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Evaluation of the macrocyclic glycopeptide A-40,926 as a high-performance liquid chromatographic chiral selector and comparison with teicoplanin chiral stationary phase

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Abstract

A new macrocyclic antibiotic of the vancomycin family, referred to by its industrial designation as A-40,926, was bonded to 5 μm silica particles and utilised as a chiral stationary phase (CSP). Since A-40,926 is structurally related to teicoplanin, the A-40,926 CSP was compared to a commercially available teicoplanin CSP. A set of 28 chiral compounds, including amino-acids and related compounds, compounds with a ring containing the stereogenic centre, compounds bearing aromatic structures near their stereogenic centres and alcohols, was tested for enantioseparation on the two CSPs. The results are compared and discussed in terms of enantioselective Gibbs energy difference. The A-40,926 CSP was able to resolve one compound that was not resolved by the teicoplanin CSP. However, it could not separate four compounds that the teicoplanin CSP did separate. It is shown that the A-40,926 CSP is complementary to the teicoplanin CSP, thereby enlarging the number of enantiomers that can be separated by the macrocyclic glycopeptide based CSPs.

Keywords: Chiral stationary phases, LC; Enantiomer separation; A-40,926; Amino acids; Teicoplanin; Antibiotics

1. Introduction

Since their introduction in 1994 [1], macrocyclic antibiotics have proven to be highly successful chiral selectors in HPLC [1–6] as well as in capillary electrophoresis [7–10]. The two most useful structural types belong to the *Ansa* family (rifamycin B and SV [2,9]) and to the glycopeptide group (vancomycin, ristocetin and teicoplanin [3–8]). These macrocycles have been found to be effective chiral selectors in both their ionized and molecular forms. They show excellent enantioselectivity for a wide variety of compounds. Thus far, the glycopeptide macrocycles are the preferred chiral selectors of this class because of their broad applicability. Indeed, they offer a unique combination of structural features

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useful in the interaction with chiral analytes. They are able to associate through (i) ionic interactions (ii) hydrogen bonding, (iii) $\pi-\pi$ and (iv) dipole–dipole interactions and (v) hydrophobic interactions via the cleft in the aglycone 'basket' and for teicoplanin, the nonyl-tail chain [11,12].

It is known that small changes in the structure of the chiral selector can induce large changes in the enantiorecognition capability of the chiral stationary phase (CSP) prepared with it. For example, derivatization of cyclodextrins produces a variety of different selectivity CSPs in HPLC [13] as well as in GC [14] and capillary electrophoresis [11,15]. Similarly, changes in the antibiotic structure such as removal of the carbohydrate moieties from the teicoplanin molecule [6,16] or investigation of glycopeptide antibiotics with slightly altered groups [17] widen the number and type of enantiomeric pairs successfully separated.

Antibiotic A-40,926 [18–21] is a member of the glycopeptide family and is structurally related to teicoplanin. A CSP was prepared with A-40,926 and its enantiorecognition capabilities were assessed with a set of enantiomeric pairs containing different functionalities. The same set of solutes has been analyzed with a commercial teicoplanin column for reference and comparison.

2. Experimental

2.1. Chemicals

The stationary phase base was the LiChrospher Si 100 silica gel (5 μm particle size, 10 nm pore diameter, 400 m^2/g specific surface area, Merck, Darmstadt, Germany). (3-Aminopropyl) triethoxysilane, dry toluene, dry pyridine and 1,6-diisocyanatohexane were obtained from Fluka (Sigma–Aldrich, Buchs, Switzerland). Table 1 lists the racemic compounds that were tested on the CSPs with their chemical structure and a reference number used in the other tables. They were all obtained from Sigma–Aldrich (St. Louis, MO). The chromatographic solvents methanol and acetonitrile, and the buffer additives [acetic acid, ammonium acetate, and triethylamine (TEA)] were from Fisher Scientific (Fair Lawn, NJ). A-40,926 was a gift of the Lepetit Research Centre (Gerenzano, Italy).

2.2. Preparation of the A-40,926 chiral stationary phase

The full procedure was recently exposed [5] for the preparation of a CSP containing teicoplanin as selector. First, 5 g of LiChrospher Si 100 were dried at 150°C for an hour under vacuum (0.1 mbar). In a round bottom flask, 120 ml of toluene were added and heated to reflux to remove azeotropically any residual water. Then, 2.5 ml of (3-aminopropyl) triethoxysilane (11 mmol) were added dropwise and the mixture was heated to reflux for 4 h. After cooling, the modified silica was filtered and washed with toluene, methanol and dichloromethane and dried at 90°C (0.1 bar, 2 h). The elemental analysis gave C 4.04%, H 1.12%, N 1.29%, corresponding to 1045 μmol/g of aminopropyl groups or 2.61 μmol/m^2 based on the N percentage.

Next, 2.5 ml of 1,6-diisocyanatohexane (15 mmol) were added to an ice-bath cooled slurry of 3 g of 3-aminopropyl-LiChrospher in 50 ml of dry toluene. Then, the mixture was heated at 70°C for 2 h. After cooling, the supernatant toluene phase was removed under argon atmosphere. The excess reactant was removed by dry toluene washing. A suspension of 0.9 g of A-40,926 (0.52 mmol) in 90 ml dry pyridine was added dropwise to the wet activated silica. Next, the mixture was heated at 70°C for 12 h with stirring and under argon atmosphere. After cooling, the A-40,926 bonded silica was washed with 50 ml portions in the sequence pyridine, water, methanol, acetonitrile and dichloromethane. It was dried under vacuum (70°C, 0.1 mbar, 2 h). The elemental analysis gave C 14.2%, H 2.4% and N 3.4%, corresponding to 121 μmol/g of A-40,926 or 0.30 μmol/m^2 based on the C percentage.

2.3. Column preparation

A classical packing procedure was used [5]: 3.3 g of the bonded LiChrospher were suspended in 60 ml of a 50/50 acetone/chloroform mixture with 1% acetic acid. After 5 min of ultrasonication, the slurry was packed in a 250×4.6 mm stainless steel column at 700 bars with a Haskel DSTV-122 pump using

Table 1
Solutes used in the study[a]

