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Effect of Stereochemistry on the Transport of Aca-Linked β-Turn Peptidomimetics Across a Human Intestinal Cell Line

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Abstract—Transcellular transport is one of the most important barriers facing the development of new therapeutic agents. However, little is known about the specific effects of structure and particularly stereochemistry on cell permeability. An attractive in vitro model has been developed for the direct assessment of cell transport, using the immortalized human epithelial cell line, Caco-2. The present study assesses the effects of stereochemistry on transport in a commonly used β-turn model system. Thus, L,L- and L,D-Ala-Ala were cyclized with aminocaproic acid, resulting in macrocycles in which the dipeptides correspond to the $i + 1$ and $i + 2$ positions of a β-turn. The transport of these dipeptides across a Caco-2 cell monolayer was determined, along with corresponding acyclic models (L,L- and L,D-$CH_3CH_2C(O)$-Ala-Ala-*n*-Pr). The transport studies were carried out in the presence and absence of verapamil, a known inhibitor of the apically polarized efflux system present in Caco-2 cells. Both apical→basolateral and basolateral→apical transport were measured. Measurements made in the presence of verapamil showed that the cyclic peptides experienced a ca. 4–5-fold difference in intrinsic flux depending on stereochemistry, with the L,D isomer being transported at a higher rate. These differences disappeared in the acyclic cases examined (permeability coefficient ratios of the L,D/L,L isomers were 1.04–1.13). These observations are discussed in terms of the conformations and hydrogen-bonding characteristics of the compounds as determined by NMR spectroscopy. © 1997 Elsevier Science Ltd.

Introduction

One of the most challenging aspects of modern pharmaceutical research is the identification and development of agents suitable for oral delivery. The oral bioavailability of a drug candidate largely depends on its ability to cross a variety of biological barriers including the intestinal epithelium and, for agents acting in the central nervous system, the blood–brain barrier. Historically, the optimization of bioavailability has been done relatively late in the drug discovery and development process, after a significant effort has already been expended toward the optimization of the desired in vitro biological activity of a given agent. The cellular permeability of a solute is a complex function of its size, lipophilicity, hydrogen bond potential, charge, and conformation.[1] An improved and pragmatic understanding of these factors would greatly facilitate the efficiency of the invention of new medicinal agents.

This situation is, if anything, more pronounced in the case of peptide and protein pharmaceuticals.[2] As a class, peptides are poorly absorbed compared to traditional 'small-molecule' pharmaceuticals, largely because their generally greater polarity is inconsistent with passive diffusion through a lipid bilayer membrane. This property, along with the tendency of a peptide or protein to undergo rapid metabolism and excretion, has been the driving force behind continuing efforts to develop peptidomimetic molecules that share the biological activity of their peptide leads and the favorable pharmacokinetic properties of more traditional drugs. Although a number of orally active peptides and peptidomimetics have been introduced,[3] such agents are typically developed while trying to maintain both potency and bioavailability.

A shared goal of these laboratories is to identify which properties of peptides and peptidomimetics lead to facile transport through biological membranes. Inherent in the development of such a 'structure–transport relationship' (STR) is the ability to readily measure transport of a series of systematically modified structures through membranes or cell monolayers. An increasing body of research has addressed the role that peptide conformation can play in transcellular transport.[4] In this paper, we describe a study that demonstrates a significant stereochemical effect on the transport of a series of related β-turn peptidomimetics across a cell monolayer based on the human intestinal cancer cell line, Caco-2.

Background

The possibilities for passage of a molecule across a cell monolayer include transcellular and paracellular routes, in addition to mechanisms with active transport or

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efflux mechanisms (Fig. 1). The paracellular flux of solutes across a healthy intestinal mucosa is minimal because of the tight junctions which exist between these highly polarized epithelial cells.[5] For solutes that have the correct physicochemical properties (e.g., size, charge, lipophilicity, hydrogen bonding potential, solution conformation), the transcellular passive route (pathway B) of diffusion can predominate.[1] However, this passive flux of a peptidomimetic across the intestinal mucosa may be limited by the substrate properties of the molecule for a recently identified apically polarized efflux mechanism (pathway B′).[1e,f,h,6] This efflux mechanism, which may be mediated by a member of the *p*-glycoprotein family, limits the transcellular flux of lipophilic solutes by preferentially facilitating their efflux from the mucosal cell across the apical membrane (i.e., toward the intestine). The flux of molecules that resemble di- and tripeptides across the intestinal mucosa can be facilitated by additional transporters as well (pathway C).[1e,7]

