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Highlighting the possible secondary interactions in the role of balhimycin and its analogues for enantiorecognition in capillary electrophoresis

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ABSTRACT

It is believed that the enantiorecognition mechanism based on macrocyclic antibiotics involves multi-modal interactions *via* hydrogen bonding, π–π interaction, steric hindrance, hydrophobic interaction and so on. A variety of enantiomeric *N*-benzoylated amino acids were separated using balhimycin (**A**) or its analogues bromobalhimycin (**B**) and dechlorobalhimycin (**C**) as chiral mobile phase additive using a CE method, which combined the partial filling technique with the dynamic coating technique and the co-EOF electrophoresis technique. The enantioresolution and the migration time were highly relevant to the structure of analytes, especially to the substitutions on the *N*-tagged benzoyl moiety of the amino acids. A steric effect and π–π interaction based mechanism is proposed in order to explain some observed enantioresolution differences between positional isomers. Notably dechlorobalhimycin exhibited the best enantioresolution for several *N*-benzoylated derivatives of leucine, which was rarely observed for *N*-dansylated amino acid derivatives. The hydrophobicity difference of the aglycone pocket among three chiral selectors was assumed to account for this behaviour.

1. Introduction

Over the years, vancomycin group glycopeptide antibiotics, such as vancomycin and balhimycin, have been successfully used as a versatile class of chiral selectors for enantioseparation by high performance liquid chromatography (HPLC) [1,2], capillary electrochromatography [3–6] and capillary electrophoresis [7–11], since their enantioselectivity was first demonstrated by Armstrong et al. [8,9,12]. Based on the molecular structure of vancomycin and well-known knowledge on enantiorecognition mechanisms for other chiral selectors such as cyclodextrin, Armstrong and co-workers presumed that the primary interaction of glycopeptide-based CE chiral separation is due to charge–charge or electrostatic interactions and proposed secondary interactions, including hydrogen bonding, steric repulsion, hydrophobic, dipole–dipole and π–π, might also play a role. Unfortunately, little was known about the geometry, secondary structure, and conformation of the vancomycin-type glycopeptide antibiotics at that time, and therefore, no more details were given. Lately, further studies have been carried out in order to investigate the complex form of vancomycin-type glycopeptides with carboxylate-containing ligands using nuclear magnetic resonance (NMR) spectroscopy, X-ray, molecular models and computer simulations [13–29]. These investigations have been very successful at establishing accurate chemical structures and the binding mode of vancomycin group glycopeptide antibiotics. It demonstrated that the high specific aglycon cavity, formed by cyclisation of five aromatic side-chains, is the main binding sites and is responsible for the antibiotics activity. In addition to these ligand–receptor interactions, two glycopeptide antibiotic molecules can form a tight dimer with anti-parallel hydrogen bonds between the peptide backbones as well as between the vancosamine residues and the peptide backbone [24,26,29]. The cooperativity between dimerisation and ligand binding of glycopeptides, e.g. balhimycin, has also been confirmed by NMR spectroscopy [24,28,29]. The molecular bases for the binding between vancomycin-type glycopeptides and carboxylate-containing ligands are thought to be conferred through hydrogen bonds, which similarly have been extensively studied by NMR spectroscopy [25,27,30,31] and X-ray crystallography [14,16,19–23]. Williams et al. have shown that productive binding is crucially dependent on the binding of the carboxylate, which is stereoselective [17,18,24,28]. Additionally, the adjacent hydrophobic interactions and amide–amide hydrogen bonds can increase the binding affinity of the carboxylate anion into an antibiotic receptor pocket [15,29].

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The mechanism of dimerisation and cooperativity effect has also been extensively applied to elucidate the enantiorecognition of vancomycin-type glycopeptide antibiotics [32–39]. Many groups attributed the influence of the linking position and type of sugar moieties of vancomycin-type glycopeptide antibiotics on their enantiorecognition ability to the cooperativity between dimerisation and ligand binding [36,40]. Recently, our group first developed the use of balhimycin as a chiral mobile phase additive (CMA) and compared the enantiorecognition ability of balhimycin and dechlorobalhimycin for dansyl amino acids. The two chlorine substituents of each balhimycin monomer, which mutually penetrate into the cavity of the adjacent molecule of the dimer, are assumed to promote dimerisation and as a consequence also enantioresolution [41,42]. However, only a few studies have been reported about the influence of other secondary interactions between vancomycin-type glycopeptide antibiotics and carboxylate-containing ligands on the enantiorecognition process [43–46].

In this report four amino acids, whose *N*-dansylated derivatives showed good enantioresolution with balhimycin as CMA, were *N*-terminally derivatised with various substituted benzoyl residues and their effect on the enantioresolution and the migration time was compared in order to study the secondary interactions involved in the enantiorecognition process on balhimycin. These *N*-benzoylated amino acids are first time enantioseparated with balhimycin as chiral selector. The substitutions on the *N*-tagged benzoyl moiety of the amino acids exhibited significant effect on the enantioresolution, which provide a chance for highlighting the possible secondary interactions in the role of balhimycin and its analogues for enantiorecognition in capillary electrophoresis. Additionally, the enantiorecognition ability of balhimycin, dechlorobalhimycin and bromobalhimycin towards 31 selected *N*-benzoylated amino acids was also compared. Compared to our previous results [41,42], different orders of the enantiorecognition ability of three CMAs were firstly observed for some *N*-benzoylated derivatives of leucine. A mechanistic hypothesis of "multimodal interactions" was advanced.

2. Materials and methods

2.1. Instrumentation

If not otherwise stated, the CE experiments were carried out on a HP^{3D} CE system (Hewlett-Packard, Waldbronn, Germany) equipped with an auto-sampler, an on-column diode-array detector and a temperature control system (15–60 °C ± 0.1 °C). A CE ChemStation (Hewlett-Packard) was used for instrument control, data acquisition and data handling. Chromatograms were transferred to an ASCII file and redrawn using Microcal Origin 6.0. The experiments for identifying the elution order of enantiomers were carried out on a Beckman P/ACE MDQ system (Beckman Instruments, Munich, Germany) equipped with diode-array UV detector. The fused-silica capillaries with a dimension of 50 μm I.D. (375 μm O.D.) and 33.5 cm total length (25 cm effective length) were purchased from Composite Metal Services Ltd. (Hallow, Worcestershire, UK).

