Facile Discrimination of Aldose Enantiomers by Reversed-Phase HPLC

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One-pot reactions of aldoses with L-cysteine methyl ester and *o*-tolyl isothiocyanate yielded methyl 2-(polyhydroxyalkyl)-3-(*o*-tolylthiocarbamoyl)-thiazolidine-4(*R*)-carboxylates. Direct HPLC analysis of the reaction mixture and UV detection at 250 nm discriminated D- and L-enantiomers of aldoses. The reaction was applied to the determination of absolute configuration the sugar residues of an aroma precursor.

Key words aldose; absolute configuration; HPLC; cysteine; isothiocyanate

Aldoses are one of the most important structural components of biomolecules, such as polysaccharides, nucleic acids, glycolipids and glycoproteins. In addition, numerous secondary metabolites in plants, such as terpenoids, steroids, and flavonoids, exist as glycosides, which conjugate with aldoses. Aldoses are optically active compounds, and confirmation of absolute configuration is required in natural product chemistry. Measurement of specific rotations of pure samples is the most reliable method, although this is impractical in many cases because only limited amounts of samples are available. Analysis using a column with a chiral stationary phase developed for the separation of enantiomers, or an HPLC system equipped with an optical rotation detector and a column specified for sugar analysis, can be applied[1,2]; however, the latter method is not applicable to mixtures of D- and L-enantiomers. Identification of sugars with small optical rotation may be also difficult. Methods using capillary electrophoresis have also been developed,[3,4] but these methods require specialized equipment or columns that are unfamiliar to most organic chemistry laboratories. Some methods based on conversion of aldose enantiomers to diastereomeric derivatives through coupling to an optically active reagent have been developed[5,6]; however, there are not many methods applicable to the widely used HPLC systems equipped with a UV detector and C_{18} reversed-phase column. This paper describes a new method to discriminate between aldose enantiomers using a usual HPLC system.

Results and Discussion

Hara *et al.* developed an excellent method using gas chromatography, in which enantiomeric aldoses were converted to trimethylsilyl ethers of methyl 2-(polyhydroxyalkyl)-thiazolidine-4(*R*)-carboxylates.[7] In order to apply this method to HPLC analysis, we converted the thiazolizine derivatives to arylthiocarbamate (**3**, **4**) by reaction with arylisothiocyanates.

The reaction procedure is very simple: sugar samples, such as D- and L-glucoses (**1**, **2**, respectively), are heated with L-cycteine methyl ester in pyridine at 60 °C for 60 min, then arylisothiocyanate was added to the reaction mixture and further reacted at 60 °C for 60 min. Then, the reaction mixture was directly analyzed by standard C_{18} HPLC and detected by a UV detector (at 250 nm). When phenylisothiocyanate was used, the retention time (t_R) of the derivatives of D- and L-glucoses were 16.35 and 15.37 min, respectively. The derivatives of D- and L-glucoses (**3**, **4**, respectively) were isolated and their structures were determined by ^{1}H- and ^{13}C-NMR spectra and FAB-MS analyses. Although the production of two diastereomers, which have an opposite configuration at the sugar C-1 position, was expected from each enantiomer, the ^{1}H- and ^{13}C-NMR spectra showed that one of the two possible diastereomers was produced preferably in the case of glucose.

When the reaction mixture was stored at room temperature for a few days, the derivatives decomposed to give thiohydantoin compounds by elimination of methanol. The derivative of L-glucose (**4**) was more unstable than that of D-glucose (**3**), and a hydantoin derivative (**5**) was produced. The resulting hydantoin derivatives of D- and L-enantiomers gave similar t_R values (8.56, 8.57, respectively), indicating that the hydantoin derivatives were not suitable for the discrimination of enantiomers.

