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High-performance liquid chromatographic enantioseparation of unusual isoxazoline-fused 2-aminocyclopentanecarboxylic acids on macrocyclic glycopeptide-based chiral stationary phases

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ABSTRACT

The enantiomers of four unusual isoxazoline-fused 2-aminocyclopentanecarboxylic acids were directly separated on chiral stationary phases containing macrocyclic glycopeptide antibiotics teicoplanin (Astec Chirobiotic T and T2), teicoplanin aglycone (Chirobiotic TAG), vancomycin (Chirobiotic V) and vancomycin aglycone (Chirobiotic VAG) as chiral selectors. The effects of the mobile phase composition, the structure of the analytes and temperature on the separations were investigated. Experiments were performed at constant mobile phase compositions in the temperature range 5–45 °C to study the effects of temperature, and thermodynamic parameters were calculated from plots of $\ln k$ or $\ln \alpha$ versus $1/T$. Some mechanistic aspects of the chiral recognition process are discussed with respect to the structures of the analytes. It was found that the enantiomeric separations were in most cases enthalpy-driven. The sequence of elution of the enantiomers was determined in all cases.

1. Introduction

By virtue of their pharmacological potential, alicyclic β-amino acids are of great interest among synthetic and medicinal chemists. The naturally occurring β-amino acid cispentacin (1*R*,2*S*-2-aminocyclopentanecarboxylic acid), icofungipen (1*R*,2*S*-2-amino-4-methylenecyclopentanecarboxylic acid) and oryzoxymycin (2*S*,5*R*,6*R*-2,6-amino-5-hydroxy-1,3-cyclohexadiene-1-carbonyloxypropionic acid) are bioactive compounds with antibacterial and antifungal activities. A number of cyclic, conformationally restricted β-amino acids have been used as building blocks for the synthesis of new peptides, which might be of importance in the synthesis of peptide-based drugs [1–3].

Amino acids containing an isoxazoline moiety have revealed anti-influenza activities and antifungal properties. Several isoxazole carboxylic acids, such as conformationally constrained aspartate and glutamate analogs, have been reported as enzyme inhibitors or agents possessing neuroprotective activities [4–7]. Isoxazoline-fused amino acids have served as important precursors for the synthesis of novel multisubstituted cyclopentene derivatives with antiviral activities [8–10].

The wide-ranging utility of these compounds requires analytical methods to check on the stereochemistry of the final product. One of the most frequently applied techniques is chiral high-performance liquid chromatography (HPLC). HPLC enantioseparations of β-amino acids have been performed by both indirect and direct methods. In the past decade, chiral derivatizing agents such as Marfey's reagent, 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate and *N*-(4-nitrophenoxycarbonyl) phenylalanine methoxyethyl ester [11], chiral stationary phases (CSPs) such as macrocyclic glycopeptides [12,13], quinine derivatives [14] and (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid-derivatives [15–19], a rapid double derivatization technique with gas chromatography [20] and (18-crown-6)-2,3,11,12-tetracarboxylic acid as a chiral NMR solvating agent have been used for the analysis of β-amino acids enantiomeric composition [21].

Enantioselective retention and separation are influenced by temperature [22–29]. In order to investigate the thermodynamic functions of enantioselective adsorption, van't Hoff plots were constructed, which may be interpreted in terms of the mechanistic aspects of chiral recognition:

$$\ln k = -\frac{\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R} + \ln \phi \quad (1)$$

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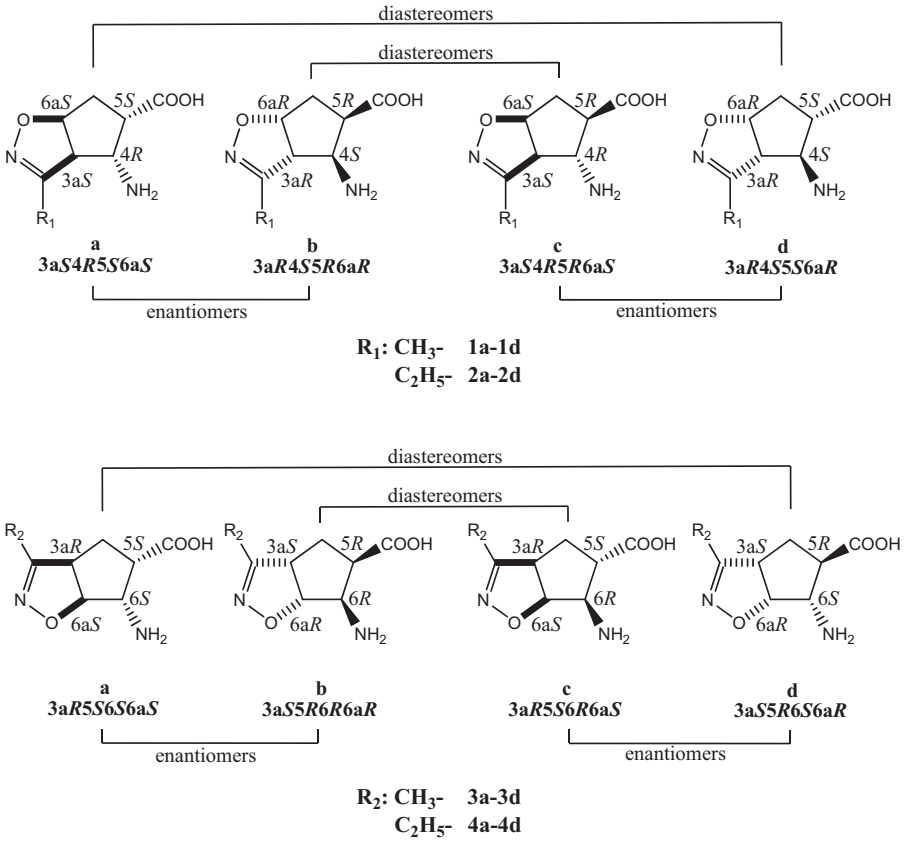

Fig. 1. Structures of isoxazoline-fused 2-aminocyclopentanecarboxylic acids.

in which k is the retention factor, ΔH° is the enthalpy of transfer of the solute from the mobile phase to the stationary phase, ΔS° is the entropy of transfer of the solute from the mobile phase to the stationary phase, R is the gas constant, T is temperature and ϕ is the ratio of volume of stationary phase (V_S) and mobile phase (V_M). The corresponding $\Delta(\Delta H^\circ)$ and $\Delta(\Delta S^\circ)$ values for the separated enantiomers can be determined from a modification of Eq. (1):

$$\ln \alpha = -\frac{\Delta(\Delta H^\circ)}{RT} + \frac{\Delta(\Delta S^\circ)}{R} \quad (2)$$

where α is the selectivity factor ($\alpha = k_2/k_1$). If $\Delta(\Delta H^\circ)$ is invariant with temperature (*i.e.* a linear van't Hoff plot is obtained), this expression shows that a plot of $R \ln \alpha$ versus $1/T$ has a slope of $-\Delta(\Delta H^\circ)$ and an intercept of $\Delta(\Delta S^\circ)$.

