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Selective Detection of Sugar Phosphates by Capillary Electrophoresis/Mass Spectrometry and Its Application to an Engineered *E. coli* Host

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A highly selective method employing capillary electrophoresis and electrospray mass spectrometry (CE-ESMS) with precursor ion scanning for fragment ions characteristic of phosphate-linked sugars was developed for the determination of "unnatural" sugar phosphates generated in vivo, as part of a natural product glycorandomization study. Cell lysates from an engineered E. coli *host were probed for "natural" and "unnatural" sugar phosphates resulting from in vivo galactokinase (GalK) bioconversions, and tandem mass spectrometry experiments were performed to confirm the identities of the sugar phosphates. Among the 22 cell lysates that were studied, 13 were found to contain the expected natural and "unnatural" sugar phosphates. This was in agreement with the GalK in vitro conversion yields, in which an in vitro yield of* $\leq 15\%$ *coincided with a lack of observable in vivo bioconversion. In addition, the CE-ESMS and precursor ion scanning method was capable of separating sugar phosphate regioisomers such as hexose-6-phosphate and hexose-1-phosphate.*

Introduction

Natural product glycorandomization is a chemoenzymatic strategy used for the synthesis of glycosylated secondary metabolites that could serve as novel therapeutic drugs.[1–3] The strategy involves the chemical synthesis of a repertoire of unique sugar precursors and three promiscuous enzymes that activate (anomeric sugar kinases and nucleotidyltransferases) and attach (glycosyltransferases) these carbohydrate libraries to various complex natural product aglycons. Many of the world's lead compounds in drug discovery are derived from glycosylated secondary metabolites,[4–8] so the potential to synthesize novel glycosylated secondary metabolites or to alter existing ones is significant.

In vitro glycorandomization has been shown to be a highly successful approach for attaching natural and "unnatural" sugar phosphate precursors to various aglycons to produce glycorandomized natural product derivatives that display biological properties that are enhanced and/or distinct from the parent natural product.[1–3] However, a number of factors could limit this multienzyme, single-vessel in vitro approach. Firstly, the scaling up of the process could very much be limited by the cost of substrates/cofactors. Secondly, the application of in vitro glycorandomization to the majority of glycosylated natural products is heavily dependent upon the level of expression of appropriate glycosyltransferases and on establishing optimal in vitro conditions for an active enzyme. In some cases, achieving these conditions can be severely dictated by the solubility of the aglycon acceptors, which are natural product aglycons. Finally, any feedback inhibition that results in the accumulation of by-products from each reaction might interfere with other steps within the process. However, these limitations could be circumvented by converting the in vitro glycorandomization process into an in vivo one. More specifically, by expressing a promiscuous sugar-1-kinase (GalK) and a nucleotidyltransferase (E_p) in tandem, one could create an unnatural NDP-sugar factory in a nonproducing host (e.g., *E. coli*) that expresses a given glycosyltransferase (or glycosyltransferase library). This in turn would provide a vehicle for glycorandomization through feeding of host with appropriate aglycon acceptors and unnatural sugars.[1,9]

One key to achieving in vivo glycorandomization is the ability to introduce unnatural sugars to the host. The engineered M173L/Y371H-GalK mutant has demonstrated remarkable substrate flexibility compared to the wide-type enzyme.[1,9] In addition, a fluorescence-based assay has revealed the ability of the engineered *E. coli* Y371H/M173l-GalK-overproducing strain to process two unnatural azidosugars in vivo.[9] However, this study was extremely limited, as it was based on the incorporation of azidosugars followed by the downstream fluorescent labeling of substrates and products through Huisgen 1,3-dipolar cycloaddition. In the current study, we developed a generic and selective capillary electrophoresis/electrospray ionization mass spectrometry (CE-ESMS) method for the analysis of intra-

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cellular sugar phosphates as a means to assess the ability of the engineered M173I-Y371H GalK *E. coli* strain to generate natural and "unnatural" sugar-1-phosphate libraries in vivo. A selective CE-ESMS analytical method for sugar phosphates would not only be useful in this study but would also be highly relevant in studies of unique glycoconjugate biosynthesis pathways, since sugar phosphates are key precursors in carbohydrate biosynthesis pathways.[1–3]

Sugar phosphates are inherently polar compounds and possess a phosphate functional group, and the use of CE-ESMS in the negative mode would thus be a suitable choice of analytical technique.[10,11] CE offers several advantages over other commonly used separation techniques, such as liquid chromatography (LC), in this context because it: 1) is particularly suited towards analyzing highly polar ionic molecules; 2) demonstrates better separation efficiency, which is ideal for resolving complex biological cell extracts and; 3) requires only small amounts of sample (in the low nL range).[12–17] In addition, the determination of the anomeric configurations of sugar phosphates by mass spectrometry can pose a challenge because regioisomers have identical *m/z* values. The use of CE with indirect UV has permitted the analysis of regioisomers,[18] but with regard to mass spectrometry, the differentiation of anomeric isomers has generally required tandem MS[11] or derivatization of the isomers with an ion/molecule reagent to produce diagnostic ions.[10] Here, we have evaluated the selectivity of this current CE-ESMS method as a tool to separate and identify regioisomers without the need for sample derivatization.

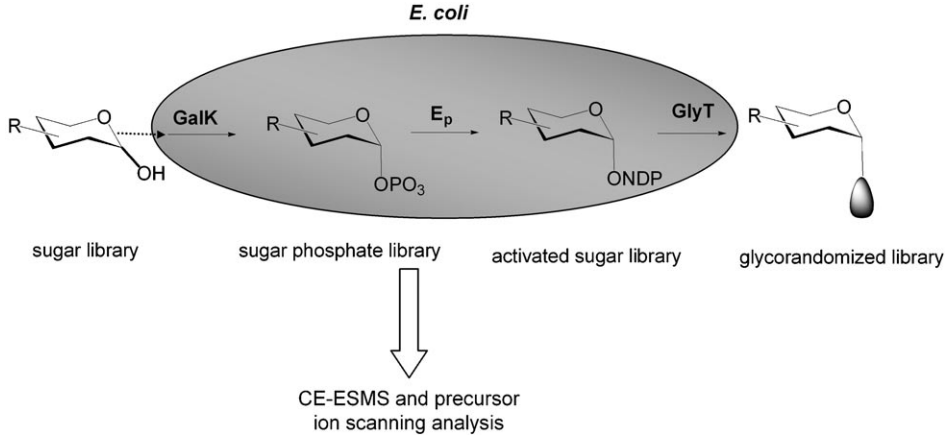
Results and Discussion

Selective detection and identification of sugar phosphates by CE-ESMS

As shown in Scheme 1, the synthesis of sugar phosphates by use of the GalK enzyme is the first step in the in vivo glycorandomization process. Accordingly, it would be expected that an accumulation, within host cells, of sugar phosphates corresponding to the sugar (natural and unnatural) that is presented to the engineered GalK-*E. coli* strain should be found. Given that phosphate-linked sugars fragment to produce characteristic phosphate ions at *m/z* 97 ($[H_2PO_4]^-$) or 79 ($[PO_3]^-$), there is the potential for selective detection of intracellular sugar phosphates by mass spectrometry with precursor ion scanning,[19,20] and this in turn provides a means to assess the promiscuity/bioconversion rate of the GalK enzyme with libraries of unnatural sugars.

