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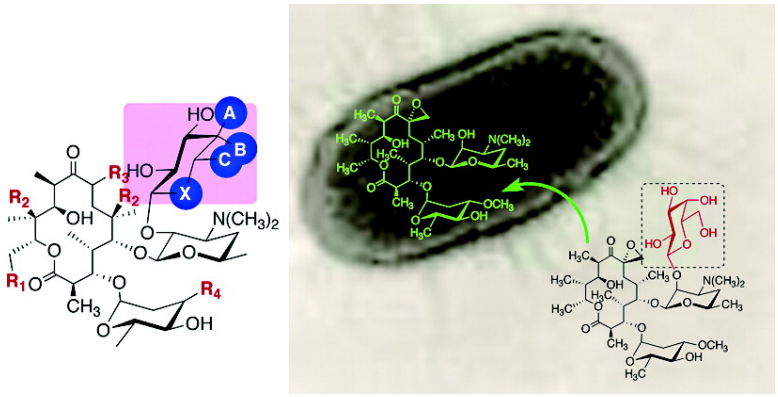

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Probing the Breadth of Macrolide Glycosyltransferases: In Vitro Remodeling of a Polyketide Antibiotic Creates Active Bacterial Uptake and Enhances Potency

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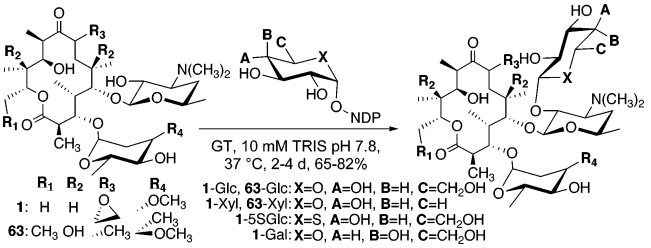
Macrolides constitute an important group of antibiotics that target primarily Gram-positive prokaryotes and collectively have been classed as "the last line of defense" against rapidly emerging resistant pathogen strains.[1] They comprise a macrocyclic polyketide backbone to which glycans are appended that alter activity, specificity, and resistance mechanisms.[2−4] Antibiotic glycan alteration (so-called glycorandomization[5−8]) is a potentially powerful strategy in combating emerging bacterial resistance. Rare, elegant examples of *in vitro* glycan modification of antibiotics have largely focused on cyclic nonribosomal peptides, such as vancomycin.[8−12] Although *in vivo* approaches have been explored,[13−18] to our knowledge, no *in vitro* studies have examined macrolide antibiotics.

Glycosyltransferases (GTs) are powerful glycosylation catalysts; however, their exquisite substrate specificity[19−21] typically curtails application to appending preferred sugar donor to preferred aglycone acceptor. Indeed, while some antibiotic-modifying GTs, such as GtfE,[11] show good variance in sugar substrates, other attempts to identify flexible GTs have instead highlighted stringency.[7] There is a need for GTs with broad tolerance as tools in antibiotic remodeling and methods for their ready identification and characterization. The inverting, family 1 (GT-1)[22] GTs from *Streptomyces lividans* (MGT)[23] and oleandomycin (**1**)-producing bacterium *S. antibioticus*[24,25] (OleD, OleI) catalyze glucose (Glc) transfer from UDP-Glc to **1**, which inactivates it. Their differing specificities[26] and membership of GT-1 suggested utility, little was known, however, about full substrate tolerance. Recombinant expression[27] and purification[28] from *Escherichia coli* C41(DE3) gave valuably[29] high protein levels (∼40 mg/L).[30] Full kinetic parameters were determined using mass spectrometric monitoring[30] coupled with pseudo-spiking calibration, allowing ready acquisition of biocatalytic data.[31] Reciprocal regression analysis employed rapid equilibrium assumption and assumed no a priori substrate role. OleI operates via a compulsory ordered Bi−Bi mechanism ($K_A/K_B \sim 20$[32]) in which **1** binds first,[33] and kinetic constants gave good to fair agreement[34,35] with previous partial kinetic characterization [OleI: k_{cat} 0.042 s^{-1}, $K_{I(1)}$ 18, $K_{M(1)}$ 4.8, $K_{M(UDPGlc)}$ 97μM]. OleD and MGT [OleD: k_{cat} 0.044 s^{-1}, $K_{I(1)}$ 165, $K_{I(UDPGlc)}$ 182, $K_{M(1)}$ 32 ± 8, $K_{M(UDPGlc)}$ 36μM; MGT: k_{cat} 0.8 s^{-1}, $K_{I(1)}$ 172, $K_{I(UDPGlc)}$ 65, $K_{M(1)}$ 1305, $K_{M(UDPGlc)}$ 497μM] operate via random Bi−Bi mechanisms ($K_A/K_B = 1.1$ and 2.6, respectively).[36] Encouragingly, K_M values for MGT > OleD or OleI suggested nonspecificity and operation in vivo at higher ambient substrate concentrations than that of OleD and OleI.

