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Thiazolidine Peracetates: Carbohydrate Derivatives that Readily Assign *cis*-, *trans*-2,3- Monosaccharides by GC/MS Analysis.

Neil. P. J. Price,[1]* Trina M. Hartman,[1] Karl E. Vermillion.[2]

U.S. Department of Agriculture, National Center for Agricultural Utilization Research, [1]Renewable Product Technology and [2]Functional Foods Research Units, 1815 N. University St., Peoria, IL 61604, USA.

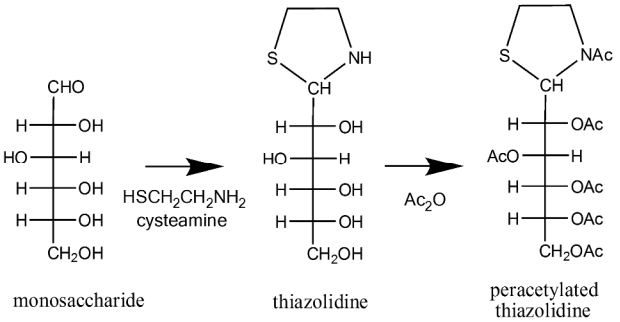
ABSTRACT: A novel group of carbohydrate derivatives is described that uniquely assign *cis*/*trans*-2,3 aldose stereoisomers at low nanomolar concentrations. Aldopentoses or aldohexoses, or component aldoses from hydrolysis of polysaccharides or oligosaccharides, react with cysteamine in pyridine to give quantitative formation of thiazolidines, which are subsequently peracetylated in a one-pot reaction. The non-polar thiazolidines peracetate (TPA) derivatives are analyzed by GC/EI-MS, each aldose giving rise to two TPA geometric isomers. The quantitative ratio of these diastereomers is dependent upon whether the parent monosaccharide is *cis*-2,3- (Rib, Lyx, Man, All, Gul, and Tal), or *trans*-2,3-aldose (Xyl, Ara, Glc, Gal, Ido, and Alt). TPAs generate observed EI-MS fragments ions characteristic of C1 – C2 and C3 – C4 bond cleavage of the parent sugars. This has been used to estimate the extent of metabolic labelling of microbial cell-wall carbohydrates, especially into the defining anomeric carbons and during aldolase/ketolase-catalyzed rearrangements.

INTRODUCTION

The compositional determination of glycoconjugates, polysaccharides, or oligosaccharides at low concentrations generally relies on acid hydrolysis to release the component monosaccharides followed by derivatization and analysis by gas chromatography-mass spectrometry (GC/MS).[1-4] The chemical derivatization of the component monosaccharides is to provide non-polar compounds with low volatility for improved chromatographic properties. Three types of derivatives are currently in general usage: alditol peracetates (AP),[5] aldononitrile peracetates (PAAN),[6] and trimethylsilyl methyl glycosides (TMS-MG).[7] These each have various advantages, and are often used in combination, and are readily detected by GC (for example, with flame ionization detection) or by GC/MS.

The electron impact mass spectral (EI-MS) data obtained by GC/MS is useful to distinguish the monosaccharide derivatives from potential non-carbohydrate components or contaminants, but neither this nor collision-induced dissociation mass spectrometry (CID-MS) provides much additional structural information. This is because the monosaccharides are geometric isomers of each other, and generally difficult to distinguish other than by chromatographic retention times (requiring known standards) or by resorting to less sensitive techniques such as nuclear magnetic resonance (NMR) spectroscopy. In addition, the EI-MS fragmentation pathways for AP, PAAN, and TMS-MG derivatives occurs with ionization at the non-reducing end of the parent monosaccharides, and therefore provides little information about the characteristic anomeric carbon (carbon C1) of the sugars.[8] We have previous described dialkyldithioacetal acetate (DADTA) derivatives that have this desirable property, and which generate well-resolved base ion peaks arising from C1-C2 bond cleavage of the parent monosaccharide with charge retention at the C1 thiol groups.[8] However, we also noted that the stability of the carbohydrate DADTA derivatives is poor and not suitable for prolonged storage, and that the thioalkane reagents needed (for example, ethanethiol) are both volatile and highly odorous to work with in the laboratory.[8]

In the present work we describe a new type of carbohydrate derivatives that are suitable for analysis by GC/MS. Reaction of free aldoses (pentoses or hexoses), or the component aldoses arising from acid hydrolysis of polysaccharides or oligosaccharides, with excess cysteamine hydrochloride in pyridine resulted in the quantitative formation of thiazolidines (Scheme 1).



Scheme 1. One-pot preparation of thiazolidine peracetates (TPA) from aldose sugars.

