

Enzymatic Synthesis of Uridine-5'-O-(2-thiodiphosphoglucose) and Related Sugar Phosphorothioates

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Methods have been developed for the enzymatic synthesis of α -glucose 1-thiophosphate, α -galactose 1-thiophosphate, glucose 6-thiophosphate, uridine-5'-O-(2-thiodiphosphoglucose), and uridine-5'-O-(2-thiodiphosphogalactose). The purified compounds were shown to be substrates for sucrose phosphorylase, galactokinase, UDP-glucose pyrophosphorylase, galactose-1-phosphate uridylyltransferase, hexokinase, glucose-6-phosphate dehydrogenase, UDP-glucose dehydrogenase, and UDP-galactose-4-epimerase. The formation of uridine-5'-O-(2-thiodiphosphoglucose) from α -glucose 1-thiophosphate and UTP as catalyzed by UDP-glucose pyrophosphorylase produces only one of the two possible epimers at the β -thiophosphoryl position. The absolute stereochemistry has not been determined. Uridine-5'-O-(2-thiodiphosphoglucose) was not utilized as an alternate substrate in the transfer of a glucosyl group in the reactions catalyzed by glycogen synthetase and sucrose synthetase. The incubation of cyanate and thiophosphate at pH 5.0 was found to result in the rapid removal of sulfur from the thiophosphate. The reaction is stoichiometric with respect to cyanate and thiophosphate. The data are consistent with the rapid formation and hydrolysis of thiocarbamoyl phosphate. © 1988 Academic Press, Inc.

INTRODUCTION

The introduction of a sulfur atom as a replacement for an oxygen atom in phosphate esters has provided a large number of useful analogs of interesting metabolic intermediates (1). These sulfur analogs differ chemically and physically only very subtly from their oxygen counterparts. However, these differences have been exploited to produce analogs that are useful probes for the elucidation of enzyme reaction mechanisms (2, 3). For example, the nucleoside phosphorothioates that are chiral at the phosphorus center have been particularly useful in the determination of the steric course of many enzymes that utilize nucleotide triphosphates for phosphoryl or adenylyl transfer (4-6). Several recent reviews have summarized the application of phosphorothioates to mechanistic problems in molecular enzymology (3, 7-9).

In this paper we have explored several possible synthetic routes to a wide variety of other nucleoside and sugar phosphorothioates. These compounds have been synthesized enzymatically, and the interactions as inhibitors and alternate

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substrates with these enzymes have been measured. The most interesting of these compounds are UDP-glucose and UDP-galactose analogs substituted at the β -phosphoryl group with sulfur (UDP(β S)-glucose and UDP(β S)-galactose).

MATERIALS AND METHODS

Chemicals and enzymes. Sodium thiophosphate and potassium cyanate were purchased from Alfa Chemical Co. ^{31}P NMR analysis of the thiophosphate indicated that it was contaminated with approximately 15% of phosphate. Adenosine-5'-O-(3-thiotriphosphate) was obtained from Boehringer, Mannheim. Sucrose synthetase was partially purified from wheat germ according to the method of Singh *et al.* (10). All other compounds and enzymes were obtained from either Sigma or Aldrich.

Synthesis of glucose 1-thiophosphate (II). The formation of glucose 1-thiophosphate was catalyzed by sucrose phosphorylase using sucrose (I) and thiophosphate as substrates. The reaction mixture contained 3.0 mM sodium thiophosphate, 10 mM sucrose, 3.0 mM MgSO_4 , 200 mM Hepes, pH 7.5, and 60 units of sucrose phosphorylase in a volume of 100 ml at 25°C. After 25 h the reaction was quenched by passage through a PM-30 ultrafiltration membrane filter (Amicon Corp.). The filtrate was diluted and applied to a DE-52 anion-exchange (Whatman) column (2.5 \times 50 cm). The glucose 1-thiophosphate (II) was eluted using a 2-liter gradient of 10–300 mM triethylamine/ HCO_3^- , pH 7.5. The fractions were assayed with dithionitrobenzene (DTNB) (11, 12). Those fractions containing glucose 1-thiophosphate were pooled, evaporated to dryness, and stored frozen. The yield was 165 μmol (55%). The ^{31}P NMR spectrum of the purified material indicated a single resonance at -40.3 ppm ($J_{\text{HP}} = 9.7$ Hz for the proton-coupled spectrum).

