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A Mechanistic Study of Enantiomeric Separation with Vancomycin and Balhimycin as Chiral Selectors by Capillary Electrophoresis. Dimerization and Enantioselectivity

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The role of the sugar moiety of glycopeptide antibiotics in chiral recognition was investigated with capillary electrophoresis. Two glycopeptide antibiotics, vancomycin and balhimycin, were employed as models since they possess the same aglycon and almost identical sugar moieties, however, with different attachment sites to the aglycon. The observed enantioselectivity of balhimycin for dansylated α-amino acids is 2.6 times higher than that of vancomycin. Blocking of the sugar amino group of balhimycin by N-carbamoylation reaction with KOCN led to a significantly decreased enantioselectivity compared to vancomycin, which remained almost the same upon carbamoylation. These results suggest a major role of the amino sugar together with its site of attachment to the aglycon. A dimerization-based mechanism is proposed to explain this phenomenon due to the fact that the dimerization properties of glycopeptides are similarly related to their glycosylation patterns; e.g., the dimerization constant of balhimycin is 78 times higher than that of vancomycin. Furthermore, the dimerization of glycopeptides promotes their affinity to carboxyl-containing ligands via cooperativity effects between the dimerization and the formation of glycopeptide−ligand complexes. The higher dimer stability probably leads to a more favorable conformation for chiral recognition. Thus, it is concluded that a weakened dimerization of N-carbamoylated balhimycin results in a decreased enantioselectivity.

Glycopeptide antibiotics, including vancomycin, ristocetin A, teicoplanin, and avoparcin, have been widely accepted as a new family of chiral selectors for enantiomeric separation by high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE), since their enantioselectivity was demonstrated by Armstrong et al.[1−4] Various chiral compounds have been separatedusing glycopeptide antibiotics as chiral selectors by HPLC and CE.[1−9] In some enantiomeric separations, very high chiral resolution factors were obtained,[10] and in order to understand the mechanism of chiral recognition of glycopeptide antibiotics based chiral selectors, several fundamental studies have been reported.

Gasper et al.[10] compared the structurally related glycopeptide antibiotics, vancomycin, ristocetin A, and teicoplanin, which were used as chiral additives in the run buffer for enantioseparation by CE. Both experimental and modeling studies were performed to elucidate their similarities and differences in chiral recognition and electrophoretic behaviors. Nair et al.[11] demonstrated that the secondary amino group of vancomycin plays a key role in chiral recognition. It was observed that vancomycin lost its enantioselectivity when the secondary amino group of *N*-methylleucine was complexed with Cu^{2+}. Berthod et al.[12] quantitatively investigated the role of sugar moieties in chiral recognition of teicoplanin-based HPLC chiral stationary phases (CSPs). In these experiments, two CSPs were prepared with teicoplanin and its aglycon in the same way. Four sets of enantiomers with different structural features were evaluated on both CSPs, and their differences in enantioselectivity were compared. It was found that α-amino acids were much better resolved by the aglycon CSP than by the teicoplanin CSP. in the case of teicoplanin, this means that the sugar moieties of teicoplanin are not needed for enantioseparations of most of α-amino acid enantiomers. However, some non-amino acid enantiomers were better resolved or only resolved on teicoplanin CSP.

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(1) Armstrong, D. W.; Tang, Y. B.; Chen, S. S.; Zhou, Y. W.; Bagwill, C.; Chen, J. R. *Anal. Chem*. **1994**, *66*, 1473−1484.

(2) Armstrong, D. W.; Rundlett, K. L.; Chen, J. R. *Chirality* **1994**, *6*, 496−509.

(3) Armstrong, D. W.; Liu, Y. B.; Ekborg-Ott, K. H. *Chirality* **1995**, *7*, 474−497.

(4) Armstrong, D. W.; Gasper, M. P.; Rundlett, K. L. *J. Chromatogr., A* **1995**, *689*, 285−304.

(5) Kang, J. W.; Yang, Y. T.; You, J. M.; Ou, Q. Y. *J. Chromatogr., A* **1998**, *825*, 81−87.

(6) Vespalec, R.; Corstjens, H.; Billiet, H. A. H.; Frank, J.; Luyben, K. C. *Anal. Chem*. **1995**, *67*, 3223−3228.

(7) Wan, H.; Blomber, L. G. *Electrophoresis* **1996**, *17*, 1938−1944.

(8) Ward, T. J.; Farris, A. B., III. *J. Chromatogr., A* **2001**, *906*, 73−89.

(9) Ward, T. J.; Dann, C. III; Brown, A. P. *Chirality* **1996**, *8*, 77−83.

(10) Gasper, M. P.; Berthod, A.; Nair, U. B.; Armstrong, D. W. *Anal. Chem*. **1996**, *68*, 2501−2514.

