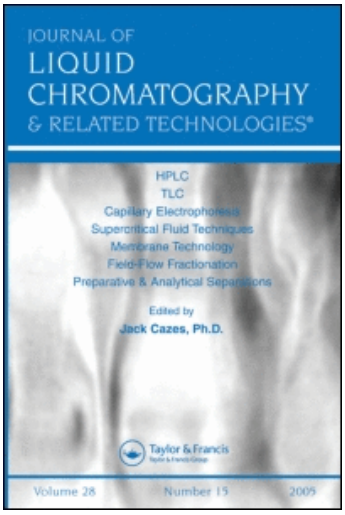


**Journal of Liquid Chromatography & Related Technologies**
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

# REVERSAL OF ENANTIOMERIC ELUTION ORDER ON MACROCYCLIC GLYCOPEPTIDE CHIRAL STATIONARY PHASES

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Online publication date: 31 October 2001

J. LIQ. CHROM. & REL. TECHNOL., 24(17), 2673–2684 (2001)

# REVERSAL OF ENANTIOMERIC ELUTION ORDER ON MACROCYCLIC GLYCOPEPTIDE CHIRAL STATIONARY PHASES

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## ABSTRACT

The macrocyclic glycopeptides, vancomycin, teicoplanin, and ristocetin A are naturally occurring chiral molecules that have been developed into one of the most useful classes of chiral stationary phases (CSPs) for HPLC. Since these chiral selectors are structurally related, they tend to have similar, but not identical, enantioselectivities for most compounds. CSPs, of this type, with opposite enantioselectivities are rare. Two exceptions have been found to this. The oxazolidiones (starting materials for asymmetric synthesis) and dansyl amino acids all show a reversal in enantioselective retention on one of these three related CSPs. By using

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the HPLC assays developed for these compounds, the levels of enantiomeric impurities can be measured down to ~0.01%. The enantiomeric purity of commercial oxazolidiones was determined.

## INTRODUCTION

The macrocyclic glycopeptides are the newest and fastest growing class of chiral selectors for HPLC.(1-24) Figure 1 gives the structure of three related glycopeptides (i.e., vancomycin, teicoplanin, and ristocetin A) that are available as chiral stationary phases (CSPs). In addition, the aglycone portion of teicoplanin (i.e., teicoplanin with the carbohydrate moieties removed) was recently produced as a separate chiral stationary phase.(24)

The aglycone portion of all the macrocyclic glycopeptides contain either three or four fused macrocyclic rings (Figure 1). Together, these fused rings form a semirigid basket-shaped entity. Each aglycone basket has associated with it: an amine moiety, a carboxylic acid group (which is esterified in ristocetin A) and phenolic moieties. These groups, along with an amino-saccharide, control the charge of these molecules. In addition, the aglycone contains several amide or peptide bonds (Figure 1). Each aglycone has one or more carbohydrate moieties attached at various locations. A single disaccharide is attached to vancomycin, while teicoplanin has three monosaccharides associated with it (Figure 1). Compared to the aglycone portion of these molecules, the carbohydrate moieties are relatively free to alter their orientation.

As a class of chiral selectors, the macrocyclic glycopeptides have very broad enantioselectivity, and can be used in all chromatographic modes (i.e., reversed phase, normal phase and polar organic modes). The teicoplanin-based CSP (Chirobiotic T) is now the preferred means of resolving native amino acids (both natural and synthetic types).(1,5,9,10) A distinct amino acid and carboxylic acid binding site has been identified for these related macrocycles.(5,7,24) Furthermore, it appears that there are other binding sites for neutral and cationic chiral analytes.(24)

Clearly, vancomycin, teicoplanin, and ristocetin A are similar, structurally related molecules. They have the same biological function, which is to bind to D-alanyl-alanine moieties in the cell wall of Gram positive bacteria.(1,2,3,8) Although they are related, these macrocycles are far from being identical. Thus, they have somewhat similar, but not identical selectivities. This property gave rise to the operating "principle of complimentary separations." This means that if only a partial enantioseparation can be obtained on one CSP, then it is likely that a baseline separation will be achieved on one of the related CSPs.(4,6)

Given the similarities of the glycopeptide chiral selectors, it is not surprising that enantiomeric elution order appears to be the same on all of these CSPs.

