## Retention and Selectivity of Teicoplanin Stationary Phases after Copper Complexation and Isotopic Exchange

# Alain Berthod,\*,<sup>†</sup> Alain Valleix,<sup>‡</sup> Veronique Tizon,<sup>†</sup> Estelle Leonce,<sup>‡</sup> Celine Caussignac,<sup>‡</sup> and Daniel W. Armstrong<sup>§</sup>

Laboratoire des Sciences Analytiques, CNRS, Université de Lyon 1, 69622 Villeurbanne, France, CEA-Saclay, SMM, Bat. 547, 91191 Gif-sur-Yvette, France, and Department of Chemistry, Gilman Hall, Iowa State University, Ames, Iowa 50011-3111

Teicoplanin is a macrocyclic glycopeptide that is highly effective as a chiral selector for LC enantiomeric separations. Two possible interaction paths were investigated and related to solute retention and selectivity: (1) interactions with the only teicoplanin amine group and (2) role of hydrogen bonding interactions. Mobile phases containing 0.5 and 5 mM copper ions were used to try to block the amine group. In the presence of copper ions, it was found that the teicoplanin stationary phase has a decreased ability to separate most underivatized racemic amino acids. However, it maintained its ability to separate enantiomers that were not  $\alpha$ -amino acids. It is established that there is little copper-teicoplanin complex formation. The effect of Cu<sup>2+</sup> on the enantioseparation of some  $\alpha$ -amino acids appears to be due to the fact that these solutes are good bidentate ligands and form complexes with copper ions in the mobile phase. Isotopic exchange with deuterium oxide was performed using acetonitrile-heavy water mobile phases. It was found that the retention times of all amino acids were lower with deuterated mobile phases. The retention times of polar or apolar molecules without amine groups were higher with deuterated mobiles phases. In all cases, the enantioselectivity factors were unaffected by the deuterium exchange. It is proposed that the electrostatic interactions are decreased in the deuterated mobile phases and the solute-accessible stationary-phase volume is somewhat swollen by deuterium oxide. The balance of these effects is a decrease in the amino acid retention times and an increase in the apolar solute retention time. The enantioselectivity factors of all of the molecules remain unchanged because all of the interactions are changed equally. We propose a new global quality criterion (the E factor) for comparing and evaluating enantiomeric separations.

The development of broadly applicable, high-efficiency enantiomeric separations is one of the great successes of separation science<sup>1.2</sup> in the latter part of the 20th century. When liquid chromatography is used, three basic experimental approaches are possible: (i) the enantiomeric pair is derivatized with a pure enantiomer to form diastereoisomers that can be separated by classical chromatography, (ii) a chiral additive can be added to the mobile phase to form chiral complexes that can be separated by a classical stationary phase, and (iii) chiral stationary phases (CSP) are prepared. This latest solution proved to be the most useful. It is now the most common way to separate enantiomeric pairs.

Chiral stationary phases can be prepared using (i) naturally occurring chiral molecules, (ii) semisynthetic chiral molecules (i.e., natural molecules that have been synthetically altered), and (iii) totally synthetic chiral molecules. In general, the enantioselective separation mechanisms are best understood for the smaller chiral selectors of known structure and limited functionality. This includes molecules from all three of the aforementioned classes. For example, some natural amino acids, like proline, make effective ligand-exchange CSPs, separating chiral molecules that can act as bidentate ligands to Cu<sup>2+,3,4</sup> Amino acid derivatives, alkaloids, and other derivatized small molecules are among the more successful  $\pi$ - $\pi$ -complex CSPs.<sup>5-9</sup> In addition, there are totally synthetic  $\pi$ -complex CSPs. Cyclodextrin-based CSPs can consist either of the natural molecule or the derivatized macrocycle.1 Larger, often polymeric, chiral molecules often have undetermined structures and large numbers of interaction sites.<sup>10-13</sup> Frequently, they have broad enantioselevities, but a basic understanding of their chiral recognition mechanism is limited. These

- (1) Armstrong, D. W. LC-GC 1997, (May) 20.
- (2) Maier, W. M.; Franco, P.; Lindner, W. J. Chromatogr. A 2001, 906, 3.
- (3) Davankov, V. A.; Semechkin, A. V. J. Chromatogr. 1977, 141, 313.
- (4) Davankov, V. A. In Giddings, J. C., Cazes, J., Brown, P. J. (Eds.); Advances in Chromatography, Marcel Dekker: New York, 1983, Vol. 22, pp 71–103.
- (5) Klemm, L. H.; Reed, D. J. Chromatogr. 1960, 3, 364.
- (6) Mikes, F.; Boshart, G. J. Chromatogr. 1978, 149, 455.
- (7) Pirkle, W. H.; Hyun, M. H. J. Chromatogr. 1985, 322, 309.
- (8) Oi, N.; Kitahara, H. J. Chromatogr. 1983, 265, 117.
- (9) Lämmerhofer, M.; Svet, F.; Fréchet, J. M. J.; Lindner, W. Anal. Chem. 2000, 72, 4623.
- (10) Lindner, W.; Mannschreck, A. J. Chromatogr. 1980, 193, 308.
- (11) Okamoto, Y.; Kawashima, M.; Yamamoto, K.; Hakada, H. Chem. Lett. 1984, 739.
- (12) Hernansson, J. J. Chromatogr. 1983, 269, 71.
- (13) Allenmark, S. G.; Bomgren, B.; Boren, H. J. Chromatogr. 1983, 269, 63.

Analytical Chemistry, Vol. 73, No. 22, November 15, 2001 5499

<sup>&</sup>lt;sup>†</sup> Université de Lyon.

<sup>&</sup>lt;sup>‡</sup> CEA-Saclay.

<sup>§</sup> Iowa State University.



**Figure 1.** The teicoplanin molecule with its 4 macrocycles, its 7 aromatic rings, 4 phenolic groups, 6 amido groups, 3 ether links, 2 chlorine atoms, a free primary amine, a carboxylic acid group, and 3 sugar moieties with 10 primary or secondary alcoholic groups and 2 amido groups, one of which bears a 9-carbon-atom alkyl chain. Twenty-five protons can be exchanged for deuterium atoms; they are noted as X's on the figure.

polymeric chiral selectors can be naturally occurring molecules, such as proteins; semisynthetic molecules, such as derivatized carbohydrates and cross-linked derivatized tartaric acid;<sup>10,14</sup> or totally synthetic polymers.<sup>15</sup>

The macrocyclic glycopeptide antibiotics have recently emerged as one of the more useful classes of chiral selectors for HPLC enantioseparations.<sup>16</sup> Although only of intermediate size, these molecules most closely rival proteins in their diversity of structure, number, functional groups, and variety of possible interactions. Yet the macrocyclic glycopeptides are sufficiently small that their structure is known, and they can be thoroughly examined by most conventional chemical diagnostic methods. The CSP based on teicoplanin has found widespread use.<sup>17,18</sup> The teicoplanin-containing columns were able to resolve all naturally occurring amino acids in their native form with common methanol–water mobile phases.<sup>17</sup> They were also able to separate a wide variety of other amino acids and small peptides and most other enantiomers containing a carboxylic group attached to the stereogenic center.<sup>1,17</sup>