The assessment of transport has been greatly facilitated in recent years by the development of in vitro models of biological membranes.[8] Caco-2, an immortalized human epithelial cancer cell line, has proven a particularly apt model.[8,9] This cell line, which readily forms highly polarized cell monolayers, has been shown by many academic and industrial laboratories to be an appropriate model of the intestinal mucosa and very useful for conducting drug transport and drug metabolism studies in vitro. To make this cell culture system more convenient for pharmaceutical scientists, our laboratory developed an apparatus that can be used to conduct Caco-2 transport and metabolism studies.[10]

Preliminary work with the Caco-2 system has begun to address the physicochemical issues that determine peptide and peptidomimetic transport through the epithelium. For example, Burton and co-workers have investigated the effect of *N*-methylation on a series of peptides based on phenylalanine.[1f,h] As Ac-Phe-Phe-Phe-NH_2 was subject to increasing *N*-methylation, the permeability of the peptide increased to a greater extent than its polarity expected based on octanol–water partition coefficients (PCs). In contrast, increasing amide bond content along the series Ac-$(Phe)_n$-NH_2, where n = 1–3 resulted in decreased permeability despite increasing PCs in the same direction. These observations suggested that a primary factor in determining transport is the relative energy needed to desolvate the amide group so that it can diffuse across the cell membrane.

An important role of hydrogen bonding in peptides and peptidomimetics is the stabilization of turns; in turn, turns are important players in ligand–receptor interactions.[11] Accordingly, our laboratory has begun to systematically examine the effect of the hydrogen-bonding potential of peptides on their ability to undertake cellular transport via various routes.[4] Preliminary results indicate the introduction of short-range cyclizations or other prodrug moieties can substantially facilitate transport. So far, these results are consistent with the idea that the formation of intramolecular hydrogen bonds is an important determining factor, although other considerations such as reductions in the average hydrodynamic radius of the molecule can also play a role. The importance of hydrogen bonding in β-turn formation, and the use of β-turns as templates for a wide range of peptidomimetics,[12] prompted us to more closely investigate the role of hydrogen bonding, configuration, and conformation in the transport of simple β-turn models.

We[13] and others[14] have investigated dipeptides cyclized with 6-aminocaproic acid (Aca) or its derivatives as small-molecule models of β-turns. In this study, the transepithelial transport profiles of *cyclo*(L-Ala-L-Ala-Aca) (**1a**) and *cyclo*(L-Ala-D-Ala-Aca) (**1b**) were compared using the Caco-2 cell culture system. Cellular permeability was measured from both the apical (AP)→basolateral (BL) and BL→AP directions to determine if these peptides were substrates for the efflux system. As controls, transepithelial transport profiles of linear molecules, $CH_3CH_2C(O)$-L-Ala-L-Ala-$CH_2CH_2CH_3$ (**2a**) and its L,D isomer (**2b**) were also determined. All of the compounds were also characterized with respect to solution-phase conformation (NMR, CD) and solubility (PC) characteristics.

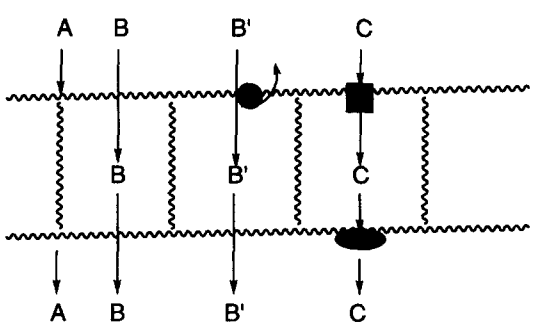
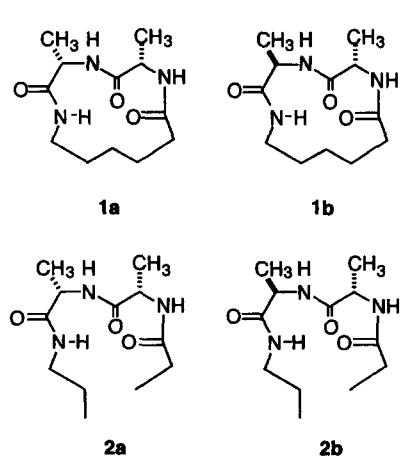


