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Experimental and Kinetic Studies of the *Escherichia coli* Glucuronylsynthase: An Engineered Enzyme for the Synthesis of Glucuronide Conjugates

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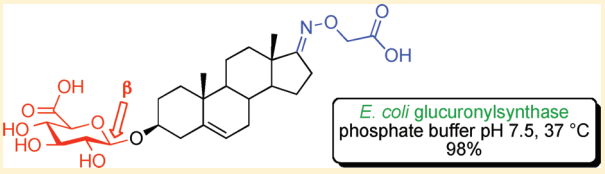
Supporting Information

ABSTRACT: The detection and study of glucuronide metabolites is essential in many fields including pharmaceutical development, sports drug testing, and the detection of agricultural residues. Therefore, the development of improved methods for the synthesis of glucuronide conjugates is an important aim. The glycosynthase derived from *E. coli* β-glucuronidase provides an efficient, scalable, single-step synthesis of β-glucuronides under mild conditions. In this article we report on experimental and kinetic studies of the *E. coli* glucuronylsynthase, including the influence of acceptor substrate, pH, temperature, cosolvents, and detergents, leading to optimized conditions for glucuronide synthesis. Enzyme kinetics also reveals that both substrate and product inhibition may occur in glucuronylsynthase reactions but that these effects can be ameliorated through the judicious choice of acceptor and donor substrate concentrations. An investigation of temporary polar substituents was conducted leading to improved aqueous solubility of hydrophobic steroidal acceptors. In this way the synthesis of the steroidal metabolite dehydroepiandrosterone 3-β-D-glucuronide was achieved in three steps and 86% overall yield from dehydroepiandrosterone.



Conjugation with glucuronic acid during phase II metabolism is a major pathway for the elimination of hydrophobic xenobiotic and endogenous compounds in mammalian systems.[1] The identification, quantification, and pharmacological evaluation of these metabolites is essential for many fields including drug development,[1] sports drug testing,[2] and the detection of agricultural residues.[3] This creates a significant demand for the synthesis of glucuronide conjugates **1** as standards.

The preparation of glucuronides provides a significant challenge for existing methods of glucuronylation (Scheme 1).[4] Chemical methods[5] of glucuronylation based on the Koenigs–Knorr reaction or more recent procedures often suffer from poor yields and side reactions due to the low reactivity of glucuronic acid derived glycosyl donors[4,5] and require one or more deprotection steps to liberate free glucuronide. Enzymatic methods[6] of synthesis employ uridine 5′-diphosphoglucuronosyl transferases (UGTs), a superfamily of enzymes responsible for glucuronylation in the body.[1] This procedure provides a mild and stereospecific synthesis in a single step. However, UGTs are substrate-specific to the acceptor alcohol, and practical considerations often limit this procedure to small scale syntheses. Given the limitations associated with existing methods, the development of improved glucuronylation protocols is an important goal.

Recently we reported a distinct strategy for the synthesis of glucuronides based on the glycosynthase derived from *Escherichia coli* β-glucuronidase (EC 3.2.1.31).[7] *Escherichia coli* β-glucuronidase is a member of the retaining β-glycosidase family 2 and catalyzes the hydrolytic cleavage of terminal β-glucuronide residues. The enzyme displays substrate promiscuity and has been widely exploited in the field of analytical chemistry for the deconjugation of glucuronide metabolites.[8] The enzyme active site contains two key catalytic residues. The side chain of glutamic acid 504 (E504)[9] acts as a nucleophile, and glutamic acid 413 (E413) is responsible for general acid/base catalysis in a double displacement mechanism (Scheme 2a). As demonstrated for a range of other retaining β-glycosidase enzymes,[10,11] mutation of the *E. coli* β-glucuronidase catalytic nucleophile to a non-nucleophilic glycine (E504G), alanine (E504A), or serine (E504S) residue disables the hydrolytic pathway (Scheme 2b). However, the resulting glycosynthase enzyme, or glucuronylsynthase, can catalyze the formation of glucuronide product **1** from α-D-glucuronyl fluoride **2** and an acceptor alcohol substrate. Our previous report[7] detailed the application of a spectrophotometric screening protocol using the wild-type *E. coli* β-glucuronidase to identify likely acceptor alcohol substrates for the glycosynthase mediated reaction, expression of the enzyme, and confirmation of these screening hits with the *E. coli* glucuronylsynthase mutants. In this paper we report details of the glucuronylsynthase development together with a kinetic study of this enzyme mediated transformation and practical improvements to the process for the synthesis of steroidal glucuronides.

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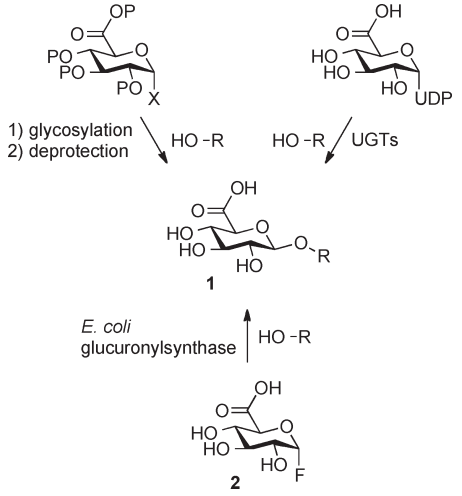
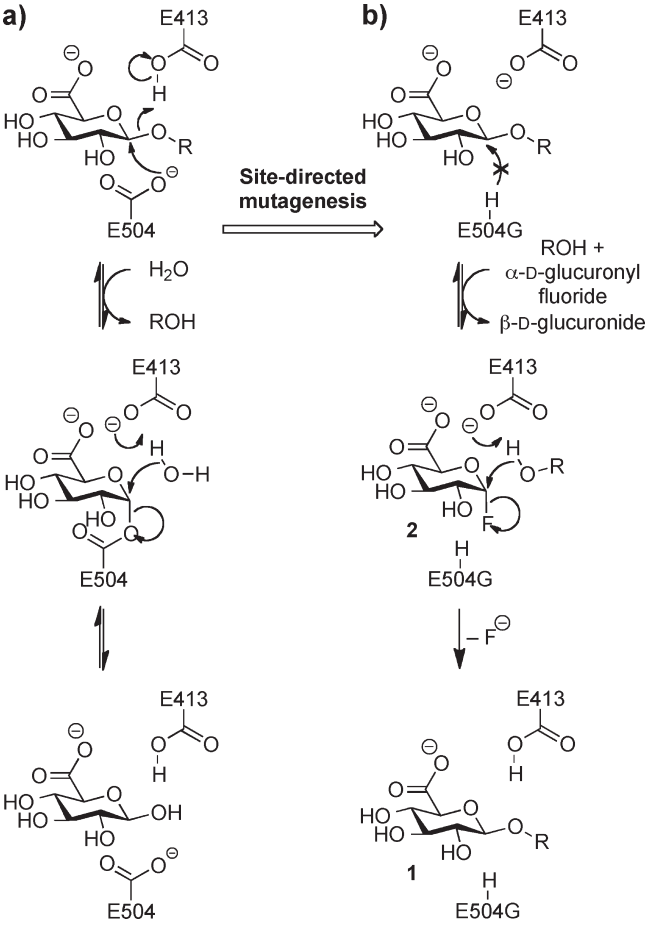
Scheme 1. Preparation of Glucuronide Conjugates 1



