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Monitoring the hydrophilicity/hydrophobicity of amino acid side-chains in the non-polar and polar faces of amphipathic α-helices by reversed-phase and hydrophilic interaction/cation-exchange chromatography

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Abstract

The ability to monitor precisely the hydrophobicity/hydrophilicity effects of amino acid substitutions in both the non-polar and polar faces of amphipathic α-helical peptides is critical in such areas as the rational de novo design of more effective antimicrobial peptides. The present study reports our initial results of employing the complementary separation modes of reversed-phase high-performance liquid chromatography (RP-HPLC) and hydrophilic interaction/cation-exchange chromatography (HILIC/CEX) to monitor the effect on apparent peptide hydrophilicity/hydrophobicity and amphipathicity of substituting single L- or D-amino acids into the centre of the non-polar or polar faces of a 26-residue biologically active amphipathic α-helical peptide, V_{681}. Our results clearly show that RP-HPLC and HILIC/CEX are best suited for resolving amphipathic peptides where substitutions are made in the non-polar and polar faces, respectively. Further, RP-HPLC and HILIC/CEX were demonstrated to be excellent monitors of hydrophilicity/hydrophobicity variations where amino acid substitutions were made in these respective faces. We believe these complementary high-performance modes offer excellent potential for rational design of novel amphipathic α-helical biologically active peptides.
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1. Introduction

The ever-increasing development of bacterial resistance to traditional antibiotics has precipitated an urgent requirement for new antibiotics possessing novel modes of action as well as different cellular targets compared to existing antibiotics in order to decrease the likelihood of development of resistance. Antimicrobial peptides may represent such a new class of antibiotics and their design and structure–activity relationships have become an area of active research in recent years [1,2]. From numerous studies on both natural and synthetic α-helical and β-sheet cationic antimicrobial peptides (so-called due to their possession of a net positive charge resulting from the presence of excess arginine and/or lysine residues), factors believed to be important for antimicrobial activity have been identified: the presence of both hydrophobic and basic residues, as well as a defined secondary structure (α-helix or β-sheet), either preformed or inducible, and an amphipathic nature, which segregates basic and hydrophobic residues to opposite sides of the molecule in lipid or lipid-mimicking environments [1–10]. This amphipathic structural feature is believed to play a critical role in the antimicrobial mechanism of action, with the hydrophilic (positively charged) domain of the peptide proposed to initiate peptide interaction with the negatively charged bacterial surface and the negatively charged head groups of bilayer phospholipids. The hydrophobic domain of the amphipathic peptide would then permit

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the peptide to enter the interior of the membrane [6,11–13]. Subsequent bilayer disruption or concomitant channel formation in, for example, the bacterial cytoplasmic membrane may lead to the leakage of cell contents and cell death [11–14].

For amphipathic α-helical peptides specifically, antimicrobial potency depends upon peptide amphipathicity, hydrophobicity, and helicity, such features only coming into play when the helical structure is induced in a hydrophobic environment such as a bacterial cell membrane [15] or, conversely, disrupted in an aqueous environment [16]. Clearly, the ability to monitor the hydrophilicity/hydrophobicity effects of amino acid substitutions in both the non-polar and polar faces of potentially useful antimicrobial amphipathic α-helical peptides is critical in the design process for such molecules. Reversed-phase high-performance liquid chromatography (RP-HPLC) has, in addition to being an effective separation tool for peptide separations, also proved to be a useful physico-chemical probe of peptide and protein structure [17–25]. The latter use includes the monitoring by RP-HPLC of the hydrophilicity/hydrophobicity of the non-polar face of amphipathic α-helical molecules, due to the interaction of this non-polar face (the preferred binding domain) with the hydrophobic stationary phase. In an analogous manner, we propose that mixed-mode hydrophilic interaction/cation-exchange chromatography (HILIC/CEX) may provide the required tool to monitor hydrophilicity/hydrophobicity of the polar face of amphipathic α-helical molecules, due to the interaction of this polar face with the hydrophilic/charged cation-exchange matrix. Although this novel high-performance mode was originally developed in the authors' laboratory strictly as a novel separation approach for peptide separations [27–33], subsequently adapted by other researchers for protein separations [34–38], we believe that HILIC/CEX can transcend its original development as simply a complementary separation technique to RP-HPLC and aid in the rational design of potentially valuable amphipathic α-helical antibiotics.

Thus, the present study represents the first report describing the employment of these complementary separation modes to monitor the effects on apparent peptide hydrophilicity/hydrophobicity and amphipathicity of substituting single L- or D-amino acids into the centre of the non-polar or polar faces of a 26-residue biologically active amphipathic α-helical peptide denoted V_{681} [39,40].

2. Experimental

2.1. Materials

HPLC-grade water was prepared by an E-pure water purification system from Barnstead International (Dubuque, IA, USA). Trifluoroacetic acid (TFA) was obtained from Halocarbon Products (River Edge, NJ, USA). Acetonitrile was obtained from EM Science (Gibbstown, NJ, USA). ACS-grade orthophosphoric acid and triethylamine (TEA) were obtained from Anachemia (Toronto, Canada). Sodium perchlorate was obtained from BDH (Poole, UK).

2.2. Analytical HPLC columns and instrumentation

RP-HPLC runs were carried out on a Zorbax SB300-C_8 column (150 mm × 2.1 mm i.d., 5 μm particle size; 300 Å pore size) from Agilent Technologies (Little Falls, DE, USA). Mixed-mode HILIC/CEX runs were carried out on a poly(2-sulfoethyl aspartamide)-silica (Poly-Sulfoethyl A) strong cation-exchange column (200 mm × 2.1 mm i.d., 5 μm, 300 Å) from PolyLC (Columbia, MD, USA).

All analytical HPLC runs were carried out on an Agilent 1100 Series liquid chromatograph.

2.3. HPLC run conditions

RP-HPLC: linear AB gradient elution (1% B/min) at a flow-rate of 0.3 ml/min, where eluent A is 0.05% aq. TFA, pH 2.0, and eluent B is 0.05% TFA in acetonitrile; temperature, 25 °C.

HILIC/CEX: linear AB gradient elution of 5 mM $NaClO_4$ to 250 mM $NaClO_4$ in 60 min at a flow-rate of 0.3 ml/min, where buffer A is 5 mM triethylammonium phosphate (TEAP), pH 4.5, containing 5 mM $NaClO_4$ and buffer B is 5 mM aq. TEAP, pH 4.5, containing 250 mM $NaClO_4$, both buffers containing 70% (v/v) acetonitrile at temperatures of 25 °C or 65 °C.

Samples injected onto the columns contained 5–10 nmol of each peptide. The gradient delay time for the HPLC system was 1.2 min. Samples were dissolved in the starting eluents for both HPLC modes. Peptide elution order was definitively established by spiking with individual peptides and individual injections. The reproducibility of the peptide separations in both HPLC modes was confirmed via duplicate (RP-HPLC) and triplicate (HILIC/CEX) runs.

2.4. Peptide synthesis and purification

Peptide synthesis was carried out by standard solid-phase synthesis methodology using N^{α}-*tert*-butyloxycarbonyl (*t*-Boc) chemistry on methylbenzhydrylamine (MBHA) resin (0.97 mmol/g) as described previously [16]. The crude peptides were purified by preparative RP-HPLC on an Applied Biosystems 400 solvent-delivery system connected to a 783A programmable absorbance detector. Amino acid analyses of purified peptides were carried out on a Beckman Model 6300 amino acid analyzer (Beckman Instruments, Fullerton, CA, USA) and the correct primary ion molecular masses of peptides were confirmed by mass spectrometry on an electrospray Mariner Biospectrometry Workstation (Applied Biosystems, Foster City, CA).