A CE-ESMS method for the selective detection of sugar phosphates was developed by using a number of commercially available sugar phosphate standards, including α-D-glucosamine-1-phosphate, α-D-galactosamine-1-phosphate, α-D-glucose-1-phosphate, D-glucose-6-phosphate, β-D-glucose-1-phosphate, *N*-acetyl-α-D-glucosamine-1-phosphate, α-D-galactose-1-phosphate, D-galactose-6-phosphate, D-fructose-1-phosphate, and D-fructose-6-phosphate. The main challenge in sugar phosphate analysis by MS alone is that sugar phosphate isomers have the same *m/z* values and display similar MS-MS fragmentation patterns.[10] Therefore, in order to differentiate between sugar phosphate isomers, it would be necessary to introduce a separation step prior to their analysis by mass spectrometry. As mentioned earlier, CE is a particularly useful separation technique in this study, as it is suited to the separation of charged and polar molecules and has demonstrated the capability to separate regioisomers such as α-D-glucose-1-phosphate and D-glucose-6-phosphate on the basis of subtle differences in their pK_a values.[18] On the other hand, stereoisomers that have the same pK_a value would be expected to remain unresolved by CE.

As shown in Figure 1C, tandem mass spectrometry experiments on the commercially available sugar phosphates α-D-glucose-1-phosphate and α-D-glucosamine-1-phosphate confirmed that fragment ions at *m/z* 97 ($[H_2PO_4]^-$) or 79 ($[PO_3]^-$) were characteristic of sugar phosphates and could be exploited in the CE-ESMS and precursor ion scanning approach for selective detection of the sugar phosphate standards. Through the use of morpholine/formate as the separation buffer, the CE conditions were optimized for separation of the three commercially available sugar phosphate standards—α-D-glucose-1-phosphate, α-D-glucosamine-1-phosphate, and *N*-acetyl-α-D-glucosamine-1-phosphate—and the chemically synthesized 6-azido-6-deoxy-α-D-glucose-1-phosphate, and separation was achieved on the basis of their differences in the electrophoretic mobilities (Figure 1A and B). It was observed in these preliminary experiments that α-D-glucosamine-1-phosphate migrated more rapidly than the α-D-glucose-1-phosphate (Figure 1A and B); this is to be expected since the amine group would impart a greater overall charge to the sugar phosphate. While



Scheme 1. Schematic of the in vivo glycorandomization process. The enzyme GalK is a double mutant with site mutations at Met173Leu and Tyr371His.

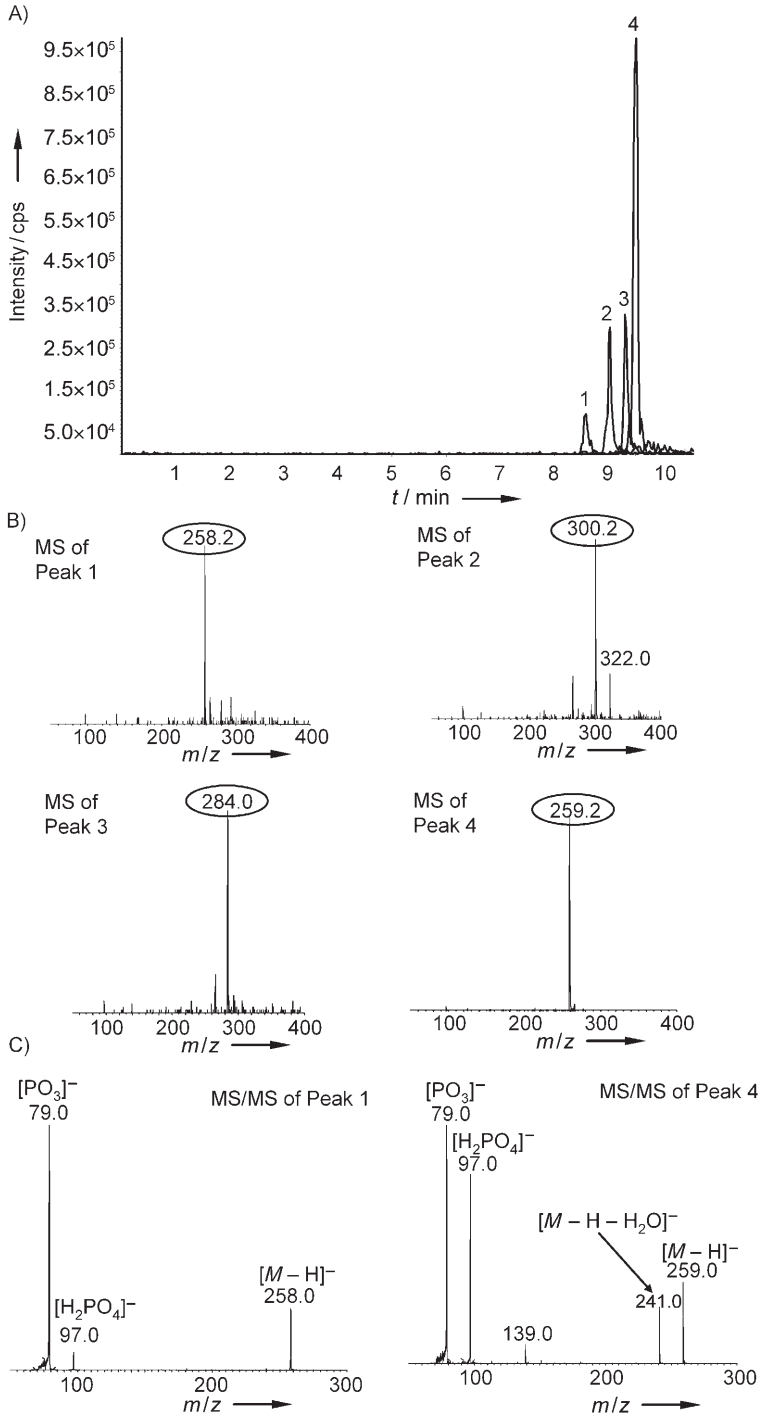


Figure 1. CE-ESMS analysis of four sugar phosphate standards. A) Precursor ion scanning of *m/z* 97. The standards are: 1) commercially available α-D-glucosamine-1-phosphate; 2) commercially available *N*-acetyl-α-D-glucosamine-1-phosphate; 3) chemically synthesized 6-azido-6-deoxy-α-D-glucose-1-phosphate; 4) commercially available α-D-glucose-1-phosphate. B) Corresponding MS spectra, and C) MS/MS spectra for α-D-glucosamine-1-phosphate and α-D-galactose-1-phosphate showing the characteristic phosphate anions at *m/z* 97 ($[H_2PO_4]^-$) and 79 ($[PO_3]^-$). The concentration of each standard is 100 μM and hydrodynamic pressure injection of 50 mbar for 5 s was employed.

