CHAPTER SEVENTEEN

Labeling Monosaccharides With Stable Isotopes

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Abstract

Chemical and chemi-enzymic methods are discussed for the preparation of monosaccharide isotopomers that are singly and multiply labeled with $^{13}$C, $^2$H, $^{17/18}$O, and $^{15}$N isotopes. The discussion focuses primarily on chemical methods to incorporate stable isotopes into monosaccharides and not on methods to assemble labeled monosaccharides into more complex biomolecules such as oligosaccharides or oligonucleotides. Two primary isotope insertion reactions are considered: cyanohydrin reduction (CR) and molybdate-catalyzed epimerization (MCE). Both methods are described in detail, including discussions of their mechanistic features, and their advantages and limitations. The integration of CR, MCE, and other chemical synthetic processes with enzyme-mediated synthesis is also discussed to illustrate how a wide range of singly and multiply labeled monosaccharides can be prepared for subsequent use in the assembly of more complex isotopically labeled biomolecules.

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1. INTRODUCTION

Carbohydrates are found throughout nature, serving as major energy sources in biological catabolism; molecular scaffolds for cell rigidity; barriers against bacterial and viral invasion in respiratory, intestinal and urinary tracts of vertebrates; defense molecules against tissue damage from freezing; mediators of cell–cell recognition during cell development; and modulators of protein folding, stability, and turnover in eukaryotic cells to name only a few of their biological functions (Taylor & Drickamer, 2011). These functions are made possible by their diverse structures and structural properties. The latter diversity poses significant challenges for the labeling of saccharides with isotopes; unlike amino acids and nucleotides, where a relatively small number of building block molecules are involved (20 and 8, respectively, to assemble native polynucleotides and oligonucleotides), many different types of monosaccharides are observed in nature, and they are assembled in multiple ways to give an incredibly large number of different types of oligo- and polysaccharides. Not only do labeling methods for saccharides need to have maximal flexibility to accommodate a large chemical space, but decisions on where to label monosaccharide building blocks and with which isotopes are further complicated by the type of scientific question being posed. There are no magic formulas to achieve labeling simplicity, except possibly to label all sites (e.g., uniformly label with $^{13}$C), and hope this strategy yields the desired information. Often it does not. Uniform labeling is attractive because it is comparatively fast and inexpensive. In contrast, deliberate labeling at one or more specific sites in a saccharide is more demanding of time, money, and synthetic expertise, but often leads to very precise information. The high cost of selective labeling is thus often more than offset by the simplicity of data analysis and interpretation.

Given the above constraints, it is not possible to discuss in one paper the many challenges faced by researchers who have a need for isotopically labeled saccharides. This chapter will thus focus narrowly on chemical methods to introduce stable isotopes into monosaccharides, with the $^{13}$C, $^2$H, $^{17/18}$O, and $^{15}$N isotopes treated exclusively. To make the treatment manageable, only monosaccharides commonly observed in vivo will be considered: D-glucose 1, D-mannose 2, D-galactose 3, D-arabinose 4, D-ribose 5, D-xylose 6, N-acetyl-D-glucosamine (GlcNAc) 7, N-acetyl-D-galactosamine (GalNAc) 8, N-acetyl-neuraminic acid (Neu5NAc) 9, and 6-deoxy-L-galactose (L-fucose) 10 (Scheme 1). This approach has advantages
because, as will be shown, chemical reactions to introduce stable isotopes into monosaccharides are relatively few in number, whereas reactions (both chemical and enzyme-catalyzed) to transform labeled monosaccharides into other labeled biomolecules, including other labeled monosaccharides, are divergent and too numerous to treat comprehensively here, although discussion of representative transformations is provided to illustrate some points. Transformative reactions, especially those that are enzyme-catalyzed, grow more numerous and practical over time, as new chemical processes are discovered, and new enzymes are isolated and made commercially available at reasonable cost.

2. TERMINOLOGY TO DESCRIBE DIFFERENT MONOSACCHARIDE ISOTOPOMERS

Simple monosaccharides contain multiple carbon, hydrogen, and oxygen atoms, and sometimes nitrogen atoms (e.g., 7–9), making possible a wide range of different isotopomers of any given monosaccharide that include $^{13}$C, $^2$H, $^{17}$O, $^{18}$O, and $^{15}$N stable isotopes. A monosaccharide can be singly (site-selective), multiply, or uniformly labeled in a given isotope. More than one type of isotope can be present in a labeled monosaccharide. Given this diversity, a general method to describe these isotopomers is helpful and such a method is described in Scheme 2. The upper case “O” and “E” symbols denote hOmonuclear and hEteronuclear labeling,
respectively. These designations are modified with subscripts to indicate the types of isotopes present in the molecule, listed in order of increasing mass. The lower case “s”, “m,” and “u” symbols designate single, multiple, or uniform labeling, respectively, of each different isotope in the molecule. This nomenclature is illustrated for different isotopomers of D-glucose 1 in Scheme 3. For example, D-[1-13C]glucose 1 (where ♦ denotes a 13C-labeled

Scheme 2 Nomenclature to describe different isotopomers of monosaccharides.

Scheme 3 Examples of terminology to identify different isotopomers of D-glucose 1.
carbon in the molecule in Scheme 3 and throughout the chapter) is denoted \( \text{OC}/s \), since it is labeled with one type of isotope \((^{13}\text{C})\) and contains only one labeled site. \( \text{D}^-[3^-2\text{H}; 1^-13\text{C}; 4^-17\text{O}]\)glucose is denoted as \( \text{E}_{\text{DCO}}/s \), since it contains three different isotopes \((^2\text{H}, ^{13}\text{C}, ^{17}\text{O})\) and each is present at a single site. \( \text{D}^-[3^-2\text{H}; 1,2,3,4,5,6^-13\text{C}_6; 2,3,4,6^-17\text{O}_4] \)glucose is denoted as \( \text{E}_{\text{DCO}}/\text{sum} \) since it contains three different isotopes \((^2\text{H}, ^{13}\text{C}, ^{17}\text{O})\), with single labeling by \(^2\text{H}\), uniform labeling by \(^{13}\text{C}\), and multiple labeling by \(^{17}\text{O}\).

Synthetic strategies for site-specific labeling (single and multiple) differ from those for uniform labeling, with the former generally achieved via chemical and/or chemo-enzymic processes, and the latter achieved by \textit{in vivo} biological processes. Thus, the synthesis of \( \text{D}^-[1^-13\text{C}] \)glucose is likely to involve a specific chemical process to introduce the \(^{13}\text{C}\) atom specifically at C1, whereas growing photosynthetic algal cells in a \( ^{13}\text{CO}_2 \) atmosphere leads to full incorporation of \(^{13}\text{C}\) atoms at all carbon sites (Behrens, Sicotte, & Delente, 1994). For example, in the latter case, the algal cells produce uniformly \(^{13}\text{C}\)-labeled intracellular starch, which is isolated and subsequently hydrolyzed chemically and/or enzymically to give \( \text{D}^-[1,2,3,4,5,6^-13\text{C}_6] \)glucose or uniformly \(^{13}\text{C}\)-labeled DNA, which is hydrolyzed to give uniformly \(^{13}\text{C}\)-labeled 2'-deoxyribonucleosides (Chandrasegaran, Kan, Sillerud, Skoglund, & Bothner-By, 1985). This chapter focuses mainly on purposeful (selective) methods of introducing stable isotopes into monosaccharides.