3. Results and discussion

3.1. RP-HPLC versus HILIC/CEX of amphipathic α-helical peptides

RP-HPLC has proven to be an ideal system for measuring hydrophobicity, amphipathicity, and association of α-helical and β-sheet peptides [7,16,20,21,29,30,41–51]. The non-polar face of, for example, an amphipathic α-helix represents a preferred binding domain for RP-HPLC, i.e., this face will bind preferentially to a reversed-phase hydrophobic stationary phase [17,18]. Indeed, Zhou et al. [17] clearly demonstrated that because of this preferred binding domain, amphipathic α-helical peptides are considerably more retentive than non-amphipathic peptides of the same amino acid composition. Thus, this preferential binding of the non-polar face of the amphipathic peptides to a reversed-phase matrix potentially makes RP-HPLC an effective monitor of the relative hydrophilicity/hydrophobicity of the non-polar face of such peptides. It should be noted that the potential for any non-specific, electrostatic interactions between negatively charged silanol groups on the hydrophobic stationary phase and positively charged lysine residues in the peptides should be negligible at the RP-HPLC run conditions used in this study (pH 2.0), where any such silanols will be protonated, i.e., neutral [52,53]. Indeed, application of peptide standards designed specifically to detect/monitor such non-ideal behaviour confirmed the column exhibited only ideal hydrophobic interaction behaviour under the run conditions employed [53].

The term hydrophilic interaction chromatography was originally introduced to describe separations based on solute hydrophilicity [54], with solutes being eluted from the HILIC column in order of increasing hydrophilicity, i.e., the opposite of RP-HPLC elution behaviour. Our laboratory subsequently took this concept a step further by taking advantage of the inherent hydrophilic character of ion-exchange, specifically strong cation-exchange, columns by subjecting peptide mixtures to linear salt gradients in the presence of high levels of organic modifier, specifically acetonitrile [27–33]. The presence of high levels of organic modifier not only suppresses any undesirable hydrophobic interactions between the peptides and the cation-exchange matrix [55], but also promotes desired hydrophilic interactions between the peptides and packing. Separations based on hydrophilicity are thus superimposed on top of those based on charge, resulting in mixed-mode HILIC/CEX, i.e., such an approach takes simultaneous advantage of both the charged character of peptides as well as any hydrophilic/hydrophobic properties they possess. Note that in an analogous manner to the non-polar face of an amphipathic α-helix representing a preferred binding domain for RP-HPLC, the hydrophilic face of the α-helix would represent a preferred binding domain for a hydrophilic stationary phase such as the strong cation-exchange matrix employed for HILIC/CEX in the present study. Evidence for such hydrophilic preferred binding domains has been reported previously by our laboratory for both amphipathic α-helical peptides [29] and cyclic amphipathic β-sheet peptides [30]. Hence, we believe that HILIC/CEX has excellent potential for monitoring the relative hydrophilicity/hydrophobicity of the polar face of such peptides.

We believe it is important to distinguish the difference in using ion-exchange stationary phases in a non-HILIC versus a true HILIC mode, such a distinction depending on the level of organic modifier in the run solvents. Thus, in (1), a non-HILIC separation, the presence of organic modifier may be simply required to eliminate non-specific hydrophobic interactions with the matrix, improve solubility of solutes being separated or perhaps enhance ionic interactions and hydrophilic effects to improve separation of some of the individual components of a solute mixture. Reports of non-HILIC mode separations in the presence of varying levels of organic solvent have been reported for various solutes including amino acids [56], peptides [57,58], proteins [59–61], and carbohydrates [62]. However, our definition of, (2), a separation carried out on an ion-exchanger in HILIC mode is the point at which the minimum organic modifier concentration required to reverse the solute elution order of a particular solute mixture relative to RP-HPLC is reached. This minimum concentration will depend on the nature and hydrophilicity/hydrophobicity of the solute components under consideration. Below this minimum concentration, hydrophilic effects may be present that affect resolution of just some of the mixture components. However, above this minimum concentration, the resolution of all sample components is affected, with higher concentrations potentially able to improve the separation still further. In fact, at very high concentrations of organic modifier, the HILIC mode is so dominant over the ion-exchange mode that a peptide with a greater net positive charge can be eluted prior to a lesser-charged peptide [28,32].

3.2. Synthetic peptide analogues based on V_{681}

V_{681} is a biologically active amphipathic α-helix with potent antimicrobial, as well as hemolytic, properties [39,40]. Such a peptide represents an excellent model to investigate the effects of introducing D-amino acids into the centre of its non-polar or polar face in an effort to modulate the hydrophilicity/hydrophobicity of the polar and non-polar faces of the peptide. The helix-disrupting properties of D-amino acids when substituted into an α-helix made up entirely of L-amino acids is well known [16,63–66]. Indeed, our laboratory determined a set of stereochemistry stability coefficients based on substitution of D-amino acids into an 18-residue amphipathic α-helix otherwise made up entirely of L-amino acids [16].

Fig. 1 shows the sequences of the synthetic peptides, based on the native V_{681}, with substitution positions at position 11 (denoted S11X peptides, where Ser11 is being substituted) in the hydrophilic face of the amphipathic α-helix or position 13 (denoted V13X peptides, where Val13 is being substituted) in the hydrophobic face of the amphipathic α-helix. For