the use of CE-ESMS and total ion scanning could be employed to detect for intracellular sugar phosphates within the engineered GalK-*E. coli* host, the cell lysate is a highly complex biological sample, and a more selective detection method that filters out ions that do not contain phosphate functional groups would thus be highly desirable. To that end, the use of precursor ion scanning would be an invaluable acquisition mode for detection of phosphate-linked sugars in the complex mixtures, and by selecting the phosphate ion at *m/z* 97, it was possible to achieve selective detection of each of the four sugar phosphates as precursor ions bearing the phosphate-linked sugars within a mixture of the four standards (Figure 1), while providing an added level of sensitivity.

Detection limit and dynamic range of CE-ESMS

The sensitivity and the linearity range of the CE-ESMS and precursor ion scanning method were determined with the sugar phosphate mixture (Figure 1). Excellent linearity was achieved for the four standards, and detection limits ranging between 2.5 and 10 μM were observed (signal-to-noise ratio of 3:1; Figure 2 and Table 1), which correspond to approximately 5 to 20 fmol of the respective sugar phosphate standard. The detection limits and linearity ranges for the sugar phosphates in cell lysates were also determined by spiking the individual standards in the negative control (engineered M173I-Y371H GalK overproduction strain in the absence of sugar) and were found to be within the same range as those observed for the aqueous sugar phosphate standards (Figure 2 and Table 1). The detection limit could not be determined for α-D-glucose-1-phosphate in the cell lysates due to the presence of an endogenous hexose phosphate (Figure 5B).

The CE-ESMS conditions for the different sugar phosphate standards having been optimized, the method was then evaluated as a tool for separating regioisomers. Four pairs of sugar phosphate isomers—including D-fructose-1-phosphate versus D-fructose-6-phosphate, α-D-galactose-1-phosphate versus D-galactose-6-phosphate, α-D-glucose-1-phosphate versus D-glucose-6-phosphate, and α-D-glucose-1-phosphate versus β-D-glucose-1-phosphate—were employed. By using CE-ESMS with precursor ion scanning for fragment ions characteristic of phosphate anions, three of the four regioisomeric pairs were detected and found to be adequately separated (Figure 3). These data revealed the capability of this CE-ESMS method for separating positional isomers (Figure 3A–C), and in all three cases it was observed that the hexose-6-phosphate migrated slightly more rapidly than the hexose-1-phosphate under the selected CE-ESMS conditions. This observation was consistent with earlier findings in which the separation of sugar phosphates on the basis of differences in their pK_a values was achieved by CE with indirect UV.[18] In the case of β-D-glucose-1-phosphate and α-D-glucose-1-phosphate, where there was no difference in the positions of the phosphate

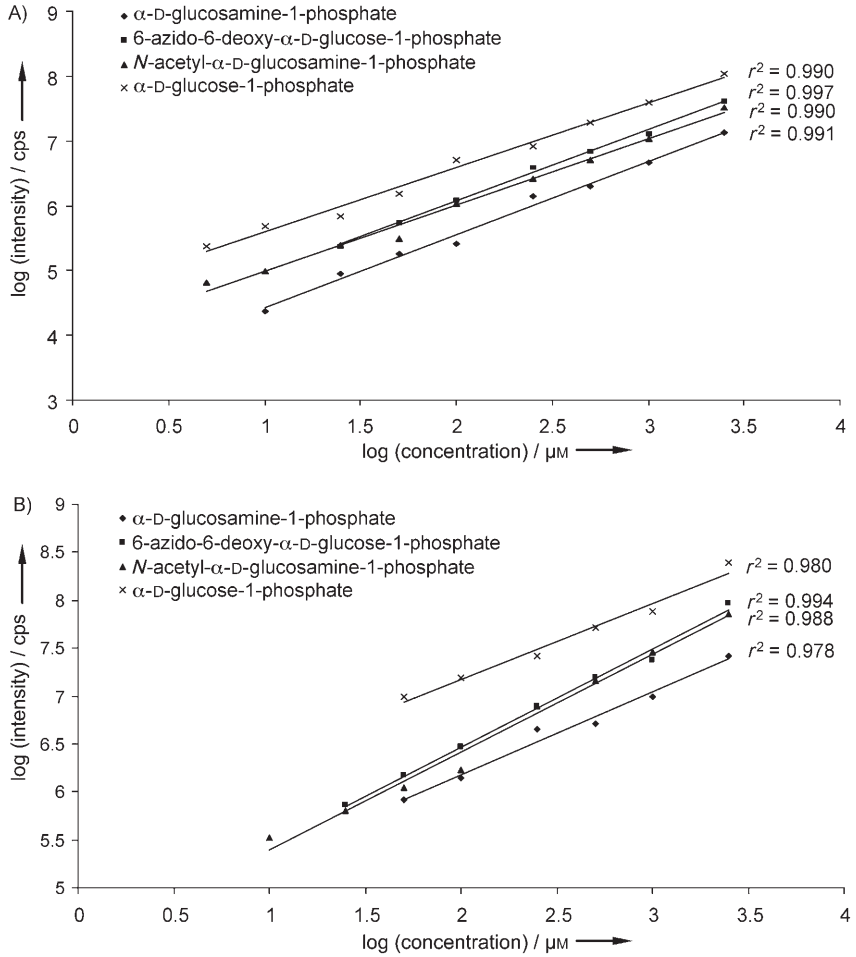


Figure 2. Linear curves for selected sugar phosphate standards displayed in logarithmic scale. A) Authentic standards, and B) standards spiked in the negative control cell lysate.

Table 1. Limits of detection and linearity for selected sugar phosphates determined by CE-ESMS and precursor ion scanning method (numbers in square brackets indicate the corresponding values detected in lysates).