The goal of this work was to investigate two particular interactions involved in the teicoplanin retention mechanism and to evaluate the impact on the enantioselectivity factors of various solutes. The first point focuses on the only primary amine group of the teicoplanin molecule. The second part focuses on hydrogen

- (14) Allenmark, S. G.; Anderson, S.; Moller, P.; Sanchez, D. Chirality 1995, 7, 248.
- (15) Okamoto, Y.; Honda, S.; Okamoto, I.; Yuki, H.; Murata, S.; Naori, R.; Tayaka, H. J. Am. Chem. Soc. **1981**, 103, 6971.
- (16) Armstrong, D. W.; Tang, Y.; Chen, S.; Zhou, Y.; Bagwill, C.; Chen, J. R. Anal. Chem. **1994**, 66, 1473.
- (17) Berthod, A.; Liu, Y.; Bagwill, C.; Armstrong, D. W. J. Chromatogr. A 1996, 731, 123.
- (18) Armstrong, D. W.; Liu, Y.; Ekborg-Ott, K. H. Chirality 1995, 7, 474.

bonding interactions that are thought to be essential for chiral recognition. These two types of interactions were evaluated using copper complexation<sup>19</sup> and deuterium isotope exchange, respectively.

#### **EXPERIMENTAL SECTION**

The Teicoplanin Chiral Stationary Phase. Teicoplanin, C<sub>88</sub>H<sub>97</sub>Cl<sub>2</sub>N<sub>9</sub>O<sub>33</sub>, MW 1878, contains a heptapeptide aglycone with seven aromatic rings, four phenolic groups, six amido groups, three ether links, and two chlorine atoms. Three saccharide units are attached to the heptapeptide. They bear 10 primary or secondary hydroxyl groups and two amido groups, one of which bears a nine (or ten)-carbon atom alkyl chain. Figure 1 shows the structure of the prevalent teicoplanin isomer, the A<sub>2</sub>-2 form, making up  $\sim$ 85% of the natural product. The four other teicoplanin isomers have the same aglycone core. They differ by the alkyl chain on the acyl glucosamine unit. A2-2 bears an 8-methylnonanoyl chain. 8-Methyldecanoyl, 9-methyldecanoyl, n-decanoyl, and (Z)-4-decanoyl variations are found on the alkyl chain of the minor teicoplanin isomers.<sup>20</sup> Teicoplanin, as all macrocyclic glycopeptides, is patented for chiral separations (agreement between University of Missouri, D.W. Armstrong, and the Astec Company, Whippany, NJ 07981). The teicoplanin coverage of the silica particles is between 0.35 and 0.45  $\mu$ mol/m<sup>2</sup>.<sup>21</sup> The average teicoplanin content of a 25-cm column is ~0.4 mole or 750 mg. Two 25-cm Chirobiotic T columns (Astec), serial number (S/N) A155-6-C and A155-6-D, were used for the copper ion study, and

- (20) Product 9269. The Merck Index, 12th ed.: Merck & Co.: 1996, p 1559.
- (21) Berthod, A.; Chen, X.; Kullman, J. P.; Armstrong, D. W.; Gasparrini, F.; D'Aquarica, I.; Villani, C. Anal. Chem. 2000, 72, 1767.

<sup>(19)</sup> Nair, U.; Chang, S. C.; Armstrong, D. W.; Rawjee, Y. Y.; Eggleston, D. S.; McArdle, J. V. *Chirality* **1996**, *8*, 590.

two other 25-cm columns, S/N 111-21 and 134-33 were used for the isotopic exchange study.

**Materials.** *Chemicals.* Acetic acid, triethylamine and most of the racemic amino acids and pure enantiomers were obtained from the Sigma-Aldrich-Fluka Co. (Saint Quentin Fallavier, France). They were used as received. The test molecules are listed in Table 1, along with their structures and properties. Vancomycin was obtained from Sigma Chemical Co. (St Louis, MO). Teicoplanin was a gift donated by Marian Merrill Dow Research Institute (Cincinnati, OH). Copper acetate was from Prolabo (Paris, France). Methanol and acetonitrile were from SDS (Marseille– Peypin, France). Deuterium oxide (or heavy water) and CH<sub>3</sub>OD were products of Euroisotop (Saint Aubin, France), a subdivision of the French Center for Atomic Energy (CEA, Gif sur Yvette, France).

*Apparatus.* A Shimadzu LC6A chromatograph was used. It included a LC-6A or a LC-10AS pump, a SPD-6A UV detector, and a CR-5A integrator. A Hitachi U-2000 UV—vis spectrophotometer was used for the study of the complexation of copper with vancomycin or teicoplanin.

*Protocol.* For the copper ion experiments, methanol–water, 60-40% v/v, was used at 1 mL/min flow rate. copper acetate (0.5 or 5 mM) was added for teicoplanin complexation. The mobile phases were buffered with 0.25%, v/v, triethylamine (18 mM) and the necessary amount of acetic acid to obtain a pH of 4.1 or 7.1. For the isotopic exchange, different mobile phases were used: acetonitrile–water, 80-20% v/v or 15-85% v/v, and methanol–water, 20-80% v/v. The corresponding mobile phases were prepared with acetonitrile and heavy water and CH<sub>3</sub>OD and heavy water. No additive was added to the deuterated or normal mobile phases (no buffer, no salts).

#### **RESULTS AND DISCUSSION**

**Copper II Ion Complexation.** Two Chirobiotic T columns containing CSPs from the same synthetic batch were used in this study. One was exposed to copper containing mobile phases, the second one served as a reference. All mobile phases contained 60%, v/v, methanol and were buffered at pH 4.1 or 7.1 by adding 0.25%, v/v, triethylamine (18 mM TEAA) and adjusting the pH by adding acetic acid dropwise.

Copper Ion Adsorption. The amount of copper II ion adsorbed by the Chirobiotic T column was estimated following their 230nm UV absorbency. The column was first equilibrated with a copper-free methanol-water mobile phase. Next, the pumping system was disconnected from the column and rinsed with the 0.5 mM copper containing mobile phase. The column was reconnected, and the recorder was started. The dead volume of the column was measured to be 2.4 mL. The amount of copper ion contained in the dead volume was deducted from the amount of copper found to be adsorbed by the CSP. The corrected copper amount adsorbed at pH 4.1 was only 9  $\mu$ mol for the column. This amount is very low; it corresponds to only one copper ion for 45 teicoplanin molecules. This result is very different from what was obtain with the vancomycin chiral selector that formed a strong 1:1 complex with copper ions at pH 5.19 Later, the same experiment was performed using the 5 mM Cu II pH 4.1 methanol-water, 60-40% v/v, mobile phase. The amount of adsorbed copper ion was  $\sim$ 50  $\mu$ mol. This corresponds to about one copper ion for eight teicoplanin molecules. This amount is still low. It is not possible

to work with a higher copper ion concentration because of the UV absorbency of the mobile phase. The copper ion adsorption seems reversible. When the column is washed with a copper-free mobile phase, most of the adsorbed copper ions are removed from the CSP.