(11) Nair, U. B.; Chang, S. S. C.; Armstrong, D. W.; Rawjee, Y. Y.; Egglester, D. S.; Mcardle, J. V. *Chirality* **1996**, *8*, 590−595.

(12) Berthod, A.; Chen, X. H.; Kullman, J. P.; Armstrong, D. W.; Gasparrini, F.; D'Acquarica, I.; Villani, C.; Carotti, A. *Anal. Chem*. **2000**, *72*, 1767−1780.

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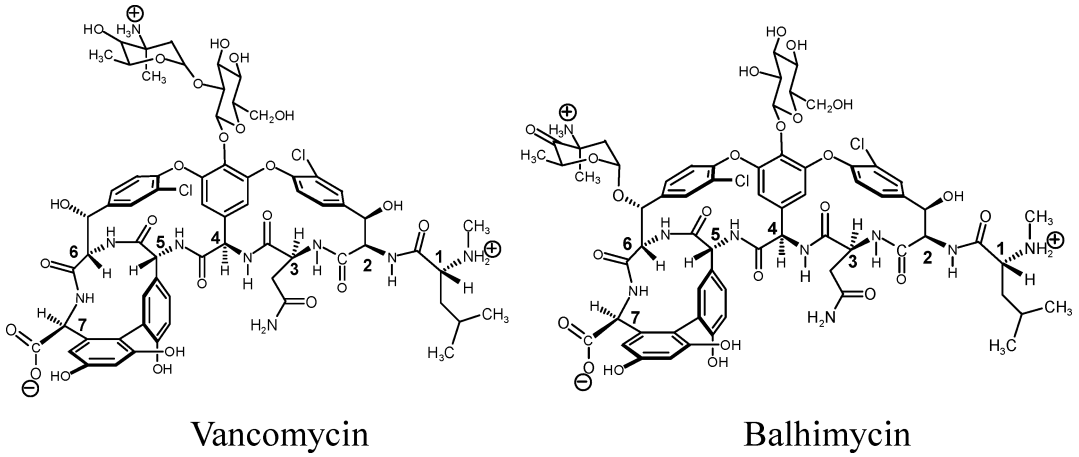

Figure 1. Structures of balhimycin and vancomycin.

In a further contribution, Berthod et al.[13] traced the possible interactions related to the retention and enantioselectivity of teicoplanin-based CSP through two experiments with HPLC. In one experiment, Cu^{2+} ions were added to the mobile phase to block the N-terminal amino group via complex formation. It was found that the enantioselectivity of teicoplanin-based CSP was decreased for separations of most underivatized racemic amino acids upon forming the complex with Cu^{2+}. However, the complex formation had no significant influence on separation of enantiomers other than α-amino acids. In a second experiment, an acetonitrile/D_2O mobile phase was used to evaluate the hydrogen-bonding interactions through isotopic exchange. It was found that the retention times of all amino acids enantiomers were decreased; however, the retention times of enantiomers without amino groups were increased. In all cases, the deuterium exchange did not influence the enantioselectivity of the CSP. Recently, Peyrin et al.[14−18] investigated the retention behavior of amino acid enantiomers and the enantioselective mechanism of teicoplanin- and vancomycin-based CSPs. It was demonstrated by displacement and perturbation studies that the enantioselectivity of glycopeptide antibiotic-based CSPs is mainly due to the interaction between the aglycon binding pocket and amino acid enantiomers.[14−16] Furthermore, Peyrin et al.[17] investigated the effect of vancomycin dimerization on enantioselectivity of vancomycin for D,L-dansylvaline enantiomers by HPLC. A theoretical model, based on multiple equilibria between analytes, vancomycin, and the silica stationary phase, was derived to analyze the obtained data. The authors demonstrated that vancomycin dimerization enhanced the enantioselectivity by a factor of 3.7 for D,L-dansylvaline enantiomers. In a further contribution by Peyin et al.,[18] the role of the heterodimers formed between vancomycin (mobile-phase additive) and ristocetin (stationary phase) in the enantioseparation of D,L-tryptophan and D,L-dansyltryptophan was investigated. The retention of enantiomers was increased with increasing vancomycin concentrations due to the formation of heterodimers. Probably due to the change of the conformation upon heterodimerization, the enantioselectivity for D,L-tryptophan enantiomers was significantly increased. However, in the case of D,L-dansyl tryptophan, a decreased enantioselectivity was observed, probably due to the fact that two glycopeptides possess antagonistic enantioselectivity.