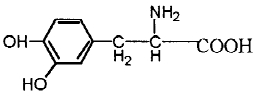
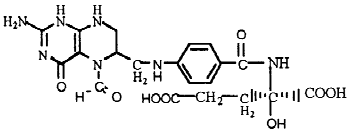
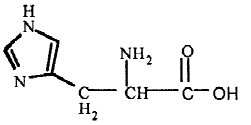
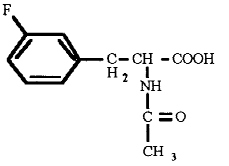
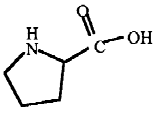
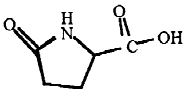
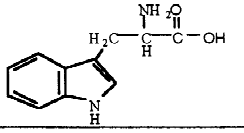
Code	solute	formula		m.w.	use
Class A - Amino Acids					
A1	Aspartic acid	COOH-CH_2-CH(NH_2)-COOH	$C_4H_7NO_4$	133	
A2	Dopa		$C_9H_{11}NO_4$	197	antiparkisonian
A3	Folinic acid		$C_{20}H_{23}N_7O_7$	473	antianemic
A4	Histidine		$C_6H_9N_3O_2$	155	essential AA
A5	Leucine	$(CH_3)_2$CH-CH_2-CH(NH_2)-COOH	$C_6H_{13}NO_2$	131	essential AA
A6	Phenylalanine	ϕ-CH_2-CH(NH_2)-COOH	$C_9H_{11}NO_2$	165	essential AA
A7	N-acetyl-m-fluoro-phenylalanine		$C_{11}H_{12}FNO_3$	225	
A8	Proline		$C_5H_9NO_2$	115	
A9	2-pyrrolidone-5-carboxylic acid		$C_5H_7NO_3$	129	
A10	Threonine	CH_3-CHOH- CH(NH_2)-COOH	$C_4H_9NO_3$	119	essential AA
A11	Tryptophan		$C_{11}H_{12}N_2O_2$	204	essential AA

Table 1. Continued

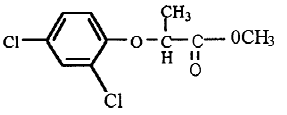
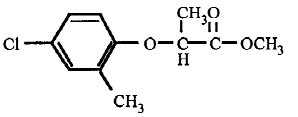
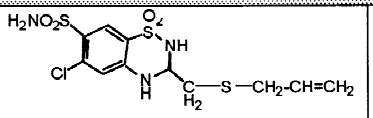
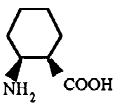
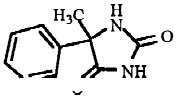
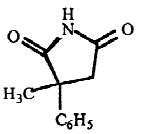
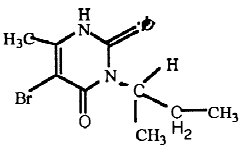
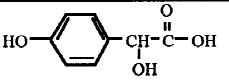
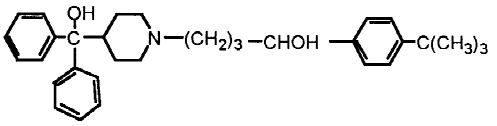
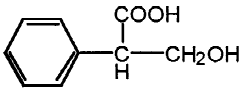
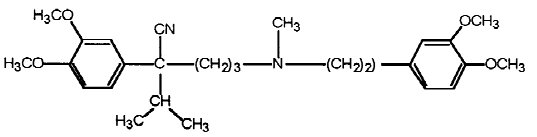
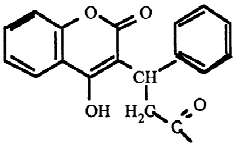
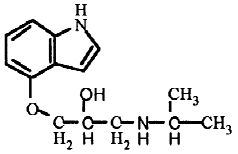
Code	solute	formula	m.w.	use
Class A' – Structurally Similar to Amino Acids				
A12	Dichlorprop-methyl		235	herbicide
A13	Mecoprop-methyl		214.5	herbicide
Class B – Stereogenic Center in a Ring				
B1	Althiazide		384	diuretic
B2	Cis-2-amino cyclohexane carboxylic acid		345	
B3	5-methyl-5-phenyl hydantoin		190	
B4	α–methyl α–phenyl succinimide		189	antiurolithic
Class C – Aromatic Ring Connected the Stereogenic Center				
C1	Bromacil		261	herbicide
C2	4-Hydroxy mandelic acid		168	urinary antiseptic

Table 1. Continued

Code	solute	formula		m.w.	use
C3	Terfenadine		$C_{32}H_{41}NO_2$	472	antihistaminic
C4	Tropic acid		$C_9H_{10}O_3$	166	
C4	Verapamil		$C_{27}H_{38}N_2O_4$	454.5	antianginal
C5	Warfarin		$C_{19}H_{16}O_4$	308	rodenticide
		NH_2-CO-CH_2-ϕ			
D3	Carnitine	$(CH_3)_3N^+$-CH_2-CHOH-CH_2-COO^-	$C_7H_{15}NO_3$	161	fat fighter
D4	Pindolol		$C_{14}H_{20}N_2O_2$	248	antihypertensive
D5	Phenyl propanolamine (Norephedrine)	ϕ-CHOH-$CH(CH_3)$-NH_2 (HCl)	$C_9H_{14}ClNO$	187.5	nasal decongestant

[a] ϕ = aromatic ring.

methanol as the pressurizing agent. The A-40,926 column efficiency was in the 40 000 plates/meter range checked with a hexane/chloroform 90/10 mobile phase and acetophenone ($k'>5$). The teicoplanin column utilized 5 μm silica particles (*Chirobiotic T* 250×4.6 mm column, from Astec, Whippany, NJ). The teicoplanin column had 48 000 plates/meter (100.μmol/g). The dead volume was determined by injecting 100% methanol.

2.4. Chromatographic system

The 28 compounds listed in Table 1 were eluted on the two 25 cm columns with 9 different mobile phases, at 22°C. The results are listed in Tables 2 and 3. The chromatographic system used a Shimadzu SCL-10A System Controller and a SIL-10A Auto Injector, a Shimadzu LC-10AT pump, and a Shimadzu SPD-10A UV detector with a Chromatopac CR 501 integrator (Shimadzu, Kyoto, Japan, obtained from Delta Instruments, Mountain View, MO).

3. Results and discussion

3.1. The A-40,926 macrocyclic glycopeptide

A-40,926 [18–21] is a complex of glycopeptides produced by the *Actinomadura* strain ATCC 39726, from which factors A and B are the major recoverable species. The chemical structure of the prevalent component of A-40,926 glycopeptide complex (factor B, >70%) is reported in Fig. 1. The molecular formula of A-40,926 factor B is $C_{83}H_{88}Cl_2N_8O_{29}$, m.w. 1731. Fig. 1 gives its structure as well as that of the teicoplanin A_2-2 molecule ($C_{88}H_{97}Cl_2N_9O_{33}$, m.w. 1878). The similarities are evident. These two molecules clearly belong to the same family. The five differences between the molecules are:

(i) the β-D-*N*-acetyl-glucosamine unit of teicoplanin is not present in A-40,926, instead there is a simple hydroxyl group;

(ii) the primary alcohol of the *N*-acyl-glucosamine unit of teicoplanin is oxidized to a carboxylic acid in A-40,926;

(iii) the primary amine group on the aglycone portion of teicoplanin is a methyl-substituted secondary amine in A-40,926;

(iv) the chlorine substituent of phenyl ring 2 of teicoplanin is not present in A-40,926, which, in turn, possesses a chlorine atom on its phenyl ring 3;

(v) the 9 carbon chain of teicoplanin (8-methyl nonanoate) is a 10 carbon chain in A-40,926 (10-methyl undecanoate).