Scheme 2. Proposed Mechanism of Action of (a) *E. coli* Wild-Type β-Glucuronidase and (b) *E. coli* E504G Glucuronylsynthase



RESULTS AND DISCUSSION

The development of the *E. coli* glucuronylsynthase system required reliable access to both the mutant enzymes and the α-D-glucuronyl fluoride **2** donor sugar. The wild-type and three putative glycosynthase mutant enzymes, E504G, E504A, and E504S, were overexpressed in a glucuronidase deficient strain of *E. coli* (GMS407(DE3)) as previously described.[7] A minor modification of replacing the method of cell lysis from freeze−thaw cycles to French pressure cell press improved the yield of purified protein from 18 to 25 mg L^{-1} of culture. The enzymes produced by this protocol are histidine tagged (His_6), allowing for purification by nickel affinity chromatography. To assess if this modification has a deleterious effect on enzyme activity, the kinetic competence of the wild-type enzyme was investigated by colorimetric assay using *p*-nitrophenyl β-D-glucuronide as substrate and monitoring enzyme mediated hydrolysis at 410 nm.[12] The observed K_m of 0.17 ± 0.02 mM and k_{cat} of 38 ± 1 s^{-1} (21 °C, 50 mM phosphate buffer, pH 7.5) compare well with reported literature values determined under similar conditions for hydrolysis by a similar histidine tagged glucuronidase construct (K_m = 0.20 mM, k_{cat} = 68 s^{-1}, Tris-HCl buffer, pH 7.6)[13] and also purified native enzyme (K_m = 0.22 mM, 37 °C, 76 mM phosphate buffer, pH 7.0).[14]

The second key component required for the glucuronylsynthase system is the α-D-glucuronyl fluoride **2** donor sugar. At the outset of this study one reported synthesis[15] provided the potassium salt of this sugar in 65% yield and employed the TEMPO-mediated oxidation of the primary hydroxyl group of α-D-glucosyl fluoride **3**.[16] Purification was achieved by ion exchange, followed by recrystallization to remove potassium acetate buffer salt. In our hands the recrystallization of the sugar from significant amounts of potassium acetate salt proved challenging. This difficulty was circumvented by the use of ammonium hydrogen carbonate as eluting buffer in the anion exchange chromatography, which was readily removed on lyophilization to afford the ammonium salt as an off-white powder. This was further purified by recrystallization from aqueous ethanol to give the α-D-glucuronyl fluoride **2** as the ammonium salt[7] in 87% yield on a multigram scale (Scheme 3). The identity of sugar **2** was confirmed by single crystal X-ray analysis from crystals grown in ethanol/water/acetone, which clearly showed the presence of the expected ammonium counterion.[17] Analysis of the material by ^{13}C NMR in D_2O solvent confirmed the absence of residual hydrogen carbonate buffer (δ ~160).

In our previous investigations the aglycone specificity of the *E. coli* β-glucuronidase and of the putative glycosynthase enzymes was determined using an established spectrophotometric screening protocol against a panel of 123 alcohol acceptors.[7,11] The 13 alcohols identified by this screen included a range of primary and cyclic secondary aliphatic alcohols, substituted benzyl alcohols, and isomeric naphthalene methanols. None of the 54 carbohydrates tested in the screen were identified as acceptors. Preparative scale synthesis of glucuronide conjugates was conducted from each of the 13 alcohol hits shown in Scheme 4. Of the three glucuronylsynthase mutants, the E504G proved most effective. The E504A mutant provided lower yields in the cases examined, and the E504S mutant did not promote glucuronide synthesis.

Reactions conducted on a small selection of alcohols not identified by the screen also hinted at a broader substrate scope. Phenol, an acceptor present in the initial screen but not identified as a hit, was glucuronylated in a low 13% yield (Scheme 4). Ethanol, an acceptor not included in the original screen, failed to afford detectable amounts of the glucuronide product. Of great

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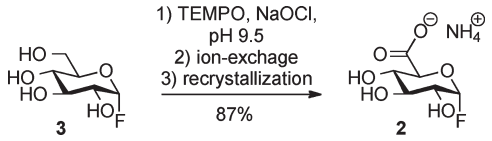
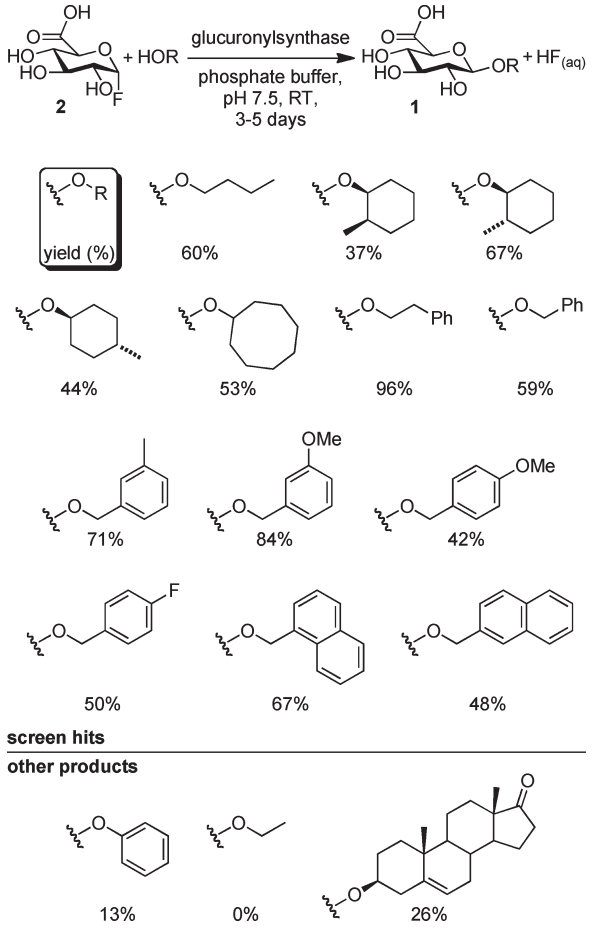
Scheme 3. Synthesis of α-D-Glucuronyl Fluoride 2



Scheme 4. Product Yields for E504G Glucuronylsynthase Mediated Synthesis



interest, the steroid dehydroepiandrosterone (DHEA), an acceptor not included in the original screen, was observed to give a modest 26% yield of the glucuronide product.