**3. INTRODUCING \(^{13}\text{C}\) INTO MONOSACCHARIDES**

Several factors must be weighed when selecting chemical reactions to introduce \(^{13}\text{C}\) site-selectively into monosaccharides. These factors include (a) the simplicity of the reaction; (b) the reliability of the reaction; (c) the reaction yield; (d) the scalability of the reaction; (e) the availability and cost of the \(^{13}\text{C}\)-labeled reagent; and (f) options for recycling labeled by-products. Factors (a)–(c) address the ability of the reaction to give consistently acceptable yields of the desired product with minimal investment of labor and reagents. Factor (d) is relevant if large quantities of a labeled product are required. Some reactions work well on smaller scales (milligram to gram amounts) but are not easily adapted to prepare labeled compounds in kilogram or greater amounts. Factor (e) is often overlooked but is crucial in minimizing the cost of labeling. For \(^{13}\text{C}\), the most cost-effective reagent is \(^{13}\text{CO}\), since it is the primary form in which \(^{13}\text{C}\) is enriched (Scheme 4); all other \(^{13}\text{C}\)-labeled one-carbon (secondary) reagents derive from \(^{13}\text{CO}\). The farther downstream the secondary labeled reagent is from \(^{13}\text{CO}\), the more
costly it tends to be on a molar basis. Factor ($f$) is also often overlooked, but due to the relatively high cost of labeling, labeled by-products ought to be convertible into the desired compound, or recycled into a reagent for subsequent use in other labeled syntheses.

### 3.1 Cyanohydrin Reduction

One of the most convenient reactions to introduce $^{13}$C into aldoses is cyanohydrin reduction (CR) (Scheme 5) (Serianni, Clark, & Barker, 1979; Serianni, Nunez, & Barker, 1979). This method utilizes K$^{13}$CN (or Na$^{13}$CN) as the labeled reagent, which is relatively inexpensive and...
commercially available in large quantities to support large-scale-labeled synthesis. The CR method is often confused with the well-known Kilani–Fischer (KF) synthesis of aldoses (Fischer, 1889; Kiliani, 1885), which is also shown in Scheme 5. In the KF method, a starting aldose reacts with KCN under alkaline conditions to produce a C2-epimeric pair of cyanohydrins (α-hydroxynitriles or aldononitriles) that subsequently undergo in situ hydrolysis to the corresponding C2-epimeric aldonate salts. The epimeric aldonates are separated by anion-exchange chromatography (Angelotti, Krisko, O’Connor, & Serianni, 1987), the aldonic acids are lactonized, and the lactones are reduced to aldoses with sodium amalgam (Na(Hg)) (Fischer, 1889) or sodium borohydride (NaBH₄) at pH 3–4 (Wolfrom & Wood, 1951). Early on, the KF method was used to synthesize [1-13C]aldoses (Walker, London, Whaley, Barker, & Matwiyoff, 1976), but it was soon realized that the method suffers from several limitations: (a) aldonate yields after chromatography vary widely; (b) some aldonates cannot be lactonized; (c) lactone reduction with Na(Hg) is cumbersome and often gives low yields; and (d) over-reduction to alditols often occurs with both Na(Hg) and NaBH₄.

The CR method eliminates the limitations of the KF method by conducting the cyanide condensation at an optimal solution pH that allows >90% conversion to the aldononitriles while keeping aldononitrile hydrolysis to a minimum (Serianni, Nunez, & Barker, 1980). The pH-stabilized aldononitriles are then reduced catalytically with a Pd/BaSO₄ catalyst under H₂ to give the C2-epimeric aldoses directly (Serianni, Nunez, et al., 1979). The pH of the heterogeneous catalytic reduction (hydrogenolysis) reaction is critical; the reaction is conducted at low pH (∼pH 4.2; adjusted with acetic acid) to promote the hydrolysis of the putative imine intermediate before it has an opportunity to be reduced to the amine. In most syntheses, the cyanohydrins are not isolated, but are reduced in situ to the C2-epimeric aldoses, which are separated by chromatography on a cation-exchange resin with Ca²⁺ as the counter-ion and distilled water as solvent (Angyal, Bethell, & Beveridge, 1979). In some cases, the C2-epimeric aldononitriles can be separated by chromatography prior to reduction (Serianni, Pierce, & Barker, 1979).

Since lactonization is not required by the CR method, short-chain aldononitriles can be reduced to short-chain aldoses. For example, formaldehyde and glycolaldehyde can serve as electrophiles, producing [1-13C]glycolaldehyde \(11^1\) and \(1\text{L-}[1-13C]\)glyceraldehyde \(12^1\) (superscripts denote the position of the \(^{13}\text{C}\) label), respectively, if \(\text{K}^{13}\text{CN}\) is used as the labeled reactant (Serianni, Clark, et al., 1979). In these cases, it was found that
conducting the Pd/BaSO₄ reduction at a pH below 4.3 is necessary to achieve a high-yield conversion to the aldose. Usually a pH of ~1.7 is used for these reductions. Presumably, the 2- and 3-carbon acyclic imines are more susceptible to over-reduction to amines compared to longer chain imines, the latter being partially protected from over-reduction by cyclization to glycosylamines (Scheme 6). The lower pH ensures full protonation of the acyclic imines, thus increasing their electrophilic character and making them more susceptible to attack by solvent water.

Scheme 6 Cyanohydrin reduction for a two-carbon (A) and five-carbon (B) [1-¹³C]α-hydroxynitrile (cyanohydrin). For C5 nitrile (B), cyclization of the acyclic imine intermediate (C) to give a cyclic glycosylamine (D) appears to protect the imine from over-reduction to the 1-amino-1-deoxy-alditol (E).
Complications can arise when unlabeled formaldehyde is used as the electrophile in CR with K\textsuperscript{13}CN (Scheme 7). Two-electron reduction of the two-carbon nitrile \textsuperscript{13}CN gives an intermediate protonated imine, which can partition into three reaction pathways. One leads to the undesired over-reduction product (primary amine). The second leads to the desired hydrolysis product, the 2-carbon aldehyde \textsuperscript{11}CN. However, \textsuperscript{11}CN appears to undergo subsequent slow isomerization under the reaction conditions via a putative symmetric ene-diol intermediate to give [2-\textsuperscript{13}C]glycolaldehyde \textsuperscript{11}CN. This isomerization leads to \textsuperscript{13}C-label scrambling in the desired product. In a similar manner, the protonated imine may also rearrange to give [2-\textsuperscript{13}C]2-aminoacetaldehyde. Reactions of this type often show \textsuperscript{13}C-label scrambling in the product glycolaldehyde if not conducted properly, and by-products containing \textsuperscript{13}C-labeled amino carbons are often observed. Label scrambling is avoided when [\textsuperscript{13}C]formaldehyde and K\textsuperscript{13}CN are used in CR.

