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Short Communication

1-Thio-β-D-galactose as a chiral derivatization agent for the resolution of D,L-amino acid enantiomers

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Abstract

o-Phthaldialdehyde in combination with 1-thio-β-D-galactose is a powerful chiral reagent for the precolumn derivatization of primary amino acids. The diastereomers formed can be efficiently resolved on conventional reversed-phase columns. Comparison of 1-thio-β-D-galactose with 1-thio-β-D-glucose revealed that even the change of the configuration of the remote OH group on the sugar moiety influences the resolution of D,L-amino acid derivatives.

1. Introduction

Fungi and bacteria produce biologically active compounds in which D-amino acids are sometimes present. The slow racemization of L-amino acids in biological material leads to D-amino acids. These acids also form undesirable side-products, for example, in peptide synthesis. In all instances an appropriate analytical method is necessary to control the enantiomeric purity of either natural or synthetic products. A number of methods have been developed for this purpose, based on chiral stationary phases which are usually designed to resolve a group of enantiomers [1]. A different approach consists in the use of chiral derivatization agents, the target reaction of which produces diastereomers which can be resolved on conventional columns; several such reagents have already been described for the determination of D,L-amino acids [2–5]. Sugars represent a natural source of easily accessible chiral compounds, the derivatives of which retain their chirality and might be used, therefore, as suitable derivatization agents. The derivatization of D,L-amino acids with the sodium salt of 1-thio-β-D-glucose has been described [6]. The aim of this work was to evaluate the effect on chiral recognition of changing the OH group configuration on the C-4 atom of the sugar moiety.

2. Experimental

2.1. Instrumentation and chromatographic conditions

A Varian Vista 5500 liquid chromatographic

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system equipped with a Rheodyne Model 7126 injection valve, a DS 604 data station and a Fluorichrom filter fluorescence detector was used. The excitation wavelength was 360 nm and the emission wavelength was a bandpass above 420 nm. The analytical columns were LiChrosorb RP-8 (5 μm) (250 × 4 mm I.D.) from Merck (Darmstadt, Germany) and a Separon SGX C8 (5 μm) (150 × 3 mm I.D.) glass column from Tessek (Prague, Czech Republic). A back-pressure terminator (Varian, Sunnyvale, CA, USA), set at 0.6 MPa, was used to prevent the formation of bubbles.

Solvent A was 0.05 *M* sodium acetate adjusted to pH 6.10 with acetic acid and solvent B was 0.1 *M* sodium acetate (pH 7.60)–methanol (1:9). Both solvents were carefully degassed prior to use. Isocratic elution with solvent A was carried out for 8 min, then a 47-min linear gradient to 45% A and 55% B was applied, followed by a column wash with a linear gradient to 100% B in 5 min. A constant flow-rate of 1.20 ml/min was maintained during the analysis. The column was thermostated at 35°C. Stability studies were performed with a Bio-Rad (Richmond, CA, USA) AS-100 HRLC automatic sampling system and a Hypersil ODS (60 × 4.6 mm I.D.) column from Hewlett-Packard (Amstelveen, Netherlands) under isocratic conditions.

2.2. Chemicals and derivatization procedure

The sodium salts of 1-thio-β-D-galactose and 1-thio-β-D-glucose were prepared as described previously [7,8]; *o*-phthaldialdehyde (Calbiochem, Los Angeles, CA, USA), amino acids (Calbiochem or Sigma, St. Louis, MO, USA), methanol, boric acid, potassium hydroxide and sodium acetate (Lachema, Brno, Czech Republic) were used.

Stock solutions were prepared weekly with 50 mg of sodium salts of thiosugars in 1 ml of water, 50 mg of *o*-phthaldialdehyde (OPA) in 1.25 ml of methanol and amino acids (4 m*M* in water). Borate buffer was prepared by dissolving 0.50 g of boric acid in 19 ml of water and adjusting the pH to 9.30 (8.20 or 10.40) with potassium hydroxide solution (45 g of potassium hydroxide in 100 ml of water). A 10-μl sample of the amino acid solution, 100 μl of the borate buffer, 50 μl of the thiosugar solution and 50 μl of the OPA solution (thiosugar/OPA = 2.2) were added consecutively to a small glass vessel with Hamilton syringes, and the mixture was thoroughly stirred. After 60 s a 10-μl aliquot was analysed.