The isolated yields from these early experiments showed that the enzyme provided high yields of glucuronide products for some substrates, but this was not universal (Scheme 4). The yields varied considerably for some closely related acceptors such as 3-methoxybenzyl alcohol (84%) and 4-methoxybenzyl alcohol (42%). In a number of instances cosolvents or detergents were used to boost reaction yield for substrates with low aqueous solubility. However, the beneficial effects of cosolvents and detergents were not universally observed. Finally the variation of reaction variables such as substrate concentration provided some surprising results. In the case of 3-methoxybenzyl alcohol doubling the substrate concentration was expected to increase enzyme activity and therefore chemical yield. In fact doubling the

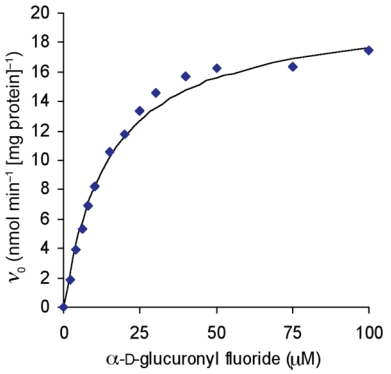


Figure 1. Plot of initial rate versus concentration for varying α-D-glucuronyl fluoride **2** donor with fixed (97 mM) 2-phenylethanol **4** acceptor (21 °C, 100 mM phosphate buffer, pH 7.5). The line represents the data fitted (least-squares) to the Michaelis−Menten equation.

substrate concentration led to a decrease in the isolated yield of glucuronide product from 84% to 64% based on the acceptor alcohol. In order to understand the factors governing this process and to optimize glucuronide synthesis, we embarked on a study of the kinetics of this enzyme catalyst.

The enzyme kinetics of the glucuronylsynthase reaction was conveniently investigated by an automatically sampled reverse-phase HPLC-UV assay monitored at 211 nm. Both alcohol acceptor and glucuronide product concentrations were determined relative to an internal standard over time to give a measure of initial rate (v_0). The first kinetic measurements were conducted at a fixed concentration of 2-phenylethanol **4** acceptor (97 mM), due to limited solubility of this substrate in the aqueous buffer, and a varying concentration of α-D-glucuronyl fluoride **2**. At this concentration of alcohol acceptor, the α-D-glucuronyl fluoride **2** donor gave apparent values of K_m^{app} = 15.0 ± 1 μM and k_{cat}^{app} = 0.024 ± 0.01 s^{-1} by least-squares fitting to the Michaelis−Menten equation (Figure 1). Because of the kinetically subsaturating concentration of the acceptor alcohol, the donor K_m^{app} and k_{cat}^{app} values provide an estimate of the kinetic parameters for this substrate. Nevertheless, the donor K_m^{app} is low relative to other glycosynthase enzymes[18] and well below the concentration required for preparative enzyme-mediated synthesis. The k_{cat}^{app} or apparent turnover number for this donor, although an underestimate of k_{cat}, is also low relative to other glycosynthases, leaving considerable scope for improving the catalytic efficiency of the enzyme.[18]

The kinetic parameters for 2-phenylethanol **4** donor were also investigated at a fixed saturating concentration of α-D-glucuronyl fluoride **2** donor (1 mM). Under these conditions the acceptor alcohol did not kinetically saturate the enzyme due to the limited aqueous solubility of the acceptor alcohol. An estimate of the kinetic parameters for 2-phenylethanol **4** was obtained by fitting the available data to the Michaelis−Menten equation. This indicated a K_m of 140 ± 10 mM, significantly higher than the highest acceptor substrate concentration tested (107 mM). An estimate of the specificity constant k_{cat}/K_m of 0.30 ± 0.02 M^{-1} s^{-1} was obtained from the slope of the plot of initial rate against acceptor concentration at low acceptor concentration (Figure 2, Table 1).

The significant difference in estimated Michaelis constants (K_m) for the donor and acceptor substrates is noteworthy. This may reflect the binding interactions for glucuronide substrates at the wild-type β-glucuronidase and the role of this enzyme in

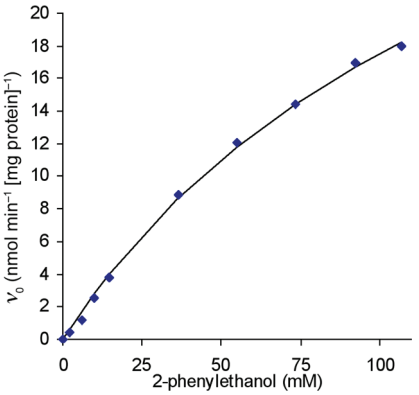
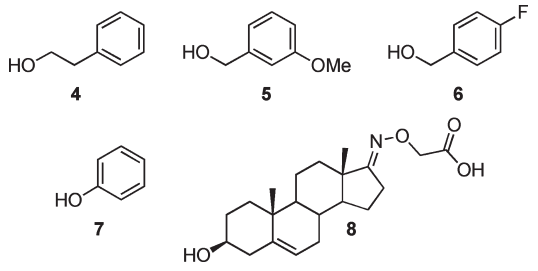


Figure 2. Plot of initial rate versus concentration for varying 2-phenylethanol **4** acceptor with fixed saturating (1 mM) α-D-glucuronyl fluoride **2** donor (21 °C, 100 mM phosphate buffer, pH 7.5). Line represents data fitted (least-squares) to the Michaelis−Menten equation.

Table 1. Estimated Kinetic Parameters for Alcohol Acceptors 4−8 with Constant Saturating (1 mM) α-D-Glucuronyl Fluoride 2 Donor[a]



acceptor	k_{cat}/K_m (M^{-1} s^{-1})	k_{cat}^{max} (s^{-1})	$concn^{max}$ (mM)
4	0.30 ± 0.02	0.020	107[b]
5	1.3 ± 0.1	0.023	55
6	0.62 ± 0.04	0.0081	37
7	0.22 ± 0.01	0.0029	30
8	4.3 ± 0.6	0.0014	0.55

[a] 100 mM phosphate buffer, pH 7.5, 21 °C. [b] The solubility limit of 2-phenylethanol **4** in aqueous buffer at 21 °C.

E. coli, the producing organism. *Escherichia coli* is an enteric organism that employs a glucuronide transporter/β-glucuronidase enzyme system to harvest the glucuronide residue as a carbon source from biliary metabolites excreted in the gut.[19] These glucuronide conjugates are formed from a wide variety of xenobiotic and some endogenous compounds so the primary recognition of the glucuronide conjugate by wild-type β-glucuronidase is expected for the target carbohydrate residue over the variable aglycone portion. For the glucuronylsynthase enzyme, the function of the wild-type enzyme is reflected in a low apparent K_m for the α-D-glucuronyl fluoride **2** donor (K_m^{app} 15.0 ± 1 μM) and a much higher estimated K_m for the 2-phenylethanol acceptor **4** (K_m 140 ± 10 mM).