In order to find more stable thiocarbamoyl-thiazolidine derivatives, seven isothiocyanates (phenyl, benzyl, benzoyl, 3,4-dimethoxyphenyl, β-phenylethyl, *p*-tolyl, and *o*-tolyl isothiocyanates) were compared. Except for benzoylisothiocyanate, all compounds yielded thiocarbamate derivatives, and the time differences ($\Delta\delta_{D-L}$) were enough to distinguish between D- and L-enantiomers. The smallest peak areas of the thiohydantoin derivatives were observed when *o*-tolyl isoth-

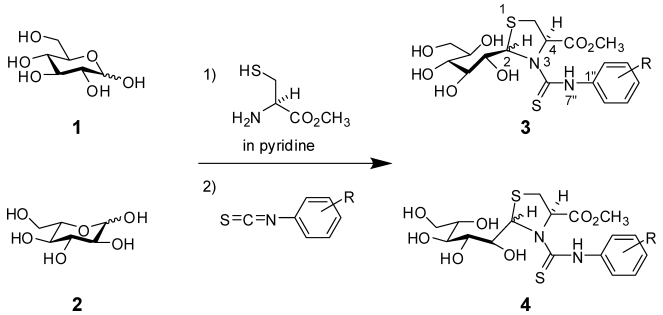

Chart 1. Reaction of Aldoses with L-Cysteine Methyl Ester and Arylisothiocyanate

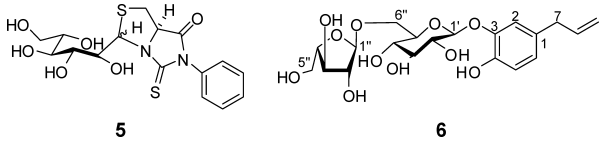

Chart 2. Structures **5** and **6**

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iocyanate was used. It is likely that its methyl group in the *ortho* position hindered thiohydantoin formation.

The t_R values of typical D- and L-aldoses after treatment with cysteine methyl ester and *o*-tolyl isothiocyanate are listed in Table 1.[8] Thiohydantoin derivatives were estimated to represent less than 20% of all thiocarbamoyl-thiazolidine derivatives, based on their peak areas. This method could not be applied to ketoses, such as fructose, which did not show any peaks on HPLC analysis. Interestingly, the migration order of D- and L-enantiomers depended on the structure of aldoses.

When D-cysteine methyl ester was used, the t_R values of the D- and L-enantiomers were reversed. Using this method, the retention time of the derivatives of the enantiomeric sugars can be predicted. Therefore, only one of the enantiomers is required to estimate the t_R values of D- and L-aldoses when L- and D-cysteine methyl esters are available, In our study, D-cysteine methyl ester was supplied *in situ* by the reduction of D-cysteine dimethyl ester with dithiothreitol. Indeed, the t_R values of D-rhamnose, D-apiose, L-glucuronic acid and *N*-acetyl-L-glucosamine in Table 1 were obtained by reaction of sugar enantiomers with D-cysteine methyl ester, because of a lack of authentic samples. A linear calibration curve of the derivative of D-glucose was obtained in the range of 0.2—5 mmol/l. However, the different sugars gave different peak areas (Table 1), indicating that the yields of the derivatization depended on the aldose structures. Therefore, estimation of the sugar molar ratio by comparing the peak area should be done carefully: calibration curves should be prepared for each sugar. Mannose, rhamnose and apiose showed large Δt_R values compared with other aldoses. This was probably caused by the difference in aldose C-1 configuration.

Next, we applied the method to structure determination of an aroma precursor isolated from the leaves of *Illicium anisatum*. The plane structure was determined to be 1-(3′,4′-dihydroxyphenyl)-2-propene 3′-*O*-(4-*O*-α-arabinopyranosyl)-β-glucopyranoside (**6**) by usual NMR techniques, including 2 dimensional NOESY spectrum, confirming the location of sugar moieties. In order to determine the absolute configuration, glycosides were hydrolyzed by heating in 0.5 M HCl and neutralized with Amberlite IRA400. After drying *in vacuo*, the residue was dissolved in pyridine and derivatized as described above. Direct HPLC analysis of the reaction mixture exhibited peaks at 19.58 and 17.52 min, which were coincided with derivatives of L-arabinose and D-glucose, confirming the absolute configuration of the sugar components.

Although the method described here is a modification of that developed for gas-liquid chromatography, C_{18}-reversed phase HPLC equipped with a UV detector is now a more common analytical system in laboratories worldwide. Our method is very simple and does not require any specialized columns or detectors, which are usually expensive. In addition, the reagents used for derivatization are inexpensive and readily available. Further optimization of the reaction conditions and application of the method to structure determination of natural products are now in progress.