The CR method is general in that almost any aldose can be used as the electrophile. For example, 5-deoxy-L-lyxose (Snyder & Serianni, 1987a) can be used to prepare 6-deoxy-L-[1-\textsuperscript{13}C]galactose (\textsuperscript{10}CN) (L-[1-\textsuperscript{13}C]fucose) and 6-deoxy-L-[1-\textsuperscript{13}C]talose using the same reaction protocol as that used for the simple aldopentose, D-lyxose. 2-Deoxy-D-ribose
(2-deoxy-D-erythro-pentose) can be used to prepare 3-deoxy-D-[1-13C]glucose and 3-deoxy-D-[1-13C]mannose (Scensny, Hirschhorn, & Rasmussen, 1983). While not extensively investigated, aldoses having >6 carbons can serve as reactants, but excess KCN is often needed to drive cyanohydrin formation to completion. In the same vein, the CR reaction can be applied to 2-ketoses, but excess KCN is needed to drive cyanohydrin formation. For example, 1,3-dihydroxypropanone (dihydroxyacetone) 14 can serve as an electrophile to produce the branched-chain aldose, 15, which is subsequently used to prepare DL-[2-13C]apiose 16 (Snyder & Serianni, 1987b) (Scheme 8). In general, since the cyanide condensation reaction is bimolecular, conducting the reaction at high aldose and cyanide concentrations helps drive cyanohydrin formation to completion, even when a 1:1 aldose:cyanide ratio is used.

Since excess K13CN is sometimes used to drive cyanohydrin formation, recovery of unreacted K13CN becomes a priority, especially when reactions are run on large scales. This recovery is achieved by purging reaction mixtures after cyanide addition is complete and the solution pH has been lowered from ~7.3 to ~4.2 with acetic acid. The reaction solution is aerated with N2 through a glass frit and the gas stream containing H13CN is flowed through methanolic KOH traps to recover the excess cyanide. The alcoholic K13CN solution can then be concentrated and the K13CN recovered for reuse.

The CR method has been automated through the design and use of a special reaction vessel and ancillary software to control it (Stafford, Serianni, & Varma, 1990). This work led to the recognition that CR reactions conducted in formic acid (rather than acetic acid) do not require the addition of H2 gas during the reduction step. The Pd/BaSO4 catalyst is able to cleave HCOOH in situ to give CO2 and H2, and the latter is subsequently consumed in CR.

![Scheme 8](image.png)

Scheme 8 The use of ketosugars as electrophiles in cyanohydrin reduction (CR).
3.2 Permutations of Cyanohydrin Reduction

The CR method can be adapted to introduce hydrogen isotopes at C1 of aldoses using isotopically labeled hydrogen gas in the hydrogenolysis step (Serianni & Barker, 1979). For example, the use of $^2$H$_2$ gas gives [1-$^2$H] aldoses (Scheme 9A). The reaction must be conducted in $^2$H$_2$O solvent because $^1$H–$^2$H exchange with solvent water occurs during reduction (Serianni & Barker, 1979). This approach to incorporate $^2$H at C1 complements that involving the reduction of aldonolactones to corresponding aldoses with NaB$_2$H$_4$ at pH 3–4 (Wolfrom & Wood, 1951). The latter method requires a lactone and reaction yields depend heavily on lactone ring size (1,4- vs. 1,5-lactones) and configuration. For example, the reduction of d-mannono-1,4-lactone with NaB$_2$H$_4$ gives a high yield of d-[1-$^2$H]mannose (>80%), whereas the same reaction applied to d-ribono-1,4-lactone gives d-[1-$^2$H]ribose in <30% yield. The lower yields are caused by poor reactivity of the lactone and/or by over-reduction to the alditol.

Aldoses undergo oxygen atom exchange at C1 in solvent water via involvement of the acyclic 1,1-$^g$em-diol (hydrate) tautomer (Scheme 9B) (Mega & Vann Etten, 1990). If the exchange reaction is conducted in oxygen-labeled water, then the oxygen isotope can be captured by subsequent cyanide addition and aldononitrile reduction to give [2-$^{17,18}$O]aldoses (Scheme 9B) (Clark & Barker, 1986). To ensure a high level of enrichment, the starting aldose is exchanged more than once with the labeled water; the number of exchanges depends on the reaction scale and the desired level of enrichment.

**A. Introduction of hydrogen isotopes at C1 of aldoses**

\[
\text{CHO} \quad \overset{	ext{KCN}}{\underset{	ext{Pd/BaSO}_4}{\text{Pd/BaSO}_4\text{pH 7.0–7.5}+\text{2H}_2}} \quad \text{CN} \quad \overset{\text{CHOH}}{\underset{	ext{H}_2}{\text{CHOH}}} \quad \text{C}_2\text{CHOH}
\]

**B. Introduction of oxygen isotopes at C2 of aldoses**

\[
\text{CHO} \quad \overset{\text{KCN}}{\underset{\text{Pd/BaSO}_4}{\text{Pd/BaSO}_4\text{pH 7.0–7.5}+\text{2H}_2}} \quad \text{CN} \quad \overset{\text{CHOH}}{\underset{\text{H}_2}{\text{CHOH}}} \quad \text{C}_2\text{CHOH}
\]

**C. Simultaneous incorporation of $^{13}$C, $^2$H, and $^{17}$O into aldoses**

\[
\text{CHO} \quad \overset{\text{KCN}}{\underset{\text{Pd/BaSO}_4}{\text{Pd/BaSO}_4\text{pH 7.0–7.5}+\text{2H}_2}} \quad \text{CN} \quad \overset{\text{CHOH}}{\underset{\text{H}_2}{\text{CHOH}}} \quad \text{C}_2\text{CHOH}
\]

**Scheme 9** Use of CR to introduce carbon, hydrogen, and oxygen isotopes into aldoses.
in the product. The extent of the oxygen exchange can be conveniently monitored by \(^{13}\text{C}\) NMR to ensure proper enrichment prior to entrapment of the labeled oxygen via CR (Mega & Vann Etten, 1990).

A single cycle of CR applied to a starting aldose can introduce carbon isotopes at C1, hydrogen isotopes at C1, and/or oxygen isotopes at C2 of the product aldoses (Scheme 9C) (Zhu, Zajicek, & Serianni, 2001).

While the CR permutations shown in Scheme 9 for the incorporation of hydrogen and oxygen isotopes into saccharides have merit, the CR method is most powerful for the insertion of carbon isotopes. Hydrogen and oxygen labeling via CR are most practical when they are coupled to carbon insertion. In the absence of carbon insertion, hydrogen and oxygen isotopes can be introduced via other routes since both atom types are terminal. There are many examples in the literature of this approach. Typically an alcohol is oxidized to a ketone, which is then subjected to exchange with oxygen-labeled water and/or reduced with an unlabeled or deuterated reducing agent (e.g., NaB\(^2\)H\(_4\)).

3.2.1 Generalized Laboratory Protocol—Cyanohydrin Reduction Reaction

To a 300-mL three-neck round-bottom flask in a well-ventilated fume hood is added 7.93 g (0.12 mol) of K\(^{13}\)CN, followed by distilled water (30 mL). A thermometer is secured via an adaptor into one of the necks and a magnetic stir bar is added to the reaction vessel. Another neck is fitted with a pH electrode. The solution is stirred at room temperature until all of the K\(^{13}\)CN has dissolved. The solution is then cooled to \(\sim 4 \, ^\circ\text{C}\) in an ice bath and glacial acetic acid is added dropwise to the cooled reaction solution until a pH of \(\sim 8.5\) is reached. The solution temperature is maintained near \(4 \, ^\circ\text{C}\) during the addition of the acid [Caution: The pK\(_a\) of HCN is 9.2, so the cyanide solution at pH \(\sim 8.5\) contains a significant amount of HCN, which is a highly poisonous gas. Care should be taken to avoid exposure to HCN by conducting the reaction in a fume hood, and by wearing an HCN monitor while conducting this part of the CR process].