The glycopeptide balhimycin used in the present study is a member of the vancomycin-type (type I) glycopeptide antibiotics. It has the same aglycon as vancomycin and very similar sugar moieties, however, with different attachment sites (Figure 1). In vancomycin, the sugar moiety (vancosamine) is linked to glucose at residue 4 of the aglycon, while in balhimycin the sugar is linked to the benzylic β-hydroxy group of residue 6. As a minor structural difference, in balhimycin the 4-hydroxy group of the vancosamine sugar is oxidized to a ketone (oxovancosamine). In our study, we found for separations of enantiomers of dansylamino acids under the same experimental conditions that the enantioselectivity of balhimycin is at least 2.6 times higher compared to vancomycin.

The aim of this study is to compare the enantioselectivity of balhimycin with that of vancomycin and to unravel the reason for this effect: Why does a change of the attachment site of the sugar significantly influence the enantioselectivity of glycopeptide antibiotics? The role of the sugar moiety in chiral recognition was investigated, and a mechanism was proposed to explain the enhanced enantioselectivity of balhimycin.

EXPERIMENTAL SECTION

Instrumentation. All separations were performed on a Beckman MDQ CE system (Beckman Instruments Inc., Fullerton, CA) equipped with an UV detector. Fused-silica capillaries with a dimension of 50 μm i.d. (365 μm o.d.) × 47 cm (37 cm to the detection window) were purchased from Ziemer (Mannheim, Germany). The capillary column was thermostated at 25 °C during the separation. Samples were injected by pressure at 20 mbar for 4 s. All analytes were detected with the UV detector at 214 nm. Applied voltage of −15 kV was used throughout the experiments.

Chemicals. Balhimycin (>99%) was prepared by the following approach: Balhimycin producer strain *A. mediterranei* DSM5908 was grown in 10 L of R5 medium at 30 °C for 5 days.[19,20] Liquid

(13) Berthod, A.; Valleix, A.; Tizon, V.; Leonce, E.; Caussignac, C.; Armstrong, D. W. *Anal. Chem.* **2001**, *73*, 5499−5508.
(14) Slama, I.; Ravelet, C.; Villet, A.; Ravel, A.; Grosset, C.; Peyrin, E. *J. Chromatogr. Sci.* **2002**, *40*, 83−86.
(15) Loukili, B.; Dufresne, C.; Jourdan, E.; Grosset, C.; Ravel, A.; Villet, A.; Peyrin, E. *J. Chromatogr., A* **2003**, *986*, 45−53.
(16) Peyrin, E.; Ravelet, C.; Nicolle, E.; Villet, A.; Grosset, C.; Ravel, A.; Alary, J. *J. Chromatogr., A* **2001**, *923*, 37−43.
(17) Slama, I.; Dufresne, C.; Jourdan, E.; Fahrat, F.; Villet, A.; Ravel, A.; Grosset, C.; Peyrin, E. *Anal. Chem.* **2002**, *74*, 5205−5211.
(18) Slama, I.; Jourdan, E.; Grosset, C.; Ravel, A.; Villet, A.; Peyrin, E. *J. Chromatogr., B* **2003**, *795*, 115−121.

supernatand was submitted to adsorption chromatography (Amberlite XAD-1180 resin, Serva Electrophoresis, Heidelberg, Germany) and eluted in a stepwise gradient with 40 and 60% MeOH/water (v/v; 5 L/fraction). After removal of the solvent, crude balhimycin was purified by a preparative LC−MS System (Merck, Darmstadt, Germany) using a Purospher-STAR RP-18e column (5 μm, 25 × 100 mm, Merck). Solvent A was 0.1% trifluoroacetic acid (TFA) and solvent B was acetonitrile/0.1% TFA (gradient 10−50% over 20 min with a flow rate of 15 mL/min). Detection was performed by ESI-ion trap-MS (Merck-Hitachi M-8000, Merck) and multiwavelength UV detection (Merck-Hitachi L-7410, Merck). Balhimycin was purified twice to give an analytical purity (>99%) in a yield of 158 mg.

Vancomycin hydrochloride (85%), tris(hydroxymethyl)aminomethane (Tris), orthophosphoric acid, sodium dihydrogen phosphate, sodium hydroxide, hydrochloric acid, potassium cyanate, and potassium chloride were obtained from Fluka (Buchs, Switzerland). Vancomycin hydrochloride was purified by preparative liquid chromatography to give a purity higher than 96%. Hexadimethrine bromide (HDB) and dansylated amino acids were obtained from Sigma (Steinheim, Germany). Stock solutions of Tris, phosphoric acid, and HDB with a concentration of 250 mM, 1 M, and 0.2% (w/v), respectively, were prepared and stored in the refrigerator. Except when addressed elsewhere, running buffers, consisting of 50 mM Tris and 0.001% of HDB, were freshly prepared from stock solutions. The buffer pH was adjusted to a desired value with phosphoric acid solution. Vancomycin or balhimycin was dissolved with the buffer solution giving concentrations required for experiments. All analytes were dissolved in a mixture of methanol and water (v/v; 1:1) to give an concentration of ~0.5 mg/mL.