In the present paper, direct HPLC methods are described for the separation of enantiomers new cyclic β^3-amino acids (Fig. 1), with the application of macrocyclic glycopeptide-based CSPs. For comparison purposes, most of the separations were carried out at constant mobile phase composition. The effects of the mobile phase composition, the specific structural features of the analytes and selectors and temperature on the retention are discussed on the basis of the experimental data. The elution sequence was determined in all cases.

2. Experimental

2.1. Chemicals and reagents

Racemic isoxazoline-fused cispentacin stereoisomers were prepared by 1,3-dipolar cycloaddition of nitrile oxides to *N*-Boc-protected ethyl 2-amino-3-cyclopentenecarboxylate as dipolarophile. The nitrile oxides were generated from nitroethane or 1-nitropropane in the presence of Boc_2O and 4-dimethylaminopyridine (DMAP). When the dipolarophile underwent cycloaddition in THF at 20 °C for 15 h, two regioisomers and one diastereomer were formed [30]. The isoxazoline-fused cispentacin enantiomers were prepared from enantiomerically pure Boc-protected ethyl 2-amino-3-cyclopentenecarboxylate [31,32]. The cycloadditions were performed similarly as for the racemic compounds. Under the same experimental conditions (nitroethane/1-nitropropane, Boc_2O and DMAP), the racemic *trans* counterparts furnished selectively only one cycloadduct, which also could be prepared by epimerization at C-5 of the very minor product. The two racemic major regioisomers were epimerized at C-5 with NaOEt in EtOH to give isoxazoline-fused *trans* amino esters [33]. These reactions were extended to their preparation in enantiomerically pure form [31,32]. In the next step, the earlier-prepared racemic and enantiomerically pure isoxazoline-fused ethyl 2-amino-cyclopentanecarboxylates were hydrolysed in the presence of HCl/H_2O (2:1) in dioxane to give the corresponding amino acid derivatives (**1a**, **1d**; **2a**, **2d**; **3a**, **3c**; **4a**, **4c**).

Methanol (MeOH) of HPLC grade was purchased from Scharlau (Sentmenat, Spain). Triethylamine (TEA), glacial acetic acid (AcOH) and other reagents of analytical reagent grade were from Sigma–Aldrich (St. Louis, MO, USA). The Milli-Q water was further purified by filtration on a 0.45-μm filter, type HV, Millipore (Molsheim, France).

All compounds mentioned in Fig. 1 were evaluated with different mobile phases. Reversed-phase mobile phases consisted of 0.1% triethylammonium acetate (TEAA) (pH 4.1)/MeOH = 98/2, 90/10,

80/20, 60/40, 40/60, 20/80 and 10/90 (v/v), the polar organic phase (POM) was 100% MeOH, and the polar ionic mobile phase (PIM) was MeOH/AcOH/TEA = 100/0.1/0.1 (v/v/v).

2.2. Apparatus and chromatography

The HPLC measurements were carried out on a Waters HPLC system consisting of an M-600 low-pressure gradient pump, an M-996 photodiode-array detector and a Millenium^{32} Chromatography Manager data system; the alternative Waters Breeze system consisted of a 1525 binary pump, a 487 dual-channel absorbance detector, a 717 plus autosampler and Breeze data manager software (both systems from Waters Chromatography, Milford, MA, USA). Both chromatographic systems were equipped with Rheodyne Model 7125 injectors (Cotati, CA, USA) with 20-μl loops.

The macrocyclic glycopeptide-based stationary phases used for analytical separation were teicoplanin-containing Chirobiotic T and T2, teicoplanin aglycone-containing Chirobiotic TAG, vancomycin-containing Chirobiotic V and vancomycin aglycone-containing Chirobiotic VAG columns, 250 mm × 4.6 mm I.D., 5-μm particle size (for each column) (Astec, Whippany, NJ, USA). The differences between the Chirobiotic T and T2 columns are that they are both on 5-μm particle size silica gel, but the Chirobiotic T uses a 120 Å pore size material and Chirobiotic T2 uses a 200 Å pore material. Also, the linkage chain on Chirobiotic T2 is approximately twice as long as that on Chirobiotic T. Hence, the coverage and spacing are different for the two stationary phases. This manifests itself mainly in the form of steric interaction differences between the two columns.

2.3. HPLC operating conditions applied for validation process

To determine validation characteristics of the analytical procedure separations were carried out repeatedly for analytes **2a**, **2b** on the Chirobiotic TAG CSP with a mobile phase 0.1% TEAA (pH 4.1)/MeOH = 60/40 (v/v) at 25 °C and for analytes **3a**, **3b** on Chirobiotic VAG CSP with a mobile phase 100% MeOH at 10 °C, at a flow rate of 0.5 ml min^{-1}. Analytes **2a**, **2b** possess an ethyl group side-chain while analyte **3a**, **3b** a methyl side-chain. The injection volume was 20 μl, while the detector was set at 210 nm.

3. Results and discussion

The analytes in this study (Fig. 1) possess an isoxazoline-fused cyclopentane skeleton. Besides carboxy and primary amino groups, analogs **1** and **3** bear a methyl group on position 3, and analogs **2** and **4** an ethyl group on position 3. These differences result in different steric effects and influence the hydrophobicity, bulkiness and rigidity of the molecules, depending on how their atoms are linked and how capable they are of different interactions with the selector.