The difference in the alkyl chains (point (v) above) is not likely to be of any significance since the natural teicoplanin produced by the fungi *Actinoplanes teicomyceticus* contains five isomers differing only by small changes in the methyl position on the nonanoate chain or in carbon atom number (from nine to ten). As will be shown, the first three structural differences listed above are the most important in terms of chiral recognition and enantioselectivity. This is because they control the overall charge of the chiral selector and provide primary interaction sites and steric effects.

3.2. Analyte selection

The selected solutes were arranged in four classes (A, B, C, D) according to their molecular structure. Since macrocyclic glycopeptides are especially efficient in separating α-amino-acids, the first class, referred to as Class A (A1–A13), contains eleven amino-acids (A1–A11) and two structurally related compounds. Two non-amino-acid herbicide molecules (A12 and A13) were added as a subsection of Class A. These two herbicides have the general structure R–C*H(COOH)–OR′, with R and R′ differing by atoms or groups, which has similarity with the general structure: R–C*H(COOH)–NH–R′ of the α-amino-acids. The second class of compounds, referred to as Class B, is made up of four non-α-amino-acid enantiomeric compounds (B1–B4), where the stereogenic center is part of a ring structure that introduces some rigidity to the molecule. The six Class C compounds (C1–C6) are non-amino-acid molecules with one or more aromatic moieties directly connected to the stereogenic center, which is not a part of any ring system. An aromatic structure is not necessarily a benzene ring. It can be any other π-electron rich ring such as uracil or pyrimidinedione ring (Bromacil). Most compounds of Classes B and C were especially well

Table 2
Chromatographic results on the two CSPs with 9 different mobile phases for the class A compounds (amino-acids and related compounds)

Compound	CSP[a]	Mobile phase[b]	k'_1	k'_2	α	R_s	$-\Delta_{R,S}\Delta G^c$
A1	Teic	60-3.8	0.34	0.49	1.40	1.4	0.22
	A40	40	6.0	6.72	1.12	1.1	0.07
A2	Teic	20	0.36	0.63	1.75	1.4	0.33
	A40		3.17	4.41	1.39	1.9	0.19
	Teic	20-4.1	0.39	0.67	1.72	1.4	0.32
	A40		0.31	0.59	1.90	1.5	0.38
	Teic	40	0.48	0.86	1.79	2.3	0.34
	Teic	60	0.53	1.11	2.10	3.5	0.43
	Teic	60-3.8	0.50	0.89	1.78	2.4	0.34
	A40		0.42	1.18	2.80	2.6	0.60
	Teic	60-4.1	0.39	0.94	2.41	1.8	0.52
	A40		0.43	1.20	2.80	3.0	0.60
	Teic	85	1.16	3.54	3.05	3.5	0.65
A3	A40	20-4.1	6.00	7.98	1.33	0.7	0.17
	Teic	60-4.1	0.74	1.21	1.64	1.3	0.29
	A40		1.43	2.0	1.40	0.9	0.20
	Teic	100	5.74	8.71	1.52	1.5	0.25
A4	Teic	60-3.8	6.60	7.60	1.15	0.8	0.08
	A40		1.38	1.75	1.27	0.7	0.14
	A40	60-4.1	4.00	4.88	1.22	1.0	0.12
	A40	85	5.00	8.55	1.71	1.0	0.31
A5	Teic	20	0.36	0.74	2.05	1.4	0.42
	A40		0.38	0.82	2.16	2.0	0.45
	Teic	40	0.47	0.87	1.85	2.8	0.36
	A40		1.50	2.74	1.83	3.0	0.35
	Teic	60	0.49	1.12	2.28	2.8	0.48
	A40		0.91	2.37	2.61	3.6	0.56
	Teic	60-3.8	0.53	0.63	1.19	3.7	0.10
	Teic	85	0.68	2.10	3.09	5.5	0.66
	A40		1.40	4.23	3.02	3.7	0.65
A6	Teic	20	0.58	0.74	1.28	0.9	0.14
	A40		0.38	0.65	1.71	1.4	0.31
	Teic	20-4.1	0.63	0.73	1.16	0.8	0.09
	A40		0.38	0.65	1.71	1.4	0.31
	Teic	40	0.71	0.95	1.34	1.5	0.17
	A40		0.63	1.26	2.00	1.7	0.41
	Teic	60	0.71	1.07	1.51	1.4	0.24
	A40		0.75	1.75	2.33	2.7	0.50
	Teic	60-3.8	0.67	0.98	1.47	2.0	0.23
	A40		0.88	1.63	1.85	3.1	0.36
	Teic	60-4.1	0.50	0.80	1.60	1.6	0.28
	A40		0.87	2.23	2.56	2.8	0.55
	Teic	85	1.05	1.97	1.88	2.7	0.37
	A40		0.92	2.79	3.03	3.7	0.65
	Teic	100	1.21	2.04	1.69	2.5	0.31
A7	A40	20	0.40	1.40	3.50	1.0	0.73
	A40	20-4.1	1.50	6.26	4.17	4.8	0.84
	Teic	60-3.8	0.15	0.58	3.86	3.0	0.79
	A40		1.27	3.75	2.95	4.6	0.63

Table 2. Continued

Compound	CSP[a]	Mobile phase[b]	k'_1	k'_2	α	R_s	$-\Delta_{R,S}\Delta G$[c]
	A40	60-4.1	0.40	2.47	6.17	6.4	1.07
	Teic	85	0.31	1.82	5.90	3.9	1.04
	A40		0.14	1.20	8.60	3.5	1.26
	A40	100	0.63	3.53	5.60	6.6	1.01
	A40	95ACN	3.50	7.25	2.07	2.4	0.43
A8	Teic	40	0.81	2.94	3.63	6.8	0.76
	Teic	60	1.05	4.27	4.07	12.5	0.82
A9	Teic	60-4.1	0.29	0.59	2.03	1.5	0.42
	Teic	100	1.70	2.51	1.48	2.0	0.23
A10	A40	20	0.88	1.06	1.20	0.6	0.11
	Teic	40	0.20	0.35	1.75	0.9	0.32
	A40		0.13	0.24	1.85	0.6	0.36
	Teic	60	0.28	0.39	1.39	1.1	0.19
	A40		0.17	0.34	2.00	1.3	0.41
	Teic	60-3.8	0.33	0.43	1.29	1.2	0.15
	Teic	85	0.74	1.21	1.63	1.4	0.29
	A40		0.53	1.19	2.25	2.0	0.41
	Teic	100	1.50	2.73	1.82	2.3	0.35
A11	Teic	20	1.00	1.29	1.29	1.4	0.15
	A40		1.75	2.74	1.57	1.6	0.26
	Teic	20-4.1	0.78	1.05	1.34	1.5	0.17
	A40		1.50	2.25	1.50	1.3	0.24
	Teic	40	0.90	1.22	1.36	1.4	0.18
	A40		1.38	2.76	2.00	2.4	0.41
	Teic	60	0.85	1.56	1.83	1.5	0.35
	A40		1.18	2.36	2.00	3.2	0.41
	Teic	60-3.8	0.78	1.10	1.41	1.5	0.20
	A40		1.00	2.29	2.29	3.3	0.49
	Teic	60-4.1	0.60	0.83	1.38	1.5	0.19
	A40		0.86	2.15	2.50	3.0	0.54
	Teic	85	1.16	2.12	1.83	2.2	0.35
	A40		1.38	3.60	2.61	4.7	0.56
	Teic	100	1.25	2.24	1.79	2.1	0.34
	A40		1.35	3.51	2.33	4.1	0.50
Class A non amino-acid compounds							
A12	Teic	20-4.1	1.43	2.04	1.43	2.5	0.21
	A40		7.01	9.25	1.32	1.2	0.16
	Teic	60-3.8	0.08	0.14	1.75	0.9	0.33
	A40		4.02	4.90	1.22	0.9	0.12
A13	Teic	20-4.1	1.28	1.79	1.40	2.0	0.20
	A40		8.02	9.30	1.16	0.9	0.09
	Teic	60-3.8	0.06	0.13	2.17	0.6	0.45
	A40		2.75	3.13	1.14	0.6	0.08