Preparative chiral SFC experiments were carried out on a Mettler Toledo AG-Berger SFC^{TM} MiniGram system (Cambridge, UK). CHIRALPAK AD-H column (1 cm I.D. × 25 cm length) was purchased from Chiral Technologies Europe (Illkirch Cedex, France). Industrial grade carbon dioxide was obtained from British Oxygen Company (London, UK).

Log *D* values of three chiral selectors at pH 6.0 were calculated using Chemiformatics Lipophilicity pH profile webtool Version 2.1. pK_a values of all tested *N*-benzoylated amino acids were calculated using Chemiformatics pKa webtool Version 10.

2.2. Chemicals and reagents

Balhimycin (>99%), bromobalhimycin (>97%) and dechlorobalhimycin (>97%) were prepared by fermentation and were purified by preparative LC [47,48]. Four amino acids (leucine, methionine, threonine, and alanine), eight acyl chlorides (benzoyl chloride, 2,4-dichlorobenzoyl chloride, *p*-nitrobenzoyl chloride, *o*-chlorobenzoyl chloride, *m*-chlorobenzoyl chloride, *p*-chlorobenzoyl chloride, 3,5-dinitrobenzoyl chloride, and 3,5-dichlorobenzoyl chloride), dodecyltrimethylammonium bromide, sodium carbonate, *N*-acetyl-L-Lys-D-Ala-D-Ala (ALAA) and hexadimethrine bromide (HDB) were purchased from Sigma (Steinheim, Germany). Tris(hydroxymethyl)aminomethane (Tris), ortho-phosphoric acid, sodium hydroxide and hydrochloric acid were obtained from Fluka (Buchs, Switzerland). Tetrahydrofuran (THF), petroleum ether, HPLC grade methanol and acetonitrile (ACN) were purchased from Fisher (Leicestershire, UK). The water used throughout all experiments was purified using an Elga water purifier (Bucks, UK). All *N*-benzoylated amino acids were prepared according to the methods described by Jursic [49]. In brief, a THF (50 mL) suspension of sodium carbonate (0.43 g, 4.0 mmol), dodecyltrimethylammonium bromide (0.012 g, 0.04 mmol), the corresponding amino acid (4.0 mmol), and the corresponding acyl chloride (4.0 mmol) was stirred at room temperature for approximately 4 h. Then the reaction mixture was refluxed for approximately 8–10 h and the solid residue was separated from the THF suspension and discarded. Residual THF was evaporated, yielding a solid residue, which was then purified by crystallisation from petroleum ether.

The enantiomers of 12 *N*-benzoylated amino acids (benzoyl leucine (B-Leu), benzoyl methionine (B-Met), 3,5-dinitrobenzoyl leucine (3,5-DNB-Leu), 3,5-dinitrobenzoyl methionine (3,5-DNB-Met), *p*-nitrobenzoyl leucine (*p*-NB-Leu), *p*-nitrobenzoyl methionine (*p*-NB-Met), 3,5-dichlorobenzoyl leucine (3,5-DClB-Leu), 3,5-dichlorobenzoyl methionine (3,5-DClB-Met), *p*-chlorobenzoyl leucine (p-ClB-Leu), *p*-chlorobenzoyl methionine (*p*-ClB-Met), *m*-chlorobenzoyl leucine (*m*-ClB-Leu), *m*-chlorobenzoyl methionine (*m*-ClB-Met)) were prepared *via* preparative SFC based on a CHIRALPAK AD-H column (1 cm I.D. × 25 cm length).

2.3. Methods

Stock solutions of 250 mM Tris, 1 M phosphoric acid and 0.2% (w/v) HDB were prepared in advance and stored in the refrigerator until use. The CE running buffer, consisting of Tris (50 mM) and HDB (0.001%, w/v), was freshly prepared from stock solutions and its pH was adjusted with phosphoric acid (1 M). All racemic samples were dissolved in a mixture of methanol and water (1:1, v/v) at an approximate concentration of 0.5 mg/ml. New capillaries were flushed first with NaOH solution (1.0 M) for 1 h, followed by ACN, water, HDB solution (0.2%, w/v) and running buffer for 30 min each. Between runs, the capillaries were flushed for 2 min at high pressure with NaOH (0.1 M), ACN, HCl (0.1 M), HDB solution (0.2%, w/v) and running buffer, respectively. The capillaries were thermostated at 25 °C. The desired amount of glycopeptide chiral selector was dissolved in the running buffer. A plug of the glycopeptide chiral selector solution was then injected into the capillary using a pressure of 50 mbar for a given period of time prior to the injection of racemic analyte. All separations were detected at λ = 214 nm. The applied voltage was −15 kV and the sample was introduced by hydraulic injection using a pressure of 50 mbar for 5 s. As described before [42], the practical term enantiorecognition rather than the thermodynamic term enantioselectivity has been used throughout this work because the selectivity α could not be determined due to the uncertainty of the actual dead time. There-