Synthesis of uridine-5'-O-(2-thiodiphosphoglucose) (III). UDP(β S)-glucose (III) was synthesized using UDP-glucose pyrophosphorylase to catalyze the reaction between glucose 1-thiophosphate and UTP. The reaction mixture contained 1.0 mM glucose 1-thiophosphate, 2.0 mM UTP, 5.0 mM MgSO_4 , 100 mM Hepes, pH 7.5, 1.0 mM dithiothreitol (DTT), 100 units of inorganic pyrophosphatase, and 50 units of UDP-glucose pyrophosphorylase in a volume of 100 ml. The reaction was monitored spectrophotometrically at 254 nm by periodically applying an aliquot to a SAX anion-exchange HPLC column (Whatman) and eluting with 0.45 M KH_2PO_4 , pH 3.5. The reaction was quenched after 12 h by passage through a PM-30 ultrafiltration membrane filter and then loaded on a column of DE-52 (2.5 \times 50 cm). The product was eluted using a gradient of 10–300 mM triethylamine/ HCO_3^- , pH 7.5. Those fractions containing UDP(β S)-glucose were pooled, evaporated to dryness, and stored frozen. The yield was 85 μmol (85%). ^{31}P NMR showed -40.2 ppm (d, β -P, $J = 28$ Hz), 14.5 ppm (d, α -P, $J = 28$ Hz).

UDP(β S)-glucose was also synthesized using galactose-1-phosphate uridylyltransferase to catalyze the reaction between UDP-glucose and glucose 1-thiophosphate. The reaction mixture contained 0.75 mM glucose 1-thiophosphate, 0.5 mM UDP-glucose, 1.0 mM MgCl_2 , 5 μM glucose 1,6-bisphosphate, 125 mM Hepes, pH 7.5, 250 units of phosphoglucomutase, and 40 units of galactose-1-

phosphate uridylyltransferase. The reaction was quenched after 36 h and then applied to a column of DE-52 (2.5×50 cm). The UDP(β S)-glucose was then purified and isolated as described above. The yield was $8 \mu\text{mol}$ (40%).

Synthesis of galactose 1-thiophosphate (V). This compound was synthesized using galactokinase to catalyze the reaction between galactose (IV) and ATP(γ S). The reaction mixture contained 1.0 mM ATP(γ S), 10 mM galactose, 100 mM Hepes, pH 7.5, 1.0 mM MgSO_4 , and 20 units of galactokinase in a final volume of 45 ml at 25°C . The time course of the reaction was monitored with HPLC by quantitation of the ADP produced during the reaction. The product was isolated as described above. The isolated yield was $12 \mu\text{mol}$ (25%). ^{31}P NMR showed -41.8 ppm.

Synthesis of uridine-5'-O-(2-thiodiphosphogalactose) (VI). The reaction mixture contained 0.5 mM galactose 1-thiophosphate (V), 0.5 mM UDP-glucose, 2.5 mM MgSO_4 , 20 mM Hepes, pH 7.5, 400 units of phosphoglucomutase, and 70 units of galactose-1-phosphate uridylyltransferase in a volume of 50 ml. The rate of formation of UDP(β S)-galactose (VI) was monitored by HPLC with a SAX anion-exchange column. The product was eluted with 50 mM KH_2PO_4 , pH 3.5. UDP(β S)-galactose was purified as described above. The isolated yield was $12 \mu\text{mol}$ (50%). ^{31}P NMR showed -40.6 ppm (d, β -P, $J = 28$ Hz), 14.3 ppm (d, α -P, $J = 28$ Hz).