Sugar phosphates	Limit of detection [μM]	Dynamic range [μM]	r^2
α-D-glucosamine-1-phosphate	10 [10]	10–2500 [25–2500]	0.991 [0.978]
N-acetyl-α-D-glucosamine-1-phosphate	5 [10]	5–2500 [10–2500]	0.990 [0.994]
6-azido-6-deoxy-α-D-glucose-1-phosphate	5 [10]	25–2500 [25–2500]	0.997 [0.988]
α-D-glucose-1-phosphate	2.5 [N/A][a]	5–2500 [50–2500]	0.990 [0.980]

[a] Not applicable.

groups, no separation of the anomers was achieved (Figure 3D). It is noteworthy that, contrary to earlier reports,[11] the MS-MS fragmentation patterns of the anomeric isomer pair were similar (data not shown) and therefore cannot be used to distinguish them.

Detection of intracellular sugar phosphates in the engineered *E. coli* strain

The optimized CE-ESMS conditions for selective detection of the sugar phosphates were applied in the analysis of the *E. coli* cell lysates, as a means to investigate the efficiency of the in vivo GalK bioconversion process. The *E. coli* host was engineered to express the flexible double mutant M173I–Y371H GalK. On the basis of previous in vitro studies,[9] 22 known M173I–Y371H substrates (Table 2 and Scheme 2) were selected for in vivo bioconversion. Feeding of these substrates to the engineered GalK–*E. coli* host was expected to lead to an accumulation of each of the corresponding sugar phosphates, as a result of bioconversion by the engineered GalK enzyme. In the subsequent CE-ESMS analysis of *E. coli* cell lysates, the desired first step of the in vivo glycorandomization process—that is, production of unnatural sugar-1-phosphate in vivo—was found to be successful under normal fermentation conditions. The bioconversion of the unnatural sugar set was observed to be accomplished in a fashion that was similar to that of a negative control (M173I–Y371H GalK overproduction strain in the absence of sugar). In order to standardize cell numbers, the culture was incubated for 16 hours at 16 °C and the OD_{600} was measured to determine cell numbers in each culture before harvesting and adjusting for downstream assays. Once the cell density was standardized, the cells were lysed and the crude lysate was subjected to CE-ESMS and MS/MS analysis.

Compounds **1**, **13**, and **14** were commercially available sugars and compound **5** was chemically synthesized, and the bioconversion of these sugars by the engineered GalK-*E. coli* host was assessed in parallel to chemically synthesized sugar-1-phosphates. All other sugar phosphate standards had been generated through in vitro GalK reactions. CE-ESMS and precursor ion scanning analysis of cell lysates 1 and 15 (Figure 4) showed the expected intracellular sugar phosphates α-D-galactosamine-1-phosphate, 6-azido-6-deoxy-α-D-galactose-1-phosphate, and the endogenous hexose-phosphate to be well separated from one another, as was expected from initial method development work with the sugar phosphate standards. The peak shapes of the intracellular sugar phosphates in cell ly-

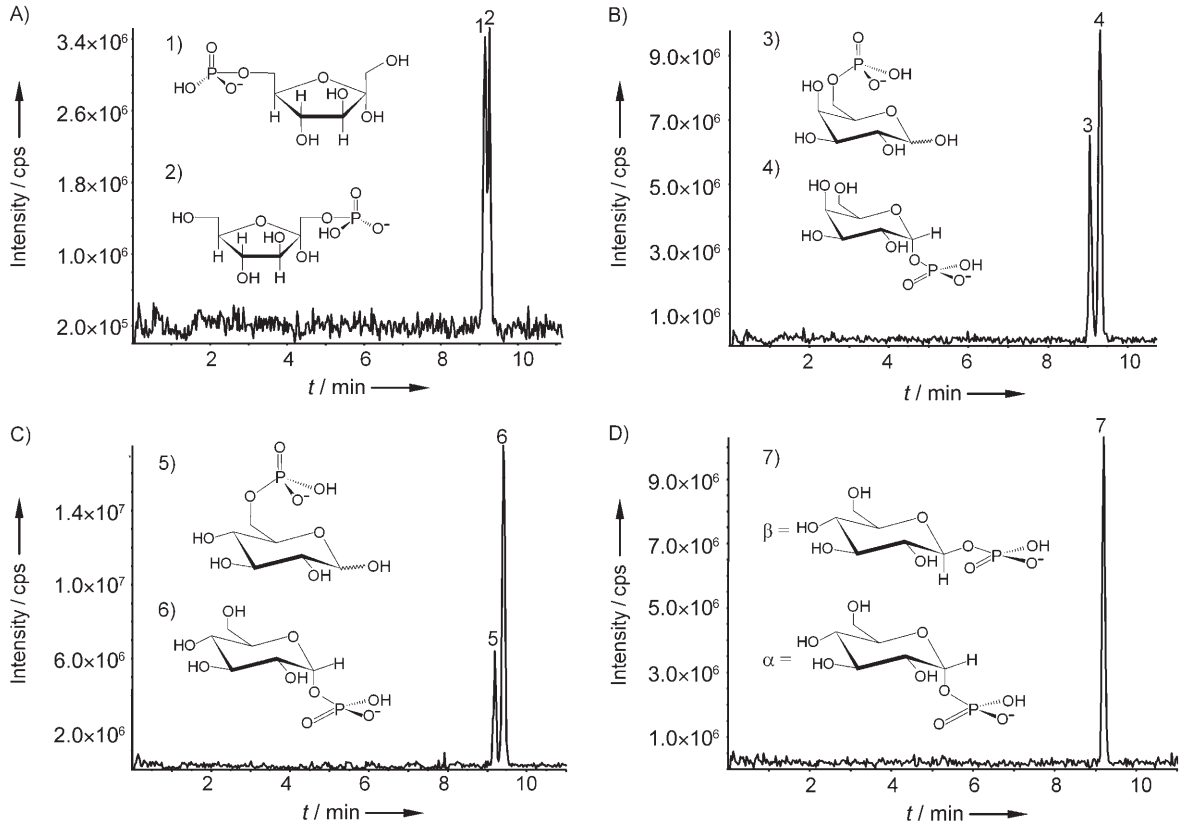
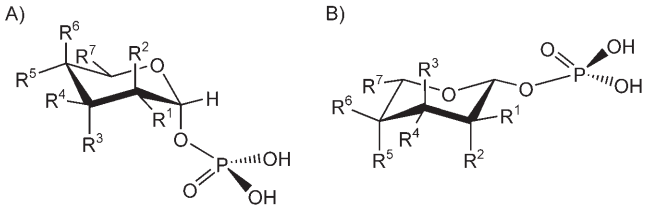


Figure 3. CE-ESMS analysis of isomer pairs of sugar phosphate standards. Concentration of each standard is 25 ng μL^{-1}, and hydrodynamic pressure injection of 50 mbar for 2 s was employed. The standards are: 1) D-fructose-6-phosphate; 2) D-fructose-1-phosphate; 3) D-galactose-6-phosphate; 4) α-D-galactose-1-phosphate; 5) D-glucose-6-phosphate; 6) α-D-glucose-1-phosphate; 7) β-D-glucose-1-phosphate; and α-D-glucose-1-phosphate.