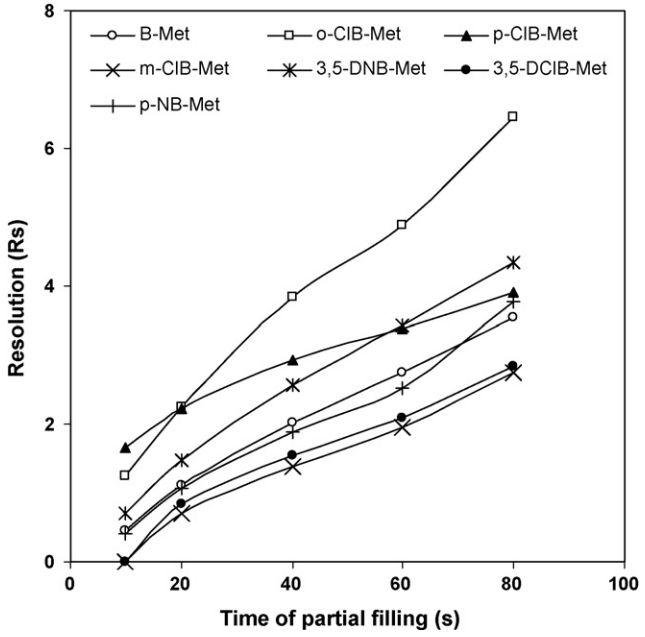

Fig. 1. Effect of plug length of balhimycin solution on the resolution factor R_s. Conditions: fused-silica capillary, 50 μm I.D. (370 μm O.D.) × 47 cm (37 cm to detection window); running buffer, 50 mM Tris–phosphate buffer solution (pH 6.0) containing 0.001%, w/v HDB; 8 mM balhimycin dissolved in running buffer was partially filled with a pressure of 50 mbar for various time; sample injection by a pressure of 50 mbar for 5 s; column was thermostated at 25 °C; applied voltage was −15 kV; detection at λ = 214 nm.

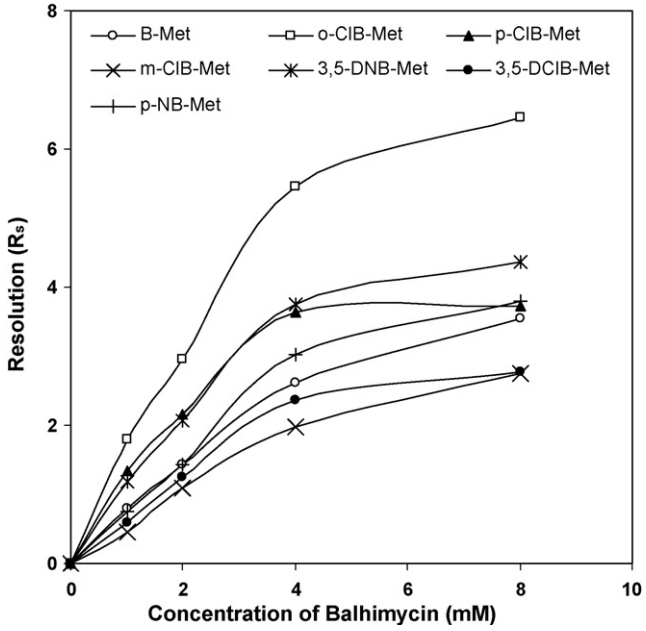

Fig. 2. Effect of balhimycin concentration on the enantioresolution factor R_s. Conditions: various amount of balhimycin dissolved in running buffer was partially filled with a pressure of 50 mbar for 80 s; other conditions as in Fig. 1.

fore, only migration times and no retention factors (k) are reported in paper.

3. Results and discussion

Based on our previous work [40–42], two series of *N*-benzoylated amino acids (methionine and leucine) were selected as test analytes because their analogous *N*-dansylated derivatives showed good enantioresolution with balhimycin as chiral selector. The same CE method, which combined the partial filling technique with the dynamic coating technique and the co-EOF electrophoresis technique, was chosen for enantioresolution in all experiments presented within this contribution. Sample was injected by pressure. The polycationic polymer HDB was added to the running buffer to dynamically form a positively charged coating on the inner wall of the capillary, minimising the adsorption of glycopeptides on the capillary wall *via* electrostatic repulsion between the coating and glycopeptide molecules. In addition, the positively charged coating reversed the EOF migrating in the same direction as the negatively charged analytes (co-EOF electrophoresis), thus appreciably shortening the separation times.

3.1. Optimisation of separation conditions

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. By varying the time of partial filling of 8 mM balhimycin from 10 to 80 s under a given pressure of 50 mbar, the effect of the plug length of balhimycin on enantioresolution (R_s) was investigated. As shown in Fig. 1, R_s of all seven racemic analytes (B-Met, *o*-chlorobenzoyl methionine (*o*-ClB-Met), *p*-ClB-Met, *m*-ClB-Met, 3,5-DNB-Met, 3,5-DClB-Met and *p*-NB-Met) increased with increasing partial filling time. Baseline enantioseparation for all eight analytes were achieved by employing a plug length of 50 mbar × 80 s. However, the impurities of balhimycin interfered with the detection of some analytes if the partial filling time of balhimycin was increased over 80 s.

Fig. 2 shows the effect of balhimycin concentration on the enantioresolution of *N*-benzoylated methionine derivatives. R_s increased dramatically with increasing balhimycin concentration from 0 to 4 mM, and then flattened with further increasing of balhimycin concentration to 8 mM. Baseline enantioresolution was achieved for all seven analytes at 4 mM balhimycin. The dependence of enantioresolution R_s of *N*-benzoylated methionine on the applied voltage was shown in Fig. 3. As expected, with an increas-

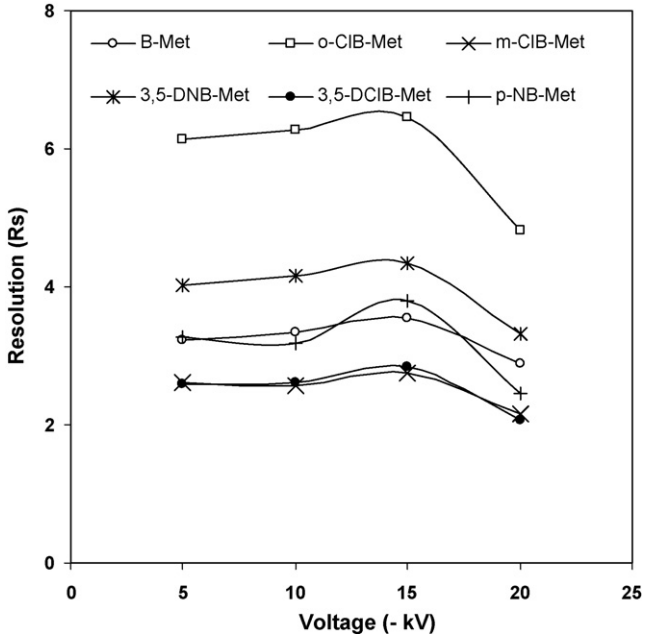

Fig. 3. Effect of applied voltage on the enantioresolution factor R_s. Conditions: 8 mM balhimycin dissolved in running buffer was partially filled with a pressure of 50 mbar for 80 s; various applied voltage in range −5 to −20 kV; other conditions as in Fig. 1.