Kinetic investigations on four additional acceptors **5**−**8** (Table 1) were conducted at a saturating concentration of α-D-glucuronyl fluoride **2** donor (1 mM). Each of these substrates showed a negative slope of initial rate against acceptor concentration

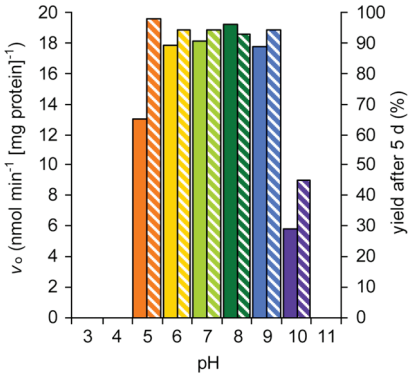


Figure 3. Dependence of initial rate (v_0, solid bars) and HPLC yield after 5 d (%, lined bars) on pH for reaction of 2-phenylethanol **4** acceptor (107 mM) and α-D-glucuronyl fluoride **2** donor (1 mM) (100 mM phosphate buffer, 21 °C).

at high acceptor concentrations, consistent with substrate inhibition. Because of substrate inhibition the data could not be fitted to the Michaelis−Menten model. Kinetic parameters for these reactions (Table 1) were estimated in two ways. An estimate of acceptor specificity k_{cat}/K_m was obtained from the slope of the plot of initial rate against acceptor concentration at low acceptor concentration. A second measure of enzyme activity, the maximum observed k_{cat} (k_{cat}^{max}), was calculated from the initial velocity at the concentration of the local maximum ($concn^{max}$).[12] It was not possible to reliably estimate K_m from this data.

The kinetic parameters for acceptors **4**−**7** give some insight into the yields observed in previous synthetic work (Scheme 4).[7] The observed yields parallel the maximum k_{cat} observed with the exception of 2-phenylethanol **4** (k_{cat}^{max} = 0.020 s^{-1}, 96%), which afforded a yield greater than that of 3-methoxybenzyl alcohol **5** (k_{cat}^{max} = 0.023 s^{-1}, 84%). Comparing estimated specificity constants (k_{cat}/K_m) for acceptor alcohols **4**−**7** gives 3-methoxybenzyl alcohol **5** (1.3 ± 0.1 M^{-1} s^{-1}) highest, followed by 4-fluorobenzyl alcohol **6** (0.62 ± 0.04 M^{-1} s^{-1}), 2-phenylethanol **4** (0.30 ± 0.02 M^{-1} s^{-1}), and then phenol 7 (0.22 ± 0.01 M^{-1} s^{-1}) as lowest. Interestingly 2-phenylethanol **4** substrate that provided the highest yield of product (96%) in previous work has among the lowest specificity constant (k_{cat}/K_m). The higher yield observed for 2-phenylethanol **4** can be attributed to the absence of substrate inhibition, which was relevant for all other acceptor alcohols under the high acceptor concentrations (~100 mM) used in preparative work. The kinetic observations also explain the reduction in yield based on acceptor alcohol that was observed on doubling the substrate concentration of acceptor 3-methoxybenzyl alcohol from 50 mM (84%) to 100 mM (64%). A higher yield is afforded by the reaction performed with substrate concentration close to the local maximum of enzyme activity ($concn^{max}$ = 55 mM). The lowest yielding acceptor phenol 7 (13%) gave the lowest values for k_{cat}^{max} and k_{cat}/K_m of the four simple alcohol acceptors investigated. This study highlights the potential for substrate inhibition under the high acceptor alcohol concentrations usually employed for preparative purposes. It indicates that operating at lower substrate concentrations or employing slow addition of substrate may be beneficial for some glucuronylsynthase reactions.

To further improve the glucuronylsynthase reaction, variables such as pH, temperature, and the effect of additives were also investigated. The pH optimum of the enzyme was determined at a fixed concentration of α-D-glucuronyl fluoride **2** donor (1 mM)

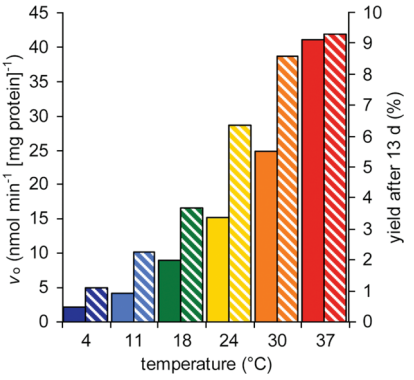


Figure 4. Dependence of initial rate (v_0, solid bars) and HPLC yield after 13 d (%, lined bars) on temperature for reaction of 3-methoxybenzyl alcohol **5** acceptor (89 mM) and α-D-glucuronyl fluoride **2** donor (89 mM) (100 mM phosphate buffer, pH 7.5).

and 2-phenylethanol **4** acceptor (107 mM). Two measurements of enzyme activity were taken at each pH value. First, the initial rate (v_0) of reaction was measured at low conversion. Second, the reactions were incubated for 5 d, and the yield of product formed at end point was determined by HPLC. High yields were obtained at pH values of 5−9 with highest initial rates across a broad plateau between pH 6 and 9 (Figure 3). The enzyme showed an attenuated initial rate at pH 5 but maintained activity at this pH to afford a high yield of product after 5 d. Studies on the hydrolytic activity of wild-type β-glucuronidase shows a similar broad pH optimum.[8,20]

Next, the enzyme activity was evaluated at several temperatures (4, 11, 18, 24, 30, 37 °C), at a fixed concentration of α-D-glucuronyl fluoride **2** donor (89 mM) and 3-methoxybenzyl alcohol **5** acceptor (89 mM). The use of 3-methoxybenzyl alcohol as acceptor alcohol and a reduced enzyme concentration (0.02 mg mL^{-1}) relative to typical synthesis experiments was also used to allow the observation of initial rates at higher temperatures and more keenly assess the influence of temperature on overall yield. Two measurements of enzyme activity were taken at each temperature value. First, the initial rate (v_0) of reaction was determined, and as expected there was an increase of initial rate with temperature (Figure 4). Second, the reactions were incubated for 13 d, and the yield of product formed was determined by HPLC. The reactions maintained at 30 and 37 °C gave similar yields after 13 d, suggesting that the higher initial reaction rate observed at the higher temperatures is attenuated over time by a higher rate of enzyme deactivation or α-D-glucuronly fluoride **2** hydrolysis.[21,22] The study indicated a temperature of 30−37 °C was preferred for the glucuronylsynthase reactions.

Next the effect of different cosolvent and detergent additives was investigated. One limitation of the glucuronylsynthase reaction is the low solubility of many of the hydrophobic acceptor alcohols relative to the glucuronide product. Additives such as detergents or cosolvents generally offer enhancement of substrate solubility but could also potentially have a deleterious effect on enzyme activity and stability.