Figure 1. Possible pathways for flux of peptide mimetics across the intestinal mucosa or the BBB. Pathway A, paracellular; pathway B, transcellular via passive diffusion; pathway B′, transcellular via passive diffusion modified by an apically polarized efflux mechanism; pathway C, transcellular via di-/tripeptide transporter.



Results

Table 1 shows the apparent permeability (P_{app}) values of the cyclic and the linear compounds in both the AP→BL and the BL→AP directions. The experiments were carried out both in the absence and presence of verapamil; the latter values are considered the 'intrinsic' permeability, that is, those unimpeded by efflux in the BL→AP direction. All of the compounds examined were substrates for this efflux system as apparent by the systematically higher values for AP→BL permeability in the presence of the inhibitor and systematically lower values for BL→AP permeability under the same conditions (albeit to different extents for each compound).

When the P_{app} values of the cyclic compounds in the AP→BL direction were compared in the absence of efflux inhibitor, *cyclo*(L-Ala-D-Ala-Aca) showed 2.8 times higher flux than that of *cyclo*(L-Ala-L-Ala-Aca). In the BL→AP direction, *cyclo*(L-Ala-D-Ala-Aca) showed 3.7 times higher flux than *cyclo*(L-Ala-L-Ala-Aca). When the P_{app} values between AP→BL and BL→AP were compared in both cyclic compounds, P_{app} values in the BL→AP direction of *cyclo*(L-Ala-D-Ala-Aca) and *cyclo*(L-Ala-L-Ala-Aca) were 3.3 and 2.5 times higher than those in the AP→BL direction, respectively. Both linear compounds **2a** and **2b** showed similar P_{app} values in both directions, and P_{app} values in BL→AP direction of both linear compounds were slightly higher than those in the AP→BL directions (1.5 and 1.7 times, respectively). When the P_{app} values were determined in the presence of verapamil (0.2 mM), P_{app} values in both directions became much closer in all compounds, supporting the view that permeability under these conditions represents the intrinsic ability of the compounds to cross the Caco-2 monolayer.

Partition coefficient (PC) values in octanol/pH 7.4 buffer are also listed in Table 1. When the log PC values of *cyclo*(L-Ala-L-Ala-Aca) and *cyclo*(L-Ala-D-Ala-Aca) were compared, *cyclo*(L-Ala-D-Ala-Aca) showed almost 1.8 times higher partition to octanol phase than that of *cyclo*(L-Ala-L-Ala-Aca). The logPC values of the linear compounds in octanol/pH 7.4 buffer were 1.6–3.3 times higher than those of the cyclic compounds; again, however, the ratio of the values for the L,D versus L,L isomer was ca. 1.8:1. Unfortunately, the insolubility of all of the compounds in isooctane made comparison of these values to those obtained in the alternative isooctane/water system (often used to estimate hydrogen bonding potential via log PC measurements) risky.

All previous data accumulated for Aca-linked dipeptides (NMR, CD, X-ray, and computations) are consistent with the adoption of turn structures centered around the dipeptide moiety.[13,14] Similar results were obtained for the compounds described herein. NMR spectra of all four compounds were recorded in DMSO-d_6. All resonances were assigned by inspection of the DQF-COSY and NOESY spectra. Both cyclic compounds showed a strong NOE from the NH proton of the Aca to the NH proton of the second Ala, the expected diagnostic NOE for a turn structure. In addition, *cyclo*(L-Ala-L-Ala-Aca) showed an NOE peak between the Ala_1 and Ala_2 NH protons, consistent with the type I β-turn. This peak was absent in the NOESY spectrum of *cyclo*(L-Ala-D-Ala-Aca), but the strong Ala_1 α–Ala_2 NH peak expected for a type II β-turn was observed. These results are fully consistent with CD spectra described previously; we also repeated these measurements in the buffer used in the transport experiments and obtained curves similar to those previously recorded in methanol and other organic solvents.[13,14] Overall, our results support the constraint of the backbone in a β-turn motif, although the hydrogen bonding patterns of γ-turns may be more important contributors than previously appreciated (see below).