2.3. Absorbance and fluorescence measurements

The derivatives for the absorbance measurements were obtained from the chromatographic fractions containing the desired enantiomers. A volume of the eluate corresponding to the peak of the each separated stereoisomer of the 1-isoindolyl-(1-thio-β-D-glycoside) was placed manually into a small vial and measured with a Varian DMS 300 double-beam UV–Vis spectrophotometer. The reference solutions were appropriate mixtures of solvents A and B. The fluorescence spectra were measured employing a Hewlett-Packard Model 1090 liquid chromatograph connected with an HP 1046 fluorescence detector. The excitation wavelength was set at 360 nm and the emission wavelength at 420 nm.

3. Results and discussion

Amino acids bearing primary amino groups form highly fluorescent compounds after derivatization with OPA in the presence of a thiol. By analogy with the reaction of mercaptoethanol [9] or glutathione [10], 1-isoindolyl-(1-thioglycosides) are assumed to be formed in the course of the reaction of amino acids, OPA and thiosugars. The reactions occurred rapidly and quantitatively at ambient temperature, reaching their maximum fluorescence within 1 min (Fig. 1). Our experience indicated that repeated injections of basic buffers may quickly cause the column performance to deteriorate. Therefore, the use of buffers with pH above 9.5 should be avoided or the derivatization mixture should be diluted with acidic buffer before injection. Similarly as with OPA–2-mercaptoethanol derivatives [11–14], thiosugar-substituted isoindoles were un-

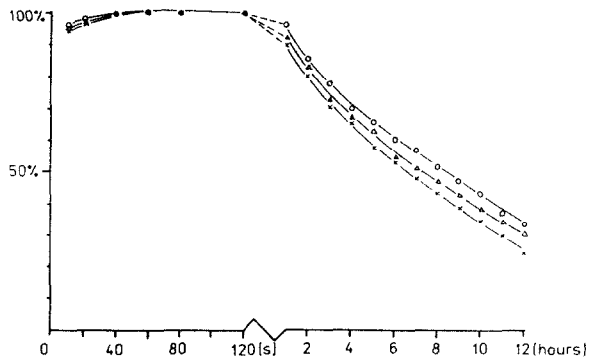

Fig. 1. Fluorescence response of OPA–1-thio-β-D-galactose derivatization as a function of reaction time and pH (× = 8.2; △ = 9.3; ○ = 10.0) and stability of the selected derivative in borate buffer at 20°C (mixture: 10 μl of 4 m*M* L-alanine, 200 μl of borate buffer, 50 μl of thiosugar solution, 50 μl of OPA solution). The ordinate (relative %) refers to the area of the chromatographic peak obtained by the analysis of the reaction mixture injected after 60 s (100%). Conditions: Hypersil ODS (3 μm) column (60 × 4.6 mm I.D.); isocratic elution with 0.05 *M* sodium acetate (pH 6.3)–methanol (65:35, v/v); flow-rate, 0.5 ml/min.

stable. The stability of these isoindoles was found to decrease with decreasing pH.

The detection limit for L-Ala, based on a signal-to-noise ratio of 2, was less than 10 pmol. The relative standard deviations of the retention times with OPA–1-thio-β-D-glucose were 0.72% (Ala), 0.48% (Val) and 0.65% (Leu) for eleven analyses of insect haemolymph [15] within 2 days. The average relative deviations for various thiosugar derivatives were less than 5% for between-day assays and less than 3% for within-day assays, which shows that the present method is highly reproducible. The determination of amino acids is relatively easy when large samples are available (>100 pmol). Problems associated with the analysis increase with diminishing sample size and are largely due to impurities in the OPA reagent, old samples of thiosugar solutions and the chemical instability of the isoindoles in the solution.