Synthesis of glucose 6-thiophosphate (VIII). This compound was synthesized by the action of yeast hexokinase on a mixture of glucose (VII) and ATP(γ S). The reaction mixture contained 1.5 mM ATP(γ S), 15 mM glucose, 15 mM MgSO_4 , 100 mM Hepes, pH 7.5, and 50 units of hexokinase in a volume of 15 ml. The reaction was complete in 5 h. The reaction was quenched and the product isolated using DE-25 as described previously. The isolated yield was $8 \mu\text{mol}$ (36%). ^{31}P NMR showed -41.26 and -41.21 ppm (α and β anomers).

Enzyme assays. The rates of the enzyme-catalyzed reactions with either the natural substrate or the thio analogs were obtained by monitoring the product of the reaction via HPLC, NMR, or uv spectroscopy. The Gilson HPLC system was equipped with a uv detector operating at 254 nm. In general, $20\text{-}\mu\text{l}$ samples were applied to a Whatman SAX anion-exchange column and then eluted with various concentrations of KH_2PO_4 (50–500 mM), pH 3.5, at a flow rate of 1.0 ml/min. ^{31}P NMR measurements were obtained with either a Varian XL-200 or XL-400 multinuclear NMR spectrometer operating at frequencies of 81 and 162 MHz, respectively. All chemical shifts were referenced relative to an internal standard of phosphate at pH 9.0. Upfield resonances are listed as positive chemical shifts. Ultraviolet spectral changes were recorded on a Gilford Model 260 or 2600 spectrophotometer.

Sucrose synthetase. This enzyme was assayed with the HPLC apparatus. Each assay mixture contained 4 mM DTT, 50 mM fructose, 50 mM Hepes, pH 7.3, 1 unit of sucrose synthetase, and 2.0 mM of either UDP-glucose or UDP(β S)-glucose in a volume of 4.0 ml. The time course was monitored by following the rate of formation of either UDP or UDP(β S) using 50 mM KH_2PO_4 , pH 3.5, as the eluting buffer.

Glycogen synthetase. Rabbit muscle glycogen synthetase was assayed in a

reaction mixture that contained 6 mg of glycogen, 50 mM Hepes, pH 7.5, 50 μ M glucose 6-phosphate, 1.0 mM of either UDP-glucose or UDP(β S)-glucose, and 1 unit of glycogen synthetase in a volume of 3.0 ml. The formation of UDP or UDP(β S) was determined using HPLC and 250 mM KH_2PO_4 as the eluting buffer.

UDP-glucose pyrophosphorylase. The forward reaction was monitored in a reaction mixture containing 4.0 mM UTP, 4.0 mM MgCl_2 , 3.0 mM dithioerythritol (DTE), 50 mM Hepes, pH 7.5, 15 units of inorganic pyrophosphatase, and 2.0 mM of either glucose 1-phosphate or glucose 1-thiophosphate. The reaction was initiated by the addition of 0.25–2.0 units of UDP-glucose pyrophosphorylase. The rate of the reaction was determined by quantitation of the UDP-glucose or UDP(β S)-glucose concentration by HPLC techniques.

The reverse reaction was measured in a solution containing 6 mM potassium pyrophosphate, 5 mM MgSO_4 , 50 mM Hepes, pH 7.5, and 1.2 mM of either UDP-glucose or UDP(β S)-glucose in a volume of 0.5 ml. The reaction was initiated by the addition of 0.1 unit of UDP-glucose pyrophosphorylase. The rate of formation of UTP was measured with HPLC using 0.45 M KH_2PO_4 as the eluting buffer.

Glucose-6-phosphate dehydrogenase. The reaction mixture contained 0.3 mM NAD, 100 mM Tris-HCl, pH 8.0, 0.25 units of glucose-6-phosphate dehydrogenase, and 0.67 mM glucose 6-phosphate or glucose 6-thiophosphate in a volume of 0.5 ml. The rate of formation of NADH was monitored spectrophotometrically at 340 nm.