The effect of different additives on the initial velocity of the enzyme was determined at a saturating concentration of α-D-glucuronyl fluoride **2** donor (1 mM) and a fixed concentration of 2-phenylethanol **4** acceptor (94 mM). All reactions with additives demonstrated a decrease in enzyme activity (Figure 5). Detergents maintained the highest activity with no velocities

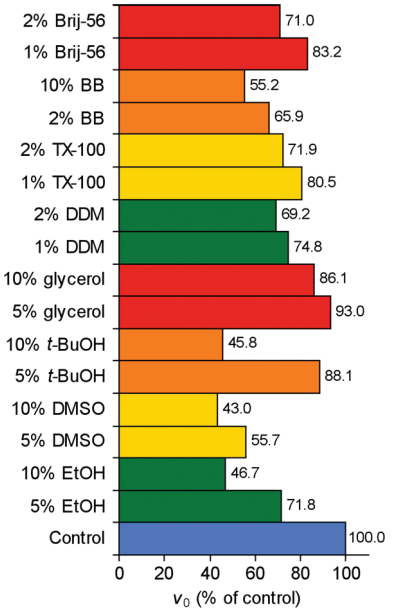
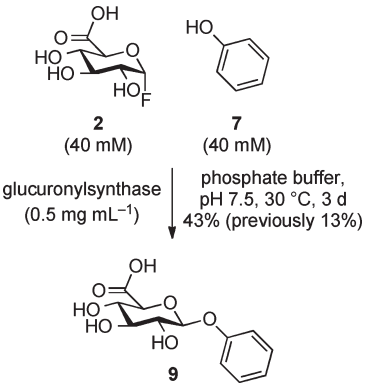


Figure 5. Effect of additives on the initial rate (v_0 as a percentage of the initial velocity for the control) for reaction of 2-phenylethanol **4** acceptor (94 mM) and α-D-glucuronyl fluoride **2** donor (1 mM) (100 mM buffer, pH 7.5, 21 °C). Brij-56, poly(ethylene glycol) hexadecyl ether; BB, commercial protein extraction reagent; TX-100, triton X-100; DDM, dodecyl β-D-maltoside.

Scheme 5. Synthesis of Phenyl β-D-Glucuronide 9

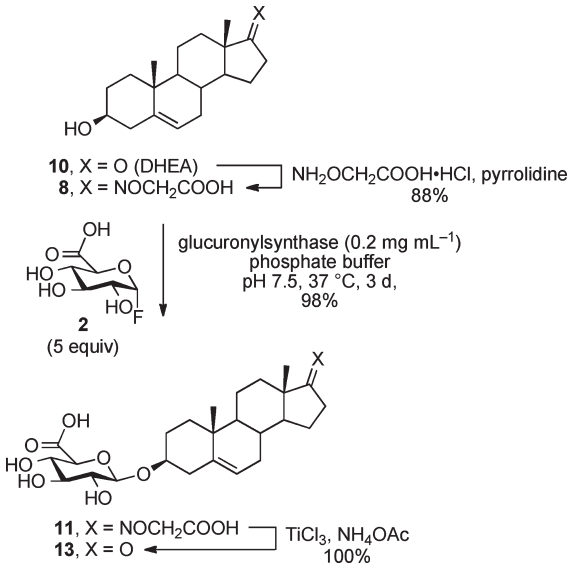


falling below 70% of the control (no additives). Organic cosolvents were more detrimental to the enzyme, albeit 5% *tert*-butanol and glycerol demonstrated enzymatic activities greater than 80% of the control.[23]

It should be noted that in the cases where the higher acceptor concentrations can be achieved with the aid of cosolvents, this may occur at the cost of lower enzyme activity. Furthermore, high substrate concentrations may not always be advantageous given the observation of substrate inhibition. In summary, the optimal conditions of enzyme mediated synthesis of glucuronides occur at pH 6−9 and temperatures of 30−37 °C. Because of the influence of substrate inhibition, the kinetic behavior of individual acceptors must be investigated in order to optimize additional variables such as acceptor concentration or cosolvent composition.

The reaction of phenol **7** as acceptor alcohol was investigated to gauge the combined effect of the optimized conditions on the

Scheme 6. Synthesis of DHEA 3-β-Glucuronide 13



reaction outcome. This substrate served as a useful benchmark, as it was not identified by the spectrophotometric screening protocol used to identify potential acceptors and the previous synthetic procedure had provided phenyl glucuronide in a low 13% yield. Repeating this reaction with 40 mM substrate concentration, just above the local maximum of enzyme activity, and at 30 °C in pH 7.5 buffer afforded a phenyl β-D-glucuronide **9** in an improved yield of 43% (Scheme 5).

To further test the scope of the glucuronylsynthase reaction, we turned our attention to the synthesis of steroid glucuronides. In previous work the glucuronylation of dehydroepiandrosterone (**10**, DHEA) was achieved in 5% in phosphate buffer, 17% with 25% v/v DMSO cosolvent and 26% with 2.5% w/v dodecyl maltoside (DDM) nonionic detergent additive.[7] However, in each case the reaction was performed as a suspension of DHEA **10** in the reaction mixture due to the low aqueous solubility of the hydrophobic steroid. The solubility of DHEA **10** has been estimated as 90 μM in aqueous buffer.[24]

To address the challenge of working with hydrophobic acceptor alcohols, the temporary introduction of polar substituents was investigated as a strategy to boost solubility into a suitable range without the need for organic cosolvents or detergent additives. A three-step process was envisaged involving (1) introduction of a polar substituent, (2) enzyme catalyzed glucuronylation, and (3) cleavage of the polar substituent to give the steroid glucuronide. A range of derivatives were explored with the *O*-(carboxymethyl)oxime derivative appearing most suitable for this purpose.[25] The oxime unit is readily generated by condensation of commercially available *O*-(carboxymethyl) hydroxylamine reagent with a carbonyl compound promoted by pyrrolidine (Scheme 6). The charged derivative CMO−DHEA **8** thus formed in 88% had significantly greater solubility than the parent steroid and could be fully dissolved in aqueous buffer up to 2 mM at 21 °C.

The higher aqueous solubility allowed for completion of an enzyme kinetic study with a varying concentration of CMO−DHEA **8** and a saturating concentration of α-D-glucuronyl fluoride **2** donor (1 mM) (Table 1). In common with a number

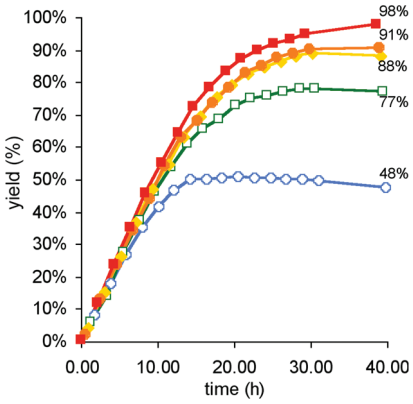


Figure 6. Reaction yield against time for reaction of CMO−DHEA **8** acceptor (1.4 mM) and varying equivalents of α-D-glucuronyl fluoride **2** donor (100 mM phosphate buffer, pH 7.5, 37 °C). (blue circles, 1 equiv; green squares, 2 equiv; yellow diamonds, 3 equiv; orange circles, 4 equiv; red squares, 5 equiv).

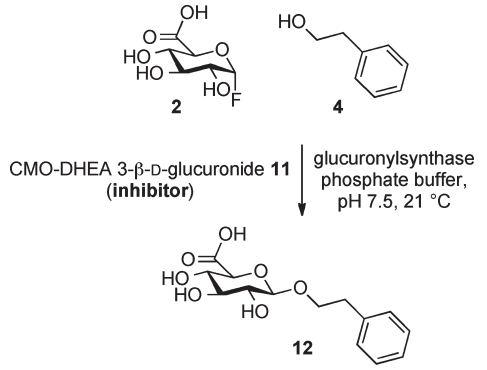
of the simple acceptor alcohols, a plot of initial rate against acceptor concentration showed a negative slope at high acceptor concentrations, consistent with substrate inhibition.[12] Relative to the simple alcohols this substrate gave the highest estimated k_{cat}/K_m (4.3 ± 0.6 M^{-1} s^{-1}) and smallest k_{cat}^{max} (0.0014 s^{-1}) at a concentration of 0.55 mM, with this local maximum occurring below the solubility limit (2 mM).