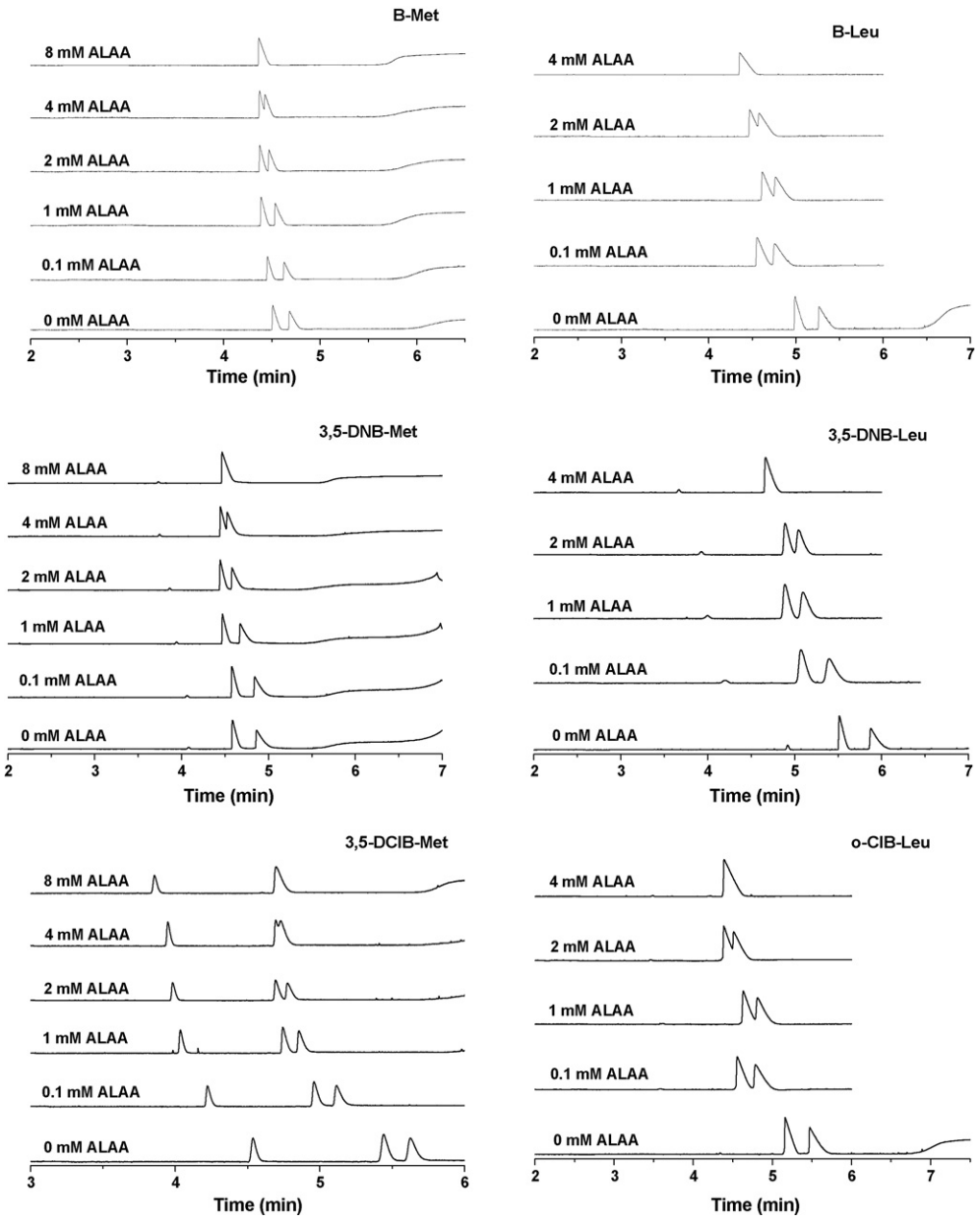

Fig. 4. Electropherograms showing the change in enantioresolution with different amounts of ALAA in chiral selector solution. Conditions: 4.0 mM balhimycin with different amount ALAA dissolved in running buffer was partially filled with pressure of 50 mbar for 80 s; other conditions as in Fig. 1.

ing of the applied voltage, the migration time of two enantiomers decreased and the peak efficiencies also changed. For example, the theoretical plates for the first eluted peak of *p*-NB-Met at various applied voltage (−5, −10, −15 and −20 kV) were around 62,000, 74,000, 100,000 and 60,000 plates/meter, respectively. R_s increased slightly as the applied voltage increased from −5 to −15 kV, and then decreased dramatically when the applied voltage further increased to −20 kV. The effect of buffer pH on the enantioresolution of *N*-benzoylated amino acids was also investigated in the range of buffer pH 5.0–8.0. Similar to results reported for dansyl amino acids in previous work [41,42], the highest enantioresolution R_s was observed at around pH 6.0 (data not shown). For eight *N*-benzoylated leucine derivatives, similar enantioresolution variations with four parameters (applied voltage, buffer pH, the concentration and the plug length of chiral selector solution) were observed. Therefore, a plug length of 50 mbar × 80 s of 4 mM chiral selector, pH 6.0 buffer and −15 kV applied voltage were selected for the following experiments.