These are readily peracetylated in a one-pot reaction with acetic anhydride using the pyridine solvent from the prior

reaction as the base catalyst. Following water:ethyl acetate partitioning the non-polar thiazolidines acetate (TPA) derivatives are submitted to analysis by GC/EI-MS. Each aldose gives rise to two TPA geometric isomers that are readily separated by the GC chromatography. We report that the quantitative ratio of these two isomers is dependent upon whether the parent monosaccharide is a *cis*-2,3- (Rib, Lyx, Man, All, Gul, and Tal), or a *trans*-2,3-aldose (Xyl, Ara, Glc, Gal, Ido, and Alt). Hence, this method is able to distinguish *cis*/*trans*-2/3 aldose isomers at low concentration by GC/MS analysis alone. In addition, the high stability of the base ions generated by GC/EI-MS (*m*/*z* 130 and 88) are characteristic for these derivatives, and like the previously described DADTA arise from C1-C2 bond cleavage with charge retention at the C1 anomeric carbon of the parent sugar. We therefore also demonstrate the potential for using this selective ionization pathway of the new TPA derivatives for metabolic flux analysis following incorporation of stable isotope ^{13}C-labeled metabolites into the anomeric carbon of multiple aldose metabolic end-products. The new TPA derivatization procedure is demonstrated for 32 neutral aldoses and aminosugars, and is applied for the analysis of four complex oligosaccharide and polysaccharide sugars.

RESULTS AND DISCUSSION

(i) **Preparation and Analysis of Carbohydrate Thiazolidine Peracetates.** The thiazolidine peracetate derivatives (TPAs) were prepared from aldoses as described previously.[9] Carbohydrate samples were dissolved in a small quantity (typically 1 mL) of saturated cysteamine hydrochloride dissolved in pyridine. The thiazolidines are formed on heating (60 °C, 15 min), and subsequently peracetylated *in situ* by adding an equal volume of acetic anhydride and reheating. The excess reagents are removed by evaporation, and the TPAs are re-dissolved in ethyl acetate. An important aspect of the thiazolidines is that they are derived from the open chain form of the original sugars, and therefore the analysis is not complicated by pyranosyl or furanosyl cyclic forms. The peracetylation also has several advantages. The thiazolidine reaction is reversible via a Schiff's base ring opening of the thiazolidine group,[9-11] but once *N*-acetylated on the thiazolidine amino group the reverse reaction is precluded. The peracetylation step also allows a direct assessment of the completeness of the thiazolidine reaction, since any unreacted sugars should be immediately apparent in the GC/MS analysis as peracetylated sugars. This provides additional confidence that the conversion of the sugars to TPA is indeed quantitative.

To further assess the quantitative linearity of the described GC method, and to determine the minimum detection limit, a representative hexoaldose (D-glucose) and pentoaldose (D-arabinose) were evaluated over the concentration range 0.2 – 2.0 nmoles injected (supplementary Fig. S.1). These assessments were initiated with stock dilutions of the sugars (0.4 – 4.0 μmoles.mL^{-1}) and the estimates include the serial dilutions, TPA derivatization reactions, and solvent extraction steps, as well as the GC response. Both TPA derivatives were linear over 0.4 – 4.0 nmole range with R-squared regression values of 0.9999 (Glc-TPA) and 0.9935 (Ara-TPA). At concentrations less than 0.4 μmoles.mL^{-1} (0.2 nmoles injected) the regression values dropped to 0.9989 and 0.9772, respectively, indicating a minimal detection limit of 0.4 nmoles injected onto the column. We also note that these quantitative lower limit values were similar to those obtained for the GC analysis of Glc and Ara as either alditol acetate or aldononitrile acetate derivatives.[8]

(ii) **Gas Chromatography/Mass Spectrometry (GC/MS) Assignment of the 2,3-Stereochemistry of Monosaccharides**. All of the eight neutral hexose and the four pentose aldose sugars were converted into TPAs and analyzed by GC/MS (Table 1). As expected, each monosaccharide gives rise to two GC peaks corresponding to the 1*S* and 1*R* stereoisomers of the thiazolidine ring (Fig. 1).

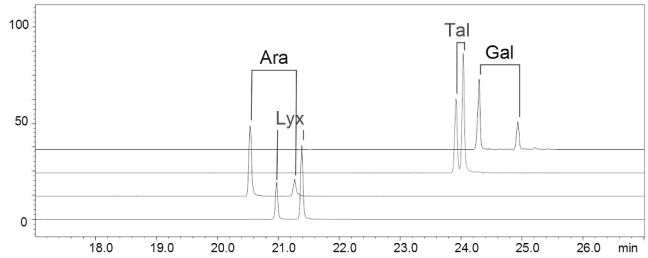
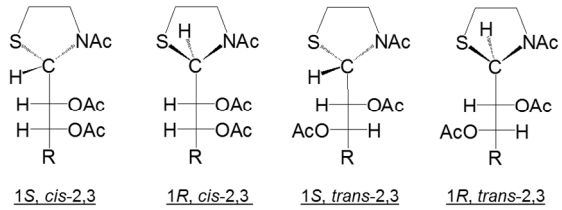


Fig. 1. Chromatographs of *cis*-2,3 (Tal, Lyx) and *trans*-2,3 (Gal, Ara) TPAs. A/B peak height ratios are small for cis-2,3 and large for trans-2,3 TPA derivatives, where A and B is the order of elution from the GC.