Solute Retention and Enantioselectivity. Table 2 lists the chromatographic results obtained with the methanol-water 60-40%, v/v, mobile phase both with and without copper ions. Different behaviors were noted. The native  $\alpha$  amino acids (such as cysteine, tryptophan, or phenylalanine) usually showed a significant increase in their retention volume accompanied by a decrease in the corresponding enantioselectivity and resolution factors. The separation of the enantiomers of cysteine was not possible when copper ions were present in the mobile phase and its retention volume was twice that of the most-retained peak with the copper-free mobile phase (Table 2). Other compounds, such as N-acetyl-3-fluorophenylalanine, carnitine, or warfarin, do not show any change when copper ions are added to the mobile phase. Some compounds show an intermediate behavior with a slightly increased retention volume and a slightly reduced enantioselectivity and resolution factor.

To have a single global parameter to compare the chromatographic separation of enantiomers, we define an "E", or effectiveness, term as

$$E = \alpha R_{\rm s} / k_1 \tag{1}$$

where  $\alpha$  is the enantioselectivity factor,  $R_s$  is the enantioresolution, and  $k_1$  is the retention factor of the first eluted enantiomer. *E* is an all-inclusive quality criterion. It is designed for just a quick screening of chromatographic variations.  $R_s$  is by itself a function of the *k* and  $\alpha$  parameters. But often, changes in  $\alpha$  and *k* factors were associated with constant  $R_s$  factors because of changes in efficiency. The *E* factor has no thermodynamic or physical meaning other than a quality criterion. High values of *E* indicate short analysis times with well-separated peaks. *E* values are useful in comparing enantioseparations on different columns or using different conditions with the same column. If the value of *E* increases, then the chromatographic quality increases and vice versa. Separations with higher *E* values are the most effective separations.

Figure 2 shows the relative value of the *E* factor (i.e., the *E* factor for the given mobile phase divided by the *E* factor of the copper free mobile phase) for all of the compounds of Table 2. Figure 2 clearly shows that the chromatographic figures of merit of all amino acids but two are dramatically degraded by the presence of copper ions in the mobile phase. The two exceptions are aspartic acid and *N*-acetyl-3-fluorophenylalanine. The other result shown in Figure 2 is that the non-amino acid compounds are unaffected or much less affected by the copper ions in the mobile phase. The 40% decrease observed with *p*-hydroxymandelic acid seems significant: mandelic acid is an  $\alpha$ -hydroxy carboxylic acid that is as effective as  $\alpha$  amino acids at chelating Cu<sup>2+</sup> in the mobile phase. However, the error on the very low retention factor,  $k_1 < 0.2$ , of this compound is high, which results in a high variation of its *E* factor.

Amine Groups and Copper Complexation. In a recent work, we demonstrated the role of the amine group of vancomycin, another

### Table 1. Enantiomeric Pairs Used as Test Solutes

Compounds	Structure	Formula	m.w.	use
α-amino acids alanine	<b>R-m</b> oiety <sup>a</sup> -CH <sub>3</sub>	C <sub>3</sub> H <sub>13</sub> NO <sub>2</sub>	89	naturally occurring
asparagine	-CH <sub>2</sub> -CO-NH <sub>2</sub>	$C_4H_8N_2O_3$	132	naturally occurring
aspartic acid	-CH2-COOH	C <sub>4</sub> H <sub>7</sub> NO <sub>4</sub>	133	naturally occurring
cysteine	-CH <sub>2</sub> -SH	C <sub>3</sub> H <sub>7</sub> NO <sub>2</sub> S	121	naturally occurring
dopa	-СН <sub>2</sub> -	$C_9H_{11}NO_4$	197	antiparkinsonian
glutamic acid	-CH <sub>2</sub> -CH <sub>2</sub> -COOH	C <sub>5</sub> H <sub>9</sub> NO <sub>4</sub>	147	naturally occurring
leucine	-CH <sub>2</sub> -CH(CH <sub>3</sub> )-CH <sub>3</sub>	C <sub>6</sub> H <sub>13</sub> NO <sub>2</sub>	131	essential aa
methionine	-CH <sub>2</sub> -CH <sub>2</sub> -S-CH <sub>3</sub>	C <sub>5</sub> H <sub>11</sub> NO <sub>2</sub> S	149	essential aa
phenylalanine	-CH <sub>2</sub> -	$C_9H_{11}NO_2$	165	essential aa
N-acetyl-3-fluoro phenylalanine	-CH <sub>2</sub> - ()-F	C <sub>11</sub> H <sub>11</sub> NFO <sub>3</sub>	224	artificial aa
tryptophan	-CH <sub>2</sub> -indole	$C_{11}H_{22}N_2O$	204	essential aa
tyrosine	СН₂-⟨◯⟩-ОН	$C_9H_{11}NO_3$	181	naturally occurrin
valine	-CH(CH <sub>3</sub> )-CH <sub>3</sub>	C <sub>5</sub> H <sub>11</sub> NO <sub>2</sub>	117	essential aa
non α-amino a carnitine	acid compounds $H_3C - N^+ - COO^-$	C <sub>7</sub> H <sub>15</sub> NO <sub>3</sub>	161	betaine amino aci fat fitting agent
coumachlor		C <sub>19</sub> H <sub>15</sub> ClO <sub>4</sub>	343	rodenticide
4-hydroxy mandelic acid	но соон	C <sub>8</sub> H <sub>8</sub> O <sub>4</sub>	168	antiseptic
5-methyl-5-phenyl hydantoin	ÓH H <sub>3</sub> C H <sub>3</sub> C NH	$C_{10}H_{10}N_2O_2$	190	anticonvulsant
2-methyl-4-phenyl indanone		C <sub>16</sub> H <sub>13</sub> O	221	-
γ-(2-naphthyl)- butyrolactone		$C_{14}H_{12}O_2$	212	
phenylphthalide		$C_{14}H_{10}O_2$	210	
warfarin		$C_{19}H_{16}O_4$	308	anticoagulant

 $^a$  The general structure of amino acids is  $^+\mathrm{NH_3}\text{-}\mathrm{CHR}\text{-}\mathrm{COO}^-$  with the R group structure listed.