The CMO−DHEA **8** glucuronylation reaction was followed by HPLC. Using 1 equiv of α-D-glucuronyl fluoride **2** donor gave a reaction profile that reached a 48% HPLC yield (Figure 6). Increasing equivalents of α-D-glucuronyl fluoride **2** afforded higher yields of product with 5 equiv giving 98% HPLC yield. These observations suggested the operation of product inhibition at higher conversion, a phenomenon that could be overcome with increasing concentrations of α-D-glucuronyl fluoride **2** donor.[26]

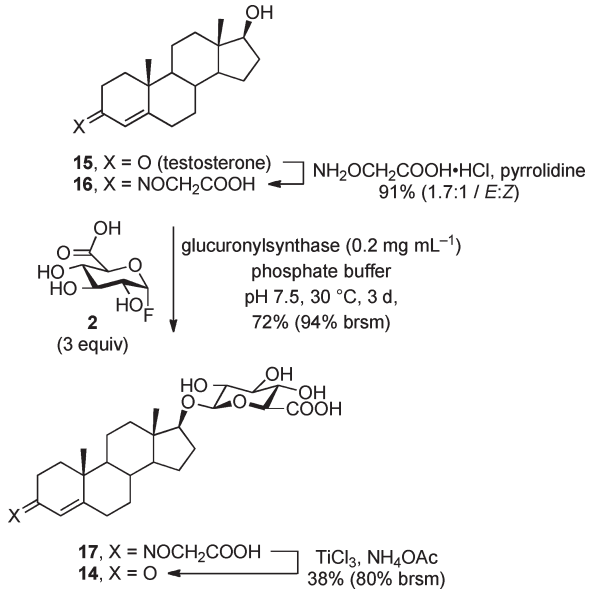
To assess this product inhibition, the influence of increasing concentrations of CMO−DHEA 3-β-D-glucuronide **11** as inhibitor on the glucuronylsynthase reaction between 2-phenylethanol **4** (88 mM) and α-D-glucuronyl fluoride **2** at several concentrations was investigated (Scheme 7). The analysis of reciprocal plots indicated mixed, predominantly competitive inhibition (K_{ic} = 71 μM, K_{iu} = 180 μM) for CMO−DHEA 3-β-D-glucuronide **11**.[27,28] The observed competitive inhibition constant is higher than the apparent Michaelis constant (K_m^{app} = 15 ± 1 μM) for α-D-glucuronyl fluoride **2**. The implication of this finding is that higher concentrations of α-D-glucuronyl fluoride **2** should successfully compete for occupancy of the enzyme active site and maintain enzyme activity as product concentration increases. This analysis is borne out in the results presented in Figure 6 with 5 equiv (7 mM) of α-D-glucuronyl fluoride giving a 98% HPLC yield of CMO−DHEA 3-β-D-glucuronide **11**.

To further assess the potential for product inhibition in the glucuronylsynthase reaction, the reverse experiment was conducted. The inhibition derived from increasing concentrations of 2-phenylethyl β-D-glucuronide **12** on the glucuronylsynthase reaction involving CMO−DHEA **8** (0.6 mM) and α-D-glucuronyl fluoride **2** at several concentrations was investigated. In this instance no inhibition was observed for 2-phenylethyl β-D-glucuronide **12** between 0 and 75 mM.[29] Interestingly for the reaction of 2-phenylethanol **4** the absence of observable substrate and product inhibition correlates to a high yield of the 2-phenylethyl

Scheme 7. Study of Glucuronylsynthase Reaction Inhibition by CMO−DHEA 3-β-D-Glucuronide 11



Scheme 8. Synthesis of Testosterone 17-β-D-Glucuronide 14



β-D-glucuronide **12** product (96%) using 1.2 equiv of α-D-glucuronyl fluoride donor. For the reaction of CMO−DHEA **8** both substrate and product inhibition are observed in this study. In the former case this can be overcome by selection of a suitable substrate concentration and in the latter case by maintaining a high concentration of the α-D-glucuronyl fluoride donor **2**.

Performing the glucuronylation of CMO−DHEA **8** on a preparative scale under optimized conditions and using 5 equiv of α-D-glucuronyl fluoride donor and 1.9 mM acceptor alcohol gave the glucuronide product **11** in 98% yield (Scheme 6). A single crystal X-ray structure of the disodium salt of the glucuronide **11**, grown from ethyl acetate/methanol/water solution confirmed the expected structure.[30] The oxime exists solely as the *E*-isomer and the glycosidic linkage presents as the β anomer. Cleavage of the oxime unit was readily achieved using titanium(III) chloride in aqueous ammonium acetate buffer to regenerate the ketone and afford DHEA 3-β-D-glucuronide **13** in quantitative yield. This three-step sequence afforded the target glucuronide (45 mg) in 86% yield, which compares favorably with the previously reported single-step glucuronylsynthase mediated synthesis (26%).[7] The reaction also compares favorably with the two-step Koenigs−Knorr glycosylation followed by base-promoted deprotection that is reported to afford DHEA 3-β-D-glucuronide **13** in 20% yield.[31] The UGT catalyzed synthesis of DHEA 3-β-D-glucuronide **13** has not been reported.

The synthesis of testosterone 17-β-D-glucuronide **14** proceeded in a similar manner (Scheme 8). Testosterone **15** was converted to the oxime derivative **16** in 91% yield as a 1:1.7 mixture of *Z* and *E* (major) isomers. This charged derivative **16** had a solubility significantly greater than that of the parent steroid **15** and could be fully dissolved in aqueous buffer up to 10 mM at 21 °C. This smoothly underwent glucuronylation under optimized conditions and using 3 equiv of α-D-glucuronyl fluoride **2** to give the glucuronide product **17** in 72% yield. Partial cleavage of the oxime was achieved using titanium(III) chloride in aqueous ammonium acetate buffer to regenerate the ketone and afford testosterone 17-β-D-glucuronide **14** in 38% yield. The mild reaction conditions allowed recovery of unreacted oxime **17** (53%). No cleavage of the glycosidic bond was observed under the mild reaction conditions and no byproduct was observed from over-reduction of the unsaturated ketone or imine functionality.[32] This three-step sequence afforded the target glucuronide in 25% yield. This contrasts with the two-step Koenigs−Knorr glycosylation followed by base-promoted deprotection, which is reported to afford testosterone 17-β-D-glucuronide **14** in 17% yield.[31] The method can also be compared to a recent report that described the UGT-mediated synthesis of 11 steroid glucuronides.[6a] The steroid glucuronides were afforded in 13−77% yield to provide 1.1−6.5 mg of product. Pertinent to this study was the synthesis of testosterone 17-β-D-glucuronide **14** in a yield of 77% (6.5 mg). However, drawbacks of this method include the high levels (1 mg mL^{-1}) of rat liver microsomal enzyme that is obtained through animal sacrifice. By contrast the glucuronylsynthase affords testosterone 17-β-D-glucuronide in a yield of 24% (16 mg) over three steps. Furthermore the method uses soluble enzyme (0.2 mg mL^{-1}) that is readily obtained on a large scale by standard methods of protein expression and nickel affinity purification.