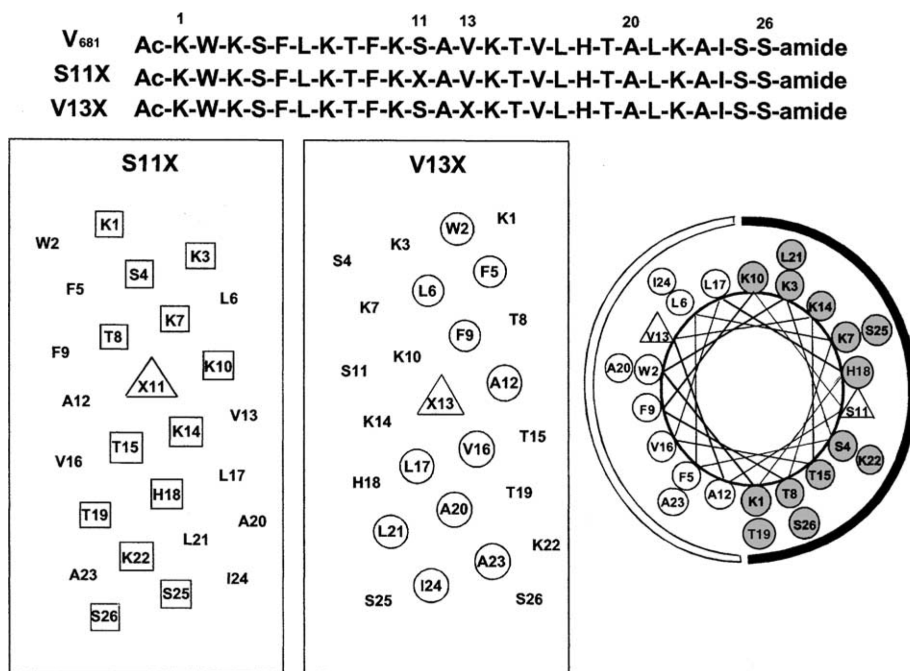

Fig. 1. Synthetic amphipathic α-helical peptides. Top: sequence of "native" peptide, denoted V_{681}, and sequence of peptide analogues, where X at position 11 (S11X series) or X at position 13 (V13X series) is substituted by L-Leu (analogue denoted L_L, etc. for other L analogues), D-Leu (analogue denoted L_D, etc. for other D-analogues), L-Val, D-Val, L-Ala, D-Ala, L-Ser, D-Ser, L-Lys, D-Lys or Gly (denoted G). Bottom left and middle: helical net representations of the peptide sequences, showing the hydrophilic face of the amphipathic α-helix of the S11X series and the hydrophobic face of the amphipathic α-helix of the V13X series; the substituted site at position 11 (S11X series) and position 13 (V13X series) is highlighted by a triangle. Bottom right: helical wheel representation of the model peptide sequences; the substituted sites at position 11 (S11X series) and position 13 (V13X series) are highlighted by triangles; residues in the hydrophilic face are shaded. The closed arc denotes the hydrophilic face; the open arc denotes the hydrophobic face. The helical wheel representation is based on the properties of the α-helix of 3.6 residues turn and that each residue will then appear 100° from the adjacent residue on the cross-section of the α-helix. Ac denotes N^{α}-acetyl and amide denotes C^{α}-amide.

the S11X peptides, the substitution position (denoted as X11 in the helical net and helical wheel presentations of Fig. 1) was chosen as being as central as possible in the hydrophilic face of the amphipathic α-helix, this face being comprised solely of polar residues, i.e., Thr and Ser residues (containing uncharged, polar side-chains) and Lys and His residues (containing basic, potentially positively charged side-chains). For the V13X peptides, the substitution position (denoted as X13 in the helical net and helical wheel presentations in Fig. 1) was chosen as being as central as possible in the hydrophobic face of the amphipathic α-helix, this face being comprised solely of non-polar residues, i.e., Ala (containing a small, slightly hydrophobic side-chain), Val (containing a larger, moderately hydrophobic side-chain), Leu and Ile (both containing bulky, strongly hydrophobic side-chains), and Phe and Trp (both containing aromatic, hydrophobic side-chains). Overall, the sizes of the polar and non-polar faces of the helix are essentially identical, enabling a good comparison of the effectiveness of HILIC/CEX and RP-HPLC, respectively, to monitor the hydrophilicity/hydrophobicity of these faces. In addition, these peptide analogues offer a concomitant opportunity to gauge the relative effectiveness of HILIC/CEX and RP-HPLC to separate amphipathic α-helical peptide analogues with substitutions made in the polar or non-polar faces of the amphipathic α-helix.

For the present initial study, L- and D-amino acids chosen for substitution at position 11 (polar face) or position 13 (non-polar face) of the peptide sequence (Fig. 1) represented a range of side-chain properties: the three non-polar residues, Ala, Val, and Leu contain side-chains of increasing size and hydrophobicity (A_L, $A_D < V_L$, $V_D < L_L$, and L_D); Ser (S_L, S_D) contains a small, polar (i.e., hydrophilic) side-chain; and Lys (K_L, K_D) contains a positively charged side-chain. Finally, the V_{681} analogues substituted with Gly at positions 11 or 13 (denoted G) represent the situations where no side-chain is present at the centre of the hydrophilic (position 11) or hydrophobic (position 13) faces of the helix.

Clearly, in order for both RP-HPLC and HILIC/CEX to be effective as monitors of hydrophilicity/hydrophobicity of the two faces of amphipathic α-helices, it is important that (1) the peptides under consideration must have a high potential to form α-helices; (2) this secondary structure must be present under the HPLC run conditions employed; and (3) the peptides must be eluted as single-stranded monomeric α-helices. V_{681} is known to have a high potential to form an α-helix [39,40], as determined by circular dichroism spectroscopy. The α-helix-inducing properties of trifluoroethanol (TFE) are well documented [67,68]. Indeed, Monera et al. [69], when carrying out temperature denaturation of α-helices in the presence of just 30% TFE, demonstrated that TFE not

only induces α-helical structure but also stabilizes it. Chen et al. [16] demonstrated that, even where helix disrupting D-amino acids are substituted into α-helical peptides, high helicity (generally comparable to their L-amino acid substituted analogues) may still be attained in the presence of 50% TFE. This was also the case in the present study, with high helicities for all L- and D-peptide analogues being obtained in 50% (v/v) TFE (data not shown).

Since all the peptides with L- or D- substitutions are maximally induced into their α-helical conformation (with the exception of Pro) in the presence of a hydrophobic environment, CD spectroscopy cannot be used to measure the α-helix disrupting properties of the substitutions based on α-helical structure.

It is also well documented that non-polar solvents and hydrophobic matrices characteristic of RP-HPLC both induce and stabilize α-helical structure [17,22–24,26]. For instance, a classic example reported by Blondelle et al. [22,24] demonstrated an excellent correlation between the CD ellipticities of peptides bound to a set of C_{18}-coated quartz plates and their RP-HPLC retention times. Indeed there is no evidence that the hydrophobic matrix characteristic of RP-HPLC destabilizes α-helical structure, quite the opposite, in fact. In addition, the ability of acetonitrile, the organic modifier traditionally employed for the majority of peptide separations by RP-HPLC [70], to induce α-helical structure in potentially helical molecules has also been demonstrated [71,72].

Excellent examples of the disruption of any tertiary/quaternary structure of amphipathic α-helical peptides by organic modifiers has been clearly demonstrated by size-exclusion chromatography of model amphipathic α-helical coiled-coil peptides by Lau et al. [72] and Mant et al. [73]. Coupled with similar disruption of such higher levels of peptide structure, or, indeed, any potential for peptide aggregation, by hydrophobic stationary phases [41,71], the peptides used in the present study can be confidently expected to be eluted as single-stranded amphipathic α-helices during RP-HPLC.

In a manner similar to RP-HPLC, under characteristic conditions of HILIC/CEX (high acetonitrile concentration in the mobile phase; 70% (v/v) in the present study), the peptide analogues would also be expected to be α-helical, allowing interaction of the hydrophilic face with the ion-exchange matrix [29]. Finally, it should be noted that the substitution sites at position 11 (hydrophilic face) and position 13 (hydrophobic face) of the peptides (Fig. 1) ensures intimate interaction of the substituting side-chain with the ion-exchange or reversed-phase stationary phase, respectively; concomitantly, this is designed to maximize any observed effects on HILIC/CEX or RP-HPLC retention behaviour, respectively, when substituting different residues at these sites.