Table 2. Structures of intracellular sugar phosphates investigated (refer to Scheme 2 for generic α and β chemical structures).

Cell lysate	Sugar phosphate	R^1	R^2	R^3	R^4	R^5	R^6	R^7
1	α-D-galactosamine-1-p	$-NH_2$	–H	–H	–OH	–H	–OH	$-CH_2OH$
2	α-D-talose-1-p	–H	–OH	–H	–OH	–H	–OH	$-CH_2OH$
3	2-deoxy-α-D-galactose-1-p	–H	–H	–H	–OH	–H	–OH	$-CH_2OH$
4	3-deoxy-α-D-glucose-1-p	–OH	–H	–H	–H	–OH	–H	$-CH_2OH$
5	6-azido-6-deoxy-α-D-glucose-1-p	–OH	–H	–H	–OH	–OH	–H	$-CH_2N_3$
6	6-bromo-6-deoxy-α-D-galactose-1-p	–OH	–H	–H	–OH	–H	–OH	$-CH_2Br$
7	6-deoxy-α-D-galactose-1-p	–OH	–H	–H	–OH	–H	–OH	$-CH_3$
8	6-deoxy-6,6-difluoro-α-D-galactose-1-p	–OH	–H	–H	–OH	–H	–OH	$-CHF_2$
9	6-deoxy-6-fluoro-α-D-glucose-1-p	–OH	–H	–H	–OH	–OH	–H	$-CH_2F$
10	6-deoxy-6-thio-α-D-galactose-1-p	–OH	–H	–H	–OH	–H	–OH	$-CH_2SH$
11	β-L-altrose-1-p	–H	–OH	–OH	–H	–H	–OH	$-CH_2OH$
12	β-L-glucose-1-p	–OH	–H	–H	–OH	–H	–OH	$-CH_2OH$
13	α-D-galactose-1-p	–OH	–H	–H	–OH	–H	–OH	$-CH_2OH$
14	α-D-glucose-1-p	–OH	–H	–H	–OH	–OH	–H	$-CH_2OH$
15	6-azido-6-deoxy-α-D-galactose-1-p	–OH	–H	–H	–OH	–H	–OH	$-CH_2N_3$
16	6-chloro-6-deoxy-α-D-galactose-1-p	–OH	–H	–H	–OH	–H	–OH	$-CH_2Cl$
17	2-deoxy-α-D-glucose-1-p	–H	–H	–H	–OH	–OH	–H	$-CH_2OH$
18	6-deoxy-α-D-glucose-1-p	–OH	–H	–H	–OH	–OH	–H	$-CH_3$
19	4-deoxy-α-D-galactose-1-p	–OH	–H	–H	–OH	–H	–H	$-CH_2OH$
20	α-D-allose-1-p	–OH	–H	–OH	–H	–OH	–H	$-CH_2OH$
21	α-D-glucosamine-1-p	$-NH_2$	–H	–H	–OH	–OH	–H	$-CH_2OH$
22	6-amino-6-deoxy-α-D-galactose-1-p	–OH	–H	–H	–OH	–H	–OH	$-CH_2NH_2$

sates detected by CE-ESMS, however, were generally broader and are likely to be due to the complexity of the sample matrix. The MS/MS fragmentation patterns revealed the two diagnostic phosphate anions at *m/z* 79 and *m/z* 97, a loss of water molecule from the sugar phosphate ion, and fragment ions at *m/z* 139 and *m/z* 199 resulting from cleavage across



Scheme 2. Generic structures of intracellular sugar phosphates investigated. A) α-D-Hexose-1-phosphates, and B) β-L-hexose-1-phosphates.

the sugar ring (Figure 4).[8,9] It is noteworthy that the loss of the intact sugar molecule from the precursor ion $[M-H]^-$ is also a typical MS/MS fragmentation pattern observed in sugar phosphate analysis.

A negative control consisting of the engineered M173I–Y371H GalK overproduction strain in the absence of sugar was prepared and used to determine the level of endogenous sugar phosphates. Small amounts of endogenous hexose phosphate were observed at *m/z* 259 in the negative control (Figure 5B) and, in addition to this, ions at *m/z* 289 and *m/z* 323 were observed; these are suspected to be a heptose phosphate and uridine monophosphate (UMP) anions, respectively. CE-ESMS and precursor ion scanning revealed that the ion intensity of hexose phosphate in the lysate expressed with D-galactose-1-phosphate was at least 20 times higher than in the negative control (Figure 5A), supporting readily occurring in vivo conversion. MS analysis revealed successful in vivo GalK bioconversion for 13 of the 22 sugar analogues. Table 3 illustrates a complete summary of the MS analysis for each investigated sugar substrate, including the determined *m/z* value and confirmation of the observed sugar phosphates by MS/MS, together with the corresponding in vitro conversion. Except for compounds **1**, **5**, **13**, and **14**, where standards are commercially available or were chemically synthesized, the sugar phosphates were synthesized by in vitro GalK reaction and were subjected to MS/MS analysis. The in vitro GalK assays were assessed under the standard reaction conditions as described previously [sugar (8 mM), ATP (15 mM), $MgCl_2$ (5 mM), GalK enzyme (15 μM), reaction time = 180 min].[9] The characteristic phosphate fragment ions at *m/z* 79 and *m/z* 97, as well as a loss of water from the precursor ion and the fragment ion resulting from the intact sugar loss, were observed in each case (data not shown). Analysis of the cell lysates revealed direct