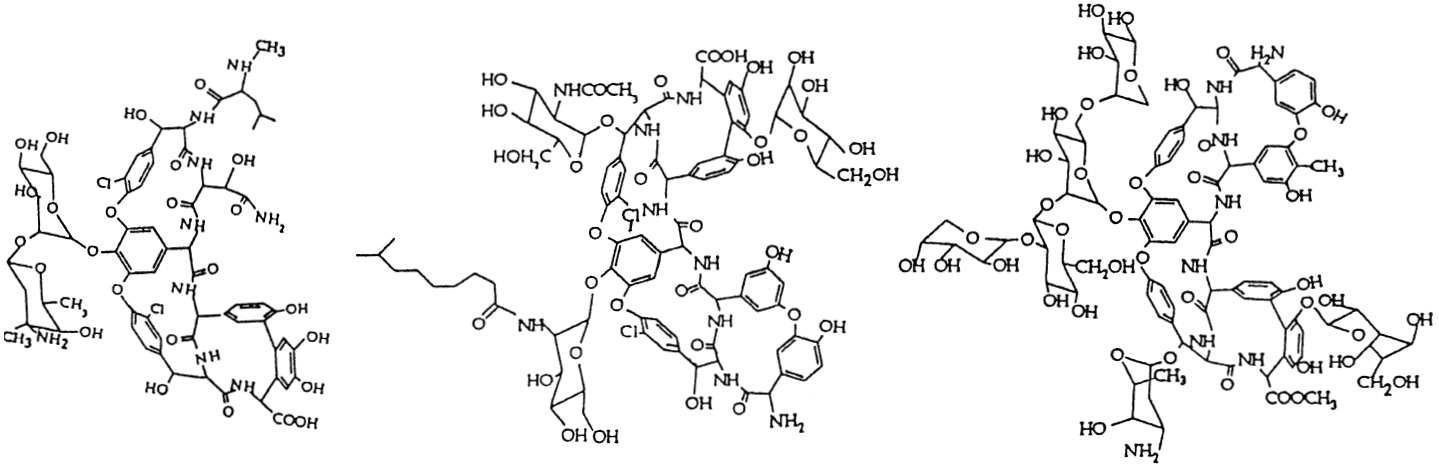



***Figure 1***. Schematic showing the structures of the related macrocyclic glycopeptides: vancomycin, teicoplanin, and ristocetin A.

Since these macrocyclic glycopeptides are complex, natural molecules, their enantiomers are unavailable. Consequently, reversing the enantioselectivity of a separation on these CSPs is difficult, and would be considered unusual. After performing thousands of separations on these CSPs over the last few years, we have found a few cases in which the enantioselectivity of a separation could be reversed either by using a related glycopeptide CSP or, in one case, by altering the mobile phase composition. This behavior has not been reported previously for this class of CSPs.

## EXPERIMENTAL

### Materials

The HPLC-grade solvents [methanol, reagent alcohol, acetonitrile, glacial acetic acid, triethylamine (99+% pure) and hexane] were purchased from Fisher Scientific (Fair Lawn, NJ, USA). All racemates and single enantiomers of derivatized amino acids and neutral molecules used in this study were purchased from Aldrich (Milwaukee, WI, USA) and Fluka (Milwaukee, WI, USA). All HPLC Chirobiotic columns [Chirobiotic V (vancomycin), Chirobiotic T (teicoplanin), Chirobiotic R (ristocetin A)] were (stainless-steel 25 cm x 4.6 mm) obtained from Advanced Separation Technologies, Inc. (Whippany, NJ).



### Methods

The separations were performed on Shimadzu (Columbia, MD) HPLC systems equipped with Model LC-6A pumps, Model SPD-6A, and SPD-6AV UV detectors, SCL-6A and SCL-6B system controllers, CR-601 and C-R3A Chromatopac integrators, and Rheodyne (Cotati, CA, USA) manual injectors. All samples were dissolved in methanol with concentration of 1 mg/mL and all separations were achieved at room temperature (~22ºC).

Mobile phases were prepared by mixing the indicated volumes of solvents or deionized and filtered water and degassed with a Crest Ultrasonic sonicator (Trenton, NJ, USA). The HPLC mobile phase flow rate was 1 mL/min and UV detection wavelengths were 254 nm for compounds containing aromatic rings and 220 nm for all others. The pH value of buffer mobile phase was measured with an Orion (Boston, MA, USA) pH meter Model 410A. Elution orders were determined by spiking a single pure enantiomer into the solution of the corresponding racemic compound.

## RESULTS AND DISCUSSION

There are few reports on the reversal of enantiomeric retention on CSPs containing natural chiral selectors.(25,26) These CSPs usually are either protein-based or linear derivatized carbohydrates.(25,26) The reversal of enantiomeric elution usually was the result of a change in mobile phase composition, although temperature effects also could be relevant. A solvent induced confirmational change in the chiral selector often was given as the reason for the change in selectivity. Note, that the changes in solvent composition that were reported were not drastic changes, such as going from the reversed phase mode to the normal phase mode (where the mechanism changed). Rather, they are milder changes, such as altering the organic modifier type or altering the pH in a reversed phase separation.