#### Table 2. Chromatographic Results Obtained with the Copper II Containing Mobile Phases<sup>a</sup>

		MeOH-	H <sub>2</sub> O 60-	-40		+ copper 5 mM						
solute	<i>K</i> <sub>1</sub>	$\alpha_{12}$	Rs	E	<i>K</i> <sub>1</sub>	α <sub>12</sub>	Rs	E	<i>K</i> <sub>1</sub>	α <sub>12</sub>	Rs	Ε
alanine	0.73	1.9	4.2	11.0	0.77	1.8	3.5	8.26	0.79	1.8	2.7	6.19
asparagine	0.83	1.6	2.9	5.56	1.04	1.5	1.2	1.72	1.17	1.5	1.1	1.36
aspartic acid	0.39	1.5	0.6	2.29	0.49	1.4	0.7	2.01	0.55	1.4	1.0	2.48
cysteine	2.14	1.2	0.5	0.275	3.96	1.0	0.0	0.0	4.91	1.0	0.0	0.0
dopa	0.83	1.9	3.7	8.59	1.26	1.6	1.3	1.63	1.45	1.5	1.1	1.14
glutamic acid	0.50	1.7	2.0	6.72	0.60	1.6	1.2	3.10	0.67	1.5	1.1	2.45
leucine	0.70	2.2	4.4	13.5	0.81	2.0	2.7	6.58	0.92	1.9	2.3	4.76
methionine	0.83	2.1	4.3	10.7	1.00	1.9	2.4	4.63	1.16	1.9	1.9	3.04
phenylalanine	0.99	1.5	2.5	3.67	1.83	1.2	0.4	0.26	2.25	1.2	0.3	0.16
<i>N</i> -acetyl-3-fluorophenylalanine	0.65	5.4	7.3	60.8	0.58	5.4	6.9	63.8	0.60	5.4	7.3	66.1
tryptophan	1.15	1.5	2.4	3.10	2.94	1.1	0.2	0.08	2.79	1.2	0.2	0.08
<i>m</i> -tyrosine	0.85	2.3	4.1	10.8	1.66	1.7	1.4	1.42	2.02	1.6	1.4	1.10
valine	0.65	1.7	3.2	8.18	0.74	1.6	2.0	4.20	0.81	1.5	1.2	2.25
carnitine	2.12	1.05	0.4	0.20	2.15	1.05	0.5	0.22	2.13	1.05	0.4	0.20
coumachlor	1.41	1.2	1.2	1.01	1.19	1.2	1.2	1.23	1.13	1.3	1.4	1.65
5-methyl-5-phenyl hydantoin	0.58	1.9	2.8	9.00	0.54	1.8	2.9	9.85	0.55	1.8	2.6	8.68
<i>p</i> -hydroxy mandelic acid	0.15	4.7	3.8	125	0.19	3.8	3.5	71.8	0.19	4.0	3.6	75.8
warfarin	0.69	1.1	0.5	0.81	0.61	1.1	0.4	0.73	0.62	1.1	0.6	1.09

<sup>a</sup> Column 1 = Chirobiotic T (S/N: A-155-6C) 25 cm, 4.6 mm i.d. All mobile phases contain 18 mM TEAA buffer at pH 4.1.



**Figure 2.** The *E* ratio (=  $\alpha R_s/K_1$ ) for the compounds of Table 2 showing the effect of copper ions on the chromatographic figures of merit obtained with the Chirobiotic T column 1. Relative values, reference mobile phase: MeOH-H<sub>2</sub>O, 60-40% v/v, pH 4.1.

macrocyclic glycopeptide antibiotic bearing some similarities with teicoplanin,<sup>22</sup> by blocking it using copper II ions.<sup>19</sup> The first marked difference between vancomycin and teicoplanin is that the former was able to establish a strong 1:1 complex with copper II ions.<sup>19</sup>

(22) Gasper, M. P.; Berthod, A.; Nair, U. B.; Armstrong, D. W. Anal. Chem. 1996, 68, 2501. Teicoplanin does not appear to form such a complex. This was checked by studying the copper complexation with the free macrocyclic glycopeptides by UV–vis spectroscopy. Small amounts of dimethyl sulfoxide (DMSO) had to be used, because the glycopeptides were not soluble at high concentrations in pure water. A 0.01 M Cu<sup>2+</sup> ion, 0.01 M vancomycin solution in DMSO–

#### Table 3. Effect of pH on the Chromatographic Results<sup>a</sup>

		column	1, pH 4	.1	column 1, pH 7.1				column 2, pH 7.1 Cu 5 mM			
solute	<i>K</i> <sub>1</sub>	$\alpha_{12}$	Rs	E	<i>K</i> <sub>1</sub>	α <sub>12</sub>	Rs	E	K <sub>1</sub>	$\alpha_{12}$	Rs	Ε
alanine	0.76	1.9	4.2	10.3	1.20	2.0	1.3	2.12	19.4	1.3	0.2	0.01
asparagine	0.87	1.6	2.8	5.07	1.66	1.0	0.0	0.0	1.65	2.2	0.9	1.21
aspartic acid	0.40	1.5	1.1	4.00	0.35	1.4	0.2	0.80	1.33	1.0	0.0	0.0
cysteine	2.41	1.1	0.6	0.28	13.0	1.0	0.0	0.0	>50	_	_	_
glutamic acid	0.51	1.7	1.7	5.62	0.47	1.5	0.6	1.87	30.9	1.3	0.5	0.02
leucine	0.73	2.2	4.6	13.6	2.85	1.0	0.0	0.0	28.2	1.1	0.2	0.01
methionine	0.86	2.1	4.6	11.3	4.25	1.0	0.0	0.0	40.2	1.4	1.9	0.07
phenylalanine	1.03	1.5	2.3	3.23	2.44	1.0	0.0	0.0	10.2	2.0	1.1	0.21
N-acetyl-3-fluoro phenylalanine	0.74	5.4	7.8	57.0	0.61	6.4	6.9	72.4	0.60	4.1	4.5	31.5
tryptophan	1.19	1.5	2.1	2.58	2.70	1.0	0.0	0.0	23.2	1.4	1.1	0.07
<i>m</i> -tyrosine	0.90	2.2	4.3	10.7	2.63	2.1	0.9	0.71	14.0	1.5	1.0	0.11
valine	0.65	1.7	2.9	7.55	1.78	1.8	1.9	1.88	24.0	1.1	0.2	0.01
carnitine	2.11	1.05	0.5	0.25	1.66	1.1	0.6	0.39	17.4	1.6	0.7	0.06
coumachlor	1.51	1.2	1.7	1.36	1.55	1.2	1.2	0.90	1.19	1.2	1.2	1.21
5-methyl-5-phenyl hydantoin	0.58	1.9	2.8	9.00	0.65	2.0	3.9	11.7	0.78	1.6	1.3	2.67
<i>p</i> -hydroxy mandelic acid	0.15	5.1	3.0	103	0.16	5.1	3.8	118	1.00	1.4	0.3	0.42
warfarin	0.65	1.1	0.6	1.05	0.25	1.2	0.4	1.86	0.75	1.0	0.0	0.0

<sup>&</sup>lt;sup>*a*</sup> Mobile phase: methanol–water, 60-40% v/v, with 18 mM TEAA buffer at indicated pH, 1 mL/min. Column 1 = Chirobiotic T, S/N = A-155-6C, 25 cm, 4.6-mm i.d.; column 2 = Chirobiotic T, S/N = A-155-6D, 25 cm, 4.6-mm i.d.

water, 20-80% v/v, buffered at pH 4 with 0.02 M TEAA shows an absorption band at 550 nm (purple color) at the side of the copper ion absorption band (790 nm). The 550-nm band corresponds to the vancomycin–copper complex.<sup>19</sup> In 0.01 M Cu<sup>2+</sup> and 0.02 M teicoplanin at pH 4 with 0.02 M TEAA in 20%, v/v, DMSO solution, the teicoplanin solution shows only the copper ion absorption band at 790 nm (blue color). Copper ion complexation with amino acids was checked with glycine. A 0.01 M Cu<sup>2+</sup> solution with 0.2 M glycine (DMSO, 20% v/v, pH 4 with 20 mM TEAA) shows a single absorption band at 650 nm, which is for the Cu–glycine complex.