Unexpected, these two isomers were not produced in equal amounts, but rather one peak was generally larger than the other (Fig.1, Table 1). Examination of all of the sugar TPAs showed a trend, where the earlier eluting GC peak (Peak A) is greater for *trans*-2,3 monosaccharide TPAs, and smaller for *cis*-2,3 TPAs (Table 1). Hence, it is possible to distinguish the 2,3-stereochemistry of the TPAs (Scheme 2) by GC analysis alone, irrespective of the retention times.



Scheme. 2. 1*S*, 1*R* stereoisomers of *cis*-2,3 and *trans*-2,3 carbohydrate TPAs.

To evaluate this more closely, we analyzed glyceraldehyde, erythrose, three 6-deoxy sugars (fucose, rhamnose, and quinovose), two 2-deoxy sugars, two mono-methoxy sugars, and six amino sugars (Table 1). Erythrose and the 6-

Table 1. Relative retention times and peak height data for *cis*- and *trans*-2,3 TPAs.

		Peak Height			Peak Retention Time (min)		
Monosaccharide TPA	**2,3 - *cis*/*trans***	**Peak A***	**Peak B**	**Pattern**	**Peak A**	**Peak B**	**Difference**
Xylose TPA	*trans*	**37915**	21126	large/small	20.89	21.32	0.43
Arabinose TPA	*trans*	**92014**	20134	large/small	20.53	21.26	0.72
Ribose TPA	*cis*	147925	**204874**	small/large	20.29	20.60	0.31
Lyxose TPA	*cis*	419017	**841008**	small/large	20.97	21.38	0.41
Mannose TPA	*cis*	905383	**2090429**	small/large	24.36	24.69	0.33
Glucose TPA	*trans*	**2162551**	1955817	large/small	24.27	24.52	0.24
Galactose TPA	*trans*	**6764241**	2927075	large/small	24.29	24.93	0.64
Allose TPA	*cis*	1095563	**1860949**	small/large	23.67	23.81	0.14
Gulose TPA	*cis*	1248147	**2945696**	small/large	24.48	24.73	0.25
Talose TPA	*cis*	36208	**1232949**	small/large	23.92	24.03	0.11
Altrose TPA	*trans*	**887460**	551104	large/small	24.06	24.53	0.47
Idose TPA	*trans*	**38645**	31914	large/small	24.38	24.61	0.22
Glyceraldehyde TPA	-	**968181**	858085	large/small	12.15	12.65	0.50
Erythrose TPA	*cis*	93326	**111155**	small/large	16.78	17.08	0.30
Rhamnose TPA	*cis*	113170	**269718**	small/large	20.57	20.88	0.31
Fucose TPA	*trans*	**603701**	161772	large/small	20.28	21.14	0.86
Quinovose TPA	*trans*	**1429709**	1115069	large/small	20.68	21.01	0.33
2-deoxy glucose TPA	-	**126790**	109307	large/small	23.05	23.25	0.20
2 deoxy galactose TPA	-	80950	-	-	23.39	-	-
2-*O*-Me glucose TPA	*trans*	**2606791**	2522815	large/small	23.40	24.16	0.77
3-*O*-Meglucose TPA	*trans*	**83635**	79875	large/small	23.68	23.73	0.05
Mannosamine TPA	*cis*	**1646740**	368569	large/small	26.47	27.18	0.71
ManNAc TPA	*cis*	3404070	**3564379**	small/large	26.45	27.23	0.78
Glucosamine TPA	*trans*	**1458540**	424724	large/small	26.00	27.02	1.03
GlcNAc TPA	*trans*	**5211330**	4424536	large/small	25.95	27.02	1.07
Galactosamine TPA	*trans*	**19889**	4342	large/small	25.86	27.23	1.36
GalNAc TPA	*trans*	**17381560**	275652	large/small	25.78	27.20	1.42

* peaks A and B are as labeled in Figure 1.

deoxy sugars followed the same *cis*/*trans*-2,3 rules observed for the neutral hexoses and pentoses (Table 1). For glyceraldehyde the difference in peak A/peak B height ratios is relatively small. However, the 2-deoxy sugar TPAs did not conform to the *cis*/*trans*-2,3 peak ratio rules. 2-DeoxyGal TPA gave a single peak, presumably due to unresolved diastereomers. However, 2-*O*-MeGlc and 3-*O*-MeGlc both have large peak A/peak B ratios characteristic of *trans*-2,3 TPA derivatives.