CONCLUSION

Improved procedures for the glucuronylsynthase catalyzed synthesis of glucuronide conjugates have been developed. The influence of acceptor substrate, pH, temperature, cosolvents, and detergents on the enzyme activity has been investigated by HPLC/UV. Substrate inhibition was observed for the majority of acceptor alcohols investigated, indicating that the substrate concentration is an important consideration for optimizing enzyme activity. The operation of mixed, predominantly competitive inhibition was observed for the product CMO−DHEA 3-β-D-glucuronide **11** in the glucuronylsynthase reaction of 2-phenylethanol **4** as acceptor. This inhibition could be overcome through the use of higher concentrations of α-D-glucuronyl fluoride **2** donor. The temporary introduction of polar substituents was investigated as a way to increase the solubility of hydrophobic steroidal substrates. In this way the synthesis of DHEA 3-β-D-glucuronide **13** was achieved in three steps and in 86% overall yield from DHEA **10**. The glucuronylsynthase reaction provides a practical alternative to existing methods for the synthesis of glucuronide metabolites. Further engineering

will be directed to increasing the substrate scope and improving the catalytic efficiency of the glucuronylsynthase enzyme.

EXPERIMENTAL SECTION

17-Carboxymethoximino-dehydroepiandrosterone (CMO−DHEA) 8.[33] Pyrrolidine (600 μL, 7.19 mmol) was added to DHEA **10** (1.00 g, 3.47 mmol) dissolved in dry methanol (40 mL) at 4 °C. After 1 h the solution had turned yellow. Carboxymethoxylamine hemihydrochloride (800 mg, 7.32 mmol) was dissolved in a dry solution of methanol (10 mL) and pyrrolidine (600 μL, 7.19 mmol) and transferred to the DHEA mixture via cannula with methanol washing (5 mL). The solution immediately cleared and was heated to reflux. The reaction was complete after 6 h. The solvent was removed under reduced pressure, and water (100 mL) was added to the residue. The pH was adjusted to 2 with aqueous hydrochloric acid (2 M), and the white precipitate was extracted with ethyl acetate until all precipitate had dissolved (5 × 150 mL with sonication required). The organic extracts were combined, washed with water (200 mL), dried over magnesium sulfate, and then evaporated to dryness. Cold chloroform (5 mL) was added to the resulting yellow-white residue (1.15 g). The white precipitate was filtered and washed with cold chloroform (2 × 3 mL) to afford 17-carboxymethoximino-dehydroepiandrosterone **8** (1.10 g, 88%); mp 215−217 °C (decomp); $[\alpha]^{20}_{D}$ −36 (*c* 1.0, DMSO) {lit.[33] $[\alpha]^{24}_{D}$ −37.9 (*c* 1, EtOH)}; R_f 0.38 (7:2:1 ethyl acetate/methanol/water). IR (KBr): 3351 (broad, O−H), 2946, 2910, 2865, 2505 (C−H), 1680 (C═O). ^{1}H NMR (800 MHz, d_6-DMSO): δ 5.28 (1H, s), 4.61 (1H, s, broad), 4.42 (2H, s), 3.26 (1H, obscured, m), 2.46 (1H, dd, *J* = 18.9, 9.0 Hz), 2.38 (1H, m), 2.16 (1H, dd, *J* = 12.9, 2.4 Hz), 2.09 (1H, t, *J* = 12.1 Hz), 1.98 (1H, m), 1.82−1.70 (3H, m), 1.67 (1H, d, *J* = 12.2 Hz), 1.58 (2H, m), 1.51 (1H, m), 1.42 (1H, m), 1.38−1.28 (3H, m), 1.12 (1H, m), 1.02−0.91 (2H, m), 0.96 (3H, s), 0.85 (3H, s), COOH not observed. ^{13}C NMR (200 MHz, d_6-DMSO): δ 171.3, 170.6, 141.4, 120.1, 70.0, 69.8, 53.5, 49.8, 43.5, 42.2, 36.9, 36.2, 33.8, 31.4, 30.82, 30.78, 25.7, 22.8, 20.2, 19.2, 16.8. LRMS (+ESI) *m*/*z*: 384 ($[M + Na]^+$, 100%). LRMS (−ESI) *m*/*z*: 360 ($[M - H]^-$, 100%).; HRMS (+ESI) calcd for $C_{21}H_{31}NO_4Na^+$ ($[M + Na]^+$) 384.2151, found 384.2151.

CMO−DHEA 3-β-D-Glucuronide 11. Glucuronylsynthase (4.14 mL, 1.45 mg/mL) was added to a solution containing CMO−DHEA **8** (20 mg, 0.055 mmol, final concentration 1.9 mM) and α-D-glucuronyl fluoride **2** (57.5 mg, 0.270 mmol) in 100 mM sodium phosphate buffer pH 7.5 (25 mL). The reaction was incubated at 37 °C without agitation for 3 days and then dried onto reverse-phase silica. The dried residue was subjected to reverse-phase flash chromatography (25% aqueous acetonitrile + 0.1% formic acid) to isolate CMO−DHEA 3-β-D-glucuronide **11** (29 mg, 98%) as a colorless solid; $[\alpha]^{20}_{D}$ −62 (*c* 1.0, DMSO); R_f 0.02 (7:2:1 ethyl acetate/methanol/water). IR (KBr): 3423 (O−H), 2942 (C−H), 1745 (C═O). ^{1}H NMR (800 MHz, D_2O): δ 5.51 (1H, s), 4.59 (1H, d, *J* = 7.3 Hz), 4.38 (2H, s), 3.72−3.69 (2H, m), 3.52−3.50 (2H, m), 3.27 (1H, t, *J* = 7.1 Hz), 2.63 (1H, dd, *J* = 19.2, 8.3 Hz), 2.52 (1H, m), 2.47 (1H, d, *J* = 12.6 Hz), 2.29 (1H, t, *J* = 12.0 Hz), 2.10 (1H, d, *J* = 11.3 Hz), 2.00−1.92 (2H, m), 1.90−1.84 (2H, m), 1.71−1.60 (4H, m), 1.55 (1H, m), 1.46−1.41 (2H, m), 1.27 (1H, m), 1.10 (1H, t, *J* = 13.0 Hz), 1.06 (3H, s), 1.06−1.02 (1H, m), 0.95 (3H, s). ^{13}C NMR (200 MHz, D_2O): δ 178.9, 177.3, 177.2, 143.3, 123.4, 101.7, 81.0, 77.7, 77.7, 74.4, 73.3, 72.9, 55.0, 51.2, 45.6, 39.5, 38.1, 38.0, 34.9, 32.29, 32.27, 30.3, 27.8, 24.2, 21.6, 20.3, 17.7. LRMS (−ESI) *m*/*z*: 536 ($[M - H]^-$, 100%), 558 ($[M - 2H + Na]^-$, 30). HRMS (−ESI) calcd for $C_{27}H_{38}NO_{10}$ ($[M - H]^-$) 536.2496, found 536.2495; calcd for $C_{27}H_{37}NO_{10}Na$ ($[M - 2H + Na]^-$) 558.2315, found 558.2302.