If the chiral recognition of amino acids is dramatically decreased when copper ions are present, and these ions do not form a complex with teicoplanin, it must be the copper-amino acid association in the mobile phase that is responsible for the loss of enantioselectivity. Davankov<sup>3,4,23</sup> used such copper ion complexation to separate amino acids by ligand exchange since the early 1970s. In our case, however, the amino acid copper ion complexation can be detrimental for chiral recognition by the teicoplanin selector. Analytes that can act as bidentate ligands to Cu<sup>2+</sup>, are the most affected by the addition of copper ion to the mobile phase. The solute N-acetyl-3-fluorophenylalanine cannot form a complex with a copper ion, because its amine group is acetylated. Thus, its enantioselective separation is not affected by the copper ions (Table 2 and Figure 2). Aspartic acid has two carboxylic acid groups. The amino group may not be available for copper complexation, because it is blocked by the second carboxylic acid group of the molecule. Alternatively, copper complexation may occur through the amine and one carboxylic acid group, leaving the other to associate with the CSP. Carnitine is a  $\gamma$ -amino acid (betaine). In addition, the amine group of carnitine is a quaternary ammonium group that is less capable of forming a copper complex than the primary amine group of the α-amino acids. This may explain why the enantiorecognition of carnitine by the teicoplanin selector is not affected by copper ions. Conversely, *p*-hydroxymandelic acid behaves more like the  $\alpha$ 

amino acids because, as an  $\alpha$ -hydroxy acid, it also chelates copper II.

pH Effect. The ionization state of both the teicoplanin chiral selector and the amino acid solutes is dependent on the mobile phase pH. Mobile phases of methanol-water, 60-40% v/v, with or without 5 mM copper nitrate were buffered at pH 7.1 with 0.25%, v/v, triethylamine and acetic acid (18 mM TEAA). A second Chirobiotic T column was used to show the chromatographic changes due only to pH variations. Table 3 lists the results obtained at pHs 4.1 and 7.1. The results listed for pH 4.1 should be compared with those listed in Table 2 and obtained using column 1 in the same conditions. A less than 10% variation is observed, so the results obtained on column 2, not exposed to copper ions, can be used to compare with results obtained with column 1 used with the copper containing mobile phases (Table 3). The retention times of most amino acids, obtained with a pH 7.1 mobile phase, were much higher than the corresponding times obtained with the pH 4.1 mobile phases. For all amino acids except N-acetyl-3-fluorophenylalanine, the pH 7.1 mobile phase produced lower resolution than the pH 4.1 mobile phase. This effect is known<sup>17,18</sup>, and working at pH 4.1 is recommended for optimum resolution, and pHs lower than 3.8 or higher than 8 should be avoided, because these extremes decrease column stability.<sup>24</sup>

At pH 7.1, 5 mM copper II ion concentration was added to the 60-40%, v/v, methanol-water mobile phase. The chromatographic results obtained with column 1 are listed in Table 3 and are compared to the copper free mobile phase using our defined *E* criterion (eq 1). In all cases, a dramatic loss of resolution was observed when copper ions were present in the mobile phase at pH 7.1. For many compounds, the copper ions induce an increase in the retention factors of ~1 order of magnitude. This occurs for alanine, cysteine, glutamic acid, leucine, methionine, tryptophan, valine, and carnitine (Table 3). The chelated analytes behave differently and possibly more like a cationic species with regard to an increasingly more effective ion-exchange-type stationary

<sup>(23)</sup> Davankov, V. A. In *Chiral Separations by HPLC*; Krstulovic, A. M., Ed.; Ellis Horwood: Chichester, 1989; Chapter 15, pp 446–475.

<sup>(24)</sup> *Chirobiotic Handbook*, 2nd ed.; Astec: Whippany, NJ **1997**, freely obtained on request at *www.astecusa.com*.

phase. The carboxylic groups of the amino acid solutes are negatively charged at pH 7.1, and the amino group is neutral. Copper ions and such negatively charged amino acid solutes may form a complex with a positive charge. This complex could be more retained on the teicoplanin stationary phase, which happens to have a negative character at pH 7.1 (Figure 1). This increase in the retention factors increases somewhat the enantioselectivity factor, but the resolution is significantly decreased (Table 3) because of poor efficiency. The change in the ionization state of the teicoplanin stationary phase has an essential relevance to its chiral recognition capability, because the enantioselectivity and resolution factors of the nonionizable solutes, coumachlor, methyl phenyl hydantoin, and warfarin are also affected, but to a much lesser extent, by the 4.1 to 7.1 pH change (Table 3). An increase of the ionic strength of the mobile phase would decrease the retention times of the chelated analytes. This is not likely to change the enantioresolution factors.

Copper Adsorption Reversibility. After completion of the experiment with copper containing mobile phases, column 1 was rinsed with ~10 column volumes of distilled water (30 min @ 1 mL/min), followed by 10 column volumes of pure methanol and 12 h (overnight @ 0.1 mL/min) with the initial methanol–water, 60-40% v/v, 0.25% triethylamine adjusted to pH 4.1 by acetic acid. The retention and enantioselectivity factors of the rinsed column 1 were within 10% of those of column 1 before exposure to copper ion (Table 2) and column 2 (Table 3). However, the resolution factors were ~30% lower than the initial values as a result of a significant decrease (about -50%) of peak efficiency. It can be concluded that the copper adsorption is for the most part reversible, because there is no strong teicoplanin–copper II complexation. But it is not possible to say if the efficiency loss is due to copper exposure or normal wearing of the column.

The conclusion of the first part of this work is that, contrary to what was obtained with the vancomycin chiral stationary phase,<sup>19</sup> it is not possible to use copper II ions to evaluate the role of the teicoplanin primary amine in the chiral recognition mechanism of amino acids. The positive results are that teicoplanin does not form strong complexes with Cu<sup>2+</sup> and that analytes with free carboxylic groups are highly recognizable on the CSP. Making the COOH group unavailable via Cu complexation greatly reduces enantioselectivity.