Methods. A new capillary column was flushed with 0.1 M NaOH solution for 30 min, followed by flushing with water, 0.2% HDB solution, and running buffer for 5 min each. Before each run, the capillary was rinsed with 0.1 M NaOH, acetonitrile, 0.1 M HCl, 0.2% HDB, and run buffer for 2 min. Washing the capillary with 0.2% HDB solution produced a positively charged coating covering the capillary wall completely. Prior to injection, a plug of vancomycin or balhimycin solution was charged into the capillary by pressure at 70 mbar for 40 s. All samples were injected by pressure at 20 mbar for 4 s.

For measuring the p*I* of vancomycin and balhimycin, DMSO solution in water (0.1%, v/v) was added to vancomycin or balhimycin solutions as an EOF marker. The obtained apparent electrophoretic mobilities were converted into their inherent electrophoretic mobilities by extracting the EOF.

The N-carbamoylation reaction was used to modify the amino group of the sugar moiety of both vancomycin and balhimycin.[21] Briefly, a sodium phosphate buffer (40 mM, pH 6.0) containing 2 mM KOCN and 0.001% HDB was used to dissolve balhimycin or vancomycin giving a concentration of 2.0 mM, respectively. The resulting mixtures were kept at room temperature for 1 h prior to separation. It was demonstrated by HPLC−MS that almost all the primary amino groups in both vancomycin and balhimycin were converted into the *N*-carbamoyl amino group. The running buffer was the same buffer but devoid of 2 mM KOCN. In comparative experiments, the buffer containing 2 mM KCl was used for dissolving the chiral additives so that all used buffers had an identical ionic strength. Thus, the influence of the ionic strength on the enantioselectivity was identical.

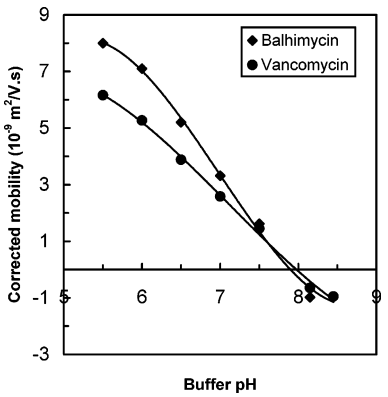

Figure 2. Dependence of the corrected mobilities on buffer pH. Conditions: fused-silica capillary, 50 μm i.d (365 μm o.d) × 47 cm (37 cm to detection window); buffer, 50 mM Tris−phosphate buffer containing 0.001% (w/v) HDB; samples injected by pressure at 20 mbar/4 s; capillary thermostated at 25 °C; UV 214-nm detection; applied voltage, −15 kV.

RESULTS AND DISCUSSION

Dynamic Modification of the Capillary Wall with a Polycationic Polymer Solution and Validation. In our previous work,[5] the polycationic polymer HDB (0.001%, w/v) was added to the run buffer to form a positively charged coating on the capillary wall. The resulting coating plays two important roles. First, it minimizes the adsorption of vancomycin on the capillary wall by electrostatic repulsion between vancomycin molecules and HDB, thereby improving the separation performance. Second, it reverses the direction of the EOF so that the EOF migrates in the same direction as the negatively charged analytes (coelectroosmotic flow electrophoresis), thus appreciably shortening separation times. It was found that rinsing the capillary with a HDB solution in high concentrations (0.2%, w/v) for 2 min before rinsing the capillary with the running buffer containing 0.001% HDB rendered good reproducibility in terms of intraday (6 injections, RSD = 0.27 and 3.9% for migration times of enantiomers and resolution factor, respectively) and interday reproducibility (4 days, RSD = 1.33 and 5.2% for migration times of enantiomers and resolution factor, respectively).

Comparison of the Enantioselectivity between Vancomycin and Balhimycin: Effect of the Buffer pH. The buffer pH is one of the important parameters influencing the enantioselectivity of glycopeptide antibiotics.[10] Therefore, the effect of buffer pH on the electrophoretic mobility of vancomycin and balhimycin was compared for a pH range from 5.5 to 8.5. Since the EOF is also pH dependent, EOF mobility had to be subtracted from apparent electrophoretic mobility. Thus, the corrected electrophoretic mobilities of glycopeptide antibiotics were plotted against buffer pH. As shown in Figure 2, the resulting curves are different,

(19) Nadkarni, S. R.; Patel, M. V.; Chatterjee, S.; Vijayakumar, E. K.; Desikan, K. R.; Blumbach, J.; Ganguli. B. N. *J. Antibiot.* **1994**, *47*, 334−341.
(20) Hopwood, D. A.; Bibb, J. M.; Chater, K. F.; Kieser, T.; Bruton, C. J.; Kieser, H. M.; Lydiate, D. J.; Smith, C. P.; Ward, J. M.; Schrempf, H. *Genetic Manipulation of Streptomyces: A Laboratory Manual*; The John Innes Foundation: Norwich, U.K,, 1985.
(21) Sheldrick, G. M.; Paulus, E.; Vertesy, L.; Hahn, F. *Acta Crystallogr.* **1995**, *B51*, 89−98.