The amino sugars give the same TPA products irrespective of whether they are 2-*N*-acetylated (ManNAc, GlcNAc, GalNAc) or not (ManN, GlcN, GalN), and hence have the same GC retention times (Table 1). The GlcN-TPA and GalN-TPA conform to the observed 2,3-*cis*/*trans* rules, confirming the results seen for the neutral sugars, but not the ManN-TPA, which was distinctly different to the ManNAc-TPA (Table 1). To confirm this, we prepared thiazolidines from ManN, ManNAc, and ManNAc after de-*N*-acetylation by acid hydrolysis (Supplementary Fig. S.2.). After the subsequent peracetylation step all three samples give the identical ManNAc-TPA derivative, but only the ManNAc-TPA has the intact 2-*N*-acetyl group during the Schiff base/thiazolidine ring closure. The peak A/B ratio is clearly smaller for the ManNAc-TPA derived from ManNAc (supplementary Fig. S.2.). This indicates an involvement by the 2-*N*-acetyl in the specificity of the thiazolidine ring closure step, possible due to steric hindrance, and prior to the subsequent per-*O*-acetylation.

(iii) **TPA Derivatives for the Analysis of Oligo- and Polysaccharides.** Having established the efficacy of the TPA derivatives for GC analysis of monosaccharides the procedure was subsequently evaluated for the analysis of oligo- and polysaccharides (Fig. 2). Acid hydrolyzed samples were evaporated to dryness and converted to TPA derivatives by the one-pot procedure. Raffinose and stachyose were chosen because they both contain glucose and galactose residue, but in a different ratio (1:1 and 1:2, respectively), plus a terminal ketose sugar (fructose) that cannot form as C1-TPA derivative. For these examples the faster eluting TPA stereoisomers (Glc1 and Gal1) gave overlapping peaks, but the slower isomeric peaks (Glc2 and Gal2) are well resolved (Fig 2. A and B). The Gal2 TPA is more intense for stachyose, consistent with the greater quantity of galactosyl residues present in that oligosaccharide (Fig. 2. B).

Frost grape polysaccharide (FGP) is a recently discovered, complex arabinogalactan isolated from native grapevine species, with emulsion properties and high viscosity.[12,13] Compositional analysis of FGP as al-

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dononitrile acetates showed that it typically consists of Ara, Gal, Xyl, and Man in a ratio 5: 3: 1: 0.3.7 The present TPA analysis confirms these findings (Fig. 2. C). Two GC peaks are evident for each of the pentose residues (Ara1 and 2, Xyl1 and 2) and two peaks for the hexoses (Gal1 and 2, Man1 and 2). Although Ara2 and Xyl2, and Gal1 and Man1 TPAs are not fully resolved by the chromatography, these components are readily identified by the baseline separation of the corresponding second peaks.

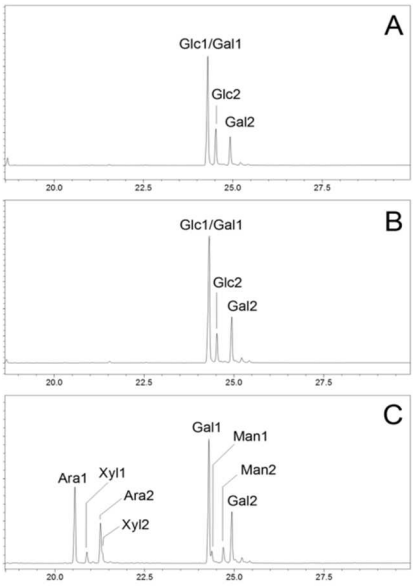

Fig. 2. TPA analysis of oligo- and polysaccharides after acid hydrolysis. A. raffinose (Glc, Gal; 1:1); B. stachyose (Glc, Gal; 1:2); C. Frost grape polysaccharide (Ara, Xyl, Gal, Man). TPA derivatives were analyzed by GC-MS.

(iv) **EI-MS Ion Fragmentation Spectra of Thiazolidine Peracetates.** Electron impact (EI) ionization is a 'hard' ionization method that leads to extensive carbon-carbon bond fragmentations that are often helpful for structure determinations.[14,15] The extent of these fragmentation pathways are readily traced by using stable isotopes, leading to selective mass increase of specific fragment ions.[16,17] EI-MS spectra were obtained on the hexosyl TPA derivatives of [U-^{13}C]glucose, [1-^{13}C]glucose, [2-^{13}C]glucose, and unlabeled glucose control (Fig. 3), and compared with those for the pentosyl derivatives of [U-^{13}C]ribose, [1-^{13}C]ribose, and unlabeled ribose (supplementary Figs. S.3. and S.4.).