[a] Teic = teicoplanin chiral stationary phase (CSP), A40 = A-40,926 CSP.

[b] Mobile phase code is: 20 = 20% methanol–80% water v/v; 20-4.1 = 20% methanol–80% aqueous buffer (pH 4.1 by TEAA, 1%); 40 = 40% methanol–60% water v/v; 60 = 60% methanol–40% water v/v; 60-3.8 = 60% methanol–40% aqueous buffer (pH 3.8 by acetic acid); 60-4.1 = 60% methanol–40% aqueous buffer (pH 4.1 by TEAA, 1%); 85 = 85% methanol–15% aqueous ammonium acetate (2.5×10^{-2} *M*); 100 = 100% methanol with 0.1% TEA and 0.1% v/v acetic acid; 95ACN = 95% acetonitrile–5% methanol with 0.2% TEA and 0.3% acetic acid.

[c] Difference in enantioselective Gibbs energy ($\Delta_{R,S}\Delta G$) in kcal/mol ($=RT \ln \alpha$), $T = 22$°C.

Table 3
Chromatographic results on the two CSPs with 9 different mobile phases for the class B, C, and D compounds

Compound	CSP[a]	Mobile phase[b]	k'_1	k'_2	α	R_s	$-\Delta_{R,S}\Delta G^c$
Class B compounds: a ring rigidifies the structure							
B1	Teic	20	4.16	4.83	1.16	1.0	0.09
	Teic	20-4.1	3.05	3.24	1.06	0.8	0.03
B2	A40	20	2.03	2.44	1.20	1.1	0.11
	A40	40	2.36	2.71	1.15	1.1	0.08
	A40	60	1.73	1.99	1.15	1.0	0.08
	Teic	85	1.33	1.46	1.10	0.9	0.06
	A40		1.57	2.43	1.55	2.4	0.26
	Teic	100	2.80	2.97	1.06	0.6	0.03
B3	Teic	20	1.55	3.55	2.29	4.1	0.49
	A40		4.13	7.89	1.91	3.8	0.38
	Teic	20-4.1	1.00	2.17	2.17	3.0	0.45
	A40		4.02	7.16	1.78	4.2	0.34
	Teic	40	0.68	1.62	2.38	4.5	0.51
	A40		2.11	4.55	2.16	4.9	0.45
	Teic	60	0.39	0.98	2.52	2.5	0.54
	A40		0.92	1.17	1.27	4.0	0.14
	Teic	60-3.8	0.36	0.74	2.05	2.4	0.42
	A40		0.90	2.10	2.33	4.8	0.50
	Teic	60-4.1	0.25	0.60	2.41	2.0	0.52
	A40		0.80	1.61	2.00	3.6	0.41
	Teic	85	0.08	0.21	2.62	2.5	0.56
	A40		0.43	1.00	2.33	3.2	0.50
	Teic	100	0.17	0.42	2.46	1.5	0.53
	A40		0.38	1.08	2.83	4.8	0.62
	Teic	95ACN	0.66	0.89	1.35	1.2	0.18
B4	Teic	20	1.32	1.58	1.20	1.3	0.11
	A40		11.0	12.0	1.09	0.7	0.05
	Teic	20-4.1	1.08	1.23	1.14	1.2	0.08
	A40		4.05	4.58	1.13	1.0	0.07
	Teic	40	0.61	0.74	1.21	0.9	0.11
	A40		5.01	5.31	1.06	0.6	0.03
	A40	60	0.65	0.78	1.20	0.5	0.10
Class C compounds: non amino-acid compounds with at least one aromatic ring							
C1	Teic	20	2.03	2.84	1.40	2.1	0.20
	A40		9.51	12.7	1.34	2.5	0.17
	Teic	20-4.1	1.88	2.45	1.30	2.1	0.15
	A40		7.20	10.4	1.44	2.8	0.21
	Teic	40	0.74	0.95	1.28	1.4	0.14
	A40		2.50	3.25	1.30	2.3	0.15
	A40	60	0.71	0.88	1.25	0.9	0.13
	A40	60-3.8	0.62	0.77	1.25	0.8	0.13
	A40	60-4.1	0.62	0.81	1.30	1.2	0.15
C2	Teic	20-4.1	0.23	1.15	5.00	5.0	0.94
	A40		0.06	0.18	3.00	2.0	0.64
	A40	60-3.8	1.13	2.01	1.78	1.1	0.34
	Teic	60-4.1	0.10	0.51	5.10	2.12	0.96
	A40		0.36	1.44	4.00	2.6	0.81
	Teic	85	0.16	0.65	4.05	3.0	0.82
C3	A40	20	4.80	7.20	1.50	3.4	0.24

Table 3. Continued

Compound	CSP[a]	Mobile phase[b]	k'_1	k'_2	α	R_s	$-\Delta_{R,S}\Delta G^c$
C4	Teic	20-4.1	0.42	0.47	1.12	0.8	0.07
	Teic	100	0.42	0.61	1.46	0.6	0.22
C5	Teic	20-4.1	10.8	11.4	1.05	0.7	0.03
	A40	60-4.1	6.25	6.88	1.10	0.5	0.06
	A40	100	7.63	8.16	1.07	0.7	0.04
C6	A40	40	8.00	9.44	1.18	0.7	0.10
	Teic	60-3.8	0.47	0.61	1.30	1.3	0.15
Class D compounds: R–CH_2–CH(OH)–CH_2–R′							
D1	A40	40	1.53	1.73	1.13	0.5	0.07
	Teic	85	5.84	6.89	1.18	1.5	0.10
	A40		1.42	2.67	1.88	3.8	0.37
	A40	100	2.25	5.13	2.28	4.6	0.48
D2	Teic	100	4.90	5.49	1.12	1.4	0.07
	A40		10.3	10.8	1.05	0.8	0.03
D3	Teic	85	4.73	5.11	1.08	1.0	0.05
	A40		1.00	1.27	1.27	0.8	0.14
	A40	100	2.89	3.55	1.23	1.2	0.12
D4	Teic	85	8.50	8.93	1.05	1.1	0.03
	A40		15.7	16.5	1.05	0.6	0.03
	Teic	100	2.37	2.68	1.13	1.4	0.07
	A40		6.25	6.88	1.10	1.2	0.06
D5	A40	85	13.9	14.7	1.06	1.3	0.03
	Teic	100	2.61	2.71	1.04	0.7	0.02
	A40		6.51	7.16	1.10	1.4	0.06

[a] Teic = teicoplanin chiral stationary phase (CSP), A40 = antibiotic A40,926 CSP.