Sucrose phosphorylase. The rate of this reaction was determined by integration of the ^{31}P NMR signal for the phosphoryl group of either glucose 1-phosphate or glucose 1-thiophosphate. The reaction mixture contained 50 mM phosphate or thiophosphate, 100 mM sucrose, 30% D_2O , 150 mM Hepes, pH 7.5, and 2–6.5 units of sucrose phosphorylase.

Phosphoglucomutase. The transfer of the phosphoryl group from C-1 to C-6 with either glucose 1-phosphate or glucose 1-thiophosphate was monitored with ^{31}P NMR spectroscopy. The 3-ml reaction mixture contained 30 mM glucose 1-phosphate or glucose 1-thiophosphate, 20 mM cysteine, 1.5 mM MgCl_2 , 10 μ M glucose 1,6-bisphosphate, 100 mM Hepes, pH 7.5, and 25% D_2O . The reaction was initiated with either 5 or 15 units of phosphoglucomutase.

Galactose-1-phosphate uridylyltransferase. The reaction mixture contained 1.0 mM glucose 1-thiophosphate, 0.8 mM UDP-glucose, 100 mM Hepes, pH 7.5, 1.0 mM MgCl_2 , and 20 units of galactose-1-phosphate uridylyltransferase. The rate of formation of UDP(β S)-glucose was measured by HPLC.

UDP-galactose-4-epimerase. The reaction mixture contained 3.3 mM UDP-glucose in a buffer containing 1.0 mM EDTA, 50 mM phosphate, 150 mM Tris, 25% D_2O , pH 9.0, and 0.01 unit of UDP-galactose-4-epimerase in a total volume of 3.0 ml at 25°C. The rate of formation of UDP-galactose was monitored by ^{31}P NMR spectroscopy. The spectrum was recorded every 10 min. Similar experiments were performed with 3.3 mM UDP(β S)-glucose and 7.5 units of UDP-galactose-4-epimerase, and the spectra were recorded at 60-min intervals. Similarly, the rate of formation of UDP-glucose was completed using 3.3 mM UDP-galactose and 0.1 unit of UDP-galactose-4-epimerase and the spectrum was recorded every 10 min. A similar experiment was also performed with 1.5 mM UDP(β S)-galactose

and 20 units of UDP-galactose-4-epimerase and the spectrum was recorded every 2 h.

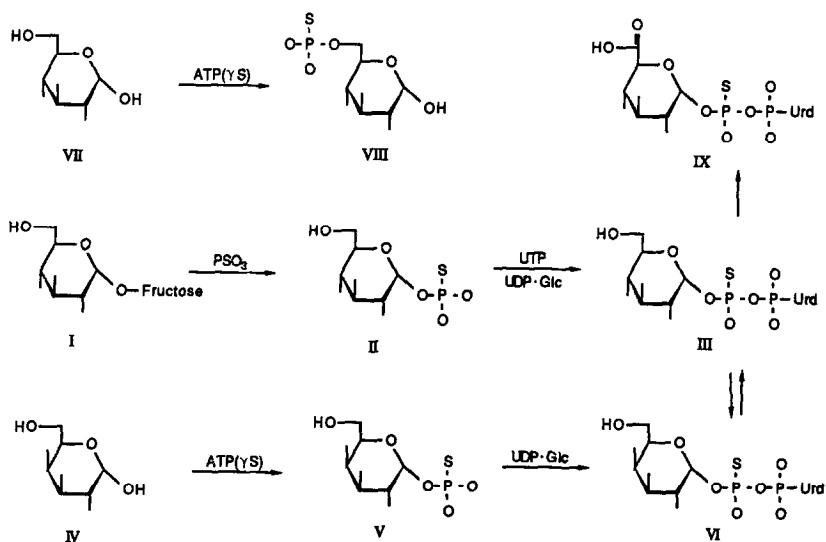
UDP-glucose dehydrogenase. The reaction mixture contained 0.2 mM of either UDP-glucose or UDP(β S)-glucose, 1.2 mM NAD, 50 mM Hepes, pH 7.5, and 0.2 unit UDP-glucose dehydrogenase in a total volume of 3.0 ml and at 25°C. The rate of formation NADH was monitored spectrophotometrically at 340 nm.