All data relating to the separation of the compounds, including the retention factors, separation factors and resolutions for each analyte on the five different Chirobiotic columns, are given in Tables 1 and 2. All the compounds mentioned in Tables 1 and 2 were evaluated with different mobile phases, with eluent compositions varied between 0.1% TEAA (pH 4.1)/MeOH = 98/2 and 10/90 (v/v), 100% MeOH and MeOH/AcOH/TEA = 100/0.1/0.1 (v/v/v). To simplify the presentation, Tables 1 and 2 basically list only the columns and chromatographic results obtained when the enantiomeric separation was achieved, but for purposes of comparison some results are presented when no separation occurred. At a given mobile phase composition, the retention factors were lower on the teicoplanin CSPs (T and T2) than on the aglycone CSP (TAG) (except for analytes **1c**, **1d**; **2c**, **2d** and **4a**, **4b** on Chirobiotic T in POM, **1c**, **1d**; **2c**, **2d** on Chirobiotic T2 in PIM and POM and **3c**, **3d** on Chirobiotic T in PIM; Table 2). Similar trends, with higher k' values on Chirobiotic TAG than on a Chirobiotic T column, were observed by Berthod et al. [34], D'Acquarica et al. [12] and Péter et al. [35–37] for unusual α-amino acids and cyclic β-amino acids. Comparison of the data for the Chirobiotic T and T2 columns revealed that the retention factors on Chirobiotic T were in most cases somewhat larger (Tables 1 and 2). Slightly higher k' values on Chirobiotic T2 than on Chirobiotic T were observed by Péter et al. [37] for β^3-homoamino acids.

The effects of the MeOH content of the mobile phase in reversed-phase mode were investigated on all five CSPs. In most cases, a U-shaped retention curve was observed for all analogs. At higher water content, the retention factor increased with increasing water content; this was probably due to enhanced hydrophobic interactions between the analyte and the CSP in the water-rich mobile phases. In the reversed-phase mode, one of the most important interactions between the analyte and the CSP is the hydrophobic interaction inside the "basket" of the glycopeptide. Here, an increase in k' at high water content was observed for all analytes. When the MeOH content of the mobile phase exceeded ~50%, the retention factor increased. This suggests that the retention behavior may be controlled by a mechanism of hydrophilic interaction chromatography (HILIC) at high MeOH contents. In this study, as earlier [38], the inflection points and the slopes of the U-shaped curves at higher and lower MeOH concentrations differed somewhat for each compound. Different extents of solvation of the stationary phase during HILIC and under the reversed-phase conditions may explain the observed retention behavior. As regards the variations in the separation factors (α) and resolutions (R_S) with change of MeOH content, no general trends were observed.

Use of the MeOH/AcOH/TEA = 100/0.1/0.1 (v/v/v) mobile phase system generally resulted in lower retention than with 100% MeOH (except for analytes **2a**, **2b** on Chirobiotic T2, **3a**, **3b** on Chirobiotic T and TAG, and **4a**, **4b** on Chirobiotic TAG). However, in a few cases (especially for analytes **3** and **4**), despite the lower k' values, higher α and R_S values were obtained, indicating that the level of chiral discrimination was better in the polar ionic mode (Table 2).

The structures of the analytes influenced the chiral recognition. In the reversed-phase mode methyl or ethyl substitution exhibited a slight effect on retention and in most cases retention factors of methyl or the ethyl substituted analogs did not differ considerably. The position of the methyl or ethyl group influenced the α and R_S values. On the teicoplanin-containing CSPs for methyl-substituted analogs better α and R_S values were obtained in most cases for enantiomers **1a**, **1b** than for **3a**, **3b**, while **3c**, **3d** were better separated than **1c**, **1d**. Similarly, for ethyl-substituted analogs, **2a**, **2b** and **4c**, **4d** exhibited better separation than **4a**, **4b** and **2c**, **2d**, respectively, on Chirobiotic T, T2 and TAG CSPs.

For comparison of the performances of the macrocyclic glycopeptide-based columns, separations were carried out with the same mobile phases (Table 1). Of the three teicoplanin-based columns Chirobiotic TAG seemed to be more effective in the separation of all enantiomers with exception pair of **2a**, **2b** for which Chirobiotic T2 and **3a**, **3b** for which Chirobiotic T was better. Comparison of the teicoplanin and vancomycin-based selectors demonstrated that the vancomycin-based CSPs were effective only in the separation of the enantiomers of **3a**, **3b** and **4a**, **4b** (the enantiomers of **1** and **2** were not separable on Chirobiotic V and VAG).

In summary, the macrocyclic glycopeptide-based CSPs display a complementary character. Elution sequences were determined in all cases. For analytes **1**–**4** on the Chirobiotic T, T2, TAG, V and VAG columns, no consistent elution sequence was observed. Neither the configuration of the carbon atom attached to the carboxyl group nor

Table 1
Retention factors of the first eluting enantiomer (k_1'), separation factors (α), resolutions (R_S) and elution sequences of regio- and stereoisomers of isoxazoline-fused cispentacin analogs in reversed-phase mode.