Table 1. Retention Times of the Thiocarbamoyl-thiazolidine

Aldoses		t_R (min)	$\Delta t_{R(D-L)}$	Relative peak area[a]
Hexose				
Glucose	D	17.48	1.33	1.0
	L	16.15		0.8
Mannose	D	11.20	−6.21	1.1
	L	17.41		1.3
Galactose	D	15.45	−0.49	2.0
	L	15.95		1.1
Rhamnose	D[b]	15.85	−13.61	0.5
	L	29.47		1.5
Fucose	D	23.76	−2.41	2.9
	L	26.17		2.0
Glucuronic acid	D	18.17	0.53	2.0
	L[b]	17.64		0.7
N-Ac-glucosamine	D	11.04	−3.89	0.2
	L[b]	14.93		0.1
Pentose				
Arabinose	D	20.83	1.31	1.7
	L	19.52		3.3
Xylose	D	20.36	1.43	2.4
	L	18.93		1.8
Apiose	D[c]	28.57	12.88	0.5
	L[c]	15.69		1.3
Ribose	D	20.96	6.15	2.4
	L	14.81		2.0

a) Based on the peak area at 250 nm of D-glucose. *b*) The t_R were obtained by using D-cystine and dithiothreitol. *c*) D-Apiose and L-apiose were synthesized from L- and D-riboses.

Experimental

Optical rotations were measured with a JASCO DIP-370 digital polarimeter. ^{1}H- and ^{13}C-NMR spectra were recorded in pyridine-d_5 or CD_3OD with with a JEOL JMN-AL400 (JEOL Ltd., Japan) operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C. Coupling constants were expressed in Hz, and chemical shifts were given on a δ (ppm) scale with tetramethylsilane as an internal standard. MS were recorded on a JEOL JMS DX-303 spectrometer, and glycerol was used as a matrix for FAB-MS measurement. Column chromatographies were performed with Silica gel 60 (Merck) and Chromatorex ODS (100—200 mesh, Fuji Silysia Chemical Ltd.). TLC was performed on precoated Kieselgel 60 F_{254} plates (0.2 mm thick, Merck) with chloroform–methanol–water (40 : 10 : 1 or 14 : 6 : 1, v/v). and spots were detected by ultraviolet (UV) illumination and by spraying 5% sulfuric acid reagent. Analytical HPLC was performed on a 250×4.6 mm i.d. Cosmosil 5C_{18}-AR II column (Nacalai Tesque Inc.) at 35 °C with isocratic elution of 25% CH_3CN in 50 mM H_3PO_4 for 40 min and subsequent washing of the column with 90% CH_3CN at a flow rate 0.8 ml/min. Peaks were detected with a Jasco MD-910 photodiode array detector.

D-Derivative (3) D-Glucose (**1**) (720 mg) and L-cysteine methyl ester hydrochloride (900 mg) was dissolved in pyridine (5 ml) and heated at 60 °C for 60 min, and then phenylisothiocyanate (0.9 ml) was added to the mixture and heated at 60 °C for 60 min. After evaporation of the solvent by rotary evaporator, the residue was separated by silica gel column chromatography with $CHCl_3$–MeOH–water (40 : 10 : 1) to yield **3** (608 mg), a white amorphous powder, $[\alpha]_D^{25}$ +81.7° (*c*=0.1, MeOH), FAB-MS *m/z*: 433 $[M+H]^+$, IR ν_{max} cm^{-1}: 3317, 1766, 1497, 1403, UV λ_{max}: 251 nm,^{1}H-NMR (400 MHz, pyridine-d_5) δ: 11.36 (1H, s, H-7″), 7.59 (2H, br d, *J*=7.5 Hz, H-2″, 6″), 7.20 (2H, br t, *J*=7.5 Hz, H-3″, 5″), 7.03 (1H, br t, *J*=7.5 Hz, H-4″), 6.53 (1H, t, *J*=7.5 Hz, H-4), 6.33 (1H, d, *J*=10.0 Hz, H-2), 5.03 (1H, dd, *J*=3.0, 1.5 Hz, glucose H-3), 4.82 (1H, dd, *J*=10.0, 1.5 Hz, glucose H-2), 4.63 (1H, dd, *J*=8.0, 3.0 Hz, glucose H-4), 4.58 (1H, ddd, *J*=8.0, 5.3, 3.8 Hz, glucose H-5), 4.52 (1H, dd, *J*=11.0, 3.8 Hz, glucose H-6a), 4.37 (1H, dd, *J*=11.0, 5.3 Hz, glucose H-6b), 3.53 (3H, s, OMe), 3.44 (1H, dd, *J*=12.0, 7.5 Hz, H-5a), 3.42 (1H, dd, *J*=12.0, 7.5 Hz, H-5b), ^{13}C-NMR (100 MHz, pyridine-d_5) δ: 183.8 (C-8″), 171.6 (COO), 141.0 (C-1″), 128.6 (C-3″, 5″), 124.7 (C-4″), 124.3 (C-2″, 6″), 78.6, 75.7, 73.2, 70.9, 69.9, 68.6, 64.9 (C-2, C-4, glucose-C), 52.4 (OMe), 31.9 (C-5). *Anal.* Calcd for $C_{17}H_{24}N_2O_7S\cdot H_2O$: C, 45.32; H, 5.82; N, 6.22. Found: C, 45.24; H, 5.43; N, 6.44.