RESULTS AND DISCUSSION

The replacement of an oxygen atom with a sulfur atom in a variety of phosphate esters has provided a rich source of useful analogs of some common metabolites and cofactors (1). For example, the five possible thio-substituted analogs of ATP have been widely utilized as inhibitors of enzyme-catalyzed reactions and in the stereochemical determination of divalent metal ion binding to nucleotide triphosphates (4-6). Sulfur substitution at the various phosphoryl groups of nucleoside triphosphates has also enabled the overall net stereochemical course of the chemical reaction at the phosphorus center of many kinases and adenylyltransferases to be determined. Shown in Scheme 1 are some of the sugar phosphates into which we have successfully introduced a sulfur atom as a replacement for an oxygen. All of these compounds have been synthesized enzymatically.

α -Glucose 1-Thiophosphate (II)

The reaction scheme starts with the synthesis of α -glucose 1-thiophosphate (II) from thiophosphate and sucrose (I) in a reaction catalyzed by sucrose phosphorylase. Thiophosphate is a very good alternate substrate for sucrose phosphorylase.



SCHEME 1

TABLE 1
Relative Activities of Thio Analog Substrates

Compound	Concentration (mM)	Enzyme	Relative rate
Thiophosphate	50	Sucrose phosphorylase	0.24
α -Glc 1-(thio)P	30	Phosphoglucomutase	≤ 0.01
α -Glc 1-(thio)P	20	UDP-Glc pyrophosphorylase	0.13
α -Glc 1-(thio)P	1.0	Gal-1-P uridyltransferase	0.05
UDP(β S)-glucose	2.0	Sucrose synthetase	≤ 0.001
UDP(β S)-glucose	1.0	Glycogen synthetase	≤ 0.002
UDP(β S)-glucose	0.20	UDP-Glc dehydrogenase	0.87
UDP(β S)-glucose	3.3	UDP-Gal-4-epimerase	0.0003
Glc 6-(thio)P	0.67	Glucose-6-P dehydrogenase	0.37

At a concentration of 50 mM thiophosphate, the reaction rate is 24% of the rate when an identical concentration of phosphate is utilized as a substrate (see Table 1).

α -Glucose 1-phosphate was tested as a substrate for phosphoglucomutase. This was attempted by monitoring the conversion to glucose 6-thiophosphate using ^{31}P NMR spectroscopy. The thiophosphoryl group of α -glucose 1-thiophosphate resonates at -40.3 ppm and the expected chemical shift for the glucose 6-thiophosphate is -41.2 ppm (see below). After incubation for 24 h with 15 units of phosphoglucomutase we could not detect any transfer from C-1 to C-6. The estimated upper limit for the reactivity of α -glucose 1-thiophosphate for phosphoglucomutase is $\leq 1\%$ of the rate with glucose 1-phosphate.

UDP(β S)-Glucose (III)

α -Glucose 1-thiophosphate (II) was found to be a substrate for UDP-glucose pyrophosphorylase and galactose-1-phosphate uridyl transferase. In both of these reactions the final product was UDP(β S)-glucose. The isolated yields of the thio-substituted UDP-glucose product ranged from 55 to 80% after chromatography on DEAE-cellulose. The relative rate for the utilization of glucose 1-thiophosphate as compared with glucose 1-phosphate is 13% with UDP-glucose pyrophosphorylase and 5% when the reaction is catalyzed with galactose-1-phosphate uridyltransferase.

UDP(β S)-glucose was found to be a substrate for the UDP-glucose dehydrogenase reaction. At a substrate concentration of 0.20 mM the velocity for the oxidation of UDP(β S)-glucose was 87% of the rate for UDP-glucose. In addition, the UDP(β S)-glucose was transformed to UDP(β S)-galactose by the action of UDP-galactose-4-epimerase. However, at a substrate concentration of 3.3 mM the rate of the epimerization reaction is 3000 times slower than it is with UDP-glucose.

