11.4 Hz, H-5α), 4.43 (dd, 1 H, J=7.2, 12.0 Hz, H-5′β), 4.87 (s, 1 H, H-1α), 5.06 (d, 1 H, J= 4.8 Hz, H-1β). Characteristic peaks due to the alkyl chains and ring protons have been merged with that of the protons present in **1**; mass spectrum (ESI-MS), m/z=571.4 $(M+Na)^+$ ($C_{27}H_{48}NaO_{11}$ requires 571.3).

Acknowledgment We thank the University of Toledo for financial support through New Faculty Research Grants, an Interdisciplinary Research Initiation Award (to SJS), and a deArce Memorial Fellowship (to DRR).

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