3.2. *Involvement of the* D-*Ala*-D-*Ala binding site of glycopeptide in enantioresolution*

In order to determine the binding site responsible for enantioseparation of *N*-benzoylated derivatives of methionine and leucine, a displacement study described before [37,41] was carried out by using peptide ALAA as a competitive blocking ligand for the aglycone pocket of balhimycin. All 15 *N*-benzoylated derivatives of methionine and leucine were tested in this competitive experiment and typical electropherograms are shown in Fig. 4. With an increase of the ALAA concentration in the 4 mM balhimycin solution from 0 to 8 mM, the enantioresolution of all analytes apparently decreased. A small amount (0.1 mM) of ALAA appreciably increased the enantioresolution of some analytes (3,5-DClB-Met, B-Met, and *o*-ClB-Met) by 4%, 9% and 6%, respectively. This observation is comparable to that of *N*-dansylated amino acids [41]. They may be rationalised by the competition between analyte and ALAA for the same binding position on the balhimycin and the co-operative

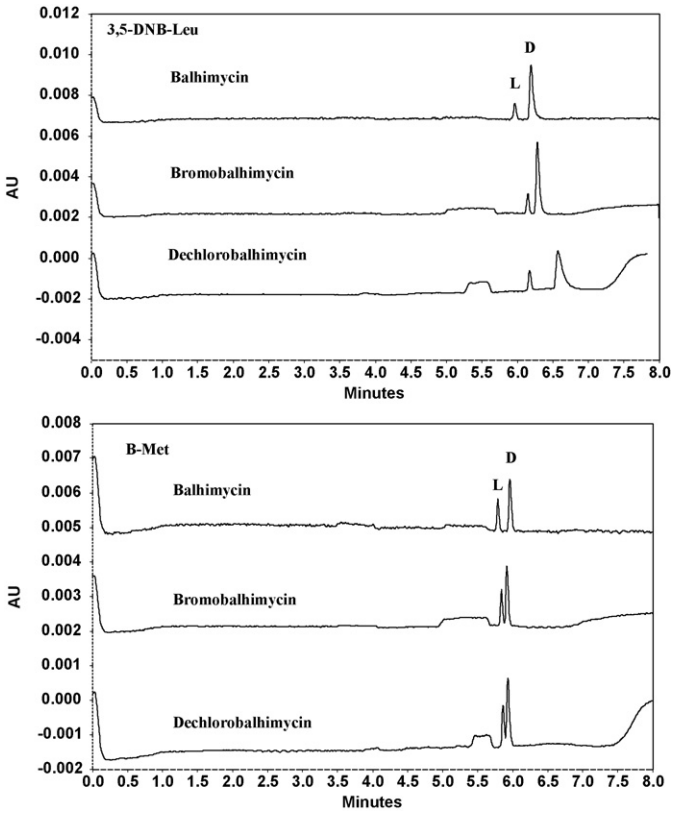

Fig. 5. Enantioresolution of enantio-enriched *N*-benzoylated derivatives of amino acids by CE. Conditions: 8 mM balhimycin dissolved in running buffer was partially filled with a pressure of 70 mbar for 40 s; other conditions as in Fig. 1.

binding of a single molecule of ALAA to one-half of a balhimycin dimer, which then binds to the analytes with increased efficiency. In a subsequent experiment, the enantiomeric elution order of *N*-benzolyated derivatives of methionine and leucine was confirmed by enantioseparation of 12 *D*-form enantiomer enriched *N*-benzoylated amino acids samples. The enantiomers of 12 *N*-benzolyated derivatives of methionine and leucine were prepared *via* preparative SFC based on a CHIRALPAK AD-H column. For all 12 tested analytes, the *L*-enantiomer was always eluted first (Fig. 5) with balhimycin or its analogues as **CMA**. This means that the *D*-enantiomer undergoes a stronger interaction with balhimycin. As a consequence, this data suggested that similar to *N*-dansylated amino acids, the main site of interaction between *N*-benzoylated amino acid derivatives and balhimycin is the cavity formed by the aglycon and this cavity is responsible for the preferential inclusion of the *D*-enantiomer.

3.3. Investigation of the enantiorecognition mechanism

It was noticed that the R_s value of *N*-benzoylated derivatives of methionine or leucine is around three times lower at least than that of their relative *N*-dansylated derivatives under identical separation conditions. This might be attributed to the larger steric effect of the dansyl moiety than that of the benzoyl moiety. In order to further investigate the enantiorecognition interaction between the glycopeptide antibiotic balhimycin or its bromo and dechloro analogues with *N*-benzoylated amino acid derivatives in capillary electrophoresis, two more amino acids (alanine and threonine), whose *N*-dansylated derivatives exhibited the high enantioresolution with balhimycin as CMA, were chemically derivatised with various benzoyl moieties. The tagging reagents used to derivatise the amino acids included benzoyl (B), *p*-nitrobenzoyl (*p*-NB), 3,5-dinitrobenzoyl (3,5-DNB), *o*-chlorobenzoyl (*o*-ClB), *m*-chlorobenzoyl (*m*-ClB), *p*-chlorobenzoyl (*p*-ClB), 3,5-dichlorobenzoyl (3,5-DClB) and 2,4-dichlorobenzoyl (2,4-DClB). Table 1 shows the migration time and enantioresolution data of all 31 *N*-benzoylated amino acids (methionine, leucine, alanine and threonine) with balhimycin (**A**), bromobalhimycin (**B**) and dechlorobalhimycin (**C**) as CMA, respectively. At the selected separation conditions, 28 out of 31 *N*-benzoylated amino acids exhibited enantioseparation with at least one of the three CMA's (Fig. 6 and Table 1). However, it turned out difficult to deduce a general rule from these data. This is possibly because of the fact that the chiral discrimination process of balhimycin or its analogues for *N*-benzoylated amino acid derivatives involves multimodal interactions. Nevertheless, when the data in Table 1 are more closely examined, there are still some interesting results that can be observed.