All of the monosaccharides tested gave rise to dominant *m/z* 130 ions due to C1 – C2 bond cleavage of the parent sugars. This correspond to the stable thiazolidinium ion $[C_3H_5SNAc]^+$ (calculated mass 130.0327 Da), and that fragments further by a loss of ketene to give $[C_3H_6SN]^+$ (calculated mass 88.0221 Da). These ions contain the carbon-1 of the parent monosaccharide, and hence for the isotopically-labeled [U-^{13}C]glucose, [1-^{13}C]glucose, [U-^{13}C]ribose, and [1-^{13}C]ribose TPA derivatives these are observed at *m/z* 131 and *m/z* 89, respectively. Molecular ions were not observed, but secondary fragmentations occur by neutral eliminations of acetate to give $[M-120]^+$ (Fig. 3, supplementary Fig. S.4). Hence, $[M-120]^+$ ions *m/z* 377, 372, 372, and 371 are observed for [U-^{13}C]Glc-TPA, [1-^{13}C]Glc-TPA, [2-^{13}C]Glc-TPA, and unlabeled Glc-TPA, respectively (Fig. 3).

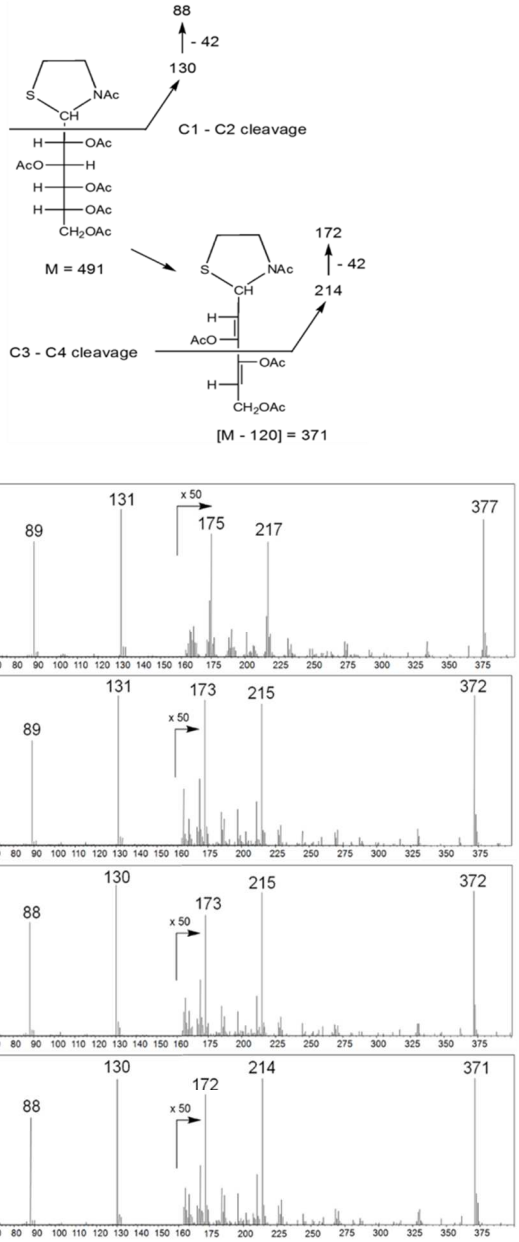

Fig. 3. EI-MS ion fragmentation spectra of thiazolidine peracetates of selectively isotopically labeled glucose. A. [U-^{13}C]Glc; B. [1-^{13}C]Glc; C. [2-^{13}C]Glc; D. unlabeled Glc control. E. Deduced ion fragmentations giving *m/z* 130 and 88 from C1-C2 bond cleavage, and *m/z* 214 and 172 from C3-C4 cleavage. The [M-120] ion arises from successive neutral losses of acetic acid.

Similarly, *m/z* 304, 300, and 299 $[M-120]^+$ ions are seen for [U-^{13}C]Rib-TPA, [1-^{13}C]Rib-TPA, and the un-

labeled Rib-TPA (supplementary Fig. S.4). The $[M-120]^+$ ions fragment further by cleavage across the C3 – C4 bond of the parent sugar with retention of charge at the derivatized end (Fig. 3, supplementary Fig. S.4). Hence, the Glc-TPA series give rise to C3 – C4 cleavage ions at *m/z* 217, 215, 215, and 214, respectively, and similarly for the Rib-TPAs at *m/z* 217, 215, and 214. These ions subsequently also undergo a neutral ketene loss (-42 Da) to generate significant ions at *m/z* 175, 173, and 172 (Fig. 3, supplementary Fig. S.4).