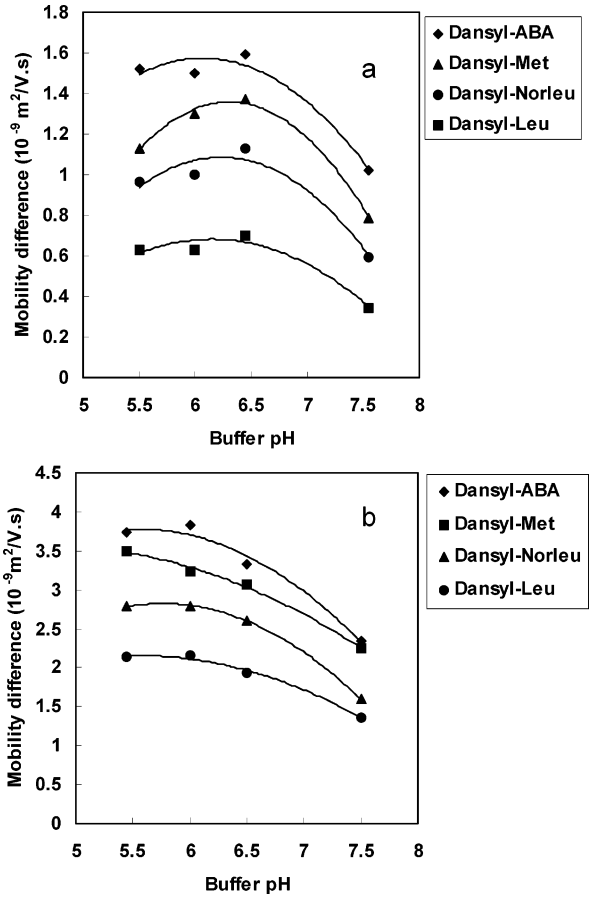

Figure 3. Dependence of the mobility differences of dansylamino acid enantiomers on buffer pH. Conditions: fused-silica capillary, 50 μm i.d. (365 μm o.d.) × 47 cm (37 cm to detection window); buffer, 50 mM Tris−phosphate buffer containing 0.001% (w/v) HDB; concentration of the chiral selectors, 2.0 mM each; plug length of the chiral selector solution, 70 mbar/40 s; samples injected by pressure at 20 mbar/4 s; capillary thermostated at 25 °C; UV 214-nm detection; applied voltage, −15 kV. Dansyl-ABA = dansyl-α-aminobutyric acid. (a) vancomycin; (b) balhimycin.

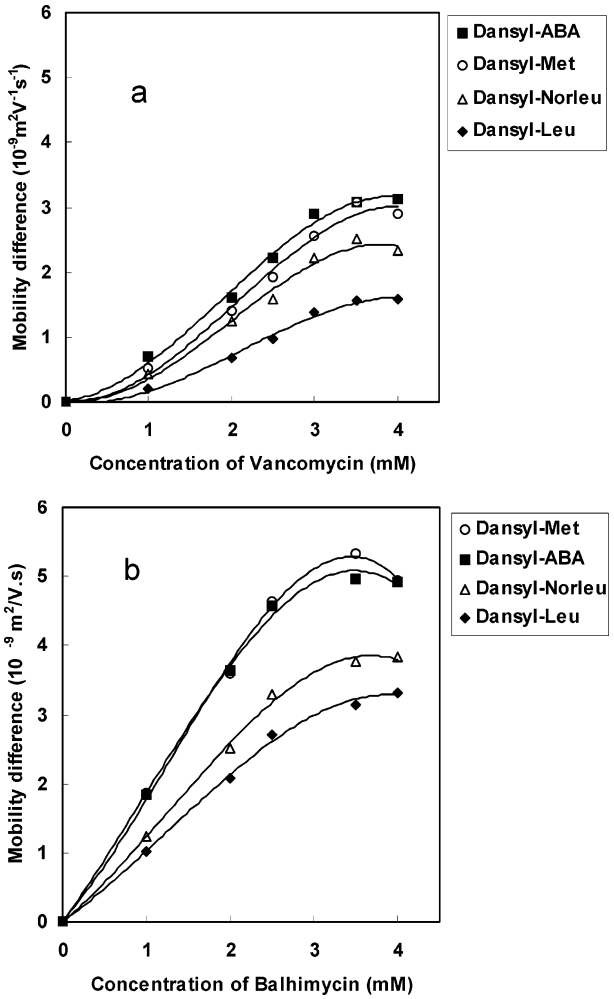

Figure 4. Dependence of the mobility differences of dansylamino acid enantiomers on concentration of chiral selectors. Conditions: buffer, 50 mM Tris−phosphate buffer containing 0.001% (w/v) HDB at pH 6.0. Other conditions as in Figure 3. Dansyl-ABA = Dansyl-α-amino butyric acid. (a) vancomycin; (b) balhimycin.