In previous publications [41,42], we have noticed that balhimycin exhibited the best enantiorecognition ability for *N*-dansylated amino acids compared to bromobalhimycin and dechlorobalhimycin. It was assumed that the two chlorine substituents of each balhimycin monomer can promote the dimerisation and as a consequence also enantioresolution by mutually penetrating into the cavity of the adjacent molecule of the dimer. However, this was not the generally observed trend for the enantioseparation of *N*-benzoylated amino acid derivatives. For all *N*-benzoylated derivatives of Met, Thr and Ala, balhimycin showed the highest enantioresolution and dechlorobalhimycin showed the lowest enantioresolution, which was consistent with that for *N*-dansylated amino acids [41]. However, different orders were observed for *N*-benzoylated derivatives of leucine. For example, the order of enantiorecognition ability for *N*-benzoyl leucine is **A** > **C** > **B**. Similar trends were observed for the three chlorobenzoyl leucines (*p*-ClB-Leu, *m*-ClB-Leu and *o*-ClB-Leu). When the benzoyl moiety was substituted by sterically demanding nitro groups or two chlorine atoms, dechlorobalhimycin showed the highest enantioresolution and the order of enantiorecognition ability now becomes **C** > **A** > **B**. This unexpected behaviour could be attributed to the side chain effect of leucine.

Compared to other three tested amino acids, leucine has a larger and more hydrophobic side chain. The steric effect and hydrophobic interaction could contribute to enantiorecognition for benzoyl amino acids. Chen and co-workers have found that the side chain size of the amino acids or tagging reagent has a great influence on enantioselectivity for compounds separated on the teicoplanin column or the vancomycin column [43,50]. In his work, the amino acids with aromatic or bulky side-chain group are better resolved after derivatisation with a reagent such as methylioisocyanate under the same chromatographic conditions. However, the increase in the bulkiness of the *N*-tagging moiety could negatively affect the enantioresolution. A similar trend was noted in our work. For example, the benzoyl amino acids with bulky amino acid side-chains (B-Leu and B-Met) are better enantioresolved than the benzoyl amino acids with smaller amino acid side-chains (B-Thr and B-Ala) under the same separation conditions. Their R_s values with 4 mM balhimycin as CMA are 1.18 (B-Leu), 1.09 (B-Met), 0.51(B-Thr) and 0.00 (B-Ala), respectively. This trend becomes weaker or disappears if the benzoyl moiety was substituted with $-NO_2$ or –Cl group. This indicated that the amino acid side chain size also plays an important role for the enantiorecognition of *N*-benzoylated amino acid derivatives with balhimycin as CMA. However, the conformation or size difference of the aglycone pocket among three chiral selectors would not be expected to be significant, and thus the size effect is unlikely to account for the differences in the enantiorecognition ability of the three chiral selectors for *N*-benzoylated derivatives of leucine. The hydrophobic interactions formed between the non-polar side chain

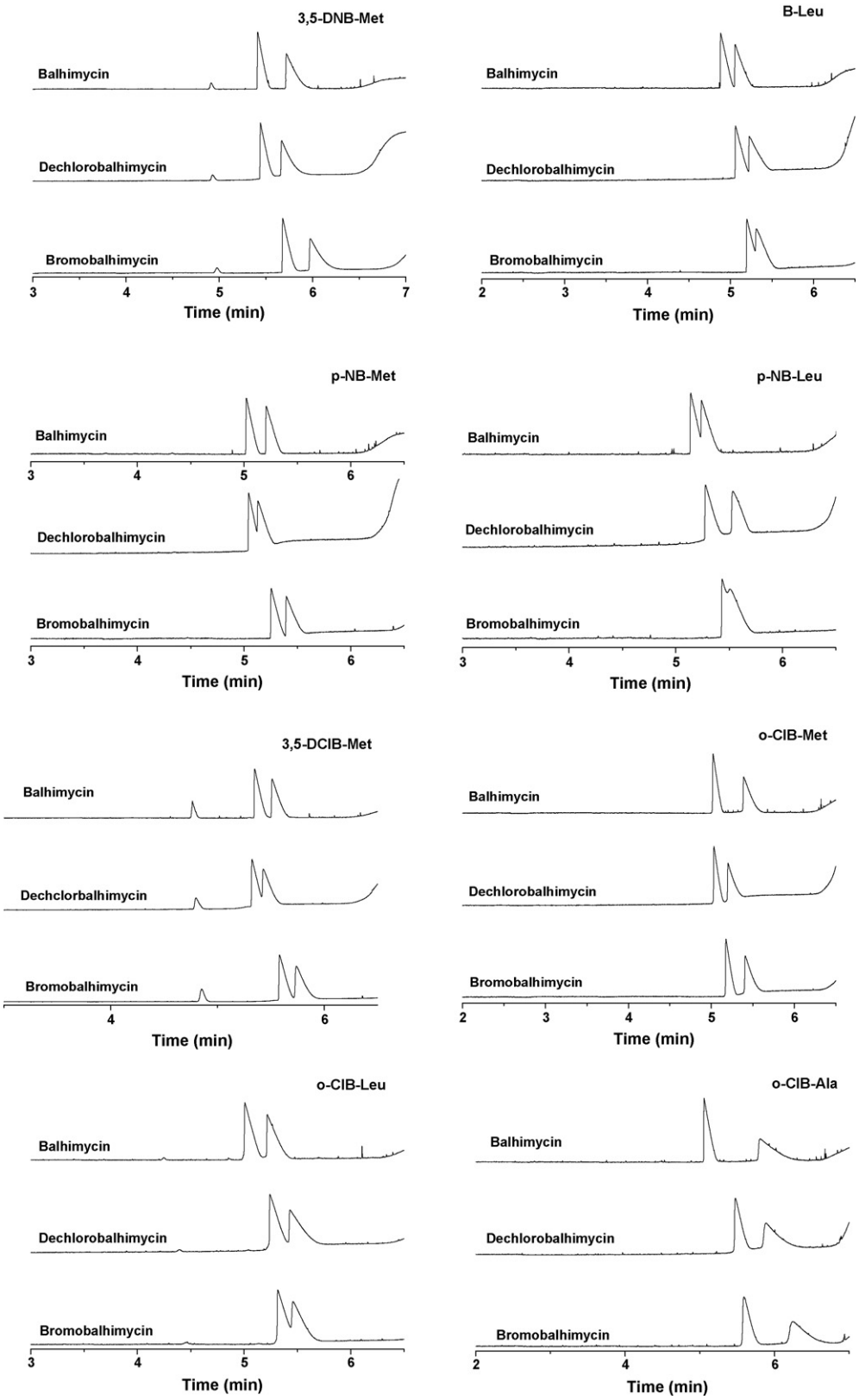

Fig. 6. Electropherograms for comparison of the enantioresolution ability between balhimycin (**CMA A**), bromobalhimycin (**CMA B**) and dechlorobalhimycin (**CMA C**). Conditions: 50 mM Tris–phosphate running buffer (pH 6.0) containing 0.001% (w/v) HDB; 4 mM chiral selector solution was partially filled with a pressure of 50 mbar for 70 s; other conditions as in Fig. 1.