3.3. RP-HPLC of amphipathic α-helical peptide analogues of V_{681}

Fig. 2 shows the reversed-phase elution profiles of mixtures of the L-amino acid substituted analogues (Fig. 2A and

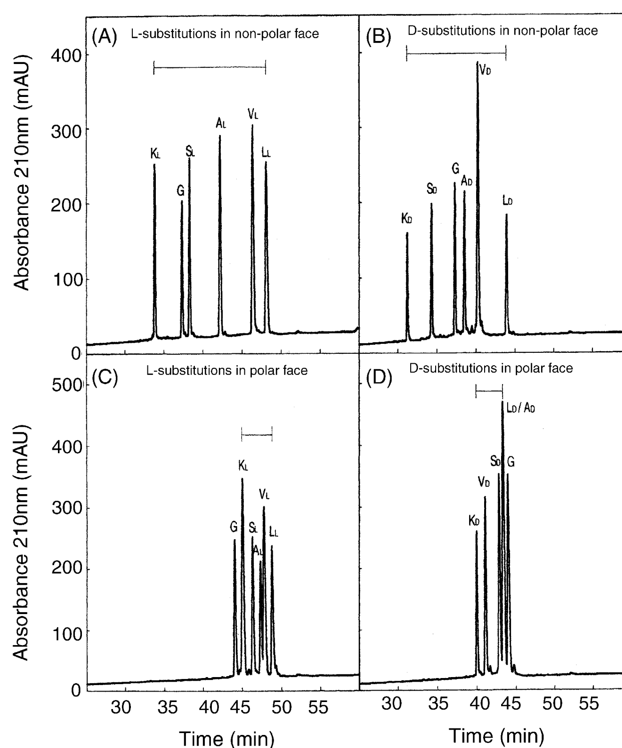

Fig. 2. RP-HPLC of amphipathic α-helical peptides. Column: reversed-phase Zorbax SB300-C_8 (150 mm × 2.1 mm i.d.); see Section 2. Conditions: linear AB gradient (1% B/min) at a flow-rate of 0.3 ml/min, where eluent A is 0.05% aq. TFA, pH 2.0, and eluent B is 0.05% TFA in acetonitrile; temperature, 25 °C. Bars denote elution ranges for the five L- or D-amino acid substituted peptides. The sequences of the peptides are shown in Fig. 1.

C) and D-amino acid substituted analogues (Fig. 2B and D) when substitutions were made in the non-polar face (Fig. 2A and B) or polar face (Fig. 2C and D) of the amphipathic α-helix. Clearly, for both the L- and D-amino acid substituted peptides, the peptide mixtures are better separated when substitutions are made in the non-polar face (the preferred binding domain for RP-HPLC; Fig. 2A and 2B, respectively) compared to the polar face (Fig. 2C and 2D, respectively). Thus, for L-substitutions in the non-polar (Fig. 2A) and polar (Fig. 2C) faces, the elution ranges for the first and last eluted L- or D-peptides (i.e., K_L to L_L) were 14.3 min and 3.8 min, respectively; for D-substitutions in the non-polar (Fig. 2B) and polar (Fig. 2D) faces, these values (i.e., K_D to L_D) were 12.7 min and 3.4 min, respectively. In addition, the D-substituted analogues were consistently eluted faster than their L-amino acid counterparts when substituted into either the polar (Table 1; Fig. 2) or non-polar (Table 2; Fig. 2) faces of the α-helix, despite the fact that each L-/D-substituted peptide pair has the same inherent hydrophilicity/hydrophobicity. This observation can be rationalized as being due to disruption of the amphipathic α-helix following introduction of the D-amino acid [16,63–66]. The overall effect on the non-polar face would be a decrease in the apparent hydrophobicity of this face when the helix is substituted (on either face) with a D-amino acid compared to

Table 1
RP-HPLC and HILIC/CEX retention data

Amino acid substitution in polar peptide face[a]

Amino acid[b] substitution	RP-HPLC (t_R, min)[c]		Δt_R (D–L)[d] (min)	HILIC/CEX (t_R, min)[c]		Δt_R (D–L)[d] (min)
	$S11X_L$	$S11X_D$		$S11X_L$	$S11X_D$	
L	48.8	43.4	−5.4	39.4	36.8	−2.6
V	47.8	41.1	−6.7	40.4	37.4	−3.0
A	47.3	43.4	−3.9	43.1	41.3	−1.8
S	46.3[e]	42.9	−3.4	45.5[e] [e]	42.9	−2.5
K	45.0	40.0	−5.0	58.6	54.7	−3.9

[a] Denotes that amino acid substitutions are made in the polar face (S11X series) of amphipathic α-helical peptides shown in Fig. 1; X_L and X_D denote L- or D-amino acids are substituted at position X of this peptide series.
[b] Denotes substitution of L-Ser with L-Leu, D-Leu, L-Val, D-Val, L-Ala, D-Ala, L-Ser, D-Ser, L-Lys or D-Lys.
[c] Retention times of RP-HPLC and HILIC/CEX runs taken from Figs. 2 and 3, respectively.
[d] $\Delta t_R = t_R$, L-substituted analogue minus t_R, D-substituted analogues.
[e] t_R values for native peptide which has three denotions: $V_{681} = S11S_L = V13V_L$.

its L-diastereomer and, hence, a decrease in retention time of the former compared to the latter. From Tables 1 and 2, subtraction of the RP-HPLC retention time of the D-amino acid substituted analogues from their L-amino acid substituted counterparts produces a retention time difference (Δt_R) representing either the disruption of the polar face (Table 1; S11X series) or non-polar face (Table 2; V13X series) by substitution of D-amino acids into these respective faces of the amphipathic α-helix.

From Fig. 2, peptides were eluted in the order of increasing hydrophobicity of the substituted side-chain (K < S < A < V < L [74]) in the non-polar face for both the L and D analogues, i.e., the elution order for the two peptide mixtures was the same, except for the relative position of the Gly–analogue (eluted between K_L and S_L in Fig. 2A and between S_D and A_D in Fig. 2B). Also from Fig. 2C, the elution order of the L–analogues is again in order of increasing hydrophobicity of the side-chain substituted into the polar face of the α-helix ($K_L < S_L < A_L < V_L < L_L$), with G now eluted prior to K_L. However, this elution order was not observed for the analogues with D-amino acids substituted into the polar face of the amphipathic α-helix (Fig. 2D), where A_D and L_D are co-eluted and V_D, with a hydrophobic side-chain, is eluted prior to S_D, which contains a polar, hydrophilic side-chain. Such an observation is likely due to the varying magnitude of disruption of the preferred non-polar binding domain of the peptide helix when different D-amino acids are substituted into the sequence, i.e., different D-amino acids disrupt the non-polar face to different extents, resulting in the RP-HPLC elution order shown in Fig. 2D. It is interesting to note that the elution of V_D significantly prior to S_D reflects the observation by Chen et al. [16] that amino acids, such as Val, with β-branched side-chains showed the greatest reduction in apparent side-chain hydrophobicity due to D-amino acid substitutions into the centre of the non-polar face of an amphipathic α-helix. Similarly, when substituted into the centre of the non-polar face of V_{681} in the present study, the presence of D-Val was the most disruptive of the hydrophobic preferred binding domain of the α-helix as measured by RP-HPLC (Δt_R, $V_D - V_L$ = −6.1 min) relative to other D-substituted analogues (Table 2). Interestingly, when D-Val is substituted in the centre of the polar face of V_{681}, it remains the most disruptive D-amino

Table 2
RP-HPLC and HILIC/CEX retention data

Amino acid substitution in non-polar peptide face[a]

Amino acid[b] substitution	RP-HPLC (t_R, min)[c]		Δt_R (D–L)[d] (min)	HILIC/CEX (t_R, min)[c]		Δt_R (D–L)[d] (min)
	$V13X_L$	$V13X_D$		$V13X_L$	$V13X_D$	
L	48.1	43.9	−4.2	45.5	42.0	−3.5
V	46.3[e]	40.2	−6.1	45.4[e] [e]	40.2	−5.3
A	42.4	38.5	−3.9	44.3	41.5	−2.8
S	38.3	34.3	−4.0	44.3	42.0	−2.3
K	33.8	31.2	−2.6	49.5	49.0	−0.5

[a] Denotes that amino acid substitutions are made in the non-polar face (V13X series) of amphipathic α-helical peptides shown in Fig. 1; X_L and X_D denote L- or D-amino acids are substituted at position X of this peptide series.
[b] Denotes substitution of L-Val with L-Leu, D-Leu, L-Val, D-Val, L-Ala, D-Ala, L-Ser, D-Ser, L-Lys or D-Lys.
[c] Retention times of RP-HPLC and HILIC/CEX runs taken from Figs. 2 and 3, respectively.
[d] $\Delta t_R = t_R$, L-substituted analogue minus t_R, D-substituted analogues.
[e] t_R values for native peptide which has three denotions: $V_{681} = S11S_L = V13V_L$.

acid with a Δt_R value even larger than that on the non-polar face (Δt_R, $V_D - V_L = -6.7$ min) (Table 1). This suggests that D-Val can disrupt the α-helix better on the polar face than on the non-polar face. This was also observed for D-Leu (Δt_R of −5.4 min on the polar face versus −4.2 min on the non-polar face) and D-Lys (Δt_R of −5.0 min on the polar face versus −2.6 min on the non-polar face) (Table 1 and Table 2). D-Ser was the only example where the α-helix disruption was better on the non-polar face (Δt_R of −4.0 min on the non-polar face versus −3.4 min on the polar face) (Tables 1 and 2).

