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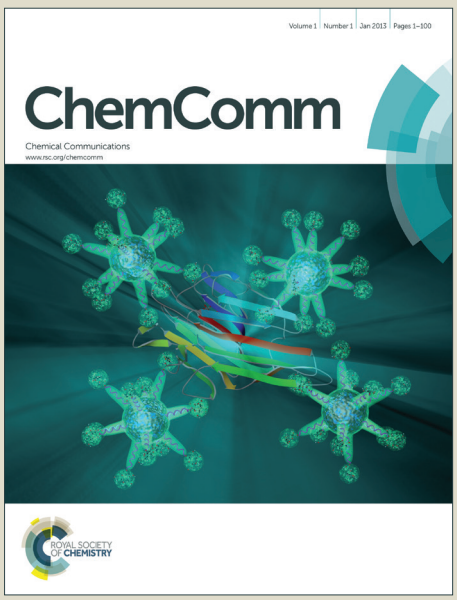

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COMMUNICATION

Improved one-pot multienzyme (OPME) systems for synthesizing UDP-uronic acids and glucuronides†

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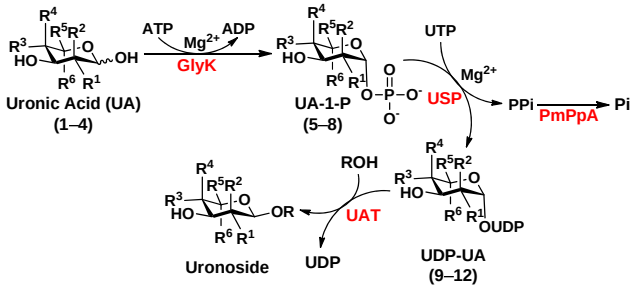
***Arabidopsis thaliana* glucuronokinase (AtGlcAK) was cloned and shown to be able to use various uronic acids as substrates to produce the corresponding uronic acid-1-phosphates. AtGlcAK or *Bifidobacterium infantis* galactokinase (BiGalK) was used with a UDP-sugar pyrophosphorylase, an inorganic pyrophosphatase, with or without a glycosyltransferase for highly efficient synthesis of UDP-uronic acids and glucuronides. These improved cost-effective one-pot multienzyme (OPME) systems avoid the use of nicotinamide adenine dinucleotide (NAD^+)-cofactor in dehydrogenase-dependent UDP-glucuronic acid production processes and can be broadly applied for synthesizing various glucuronic acid-containing molecules.**

Uronic acids such as D-glucuronic acid (D-GlcA), D-galacturonic acid (D-GalA), D-mannuronic acid (D-ManA), L-iduronic acid (L-IdoA), L-guluronic acid (L-GulA), etc. are common residues in important glycoconjugates and polysaccharides. For example, D-GlcA is an essential component in glycosaminoglycans (GAGS) such as hyaluronan, chondroitin sulfate, dermatan sulfate, heparan sulfate, and heparin. Epimerization of D-GlcA to L-IdoA at the polysaccharide level is an important post-glycosylational modification in heparan/heparin sulfate and dermatan sulfate.[1] Human UDP-glucuronosyltransferases catalyze the addition of D-GlcA to non-polar toxic compounds (so called glucuronidation) as one of detoxification mechanisms.[2] D-GlcA or modified D-GlcA (e.g. 4-*O*-methylated D-GlcA) is a component of plant-produced hemicelluloses such as glucuronoxylan[3-8] as well as xanthan and gellan produced by Gram-negative bacteria.[9, 10] D-GalA or modifed D-GalA (e.g. D-GalA methyl ester) is a component in plant cell wall pectins such as homogalacturonans and rhamnogalacturonans[11-13] as well as in the capsular polysaccharide of cyanobacteria and bacteria and lipopolysaccharide (LPS) of Gram-negative bacteria.[12, 14] D-ManA and L-GulA are the building blocks of alginates produced by brown algae[15] and bacteria.[16] Similar to the formation of L-IdoA from D-GlcA, L-GulA is produced from D-ManA at the alginate polymer level catalyzed by a C5-epimerase.