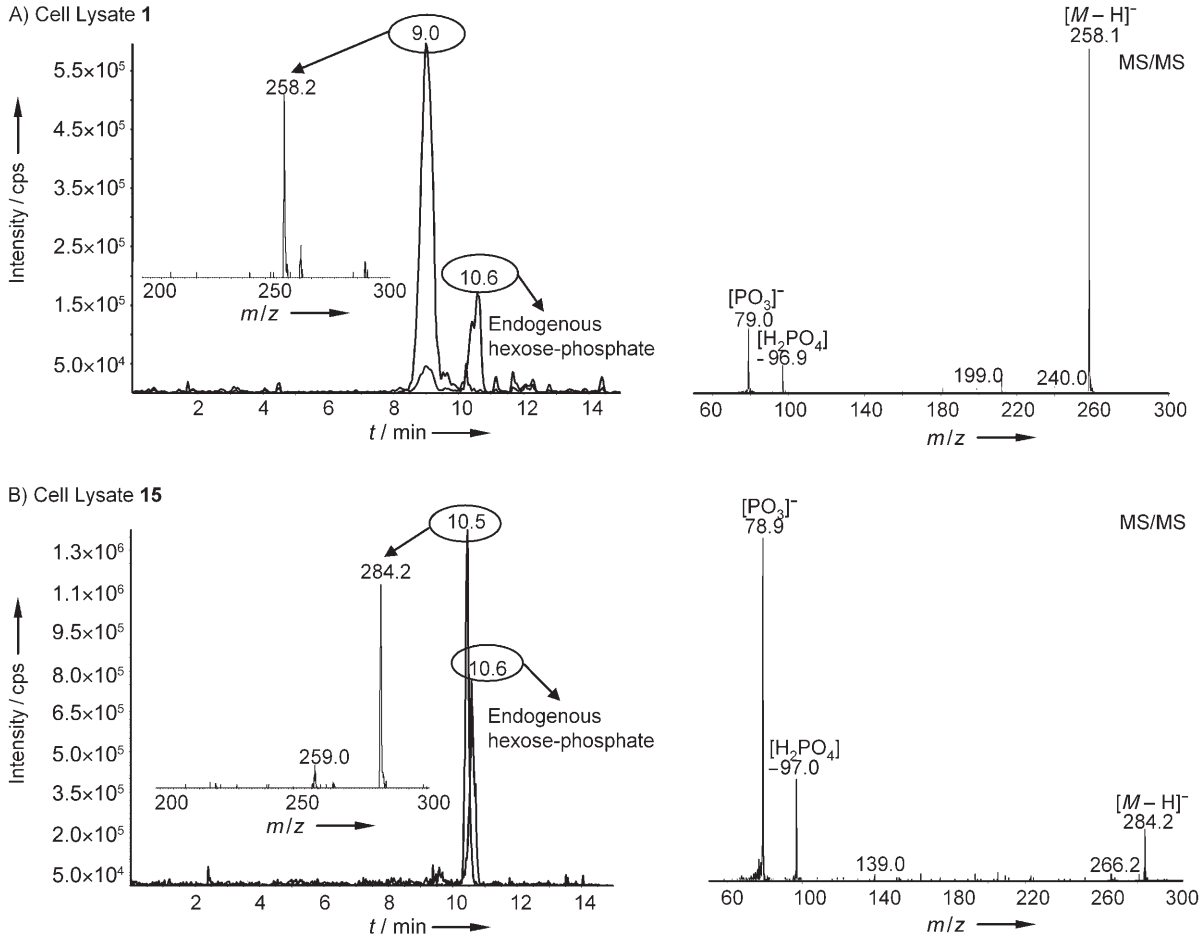


Figure 4. Evaluation of the in vivo GalK bioconversion process of the engineered *E. coli* host. CE-ESMS and precursor ion scanning for fragment ions characteristic of phosphate-linked sugars, together with MS/MS experiments, revealed the presence of the bioconversion products A) α-D-galactosamine-1-phosphate in cell lysate 1, and B) 6-azido-6-deoxy-α-D-galactose-1-phosphate in cell lysate 15.

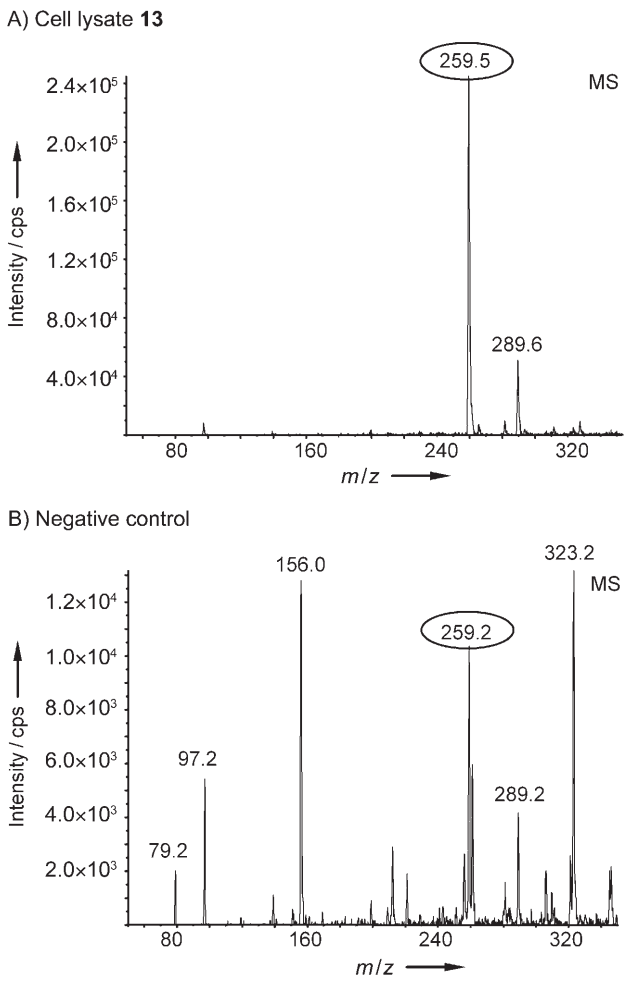


Figure 5. CE-ESMS and precursor ion scanning analysis for fragment ions characteristic of phosphate-linked sugars revealed: A) significantly greater levels of α-D-galactose-1-phosphate in cell lysate 13 synthesized in vivo by the GalK bioconversion process, in relation to B) endogenous levels of α-D-galactose-1-phosphate detected in the negative control.

correlations between the sugar phosphates detected in vivo and the yields obtained by in vitro conversion (Table 3), in which an in vitro yield of $\leq$15% coincided with a lack of observable in vivo bioconversion. The natural substrate α-D-galactose-1-phosphate was the best substrate, resulting in an in vitro conversion rate of 82%. While the transport of "unnatural" sugars into this typical engineered *E. coli* host is less than optimal (e.g., typically $<$15%), the results are notable for a variety of reasons. Firstly, the consistency between the in vivo and the in vitro yields suggests that other potential sugars that might be competing for the mutant GalK do not greatly influence the production of the desired product. Should such competition become a problem with future "unnatural" sugars, this system is easily amenable to minimal media growth. Secondly, the reflected yields also suggest that the "unnatural" sugars are not being metabolized via alternative "dead-end" metabolic pathways, some of which could be genetically excised if problematic to future development. Most importantly, the result illustrates that "unnatural" sugars are able to enter the heterologous *E. coli* host and are clearly accessible to the engineered, heterologously expressed, promiscuous sugar kinase.