[b] Mobile phase code is: 20 = 20% methanol–80% water v/v; 20-4.1 = 20% methanol–80% aqueous buffer (pH 4.1 by TEAA, 7%); 40 = 40% methanol–60% water v/v; 60 = 60% methanol–40% water v/v; 60-3.8 = 60% methanol–40% aqueous buffer (pH 3.8 by acetic acid); 60-4.1 = 60% methanol–40% aqueous buffer (pH 4.1 by TEAA, 1%); 85 = 85% methanol–15% aqueous ammonium acetate (2.5×10^{-2} *M*); 100 = 100% methanol with 0.1% TEA and 0.1% v/v acetic acid; 95ACN = 95% acetonitrile–5% methanol with 0.2% TEA and 0.3% acetic acid.

[c] Difference in enantioselective Gibbs energy ($-\Delta_{R,S}\Delta G$) in kcal/mol ($=RT \ln \alpha$), $T = 22$°C.

resolved by the cyclodextrins based CSPs [11,13–15]. Class D compounds are five non-amino-acid solutes that contain an alcohol moiety connected to the stereogenic center (i.e., a general structure: R–CH_2–C*HOH–CH_2–R). Acetyl carnitine also was included in this group even though its hydroxyl group was acetylated. These compounds were selected because of previously noted interactions with the teicoplanin CSP [4,5,11,12]. Table 1 lists the compounds arranged according to their molecular structure and alphabetic order within classes. The name, molecular weight and use are given with the molecular structure. Table 2 lists the results for the Class A compounds (α-amino-acids) using the compounds codes listed in Table 1. Similarly, Table 3 lists the results obtained for the three other compounds classes. The retention factors (k'_1 and k'_2), selectivity (α), resolution factor (R_s) and the enantioselective free energy differences ($-\Delta_{R,S}\Delta G$) are listed for each compound, in addition to the mobile phase used.

3.3. Mobile phases and presentation of the results

All compounds in Table 1 were evaluated with seven different RPLC mobile phases ranging from

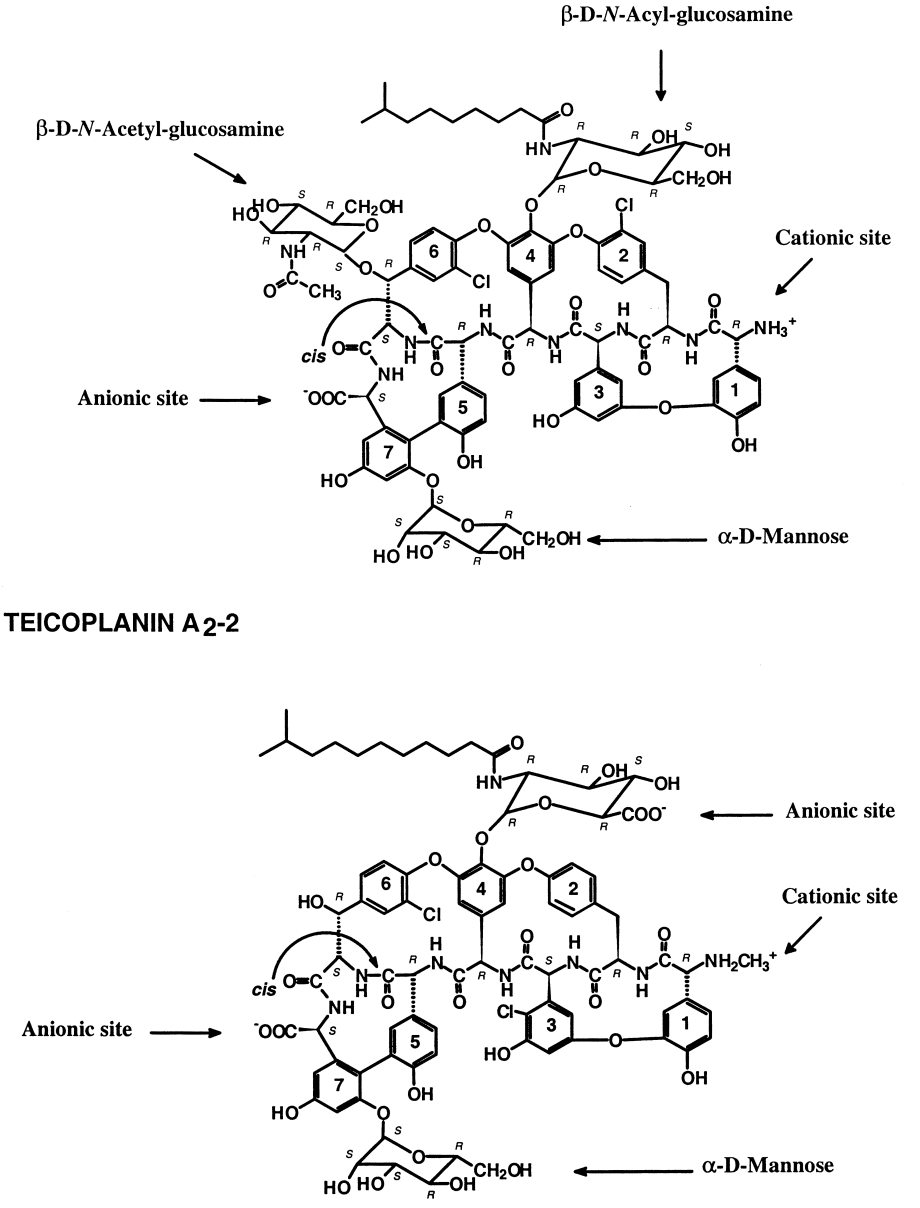


Fig. 1. The structures of the macrocyclic antibiotics teicoplanin (*top*) and A-40,926 (*bottom*). The numbering of the aromatic rings is linked to their connection to the polypeptide macrocycle starting at the amine group (cationic site).

20% to 85% methanol/water (v/v). Also, two polar organic mobile phases were evaluated. They were 1) 100% methanol with 0.1% acetic acid and 0.1% TEA and 2) acetonitrile/methanol 95/5 (v/v) with 0.2% triethylamine and 0.3% acetic acid added (the latter having a much lower hydrogen bonding capability). On each of the two CSPs, 252 chromatograms were obtained (28 compounds times 9 mobile phases). To simplify the presentation of the results, Tables 2 and 3 list only the chromatographic results obtained when an enantiomeric separation was achieved. This was the case for 162 chromatograms. Of the 162

successful enantioseparations, 83 were obtained with the A-40,926 CSP (51.2%) and the remaining 79 were obtained on the teicoplanin CSP (48.8%).