In nature, the key enzymes that catalyze the synthesis of uronic acid-containing structures are uronosyltransferases (UATs). They use uridine 5'-phosphate (UDP)- or guanosine 5'-diphosphate (GDP)-activated uronic acids, such as UDP-GlcA, UDP-GalA, or GDP-ManA, as donor substrates. UDP-GlcA is commonly synthesized from UDP-glucose (UDP-Glc) by an NAD^+-dependent oxidation process catalyzed by a UDP-glucose dehydrogenase (Ugd).[17, 18] Alternatively, *Arabidopsis thaliana* UDP-sugar pyrophosphorylase (AtUSP) has been shown to catalyze the synthesis of UDP-GlcA, UDP-Glc, UDP-galactose, UDP-xylose, and UDP-L-arabinose directly from the respective sugar-1-phosphates.[19, 20] Synthesizing UDP-GlcA directly from GlcA-1-P by AtUSP avoids the use of expensive cofactor NAD^+ required by Ugd and is a more effective approach. AtUSP has also been used in a OPME system for small-scale synthesis of UDP activated Gal, Glc, 6-deoxy-Gal, and 4-N_3-Gal and for preparative-scale synthesis of dUDP-Glc and dTDP-Glc.[21] Nevertheless, AtUSP has not been used in preparative-scale synthesis of UDP-uronic acids. On the other hand, tritium labelled UDP-GalA has been synthesized from UDP-GlcA using a UDP-GlcA 4-epimerse-catalyzed reaction.[22] A more direct approach for synthesizing UDP-GalA from GalA-1-phosphate has been achieved in a preparative scale using *Pisum sativum* sprout sugar-pyrophosphorylase.[12] GDP-ManA has been synthesized from GDP-mannose using a GDP-mannose dehydrogenase from algae *Ectocarpus siliculosus*.[23] UDP-IdoA is not naturally produced, but is a potential substrate for glycosyltransferase-catalyzed synthesis of heparan sulfate (HS) or heparin and has been chemically synthesized.[24]



Scheme 1. A one-pot multienzyme (OPME) strategy for chemoenzymatic synthesis of uronosides. GlyK, glycokinase; USP, UDP-sugar pyrophosphorylase; PmPpA, *Pasteurella multocida* inorganic pyrophosphatase;[25] UAT, uronosyltransferase. D-GlcA (**1**), GlcA-1-P (**5**), and UDP-GlcA (**9**): $R^1 = R^3 = OH$, $R^2 = R^4 = R^6 = H$, $R^5 = CO_2H$; D-GalA (**2**), GalA-1-P (**6**), and UDP-GalA (**10**): $R^1 = R^4 = OH$, $R^2 = R^3 = R^6 = H$, $R^5 = CO_2H$; D-ManA (**3**), ManA-1-P (**7**), and UDP-ManA (**11**): $R^2 = R^3 = OH$, $R^1 = R^4 = R^6 = H$, $R^5 = CO_2H$; L-IdoA (**4**), IdoA-1-P (**8**), and UDP-IdoA (**12**): $R^1 = R^3 = OH$, $R^2 = R^4 = R^5 = H$, $R^6 = CO_2H$.

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Motivated by synthetic challenges of uronic acid-containing structures, we sought to develop an efficient one-pot multienzyme (OPME) chemoenzymatic approach for synthesizing uronosides (e.g. heparosan oligosaccharides and derivatives). As shown in Scheme 1, the idea is to chemically synthesize diverse uronic acids (UA) which can be activated by a suitable monosaccharide-1-phosphate kinase (glycokinase or GlyK) to produce uronic acid-1-phophates (UA-1-P). With a suitable UDP-sugar pyrophosphorylase (USP), various UDP-uronic acids (UDP-UA) can be obtained and used as donor substrates for uronosyltransferases (UATs) for the synthesis of uronosides. An inorganic pyrophosphatase from *Pasteurella multocida* (PmPpA)[25] can be included to break down the pyrophosphate formed in the USP-catalyzed reaction to drive the reaction towards the formation of UDP-UA. Ideally, the multiple enzymes involved can be mixed together in one-pot with all substrates needed for the production of desired uronosides without isolating intermediates.

To test the idea, D-ManA (**3**) and L-IdoA (**4**) were chemically synthesized (see Supporting Information) and used together with commercially available D-GlcA (**1**) and D-GalA (**2**) as potential substrates for glycokinases. *Bifidobacterium infantis* galactokinase (BiGalK) was shown previously to be able to catalyze the conversion of D-GalA (**2**) to GalA-1-phosphate (**6**).[26] However, its activity towards the formation of other uronic acid-1-phosphates was not determined. On the other hand, *Arabidopsis thaliana* glucuronokinase (AtGlcAK) was shown to catalyze the formation of GlcA-1-phosphate (**5**) efficiently but D-GalA (**2**) was reported not a suitable substrate.[27] We cloned AtGlcAK (see Supporting Information) and it was tested with BiGalK for their activities in using D-GlcA (**1**), D-GalA (**2**), D-ManA (**3**), and L-IdoA (**4**) as substrates for preparative-scale synthesis of the corresponding sugar-1-phosphates (**5**–**7**) (Table 1) and L-IdoA-1-phosphate (**8**).