Conclusions

CE-ESMS and precursor ion scanning for fragment ions characteristic of the phosphate anion was shown to be a highly selective and rapid method for identifying the unique sugar phosphates produced in vivo by GalK bioconversion. The identity of each sugar phosphate in the cell lysates was confirmed by performing tandem MS experiments, and a typical MS/MS spectrum was found to consist of the diagnostic phosphate anions, a loss of a water molecule, and the characteristic loss of the intact sugar molecule from the precursor ion. The sample preparation requirements for the cell lysates prior to their analysis by CE-ESMS are limited; this reduces the potential loss of the intracellular sugar phosphate precursors during typical sample preparation procedures. Among the 22 cell lysates that were examined for in vivo conversion into sugar phosphates, 13 were found to contain the expected sugar phosphates—a 60% success rate of bioconversion. A general relationship was observed between the in vitro and in vivo conversion, such that the higher the percentage of in vitro conversion, the more likely it was that the in vivo bioconversion was accomplished. This result suggests that those that were not detected were probably below the limit of detection of the current methodology. The typical limit of detection for the CE-ESMS method and precursor ions scanning of *m/z* 97 was in the low μM range and corresponds to a low fmol detection mass level.

Evidence for in vivo bioconversion was first reported in an earlier work in which 6-azido-6-deoxy-D-galactose was used as the sugar substrate.[9] The results here provide a first global assessment of in vivo bioconversion of unnatural sugars into their corresponding sugar-1-phosphate analogues by using the engineered GalK *E. coli* host. The success in production of novel sugar-1-phosphate libraries in vivo is critical to demonstrating the concept of in vivo glycorandomization. We anticipate that overproduction of the next enzyme in our short activation pathway (an engineered sugar-1-phosphate nucleotidyltransferase) will provide a corresponding set of diverse NDP-sugars in a similar manner. While these unique sugar-1-phosphate and NDP-sugar libraries will clearly serve as versatile chemical tools for glycobiology, these studies more notably support the notion of constructing short sugar activation pathways in vivo that ultimately lead to in vivo natural product glycorandomization.

Experimental Section

Materials: For the CE-ESMS analysis, morpholine was purchased from Sigma–Aldrich. HPLC-grade isopropyl alcohol (IPA) and methanol were from Fisher Scientific. Deionized water was obtained from a MilliQ water purification system. Anhydrous ethanol for protein precipitation was from Commercial Alcohol Inc. (Toronto, ON, Canada). Bare fused-silica capillary for CE-ESMS was obtained from Polymicro Technologies (Tucson, AZ, USA). The sugar phosphate

Table 3. Representative MS data for sugar-1-phosphates produced by in vivo bioconversion. Standard reaction conditions for all enzymatic reactions: [sugar] = 8 mM, [ATP] = 15 mM, [$MgCl_2$] = 5 mM, [enzyme] = 15.0 μM, reaction time = 180 min. The overall average error of in vitro conversion is ±3%.

Sugar substrate	Calcd mass	Determined *m/z* $[M-H]^-$	MS/MS confirmation	in vitro conversion [%]
D-galactosamine (**1**)	259	258	+[c]	45
D-talose (**2**)	260	259	+[c]	38
2-deoxy-D-Gal (**3**)	244	243	+[c]	68
3-deoxy-D-Glc (**4**)	244	243	+[c]	15
6-azido-D-Glc (**5**)	285	–[a]	N/A[b]	14
6-bromo-D-Gal (**6**)	322	–[a]	N/A[b]	14
6-deoxy-D-Gal (**7**)	244	243	+[c]	23
6,6-difluoro-D-Gal (**8**)	280	–[a]	N/A[b]	26
6-fluoro-D-Glc (**9**)	262	261	+[c]	12
6-thio-D-Gal (**10**)	276	–[a]	N/A[b]	25
L-altrose (**11**)	260	259	+[c]	56
L-Glc (**12**)	260	259	+[c]	30
D-Gal (**13**)	260	259	+[c]	82
D-Glc (**14**)	260	259	+[c]	42
6-azido-D-Gal (**15**)	285	284	+[c]	44
6-chloro-D-Gal (**16**)	278	–[a]	N/A[b]	12
2-deoxy-D-Glc (**17**)	244	243	+[c]	18
6-deoxy-D-Glc (**18**)	244	–[a]	N/A[b]	12
4-deoxy-D-Gal (**19**)	244	–[a]	N/A[b]	12
D-allose (**20**)	260	259	+[c]	14
D-glucosamine (**21**)	259	–[a]	N/A[b]	9.0
6-amino-D-Gal (**22**)	259	–[a]	N/A[b]	11

[a] No detectable products. [b] Not applicable. [c] Product detected.

standards were produced by in vitro GalK conversion as described previously,[9,21,22] with the exception of α-D-galactosamine-1-phosphate (**1**), α-D-galactose-1-phosphate (**13**), and α-D-glucose-1-phosphate (**14**), which were purchased from Sigma–Aldrich. All other chemicals involved in the synthesis of sugar phosphate libraries were purchased from Sigma, Fisher/Acros Organics, or Fluka.

In vitro GalK assay: The DNS assay was used to assess the substrate specificity of the purified GalK mutants as previously described.[9] The in vitro GalK assays were assessed under the standard reaction conditions as described previously [sugar (8 mM), ATP (15 mM), $MgCl_2$ (5 mM), [enzyme] = 15 μM, reaction time = 180 min].[9]

Generation of sugar-1-phosphate libraries: The GalK double mutant pGalKMLYH-*E. coli*[9] was overexpressed at 16 °C by induction of a culture (40 mL) at an OD_{600} ~0.7 with isopropyl-D-thiogalactopyranoside (IPTG; 1 mM). The induced cultures were incubated with shaking (140 rpm) for 1 h, and unnatural sugar substrate (100 mM, 1.6 mL) was added to the culture (final concentration of 4 mM). A negative control strain (M173I–Y371H GalK overproduction strain in the absence of sugar) was processed in parallel. The cultures were further incubated at 16 °C with shaking (140 rpm) for 16 h. To assess bioconversion, the final volume of each sample was adjusted to a standard cell density (determined by OD_{600}). The cells were harvested by centrifugation (15 min, 12000 rpm) and the recovered cell pellet (~400 mg) was washed until DNS analysis[22] of the wash solution revealed no remaining endogenous extracellular free monosaccharide (2×20 mL sodium phosphate buffer). The pellet was frozen, thawed, resuspended in H_2O/MeOH (1:1), and heated (100 °C, 15 min), and was then sonicated (5×45 s) on ice to deactivate enzymes. An assay of this crude extract for GalK activity revealed that all enzymes had been inactivated and, thus, any final conversion detected was the result of in vivo conversion. Cell debris was removed by centrifugation (15 min, 12000 rpm) and the cell lysate was lyophilized to give a pale white solid (~15–20 mg). The crude cell lysates and control samples were submitted for CE-ESMS and MS/MS analysis.