The absolute stereochemistry for the β -thiophosphoryl group of the thio-labeled UDP-glucose is unknown. The ^{31}P NMR spectrum showed only a single doublet for the β -thiophosphoryl group. The existence of both stereoisomers would have resulted in two pairs of doublets for the β -thiophosphoryl group since the mole-

cules would be diastereomers. The predicted chemical shift differences between the two possible isomers is approximately 0.48 ppm. This difference is based on the chemical shift difference between the two diastereomers of UDP(α S)-glucose that have been synthesized by the Frey group (13). It can therefore tentatively be concluded that only one of the two possible diastereomers are formed enzymatically from α -glucose 1-thiophosphate.

The UDP(β S)-glucose samples from each enzyme reaction were combined in an effort to determine whether the diastereomers produced from each enzyme reaction were identical. After mixing equal amounts of UDP(β S)-glucose from each enzyme-catalyzed reaction, the NMR signal for the β -thiophosphoryl group still showed a single doublet. Therefore, both enzymes catalyzed the formation of the same diastereomer. We have been unsuccessful in attempts to chemically synthesize UDP(β S)-glucose from a condensation of activated UMP and glucose 1-thiophosphate.

The UDP(β S)-glucose was tested with two enzymes to determine the relative ability of this new compound to serve as a substrate in glycosyl transfer reactions. UDP(β S)-glucose was not a substrate for either glycogen synthetase or sucrose synthetase. The relative upper limit for the transfer of the glycosyl group to fructose and glycogen was found to be 0.1 and 0.2%, respectively. However, it would be interesting to test the other diastereomer of UDP(β S)-glucose when that molecule becomes available since a large number of enzymes that utilize nucleoside phosphorothioates are very specific for the absolute configuration of the thiophosphoryl group. This selectivity is based either on the absolute configuration of the metal-nucleotide complex or on the direct interaction between the thiophosphoryl group with the enzyme. Therefore, the other diastereomer may show some reactivity.

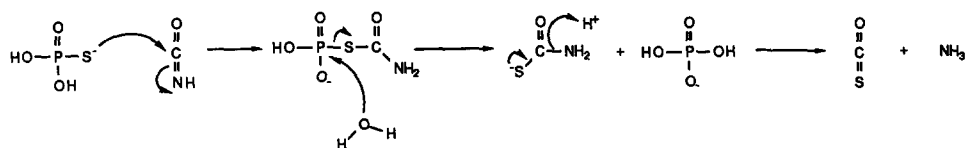
UDP(β S)-Galactose (VI)

The synthesis of the thio analog of UDP-galactose was initiated by the thiophosphorylation of galactose at C-1 with ATP(γ S) in a reaction catalyzed by galactokinase. The α -galactose 1-thiophosphate was a good substrate for galactose-1-phosphate uridylyltransferase. The reaction was initiated by the addition of α -galactose 1-phosphate and UDP-glucose. The formation of the thio-labeled UDP-galactose was enhanced by converting the glucose 1-phosphate product to glucose 6-phosphate with phosphoglucomutase. The absolute stereochemistry for the thiophosphoryl group is unknown but the ^{31}P NMR spectrum indicated that only one stereoisomer was formed.

The UDP(β S)-galactose was found to be a substrate for the UDP-galactose-4-epimerase reaction. The reaction was followed by monitoring the change in the chemical shift of the β -thiophosphoryl group upon conversion from UDP(β S)-galactose to UDP(β S)-glucose.

Glucose 6-Thiophosphate (VIII)

ATP(γ S) was found to thiophosphorylate glucose at C-6 in the presence of yeast hexokinase. The glucose 6-thiophosphate was not a substrate for phosphoglucomutase (see above) but was a substrate for glucose-6-phosphate dehydrogenase.