Full substrate specificity was probed through library screening[31] transfer from 18 sugar donors to 64 representative acceptors[30] and indicated that in addition to **1**, flavanols, coumarins, and other aromatics, such as 3,4-dichloroaniline, were acceptors for OleD, OleI, and MGT (Figure 1). This surprisingly broad acceptor plasticity[37] indicated that these GTs have not evolved to recognize a precise macrolide but rather planar, cyclic, hydrophobic molecules; as such, they display the relaxed hydrophobic specificity of the xenobiotic-modifying GTs prevalent in family GT-1. Indeed, sugar transfer to oleandomycin modifies OH-2 of the hydrophobic deoxysugar desosamine. OleD and MGT also showed activity toward benzyl α-mannoside (**53**) bearing a hydrophobic aglycone; all three failed with more hydrophilic carbohydrate acceptors. Interestingly, the novel acceptor specificity for coumarins discovered here is similar to that of GT NovM in the biosynthesis of novobiocin;[38] OleD, OleI, MGT created aminocoumarin antibiotic analogue **3**-Glc (Figure 1) with enhanced kinetic efficiency over NovM synthesis of **3**-noviose.[38]

Scheme 1. GT Remodeling of Oleandomycin **1** and Erythromycin **63**



Plasticity in both sugar and nucleotide recognition was probed with 18 natural/non-natural donors[30,31,39] against identified hit acceptors (Figure 1a). These indicated (Figure 1b) tolerance by all GTs for ring O alteration, by OleD for varied functionality at C-5 and by OleD and OleI for configurational flexibility at C-4 (e.g., ability to transfer Gal/Ara). Only OleI showed activity with UDP-Gal and **1**. OleD shows some tolerance of base variation (U → G, U → T with Xyl). All three enzymes are largely restricted in C-2,3 configuration, although some activity with non-natural UDP-Man but not GDP-Man with OleD and MGT suggested a role for the nucleotide as a determinant of specificity. OleD also transferred UDP-GlcNAc (to coumarin acceptors **2**, **4**, and **23** but not **1**). Pseudo single substrate kinetics [OleD: $K_{M(UDP5SG)}$ 37.8 μM, k_{cat} 0.0033 s^{-1}; OleI: $K_{M(UDP5SG)}$ 129 μM, k_{cat} 0.013 s^{-1}; MGT: $K_{M(UDP5SGlc)}$ 200 μM, k_{cat} 1.8 s^{-1}, [**1**] = 50 μM] for the most active non-natural donor, UDP-5S-Glc, revealed that all three GTs have smaller K_M values than for UDP-Glc, despite the change of endocyclic heteroatom. More dramatic k_{cat} effects were observed: OleI and OleD are 3- and 10-fold lower, while MGT is 2-fold *higher*. The transition state of transfer is therefore better stabilized by α-sulfur