**Role of Hydrogen Bonding Assessed by Deuterium Isotopic Exchange.** In the second part of this study, we investigated the role of hydrogen bond interactions in the retention and chiral recognition process of teicoplanin. We thought that many polar hydrogen atoms of the chiral selectors could be exchanged for deuterium atoms (X's in Figure 1). The separation of racemic pairs on such an isotopically modified chiral stationary phase should be enlightening. Heavy water (D<sub>2</sub>O) mobile phases were used in HPLC to perform MS detection<sup>25,26</sup> or NMR.<sup>27</sup> RPLC mobile phases containing water enriched with deuterium oxide were used to study the hydratation state of C18 bonded phases.<sup>28–29</sup>

#### Table 4. Deuterium Oxide Compared to Water Properties

property (unit)	water	deuterium oxide	percent change
MW (Da)	18.02	20.03	+11.20
mp (°C)	0.0	3.8	$+1.4^{a}$
bp (°C)	100.0	101.4	$+0.37^{a}$
$d^{25}$ (g cm <sup>-3</sup> )	0.997	1.104	+10.7
mol vol (cm <sup>3</sup> mol <sup><math>-1</math></sup> )	18.07	18.14	+0.4
spec. heat liq. (cal $g^{-1} \circ C^{-1}$ )	1.000	1.028	+2.8
heat fusion (kcal mol <sup>-1</sup> )	1.436	1.501	+4.5
heat vap. (kcal $mol^{-1}$ )	9.717	9.917	+2.05
dielectric constant (@ 20 °C)	80	78	-2.5
ionization constant (@ 25 °C)	14.0	14.96	+6.9
$[OH^{-}]$ or $[OD^{-}]$ (M)	$10^{-7}$	$3.3 \ 10^{-8}$	-67
refractive index	1.333	1.338	+0.4
viscosity ( $10^3$ N s m <sup>-2</sup> or cP)			
15 °Č	1.139	1.40	+23.0
20 °C	1.002	1.25	+24.7
30 °C	0.797	1.03	+29.2
40 °C	0.719	0.81	+12.7

<sup>a</sup> Percent change on absolute temperature in K.

Deuterium Oxide Properties. Heavy water (deuterium oxide) has the interesting property of being able to exchange deuterons for hydrogen atoms with any functional group containing a labile hydrogen atom. These functional groups include the -OH in alcohols, phenols, and carboxylic acids and the -NH- in amines and amides in neutral conditions.<sup>30</sup> Bonds to deuterium are slightly stronger than bonds to hydrogen, resulting in small but significant changes in reaction rates. Table 4 compares the properties of heavy and normal water. The boiling point of D<sub>2</sub>O is 101.4 °C, slightly higher than that of H<sub>2</sub>O, although the atomic weight of the former is 12% higher (20.03). The energy needed to separate the deuterium oxide molecules (heat of vaporization) is only 2% higher that the corresponding energy for water, although the D<sub>2</sub>O molecules are 12% heavier. These values show that the intermolecular hydrogen bonds are slightly stronger than the inter-D<sub>2</sub>O molecule "deuterium" bonds.

The ionization constant of  $D_2O$  is one unit higher than that of water, corresponding to a concentration of  $DO^-$  at equilibrium 3 times lower in  $D_2O$  (=  $3.3 \times 10^{-8}$  M) than in  $H_2O$  ([ $OH^-$ ] =  $10^{-7}$  M). This shows that the oxygen–deuterium  $\sigma$  bond is slightly stronger than the classical OH bond. It is clear that the dissociation constant of any ionizable group (phenol, carboxylic acid, or amine) will be different in the hydrogen form from that in the deuterium form. The fact that  $pK_a$  values were higher in  $D_2O$  than in  $H_2O$  was already mentioned in the literature.<sup>31</sup> Because this point is not the subject of this study, we chose neither to buffer nor to add any salt to the mobile phases.

Table 4 also shows that the viscosity of heavy water is  $\sim$ 25% higher than that of normal water. Then, the observed column back pressure will be 25% higher at equal flow rate. For this reason, a flow rate of 0.8 mL/min was used for all of the experiments with deuterated mobile phases and respective comparison experiments with aqueous mobile phase.

*Column Equilibration.* Figure 1 shows the exchangeable protons of the teicoplanin molecule as X's instead of Hs. Residual

<sup>(25)</sup> Edmonds, C. G.; Pomeranz, S. C.; Hsu, F. F.; McClosky, J. A. Anal. Chem. 1988, 60, 2314.

<sup>(26)</sup> Olsen, M. A.; Cummings, P. G.; Kennedy-Gabb, S.; Wagner, B. M.; Nicol, G. R.; Munson, B. Anal. Chem. 2000, 72, 5070.

<sup>(27)</sup> Fukuhara, T.; Komatu, K.; Yamamoto, S.; Takamatu, T. Bunseki Kagaku 1996, 45, 421.

<sup>(28)</sup> Marchall, D. B.; McKenna, W. P. Anal. Chem. **1984**, *56*, 2090.

<sup>(29)</sup> Bliesner, D. M.; Sentell, K. Anal. Chem. 1993, 65, 1819.

 <sup>(30)</sup> Dharmasiri, K.; Smith, D. L. Anal. Chem. 1996, 68, 2340.
 (31) Okafo, G. N.; Camilleri, P. J. Chromatogr. 1991, 549, 404.

#### Table 5. Chromatographic Results Obtained with the Deuterated Mobile Phases<sup>a</sup>

	aq	ueous m	obile ph	ase	deuterated mobile phase				$\Delta G$ (kJ/mol)		
solute	<i>K</i> <sub>1</sub>	α <sub>12</sub>	Rs	E	<i>K</i> <sub>1</sub>	$\alpha_{12}$	Rs	E	exch. $H^b$	enantiomer	isotopic
leucine <sup>c</sup>	3.22	1.29	2.4	0.96	3.1	1.29	2.5	1.04	3	0.62	0.09
phenylalanine <sup>c</sup>	3.81	1.18	2.3	0.71	3.5	1.20	2.3	0.79	3	0.42	0.19
tryptophan <sup>c</sup>	4.01	1.16	1.5	0.43	3.58	1.18	1.5	0.49	4	0.38	0.26
tyrosine <sup>c</sup>	3.49	1.20	2.2	0.75	3.35	1.20	2.2	0.79	4	0.44	0.10
coumachlor <sup>c</sup>	2.86	1.25	2.3	0.83	3.71	1.23	2.4	0.73	1	0.52	-0.62
5-methyl-5-phenyl hydantoin <sup>d</sup>	0.76	1.43	3.5	6.55	0.88	1.45	3.3	5.47	2	0.89	-0.36
<i>p</i> -hydroxy mandelic acid <sup>e</sup>	0.33	3.37	4.7	48.2	0.36	3.28	4.7	43.2	3	2.93	-0.17
2-methyl-4-phenyl-indanone <sup>e</sup>	6.61	1.08	1.3	0.21	8.54	1.08	1.3	0.16	0	0.19	-0.61
naphththyl butyrolactone <sup>f</sup>	6.63	1.12	1.4	0.24	8.63	1.09	1.4	0.18	0	0.25	-0.61
phenyl phthalide <sup>g</sup>	3.12	1.21	1.6	0.62	3.66	1.20	1.6	0.53	0	0.45	-0.39