Compound	Column	Mobile phase TEAA/MeOH (v/v)	k_1'	α	R_S	Elution sequence
1a, **1b**	T	90/10	2.63	1.00	0.00	–
		60/40	2.31	1.01	0.20	*a* < *b*
		10/90	4.76	1.07	0.80	*a* < *b*
	T2	90/10	0.93	1.10	0.70	*a* < *b*
		60/40	0.91	1.13	1.00	*a* < *b*
		10/90	2.96	1.16	1.65	*a* < *b*
	TAG	90/10	4.31	1.19	1.80	*a* < *b*
		60/40	2.73	1.26	2.25	*a* < *b*
		10/90	4.76	1.33	3.25	*a* < *b*
1c, **1d**	T	90/10	1.85	1.00	0.00	–
		10/90	7.51	1.01	0.20	*d* < *c*
	T2	90/10	1.39	1.00	0.00	–
		10/90	8.16	1.04	0.65	*d* < *c*
	TAG	90/10	3.26	1.06	0.70	*c* < *d*
		10/90	8.20	1.02	0.20	*c* < *d*
2a, **2b**	T	90/10	1.88	1.06	0.65	*a* < *b*
		60/40	1.77	1.09	0.85	*a* < *b*
		10/90	3.75	1.07	1.00	*a* < *b*
	T2	90/10	1.07	1.14	0.80	*a* < *b*
		60/40	0.95	1.18	1.20	*a* < *b*
		10/90	2.28	1.24	2.60	*a* < *b*
	TAG	90/10	5.63	1.18	1.85	*a* < *b*
		60/40	2.92	1.30	2.25	*a* < *b*
		10/90	4.65	1.18	1.90	*a* < *b*
2c, **2d**	T	90/10	2.55	1.00	0.00	–
		10/90	6.52	1.00	0.00	–
	T2	90/10	1.64	1.00	0.00	–
		10/90	6.64	1.03	0.60	*d* < *c*
	TAG	90/10	4.98	1.10	1.40	*c* < *d*
		60/40	4.52	1.09	1.55	*c* < *d*
		10/90	6.95	1.03	0.40	*c* < *d*
3a, **3b**	T	90/10	2.09	1.00	0.00	–
		10/90	5.01	1.17	1.45	*a* < *b*
	T2	90/10	1.11	1.00	0.00	–
		10/90	3.66	1.00	0.00	–
	TAG	90/10	6.09	1.09	1.00	*b* < *a*
		10/90	5.66	1.09	0.85	*b* < *a*
	V	90/10	0.30	1.15	0.55	*b* < *a*
		10/90	0.98	1.36	2.95	*b* < *a*
	VAG	90/10	0.57	1.18	1.10	*b* < *a*
		10/90	1.18	1.31	2.70	*b* < *a*
3c, **3d**	T	90/10	1.90	1.13	1.30	*d* < *c*
		10/90	5.71	1.02	0.30	*d* < *c*
	T2	90/10	1.07	1.16	1.00	*d* < *c*
		10/90	3.23	1.14	1.65	*d* < *c*
	TAG	90/10	3.18	1.06	0.60	*c* < *d*
		10/90	5.75	1.24	2.15	*c* < *d*
	V	90/10	0.16	1.25	0.60	*d* < *c*
		10/90	0.78	1.13	0.80	*d* < *c*
	VAG	90/10	0.54	1.00	0.00	–
		10/90	1.63	1.06	0.60	*d* < *c*
4a, **4b**	T	90/10	2.64	1.00	0.00	–
		10/90	4.82	1.17	1.65	*a* < *b*
	T2	90/10	1.10	1.00	0.00	–
		10/90	3.72	1.00	0.00	–
	TAG	90/10	7.86	1.09	0.90	*b* < *a*
		10/90	4.91	1.18	2.15	*b* < *a*
	V	90/10	0.45	1.09	0.45	*b* < *a*
		10/90	0.92	1.35	2.60	*b* < *a*
	VAG	90/10	0.81	1.16	1.35	*b* < *a*
		10/90	1.08	1.31	3.00	*b* < *a*
4c, **4d**	T	10/90	4.50	1.08	1.10	*d* < *c*
	T2	10/90	2.88	1.14	1.55	*d* < *c*
	TAG	10/90	4.60	1.27	2.50	*c* < *d*
	V	10/90	0.71	1.08	0.50	*d* < *c*
	VAG	10/90	1.47	1.05	0.55	*d* < *c*

Chromatographic conditions: selectors and columns. **T** and **T2**, teicoplanin, Chirobiotic T and T2; **TAG**, teicoplanin aglycone, Chirobiotic TAG; **V**, vancomycin, Chirobiotic V; **VAG**, vancomycin aglycone, Chirobiotic VAG; mobile phase, 0.1% triethylammonium acetate (pH 4.1)/MeOH (v/v); flow rate, 0.5 ml min^{-1}; detection, 210 nm.

Table 2
Retention factors of the first eluting enantiomer (k_1'), separation factors (α), resolutions (R_S) and elution sequences of regio- and stereoisomers of isoxazoline-fused cispentacin analogs in polar organic and polar ionic modes.

Compound	Column	Mobile phase MeOH (100%) MeOH/AcOH/TEA (v/v/v)	k_1'	α	R_S	Elution sequence
1a, **1b**	T	100	9.29	1.11	0.90	*b* < *a*
		100/0.1/0.1	7.78	1.03	0.35	*a* < *b*
	T2	100	5.23	1.16	0.90	*a* < *b*
		100/0.1/0.1	4.91	1.00	0.00	–
	TAG	100	11.91	1.05	0.30	*b* < *a*
		100/0.1/0.1	8.16	1.00	0.00	–
1c, **1d**	T	100	14.70	1.04	0.40	*d* < *c*
		100/0.1/0.1	7.41	1.04	0.95	*c* < *d*
	T2	100	16.65	1.04	0.65	*d* < *c*
		100/0.1/0.1	7.78	1.07	0.30	*d* < *c*
	TAG	100	7.48	1.00	0.00	–
		100/0.1/0.1	7.45	1.00	0.00	–
2a, **2b**	T	100	7.30	1.00	0.00	–
		100/0.1/0.1	6.66	1.02	0.25	*a* < *b*
	T2	100	4.19	1.24	1.40	*a* < *b*
		100/0.1/0.1	4.91	1.21	1.20	*a* < *b*
	TAG	100	12.91	1.00	0.00	–
		100/0.1/0.1	11.01	1.26	1.20	*a* < *b*
2c, **2d**	T	100	16.59	1.00	0.00	–
		100/0.1/0.1	6.41	1.00	0.00	–
	T2	100	14.49	1.17	1.00	*d* < *c*
		100/0.1/0.1	9.59	1.03	0.40	*d* < *c*
	TAG	100	10.14	1.00	0.00	–
		100/0.1/0.1	7.38	1.00	0.00	–
3a, **3b**	T	100	7.27	1.46	3.75	*a* < *b*
		100/0.1/0.1	9.13	1.21	1.65	*a* < *b*
	T2	100	7.16	1.00	0.00	–
		100/0.1/0.1	5.92	1.03	0.25	*b* < *a*
	TAG	100	11.63	1.29	1.45	*a* < *b*
		100/0.1/0.1	15.49	1.08	0.50	*b* < *a*
	V	100	1.95	1.40	2.70	*b* < *a*
		100/0.1/0.1	1.60	1.44	3.05	*b* < *a*
	VAG	100	2.41	1.34	3.50	*b* < *a*
		100/0.1/0.1	1.73	1.36	3.10	*b* < *a*
3c, **3d**	T	100	11.76	1.03	0.25	*c* < *d*
		100/0.1/0.1	7.85	1.05	0.45	*c* < *d*
	T2	100	5.57	1.29	2.00	*d* < *c*
		100/0.1/0.1	3.19	1.23	1.35	*d* < *c*
	TAG	100	14.75	1.06	0.35	*c* < *d*
		100/0.1/0.1	6.00	1.09	1.25	*c* < *d*
	V	100	1.74	1.14	1.10	*d* < *c*
		100/0.1/0.1	1.50	1.00	0.00	–
	VAG	100	3.45	1.08	0.75	*d* < *c*
		100/0.1/0.1	1.58	1.14	0.95	*d* < *c*
4a, **4b**	T	100	10.06	1.20	1.70	*a* < *b*
		100/0.1/0.1	7.67	1.11	0.95	*a* < *b*
	T2	100	7.08	1.00	0.00	–
		100/0.1/0.1	6.46	1.00	0.00	–
	TAG	100	9.25	1.24	0.90	*b* < *a*
		100/0.1/0.1	11.07	1.25	1.50	*b* < *a*
	V	100	1.81	1.38	2.20	*b* < *a*
		100/0.1/0.1	1.47	1.41	3.30	*b* < *a*
	VAG	100	2.18	1.34	3.15	*b* < *a*
		100/0.1/0.1	1.49	1.38	3.10	*b* < *a*
4c, **4d**	T	100	8.76	1.09	0.65	*d* < *c*
		100/0.1/0.1	5.74	1.12	1.15	*d* < *c*
	T2	100	4.90	1.26	1.75	*d* < *c*
		100/0.1/0.1	4.54	1.06	0.40	*c* < *d*
	TAG	100	13.94	1.03	0.30	*c* < *d*
		100/0.1/0.1	6.54	1.14	0.50	*c* < *d*
	V	100	1.51	1.09	0.45	*d* < *c*
		100/0.1/0.1	1.09	1.12	1.30	*d* < *c*
	VAG	100	3.24	1.06	0.50	*d* < *c*
		100/0.1/0.1	1.28	1.10	0.55	*d* < *c*