Non-polar face substitutions are replacing L-Val at position 13. Since, L-Ala, L-Ser, and L-Lys are all more hydrophilic than L-Val, it would be expected that such substitutions would decrease peptide retention time as observed (L-Val, t_R = 46.3 min; L-Ala, 42.4 min; L-Ser, 38.3 min; and L-Lys, 33.8 min). Similarly, L-Leu is more hydrophobic than L-Val and retention time increases to 48.1 min. For all polar face substitutions, the non-polar face remains the same, i.e., with Val at position 13 (Fig. 1) and polar face substitutions are replacing L-Ser at position 11. Since L-Ala, L-Val, and L-Leu are more hydrophobic than L-Ser, it would be expected that such substitutions would increase peptide retention time (as, indeed, was observed in the elution order of Fig. 2C), even though they do not involve the preferred binding domain for RP-HPLC. Note that a L-Val to L-Leu substitution on the polar face increases retention time and hydrophobicity by 1.0 min (Table 1), whereas the same substitution on the non-polar face increases retention time and hydrophobicity by 1.8 min (Table 2). This, almost doubling of the effect of the addition of a single CH_2 group, shows that hydrophobicity is more easily affected by substitution in the non-polar face, i.e., the preferred binding domain for RP-HPLC. Further, when you increase hydrophobicity on the polar face from L-Ser to L-Ala, L-Val, and L-Leu, the overall hydrophobicity of the peptide, as measured by RP-HPLC, is 46.3, 47.3, 47.8, and 48.8 min, respectively, i.e., L-Leu increases overall hydrophobicity by 2.5 min relative to L-Ser (Table 1). By comparison, the same change on the non-polar face accounts for a significantly greater effect where L-Leu increases overall hydrophobicity by 9.8 min relative to L-Ser (Table 2).

It is also worth noting the elution behaviour of K_L relative to analogue G when L-Lys is substituted into the non-polar face of the α-helix (Fig. 2A) compared to the polar face (Fig. 2C). It has been clearly shown in model random coil peptides [74] that substitution of a Gly residue by a positively charged Lys residue leads to a significant decrease in peptide retention time during RP-HPLC at pH 2.0, exactly as observed in Fig. 2A for the amphipathic peptides of the present study. However, from Fig. 2C, K_L is eluted after the G analogue when L-Lys is substituted into the polar face of the α-helix. Such an observation is likely due to the L-Lys being in the centre of the hydrophilic face of the amphipathic α-helix, i.e., on the opposite side of the hydrophobic preferred binding domain for RP-HPLC. Although, the positively charged Lys side-chain still affects peptide retention behaviour (note its elution in Fig. 2C prior to A_L, V_L, and L_L containing non-polar groups at the substitution site), this effect is likely diminished compared to the situation where L-Lys is substituted into the centre of the non-polar face of the helix and is, therefore, able to interact to a greater extent with the hydrophobic stationary phase. Indeed, the RP-HPLC retention behaviour of all of the polar face-substituted analogues (with identical non-polar faces) relative to their non-polar face counterparts, in terms of the much narrower elution range of the former, as well as the severely diminished contribution to apparent peptide hydrophobicity of even hydrophobic side-chains, such as Leu and Val, when substituted in the polar face of the helix, clearly underlines the presence of a preferred binding domain for RP-HPLC and represented by the non-polar face of the amphipathic α-helix. A similar overall trend can also be seen for the D-substituted analogues, albeit interpretation is complicated somewhat by the helix-disrupting properties of D-amino acids (note, for example, the slight reduction in retention time for L_D when D-Leu is substituted in the polar face (Fig. 2D; Table 1) compared to the non-polar face (Fig. 2B; Table 2).

3.4. *HILIC/CEX of amphipathic α-helical peptide analogues of V_{681}*

Fig. 3 shows the HILIC/CEX profiles of mixtures of the L-amino acid substituted analogues (Fig. 3A and C) and D-amino acid substituted analogues (Fig. 3B and D) when substitutions were made in the polar face (Fig. 3A and B) or non-polar face (Fig. 3C and D) of the amphipathic α-helix. From Fig. 3, for both the L- and D-amino acid substituted peptides, the peptide mixtures are better separated when substitutions are made in the polar face of the α-helix (the preferred binding domain for HILIC/CEX) compared to the non-polar face. Thus, for L-substitutions in the polar (Fig. 3A; Table 1) and non-polar (Fig. 3C; Table 2) faces, the elution range for the first eluted and last eluted L-substituted peptides were 19.2 (L_L to K_L) and 5.2 min (S_L to K_L), respectively; for D-substitutions in the polar (Fig. 3B; Table 1) and non-polar (Fig. 3D; Table 2) faces, these values were 17.9 (L_D to K_D) and 8.8 min (V_D to K_D), respectively. The Lys analogues, due to their extra positive charge, are clearly well separated from the remainder of the peptides in the mixtures. However, even if one were to exclude these analogues from the elution range comparison, i.e., only compare peptides of identical net positive charge, the superior separation of the analogues with substitutions in the polar face of the α-helix is still clear: for L-substitutions in the polar (Fig. 3A; Table 1) and non-polar (Fig. 3C; Table 2) faces, the elution ranges were 6.0 (L_L to S_L) and 1.2 min (L_L to S_L), respectively; for D-substitutions in the polar (Fig. 3B; Table 1) and non-polar (Fig. 3D; Table 2) faces, these values were 6.1 (L_D to S_D) and 1.8 min (L_D to V_D), respectively. Concomitant with this larger elution range for peptide analogues substituted in their polar faces (Fig. 3A and B) is improved peptide resolution compared to the analogues with substitutions made in their non-polar faces (Fig. 3C and D). Thus, for both L- (Fig. 3A)