Focusing on the amino-acid solutes only (compounds A1 to A11), of the 99 chromatograms run on the A-40,926 CSP, 40 were enantioseparated (40.4%). This figure was 43.4% on the teicoplanin column (43 separations≥0.5). Considering the 17 non-amino acid compounds (Table 3 plus compounds A12 and A13), 43 separations were successful out of the 153 tested on the A-40,926 CSP (28.1%). The corresponding number for the teicoplanin column was 36 successful separations (23.5%). From the solute point of view, 23 solutes were resolved by the two CSPs (82%), 1 was resolved by the A-40,926 CSP only (4%). This compound was terfenadine (C3). Four solutes were resolved by the teicoplanin CSP only (14%). They were proline (A8), 2-pyrrolidone-5-carboxylic acid (A9), althiazide (B1) and tropic acid (C4).

3.4. Stationary phase polarity and solute retention

Considering the nature of the bonded material on the silica surface, it is apparent that different functional groups are present on the two CSPs. For the A-40,926 CSP, the linkage between the silica surface and the antibiotic has 9 apolar methylene units and either a ureido or carbamate group. The polar groups of the A-40,926 antibiotic are 11 hydroxyl groups of which 4 are phenolic groups, 1 free secondary amino group and 2 free carboxylic acid groups. Its apolar groups are the 11 carbon chain units of its sugar alkyl chain, the row of 6 amide linkages in the macrocyclic portion of the molecule (see Fig. 1) and the 7 benzene rings attached to it. For the teicoplanin CSP, the linkage between the silica surface and the antibiotic is only three methylene units long with a carbamate link. The polar groups of the teicoplanin molecule are 14 hydroxyl groups of which 4 are phenolic groups, 1 free primary amine and 1 free carboxylic acid. The teicoplanin apolar groups are very similar to those of the A-40,926 antibiotic: 9 carbon chain units of the sugar alkyl chain, the row of 6 amide linkages and the 7 aromatic rings attached to the 'basket' (Fig. 1). Thus, it is difficult to evaluate the polarity of the two phases considering just their molecular structure. The retention factors of the different test molecules should give a better idea of the relative polarity of these stationary phases.

The retention factors of the first eluted enantiomer of the majority of the compounds of the four classes are somewhat lower with the same RPLC mobile phase on the teicoplanin CSP than on the A-40,926 CSP (Tables 2 and 3). For example, the k'_1 values of the first eluted enantiomer of pindolol (D4) are 8.50 and 15.7 with the 85% methanol mobile phase on the teicoplanin and A-40,926 CSPs, respectively. These k'_1 values both drop to 2.37 and 6.25, respectively, with the 100% methanol mobile phase (Table 3). Considering 5-methyl-5-phenyl hydantoin solute (B3), its k'_1 values are 1.55 and 4.13 with the 20% methanol mobile phase. They drop to 0.08 and 0.43 when the 85% methanol mobile phase is used. The retention factors of the first enantiomers are half or lower with the teicoplanin CSP compared to the A40296 CSP (Table 3). Considering the number of functional groups, including hydroxyls, phenols, charged amine or carboxylate groups, aromatic rings and alkyl chains (Fig. 1), it is difficult to rank the polarity of these stationary phases. It can be observed that, with the set of 28 compounds studied, the A-40,926 stationary phase is somewhat more retentive than the teicoplanin CSP in the RPLC mode. It is possible that this increased retention is due to the longer linkage chain of the A-40,926 CSP. However, on a compound per compound basis, the retention trends on both stationary phases are the same.

Fig. 2 shows the retention factors (log k') of three compounds versus the mobile phase composition. Teicoplanin retention factors are shown with thin lines, while A-40,926 retention factors are connected with bold lines. For the amino-acid phenylalanine (A6), an increase in the retention factor is observed when the mobile phase methanol content increases. This behavior was observed on other macrocyclic glycopeptide stationary phases [3,4]. It is due to the reduced solubility of amino-acids in methanol rich mobile phases. For tryptophan (A11) on the A-40,926 CSP, a decrease in retention is observed with the increase in the methanol content of the mobile phase up to 60% (v/v) methanol (Fig. 2, middle). For the methanol rich mobile phases, the retention factors of tryptophan increase on both CSPs. For most