In a separate reaction flask, the starting aldose (0.1 mol) is dissolved in \(\sim 40 \, \text{mL}\) of distilled water and the solution is cooled to \(4 \, ^\circ\text{C}\) in an ice bath.

The cooled aldose solution is added slowly to the cooled [\(^{13}\text{C}\)]cyanide solution via the third neck of the reaction vessel, making sure the reaction temperature remains below 10–15 \(^\circ\text{C}\) during the addition. After addition of the aldose solution is complete, the reaction solution is adjusted to pH \(\sim 7.5\) (HOAc or KOH) and the reaction mixture is warmed to room temperature
over ~5 min using a warm water bath. After ~15 min at pH ~7.5 and room temperature, the solution pH is lowered to ~4.2 with the addition of glacial acetic acid.

Excess labeled cyanide can be recovered by purging the reaction vessel with N₂ gas and passing the H¹³CN-laden gas stream through two or three methanolic KOH traps connected in series by Tygon™ tubing [Note: Excess cyanide must be removed from the reaction mixture prior to catalytic reduction because cyanide will poison the palladium catalyst]. After excess cyanide has been removed, the solution pH is adjusted to 4.2 with either HOAc or KOH.

Into a 250 or 500-mL Parr glass reduction vessel is added 6.2 g of 5% palladium on barium sulfate. Distilled water (~20 mL) is added, the vessel is evacuated and filled with H₂ gas, and the catalyst is pre-reduced at ~30 psi until it turns from a brown to a gray color. The reduction vessel is opened, the reaction solution containing the [1-¹³C]aldononitriles is added, the vessel is evacuated and filled with H₂ gas, and the reduction is conducted at ~30 psi until H₂ gas consumption ceases (3–4 h at room temperature with good shaking by the Parr apparatus).

After reduction is complete, the suspension is filtered to remove the catalyst, and the filtrate is treated batchwise and successively with excess Dowex 50-X8 (H⁺) cation-exchange resin and with excess Dowex 1-X8 (OAc⁻) anion-exchange resin. The deionized solution is concentrated at 30 °C in vacuo on a rotary evaporator to a volume of ~20 mL. This solution, which contains the product C2-epimeric [1-¹³C]-labeled aldoses, is applied to a chromatography column containing Dowex 50-X8 (200–400 mesh) cation-exchange resin in the Ca²⁺ form (Angyal et al., 1979). The column is eluted with distilled water and the eluting aldoses are detected with a reducing sugar assay (Hodge & Hofreiter, 1962).

### 3.3 Molybdate-Catalyzed Epimerization of Aldoses Accompanied by C1–C2 Transposition

A disadvantage of CR is that, since cyanide addition to the carbonyl is not stereospecific, two C2-epimeric aldoses form. The ratio of C2-epimeric aldononitriles depends on the solution pH of the cyanide addition reaction (Serianni et al., 1980). For example, in the KF synthesis under alkaline conditions using D-arabinose as the electrophile, the final gluco/manno ratio of aldonates favors the gluco epimer (~65%). In contrast, if cyanide addition is conducted at pH ~7.3, the gluco/manno ratio of aldononitriles favors the manno epimer (~70%) (Serianni, Nunez, et al., 1979). Experimental data
indicate that the KF ratio of aldonates is determined by the relative rates of hydrolysis of cyclic C2-epimeric imidolactone intermediates that form during the conversion of aldononitrile to aldonate (since CN addition is reversible and rapid under alkaline conditions), whereas the CR ratio of aldononitriles is determined by CN addition only (Serianni et al., 1980). Thus, from a biological viewpoint, CR applied to \( \alpha \)-arabinose gives the arguably more desirable \textit{gluco} epimer as the minor product.

Efforts to convert less-desirable C2-epimers obtained from CR into more-useful labeled products include chemical- and enzyme-catalyzed isomerization. For example, base-catalyzed aldose-2-ketose isomerization in the presence of boronic acids provides a means to chemically equilibrate an aldose with its C2 epimer and 2-ketose without significant alkaline degradation (Barker, Chopra, Hatt, & Somers, 1973; Barker, Hatt, & Somers, 1973). Aldose–ketose isomerases catalyze aldose-2-ketose exchange. For example, \( \alpha \)-\([1-\text{\textsuperscript{13}}\text{C}]\)mannose \( 2^1 \) is converted into an equilibrium mixture of \( \alpha \)-\([1-\text{\textsuperscript{13}}\text{C}]\)mannose \( 2^1 \), \( \alpha \)-\([1-\text{\textsuperscript{13}}\text{C}]\)glucose \( 1^1 \), and \( \alpha \)-\([1-\text{\textsuperscript{13}}\text{C}]\)fructose \( 17^1 \) by the action of xylose (glucose) isomerase (GI) (E.C. 5.3.1.5). In these cases, labeling integrity remains intact; the position of label in the starting aldose is faithfully transferred to the analogous position in the product(s).

The discovery of a remarkable carbon skeletal rearrangement in aldoses radically changed the strategies to prepare isotopically labeled monosaccharides and enhanced the applicability and overall efficiency of the CR method. Molybdate-catalyzed C2-epimerization (MCE) interconverts aldoses with their C2-epimers in high yield, as first shown by Bilik and coworkers (Bilik & Stankovic, 1973). It was assumed that backbone C–C bonds remain intact during the epimerization, and that breaking and forming C–H bonds underpin the mechanism (Bilik, Petrus, & Farkas, 1975). However, subsequent mechanism studies using \( ^{13}\text{C} \)-labeled substrates revealed unexpectedly that MCE proceeds via C1–C2 transposition that occurs in a stereospecific manner (Hayes, Pennings, Serianni, & Barker, 1982) (Scheme 10). The reaction can be viewed as an internal redox process where, in the forward direction, C1 is reduced and C2 is oxidized. Since C–H and C–O bonds in the C1–C2 fragment remain intact during the transposition, isotopic labels present at C1 are transferred intact to C2 and vice versa. Thus, as shown in Scheme 10, \( \alpha \)-\([1-\text{\textsuperscript{13}}\text{C};1-\text{\textsuperscript{2}}\text{H};1-\text{\textsuperscript{18}}\text{O}]\)mannose equilibrates with \( \alpha \)-\([2-\text{\textsuperscript{13}}\text{C};2-\text{\textsuperscript{2}}\text{H};2-\text{\textsuperscript{18}}\text{O}]\)glucose. Only catalytic amounts of sodium molybdate are required, the reaction is conducted in water, reaction times are normally <5 h, and the ratio of C2-epimeric aldoses is determined by their relative thermodynamic stabilities. Close inspection of
reaction mixtures reveals the presence of minor by-products that appear to arise from conventional isomerization/epimerization. Conducting MCE with resin-bound molybdate reduces the amounts of these nonspecific by-products, especially when tetroses and pentoses are used as reactants (Clark, Hayes, & Barker, 1986).

Studies show that hydroxyl groups at C1, C2, and C3 of the aldose are absolutely required for the reaction; that the acyclic hydrate form of the aldose is the reactive form; and that molybdate binds to the acyclic hydrate in a dimolybdate complex (Hayes et al., 1982). Inspection of these putative Mo–Mo complexes shows that H1 in the starting aldose and H2 in the product aldose are exposed and peripheral in the complex. This structural feature can be exploited in MCE by replacing H1 of the aldose with an R-group, thus allowing interconversion of a 2-ketose and a C2-branched-chain aldose (Hricoviniova-Bilikova, Hricovini, Petrusova, Serianni, & Petrus, 1999; Wu, Pan, Zhao, Imker, & Serianni, 2007; Zhao, Petrus, & Serianni, 2001). Two examples of this type of MCE reaction are shown in Scheme 11.