<sup>*a*</sup> Column, Chirobiotic T; S/N = 134–33; flow rate, 0.8 mL/min; no buffer added. <sup>*b*</sup> exch. H = number of hydrogen atoms exchanged by the solute for deuterium atoms. <sup>*c*</sup> Acetonitrile (ACN)-H<sub>2</sub>O 80–20% v/v and ACN-D<sub>2</sub>O 80–20% v/v. <sup>*d*</sup> Acetonitrile (ACN)-H<sub>2</sub>O 20–80% v/v and ACN-D<sub>2</sub>O 20–80% v/v. <sup>*c*</sup> Methanol-H<sub>2</sub>O 20–80% v/v and CH<sub>3</sub>OD-D<sub>2</sub>O 20–80% v/v. <sup>*f*</sup> Acetonitrile (ACN)-H<sub>2</sub>O 5–95% v/v and ACN-D<sub>2</sub>O 5–95% v/v. <sup>*g*</sup> Acetonitrile (ACN)-H<sub>2</sub>O 15–85% v/v and ACN-D<sub>2</sub>O 15–85% v/v.

silanols of the silica stationary-phase backbone also bear exchangeable protons. To equilibrate the column, the acetonitrileheavy water 80-20%, v/v, mobile phase (or the selected deuterated mobile phase) was percolated through the Chirobiotic T column for 12 h (overnight) at 0.1 mL/min ( $\sim$ 70 mL or  $\sim$ 35 column volumes without recycling). The equilibration was confirmed by the constancy of the retention times of naphthyl butyrolactone and the other enantiomers injected in triplicate. C18 columns were reported to be equilibrated in <15 min at 1 mL/ min.<sup>26</sup> In this case, only silanols had to be isotopically exchanged. Unfortunately, the equilibration time needed to return to the hydrogenated state was never indicated. In our case, exchange of deuterium atoms back to protons, it was found that the equilibration time was approximately four times longer (2 days with acetonitrile-water, 80-20% v/v, at 0.1 mL/min). Here again, the retention time of naphthyl butyrolactone was used to follow the isotopic exchange. These results also confirm that bonds to deuterium are stronger than bonds to hydrogen, that is, with the deuterium bonds forming faster than they are broken.

Solute Retention and Selectivity. Table 5 lists the results of the separation of enantiomers performed with classical hydroorganic mobile phases and the corresponding deuterated mobile phases on the teicoplanin-bonded column. Three results were obtained: (1) amino acids were eluted earlier with deuterated mobile phases than with the corresponding aqueous mobile phases; (2) non-amino acid compounds, either very polar (mandelic acid is anionic), semipolar (coumachlor), or apolar (naphthyl butyro-lactone), were eluted later with deuterated mobile phases; (3) in all of cases that were studied, the enantioselectivity and resolution factors were not affected by these changes in retention factors.

Figure 3 shows the *E* ratio (eq 1) for the compounds that were studied. The controlling factor of the effectiveness parameter, *E*, is clear in this case. The  $\alpha$ , enantioselectivity, and *R*<sub>s</sub>, resolution, factors are not significantly changed in the deuterated experiments, so the *E* ratio variations are only due to the *k*, retention factor, changes. It is shown that the maximum retention factor change is observed for naphthyl butyrolactone. This corresponds to a 24% increase in retention factor when going from an aqueous (5% acetonitrile, v/v) mobile phase to the corresponding deuterated mobile phase. A 12% decrease in retention factor is observed for tryptophan when a deuterated mobile phase is used (Table 5 and Figure 3).

Amino Acids. Experiments show that amino acids are significantly less retained in a deuterium environment than in the usual hydrogen environment (Table 5). Okafo and Camilleri observed this phenomenon on a C18 stationary phase.<sup>31</sup> They found that amino acids were less retained when a 2/1 ACN/D<sub>2</sub>O mobile phase was used instead of a 2/1 ACN/H<sub>2</sub>O mobile phase. They were using tetrabutylammonium hydroxide as an ion-pairing agent. They explained their results by stating that the amino acid's ionization was lower (higher  $pK_a$ 's). Edmonds et al. also observed the decreased retention times with small peptides and nucleosides on a C18 stationary phase with a 50/50 ACN/D<sub>2</sub>O mobile phase.<sup>25</sup> Several authors found that, when isotopomers are separated on C18 columns using an aqueous mobile phase, the most deuterated compound elutes first.<sup>32-35</sup> All amino acids have at least three exchangeable protons. A decrease in the retention factor between 0.3 and 1% per D atom was observed for different molecules.<sup>32–35</sup> This effect will account for a part of the observed decreased retention times of the amino acids. The additional difference in our case is that, unlike the C18 stationary phase, the teicoplanin stationary phase is deuterated itself to a significant extent. Its ionization state is likely to decrease in a manner similar to that of amino acids.31

The decrease in electrostatic interactions between the charged groups of the amino acid solutes and those of the teicoplanin selectors (ammonium and carboxylate groups) explain the major part of the earlier elution of amino acids with deuterated mobile phases. From a chiral mechanistic point of view, it is unfortunate that we cannot obtain clear evidence of the hydrogen bond role in the teicoplanin chiral recognition mechanism. Our results show that the separation of enantiomers is not changed by altering the magnitude of the hydrogen bonding and electrostatic interactions. We have demonstrated that the interaction of the amine group of the teicoplanin molecule (a cationic site) with the carboxylate moiety of the amino acid (an anionic site) can be a primary step of the chiral recognition mechanism.<sup>17,18,21,22</sup> In the deuterated medium, this electrostatic interaction is weakened by the higher p*K*<sub>a</sub> values of both amine and carboxylic acid groups on both the

(34) Shi, B.; Davis, B. H. J. Chromatogr. A 1996, 731, 351.

<sup>(32)</sup> Tanaka, N.; Thornton, E. R. J. Am. Chem. Soc. 1976, 98, 1617.

<sup>(33)</sup> Jinno, K.; Hirata, Y.; Hiyoshi, Y. J. High Resolut. Chromatogr. Chromatogr. Commun. 1982, 5, 102.

<sup>(35)</sup> El Tayar, N.; Van de Waterbeemd, H.; Markoulina, M.; Testa, B.; Testa, W. F. Int. J. Pharm. 1984, 19, 271.



**Figure 3.** Effect of the deuterium isotopic exchange on the chromatographic figure of merit (*E* ratio,  $E = \alpha R_s/k'$ ) of the solutes of Table 5; relative values, the aqueous phase is the reference; column, Chirobiotic T; experimental conditions, see Table 5.

amino acid solutes and the stationary phase. Because the enantioselectivity factors are not altered, it can be concluded that the other interactions, especially hydrogen bonds, necessary to obtain chiral recognition are similarly attenuated by the deuterated mobile phases.