Chromatographic conditions: selectors and columns. **T** and **T2**, teicoplanin, Chirobiotic T and T2; **TAG**, teicoplanin aglycone, Chirobiotic TAG; **V**, vancomycin, Chirobiotic V; **VAG**, vancomycin aglycone, Chirobiotic VAG; mobile phase, 0.1% triethylammonium acetate (pH 4.1)/MeOH (v/v); flow rate, 0.5 ml min^{-1}; detection. 210 nm.

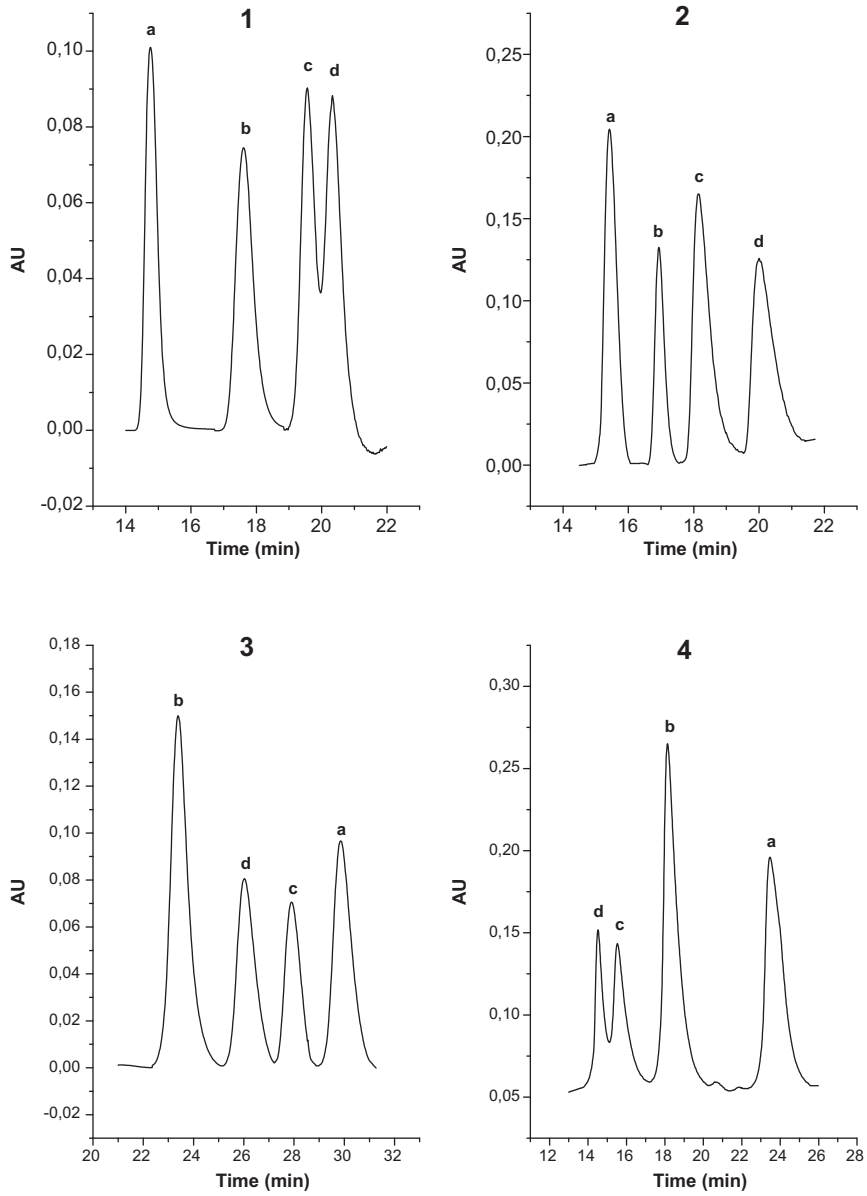

Fig. 2. Separation of four stereoisomers of isoxazoline-fused cispentacin derivatives. Chromatographic conditions: column, for analytes **1** and **2** Chirobiotic TAG, for **3** Chirobiotic VAG, and for **4** Chirobiotic V; mobile phase, for analyte **1**, 0.1% TEAA (pH 4.1)/MeOH = 60/40 (v/v), for **2**, 0.1% TEAA (pH 4.1)/MeOH (v/v) = 80/20 (v/v); for **3**, MeOH, 100%, and for **4**, MeOH/AcOH/TEA = 100/0.1/0.1 (v/v/v); temperature, 25 °C for analytes **1** and **2**, and 10 °C for analytes **3** and **4**; flow rate, 0.5 ml min^{-1}; detection, 210 nm.

that of the carbon atom attached to the amino group determined the elution sequence, and in some cases the elution sequence also differed when the mobile phase was changed from reversed-phase to polar organic or polar ionic mode.

3.1. Separation of four enantiomers of isoxazoline-fused 2-aminocyclopentanecarboxylic acids

Since the biological activities of isoxazoline-fused 2-aminocyclopentanecarboxylic acid analogs depend strongly on their configurations, it is a basic task to separate and identify not only the enantiomers, but also the diastereomers in one chromatographic run. The data listed in Table 1 reveal that separation of the four stereoisomers could be achieved in only a few cases. To attain separation of the four stereoisomers in one chromatographic run, the separation was optimized by variation of the CSPs and mobile phase composition. Fig. 2 and Table 3 illustrate the enantioseparation of all four stereoisomers of isoxazoline-fused 2-aminocyclopentanecarboxylic acid analogs.