3.3.1 Generalized Laboratory Protocol—Molybdate-Catalyzed Epimerization of Aldoses
An aqueous solution containing an aldose (0.1 M) and molybdic acid (85%) (5 mM) at pH 4.5 (adjusted with acetic acid) is incubated at 90 °C for 2–13 h
depending on the aldose. The solution is cooled and treated batchwise and separately with excess Dowex 50-X8 (H\(^+\)) cation and Dowex 1-X2 (OAc\(^-\)) anion ion-exchange resins, and the resulting deionized solution is concentrated to \(\sim\)10 mL at 30 °C \textit{in vacuo} in a rotary evaporator. This solution is applied to a chromatography column containing Dowex 50-X8 (200–400 mesh) cation-exchange resin in the Ca\(^{2+}\) form (Angyal et al., 1979). The column is eluted with distilled water and the eluting aldoses are detected with a reducing sugar assay (Hodge & Hofreiter, 1962).

![Scheme 11](image)

**Scheme 11** The application of molybdate-catalyzed epimerization (MCE) to branched-chain aldoses.

4. MULTIPLE LABELING OF ALDOSES VIA CHAIN INVERSION

CR and MCE are attractive and economical processes when there is need to introduce isotopic labels at C1 and/or C2 of an aldose. Labeling at terminal hydroxymethyl (CH\(_2\)OH) carbons of aldoses can also be achieved using aldose derivatives such as 18 (King-Morris, Bondo, Mrowca, & Serianni, 1988) in conjunction with CR. It is more time-consuming and costly to introduce isotopic labels at \textit{internal} positions of aldoses (i.e., secondary alcoholic carbons). This problem can be overcome in some cases by chain inversion (Wu, Serianni, & Bondo, 1992). An example of this approach is shown in Scheme 12. \(\text{d-}[1,2-{\text{\textsuperscript{13}}C}_2]\)mannose 2\(^{1,2}\) is prepared from two rounds of CR starting from \(\text{d-}\)erythrose, with MCE used to improve overall yield by converting the \(\text{d-}[1,2-{\text{\textsuperscript{13}}C}_2]\)glucose 1\(^{1,2}\) by-product to \(\text{d-}[1,2-{\text{\textsuperscript{13}}C}_2]\)mannose 2\(^{1,2}\). Methyl glycosidation followed
by C6 oxidation and glycoside hydrolysis gives mannuronic acid, which is reduced to give D-[5,6-\(^{13}\)C\(^2\)]mannonic acid. The labeled mannonic acid is reduced via the 1,4-lactone to give D-[5,6-\(^{13}\)C\(^2\)]mannose 2\(^{5,6}\), or cleaved to give D-[4,5-\(^{13}\)C\(^2\)]arabinose 4\(^{1,5}\), which can be used in CR and MCE to give D-[1,5,6-\(^{13}\)C\(^3\)]glucose 1\(^{1,5,6}\) and D-[2,5,6-\(^{13}\)C\(^3\)]glucose 1\(^{2,5,6}\).

### 5. LABELING AT THE INTERNAL CARBONS OF ALDOSES

Integration of CR, MCE, and enzymic reactions permits the introduction of stable isotopes at internal positions of aldoses. These approaches are illustrated in Schemes 13 and 14. In Scheme 13, D-[1-\(^{13}\)C]ribose 5\(^1\) and D-[1-\(^{13}\)C]arabinose 4\(^1\) are produced by the application of CR with K\(^{13}\)CN to D-erythrose. MCE applied to D-[1-\(^{13}\)C]ribose 5\(^1\) gives D-[2-\(^{13}\)C]arabinose 4\(^2\), which when subjected to CR with K\(^{13}\)CN gives D-[1,3-\(^{13}\)C\(^2\)]glucose 1\(^{1,3}\) and D-[1,3-\(^{13}\)C\(^2\)]mannose 2\(^{1,3}\). MCE applied to the latter gives D-[2,3-\(^{13}\)C\(^2\)]glucose 1\(^{2,3}\). The by-product D-[1-\(^{13}\)C]arabinose 4\(^1\) can be subjected to CR with K\(^{13}\)CN to give D-[1,2-\(^{13}\)C\(^2\)]glucose 1\(^{1,2}\) and D-[1,2-\(^{13}\)C\(^2\)]mannose 2\(^{1,2}\), with the yield of the former improved by the application of MCE to the latter. Alternatively, D-[1-\(^{13}\)C]arabinose 4\(^1\) can be subjected to MCE to give D-[2-\(^{13}\)C]ribose 5\(^2\), which can be used in the synthesis of labeled ribo- and 2'-deoxyribonucleosides. The initial D-[1-\(^{13}\)C]ribose 5\(^1\) can also be funneled in ribo- and 2'-deoxyribonucleosides labeled at C1'. The routes shown in Scheme 13 illustrate the value of coupling CR with MCE; even though CR produces two C2-epimeric products, MCE can be used to funnel both epimers into useful and valuable labeled compounds, thereby eliminating waste and maximizing benefit.

Aldolase-catalyzed reactions offer the opportunity to insert stable isotopes into internal carbons of aldoses (Serianni, Cadman, Pierce, Hayes, & Barker, 1982). For example, in Scheme 14, [1-\(^2\)H]glycolaldehyde is subjected to CR with K\(^{13}\)CN to give DL-[1-\(^{13}\)C;2-\(^2\)H]glyceraldehyde. The labeled aldotriose is reacted with DHAP (dihydroxyacetone phosphate; 1,3-dihydroxypropanone phosphate) in the presence of rabbit muscle aldolase (RMA) (fructose bisphosphate aldolase) (E.C. 4.1.2.13) to give DL-[4-\(^{13}\)C;5-\(^2\)H]fructose 1P (F1P) and L-[4-\(^{13}\)C;5-\(^2\)H]sorbose 1P (S1P). RMA is used to resolve an enantiomeric mixture of the aldotriose into two diastereomers. After treatment with acid phosphatase (E.C. 3.1.3.2), the resulting labeled D-fructose can be converted into D-[4-\(^{13}\)C;5-\(^2\)H]glucose by the action of glucose (xylose) isomerase (GI) (E.C. 5.3.1.5). The by-product L-[4-\(^{13}\)C;5-\(^2\)H]sorbose 1P can either be re-equilibrated with
Scheme 12  A synthetic route to prepare D-[2,5,6-13C₃]glucose 1₂,₅,₆ from D-[1,2-13C₂]mannose 2₁,₂ by chain inversion.
Scheme 13 The integration of the CR and MCE reactions to prepare D-[1,3-13C2]glucose 1 and D-[2,3-13C2]glucose 1.

Scheme 14 The use of rabbit muscle aldolase (RMA) to prepare D-[4-13C;5-2H]glucose 1 from triose precursors.
RMA to improve the yield of the desired fructo or gluco products, or dephosphorylated to give \(1-[4^{-13}\text{C};5^{-2}\text{H}]\text{sorbose}\) for use in other labeled syntheses (e.g., fructose 17; see Scheme 27). The RMA preparation should be free of contaminating triose phosphate isomerase (TIM) (E.C. 5.3.1.1) activity in order to avoid the formation of unlabeled D-fructose 1,6-bisphosphate (FBP), which would lower the reaction yield. However, if TIM is present, the FBP can be separated from the desired monophosphates by anion-exchange chromatography.