(v) **Fractional ^{13}C Incorporations at the Carbohydrate Anomeric Carbons.** The characterized EI ionization fragmentation pathways can be valuable for identifying and quantifying the extent of incorporation of isotopes into biological molecules during metabolism.[12,15] For the sugars this is particularly important for the characteristic C-1 anomeric carbons,[18] which are readily lost as CO_2 during oxidative metabolism of hexose-6-phosphate to pentose sugars (the so-called phosphopentose oxidative (PPO) pathway).[19] Moreover, C3-C4 metabolism via the aldolase-catalyzed recombination steps of the glycolytic-gluconeogenesis pathways also leads to redistribution of the metabolic carbon flux.[19]

Metabolic incorporation of ^{13}C into cellular carbohydrates was evaluated for two diverse bacterial species, *Bacillus* and *Streptomyces*. The cells were grown in culture on either [1-^{13}C]glucose or [1-^{13}C]fructose as a carbon source, washed to remove excess soluble ^{13}C-labeled precursors, and then acid hydrolyzed. The hydrozylate was dried and the acid-labile monosaccharides were converted to thiazolidine peracetates for analysis by GC/MS. Six cellular sugars were detected in the hydrozylate (Rib, Glc, Gal, Man, GlcNAc, and GalNAc), each giving rise to two diastereomer GC peaks (Table 2).

Table 2. Metabolic enrichment of 1-^{13}C-labeled precursors (Glu or Fru) into the cellular carbohydrate components of *Bacillus subtilis*.*

	Rt	1-^{13}C-labeled Glucose		1-^{13}C-labeled Fructose	
Sugar TPA	min	% *m/z* 89	% *m/z* 131	% *m/z* 89	% *m/z* 131
Rib A	20.28	10.12	12.17	10.43	12.68
Rib B	20.60	9.85	12.12	9.96	13.58
Glc/Gal A	24.23	24.27	25.99	22.21	23.99
Man A	24.33	14.90	16.34	21.73	22.54
Glc B	24.50	24.38	25.95	21.72	23.99
Man B	24.70	13.57	15.03	21.65	23.06
GalNAc A	25.73	14.98	16.11	17.72	20.66
GlcNAc A	25.86	26.37	26.81	26.31	29.02
GlcNAc B	26.95	20.24	26.13	32.91	28.37
GalNAc B	27.12	23.72	12.54	30.74	25.35

**Bacillus* cells were metabolically labeled in culture, washed to remove excess ^{13}C-labelled precursors, then acid-hydrolyzed and converted to thiazolidine peracetates for analysis by GC/MS. The ^{13}C % incorporations at C-1 were calculated from *m/z* 89/ (*m/z* 88 + 89) and *m/z* 131/ (*m/z* 130 + 131) for each of the cellular component monosaccharides (sugar TPAs) identified by the GC analysis.

The percentage ^{13}C incorporations into the anomeric carbon of these sugars were calculated from *m/z* 89/(*m/z* 88 + 89) and *m/z* 131/(*m/z* 130 + 131) for each of the cellular component monosaccharides (sugar TPAs) identified by the GC analysis (Table 2). For the *Bacillus* strain the fractional incorporations were typically 10 – 30% depending on the sugar residue and on the metabolic precursor. The ^{13}C enrichments of the ribose C-1 are noticeably lower than those of the neutral hexoses, presumably due to an active PPO pathway.[19] In general the incorporations from [1-^{13}C]Glc and [1-^{13}C]Fru were similar, although the cellular Man shows a greater enrichment from fructose than from glucose (Table 2). This was also evident for the *Streptomyces*, for which the Man *m/z* 88/89 and *m/z* 130/131 ratios are smaller when the strain is grown on [1-^{13}C]Fru (Fig. 4). A similar effect is also seen for the GlcNAc sugar from the *Streptomyces* strain (Fig. 4). The explanation for this greater enrichment of Man and GlcNAc from ^{13}C-Fru is that these sugars are most often biosynthesized in a single enzymatic step from fructose-6-phosphate, involving mannose-6-phosphate isomerase or glucosamine synthetase, respectively.[20,21]

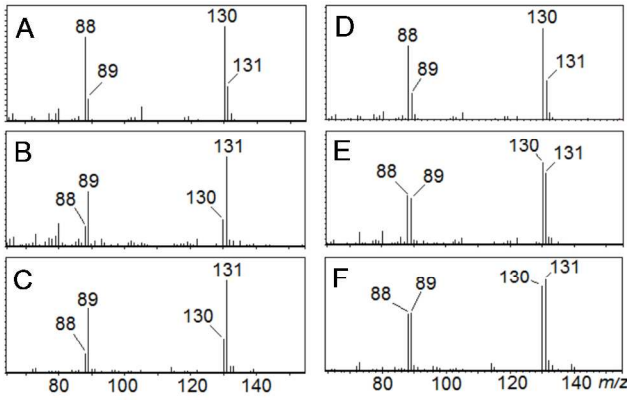

Fig. 4. ^{13}C/^{12}C mass ratio analysis of cellular sugars from *Streptomyces* cultured on [1-^{13}C]Fru (A, B, C) or [1-^{13}C]Glc (D, E, F). The EI-MS ions (*m/z* 88, 89, and *m/z* 130, 131) from the C-1 carbons of Rib-TPA (A, D), Man-TPA (B, E), and GlcNAc-TPA (C, F) are shown. Larger [M+1]/M ratios are indicative of greater ^{13}C enrichment.