The macrocyclic glycopeptides are much smaller than the biological polymers that are used as chiral selectors. Thus, they seem to be less susceptible to solvent-induced changes in enantioselectivity. Indeed, the only reverse in enantioselective retention we've documented on the vancomycin CSP (involving different mobile phases), was for N-benzyl-α-methylbenzylamine (Figure 2). In this case, it took a rather drastic change in the mobile phase (i.e., from the polar organic mode to the reversed phase mode). It is less surprising to get a change in enantioselectivity if the retention mechanism is completely altered. Despite this fact, the enantiomeric retention order of a wide variety of solutes on the macrocyclic glycopeptide CSPs is reasonably constant. Table 1 lists several classes of molecules that appear to have the same basic enantioselectivity on the vancomycin, teicoplanin, and ristocetin chiral stationary phases.

Throughout the course of our studies, it was noted that a compound would sometimes have a different enantiomeric retention order on one of the macrocyclic glycopeptides CSPs. Thus far, all compounds that have shown this behavior fall into one of two classes: 1) the oxazolidinones, and 2) dansylated amino acids (Table 2). Of these, the dansyl-amino acids show the most consistent behavior. The D-enantiomers are preferentially retained on the vancomycin and teicoplanin CSPs, while the L-enantiomer is more retained on the ristocetin CSP (Table 2). It is the retention order on the ristocetin CSP that is unusual or anomalous. In most other cases, both the native and derivatized D-amino acids enantiomers are more retained on macrocyclic glycopeptide CSPs. Indeed, the biological function of these glycopeptides is to bind to D-alanyl-D-alanine on bacterial cell walls. The dansyl-fluorophore is a relatively bulky group and it contains an amine functionality. Apparently, this combination of additional steric-bulk and the hydrogen bonding or charge effect of the amine is sufficient to alter the enantioselectivity of ristocetin A, toward dansyl amino acids.

It is known that amino acids associate with the amine moiety these macrocyclic glycopeptides via their carboxylic acid groups.(1,5,9,10) This association

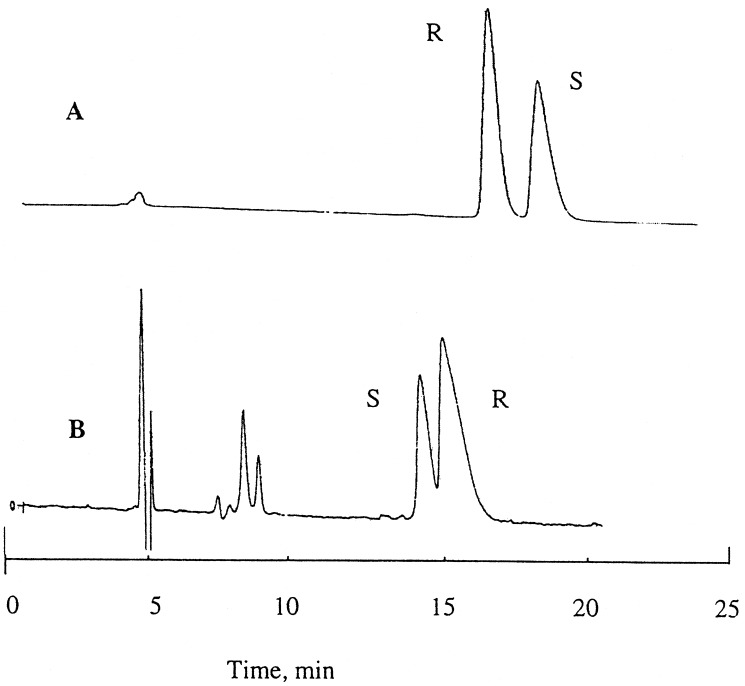



***Figure 2***. Chromatograms showing the reversal in elution order for enantiomers of N-benzyl-α-methylamine on the Chirobiotic V column (250 x 4.6 mm) resulting from a change in the mobile phase composition. Chromatogram (A) was generated in the polar organic mode (acetonitrile/0.1% TEAA buffer, pH 4.1; 20/80, by volume).



can be either electrostatic in nature or hydrogen bonding depending on the pH and/or mobile phase composition. The additional simultaneous interactions (required for chiral recognition) are thought to consist of hydrogen bonds to the amide groups of the aglycone, and in some cases, hydrophobic interactions (at

***Table 1.*** Some Classes of Compounds That Appear to Have the Same Enantiomeric Retention Order on Chirobiotic V, T, and R Chiral Stationary Phases