L-Derivative (4) L-Glucose (**2**) (300 mg) was reacted in the manner similar to described for the D-enantiomer to give **4** (98.8 mg), a white amorphous powder, $[\alpha]_D^{25}$ +222.6° (*c*=0.1, MeOH), FAB-MS *m/z*: 433 $[M+H]^+$, 401 $[M+H-MeOH]^+$, IR ν_{max} cm^{-1}: 3202, 1765, 1498, 1403, UV λ_{max} 244 nm, ^{1}H-NMR (400 MHz, pyridine-d_5) δ: 11.45 (1H, s, H-7″), 7.80 (2H, br d, *J*=7.5 Hz, H-2″, 6″), 7.22 (2H, br t, *J*=7.5 Hz, H-3″, 5″), 7.07 (1H, br t, *J*=7.5 Hz, H-4″), 6.66 (1H, t, *J*=8.0 Hz, H-4), 6.15 (1H, d, *J*=9.2 Hz, H-2),

5.18 (1H, br s, glucose H-3), 4.64 (2H, br s, glucose H-4, 5), 4.60 (1H, br d, J=9.2 Hz, glucose H-2), 4.56 (1H, dd, J=11.0, 2.7 Hz, glucose H-6a), 4.38 (1H, dd, J=11.0, 5.1 Hz, glucose H-6b), 3.60 (5H, m, OMe, H-5a, 5b), ^{13}C-NMR (100 MHz, pyridine-d_5) δ: 183.0 (C-8″), 171.7 (COO), 141.4 (C-1″), 128.5 (C-3″, 5″), 125.1 (C-2″, 6″), 125.0 (C-4″), 75.9, 75.9, 72.7, 70.7, 70.3, 68.5, 65.0 (C-2, C-4, glucose-C), 52.6 (OMe), 31.4 (C-5).

L-Thiohydantoin Derivative (5) The derivative **5** (120 mg) was obtained as a by-product of **4** as a white powder, FAB-MS m/z: 401 $[M+H]^+$, UV λ_{max} 255, 273 nm, ^{1}H-NMR (400 MHz, pyridine-d_5) δ: 7.30—7.40 (5H, m, phenyl-H), 6.70 (1H, d, J=4.8 Hz, H-2), 5.42 (1H, t, J=8.3 Hz, glucose H-3), 5.00 (glucose H-2, H-4, overlapped with HOD signal), 4.67 (1H, m, glucose H-5), 4.53 (1H, dd, J=9.3, 7.8 Hz, H-4), 4.51 (1H, dd, J=10.3, 3.9 Hz, glucose H-6a), 4.37 (1H, dd, J=10.3, 5.4 Hz, glucose H-6b), 3.53 (1H, dd, J=9.3, 7.8 Hz, H-5a), 3.36 (1H, t, J=9.3 Hz, H-5b), ^{13}C-NMR (100 MHz, pyridine-d_5) δ: 186.3 (C-8″), 172.2 (COO), 134.3 (C-1″), 129.2, 128.9, 124.3 (C-2″, 3″, 4″, 5″, 6″), 78.1, 74.2, 73.4, 71.0, 67.5, 66.3, 65.1 (C-2, C-4, glucose-C), 32.2 (C-5).