To our delight, AtGlcAK not only showed high efficiency in catalyzing the synthesis of GlcA-1-P (**5**, quantitative yield), but also was highly active in producing ManA-1-P (**7**, 95%) and was able to catalyze the synthesis of GalA-1-P (**6**) although with a lower yield (31%). In comparison, BiGalK was able to catalyze the synthesis of GalA-1-P (**6**) in a high yield (92%) but did not tolerate D-GlcA (**1**) or D-ManA (**3**) as substrates. L-IdoA (**4**) was not a substrate for either AtGlcAK or BiGalK. Therefore, L-IdoA-1-phosphate (**8**) was not obtained.

The obtained GlcA-1-P (**5**), GalA-1-P (**6**), and ManA-1-P (**7**) were used as potential substrates for two UDP-sugar pyrophosphorylases (USPs), including *Bifidobacterium longum* USP (BLUSP) which showed activity in using glucose-1-P, galactose-1-P, mannose-1-P, and their derivatives in synthesizing the corresponding UDP-sugars,[28] and a USP cloned from *Arabidopsis thaliana* (AtUSP)[19] (see Supporting Information). By mass spectrometry analysis, GlcA-1-P (**5**) was confirmed to be a substrate for BLUSP, but GalA-1-P (**6**) and ManA-1-P (**7**) were not. As shown in Table 2, UDP-GlcA (**9**) was successfully synthesized in 80% yield using a one-pot three-enzyme (OP3E) system containing AtGlcAK, BLUSP, and PmPpA. When BLUSP was replaced by AtUSP in the OP3E system, UDP-GlcA was produced in 57% yield. AtUSP was also shown to be active towards both GalA-1-P (**6**) and ManA-1-P (**7**) to produce their corresponding UDP-uronic acids as determined by mass spectrometry. Nevertheless, its use with AtGlcAK and PmPpA in one pot for the synthesis of UDP-ManA (**11**) was unsuccessful, which may be caused by the low activity of AtUSP towards ManA-1-P. The AtUSP was successfully used together with BiGalK and PmPpA in one pot for the synthesis of UDP-GalA (**10**) in 39% yield.

Table 1. Preparative-scale (20–80 mg) syntheses of uronic acid-1-phosphates catalyzed by AtGlcAK or BiGalK. ND, not detected. Isolated yields are shown.

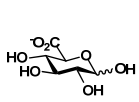
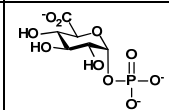
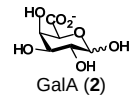
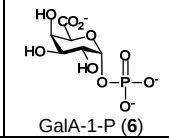
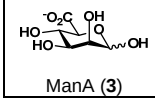
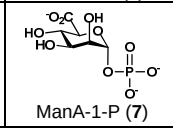
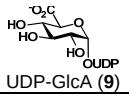
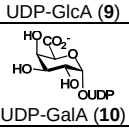
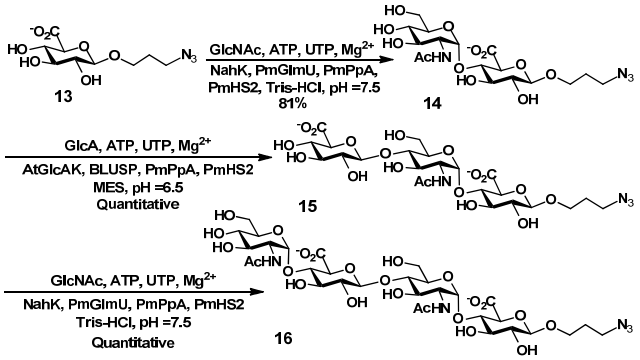
Substrate	Product	Yield (%)	
		AtGlcAK	BiGalK
 GlcA (**1**)	 GlcA-1-P (**5**)	Quantitative	ND
 GalA (**2**)	 GalA-1-P (**6**)	31	92
 ManA (**3**)	 ManA-1-P (**7**)	95	ND

Table 2. Preparative-scale (34–151 mg) one-pot three-enzyme (OP3E) synthesis of UDP-uronic acids. –, not tested. ND, not detected. Isolated yields are shown.