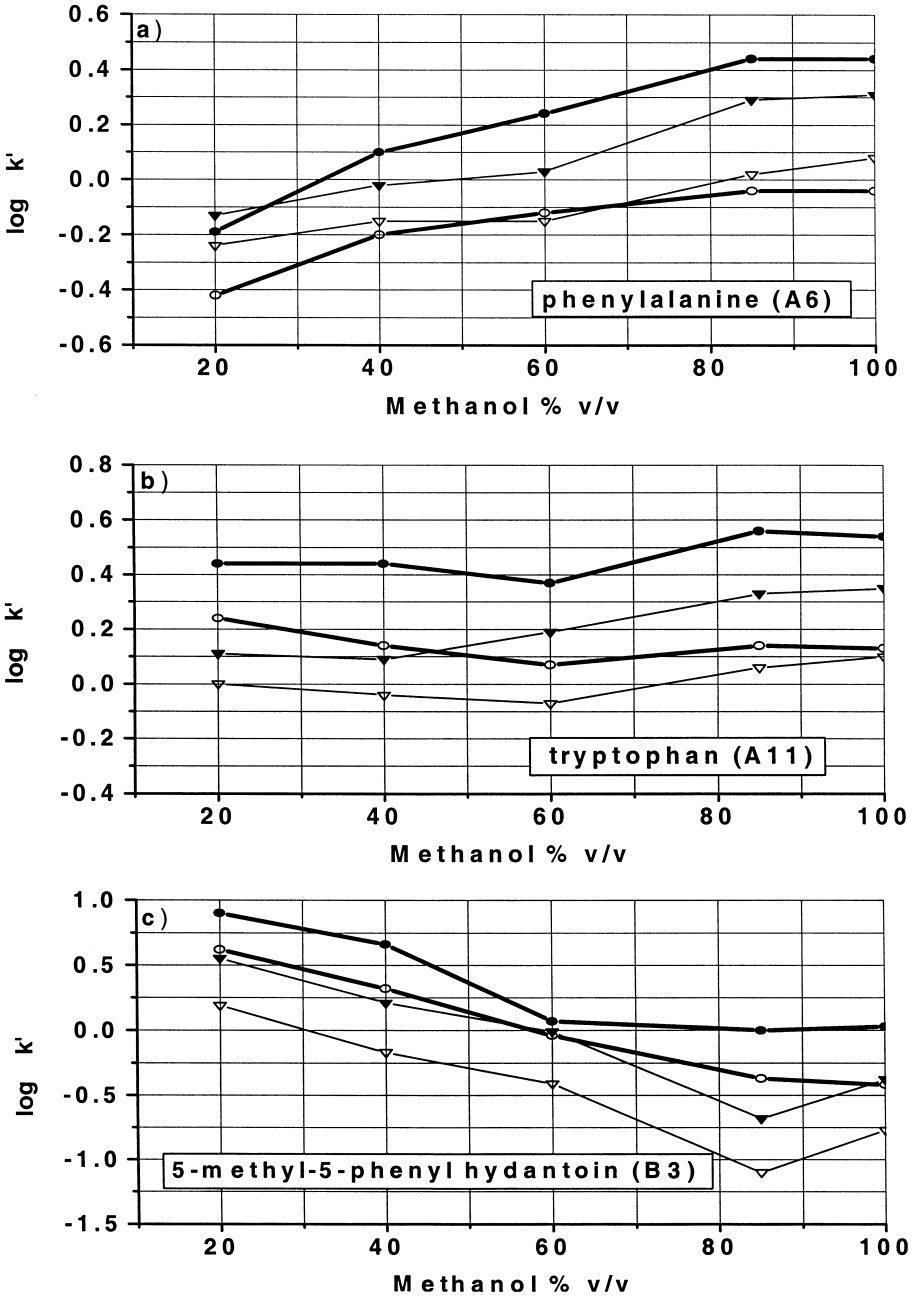


Fig. 2. Plots of log k' vs. the methanol mobile phase content for: a) phenylalanine (A6), b) tryptophan (A11), and c) 5-methyl-5-phenyl hydantoin (B3); thick lines and circles: A-40,926-CSP; thin lines and triangles, teicoplanin-CSP. ($\triangledown$) k'_1 teicoplanin; (▼) k'_2 teicoplanin; (○) k'_1 A-40,926; (●) k'_2 A-40,926.

non-amino-acid compounds, an almost linear decrease of the log k' value is observed with increasing methanol content in the mobile phase (see 5-methyl-5-phenyl-hydantoin, B3, Fig. 2, bottom). No retention factors higher than 26 (retention time 80 min) were listed in the tables because this retention time

was considered as a maximum acceptable value. When the injected solute did not elute after 90 min, the column was rinsed with pure methanol and re-equilibrated with the mobile phase for another solute.

3.5. Enantioselectivity

Tables 2 and 3 list the enantioselectivity factors ($\alpha = k'_2/k'_1$), the resolution factors (R_s) and the difference in enantioselective Gibbs energy obtained for the set of solutes. This difference in energy was estimated using [14]:

$$-\Delta_{R,S}\Delta G = RT \ln \alpha$$

The highest enantioselectivity factors obtained on the A-40,926 CSP were $\alpha = 8.6$ and 4.0 for *N*-acetyl-*m*-fluorophenylalanine (A7) and 4-hydroxymandelic acid (C2), respectively. The highest enantioselectivity factors obtained on the teicoplanin CSP were $\alpha = 5.9$ and 5.1 for the very same compounds. These α values correspond to differences in enantioselective Gibbs energy in the 0.96–1.04 kcal/mol range, which is indicative of the very high enantiorecognition capability of these two chiral selectors. Enantioselectivity factors, α, between 1.05 and 1.20, corresponding to enantioselective energies in the 0.03–0.11 kcal/mol range, at 22°C, are typical of separations reported early on [11]. The data in Tables 2 and 3 indicate that the resolution factors associated with the corresponding high enantioselectivity factors can be as high as 12.5. Fig. 3 shows the chromatogram of tryptophan (A11) on the two CSPs with the 85/15 MeOH–H_2O mobile phase. Approximately 3 min separate the first eluted L-tryptophan enantiomer from the D-enantiomer on the teicoplanin CSP. This time difference is 8 min for these enantiomers on the A-40,926 CSP. The corresponding enantioselectivity factors were 1.83 and 2.61, and the resolution factors were 2.2 and 4.7 on the teicoplanin and A-40,926 CSPs, respectively (Table 2). In this particular example, the A-40,926 CSP is the more effective column. It should be noted that, in this example and for all other separations of racemic common amino-acid pairs, the L-form is eluted first and the D-form is eluted last. This was already observed in our previous works [3,4,11].

Table 2 shows that there is an increase in the

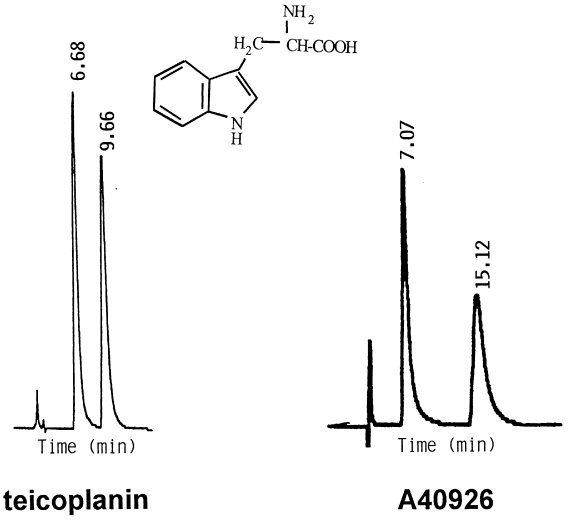


Fig. 3. Chromatogram of tryptophan (A11) on the two CSPs. Mobile phase: methanol/aqueous ammonium acetate (2.5×10^{-2} *M*) 85/15 v/v; flow-rate: 1 ml/min; UV detection @ 254 nm.

enantioselectivity factors and greater differences in the Gibbs energy for the amino-acid compounds when the methanol content in the mobile phase becomes high. For example, the enantioselectivity factors for phenylalanine (A6) are 1.28, 1.34, 1.51 and 1.88 on the teicoplanin CSP when using 20%, 40%, 60% and 85% methanol rich mobile phases, respectively. The corresponding difference in enantioselectivity Gibbs energy more than doubles starting at 0.14 kcal/mol for the 20% methanol mobile phase and ending at 0.37 kcal/mol for the 85% methanol rich mobile phase. The corresponding α values for the A-40,926 CSP are 1.71, 2.00, 2.33 and 3.03 showing a parallel increase in enantioselectivity and a doubling in enantioselectivity Gibbs energy. Conversely, the enantioselectivity factors and the corresponding energy differences for non-ionizable compounds are almost independent of the methanol content in the mobile phase (Table 3).