### 6. EXTENSION TO BIOLOGICALLY IMPORTANT ALDOSES

The preceding discussion demonstrates that CR and MCE can be used to prepare a wide range of isotopically labeled biologically important aldoses and 2-ketoses. Correlations between the starting aldose and CR product aldoses (underlined) in Scheme 1 are as follows:

(a) D-arabinose 4 → D-glucose 1 and D-mannose 2
(b) D-lyxose → D-galactose 3 and D-talose
(c) D-erythrose → D-arabinose 4 and D-ribose 5
(d) D-threose → D-lyxose and D-xylose 6
(e) 5-deoxy-L-lyxose (Snyder & Serianni, 1987a) → 6-deoxy-L-galactose (L-fucose) 10 and 6-deoxy-L-talose

The N-acetylated 2-amino-2-deoxyaldoses, GlcNAc 7 and GalNAc 8, can be prepared using CR, with glycosylamines instead of aldoses serving as electrophiles (Scheme 15) (Zhu et al., 2006). D-Aldopentoses are converted in high yield with \(\text{NH}_3\) gas into glycosylamines, which are relatively stable in alkaline solution. Glycosylamines serve as good substrates in CR (as acyclic

![Scheme 15](image)

Scheme 15 The application of CR to the synthesis of isotopically labeled GlcNAc 7.
protonated imines) to give C2-epimeric aminonitriles, and the latter reduce in high yield to give a mixture of C2-epimeric 2-amino-2-deoxyaldoses. The latter are separated by cation-exchange chromatography and converted to the N-acetylated derivatives by EEDQ-mediated acetylation or by other methods. This approach allows the incorporation of $^{13}$C, $^2$H, and/or $^{15}$N isotopes in a single CR cycle as shown in Scheme 15. Use of appropriately labeled aldopentoses broadens the options for labeling GlcNAc 7 and GalNAc 8. If arabinose 4 is used at the starting pentose, then GlcNAc 7 (and ManNAc 19) are produced (Scheme 15). If D-lyxose is used as starting aldopentose, then GalNAc 8 (and TalNAc) are produced.

The CR route shown in Scheme 15 leads to the production of ManNAc 19, which along with pyruvate, can be used in the enzymic synthesis of Neu5Ac 9 (Scheme 1) (Klepach, Carmichael, & Serianni, 2008; Klepach, Zhang, Carmichael, & Serianni, 2008; Lin, Lin, & Wong, 1997). Since pyruvate is available commercially in different isotopomeric forms, and sialic acid (Neu5NAc) aldolase (E.C. 4.1.3.3) is available commercially, stable isotopically labeled 9 can be prepared as shown in Scheme 16. Purification of 9 is achieved by chromatography on an anion-exchange column (Klepach, Carmichael, et al., 2008).

Scheme 16 Enzymic synthesis of isotopically labeled Neu5Ac 9 using sialic acid aldolase.
7. RELATIVE CARBONYL REACTIVITIES IN OSONES—SYNTHESIS OF LABELED 2-KETOSES

The reactivity of ketoses as electrophiles in CR is generally lower than that of aldoses, presumably because of the greater steric hindrance at the internal carbonyl carbon in the former. This reduced reactivity can be exploited in the reduction of 1,2-dicarbonyl sugars, commonly denoted as osones. Osones can be generated chemically (Vuorinen & Serianni, 1990a) or enzymically (Freimund, Huwig, Giffhorn, & Köpper, 1998). For example, the 1,2-dicarbonyl sugar, L-threo-pentos-2-ulose 20, is produced from L-xylose via oxidation with Cu(OAc)$_2$ in aqueous methanol (Scheme 17). The osone, D-glucosone 21, is prepared by the action of pyranose 2-oxidase (E.C. 1.1.3.10) on D-glucose 1 (Zhang & Serianni, 2012). The oxidase displays broad specificity for the aldose substrate (Freimund et al., 1998), thus lending itself to the synthesis of a wide range of osones [e.g., 3-deoxy-D-glucosone 22 (Zhang, Carmichael, & Serianni, 2011)].

Scheme 17  Selective reactivity of the carbonyl groups in osone 20 to reduction (to give ketose 23) and to cyanide addition/hydrolysis (to give vitamin C 24).
Osones can be converted in high yield to 2-ketoses by reduction with Pt/C and H₂ (Vuorinen & Serianni, 1990b) (Scheme 17). The latter reaction exploits the relative susceptibilities of C1 and C2 to nucleophilic attack, either by hydride and cyanide anions. The reduction proceeds almost exclusively at C1, giving 2-ketose products. For example, as shown in Scheme 17, 20₁ can be reduced in high yield to L-[1-¹³C]threo-2-pentulose 23₁ (L-[1-¹³C]xylulose). If only slightly more than one equivalent of hydride donor is used, only very minor amounts of aldose and/or alditol by-products are produced. This approach provides access to isotopically labeled 2-ketosugars via labeled aldoses that may not be available via enzymic methods (see below).

The selectivity of C1 in osones to attack by cyanide anion is nicely demonstrated in the synthesis of ¹³C-labeled vitamin C 24 (Scheme 17) (Drew, Church, Basu, Vuorinen, & Serianni, 1996). This process is not CR per se, but rather KF in that the initially formed aldononitriles are permitted to cyclize to imidolactone intermediates (Serianni et al., 1980), which subsequently undergo hydrolysis under acidic conditions to give [1,2-¹³C₂] ascorbic acid 24¹.². Imidolactones have been implicated as key intermediates in the KF reaction (Serianni et al., 1980), and in this case form rapidly because of the conformational (flexibility) constraints in the acyclic nitrile backbone imposed by an sp²-hybridized C3 carbon. This process yields the reduced form of 24, an α,β-unsaturated γ-lactone.

8. MANIPULATION OF THREE-CARBON BUILDING BLOCKS IN ENZYME-MEDIATED ALDOL CONDENSATION

Aldose phosphates serve as viable electrophiles in CR. For example, glycolaldehyde phosphate can be converted into DL-[1-¹³C]glyceraldehyde 3P (G3P) by CR using K¹³CN as a reactant (Serianni, Pierce, et al., 1979). DL-[1-¹³C]G3P can be treated with TIM and RMA to give a single 2-ketohexose product after the reaction reaches equilibrium: L-[3,4-¹³C₂] sorbose 1,6-bisphosphate (Scheme 18). Only the L-sorbo product is obtained because only D-G3P is a substrate for TIM, thus creating the DHAP that becomes available for RMA-mediated condensation with L-G3P.