Substrate	Product	OP3E Yield (%)			
		AtGlcAK		BiGalK	
		BLUSP	AtUSP	BLUSP	AtUSP
GlcA (**1**)	 UDP-GlcA (**9**)	80	57	–	–
GalA (**2**)	 UDP-GalA (**10**)	–	–	ND	39



Scheme 2. Sequential OPME synthesis of heparosan disaccharide (**18**), trisaccharide (**19**), and tetrasaccharide (**20**). Enzymes used: NahK, *N*-acetylhexosamine-1-phosphate kinase; PmGlmU, *Pasteurella multocida* *N*-acetylglucosamine-1-phosphate uridylyltransferase; PmPpA, *Pasteurella multocida* inorganic pyrophosphatase; PmHS2, *Pasteurella multocida* heparosan synthase 2; AtGlcAK, *Arabidopsis thaliana* glucuronokinase; BLUSP, *Bifidobacterium longum* UDP-sugar pyrophosphorylase.

Compared to the OPME approach reported previously for synthesizing UDP-GlcA from glucose-1-P using *Escherichia coli*

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glucose-1-phosphate uridylyltransferase (EcGalU) and *Pasteurella multocida* UDP-glucose dehydrogenase (PmUgd),[18] the newly established OPME system containing AtGlcAK, BLUSP or AtUSP, and PmPpA avoids the use of expensive NAD^+ cofactor and represents a direct and improved system.

The OPME UDP-GlcA generation system shown here can be readily used with a glucuronyltransferase for highly efficient synthesis of glucuronides. An example was shown for *Pasteurella multocida* heparosan synthase 2 (PmHS2)[29-31]-catalyzed synthesis of heparosan trisaccharide (**15**) from disaccharide (**14**) where D-GlcA activation and transfer were achieved in a quantitative yield by a one-pot four-enzyme (OP4E) system containing AtGlcAK, BLUSP, PmPpA, and PmHS2 (Scheme 2). BLUSP was utilized in this OP4E reaction because it offered better yield than AtUSP in the preparative-scale synthesis of UDP-GlcA and its optimal pH range (5.0–6.5)[28] was closer to that of PmHS2 (5.0–7.0)[29] than AtUSP (8.0–9.0)[19]. Both disaccharide (**13**) and tetrasaccharide (**16**) were obtained using a OP4E *N*-acetylglucosamine (GlcNAc) activation and transfer system containing an *N*-acetylhexosamine-1-phosphate kinase (NahK),[32] *Pasteurella multocida* *N*-acetylglucosamine-1-phosphate uridylyltransferase (PmGlmU),[18, 33] PmPpA, and PmHS2.[18, 29] A longer glucuronide (trisaccharide **15**) was shown a better acceptor for PmHS2 than monosaccharide **13**. Heparosan disaccharide **14** and tetrasaccharide **16** were obtained in 81% and quantitative yields, respectively. Overall, the sequential OPME reaction (Scheme 2) produced heparosan tetrasaccharide from a simple monosaccharide acceptor (**13**) and two free monosaccharides (as precursors for glycosyltransferase sugar nucleotide donors) with high efficiency (81% yield).

In conclusion, we have identified AtGlcAK as a promiscuous sugar-1-phosphate kinase that can use D-GlcA, D-GalA, and D-ManA for synthesizing the corresponding sugar-1-phosphates. Furthermore, BLUSP has been proven to be more promiscuous than what has been characterized previously. It can use GlcA-1-P as the substrate for the synthesis of UDP-GlcA. A direct and improved OPME system containing AtGlcAK with BLUSP or AtUSP in the presence of PmPpA has been developed for the synthesis of UDP-GlcA from a simple free monosaccharide D-GlcA. In addition, a OPME system containing BiGalK, AtUSP, and PmPpA has been established for the synthesis of UDP-GalA from D-GalA. Furthermore, a highly efficient OPME GlcA-activation and transfer system containing AtGlcAK, BLUSP, PmPpA, and PmHS2 has been developed which can be used with a OPME GlcNAc-activation and transfer system containing NahK, PmGlmU, PmPpA, and PmHS2 for sequential high-yield production of heparosan oligosaccharides and derivatives. The direct and improved OPME GalA-activation and transfer system avoids the use of NAD^+, the cofactor required by the UDP-Glc dehydrogenase-dependent process, and can be used for enzymatic or chemoenzymatic synthesis of other glucuronides.

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Notes and references

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† Electronic supplementary information (ESI) available: Experimental details, NMR and HRMS data. See DOI: 10.1039/b000000x/

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Efficient one-pot multienzyme (OPME) systems were established for the synthesis of UDP-GlcA, UDP-GalA, and glucuronides from simple monosaccharides.