Analytical Procedure (A) Sugar (5 mg) and L-cysteine methyl ester (5 mg) was dissolved in pyridine (1 ml) and heated 60 °C for 1 h, and then isothiocyanate (5 mg) was added to the mixture and heated further for 1h. The reaction mixture (2 μl) was analyzed by HPLC and detected at 250 nm (Table 1). (B) A 0.2 ml solution of aldose in pyridine (10 mg/ml) was mixed with a 0.2 ml solution containing D-cystine dimethyl ester (2 mg) and dithiothreitol in pyridine (2 mg) in a screw-capped vial, and heated at 60 °C for 1 h. A 0.2 ml solution of *o*-torylisothiocyanate in pyridine (10 mg/ml) was added to the mixture, which was heated at 60 °C for 1 h. The reaction mixture was directly analyzed by reversed-phase HPLC.

1-(3′,4′-Dihydroxyphenyl)-2-propene 3′-*O*-(4-*O*-α-arabinopyranosyl)-β-glucopyranoside (6) White amorphous powder, $[\alpha]_D^{13}$ −122.0° (c=0.3, MeOH), FAB-MS m/z: 467 $(M+Na)^+$, *Anal.* Calcd for $C_{20}H_{28}O_{11}\cdot 3/4H_2O$: C, 52.46, H: 6.49. Found: C, 52.57; H, 6.49. ^{1}H-NMR (CD_3OD) δ: 6.99 (1H, d, J=2 Hz, H-2), 6.75 (2H, m, H-5, 6), 5.94 (1H, ddt, J=16.8, 10.1, 6.7 Hz, H-8), 5.05 (1H, br d, J=16.8 Hz, H-9a), 5.02 (1H, br d, J=10.1 Hz, H-9b), 4.94 (1H, d, J=1.4 Hz, H-1″), 5.71 (1H, d, J=7.6 Hz, H-1′), 4.07 (1H, dd, J=1.4, 12.3 Hz, H-6′), 4.03 (1H, dd, J=1.4, 3.2 Hz, H-2″), 3.97 (1H, dt, J=3.3, 5.3 Hz, H-4″), 3.84 (1H, dd, J=3.2, 5.3 Hz, H-3″), 3.72 (1H, dd, J=3.2, 11.9 Hz, H-5″), 3.54—3.66 (3H, m, H-5′, 6′, 5″), 3.35—3.52 (3H, H-2′, 3′, 4′), 3.29 (2H, br d, J=6.7 Hz, H-7). ^{13}C-NMR (CD_3OD) δ: 146.6, 146.5 (C-3, 4), 139.2 (C-8), 133.2 (C-1), 124.9 (C-6), 119.4, 117.0 (C-2, 5), 115.7 (C-9), 109.9 (C-1″), 104.5 (C-1′), 85.9 (C-2″), 83.2 (C-4″), 78.9 (C-3″), 77.5 (C-3′), 76.9 (C-5′), 74.9 (C-2′), 71.7 (C-4′), 67.9 (C-6′), 63.0 (C-5″), 40.5 (C-7). NOESY correlations: H-1′→H-2, H-1″→H-6′, H-2 and H-6→H-7.

Determination of Sugar Configuration The glycoside (**6**) (0.5 mg, 1.1×10^{-6} mol) were hydrolyzed by heating in 0.5 M HCl (0.1 ml) and neutralized with Amberlite IRA400. After drying *in vacuo*, the residue was dissolved in pyridine (0.1 ml) containing L-cysteine methyl ester hydrochloride (0.5 mg) and heated at 60 °C for 1 h. A 0.1 ml solution of *o*-torylisothiocyanate (0.5 mg) in pyridine was added to the mixture, which was heated at 60 °C for 1 h. The reaction mixture was directly analyzed by reversed-phase HPLC. The peaks at 19.58 and 17.52 min were coincided with derivatives of L-arabinose and D-glucose.

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