implying that both glycopeptide antibiotics show somewhat different ionization properties. This may be attributed to the change of the attachment site of the sugar to the aglycon. However, both glycopeptide antibiotics have very close p*I* values, which were determined as 7.8 and 7.9 for balhimycin and vancomycin, respectively. Furthermore, it was observed that their enantioselectivity had a different response with regard to changes of buffer pH (Figure 3). For vancomycin, the optimal enantioselectivity (in terms of electrophoretic mobility difference of enantiomers) appears at pH ∼6.0, while for balhimycin the optimal enantioselectivity appears at a pH less than 6. It can be seen from Figure 3 that, at the same pH, balhimycin has a higher enantioselectivity than vancomycin.

Effect of Chiral Selector Concentrations on Enantioselectivity. The partial filling technique has been used to improve the detection sensitivity when UV-active chiral selectors such as proteins[22] and vancomycin[9,23,24] were used as chiral additives. In the partial filling technique, the separation process only occurs inside the plug of the selector solution. Therefore, the plug length represents the effective separation length of the capillary. In our case, the modified EOF was so strong that it was able to carry the plug of the chiral selector solution, which migrated against the EOF, to the detection window. Therefore, the separation was confined to a separation window determined by the plug front of the chiral selector solution.[24] A plug length produced by a pressure of 70 mbar·40 s was selected as an overall optimized parameter. The effects of various vancomycin or balhimycin concentrations on enantioselectivity were investigated in a range from 1.0 to 4 mM at pH 6.0. For all tested enantiomers, the enantioselectivity in terms of the mobility difference of enantiomers was plotted against concentrations of the chiral selector. As seen in Figure 4, the enantioselectivity increased with increases in the chiral selector concentration. Furthermore, Figure 4 shows that balhimycin has a higher enantioselectivity than vancomycin for the separation of dansylated amino acids. Interestingly, for separation of the same enantiomers, the difference in enantioselectivity between balhimycin and vancomycin decreases with high chiral selector concentrations. For example, in the case of dansyl-

(22) Valtcheva, L.; Mohammad, J.; Pettersson, G.; Hjerten, S. *J. Chromatogr.* **1993**, *638*, 263−267.

(23) Fanali, S.; Desiderio, C. *J. High Resolut. Chromatogr.* **1996**, *19*, 322−326.

(24) Kang, J. W.; Wistuba, D.; Schurig, V. *Electrophoresis* **2003**, *24*, 2674−2679.

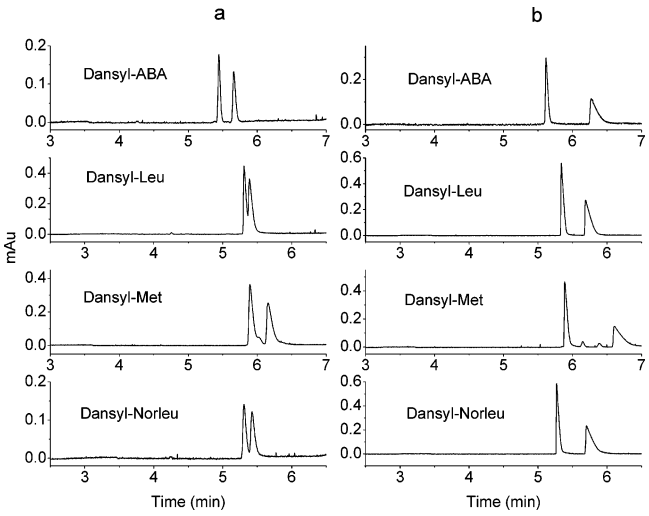

Figure 5. Electropherograms for comparison of the enantioselectivity between balhimycin and vancomycin. Conditions: buffer, 50 mM Tris−phosphate buffer containing 0.001% (w/v) HDB at pH 6.0; concentration of the chiral selectors, 2.0 mM each. Other conditions as in Figure 3. Columns: (a) vancomycin was used as chiral selector; (b) balhimycin was used as chiral selector. Dansyl-ABA = dansyl-α-aminobutyric acid.