The successful separation diastereomeric isoindoles on a conventional reversed-phase column has been reported with 2,3,4,6-tetra-O-acetyl-1-thio-β-D-glucose [2] and 1-thio-β-D-glucose [6]. In the same way, OPA–1-thio-β-D-galactose can be used as a chiral derivatization reagent. Fig. 2a

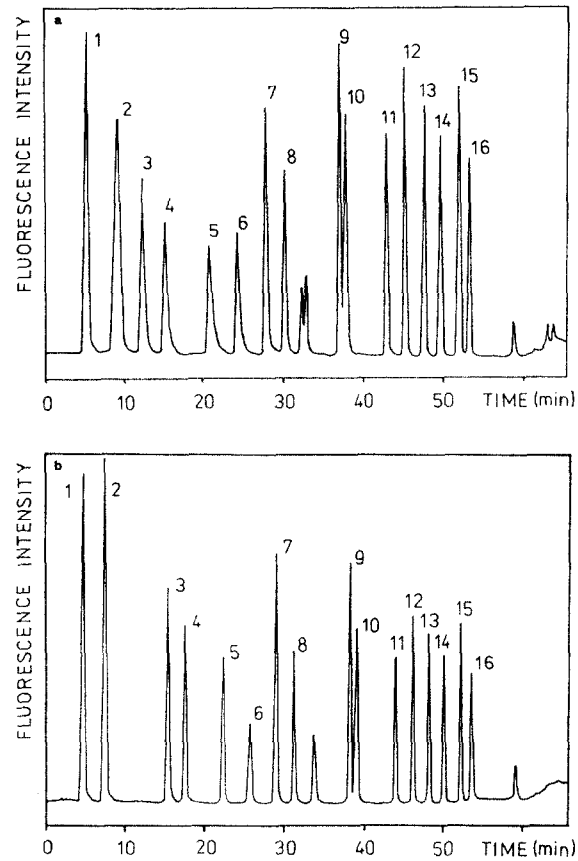

Fig. 2. Separation of amino acids enantiomers after derivatization with OPA–1-thio-β-D-galactose reagent. Conditions: mobile phase A, 0.05 *M* sodium acetate buffer (pH 6.10); B, 0.1 *M* sodium acetate (pH 7.60)–methanol (1:9, v/v); columns: (a) Merck LiChrosorb RP-8 (5 μm) (250 mm × 4 mm I.D.), isocratic elution, 100% A for 8 min, linear gradient to 45% A in 55 min and 0% A in 60 min, flow-rate 1 ml/min; (b) Tessek Separon SGX C_8 (5 μm) (150 × 3 mm I.D.), linear gradient elution from 100% A at 0 min to 30% A at 55 min, flow-rate 0.5 ml/min. Peaks: 1 = D,L-Asp; 2 = D,L-Glu; 3, 4 = L-, D-Ser; 5, 6 = L-, D-Tre; 7, 8 = L-, D-Ala; 9, 10 = L-, D-α-amino-*n*-butyric acid; 11, 12 = D-, L-Val; 13, 14 = L-, D-Phe; 15, 16 = L-, D-Leu. Each peak represents 2 nmol.

shows the separation of some amino acid enantiomers, the resolutions and elution orders for which are given in Table 1, in comparison with those obtained with 1-thio-β-D-glucose. The type of RP column is not critical to achieve the resolution of amino acid enantiomers, but because a methanol–buffer mixture causes a

Table 1
Resolution and retention times of various diastereomeric OPA–thiosugar derivatives

Amino acid	Derivatization reagent					
	1-Thio-β-D-glucose			1-Thio-β-D-galactose		
	t_r (min)		Resolution	t_r (min)		Resolution
	L-	D-		L-	D-	
Asp	5.3	4.9	0.64	4.6		0
Glu	11.3	12.6	1.24	8.9		0
Ser	16.0	18.4	2.18	12.1	15.2	3.29
Tre	23.3	26.6	5.20	20.7	24.2	4.13
Ala	30.1	32.7	4.35	27.7	30.1	4.48
Arg	31.5	32.0	0.76	29.9	30.3	0.58
Tyr	38.4	38.8	0.25	37.1	37.8	0.85
α-n-Abu	38.7	39.7	1.74	37.0	37.8	1.33
Val	46.6	45.1	2.84	45.4	43.0	4.28
Nva	46.4	47.5	1.76	44.9	45.9	1.73
Trp	46.6	47.5	1.52	45.1	46.8	3.19
Phe	48.8	50.4	3.03	47.6	49.6	3.53
Ile	52.8	51.6	1.62	52.2	51.8	1.75
Leu	53.0	53.9	1.75	51.8	53.1	2.31
Nle	53.1	54.0	1.73	52.2	53.1	1.67
Lys	56.9		0	55.5	54.2	2.61

For chromatographic conditions, see Experimental. $t_0 = 1.15$ min ($NaNO_3$); resolution = $1.177(t_2 - t_1)/[w_{1/2(1)} + w_{1/2(2)}]$.

relatively high back-pressure, the use of short columns is preferred. Particularly good resolution was obtained with 150 × 3 mm I.D. columns and a slightly modified gradient programme (Fig. 2b).