DHEA 3-β-D-Glucuronide 13.[31] CMO−DHEA 3-β-D-glucuronide **11** (50 mg, 0.093 mmol) and ammonium acetate (96 mg, 1.2 mmol) were purged with nitrogen, and then dioxane (0.5 mL) and aqueous acetic acid (50%, 38 μL) were added. In a second flask, titanium trichloride (36 mg, 0.23 mmol) was purged with nitrogen before water (6 mL) was added. The aqueous titanium trichloride was added via cannula to the stirred CMO−DHEA 3-β-D-glucuronide solution at room temperature. The reaction instantly turned black-violet upon addition and gradually changed to a white-gray as the reaction proceeded. After 2 h, the reaction was deemed complete so the solution was acidified to pH 2 and dried onto silica. The dry residue was subjected to flash chromatography (7:2:1 ethyl acetate/methanol/water +0.1% formic acid) to isolate DHEA 3-β-D-glucuronide **13** (44.8 mg, 100%) as a colorless solid; $[\alpha]^{20}_{D}$ −29 (*c* 0.70, MeOH), (lit.[31] $[\alpha]^{25}_{D}$ −35.5 (*c* 1, EtOH)); R_f 0.25 (7:2:1 ethyl acetate/methanol/water). IR (NaCl): 3369 (O−H), 2938, 2903 (C−H), 1733 (C═O), 1636. ^{1}H NMR (800 MHz, MeOD): δ 5.42 (1H, d, *J* = 4.9 Hz), 4.45 (1H, d, *J* = 7.8 Hz), 3.80 (1H, d, *J* = 9.7 Hz) 3.58−3.47 (2H, m), 3.38 (1H, t, *J* = 9.1 Hz), 3.20 (1H, t, *J* = 8.4 Hz), 2.48−1.43 (2H, m), 2.29 (1H, m), 2.16−2.07 (3H, m), 2.00−1.87 (3H, m), 1.78 (1H, m), 1.74−1.52 (5H, m), 1.40−1.23 (2H, m), 1.11−1.04 (2H, m), 1.07 (3H, s), 0.90 (3H, s). ^{13}C NMR (75 MHz, MeOD/D_2O): δ 225.5, 169.8, 142.1, 122.2, 102.1, 79.7, 77.6, 74.7, 73.5, 72.5, 52.9, 51.6, 39.5, 38.3, 37.9, 36.8, 32.7, 32.5, 31.8, 30.4, 22.7, 21.4, 19.8, 13.9, one carbon overlapping or obscured. LRMS (−ESI) *m*/*z*: 927 ($[2M - H]^-$, 43%), 463 ($[M - H]^-$, 100). HRMS (−ESI) calcd for $C_{25}H_{35}O_8$ ($[M - H]^-$) 463.2338, found 463.2342.

ASSOCIATED CONTENT

Supporting Information. CIF files for the X-ray crystal structures of α-D-glucuronyl fluoride **2** and CMO−DHEA 3-β-D-glucuronide **11**, protein expression procedures, experimental procedures, spectroscopic data and ^{1}H and ^{13}C NMR spectra for compounds **2**, **8**, **9**, **11**, **13**, **14**, **16** and **17**. This material is available free of charge via the Internet at http://pubs.acs.org.

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(21) The first order rate constant for hydrolysis (k_H) of 1.8×10^{-6} s^{-1} was determined for α-D-glucuronyl fluoride **2** by ^1H NMR analysis (unbuffered D_2O, 37 °C). Details are reported in Supporting Information. This hydrolysis rate constant (k_H) is of equivalent magnitude to other reported nonenzymatic glycosyl fluoride hydrolysis: (a) 3-*O*-cellobiosyl-α-D-glucosyl fluoride $k_H = 2.57 \times 10^{-6}$ s^{-1} (50 mM maleate buffer, pH 7.0, 0.1 mM $CaCl_2$ 35 °C) Faijes, M.; Pérez, X.; Pérez, O.; Planas, A. *Biochemistry* **2003**, *42*, 13304–13318. (b) α-D-glucopyranosyl fluoride $k_H = 8.42 \times 10^{-6}$ s^{-1} (50 mM sodium acetate buffer, pH 6.0, 40 °C) Zhang, Y.; Bommuswamy, J.; Sinnott, M. L. *J. Am. Chem. Soc.* **1994**, *116*, 7557–7563. (c) α-D-glucopyranosyl fluoride $k_H = 1.5 \times 10^{-6}$ s^{-1} (5 mM potassium phosphate buffer, 30 °C) Banait, N. S.; Jencks, W. P. *J. Am. Chem. Soc.* **1991**, *113*, 7951–7958. The hydrolysis of α-D-glucopyranosyl fluoride is catalyzed by phosphate buffer and is subject to isotope effects. (d) Banait, N. S.; Jencks, W. P. *J. Am. Chem. Soc.* **1991**, *113*, 7958–7963. We are currently investigating the influence of buffer composition on the hydrolysis of α-D-glucuronyl fluoride **2**.

(22) The HPLC reaction yields increased on average 3.1 times from day 4 to day 13 for each temperature regime. The HPLC yield of the reaction held at 37 °C for 13 d was 2.9 times greater than that determined at 4 d. Details are reported in Supporting Information.

(23) A more comprehensive study of wild-type *E. coli* β-glucuronidase activity and stability in a wide range of cosolvents and detergents is reported in Supporting Information. Similar results are observed for the influence of cosolvent and detergent activity on both the wild-type glucuronidase and the glucuronylsynthase enzymes. For the effects of cosolvents on immobilized *E. coli* β-glucuronidase activity see ref 20b.

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(25) Derivatives investigated included: hydrazone, 2-(2-pyridyl)hydrazone, oxime, *O*-(carboxymethyl)oxime.

(26) Nonenzymatic hydrolysis of α-D-glucuronyl fluoride was excluded as a significant factor in the incomplete reactions on kinetic grounds. See ref 21.

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(28) Plots of $1/v_0$ against [CMO-DHEA 3-β-D-glucuronide] (Dixon plot), [α-D-glucuronyl fluoride]/v_0 against [CMO-DHEA 3-β-D-glucuronide], and $1/i_{0.5}$ against v_0/V_{max} are reported in Supporting Information.

(29) A plot of initial rate against concentration of 2-phenylethyl β-D-glucuronide **12** (0−75 mM) for the glucuronylsynthase reaction of CMO-DHEA **8** and α-D-glucuronyl fluoride **2** is reported in Supporting Information.

(30) The ORTEP diagram and CIF file of the CMO-DHEA 3-β-D-glucuronide **11** crystal structure (CCDC 793480) is provided in Supporting Information.

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