In both linear compounds, the stronger NOEs observed are consistent with an extended, open structure: the N-terminal –CH_2C(O)NH– to Ala_1-NH, and the Ala_1 to Ala_2-CH_α to the *C*-terminal –C(O)N**H**–. However, in both compounds a very weak NOE was observed from the Ala_2-NH to the Pr-NH, indicating that a low population of turn structure(s) exists in solution, rapidly averaging with the extended form. This NOE would be found in either type of β-turn or in a γ-turn, and without additional experimental data, these cannot be distinguished. From the small size of the NOE, it can be estimated that the population of turn structure(s) does not exceed a few percent.

Table 1. Transport properties of stereoisomeric Ala-Ala pseudopeptides

	$P_{app} \times 10^7$ (cm/s)[a]				
	Without additive		With verapamil		
Compound	**AP → BL**	**BL → AP**	**AP → BL**	**BL → AP**	**log PC[b] (octanol/buffer)**
1a (cyclic L,L)	0.62 (0.04)	1.57 (0.15)	0.66 (0.15)	0.99 (0.17)	−0.59 (0.02)
1b (cyclic L,D)	1.75 (0.15)	5.82 (0.19)	3.39 (0.32)	4.10 (0.28)	−0.34 (0.07)
2a (acyclic L,L)	2.59 (0.14)	3.84 (0.43)	2.81 (0.26)	2.94 (0.36)	−0.13 (0.02)
2b (acyclic L,D)	2.40 (0.32)	4.06 (0.25)	2.91 (0.20)	3.43 (0.15)	−0.07 (0.01)

[a]Mean values (±SD), determined from three separate filter experiments.
[b]Mean values (±SD), determined from three determinations.

The acyclic L,D isomer **2b** has a CD spectrum that is somewhat similar to its cyclic counterpart **1b** (Figure 2). However, the NMR results in DMSO-d_6 described above are clearly consistent with a linear/unconstrained conformation. Literature precedent for β-turn formation in capped acyclic dipeptides has been limited to proline-containing examples like Piv-Pro-Phe-NHMe.[16] The NMR experiments were repeated in 4:6 methanol-d_4:H_2O solution to rule out the remote possibility that this isomer contains a greater amount of β-turn in aqueous solution than in DMSO.[17] Comparable NMR spectra in these two solvent systems lead us to believe that the confluence of the CD spectra of **1b** and **2b** is a coincidence arising from the L,D-configurations of the dipeptide.

Finally, the NMR properties of **1** and **2** were examined to shed some light on the hydrogen bonding tendency of the compounds (Table 2). Amide bond temperature coefficients have often been used for this purpose and were measured.[18] Recently, increasing emphasis has been placed on chemical shift values as trivially obtained and sensitive indicators of the relative strength of hydrogen bonds with carbonyl groups and the following discussion will center on those values.[19]

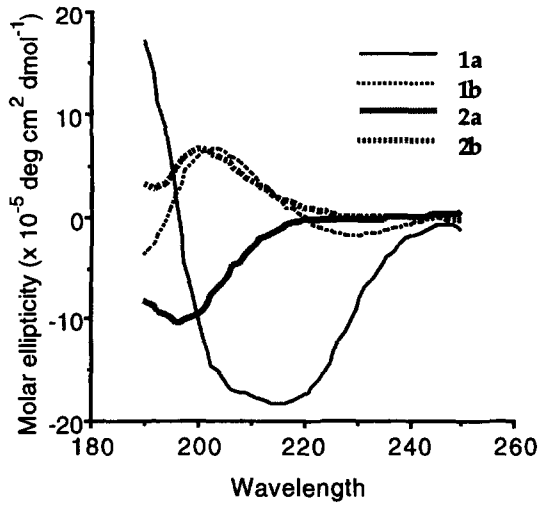


Figure 2. CD spectra of cyclic and acyclic peptides measured in 10 mM Hepes (pH 7.4 buffer) at 37 °C.