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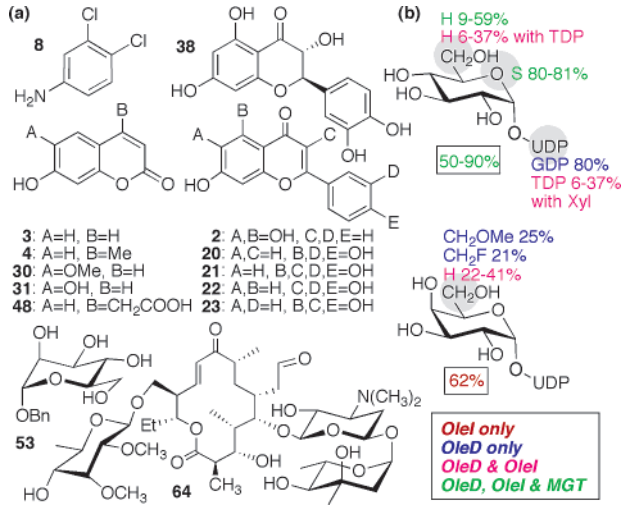

Figure 1. (a) Acceptor and (b) donor substrates of OleD, OleI % MGT. In (b), percent values indicate yields (color-coded by enzyme) for transfer of non-natural sugars (structural variation highlighted) to **1**.

in the order MGT > OleI > OleD, perhaps reflecting different conformational itinerary response to smaller C_1-S-C_6 bond angle and ring puckering[40] or different levels of developing C-1 charge.[41] This highlights UDP-5S-Glc as both mechanistic probe and non-natural donor in remodeling.

To see how this exciting substrate tolerance could be applied to other antibiotics, we also screened all donors against erythromycin (**63**) and tylosin (**64**) that contain similar dimethylaminosugar acceptor moieties (β-D-desosamine and 4-OH variant β-D-mycaminose, respectively) to oleandomycin **1**. Although OleI showed little activity, both OleD and MGT remodeled both antibiotics with Glc and Xyl (OleD: **64**-Glc 55%, **63**-Glc 74%, **63**-Xyl 65%; MGT: **64**-Glc 51%, **64**-Xyl 10%, **63**-Glc 58%, **63**-Xyl 9%).

The breadth of substrate tolerance, demonstrated here for these macrolide GTs, is highly unusual for a class of enzymes normally regarded as highly stringent. Their activities allowed synthesis (in up to 90%) of 12 novel polyketide (**1**-Glc, **1**-5SGlc, **1**-Xyl, **1**-Gal, **1**-Gal6F, **1**-GalOMe, **1**-Ara, **63**-Glc, **63**-Xyl, **64**-Glc, **64**-Xyl) and coumarin (**3**-Glc) antibiotics. These antibiotics are strong potential candidates for "glycotargeted'[42] antibiotics, in which a carbohydrate "cap" might enhance cellular uptake. Antibiotic activity screening against *E. coli* strain BL21(DE3), which displays endogenous β-galactosidase (β-G) activity and is thus able to convert **1**-Gal back to **1**, showed enhanced potency (MIC $\sim$ 400 μg/mL) over **1** and **1**-Glc (MIC > 400 μg/mL). Moreover, inhibition by **1**-Gal increased 16-fold (MIC $\sim$ 25 μg/mL) when intracellular β-G levels were enhanced by transformation with plasmid for exogenous β-G.[43] Monitoring intracellular uptake and loss from solution indicated $\sim$14 μg of **1**-Gal/mL of culture transported into *E. coli* BL21(DE3). This active uptake of **1**-Gal was explored further using *E. coli* TUNER that lacks the *lacY* gene encoding lactose permease. This bacterium took up only $\sim$4 μg/mL and was resistant to **1**-Gal, suggesting that lactose permease's recognition of Gal-appended structures[44] is responsible for the active uptake of **1**-Gal. Once internalized, **1**-Gal is hydrolyzed by β-G activity to **1**; combined active uptake and "uncapping" causes enhanced antibacterial activity.

In summary, high-level expression of three macrolide GTs created a synthetic "tool kit" with such plasticity that 12 modified antibiotics have been readily created. One, **1**-Gal, is enhanced over its parent **1** by "glycotargeting", allowing higher internalization. The clear broader potential is being explored.

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Supporting Information Available: Experimental procedures, characterization, and biological testing. This material is available free of charge via the Internet at http://pubs.acs.org.

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