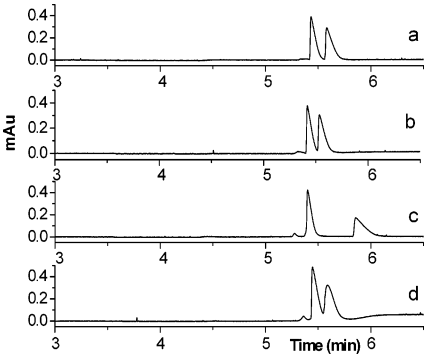

Figure 6. Electropherograms showing the change in enantioselectivity obtained before and after blocking of the sugar amino groups. Conditions: buffer, 40 mM sodium phosphate buffer containing 0.001% (w/v) HDB at pH 6.0; concentration of the chiral selectors, 2.0 mM each. Other conditions as in Figure 3. Dansyl-norleu was used as a model analyte. Chiral selector used: (a) vancomycin; (b) N-carbamoylated vancomycin; (c) balhimycin; (d) N-carbamoylated balhimycin.

Met, the enantioselectivity ratio of balhimycin to vancomycin is 3.5 at 1 mM selector concentrations, while the ratio decreased to only 1.7 at 4 mM. Electropherograms of enantioseparations using vancomycin and balhimycin as chiral selectors are shown in Figure 5, and it can be seen that enantioselectivity of balhimycin is more than 2.6 times higher than that of vancomycin under identical conditions.

Role of Sugar Moieties for Enantioselectivity. As shown by the above-mentioned experiments, the enantioselectivity of balhimycin for enantiomers of dansylamino acids was more than 2.6 times higher than that of vancomycin. Considering that balhimycin has the same aglycon as vancomycin and very similar sugar moieties (the keto sugar of balhimycin usually exists as the ketohydrate) but with different attachment sites to the aglycon (Figure 1), it is reasonable to assume that the enhanced enantioselectivity of balhimycin is mainly due to the sugar attached to residue 6 of the aglycon. Especially the amino group of the vancosamine sugar was assumed to contribute to this effect. To prove this prediction, amino groups of sugar moieties of both glycopeptides had to be blocked in order to abolish the effect of the amino group on enantioselectivity. Potassium cyanate was an ideal reagent for this purpose because of the moderate reaction conditions compatible with the electrophoretic buffer. Furthermore, the resulting *N*-carbamoyl group is sterically small, thus providing only a minor effect on the overall separation properties of glycopeptides. As expected, upon blocking of the amino groups, the enantioselectivity of modified balhimycin significantly decreased to the level of vancomycin, while the enantioselectivity of modified vancomycin was only slightly decreased. Figure 6 shows representative separations of dansyl-D,L-norleu. In addition, dansyl-Met and dansyl-α-aminobutyric acid (dansyl-ABA) were also tested and the same trends were observed (data not shown). Without blocking the amino group of sugars, the resolution factors obtained with vancomycin and balhimycin were 1.5 and 3.9, respectively. After blocking the amino group, resolution factors dropped to 1.3 and 1.2, respectively. This experiment suggested that the enhancement of the enantioselectivity of balhimycin is attributed to its sugar attachment site at amino acid 6 of the aglycon with a significant contribution of the amino group of the sugar moiety to enantioselectivity.

Thus far, it is still difficult to clearly point out the definite contribution of the amino group in the process of chiral recognition. An enantioselective ion pair interaction seems impossible since blocking the amino group of vancomycin does not influence its enantioselectivity, as shown in Figure 6. We propose that the enhanced enantioselectivity of balhimycin is related to the dimerization properties of glycopeptide antibiotics, which are known to be strongly affected by their glycosylation patterns.[25,26] This has been shown for a number of glycopeptide antibiotics, which form asymmetric back-to-back dimers linked by hydrogen bonds in aqueous solutions.[26] For this promotion by dimerization interaction, especially the sugar moieties of glycopeptide antibiotics play a key role.[26,27] This effect becomes obvious for the case of vancomycin and balhimycin, which possess the same aglycon, whereas the dimerization constant of balhimycin is 78 times higher.[25,26] The enhanced dimerization of balhimycin is attributed to the amino group of the sugar moiety attached to amino acid 6, which is able to form a hydrogen-bonding interaction with the carbonyl group of residue 2 in the opposite half of the dimer.[26,28] As seen in the X-ray single crystal structure of a vancomycin dimer, the sugar moieties are not involved in hydrogen-bonding interactions because their conformation is considerably mobile.[29] As has been suggested by Williams et al.,[30−32] the dimerization of glycopeptide antibiotics might promote their affinity to ligands