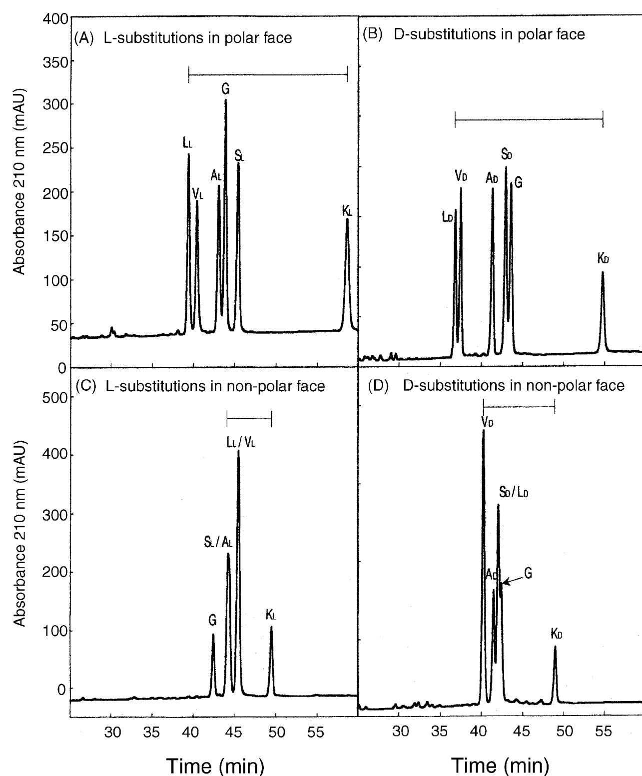

Fig. 3. HILIC/CEX of amphipathic α-helical peptides. Column: strong cation-exchange Poly-Sulfoethyl A (200 mm × 2.1 mm i.d.); see Section 2. Conditions: linear AB gradient (5 mM $NaClO_4$ to 250 mM $NaClO_4$ in 60 min) at a flow-rate of 0.3 ml/min, where buffer A is 5 mM aq. triethylammonium phosphate (TEAP), pH 4.5, containing 5 mM $NaClO_4$ and buffer B is 5 mM aq. TEAP, pH 4.5, containing 250 mM $NaClO_4$, both buffers also containing 70% (v/v) acetonitrile; temperature, 65 °C. Bars denote elution ranges for the five L- or D-amino acid substituted peptides. The sequences of the peptides are shown in Fig. 1.

and D- (Fig. 3B) amino acid substitutions in the polar face, all six peptides were satisfactorily separated. In contrast, for L-substitutions in the non-polar face (Fig. 3C), S_L and A_L were coeluted as were L_L and V_L; similarly, for D-substitutions in the non-polar face (Fig. 3D), S_D and L_D were coeluted, these coeluted peptides also being only poorly resolved from A_D and G.

From Fig. 3 and Tables 1 and 2, in a similar manner to the RP-HPLC results (Fig. 2; Tables 1 and 2), the D-substituted analogues were again consistently eluted faster than their corresponding diastereomers in HILIC/CEX. As noted previously for the RP-HPLC results, this pattern of earlier elution for the D-substituted analogues is likely due to disruption of the preferred binding domain (in this case, the hydrophilic preferred binding domain represented by the polar face of the amphipathic α-helix) by a D-amino acid substitution into the centre of either the polar or non-polar face of the helix. From Tables 1 and 2, in a similar manner to the RP-HPLC results, subtraction of the HILIC/CEX retention time of the D-amino acid substituted analogues from their L-amino acid substituted counterparts again produces Δt_R values representing either the disruption of the polar face (S11X series; Table 1) or non-polar face (V13X series; Table 2) by substitution of D-amino acids into these respective faces of the amphipathic α-helix. The D-amino acid that was most disruptive to the hydrophilic preferred binding domain of the α-helix as measured by HILIC/CEX was D-Val substituted on the non-polar face (Δt_R, $V_L - V_D = -5.3$ min; Table 2) and D-Lys substituted on the polar face (Δt_R, $K_L - K_D = -3.9$ min; Table 1). This is in contrast with the RP-HPLC results where D-Val was most disruptive of the non-polar preferred binding domain whether the substitution was on the polar or non-polar face. HILIC/CEX could be more sensitive to the D-Lys substitution on the polar face because of the mixed mode effects. The lysine residue introduces an additional charge, which affects both ion-exchange and hydrophilic interactions. Interestingly, D-Val was the second best substitution for disruption of the polar preferred binding domain when made on the polar face (Table 1).

Also from Fig. 3, peptides are eluted in the order of increasing hydrophilicity of the substituted amino acid side-chain in the polar face for both the L- (Fig. 3A) and D- (Fig. 3B) analogues, i.e., L < V < A < S < K. There is no clear pattern to the elution order of the peptides when substitutions are made in the non-polar face (Fig. 3C and D). In particular, the elution order of the D-substituted analogues is likely influenced by the degree of disruption of the polar face by different D-amino acid substitutions in the non-polar face.

Figs. 4 and 5 now illustrate the effect of temperature on HILIC/CEX of peptide analogues with L-substitutions in the polar face (Fig. 4) or the non-polar face (Fig. 5). From Fig. 4, the effect of rise in temperature from 25 to 65 °C produced a dramatic improvement in the resolution of the L-substituted analogues, as well as a significant increase in retention time. A_L and G, in particular, illustrate this improvement, being coeluted at 25 °C and resolved to baseline at 65 °C. The greater the increase in retention time of K_L (with an extra positive charge) suggests that the more hydrophilic the substituted amino acid, the greater the effect of temperature on peptide retention time in HILIC/CEX, reflecting earlier observations by this laboratory [33]. From Fig. 5, an improvement in resolution on raising the temperature from 25 to 65 °C can also be seen for the peptides where L-substitutions were made in the non-polar face, albeit not as dramatic as seen for the peptides substituted in the polar face (Fig. 4). Retention times have also again increased. The major effect of raising the temperature was to improve the resolution of the coeluted peptide pairs of S_L/A_L and L_L/V_L.

Although not shown here, raising the temperature from 25 to 65 °C for RP-HPLC of these L-substituted peptides did not significantly improve the separation of the peptide mixtures achieved at the lower temperature (Fig. 2A and C), particularly the already excellent resolution of the analogues with L-substitutions made in the non-polar face of the α-helix (Fig. 2A). The major effect was a decrease in retention times of all peptides in the two mixtures. Thus, we believed that the similar retention time range of both the L- and D-substituted analogues at 25 °C for RP-HPLC and 65°C for HILIC/CEX

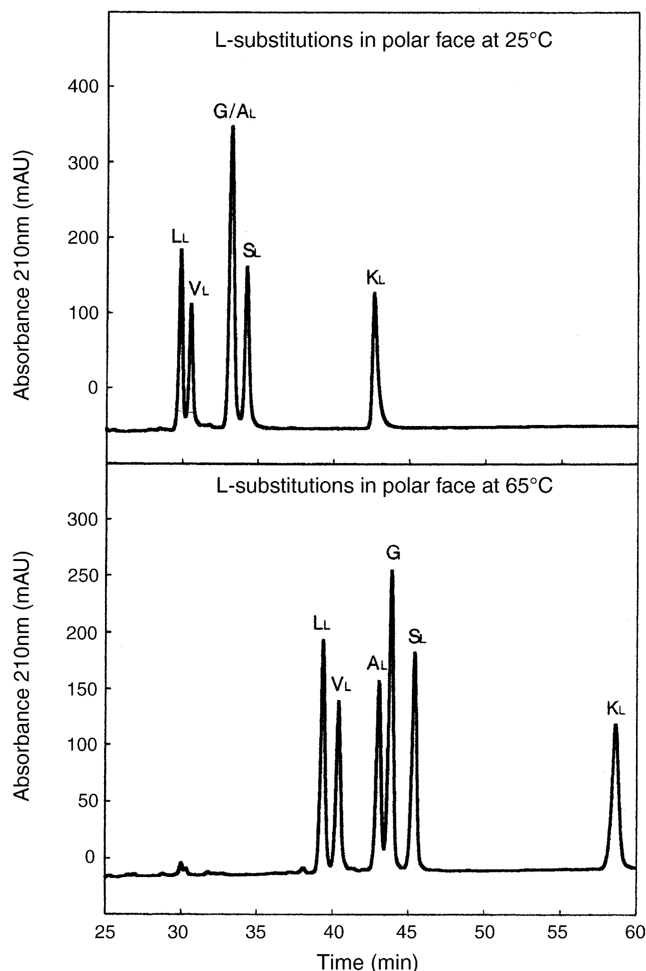

Fig. 4. Effect of temperature on HILIC/CEX of amphipathic α-helical peptides, where substitutions are made in the polar face of the α-helix. Column: see Fig. 3. Conditions: same as Fig. 3 except temperature is now 25 °C (top panel) or 65 °C (bottom panel). The sequences of the peptides (SX11 series) are shown in Fig. 1.