1. FMOC-amino acids
2. N-t-BOC-amino acids
3. N-CBZ-Amino acids
4. Nonsteroidal anti-inflammatory compounds
5. β-Adrenergic blockers
6. 4-Aryldihydropyrimidines

***Table 2.*** Members of Two Classes of Compounds Where There Is a Change in the Enantiomeric Elution Order on Macrocyclic Glycopeptide CSPs

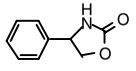
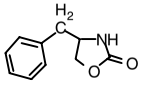
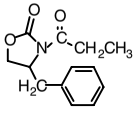
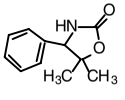
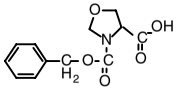
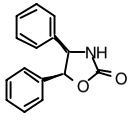
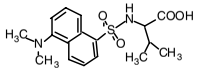
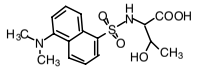
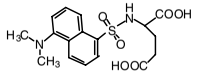
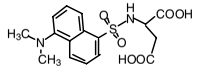
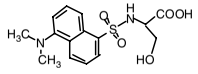
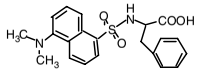
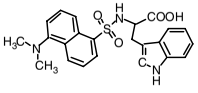
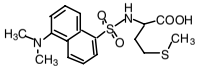
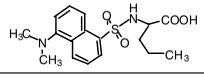
| Compound Oxazolidiones | Structure | Elution Order[a] Vancomycin V | RistocetinA R | Teicoplanin T | Mobile Phase[b] |
|---|---|---|---|---|---|
| RS - 4- phenyl - 2- oxazolidinone |  | R | S | R | T& R= 100% MeOH V=$H_2O$/MeOH(90/10) |
| RS- 4- benzyl- 2- oxazolidinone |  | R | S | R | T=100% MeOH V=$H_2O$/MeOH(90/10) R=$H_2O$/MeOH(75/25) |
| RS- 4- benzyl- 3- propionyl- oxazolidinone |  | R | Unresolved | S | T=Buffer[c]/MeOH (90/10) V=MeOH/$H_2O$(82/18) |
| RS- 5,5,dimethyl-4- phenyl-2- oxazolidionone |  | R | S | R | V, R, T = Hex/EtOH(80/20) |
| RS-3-benzyloxy carbonyl-4- oxazolidine carboxylic acid |  | R | R | S | T=Buffer/MeOH (80/20) R=Buffer/MeOH (80/20) V= EtOH/$H_2O$(40/60) |
| 4S,5R(+) - cis- 4,5- diphenyl-2- oxazolidinone |  | 4R,5S | 4S,5R | 4S,5R | V, R, T =EtOH/Hex(50/50) |

***Table 2.*** Continued

| Dansyl-Amino Acids | Structure | Elution Order[a] Vancomycin V | RistocetinA R | Teicoplanin T | Mobile Phase[a] |
|---|---|---|---|---|---|
| DL-Valine |  | L | D | L | Buffer[b]/MeOH (80/20) |
| DL - Threonine |  | L | D | L | Buffer/MeOH (80/20) |
| DL - Glutamatic acid |  | L | D | L | Buffer/MeOH (80/20) |
| DL - Aspartic acid |  | L | D | L | Buffer/MeOH (80/20) |
| DL – Serine |  | L | D | L | Buffer/MeOH (80/20) |
| DL - Phenylalanine |  | L | D | L | Buffer/MeOH (80/20) |
| DL - Trytophan |  | unresolved | D | L | Buffer/MeOH (80/20) |
| DL - Methionine |  | L | D | L | Buffer/MeOH (80/20) |
| DL - Norvaline |  | L | D | L | Buffer/MeOH (80/20) |

[a]The configuration of the first eluted enantiomer is given.
[b]The flow rate for all separation was 1.0 ml/min except for the first two compounds in this table which was 0.5 ml/min.
[c]The buffer was 1% triethylamine acetate, pH= 4.1.