(25) Mackay, J. P.; Gerhard, U.; Beauregard, D. A.; Mapleston, R. A.; Williams, D. H. *J. Am. Chem. Soc.* **1994**, *116*, 4573−4580.
(26) Shiozawa, H.; Zerella, R.; Bardsley, B.; Tuck, K. L.; Williams, D. H. *Helv. Chim. Acta* **2003**, *86*, 1359−1370.
(27) Kaplan, J.; Korty, B.; Axelsen, P. H.; Loll, P. J. *J. Med. Chem.* **2001**, *44*, 1837−1840.
(28) Schäfer, M.; Sheldrick, G. M.; Schneider, T. R.; Vertesy, L. *Acta Crystallogr.* **1998**, *D54*, 175−183.
(29) Loll, P. J.; Bevivino, A. E.; Korty, B. D.; Axelsen, P. H. *J. Am. Chem. Soc.* **1997**, *119*, 1516−1522.
(30) Mackay, J. P.; Gerhard, U.; Beauregard, D. A.; Westwell, M. S.; Searle, M. S.; Williams, D. H. *J. Am. Chem. Soc.* **1994**, *116*, 4581−4590.

via a cooperativity effect of dimerization and formation of a glycopeptide−ligand complex. As glycopeptide antibiotics prefer to tightly bind D-amino acids and peptides,[1] we conclude that this effect might also result in an increased enantioselectivity. The promotion of the enantioselectivity by dimerization of vancomycin-based CSP has been demonstrated by Peyrin et al. in the case of HPLC.[17]

The results of our experiments dealing with blocking sugar amino groups for glycopeptide antibiotics, shown in Figure 6, can be evidence for our assumption that the enhanced enantioselectivity of balhimycin might be due to its more stable dimer conformation compared to vancomycin. Further support comes from the X-ray structures of balhimycin and carbamoylated balhimycin (ureidobalhimycin), showing that the length of hydrogen bonds in the interface of the dimer significantly increases upon blocking the amino group of the sugar moiety.[21,28] Thus, the carbamoylated amino group of balhimycin does not participate in hydrogen bonding leading to a weakened dimerization. The higher dimer stability probably at the same time leads to a more favorable conformation for chiral recognition. Furthermore, a high dimer constant also results in higher equilibrium concentrations of the dimer. Theoretically, the formation of the dimer can be expressed as

$$A + A \rightleftharpoons A_2 \quad (1)$$

Then the dimerization constant is expressed as

$$K_{dimer} = [A_2]/[A]^2 \quad (2)$$

where A and A_2 represent the monomer and the dimer of the glycopeptide antibiotics, respectively. [A] and [A_2] represent the equilibrium concentration of the monomer and the dimer, respectively. The total concentration of vancomycin and balhimycin C_t can be expressed as

$$C_t = [A] + 2[A_2] \quad (3)$$

From eq 2, it can be concluded that a high dimerization constant results in high dimer concentrations and the approximate equilibrium concentration of the dimer and the monomer can be calculated by combining eq 2 and eq 3. Accordingly, the dimerization constant of vancomycin[25] and balhimycin[26] in solution are 700 and 5.5×10^4 M^{-1}. For a balhimycin solution of 2.00 mM, the concentrations of the dimer and the monomer are calculated to 0.94 and 0.13 mM, respectively. In this case, ∼94% of balhimycin molecules are present in the dimer form. In comparison, for a vancomycin solution of 2.00 mM, the concentration of the dimer and the monomer are 0.55 and 0.89 mM, respectively. Only ∼55% of the molecules exist in the dimer form. Thus, the higher dimer concentration might yield higher enantioselectivity. In accordance with our results (shown in Figure 4), that the enantioselectivity difference of balhimycin and vancomycin decreases for high selector concentrations since then more vancomycin is present in its dimer form. This may explain in part why balhimycin shows higher enantioselectivity than vancomycin at the same total concentration level.

CONCLUSION

We have investigated the role of the amino group of the sugar moiety with regard to enantioselectivity for balhimycin compared to vancomycin. By selective blockage of the amino group of the vancosamine sugar, it could be demonstrated that this functional group plays a key role in the promotion of enantioselectivity of balhimycin. This phenomenon is proposed to be related to the dimerization properties of glycopeptide antibiotics, which are mainly influenced by their glycosylation patterns. The consistency between high dimerization constants and an increased enantioselectivity can be explained by a cooperativity effect between dimerization and the formation of the glycopeptide-analyte complex. The dimer conformation of the glycopeptides with high dimerization constants might be structurally favorable for chiral recognition. Thus, it is proposed that the decreased enantioselectivity of N-carbamoylated balhimycin is attributed to a decreased dimerization compared to balhimycin. Moreover, the fact that glycopeptides with high dimerization constants result in high dimer concentrations in solution is an explanation of why balhimycin shows higher enantioselectivity.

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(31) Williams, D. H.; Maguire, A. J.; Tsuzuki, W.; Westwell, M. S. *Science* **1998**, *280*, 711−714.

(32) Shiozawa, H.; Chia, B. C. S.; Davies, N. L.; Zerella, R.; Williams, D. H. *J. Am. Chem. Soc.* **2002**, *124*, 3914−3919.