By applying the RMA reaction to an enantiomeric mixture of DL-glyceraldehyde and unlabeled DHAP in the absence of TIM, stable isotopes can be introduced into the bottom-half (C4–C6 carbons) of F1P and S1P (Scheme 19). DL-[1,2-¹³C₂]glyceraldehye 1₂₁.² is generated from
two rounds of CR. The dilabeled aldotriose is condensed with DHAP in the presence of RMA to give D-[4,5-\(^{13}\text{C}_2\)]fructose 1P and L-[4,5-\(^{13}\text{C}_2\)]sorbose 1P. Including a strong (transition state) inhibitor of TIM (e.g., 2-phosphoglycolate) in the reaction mixture minimizes the formation of unlabeled D-G3P, which would lead to the formation of unlabeled FBP as a by-product and lower the reaction yield of the desired labeled 2-ketose 1-phosphates. This problem can occur because some commercial preparations of RMA often contain contaminating TIM activity. However, if this side reaction does occur, it is possible to separate the labeled monophosphates from the unlabeled bisphosphate by anion-exchange chromatography (Serianni et al., 1982). Separation of the monophosphates is more easily achieved as the free 2-ketoses by acid phosphatase treatment (Serianni et al., 1982) and chromatography on Dowex 50 cation-exchange resin with Ca\(^{2+}\) as the counter-ion (Angyal et al., 1979).
The labeled D-fructose 17 and L-sorbose 25 are then converted into other useful labeled products (see Scheme 27).

While the routes shown in Schemes 18 and 19 are useful, better control over RMA-mediated isotope labeling of saccharides can be gained by exploiting ways to resolve the DL-mixture of glyceraldehyde generated by the application of CR to glycolaldehyde. The following discussion describes approaches to achieve this control.

Lin and coworkers (Hayashi & Lin, 1967) have shown that the enzyme, glycerol kinase (E.C. 2.7.1.30), accepts L-glyceraldehyde hydrate as a substrate, producing L-glycerol 3P. The kinase also accepts D-glyceraldehyde hydrate, but appears to bind it in an inverted orientation that positions one of the hemiacetal hydroxyl groups for phosphorylation (Scheme 20). The resulting phosphomonoester is labile upon release from the enzyme, and thus the kinase appears to behave as an “ATPase” when D-glyceraldehyde is the substrate. This behavior has been confirmed by NMR studies of reaction mixtures using 13C-labeled substrates (Serianni, Clark, et al., 1979). Glycerol kinase is thus able to resolve CR-generated enantiomers of DL-glyceraldehyde 12, a property that can be exploited as shown in the following reaction schemes.

It should be noted that an alternative approach to resolving DL-glyceraldehyde 12 enzymically might involve the use of triokinase (Frandsen & Grunnet, 1971; Rodrigues et al., 2014) (Scheme 21), which purportedly accepts only D-glyceraldehyde as substrate to give D-G3P, but this enzyme has not been fully characterized (it has been implicated in the metabolism of fructose in human liver) and is not commercially available.

![Scheme 20](image-url)
Top-half labeling (C1–C3) of 2-ketoheoses can be accomplished as shown in Scheme 22. DL-[1,2-13C2]glyceraldehyde 12 is prepared chemically by CR (Scheme 19), and glyceraldehyde kinase converts the L-enantiomer into L-[1,2-13C2]G3P, which is separated by anion-exchange chromatography from the unphosphorylated D-enantiomer. L-[1,2-13C2]G3P is chemically reduced to L-[1,2-13C2]glycerol 3P which serves as a substrate for glycerol 3P oxidase (E.C. 1.1.3.21) to give [2,3-13C2]DHAP. The labeled ketotriose is then condensed with unlabeled D-glyceraldehyde (produced chemically from D-fructose; Perlin, 1962) in the presence of RMA to give one product, D-[2,3-13C2]fructose 1P. As shown in Scheme 18, TIM activity in the last step should be kept to a minimum. To drive the oxidase reaction to completion, catalase (E.C. 1.11.1.6) is added to the reaction mixture to convert H2O2 into O2 and H2O. While glycerol 3P dehydrogenase (E.C.
1.1.1.8) could be substituted for glycerol 3P oxidase, the former enzyme requires the coenzyme, NAD$^+$, which requires recycling. Use of the oxidase (commercially available) eliminates this complication.

Top- and bottom-half labeling of 2-ketohexoses mediated by RMA can be achieved in multiple ways depending on the desired labeling pattern in the product. The simplest route is shown in Scheme 23 and involves a *symmetrically labeled glycerol intermediate*. In the example given, DL-[1,3-$^{13}$C$_2$] glyceraldehyde 121,3 is chemically reduced to [1,3-$^{13}$C$_2$]glycerol, which is acted on by glycerol kinase to give L-[1,3-$^{13}$C$_2$]glycerol 3P. The latter is treated with glycerol 3P oxidase, RMA, and TIM to give d-[1,3,4,6-$^{13}$C$_4$]FBP. Unlike the route shown in Scheme 22, TIM is required to generate the labeled G3P required by RMA. Key to this sequence is “symmetric labeling” in the DL-glyceraldehyde 12 starting material, whose reduction gives a *symmetrically labeled glycerol*. Since the latter triol is prochiral, lack of label symmetry would lead to label scrambling in the product ketose phosphate, as shown in Scheme 24. The need for labeling symmetry applies not only to $^{13}$C (as shown), but also to other isotopes (e.g., $^2$H and/or $^{17,18}$O).

A permutation of the route shown in Scheme 23 is shown in Scheme 25. In this pathway, label scrambling complications arising from the use of “asymmetrically labeled” DL-glyceraldehyde (e.g., Scheme 24) are eliminated by employing glycerol kinase early in the pathway to resolve the glyceraldehyde enantiomers. In the illustrated route, DL-[1,2-$^{13}$C$_2$]glyceraldehyde is converted to L-[1,2-$^{13}$C$_2$]G3P, which, after separation from the unphosphorylated d-glyceraldehyde enantiomer, is chemically reduced

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Scheme 23 Use of “symmetrically labeled” glycerol to prepare a symmetrically, multiply $^{13}$C-labeled ketose phosphate, d-[1,3,4,6-$^{13}$C$_4$]FBP.
Scheme 24 Illustration of $^{13}$C-label scrambling in the synthesis of tetra-$^{13}$C-labeled isoto- 

tomers of FBP when disymmetrically labeled glycerols are employed.

Scheme 25 The use of glycerol kinase, NaBH$_4$ reduction, and glycerol 3P oxidase to pre-

pare a single symmetrically, multiply $^{13}$C-labeled ketose phosphate, D-$^{[2,3,4,5-^{13}C_4]}$FBP.
to L-[1,2-13C2]glycerol 3P. The latter is converted to D-[2,3,4,5-13C4]FBP by the concerted action of glycerol 3P oxidase, catalase, RMA, and TIM. This route, like that shown in Scheme 23, produces “symmetrically labeled” 2-ketohexoses.

Further elaboration of the general labeling strategies shown in Schemes 22, 23, and 25 is shown in Scheme 26, where “asymmetric labeling” of the product 2-ketohexose is achieved. In this route, DL-[2,3-13C2]glyceraldehyde 12 is treated with glycerol kinase to give L-[2,3-13C2]G3P and unphosphorylated D-[2,3-13C2]glyceraldehyde. After purification of the phosphoester, chemical reduction with NaBH4 gives L-[2,3-13C2]glycerol 3P, which is converted to [1,2-13C2]DHAP with glycerol 3P oxidase and condensed with DL-[2-13C]glyceraldehyde in the presence of RMA to give D-[1,2,5-13C3]fructose 1P and L-[1,2,5-13C3]sorbose 1P. To achieve optimal yields, TIM activity should be eliminated, but like before (Scheme 19), this activity can be tolerated. Further processing of the D-fructose 1P and L-sorbose 1P may involve treatment with acid phosphatase (E.C. 3.1.3.2), separation of the 2-ketohexoses by chromatography, and/or the application of chemical or enzymic reactions to give the desired products.