3.2. Effects of temperature and thermodynamic parameters

In order to investigate the effects of temperature on the chromatographic parameters, a variable-temperature study was carried out on Chirobiotic T and TAG columns over the temperature range 5–45 °C (in 10 °C increments). Experimental data for the mobile phase 0.1% TEAA (pH 4.1)/MeOH = 10/90 (v/v) are listed in Table 4. A comparison of the retention factors in Table 4 reveals that all of the recorded values decreased with increasing temperature. It is evident that an increase in the separation temperature lowers the separation factor, α. However, for analytes **3a**, **3b** on the Chirobiotic TAG column, α (and R_S) increased with increasing temperature. Increasing temperature may improve the peak symmetry and efficiency, and therefore the resolution may also improve.

Table 3
Chromatographic data, retention factors and resolutions for separation of four stereoisomers of isoxazoline-fused cispentacin derivatives on Chirobiotic TAG, VAG and V columns.

Analyte	Column	Mobile phase	k_1'	k_2'	k_3'	k_4'	R_{S1}	R_{S2}	R_{S3}	Elution sequence
1	TAG	**a**	2.73	3.45	3.95	4.13	3.10	2.05	0.80	*a* < *b* < *c* < *d*
2	TAG	**b**	2.89	3.28	3.58	4.05	1.95	1.40	2.15	*a* < *b* < *c* < *d*
3	VAG	**c**	2.53	2.93	3.21	3.50	1.95	1.45	1.80	*b* < *d* < *c* < *a*
4	V	**d**	1.27	1.43	1.83	2.67	0.90	1.95	3.30	*d* < *c* < *b* < *a*

Chromatographic conditions: mobile phase, **a**, 0.1% TEAA (pH 4.1)/MeOH = 60/40 (v/v), **b**, 0.1% TEAA (pH 4.1)/MeOH (v/v) = 80/20 (v/v); **c**, MeOH, 100%, **d**, MeOH/AcOH/TEA = 100/0.1/0.1 (v/v/v); temperature, 25 °C for analytes **1** and **2**, 10 °C for analytes **3** and **4**; flow rate, 0.5 ml min^{-1}; detection, 210 nm.

Table 4
Retention factor of first-eluting enantiomer (k'), separation factor (α) and resolution (R_s) of regio- and stereoisomers of isoxazoline-fused cispentacin analogs **1**–**4** as a function of temperature.

Analyte	Column	k_1', α, R_S	Temperature (°C)				
			5	15	25	35	45
1a, **1b**	T	k_1'	5.20	4.77	4.23	3.80	3.47
		α	1.08	1.06	1.04	1.03	1.01
		R_S	0.80	0.70	0.40	0.30	0.20
	TAG	k_1'	6.18	5.39	4.76	4.19	3.59
		α	1.47	1.40	1.33	1.26	1.21
		R_S	3.40	3.35	3.30	3.15	2.65
1c, **1d**[a]	T	k_1'	8.22	7.42	6.82	6.45	5.86
		α	1.02	1.01	1.00	1.00	1.00
		R_S	0.20	0.10	0.00	0.00	0.00
	TAG	k_1'	9.02	7.96	7.02	6.07	5.46
		α	1.02	1.02	1.02	1.02	1.01
		R_S	0.30	0.25	0.20	0.20	0.15
2a, **2b**	T	k_1'	4.66	4.26	3.82	3.39	3.12
		α	1.12	1.09	1.06	1.04	1.02
		R_S	1.05	0.85	0.75	0.55	0.35
	TAG	k_1'	6.05	5.26	4.65	4.06	3.46
		α	1.25	1.22	1.18	1.14	1.12
		R_S	2.05	2.00	1.90	1.80	1.45
2c, **2d**	T	k_1'	7.65	7.01	6.52	5.97	5.56
		α	1.02	1.01	1.00	1.00	1.00
		R_S	0.15	0.10	0.00	0.00	0.00
	TAG	k_1'	8.86	7.87	6.95	5.96	5.47
		α	1.03	1.03	1.03	1.03	1.02
		R_S	0.40	0.35	0.30	0.25	0.20
3a, **3b**	T	k_1'	6.41	5.72	5.01	4.34	3.83
		α	1.29	1.21	1.17	1.12	1.08
		R_S	1.90	1.60	1.45	1.20	0.70
	TAG	k_1'	9.03	7.53	6.48	5.52	4.65
		α	1.04	1.06	1.08	1.10	1.11
		R_S	0.15	0.45	0.85	0.95	1.00
3c, **3d**	T	k_1'	6.73	6.31	5.71	5.17	4.82
		α	1.03	1.02	1.02	1.01	1.01
		R_S	0.40	0.30	0.25	0.20	0.10
	TAG	k_1'	6.02	5.40	4.62	4.10	3.73
		α	1.25	1.25	1.24	1.23	1.21
		R_S	2.40	2.30	2.15	1.95	1.90
4a, **4b**	T	k_1'	5.99	5.28	4.54	3.99	3.59
		α	1.15	1.10	1.08	1.05	1.03
		R_S	1.55	1.05	1.00	0.70	0.30
	TAG	k_1'	6.38	5.54	4.82	4.12	3.50
		α	1.19	1.18	1.17	1.17	1.16
		R_S	1.40	1.75	1.80	2.00	2.05
4c, **4d**	T	k_1'	5.67	5.33	4.89	4.42	4.12
		α	1.10	1.08	1.07	1.06	1.05
		R_S	0.85	0.75	0.65	0.60	0.50
	TAG	k_1'	6.52	5.79	5.18	4.55	4.21
		α	1.29	1.27	1.26	1.24	1.22
		R_S	2.70	2.55	2.50	2.45	2.30

Chromatographic conditions: column, **T**, Chirobiotic T, **TAG**, Chirobiotic TAG; mobile phase, 0.1% TEAA (pH 4.1)/MeOH = 10/90 (v/v); flow rate, 0.5 ml min^{-1}; detection, 210 nm.

[a] For analyte **1c**, **1d** at 55 °C: $k_{1(c)}'$, 5.63; $k_{2(d)}'$, 5.88; α, 1.05; R_S, 0.35.

Table 5
Thermodynamic parameters, ΔH°, $\Delta S^{\circ *}$, $\Delta(\Delta H^\circ)$, $\Delta(\Delta S^\circ)$, $\Delta(\Delta G^\circ)$, correlation coefficients (R^2) and T_{iso} temperature of analytes **1**–**4** on Chirobiotic T and TAG columns.