The enantiomers of most primary amino acids were baseline resolved. Some separations might be further improved by changing the pH of the buffer, because the capacity factors of some derivatives, *e.g.*, Asp, Glu and Arg, are pH dependent. Generally, the 1-thio-β-D-galactose derivatives were slightly less hydrophobic and were eluted earlier than the 1-thio-β-D-glucose derivatives. Although the thiosugars differ only in the configuration of the OH group on the C-4 atom, *i.e.*, on the remote hydroxy group with respect to the bond of the isoindole moiety, this influences the resolution of some D,L-amino acid enantiomers. As is evident from this comparison, "long-range" interactions between the amino acid residue and the sugar moiety take place, and the change in configuration of the OH group may alter the mechanism of resolution. This effect was illustrated by the different resolutions of Lys, Asp and Glu. In addition, it is well known that no reagent has general applicability, and the potential advantage of 1-thio-β-D-galactose as a chiral reagent consists in the improved resolution of some enantiomeric pairs, *e.g.*, Ser, Val or Trp. The method can possibly be applied also to the routine determination of amino acids in biological fluids or peptide hydrolysates, as most L-amino acids are baseline separated [15]. In analysis, derivatives of biogenic amines and urea were eluted later than amino acids, whereas amino sugars, *e.g.*, galactosamine or glucosamine, were eluted between Ser and Tre and thus can be determined simultaneously.

The D- and L-derivatives of amino acids usually exhibited a slightly different fluorescence response. 1-Thio-β-D-galactose and 1-thio-β-D-glucose and most L-amino acids formed derivatives the chromatographic peaks of which had about 5–20% greater areas whether or not they

were eluted first or second from the enantiomeric pair (Fig. 2). To elucidate this difference, D,L-enantiomers of Asp, Ser, Arg, Tyr, Trp and Phe were chosen as different types of amino acids for UV absorbance and fluorescence measurements of their corresponding 1-isoindolyl-(1-thioglycoside) derivatives (Fig. 3). In addition to small differences caused by the substituents themselves, all derivatives exhibited in the range 200–400 nm broad maxima at 333 nm with a shoulder at 347 nm, which is in accordance with data published for other isoindoles [9]. The fluorescence characteristics of the derivatives were measured with the excitation wavelength set at 360 nm. The emission spectra thus obtained were very similar in shape to those of UV spectra, with a broad maximum at 420 nm. This indicates the significance of the isoindolyl ring system for the course of both UV and fluorescence spectra and minimal influence of the substituents. The small differences in the fluorescence intensities of the D,L-derivatives, therefore, probably originate from the different local arrangements of substituents around the isoindolyl moiety in individual diastereomers. This effect is in fact the same as that of changing the solvent on the fluorescence intensity.

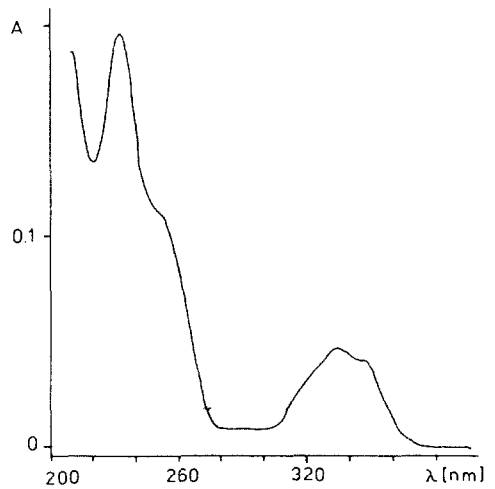

Fig. 3. UV spectrum of 1-isoindolyl-(1-thio-β-D-galactoside) derivative with L-phenylalanine.

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