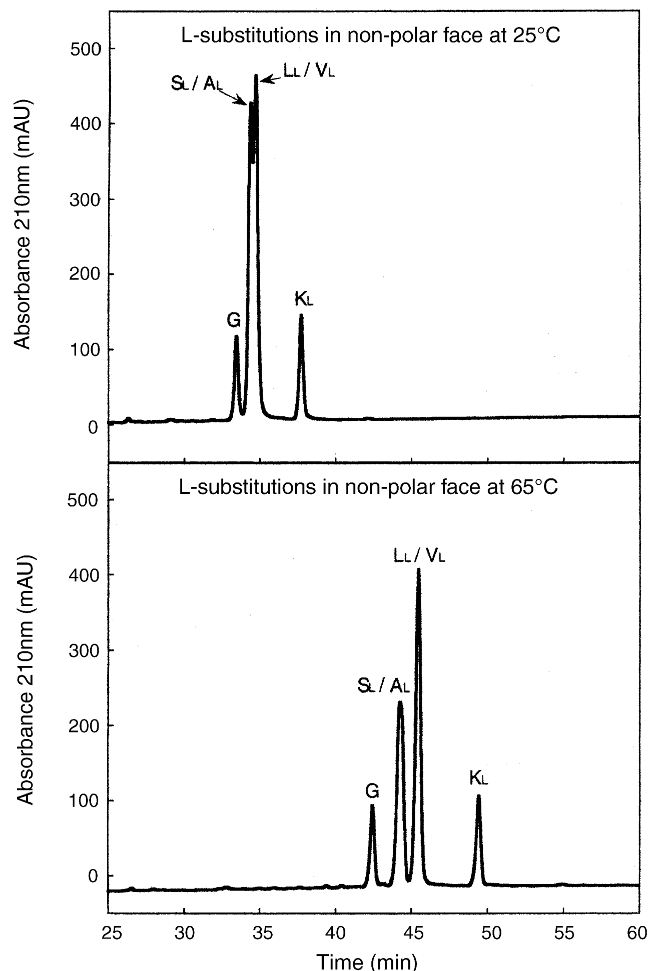

Fig. 5. Effect of temperature on HILIC/CEX of amphipathic α-helical peptides, where substitutions are made in the non-polar face of the α-helix. Column: see Fig. 3. Conditions: same as Fig. 3 except temperature is now 25 °C (top panel) or 65 °C (bottom panel). The sequences of the peptides (VX13 series) are shown in Fig. 1.

(as well as the clear advantage of employing the higher temperature for HILIC/CEX; Figs. 4 and 5) allowed a more valid comparison of the effectiveness of these two HPLC modes both for resolution of peptide mixtures and as monitors of hydrophilicity/hydrophobicity of the non-polar and polar faces, respectively, of amphipathic α-helical peptides.

3.5. RP-HPLC and HILIC/CEX as monitors of hydrophilicity/hydrophobicity of amphipathic α-helical peptides

Table 3 summarizes the effect of amino acid substitutions on the retention behaviour of the native peptide, V_{681} (also denoted as $S11S_L$ and $V13V_L$). Of interest here is the relative ranges of Δt_R values as determined by RP-HPLC or HILIC/CEX when substitutions are made in the polar face ($S11S_L$ series) or non-polar face ($V13V_L$) of V_{681}. Thus, when substitutions are made in the polar face, the Δt_R range as measured by RP-HPLC is just 8.8 min compared to 21.8 min for HILIC/CEX, highlighting the greater sensitivity of the latter HPLC mode for monitoring substitutions made in the polar face. In contrast, RP-HPLC (Δt_R range = 16.9 min) is clearly more sensitive to changes made in the non-polar face compared to HILIC/CEX (Δt_R range = 9.3 min). Such results again underline the complementary nature of these two HPLC modes.

From Fig. 6, this opposing, if complementary, nature of RP-HPLC and HILIC/CEX is quite clear. Thus, with amino acid substitutions (whether L- or D-amino acids) made in the non-polar face of the amphipathic α-helix, peptides are eluted during RP-HPLC in order of increasing hydrophobicity of the non-polar preferred binding domain. In contrast, with substitutions made in the polar face of the α-helix (whether L- or D-amino acids), peptides are eluted during HILIC/CEX in order of increasing hydrophilicity (decreasing hydrophobicity) of the polar preferred binding domain. The results shown in Fig. 6 indicate that RP-HPLC and HILIC/CEX are, indeed, potentially useful monitors of hydrophilicity/hydrophobicity

Table 3
Effects of amino acid substitutions on the retention behaviour of peptide V_{681}

Amino acid[a] substitution		RP-HPLC[b] Δt_R (min)		HILIC/CEX[b] Δt_R (min)
S11 S_L[c] → L_L		+2.5		−6.0
S_L → V_L		+1.5		−5.0
S_L → A_L		+1.0		−2.3
S_L → K_L		−1.3		+13.2
S_L → L_D	Range 8.8 min	−2.9	Range 21.8 min	−8.6
S_L → V_D		−5.2		−8.0
S_L → A_D		−2.9		−4.1
S_L → S_D		−3.4		−2.5
S_L → K_D		−6.3		+9.3
V13 V_L[c] → L_L		+1.8		0
V_L → A_L		−3.9		−1.2
V_L → S_L		−8.0		−1.2
V_L → K_L		−12.5		+4.0
V_L → L_D	Range 16.9 min	−2.4	Range 9.3 min	−3.5
V_L → V_D		−6.1		−5.3
V_L → A_D		−7.8		−4.0
V_L → S_D		−12.0		−3.5
V_L → K_D		−15.1		+3.5

[a] Denotes that amino acid substitutions are made in the polar face (S11X series) or non-polar face (V13X series) of amphipathic α-helical peptides shown in Fig. 1.

[b] Denotes change in retention time when an amino acid substitution is made in the polar face (e.g., t_R of S11S_L minus t_R of S11L_L = +2.5 and −6.0 min in RP-HPLC and HILIC/CEX, respectively) or non-polar face (e.g., t_R of V13V_L minus t_R of V13L_L = +1.8 and 0 min in RP-HPLC and HILIC/CEX, respectively) of the amphipathic α-helical peptides shown in Fig. 1.

[c] Note that denotions S11S_L and V13V_L both represent the native V_{681} peptide.

of the non-polar and polar faces, respectively, of an amphipathic α-helical peptide.