Analyte	Column	Stereo-isomer	$-\Delta H^\circ$ (kJ mol^{-1})	$-\Delta S^{\circ *}$ (J mol^{-1} K^{-1})	Correlation coefficient (R^2)	$-\Delta(\Delta H^\circ)$ (kJ mol^{-1})	$-\Delta(\Delta S^\circ)$ (J mol^{-1} K^{-1})	$-\Delta(\Delta G^\circ)$ (288 K) (kJ mol^{-1})	T_{iso} (°C)
1a, **1b**	T	1	7.7	13.7	0.9961	1.2	3.7	0.2	55
		2	8.9	17.4	0.9974				
	TAG	1	9.8	20.1	0.9949	3.5	9.5	0.8	98
		2	13.4	29.6	0.9977				
1c, **1d**	T	1	6.0	4.2	0.9940	0.7	2.2	≪0.1	36
		2	6.4	5.3	0.9930				
	TAG	1	9.4	15.4	0.9976	0.2	0.4	1.0	147
		2	9.6	15.8	0.9977				
2a, **2b**	T	1	7.6	14.4	0.9953	1.6	4.7	0.2	58
		2	9.1	19.1	0.9973				
	TAG	1	10.1	21.3	0.9942	2.1	5.8	0.5	96
		2	12.3	27.1	0.9964				
2c, **2d**	T	1	5.9	4.2	0.9985	0.6	2.0	≪0.1	37
		2	6.2	5.3	0.9995				
	TAG	1	9.1	14.6	0.9952	0.2	0.5	0.1	152
		2	9.3	15.1	0.9953				
3a, **3b**	T	1	9.6	19.0	0.9952	3.2	9.4	0.5	66
		2	12.8	28.4	0.9989				
	TAG	1	12.1	25.0	0.9980	−1.2	−4.7	0.2	−15
		2	10.9	20.3	0.9969				
3c, **3d**	T	1	6.4	7.0	0.9923	0.3	1.0	0.1	67
		2	6.7	8.0	0.9926				
	TAG	1	9.1	17.6	0.9957	0.8	0.7	0.5	745
		2	9.8	18.4	0.9966				
4a, **4b**	T	1	9.6	19.6	0.9986	2.0	6.2	0.3	55
		2	11.6	25.7	0.9993				
	TAG	1	10.9	23.5	0.9955	0.6	0.6	0.4	740
		2	11.4	24.1	0.9956				
4c, **4d**	T	1	6.1	7.2	0.9901	0.8	2.2	0.2	100
		2	6.9	9.4	0.9927				
	TAG	1	8.2	13.9	0.9973	1.0	1.3	0.6	450
		2	9.2	15.2	0.9979				

Mobile phase, 0.1% TEAA (pH 4.1)/MeOH = 10/90 (v/v); $\Delta S^{\circ *} = \Delta S^\circ + \ln \Phi$, where Φ is the phase ratio; R^2, correlation coefficient of van't Hoff plot, $\ln k - 1/T$ curves; T_{iso}, temperature of $\ln k - 1/T$ curves where enantioselectivity cancels.

Thermodynamic parameters were obtained from van't Hoff plots [Eq. (1)]. The ΔH° and $\Delta S^{\circ *}$ values calculated from the slopes and intercepts of these plots for the enantiomers on Chirobiotic T and TAG columns were negative (Table 5). The second-eluting enantiomers with (the exception of **3a**, **3b** on Chirobiotic TAG) exhibited more negative ΔH° and $\Delta S^{\circ *}$ values; it is likely that they have fewer degrees of freedom on the CSP, *i.e.* they are held at more points or are less able to move or rotate. It is widely accepted that both enantiomers undergo the same non-specific interactions, whereas the more strongly retained one is subject to additional stereospecific interactions. Moreover, it was observed that $\Delta H^\circ{}_1$ and $\Delta H^\circ{}_2$, and in parallel $\Delta S^{\circ *}{}_1$ and $\Delta S^{\circ *}{}_2$, for the Chirobiotic T column were in most cases less negative than those for the Chirobiotic TAG column. This may be due to the fact that the latter does not contain sugar units, and this may promote the interaction between the analyte and the CSP.

For the four analytes, the **c**, **d** enantiomers exhibited smaller, and the **a**, **b** enantiomers larger $-\Delta H^\circ$ and $-\Delta S^{\circ *}$ values on both CSPs. The *trans* configuration of the amino and carboxy groups in the **c**, **d** enantiomer pairs may sterically inhibit their fit and orientation in the cavity, and the separation was thermodynamically less favorable. The largest $-\Delta H^\circ$ and $-\Delta S^{\circ *}$ values for all analytes on the TAG CSP indicate that separation on it were sterically favorable.

The differences in the changes in enthalpy and entropy, $\Delta(\Delta H^\circ)$ and $\Delta(\Delta S^\circ)$, are also presented in Table 5. The $-\Delta(\Delta H^\circ)$ values on the Chirobiotic T CSP ranged from −0.3 to −3.2 kJ mol^{-1} and on the Chirobiotic TAG CSP from 1.2 to −3.5 kJ mol^{-1}. The interactions of **1a**, **1b** with the Chirobiotic TAG CSP were characterized by the highest negative $\Delta(\Delta H^\circ)$ value, while **3a**, **3b** on Chirobiotic TAG exhibited a positive $\Delta(\Delta H^\circ)$. The trends in the change in $-\Delta(\Delta S^\circ)$ showed that **1a**, **1b** on Chirobiotic TAG displayed the largest negative entropies, and on the Chirobiotic T CSP $-\Delta(\Delta S^\circ)$ ranged from −1.0 to −9.4 J mol^{-1} K^{-1} and on the Chirobiotic TAG CSP from 4.7 to −9.5 J mol^{-1} K^{-1} (Table 5). For **3a**, **3b**, similarly to $\Delta(\Delta H^\circ)$ on the Chirobiotic TAG column, $\Delta(\Delta S^\circ)$ was positive.

The thermodynamic parameter $-\Delta(\Delta G^\circ)$ suggests that TAG induces highly efficient binding to the selector, as reflected by the larger negative $\Delta(\Delta G^\circ)$ values for **1**–**4** on Chirobiotic TAG. For analytes **1**–**4** on both columns (with the exception of **3a**, **3b** on Chirobiotic TAG), selector–selectand complex formation proceeds via multiple intermolecular interactions and was generally exothermic, with a corresponding negative entropic contribution.

For **3a**, **3b** on Chirobiotic TAG, the positive $\Delta(\Delta S^\circ)$ compensated the positive $\Delta(\Delta H^\circ)$ and resulted in a negative $\Delta(\Delta G^\circ)$. In this temperature range, enantioresolution is entropically driven, and the selectivity increases with increasing temperature (Table 5).