Discussion

As described in the previous section, all of the compounds examined appeared to be substrates for the apically polarized efflux system.[6] In this paper, however, we wish to focus on the effect of structure and stereochemistry on the intrinsic permeability of this series, and will confine the following discussion to the intrinsic permeability measurements, i.e., those made in the presence of verapamil (Table 1). Under these conditions, the data for all four compounds are reasonably well correlated for measurements in both the BL→AP and AP→BL directions (the R^2 value is 0.971). As mentioned earlier, this fact supports these values as the intrinsic permeability of the compounds. In contrast, although the transport data for **1** and **2** reasonably reflect the octanol/water coefficients within each cyclic and acyclic pair, respectively, there is not a general correlation of PC data for all four compounds. This is not surprising because transport/membrane permeability is expected to depend of molecular radius in addition to bulk solvation and polarity. Qualitatively, the linear compounds **2a**, shown to be more hydrophobic by the PC data, likely expose a greater portion of the capping *N*-terminal propanoyl and *C*-terminal propyl groups to solvent relative to the cyclic compounds **1**, in which the two ends are attached and therefore less flexible.

The most striking observation is the significant difference in permeability due to the configuration change in the β-turn mimics **1** but not in the acyclic compounds **2**. In the former case, the L,D-configured **1b** is transported approximately four to five times more rapidly than its homochiral isomer. This contrasts sharply with the **2a**/**2b** P_{app} ratio, which varies only from 1.03 to 1.16 for the AP→BL and the BL→AP directions. To our knowledge, this is the first time a significant dependence of membrane permeability on molecular configuration has been explicitly measured (although such differences may well contribute to oral bioavailability measurements made for various series of drug candidates).

Although the dipeptide portion of **1a** exists mostly in a type I β-turn conformation (with the two Ala residues corresponding to the $i + 1$ and $i + 2$ positions of the curve) and the corresponding part of **1b** is in a type II conformation, it seems unlikely that the simple rotation of the Ala–Ala amide bond could be directly responsible for this difference in transport (although it may well be important in potential intermolecular interactions between the molecules and the peptide

Table 2. Selected NMR Data for compounds **1** and **2** in DMSO-d_6

	NH chemical shifts (ppm)			NH temperature coefficients (ppb)		
Compound	**Ala_1**	**Ala_2**	**'Aca'**	**Ala_1**	**Ala_2**	**'Aca'**
1a (cyclic L,L)	8.09	7.69	7.37	4.0	5.0	6.0
1b (cyclic L,D)	8.49	8.57	7.00	7.5	9.0	3.0
2a (acyclic L,L)	7.97	7.84	7.71	5.0	6.3	4.2
2b (acyclic L,D)	8.01	8.17	7.75	7.5	4.8	4.0

transporter). On the other hand, it seems certain that the relative configuration of the two stereogenic centers influences the overall conformation of the macrocycle and accordingly its ability to engage in intramolecular versus intermolecular hydrogen bonding.

The chemical shift data in Table 2 are instructive in this regard. The participation of a proton in an idealized (linear) H-bond with an amide should lead to maximum deshielding because the proton is well within the deshielding cone of the amide carbonyl group, with more upfield chemical shifts resulting from either lengthening of the C=OH distance or deviation from the plane. Alternatively, an amide proton hydrogen bonding with water should not experience such deshielding. Along these lines, the spectrum of the more highly permeable compound **1b** has significant downfield shifts from that of its isomer **1a** for both the Ala_1 NH and Ala_2 NH protons (0.40 and 0.88, respectively). Interestingly, the values for the Aca NH (with the potential to engage in the hydrogen bond most closely associated with β-turn formation, i.e., that between the *i* and *i* + 4 residues) indicate less intramolecular hydrogen bonding in **1b**, as evidenced by its upfield shift in the less permeable isomer **1a** ($\Delta\delta$ for **1a–1b** being 0.37). In contrast to this situation, the $\Delta\delta$ values between **2a** and **2b** are practically nonexistent for both the Ala_1 and Aca amides, but again reach a high value of 0.33 for the Ala_2 NH.