Such potential is highlighted in Table 4, which compares the relative efficacy of RP-HPLC versus HILIC/CEX for monitoring hydrophilicity/hydrophobicity changes when substituting L-Ser with L-Ala, L-Val, L-Leu or L-Lys in the polar or non-polar face of V_{681}. L-Substituted amino acids were chosen since replacement of an L-amino acid with another L-amino acid will have the least effect on helix conformation. In contrast, replacement of an L-amino acid with a D-amino acid will cause disruption of both the polar and non-polar faces of the helix, no matter which face the substitution is made, the extent of such disruption being dependent on the particular D-amino acid [16]. In addition, the Gly analogues were not included in Table 4 since glycine (which is also neither an L- or a D-amino acid) is a known α-helix disrupter, second only to proline in its helix-disruptive characteristics [75]. Thus, Table 4 represents the most valid demonstration of the potential effectiveness of RP-HPLC and HILIC/CEX for monitoring changes in the non-polar and polar faces, respectively, of V_{681} through L-amino acid substitutions, i.e., negligible effect on α-helix structure and a systematic increase in inherent hydrophobicity of either face through substitution of the polar uncharged Ser with non-polar side-chains (Ala < Val < Leu) [72] or a large increase in hydrophilicity through its substitution with positively charged Lys.

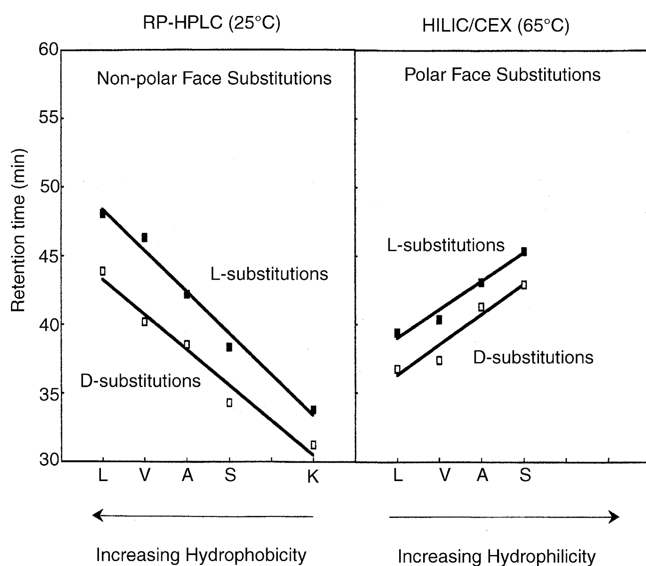

Fig. 6. Selectivity of RP-HPLC vs. HILIC/CEX of amphipathic α-helical peptides. Retention time data taken from Table 1 and Fig. 2 (RP-HPLC) and Fig. 3 (HILIC/CEX). The one-letter codes on the abscissa denote the L- or D-amino acid substituted into the non-polar face and separated by RP-HPLC at 25 °C (left panel) or polar face and separated by HILIC/CEX at 65 °C (right panel) of the V13X series or S11X series peptides, respectively (Fig. 1).

From Table 4, for the polar face substitutions on the preferred binding domain as measured by HILIC/CEX (S11X series; see Fig. 1), as the hydrophobicity of the substitution is increased (Ala < Val < Leu), there is a decrease in retention time compared to the Ser-substituted analogue (Δt_R of −2.3, −5.0, and −6.0 min, respectively); also, there is a large increase in retention time for the Lys-substituted analogue (+13.2 min). However, when these same polar face substitutions are measured by RP-HPLC (non-preferred binding domain), the Δt_R values are relatively small (ranging from just +1.0 min to +2.5 min for the non-polar side-chain

Table 4
Monitoring of effects of the same substitutions on the non-polar vs. the polar face by RP-HPLC and HILIC/CEX

Amino acid[a] substitution	RP-HPLC (pbd)[b] Δt_R(min)[c]	RP-HPLC (non-pbd)[b] Δt_R(min)[c]	HILIC/CEX (pbd)[b] Δt_R(min)[c]	HILIC/CEX (non-pbd)[b] Δt_R(min)[c]
$S_L \rightarrow L_L$	+9.8	+2.5	−6.0	+1.2
$S_L \rightarrow V_L$	+8.0	+1.5	−5.0	+1.2
$S_L \rightarrow A_L$	+3.9	+1.0	−2.3	0
$S_L \rightarrow K_L$	−4.5	−1.3	+13.2	+5.2

[a] Denotes L-Ser substitution with L-Leu, L-Val, L-Ala, or L-Lys.
[b] pbd and non-pbd denote preferred binding domain and non-preferred binding domain, respectively.
[c] Δt_R = retention time of S_L subtracted from denoted L-analogue with which it has been substituted; RP-HPLC and HILIC/CEX retention times taken from Figs. 2 and 3, respectively, and Tables 1 and 2.

substitutions and just −1.3 min for Lys), particularly, when compared to the significantly larger substitution effects detected by HILIC/CEX. Clearly, and considering the overall similar retention time ranges of RP-HPLC (Fig. 2) and HILIC/CEX (Fig. 3) of these S11X series peptides (Table 1), Tables 3 and 4 demonstrate that *HILIC/CEX monitors changes in the polar face of V_{681} more effectively than RP-HPLC*.

From Table 4, for the non-polar face substitutions (V13X series; see Fig. 1), as the hydrophobicity of the substitution is increased (Ala < Val < Leu), there is now a significant increase in RP-HPLC retention time compared to the Ser-substituted peptide, with Δt_R values ranging from +3.9 min for Ala up to +9.8 min for Leu when substituted into this preferred binding domain for RP-HPLC (compared to just +1.0 min for Ala and up to +2.5 min for Leu when substituted in the polar face and non-preferred binding domain). In addition, a Ser to Lys substitution now results in a Δt_R value of −4.5 min (compared to just −1.3 min for the polar face substitution). In contrast, HILIC/CEX is proving to be very insensitive to changes in hydrophobicity in the non-polar face (i.e., its non-preferred binding domain), as witnessed by its inability to distinguish between the Ser- and Ala-substituted peptides ($\Delta t_R = 0$ min), as well as the Val- and Leu-analogues (Δt_R = +1.2 min for both peptides). The positive charge on Lys has enabled the Ser to Lys substitution in the non-polar face to be monitored by HILIC/CEX (Δt_R = +5.2 min), albeit not as effectively as when this substitution is made in the polar face (Δt_R = +13.2 min). Thus, in contrast to the S11X series analogues, and again considering the overall similar analysis times for RP-HPLC (Fig. 2) and HILIC/CEX (Fig. 3) of the V13X series peptides (Table 2), Tables 3 and 4 also demonstrate that *RP-HPLC clearly monitors changes in the non-polar face of V_{681} more effectively than HILIC/CEX*.

4. Conclusions

The present study reports initial results of our approach to monitoring the effect on apparent peptide hydrophilicity/hydrophobicity and amphipathicity of substituting single L- or D-amino acids into the centre of the non-polar or polar faces of a 26-residue biologically active amphipathic α-helical peptide denoted V_{681}. Overall peptide hydrophobicity can be increased by amino acid substitutions in the polar or non-polar face of the amphipathic α-helix. However, our results show that, where substitutions have been made in the non-polar face, RP-HPLC is the best approach for monitoring such changes; conversely, for substitutions made in the polar face, HILIC/CEX, which resolves peptides based substantially on their hydrophilic character, was best suited for monitoring the effect of such substitutions. Our results provide insights not only into the relative efficacy of RP-HPLC versus HILIC/CEX for resolution of specific peptide mixtures, but also for gauging the apparent hydrophilicity/hydrophobicity of L- and D-amino acids substituted into the non-polar or polar faces of an amphipathic α-helical peptide. We believe these complementary RP-HPLC and HILIC/CEX methods offer excellent potential for rational design of novel amphipathic α-helical biologically active peptides, where modulation of the amphipathicity of such molecules may lead to the development of more effective antimicrobial agents.

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