It should be noted that, since RMA exhibits broad specificity with regard to the aldose substrate, the reaction routes shown in Schemes 22 and 26 can be modified to produce, for example, 2-ketopentoses if glycolaldehyde 11 is
used instead of D- or L-glyceraldehyde in the RMA reaction. Thus, for example, using [1-13C]glycolaldehyde 11 instead of DL-[2-13C]glyceraldehyde in the RMA-catalyzed step in Scheme 26 would give only one product, D-[1,2,5-13C3]xylulose 1P.

9. MANIPULATION OF ISOTOPICALLY LABELED D-FRUCTOSE 17 AND L-SORBOSE 25

The enzymic routes shown in Schemes 18–19 and 22–26 use RMA to catalyze stereospecific aldol condensation between DHAP and G3P or glyceraldehyde. Absolute configurations at C3 and C4 in the 2-ketohexose products are specified by the enzyme, such that only D-fructo and/or L-sorbo configurations (C3–C4 D-threo) form. Analogous DHAP-aldolases have been isolated and characterized that produce the other three C3–C4 absolute configurations (D-erythro; L-threo; L-erythro) (Falcicchio, Wolterink-Van Loo, Franssen, & van der Oost, 2014), and thus the other six 2-ketohexoses (L-fructo; D-sorbo; D-psico; L-psico; D-tagato; L-tagato) are accessible enzymically via the same strategies discussed for RMA. Because RMA is the most common Class I aldolase and is commercially available at reasonable cost, there is a strong incentive to use this enzyme in the three-carbon precursor assembly of 2-ketohexoses. In addition, the D-fructo and L-sorbo products can be converted into other saccharides, as shown in Scheme 27. After dephosphorylation, D-fructose 17 is converted enzymically into D-glucose 1 by glucose (xylose) isomerase (E.C. 5.3.1.5). L-Sorbose 25 is reduced to L-sorbitol (D-glucitol) chemically, and the latter oxidized to D-fructose by D-sorbitol dehydrogenase (E.C. 1.1.1.14), with the required NAD+ coenzyme recycled using the lactate dehydrogenase system. Note that in the conversion of 25 to 17, the carbon chain is inverted; for example, 13C labeling at C1 and C2 of 25 gives 17 with 13C-labeling at C5 and C6.

10. CONCLUDING REMARKS

Pioneering studies in the latter half of the twentieth century involving stable isotopically labeled carbohydrates revealed substantial shortcomings in the laboratory methods available for isotope labeling. Since then, two core developments have occurred that simplify the isotopic labeling of saccharides significantly: (1) the introduction of new high-yield isotope insertion reactions, CR and MCE, that are simple to perform, reliable, and scalable; and (2) increased commercial availability of carbohydrate-transforming
enzymes at an affordable cost. These developments have greatly improved access to, and the structural diversity of, isotopically labeled monosaccharides. These innovations have also facilitated the conversion of labeled monosaccharides into an ever-widening range of biologically relevant labeled compounds that either retain their saccharide character (e.g., enzymic assembly into stable isotopically labeled oligosaccharides (Rosevear, Nunez, & Barker, 1982)) or serve as synthetic precursors in the preparation of other types of labeled biomolecules (e.g., ribo- and 2'-deoxyribonucleosides (Kline & Serianni, 1990; Bandyopadhyay, Wu, & Serianni, 1993; Wu & Serianni, 1994); vitamins (Drew et al., 1996)). While notable progress has been made over the past 50 years, new insertion and conversion reactions—chemical, enzymic, and biological—will continue to be discovered and developed in the coming years. At the same time, applications of stable isotopically labeled saccharides and their derivatives

Scheme 27 Enzymic routes to recycle d-fructose 1P and l-sorbose 1P.
to scientific and technical problems are likely to grow, including fundamen-
tal structural and mechanistic studies, metabolic pathway elucidation (Meier, Karlsson, Jensen, Lerche, & Duus, 2011), and noninvasive medical
diagnostic testing (e.g., breath tests; Robayo-Torres et al., 2009).

It is not hyperbole to assert that current labeling technology for simple
monosaccharides allows access to virtually any $^{13}$C, $^2$H, and/or $^{17,18}$O iso-
topomer that can be imagined, although synthesis costs vary widely with the
isotopomeric target. Cost still remains an impediment to more routine use of
stable isotopically labeled saccharides in research. The two core insertion
reactions described in this report, CR and MCE, have properties and effi-
ciencies often associated with enzyme reactions, and thus are very well suited
for the synthesis of labeled compounds. Both reactions are adaptable to many
different starting aldoses and, importantly, are scalable to the kilogram level
and greater. The most significant shortcoming of CR is its lack of stereo-
specificity, but methods are available to fully utilize both C2-epimeric aldose
products, thus offsetting this shortcoming to a reasonable extent. Commer-
cial production of D-[1-$^{13}$C$^1$]glucose routinely involves individual CR
reactions that are conducted with hundreds of grams of D-arabinose and
K$^{13}$CN, thus reducing the per-gram cost of this compound appreciably.
Large-scale isolation and purification of labeled aldoses and other saccharides
involve the use of reverse osmosis to rapidly and cost-effectively reduce the
volume of aqueous solutions of labeled monosaccharides prior to their puri-
fication by chromatography. The CR and MCE reactions have also been
used in cGMP syntheses of labeled monosaccharides destined for use in
human clinical trials.

While current synthetic methods provide access to virtually any iso-
topomer of simple monosaccharides, the adage that “availability begets
need” fails if cost to the research community is prohibitive. The cost of
producing a saccharide isotopomer is largely determined by three factors:
(1) the cost of raw isotope and other reagents required in the synthesis
(2) the properties of the synthetic route (length; reliability; scalability; yield;
ease of product purification; product purity requirements; isotopic
enrichment requirements; options for recycling labeled by-products
into desirable isotopomers)
(3) the cost of labor to perform the synthesis
The above discussion has focused heavily on factor (2) for good reason:
Factor (2) is core to determining total cost because it influences the contribu-
tions made by factors (1) and (3). Lengthy reaction routes increase labor
cost. Unreliable reactions introduce uncertainty, which is reflected in
increased cost. Unscalable reactions increase per-gram cost. It is clear that suboptimal reaction routes are tightly coupled to increased cost. However, independent contributions to cost made by factor (1) are not insignificant, and future improvements in enrichment methods to produce, for example, K\(^{13}\)CN, will help reduce isotope costs. Enzymic transformations can be limited by the availability and cost of enzymes, but improvements in protein expression will reduce costs in the long run. It can be anticipated that “synthetic” enzymes based on nonpeptide scaffolds may eventually replace amino-acid-based polymeric catalysts. In many labeled syntheses, factor (3) is a major contributor to cost, and in some cases the dominant contributor. Keeping reaction routes short and simple minimizes the cost contribution from (3) by reducing synthesis times and minimizing the level of chemical expertise needed to conduct the synthesis. Likewise, it can be anticipated that further reductions in the contribution of labor to isotope cost will evolve from laboratory automation, and signs already indicate that automation will become more widespread as time evolves (Godfrey, Masquelin & Hemmerle, 2013). This automation will be most easily implemented for reaction routes that employ simple processes. Thus, there is a strong synergy between factors (2) and (3), as there is between factors (2) and (1).

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