The NMR data suggest that there is little difference in hydrogen bonding patterns between **2a** and **2b**, and that **1b** is substantially better able to involve its Ala_1 and Ala_2 amide protons in hydrogen bonding (but not its Aca-NH). If true, this strongly suggests that the improved permeability of **1b** can be largely traced to the involvement of hydrogen bonding motifs other than the classical β-turn model. Indeed, the Aca-NH is not necessary to maintain turn-like dihedral angles in these molecules because its role is taken over by the carbon backbone of the Aca linkage. γ-Turn structures that intimately involve the Ala_2-NH seem quite reasonable (Fig. 3) and consistent with the observation that this proton is subject to the greatest chemical shift variations between isomers. This issue is currently the subject of a detailed molecular dynamics study in this laboratory that will be reported elsewhere; preliminary results indicate that structures **b** and **c** appear with considerable greater frequency than **a** in a simple dynamics study carried out with the Kollman force field. Experimentally, the increased internal hydrogen bonding ability of **1b** is manifested in both the transport data and the PC measurements, which show this isomer to the less polar in a simple bilayer competition.

Conclusion

The demonstration that stereochemistry can play a decisive role in affecting the rate of molecular transport in such an important motif in peptidomimetic design has import for the many current efforts directed toward the development of orally active agents based on peptides. This work provides one example of an increase in binding attendant on D-amino acid incorporation in a cyclic peptide model that may or may not affect receptor binding when translated to biologically active cyclic peptides. The peptide literature is filled with examples of a single stereochemical change—usually an L→D amino acid modification—that simultaneously decreases binding to a circulating peptidase (for example) while retaining potency at its target receptor or enzyme.[2] Additionally, this information, coupled to the NMR work described here, strengthens the present assessment of the importance of hydrogen bonding in molecular transport across biological membranes. Current efforts are directed toward understanding the generality of these effects in both β-turn models and other classes of peptidomimetics and to establishing in detail the effect of configurational changes on the conformational space in this class of macrocyclic compounds.

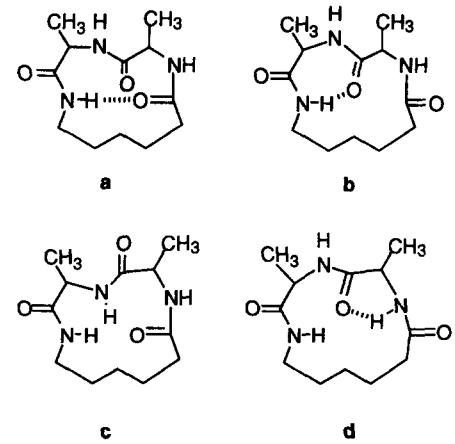


Figure 3. Some possible hydrogen bonding modes in Aca-linked dipeptides: (a) β-turn type involving the Aca NH; (b) and (c) γ-turns; and (d) a strained hydrogen bond involving the Ala_1 NH.

Experimental

Materials

[^{14}C]Mannitol (55 mCi/mmol) was purchased from American Radiolabeled Chemicals (St Louis, MO). Verapamil, 2-(*N*-morpholino)ethane sulfonic acid (MES), and Dulbecco's phosphate buffer solution (D-PBS; powder form) were purchased from Sigma Chemical (St Louis, MO). *N*-[2-Hydroxyethyl]-piperazine-*N'*-[2-ethanesulfonate] (Hepes), Hanks's balanced salt solution (HBSS), Dulbecco's modified Eagle medium (DMEM), and nonessential amino acids (NEAA) were obtained from JRH Biosciences (Lenexa, KS). Fetal bovine serum (FBS) was from Intergen Company (Cambridge, MA), and rat tail collagen (type I) was from Collaborative Research (Lexington, MA). Penicillin and streptomycin were obtained as a mixture from Irvine Scientific (Santa Ana, CA). Transwell®

clusters, PVP-free, 24.5 mm in diameter (4.71 cm^2 surface area), and 3.0-mm pore size were purchased from Costar Corporation (Bedford, MA). Acetonitrile was HPLC grade. Other chemicals were used as received.