The data were used to calculate the temperature, T_{iso}, at which point the enantioselectivity and the elution sequence changed (Table 5). In most cases, T_{iso} was considerably higher than room temperature; enthalpically driven enantioseparation was obtained. For **3a**, **3b** on Chirobiotic TAG, T_{iso} was −15 °C, positive $\Delta(\Delta H^\circ)$ and $\Delta(\Delta S^\circ)$ were observed in the investigated temperature range and the selectivity increased with increasing temperature.

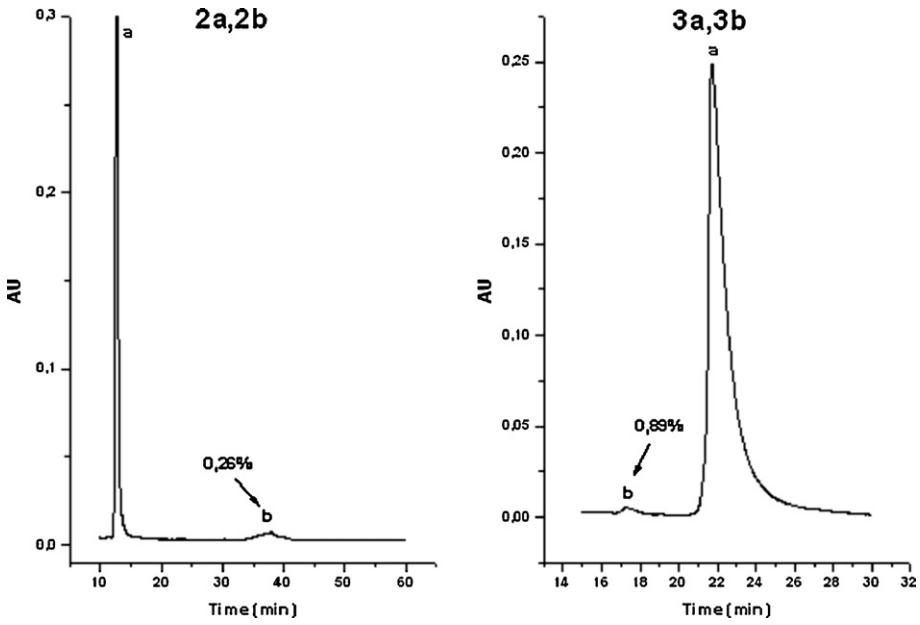

Fig. 3. Separation of minor enantiomers of **2a**, **2b** and **3a**, **3b** when it is present in an excess of the major isomer. Chromatographic conditions: column, Chirobiotic VAG for **2a**, **2b**, and Chirobiotic V for **3a**, **3b**; mobile phase for **2a**, **2b** 0.1% TEAA (pH 4.1)/MeOH (v/v) = 10/90 (v/v) and for **3a**, **3b** MeOH/AcOH/TEA = 100/0.1/0.1 (v/v/v); temperature, 25 °C; flow rate, 0.5 ml min^{-1}; detection, 210 nm.

For **1c**, **1d** and **2c**, **2d**, on the Chirobiotic T CSP, T_{iso} was 37 °C. For **1c**, **1d** on the Chirobiotic T column with the 0.1% TEAA (pH 4.1)/MeOH = 10/90 (*v*/*v*) mobile phase composition, on increase of the temperature from 5 °C to 55 °C, α first decreased; then, after a domain where no separation occurred, α slightly increased with increasing temperature at 55 °C and the elution sequence changed (Table 4). In a domain around the isoenantioselective temperature, the separation of enantiomers could not be obtained. This domain may be referred to as a "temperature-induced blind zone" in chiral recognition [39]. Outside the blind zone, enthalpically or entropically driven enantioseparation can be observed. It should be mentioned that peak inversions on temperature changes were generally observed only for separations with marginal enantioselectivity [40–43].

4. Method validation

4.1. Linearity

Solution of the racemates were prepared at six different concentration levels, from 1 to 100 μg ml^{-1} for analytes **2a**, **2b** and **3a**, **3b**, respectively. Three parallel injections of each solution were made under the chromatographic conditions described above. The peak area response of the first and the second eluting enantiomers was plotted against the corresponding concentration and the linear regression was computed by the least square method using Microsoft Excel program. Very good linearity was observed in the investigated concentration range with the following regression equations: for **2a**, $y = 1.93 \times 10^6 x + 3385$ ($R^2 = 0.9989$), for **2b**, $y = 1.85 \times 10^6 x + 3263$ ($R^2 = 0.9997$), for **3a**, $y = 9.56 \times 10^6 x + 2574$ ($R^2 = 0.9990$) and for **3b**, $y = 1.00 \times 10^7 x + 2656$ ($R^2 = 0.9987$). (The difference of the regression parameters of the enantiomers were within the standard error.)

4.2. Limit of detection (LOD) and limit of quantitation (LOQ)

LOD and LOQ were determined based on the calibration curve according to the ICH guidelines using the *S*/*N* ratio equal to 3 and 10, respectively [44]. LOD was 5 μg ml^{-1} and 2.5 μg ml^{-1}, while LOQ 16 μg ml^{-1} and 8.0 μg ml^{-1} for **2a**, **2b** and **3a**, **3b**, respectively. Further, the determination limit for the minor isomer is less than 0.1% when it is present in an excess of the major isomer (Fig. 3).

4.3. Precision

Replicate HPLC analysis showed that the relative standard deviation were no more than 5% for the peak area response and less than 1.8% for the retention time.

5. Conclusions

The enantiomeric separations of isoxazoline-fused 2-aminocyclopentanecarboxylic acid analogs were investigated using macrocyclic glycopeptide-based CSPs, *i.e.* Chirobiotic T, T2, TAG, V and VAG columns. The separations could be accomplished in reversed-phase mode using 0.1% TEAA (pH 4.1)/MeOH mobile phases with different compositions. Of the five Chirobiotic columns, Chirobiotic T and TAG appeared most suitable for the enantioseparation of isoxazoline-fused 2-aminocyclopentanecarboxylic acids. The values of thermodynamic parameters such as the changes in enthalpy, $\Delta(\Delta H^\circ)$, entropy, $\Delta(\Delta S^\circ)$, and Gibbs free energy, $\Delta(\Delta G^\circ)$, depended on the structures of the analytes and on the chiral selectors used. The elution sequence was determined in all cases, but no general predictive rule could be found to describe the elution behavior of these compounds.

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