Sample preparation of *E. coli* lysates: Dried cell lysates were first dissolved in deionized water (10 mg mL^{-1}). Each lysate (300 μL) was then mixed with anhydrous ethanol to obtain a final organic composition of 75% (*v/v*) to precipitate proteins. Insoluble material was removed by centrifugation, and lysate was evaporated to dryness on a Speedvac SC110A concentrator (ThermoSavant, Holbrook, NY). After reconstitution in deionized water (200 μL), the cell lysates were then filtered (60 min, 14000 rcf) through a Microcon YM-3 centrifugal filter unit (3000 Da NMWL, Millipore, Bedford, MA). The flow-through portion containing the small metabolites was evaporated to dryness and was reconstituted in CE buffer (100 μL) prior to CE-ESMS analysis.

CE-ESMS analysis of *E. coli* lysates: Electrophoretic separations were performed in bare, fused silica capillaries (100 cm×50 μm i.d.) with voltage and pressure (30 kV, 50 mbar) applied across the capillary inlet where the CE buffer was inserted (30 mM morpholine/formate pH 9.0) and the MS source.[19,20] CE-ESMS was carried out with an Agilent CE system (Santa Clara, CA, USA) coupled to a 4000 Q TRAP mass spectrometer (AB/MDS Sciex, Concord, ON, Canada) through a coaxial sheath flow interface supplied from AB/MDS Sciex. The sheath buffer (IPA/methanol 2:1 (*v/v*)) was delivered by syringe pump (1.25 μL min^{-1}). The 4000 Q TRAP was equipped with a TurboV source for electrospray ionization. Linear Ion Trap (LIT) mode was used both for MS full scan (1000 amu s^{-1} scan speed, 20 ms fill time) and for product ion scan (30–35 eV laboratory frame of reference). Precursor ion scanning experiment was performed by use of the phosphate anion at either *m/z* 97 $[H_2PO_4]^-$ or 79 $[PO_3]^-$ (30–35 eV laboratory frame of reference). All acquisitions were made in the negative mode. Data acquisition and processing were performed with Analyst v 1.4.1.

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[1] J. Yang, D. Hoffmeister, L. Liu, X. Fu, J. S. Thorson, *Bioorg. Med. Chem.* **2004**, *12*, 1577–1584.
[2] B. R. Griffith, J. M. Langenhan, J. S. Thorson, *Curr. Opin. Biotechnol.* **2005**, *16*, 622–630.
[3] J. M. Langenhan, B. R. Griffith, J. S. Thorson, *J. Nat. Prod.* **2005**, *68*, 1696–1711.
[4] J. S. Thorson, W. A. Barton, D. Hoffmeister, C. Albermann, D. B. Nikolov, *ChemBioChem* **2004**, *5*, 16–25.
[5] J. S. Thorson, T. J. Hosted, Jr., J. Jiang, J. B. Biggins, J. Ahlert, *Curr. Org. Chem.* **2001**, *5*, 139–167.
[6] A. C. Weymouth-Wilson, *Nat. Prod. Rep.* **1997**, *14*, 99–110.
[7] V. Kren, L. Martinkova, *Curr. Med. Chem.* **2001**, *8*, 1303–1328.
[8] P. Jones, T. Vogt, *Planta* **2001**, *213*, 164–174.

[9] J. Yang, X. Fu, J. Liao, L. Liu, J. S. Thorson, *Chem. Biol.* **2005**, *12*, 657 – 664.
[10] M. D. Leavell, J. A. Leary, *J. Am. Soc. Mass Spectrom.* **2003**, *14*, 323 – 331.
[11] B. A. Wolucka, J. S. Rush, C. J. Waechter, V. N. Shibaev, E. de Hoffmann, *Anal. Biochem.* **1998**, *255*, 244 – 251.
[12] R. Ramautar, A. Demirci, G. J. de Jong, *TrAC Trends Anal. Chem.* **2006**, *25*, 455 – 466.
[13] C. Nogami, H. Sawada, *Electrophoresis* **2005**, *26*, 1406 – 1411.
[14] K. Harada, E. Furusaki, A. Kobayashi, *J. Biosci. Bioeng.* **2006**, *101*, 403 – 409.
[15] L. Jia, S. Terabe in *Capillary Electrophoresis and its Application in Metabolome Analysis. Metabolome Analyses: Strategies for Systems Biology* (Eds.: S. Vaidyanathan, G. G. Harrigan, R. Goodacre), Springer, MA, **2004**, pp. 83 – 101.
[16] P. Britz-McKibbin, S. Terabe, *J. Chromatogr. A* **2003**, *1000*, 917 – 934.
[17] C. Simó, A. Cifuentes in *Mass Spectrometry Detection in Capillary Electrophoresis. Comprehensive Analytical Chemistry* (Eds.: M. L. Marina, A. Río, M. Valcárcel), Elsevier, New York, **2005**, pp. 441 – 517.
[18] Y. Ciringh, J. S. Lindsey, *J. Chromatogr. A* **1998**, *816*, 251 – 259.
[19] D. J. McNally, J. P. M. Hui, A. J. Aubry, K. K. K. Mui, P. Guerry, J.-R. Brisson, S. M. Logan, E. C. Soo, *J. Biol. Chem.* **2006**, *281*, 18489 – 18498.
[20] E. C. Soo, A. J. Aubry, S. M. Logan, P. Guerry, J. F. Kelly, N. M. Young, P. Thibault, *Anal. Chem.* **2004**, *76*, 619 – 626.
[21] J. Yang, L. Liu, J. S. Thorson, *ChemBioChem* **2004**, *5*, 992 – 996.
[22] J. Yang, X. Fu, Q. Jia, J. Shen, J. B. Biggins, J. Jiang, J. Zhao, J. J. Schmidt, P. G. Wang, J. S. Thorson, *Org. Lett.* **2003**, *5*, 2223 – 2226.

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