3.6. Comparison of the two chiral stationary phases

The average in enantioselective Gibbs energy difference was used to compare the enantioselectivity of the two columns. Considering only the successful chromatograms listed in Tables 2 and 3, the average

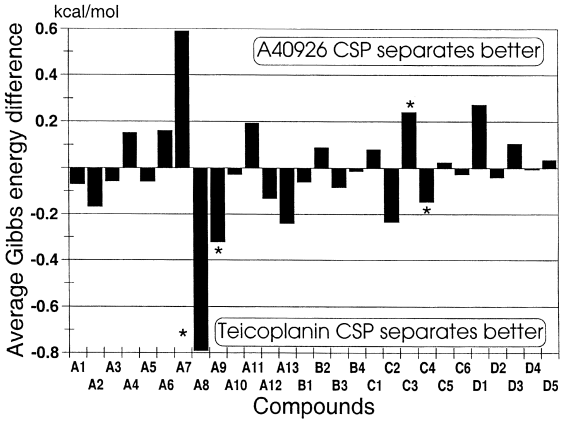


Fig. 4. Comparison of the two CSPs using the average enantioselective Gibbs energy difference (see text). The asterisks mean that the compound was separated by the particular CSP only. For compound codes, see Table 1.

energy values, presented in Fig. 4, were obtained as follows: for a given compound, the enantioselective Gibbs energy difference on the teicoplanin CSP was subtracted from the corresponding energy difference found on the A-40,926 CSP, for the same mobile phase. Then the average value for all mobile phases was calculated for each compound and used to prepare Fig. 4. For example, aspartic acid (A1, Table 2) was resolved only on A-40,926 with the 40% methanol mobile phase. The energy difference is 0.07 kcal/mol. It is 0 kcal/mol with the teicoplanin CSP (no separation). So the difference between the two CSPs and the average is also 0.07 kcal/mol. The calculation for D,L-DOPA (A2) is more demonstrative: for the mobile phase with 20% methanol, the enantioselective free energy differences on teicoplanin and A-40,926 are respectively 0.33 and 0.19; therefore 0.19 minus 0.33 is −0.14 kcal/mol. The same calculation is made for the six other mobile phase compositions, giving the following values: 0.06, −0.34, −0.43, 0.26, 0.08, −0.65 kcal/mol. When no results are listed, 0 kcal/mol (no separation) is used. Then the energy differences are averaged giving a value −0.167 kcal/mol, which is plotted in Fig. 4. A negative sign means that, from an overall point of view, the teicoplanin column is superior to the A-40,926 column for separating D,L-DOPA.

Fig. 4 shows that 9 enantiomers were better separated on the A-40,926 CSP (positive values, >0.05), including one that was separated only by this CSP (asterisk in Fig. 4), 12 solutes were better separated by the teicoplanin CSP (negative values, <0.05), including 4 that were separated only by this stationary phase (see asterisks). Seven solutes show near zero average values (± 0.05), they were equally well separated by both stationary phases. Fig. 5 shows the separation of 5-methyl-5-phenyl hydantoin (B3) on the two CSPs. There are ~2.7 min difference in retention times of the enantiomers on the A-40,926 CSP compared to ~1.1 min with the teicoplanin CSP. However, in this case, the low retention time of the first eluted B3 enantiomer on the teicoplanin CSP produces an enantioselectivity factor of 2.4 which is higher than the corresponding α value of 2.0 on the A-40,926 CSP (Table 3). As illustrated in Table 3, the corresponding resolution factors are 2.0 and 3.6 on the teicoplanin and A-40,926 CSPs, respectively. Fig. 4 shows the teicoplanin CSP is better than A-40,926 for separating

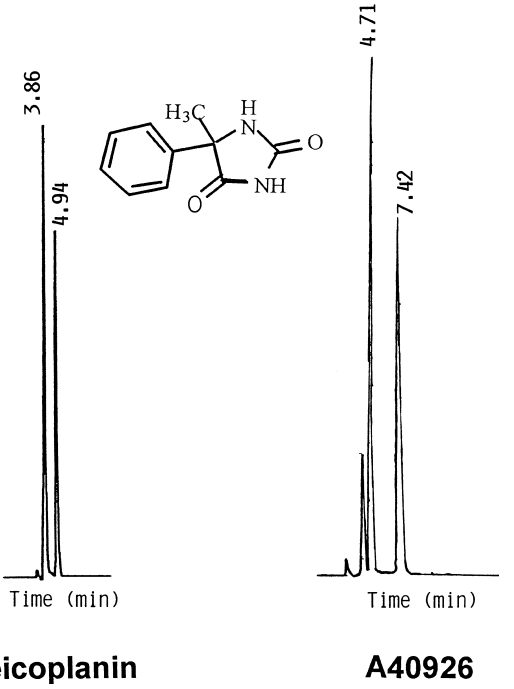


Fig. 5. Chromatogram of 5-methyl-5-phenyl hydantoin (B3) on the two CSPs. Mobile phase: methanol/aqueous buffer 1% TEAA, pH 4.1 (60/40, v/v); flow-rate: 1 ml/min; UV detection @ 254 nm.

enantiomers of compound B3 because it is based on enantioselectivity and not on resolution factors. It may be argued that from a practical point of view, resolution factors should be the criterion for comparison. There is no doubt that, from a thermodynamic point of view, the difference in enantioselective Gibbs energy is the reference criterion.

There are no clear structural differences between the solutes resolved by one CSP versus the other. The glucosamine moiety that is missing in the A-40,926 does not induce a significant change in the enantiorecognition of amino-acids. This was proven in previous studies by comparing the teicoplanin CSP with a CSP made with its aglycone only [6,16]. The glucosamine carbohydrate unit may be too far from the amino-acid binding site of teicoplanin, which is located near the free amino group on the aglycone 'basket' [11,22]. The three significant structural differences in the amino-acid binding site of the two chiral selectors are (i) the secondary amine (i.e. methyl amino group) of A-40,926, (ii) the additional carboxylic acid group on the acyl-glycosamine of A-40,926 and (iii) the chloro-substituent on ring 3 of the aglycone of A-40,926 (see Fig. 1). These slight alterations on or near the binding site for amino-acids did change their enantioselectivity, somewhat. It is improved for histidine (A4), *N*-acetyl-*m*-fluorophenylalanine (A7) and phenylalanine (A6). It is decreased for D,L-dopa (A2), folinic acid (A3), proline (A8) and 2-pyrrolidone carboxylic acid (A9). There is no significant change with threonine (A10) (Fig. 4). For the non-amino-acid compounds, the enantiomers of terfenadine (C3) could be resolved by the A-40,926 CSP and not by the teicoplanin CSP. Conversely, the chiral enantiorecognition of althiazide (B1) and tropic acid (C4) were lost on the A-40,926 CSP. Also, the separation of hydroxymandelic acid enantiomer (C2) by the A-40,926 CSP is possible only with acidic mobile phases (Table 3). The latter point may indicate that the extra carboxylic acid group of A-40,926 and/or the carboxylic group of C2 should be protonated to promote chiral enantiorecognition on the A-40,926 CSP.

The structural differences between the two CSPs presented in this work do not make one superior to the other. The total number of enantiomeric pairs that can be separated by the two CSPs is higher. These two CSPs belong to the same family of chiral selectors. They can separate similar classes of compounds. However, it is shown that there are differences in the enantioselectivity. This means that the two CSPs are complementary to one another. If an enantiomeric pair is poorly separated on one CSP, it is worthwhile to try the same conditions on the related CSP.

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