Table 1
Comparison of the enantioresolution of *N*-benzoylated derivatives of amino acids with balhimycin, bromobalhimycin and dechlorobalhimycin.

Amino acid	Reagent	**CMA A**			**CMA B**			**CMA C**		
		t_1 (min)	t_2 (min)	R_s	t_1 (min)	t_2 (min)	R_s	t_1 (min)	t_2 (min)	R_s
Alanine#	B	4.783	4.783	0.00	4.913	4.913	0.00	4.899	4.899	0.00
	p-NB	5.153	5.227	0.75	5.677	5.757	/	5.536	5.536	0.00
	3,5-DNB	6.056	6.244	1.14	6.725	6.862	/	6.585	6.585	0.00
	2,4-DClB	5.268	5.354	/	5.752	5.752	0.00	5.639	5.639	0.00
	3,5-DClB	5.919	6.121	1.47	6.582	6.792	0.74	6.484	6.484	0.00
	m-ClB	5.594	6.002	2.08	6.202	6.556	1.56	5.959	6.118	0.78
	o-ClB	5.059	5.810	2.80	5.591	6.243	2.37	5.474	5.887	1.73
	p-ClB	5.174	5.271	0.56	5.754	5.754	0.00	5.760	5.760	0.00
Leucine#	B	4.882	5.058	1.18	5.196	5.312	0.66	5.060	5.227	0.97
	p-NB	5.139	5.244	0.82	5.434	5.508	/	5.279	5.534	1.62
	3,5-DNB	5.553	5.861	1.98	5.883	6.179	1.53	5.810	6.287	2.14
	2,4-DClB	5.174	5.174	0.00	5.519	5.519	0.00	5.268	5.401	0.57
	3,5-DClB	5.746	5.894	0.82	6.052	6.177	0.55	5.932	6.102	0.94
	m-ClB	5.373	5.620	1.49	5.540	5.688	0.85	5.566	5.822	1.17
	o-ClB	5.008	5.217	1.47	5.318	5.459	0.85	5.243	5.432	1.15
	p-ClB	5.057	5.190	0.99	5.528	5.612	0.51	5.396	5.396	0.00
Methionine#	B	4.761	4.937	1.09	5.001	5.139	0.81	4.844	4.940	0.53
	p-NB	5.021	5.208	1.67	5.254	5.397	1.16	5.043	5.130	0.72
	3,5-DNB	5.413	5.721	2.35	5.680	5.975	1.93	5.443	5.672	1.63
	3,5-DClB	5.350	5.511	1.52	5.584	5.743	1.24	5.324	5.434	0.95
	m-ClB	5.283	5.479	1.47	5.520	5.671	1.06	5.288	5.401	0.87
	o-ClB	5.020	5.392	3.03	5.176	5.410	1.90	5.032	5.201	1.46
	p-ClB	4.972	5.183	1.51	5.316	5.442	1.07	5.062	5.142	0.92
Threonine*	B	5.853	5.953	0.79	6.169	6.169	0.00	6.070	6.070	0.00
	p-NB	5.897	6.054	1.25	6.249	6.249	0.00	6.154	6.154	0.00
	3,5-DNB	6.912	7.136	2.02	7.235	7.400	1.72	7.145	7.236	1.03
	2,4-DClB	6.620	6.659	/	6.825	6.825	0.00	7.028	7.028	0.00
	3,5-DClB	6.855	6.972	0.79	7.193	7.193	0.00	7.027	7.027	0.00
	m-ClB	6.235	6.342	1.03	6.711	6.711	0.00	6.607	6.607	0.00
	o-ClB	6.016	6.057	/	6.143	6.143	0.00	6.077	6.077	0.00
	p-ClB	6.049	6.049	0.00	6.415	6.415	0.00	6.221	6.221	0.00

Conditions are the same as in Fig. 6.
CMA concentration: #4 mM and *8 mM, / R_s < 0.5.

of the cell wall mucopeptide precursor ALAA and hydrocarbon portions of this antibiotic has been reported and it played a considerably important role in the binding affinity of the carboxylate anion into antibiotic receptor pocket [29,51]. Recently, Jorgensen et al. also simulated the interaction between vancomycin analogues with peptides. It was reported that two methyl group from the Leu[1] side chain of vancomycin could form a hydrophobic clustering with hydrophobic groups from ligands [15]. This could provide an explanation for the differences in the enantiorecognition ability of the three chiral selectors **A**–**C** for *N*-benzoylated derivatives of leucine. The calculated log *D* values at pH 6.0 for balhimycin, bromobalhimycin and dechlorobalhimycin were −0.355, −0.055 and −1.781 using Chemiformatics Lipophilicity pH profile webtool Version 2.1. Therefore, it would be expected that dechlorobalhimycin provides weaker hydrophobic interactions compared to balhimycin and bromobalhimycin. Based on the molecular structure of balhimycin and the well-investigated complex formation of vancomycin-type glycopeptides with carboxylate-containing ligands [14,15,19,21,22,24,35,37], the side chain of the *L*-amino acids may be easier for interacting with the two methyl group from the Leu[1] side chain of vancomycin than that of *D*-enantiomer, which could have a negative impact on the enantiorecognition ability of the chiral selector. Due to the larger hydrophobicity of the side chain of Leucine, such a negative impact could be expected to be more significant and thus the dechlorobalhimycin exhibited better or even the best enantiorecognition ability among the three chiral selectors.