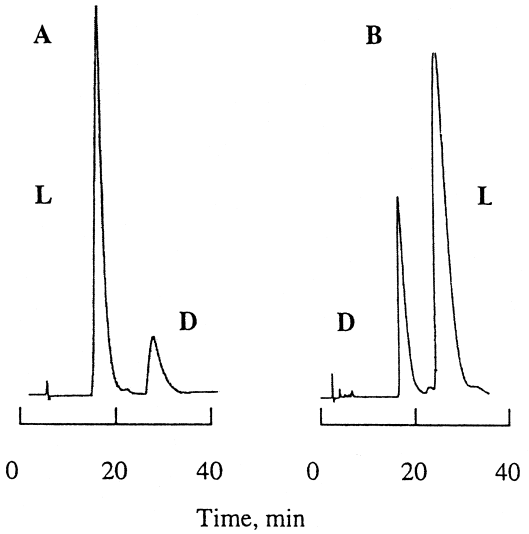



***Figure 3***. Chromatograms showing the reversal in elution order for dansyl-D, L-methionine on two different macrocyclic glycopeptide CSPs. Chromatogram A was generated using the Chirobiotic T column, and chromatogram B was generated using the Chirobiotic R column. The mobile phase compositions and other conditions are the same as given in Table 2.



least in the reversed-phase mode). Derivatizing the amine group of an amino acid with a fluorophore can alter the secondary interactions between the analyte and the aglycone. For example, the amino moiety of the amino acid would no longer be available for hydrogen bonding to the macrocyclic glycopeptide. However, the steric bulk of the fluorophore could accentuate either hydrophobic interaction (in the reversed phase mode) or steric repulsive interactions. As mentioned previously, the dansyl group also contains an amine moiety that could result in additional interactions.

The oxazolidinones do not show consistent retention behavior, as do the dansyl-amino acids (Table 2). It is not known how the oxazolidinones interact with the macrocyclic glycopeptides. Hence, the relative retentions reported can only be taken as empirical observations. The R-enantiomer always elutes before the S-enantiomer on the vancomycin CSP. However, there was no consistent pattern on either the ristocetin A or teicoplanin CSPs.

Chiral oxazolidinones are widely used in asymmetric synthesis. Since they are commercially available starting materials, it is often assumed that they are

***Table 3.*** The Enantiomeric Composition of Chiral Compounds Which Show Reversal in Enantiomer Elution Order

| Compound Name | Commercial Source | Enantiomeric Composition | | Separation Method Number[a] |
|---|---|---|---|---|
| | | Enantiomeric Contaminant(%) | Enantiomeric Excess(%) | |
| R,S - 4- phenyl - 2- oxazolidinone | Aldrich | S=0.05<br>R=99.90 | R=0.20<br>S=99.60 | 1 |
| R,S- 4- benzyl- 2- oxazolidinone | Aldrich | S=0.06<br>R=0.03 | R=99.88<br>S=99.94 | 2 |
| R,S- 4- benzyl- 3- propionyl- oxazolidinone | Aldrich | S=0.30<br>R=0.14 | R=99.40<br>S=99.72 | 3 |
| R,S- 5,5,dimethyl- 4-phenyl-2- oxazolidionone | Aldrich | R=0.03<br>S=0.08 | S=99.94<br>R=99.84 | 4 |
| R,S-3-benzyloxy carbonyl-4- oxazolidine carboxylic acid | Aldrich | S=2.38<br>R=0.85 | R=95.24<br>S=98.30 | 5 |
| 4S,5R(+) - cis- 4,5- diphenyl-2- oxazolidinone | Aldrich | 4R, 5S = 0.08<br>4S, 5R = 3.11 | 4S, 5R = 99.84<br>4R, 5S = 93.78 | 6 |

[a] Method No. 1= Chirobiotic T. Mobile phase 1% TEAA/MeOH=80/20(pH=4.1), Flow rate1mL/min.
Method No. 2 = Chirobiotic T. Mobile phase 1% TEAA/MeOH=90/10(pH=4.1), Flow rate 1mL/min.
Method No. 3 = Chirobiotic T. Mobile phase 1% TEAA/MeOH=85/15(pH=4.1), Flow rate 1mL/min.
Method No. 4 = refer to D. W. Armstrong et al./ Tetrahedron: Asymmetry 9 (1998) 2043-2064.
Method No. 5 = Chirobiotic $T_{AG}$. Mobile phase 1% TEAA/MeOH=70/30(pH=4.1), Flow rate 1mL/min.
Method No. 6 = Chirobiotic T. Mobile phase Hex/EtOH=50/50, Flow rate 1 mL/min.

enantiomerically pure. In previous work, it was shown that enantiomeric impurities were prevalent in most of the available chiral catalysts, auxiliaries, synthons, and resolving agents.(27,28) Table 3 gives the enantiomeric purity found for the oxazolidinones used in this study. Note the wide variation in the enantiomeric purity found for this class of chiral compounds. As was noted previously, the

batch-to-batch enantiomeric purity of each of these commercial chiral auxiliaries varies widely, since there is no enantiomeric quality control in their production or sale.(26,27)

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Received March 11, 2001 Manuscript 5547
Accepted May 17, 2001