MECHANISTIC STUDIES To evaluate a mechanistic basis for the diastereoselective behavior of the TPA-derivatives, NMR spectra were obtained of the D-Glc and L-Ara thiazolidines prior to the peracetylation step (Fig. 5). The thiazolidine H-1 protons were identified (4.8 – 5.0 ppm) and the ratio of diastereomers was assessed from the observed integrals. These data were compared with the C1-epimer ratios obtained by GC analysis of the corresponding Glc and Ara TPAs, which includes the subsequent peracetylation procedure. The

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diastereoselective A/B ratios were apparent from both data sets (Fig. 5).

Thus, the H-1A and H-1B of Ara-thiazolidine are observed at 4.88 ppm ($J_{1,2}$ = 7.5 Hz) and 4.95 ppm ($J_{1,2}$ = 3.9 Hz), in a percentage ratio of 78.0 : 22.0, in close agreement with the GC peak A/B ratio of 78.1 : 21.9. Similarly, the Glc-thiazolidine H-1A and H-1B NMR signals are at 4.92 ppm ($J_{1,2}$ = 6.4 Hz, 59.9%) and 4.95 ($J_{1,2}$ = 4.0 Hz, 40.1%), respectively, and are also in fair agreement with the observed GC ratio of 51.4 : 48.6 (Fig. 5). These data indicate that the peak A/B ratio differences occur prior to the peracetylation step, and is most likely determined during the thiazolidine ring formation.

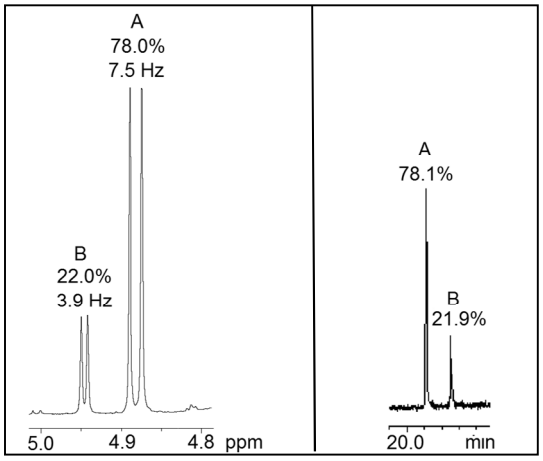

Fig. 5. Equivalence of Ara-thiazolidine (NMR signals, left) and Ara-TPA after peracetylation (GC profile, right). The C-1 epimers are labeled A or B.

The diastereoselective GC peak ratio probably arise because of the more favorable formation of one C-1 epimer over the other, most likely during the formation of the thiazolidine ring (Scheme 1). This occurs initially by formation of a Schiff's base between the aldose carbon 1 and the amino group of the cysteamine, and is followed by a nucleophilic attack by the thiol group leading to the thiazolidine ring closure. It is the facial preference for this nucleophilic addition that determines the ratio of the two TPA geometric isomers for each sugar, as seen in Table 1. This facial preference for ring closure may be determined by hydrogen bonding in the Schiff's base intermediates (supplementary Fig. S. 5.). Thus, hydrogen bonding between the thiazolidine imino nitrogen and the monosaccharide 2-OH group (2-OH – N hydrogen bonding) may result in a transiently stable 5-membered ring, or to the 3-OH group (3-OH – N hydrogen bonding) resulting in a 6-membered ring (supplementary Fig. S. 5.). Hence, these stabilized intermediates result in preferential thiazolidine ring closure, leading to a preferred diastereomer.

CONCLUSION

In this paper we have described aldose thiazolidine peracetates that are readily prepared in a one-pot reaction and that are highly suitable for analysis by GC/MS. We have characterized twenty seven different monosaccharides using these new derivatives, including all of the D-series, neutral aldopentoses and aldohexoses. As further examples, several oligosaccharides and a novel arabinogalactan polysaccharide have also been analyzed. The C1 – C2 and C3 – C4 IE-MS ion fragmentation pathways has also been established for hexose and pentose TPAs, and shown to be valuable for tracing the enrichment of stable isotopes into carbohydrate metabolism. The carbohydrate TPAs gave two diastereomeric peaks observed by GC, although unexpectedly these were diastereoselective, dependent upon the C2 – C3 stereochemistry of the parent sugar. Hence, this has further value for establishing *cis*/*trans*-2,3- sugars based on GC analysis alone, and is therefore generally applicable on a sub-nanomole scale.