Notably the migration time of *N*-benzoylated amino acids, whose benzoyl aromatic ring was substituted with various electron-withdrawing groups (−NO_2 or −Cl), are longer than that for *N*-benzoyl amino acids. Such an observation could be rationalised by the decrease in the ratio of the charge to mass of the analytes. The pK_a values of all 31 *N*-benzoylated derivatives of amino acids were calculated using Chemiformatics pK_a webtool Version 10. The pK_a values of four *N*-benzoyl amino acids (methionine, leucine, alanine and threonine) are 4.25, 4.34, 4.09 and 3.61, respectively. When the *N*-tagged benzoyl moiety was substituted with different electron-withdrawing group (–Cl or –NO_2), the pK_a values of analytes are even lower than that of its relative *N*-benzoyl amino acid. Therefore, all *N*-benzoylated amino acids will carry one negative electrostatic charge at pH 6.0 and the charge-to-mass ratio is only correlated to the molecular weight of analytes. Compared to *N*-benzoyl amino acid, its relative aromatic substituted derivates has a larger molecular weight, and therefore a lower charge-to-mass ratio, lower electrophoresis mobility and thus a longer migration time. However, it was also observed that the position of the substitutes on the benzoyl moiety affects the migration time of the solute. The compound derivatised with a chlorine group at the meta-position on the benzoyl moiety exhibited a longer migration time than the ortho or para isomers. Similarly, the 3,5-DClB derivatives showed longer migration time than its 2,4-DClB derivatives. These observations clearly cannot be explained by the change in the ratio of the charge to mass of the analytes. Previously, Chen et al. reported π–π complexations between another macrocyclic glycopeptide selector teicoplanin and *N*-benzoyl amino acids [50]. The aromatic rings within teicoplanin are thought to be π-donors or π-basic and interact strongly with π-acidic groups, such as the electron-withdrawing groups represented by nitro or chloro substituents at aromatic ring. Therefore, the highly π-acidic 3,5-DNB derivatised compounds consistently resolved better than the benzoyl derivatives for the majority of compounds examined. Such π–π complexations may also explain the influence of the position

of the derivatised group on the benzoyl moiety on the migration time of *N*-benzoylated amino acids. Compounds derivatised with electron-withdrawing group chloro at the meta-position of the benzoyl group would be expected to have greater π–π interactions since the derivative at the meta position increases the π-acidity for compounds through resonance relative to the ortho- or para-position. Therefore, *m*-ClB derivatised compounds exhibited longer migration times than *o*-ClB or *p*-ClB derivatised compounds.

While the effect of the possible π–π interactions on the migration time of *N*-benzoylated derivatives of amino acids is recognised, the impact on the enantioresolution is still questionable. It is expected that as the π acidity of the *N*-benzoylated derivatives of amino acids increases, the enantioresolution improves if other factors remain constant. From Table 1, it was noted that the highly π-acidic 3,5-DNB derivatised amino acids consistently exhibited better enantioresolution than the underivatised benzoyl amino acids. For example, 3,5-DNB-Leu has superior enantioresolution (R_s, 1.98) than B-Leu (R_s, 1.18) with balhimycin as CMA. Similarly, the higher π-acidic 3,5-DClB derivatised amino acids also exhibited better enantioresolution than the relatively weaker π-acidic 2,4-DClB derivatised amino acids. However, the effect of the monochloro-substituted position (meta, para or ortho) on the benzoyl moiety on enantioresolution does not follow this rule. It is difficult to define a general trend across all four amino acids. As expected, the compound derivatised with a chlorine substituent at the meta-position on the benzoyl moiety (*m*-ClB) exhibited enhanced enantioresolution compared to the underivatised benzoyl amino acids. However, for alanine, methionine and leucine, the best enantioresolution among the three positional isomers of chlorobenzoyl amino acids are compounds derivatised with a chlorine atom in the ortho-position of the benzoyl moiety (*o*-ClB). This behaviour may due to the steric hindrance of the ortho-substituent.

4. Conclusions

Enantioseparations of a series of *N*-benzoylated derivatives of four amino acids (leucine, alanine, methionine and threonine) were compared using a CE method, which combined the partial filling technique with the dynamic coating technique and the co-EOF electrophoresis technique. Three vancomycin-type macrocyclic antibiotics, i.e. balhimycin (**A**), bromobalhimycin (**B**) and dechlorobalhimycin (**C**), were used as CMA. The displacement study using peptide ALAA as a competitive blocking ligand for the aglycone pocket proved that the *N*-benzoylated derivatives of amino acids have the same binding position on these chiral selectors and hydrogen bonding is the primary interaction responsible for the enantiorecognition. It was also found that the size of the side chain of the amino acids or tagging reagent has a great influence on enantioselectivity for compounds. Specifically, we suggested that derivatising the aromatic ring of the *N*-tagged benzoyl moiety could alter the π–π interaction and steric effect and thus the enantioresolution and migration time. Remarkably, several *N*-benzoylated derivatives of Leucine were best enantioseparated using dechlorobalhimycin, which showed the lowest enantiorecognition ability for *N*-dansylated amino acids and *N*-benzoylated derivatives of the other three amino acids tested. A possible hydrophobic clustering between two methyl groups of Leu[1] of chiral selector and the non-polar side chain of *N*-benzoylated derivatives of leucine was assumed to be responsible for this. In summary, the enantiorecognition mechanism of balhimycin and its analogues for *N*-benzoylated derivatives of amino acids involved "multimodal interactions". The possible secondary interactions, such as hydrophobic interaction, steric hindrance and π–π interaction, could play an important role in enantiorecognition process depends on the structure of the analytes. In order to confirm this hypothesis, molecular modelling and NMR experiments may be useful tools for further studies in the further.

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