SCHEME 2

The relative rate compared at a substrate concentration of 0.67 mM was found to be 37%. Orr *et al.* (14) have previously shown that hexokinase catalyzes the smooth transfer of the thiophosphoryl group from ATP(γ S) to glucose. They have also demonstrated that glucose 6-thiophosphate is a substrate for glucose-6-phosphate dehydrogenase.

Carbamoyl Thiophosphate

Attempts were made to chemically synthesize carbamoyl thiophosphate from cyanate and thiophosphate at pH 5.0. Carbamoyl phosphate can be readily synthesized by simply mixing cyanate and phosphate and therefore has found great utility in the efficient synthesis of labeled ATP (^{32}P and ^{18}O) molecules via the coupling of carbamoyl phosphate and ADP with carbamate kinase. Upon mixing of excess cyanate and thiophosphate together at pH 5.0, all of the thiophosphate was rapidly converted to phosphate. The proposed mechanism for this transformation is illustrated in Scheme 2. Consistent with this proposal is the stoichiometric conversion of cyanate. When a twofold molar excess of thiophosphate is mixed with cyanate only 50% of the thiophosphate is converted to phosphate. The rest remains as thiophosphate. We were unable to detect the formation of thiocarbamoyl phosphate with ^{31}P NMR nor were we able to trap this molecule with ornithine transcarbamoylase. The hydrolytic reaction must therefore be very fast. Mikolajczyk has previously shown that phosphorothioate diesters react preferentially through the sulfur atom in reaction with dicyclohexylcarbodiimide (15). Therefore, the reaction scheme presented above was not totally unexpected.

SUMMARY

The thio analogs of UDP-glucose and UDP-galactose labeled at the β -phosphoryl group have been enzymatically synthesized. These compounds have proven to be substrates for UDP-glucose pyrophosphorylase, galactose-1-phosphate uridylyltransferase, UDP-galactose-4-epimerase, and UDP-glucose dehydrogenase. No activity was observed with glycogen synthetase or sucrose synthetase. These compounds should be useful as alternate substrates and inhibitors for glycosyl transfer enzymes. The results are summarized in Table 1.

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REFERENCES

1. FREY, P. A. (1982) *Tetrahedron* **38**, 1541–1567.
2. ECKSTEIN, F. (1970) *J. Amer. Chem. Soc.* **92**, 4718–4723.
3. KNOWLES, J. R. (1980) *Annu. Rev. Biochem.* **49**, 877–919.
4. BLATTNER, W., AND KNOWLES, J. R. (1979) *J. Amer. Chem. Soc.* **101**, 510–511.
5. SHEU, F. R., RICHARD, J. P., AND FREY, P. A. (1979) *Biochemistry* **18**, 5548–5556.
6. ECKSTEIN, F., AND GOODY, R. S. (1976) *Biochemistry* **15**, 1685–1691.
7. ECKSTEIN, F. (1985) *Annu. Rev. Biochem.* **54**, 367–402.
8. GERLT, J. A., CODERRE, J. A., AND MEHDI, S. (1983) *Adv. Enzymol.* **55**, 291–380.
9. FREY, P. A. (1982) *New Compr. Biochem.* **3**, 201–248.
10. SINGH, A. N., HESTER, L. S., AND RAUSHEL, F. M. (1987) *J. Biol. Chem.* **262**, 2554–2557.
11. GOODY, R. S., AND ECKSTEIN, F. (1971) *J. Amer. Chem. Soc.* **93**, 6252–6257.
12. RICHARD, J. P., AND FREY, P. A. (1982) *J. Amer. Chem. Soc.* **104**, 3476–3481.
13. SHEU, F. R., AND FREY, P. A. (1978) *J. Biol. Chem.* **253**, 3378–3380.
14. ORR, G. A., SIMON, J., JONES, S. R., CHIN, G. J., AND KNOWLES, J. R. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 2230–2233.
15. MIKOLAJCZYK, M. (1966) *Chem. Ber.* **99**, 2083–2090.