METHODS AND MATERIALS

Materials. Isotopically-labeled carbohydrates were from Omicron Biochemicals, Inc., South Bend, IN, and other reagents from Sigma-Aldrich, St. Louis, MO. Frost grape polysaccharide was obtained from Dr. Stephen Vaughn, Agricultural Research Service, Peoria, IL. Monosaccharide stock solutions were prepared at 100 mg.mL^{-1} in water and stored at -20 °C. Cysteamine reagent was prepared by dissolved cysteamine hydrochloride (1 g) in dry pyridine (30 mL). The reagent was stable for at least one week when stored in the dark at 4 °C. ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were obtained on a Bruker Avance instrument (Bruker BioSpin Corp., Billerica, MA). The spectra were recorded in D_2O, obtained from Cambridge Isotope Labs (Andover, MA). The pulse sequences were supplied by Bruker, and processing was done with Bruker TOPSPIN, version 1.3. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS) was achieved on a Bruker Microflex reflecton instrument (Bruker Daltonics, Billerica, MA) using 2, 5-dihydrobenzoic acid as the matrix. *Bacillus subtilis* MW10 was obtained for the Bacillus Genetic Stock Center, Columbus, OH, and *Streptomyces* spp. NRRL F-5065 from the ARS Microbial Culture Collection housed in Peoria, IL.

Preparation of Carbohydrate Thiazolidine Peracetates. Aqueous stock solutions (10 µL, 100 mg.mL^{-1}) of the standard sugars were evaporated to dryness in screw-capped reaction tubes. Dilution series were prepared were appropriate. Oligo- and polysaccharide samples were hydrolyzed with aqueous trifluoroacetic acid (2 M; 2 mL, 120°C; 60 min), then evaporated to dryness. Cysteamine reagent (1 mL) was added to the sugar residues and were reacted on

a hot block (60 °C; 20 min). The reactions were cooled and without evaporating were treated with acetic anhydride (1 mL, 60 °C; 20 min) to complete the peracetylation. The completed TPA reactions were cooled and evaporated to dryness. The residues were dissolved in ethyl acetate (1 mL) and washed with distilled water (2 mL). The upper, organic layer was used directly for GC/MS analysis (1 µL injection, equivalent to 1 µg of the underivatized parent sugar).

Gas Chromatography/Mass Spectrometry. GC/MS analyses were performed on a Shimadzu Model GC 2010 Plus gas chromatograph equipped with an AOC Model 20i autoinjector. The GC was interfaced with a Shimadzu Model QP2010 Ultra mass-selective detector configured in electron impact (EI) mode. Chromatography was accomplished with a fused-silica capillary Phenomenex ZB-5MSi column (30 m; 0.25 mm). Helium (18.6 mL/min) was used as the carrier gas. The oven temperature was ramped over a linear gradient from 150 °C to 300 °C at 5 °C/min, then held for 5 min. The MS acquisition was started 6 min into the run to occlude excess reagent peaks. Injector and detector/interface temperatures were 275 and 300 °C, respectively. Mass spectra were recorded in positive ion mode over the range m/z 45 – 600.

Metabolic Experiments. Bacterial cells (*Bacillus subtilis* and *Streptomyces* spp. NRRL F-5065) were cultured in liquid TYD medium (tryptone (2 $g.L^{-1}$), yeast extract (2 $g.L^{-1}$), glucose (6 $g.L^{-1}$) and $MgCl_2.6H_2O$ (0.3$g.L^{-1}$) as described previously.[12]. For labeling experiments the glucose component was substituted with a ^{13}C isotopically labeled carbon source, either [1-^{13}C]glucose or [1-^{13}C]fructose. The cultures were grown aerobically on a shaker table (200 rpm, 28 °C, 24 h). Cell pellets were harvested by centrifugation (8000 rpm, 20min) and washed several times with deionized water. The washed cells were acid hydrolyzed with aqueous trifluoroacetic acid (2 M; 2 mL, 120°C; 180 min), then centrifuged to remove cell debris. The supernatants were recovered, evaporated to dryness on an airline, and the component monosaccharides were converted to TPA derivatives and analyzed by GC/MS as described above.

ASSOCIATED CONTENT

Supporting Information. Five additional supplementary figures, and an additional reference.

AUTHOR INFORMATION

Corresponding Author

*neil.price@ars.usda.gov

Author Contributions

The manuscript was written through contributions of all authors. / All authors have given approval to the final version of the manuscript.

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ABBREVIATIONS

TPA, thiazolidine peracetate; GC, gas chromatography; EI-MS, electron impact-mass spectrometry; CID, collision induced dissociation.

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