Uncharged Solutes. Oppositely, experiments show that the uncharged solutes without or with fewer exchangeable protons are significantly more retained in a deuterium environment (Table 5). Similar results were obtained with C18 columns and various solutes.<sup>26,36</sup> Considering that these solutes are likely more retained through partitioning than through electrostatic interactions, these results can be explained by an increase in the solute accessible stationary phase volume,  $V_S$ , or by identical change of both enantiomers' distribution coefficient between phases, *K*. In countercurrent chromatography, changes in the stationary phase volume, that is a liquid phase, are very common. These changes do not affect the solute distribution coefficients, *K*, and the selectivity factors,  $\alpha$ .<sup>37</sup> Indeed, in the partition mechanism, the solute retention factor, *k*, depends on  $V_S$  and *K* according to

$$k = KV_{\rm S}/V_{\rm O} \tag{2}$$

with  $V_{\rm O}$ , the mobile phase volume. A  $V_{\rm S}$  increase produces a k increase. If the distribution constants do not change, the selectivity factor,  $\alpha = k_2/k_1$ , remains unchanged, as observed experimentally.<sup>26,36</sup> The increase in stationary phase volume may be due to a higher wetting of the organic bonded layer by the

deuterium oxide molecules. More studies outside the scope of this work are needed to establish that more deuterium oxide molecules are incorporated in the stationary phase or that  $D_2O$  molecules occupy a greater volume than  $H_2O$  molecules in the bonded organic layer of the stationary phase. The chiral mechanism involved in the separation of isomers by the teicoplanin bonded stationary phase is not affected by modifications of the stationary phase volume. It is also not changed by altering the magnitude of the hydrogen and electrostatic bonds. It is known that enantioselectivity changes when a variation in the relative number or magnitude of enantioselective versus nonenantioselective interactions occurs.<sup>38</sup>

Case of p-Hydroxymandelic Acid. This solute is a polar compound that can exchange three hydrogen atoms. Its  $pK_a$  value is likely increased in the deuterated mobile phase. However, the retention times of its two enantiomers are slightly higher in the deuterated mobile phase than in hydrogenated mobile phase (Table 5). p-Hydroxymandelic acid is sensitive to the increase in stationary phase volume, but the K distribution constants of its two enantiomers are similarly decreased by the change in electrostatic interactions (higher  $pK_a$ ) and the isotopic exchange of three hydrogen atoms. The electrostatic and isotopic effects partly cancel the stationary-phase volume effect. Compared to amino acids, p-mandelic acid lacks the amine group. This shows the significance of this amine group in the overall retention of amino acids by the teicoplanin stationary phase. For amino acids, the electrostatic and isotopic effects overcome the stationary phase volume effect.

<sup>(36)</sup> Valleix, A.; Leonce, E.; Caussignac, C.; Buisson, D.; LeGallo, E.; Tchapla, A. Abstract A106, SEP01 Symposium, June 12–14, 2001, Paris, France.
(37) Berthod, A. J. Chromatogr. 1991, 550, 677.

<sup>(38)</sup> Boehm, R. E.; Martire, D. E.; Armstrong, D. W. Anal. Chem. 1988, 60, 522.

Comparison of the Chiral and Isotopic Interactions. A convenient way to evaluate the magnitude of the interactions involved in the separation mechanism is to give a rough estimate of the Gibbs enantioselectivity energy difference,  $\Delta G_{\text{enantio}}$ . This energy, expressed in kJ/mol, corresponds to the difference in the energy interaction of the two enantiomers and the teicoplanin stationary phase.  $\Delta G_{\text{enantio}}$  is calculated using the enantioselectivity factor<sup>39</sup>

$$-\Delta G_{\text{enantio}} = RT \ln k_2' / k_1' = RT \ln \alpha \tag{3}$$

Eq 3 shows that  $\alpha$  and  $\Delta G_{\text{enantio}}$  are closely related. The values are listed in Table 5. The highest  $\alpha$  value, 3.3, was obtained for mandelic acid. It corresponds to a  $-2.9 \text{ kJ/mol} \Delta G_{\text{enantio}}$  energy difference in the teicoplanin interaction with the L and the D forms of mandelic acid (Table 5) in hydrogenated or deuterated mobile phases. Most other solutes have a  $\Delta G_{\text{enantio}}$  energy level between -0.3 and -0.6 kJ/mol (Table 5). In all cases studied, the  $\Delta G_{\text{enantio}} H_{20}$  value is not significantly different from the  $\Delta G_{\text{enantio}} D_{20}$  value.

These energy levels between two enantiomers can be compared to the energy difference for the same enantiomer when isotopic exchange is performed. The parameter,  $\Delta G_{\text{isotop}}$ , will measure the energy difference in the interaction of an enantiomeric form of a solute with the deuterated stationary phase and with the normal, proton-containing, stationary phase.

$$-\Delta G_{\rm isotop} = RT \ln k_{\rm 1H_2O} / k_{\rm 1D_2O} = RT \ln k_{\rm 2H_2O} / k_{\rm 2D_2O} \quad (4)$$

The + or - sign on the value obtained indicates that the deuterated solute has less or more affinity for the deuterated teicoplanin stationary phase, respectively. This affinity difference may be as big as -0.61 kJ/mol for methyl phenyl indanone and

(39) Berthod, A.; Li, W.; Armstrong, D. W. Anal. Chem. 1992, 64, 874.

naphthyl butyrolactone (Table 5), corresponding to a ~20% increase in retention factors of both enantiomers. In the opposite way, the ~10% decrease in retention factors observed for tryptophan gives a 0.26 kJ/mol  $\Delta G_{isotop}$  energy difference. Considering the energy values listed in Table 5, it seems that the bigger  $-\Delta G_{enantio}$  values (hydroxy mandelic acid, methyl phenyl hydantoin and leucine) correspond to the smaller  $-\Delta G_{isotop}$  value (0.17, 0.36, and -0.09 kJ/mol, respectively). A wider variety of compounds should be studied to establish firmly this possible trend. It would mean that, with teicoplanin chiral selectors, the lower the deuterium isotopic effect, the higher the enantioselectivity factor.

#### CONCLUSION

The use of mobile phases containing copper ions did not allow us to elucidate the chiral recognition role played by the primary amine group of the teicoplanin molecule. It did prove, however, that teicoplanin does not form complexes with copper ions in the same manner as amino acids and vancomycin do. The use of fully deuterated mobile phases showed that several chiral interactions are simultaneously modified, resulting in the constancy of the enantioselectivity factors. The chromatograms obtained with such deuterated mobile phases are significantly different from the chromatograms obtained with normal hydrogenated mobile phases. These changes may be critical in the case of the coupling of HPLC with MS or NMR.

#### ACKNOWLEDGMENT

Alain Valleix thanks the French Commissariat à l'Energie Atomique, Service des Molécules Marquées for its continuous support and generous supply of deuterium oxide solvent. Alain Berthod thanks the French Centre National de la Recherche Scientifique (UMR 5619, 2007, and FRE2394). Daniel Armstrong gratefully acknowledges support of this work by the National Institutes of Health, NIH RO1 GM53825-05.

Received for review April 17, 2001. Accepted September 4, 2001.

AC010444T