Synthesis

Cyclo(L-Ala-L-Ala-Aca) (**1a**) and *cyclo*(L-Ala-D-Ala-Aca) (**1b**) were prepared as previously reported.[14e] The linear peptides were prepared using solution-phase techniques. Boc-L-Ala-OH and Boc-D-Ala-OH, respectively, were reacted with *n*-$PrNH_2$ (isobutyl chloroformate (IBCF), TEA, THF, −5 °C) to give the corresponding C-terminal amides. Following N-deprotection (4 N HCl/dioxane, THF, 25 °C), Boc-L-Ala-OH was coupled to the two capped amino acids using identical coupling conditions. Following another N-deprotection, the two peptides were reacted with propionic acid (DCC, TEA, CH_2Cl_2, 0 °C) to give the target amides as white solids after purification by column chromatography. L,L-$CH_3CH_2C(O)$-Ala-Ala-*n*-Pr (**2a**): mp 267–268 °C; ^1H NMR (300 MHz, $CDCl_3$) δ 6.85 (d, J = 7.8 Hz, 1H), 6.61 (br s, 1H), 5.14 (d, J = 6.7 Hz, 1H), 4.46 (quintet, J = 7.2 Hz, 1H), 4.22–4.08 (m, 1H), 3.27–3.09 (m, 2H), 1.57-1.43 (m, 2H), 1.43 (s, 9H), 1.37 (d, J = 6.9 Hz, 3H), 1.35 (d, J = 6.9 Hz, 3H), 0.89 (t, J = 7.3 Hz, 3H); ^{13}C NMR (74.5 MHz, $CDCl_3$) δ 172.6, 171.9, 155.6, 80.4, 50.6, 48.9, 41.3, 28.3, 22.7, 19.0, 18.2, 11.3. L,D-$CH_3CH_2C(O)$-Ala-Ala-*n*-Pr (**2b**): mp 193–194 °C; ^1H NMR (300 MHz, $CDCl_3$) δ 6.93 (d, J = 7.0 Hz, 1H), 6.78 (br s, 1H), 5.26 (d, J = 6.8 Hz, 1H), 4.47 (quintet, J = 7.2 Hz, 1H), 4.14–4.07 (m, 1H), 3.20–3.13 (m, 2H), 1.56–1.42 (m, 2H), 1.42 (s, 9H), 1.37 (d, J = 7.1 Hz, 3H), 1.34 (d, J = 7.2 Hz, 3H), 0.88 (t, J = 7.4 Hz, 3H); ^{13}C NMR (74.5 MHz, $CDCl_3$) δ 172.8, 172.0, 155.6, 80.2, 50.5, 48.9, 41.2, 28.3, 22.7, 18.2, 18.1, 11.3.

Transepithelial transport studies

Caco-2 cells were plated and grown according to previously published procedures.[7b] Apical→basolateral (AP→BL) and basolateral→apical (BL→AP) flux experiments of the cyclic and linear compounds (0.5 mM) were conducted in Caco-2 cells at 37 °C in the presence or absence of verapamil (0.2 mM) according to previously published procedures.[7b] On the day of the experiment, cells were rinsed with HBSS containing 25 mM glucose and 10 mM Hepes (pH 7.4 buffer) at 37 °C. Plates were then preincubated at 37 °C for 10 min with pH 7.4 buffer on the both sides. After removal of the preincubation medium, a 1.5 mL aliquot of pH 7.4 buffer was placed in the receiver chamber. A 1.5 mL aliquot of the pH 7.4 buffer containing a compound of interest with or without verapamil was added to the donor side. Samples (200 μL) were removed at designated times from the receiver chamber and replaced with fresh pH 7.4 buffer. The samples were then acidified by addition of 0.08 N HCl (100 μL) and analyzed by HPLC.

The permeability coefficient (P_{app}) was calculated according to the following equation:

$$P_{app} = \frac{V \cdot dC}{A \cdot C_0 \cdot dt}$$

where $V \cdot (dC/dt)$ is the steady-state rate of appearance of the peptidomimetic in the receiver chamber after initial lag time, C_0 is the initial peptidomimetic concentration in the donor chamber, and A is the area of the Transwell®.

HPLC analysis

The analysis conditions for the peptidomimetics were as follows: column, C18 (Vydac, 4.6 mm × 250 mm, Hesperia, CA); mobile phase, 70 mM phosphate buffer (pH 3.5) containing 10 mM heptane sulfonic acid and 5–8% acetonitrile; detection, 210 nm; flow rate, 1 mL/min. The retention times of **1a**, **1b**, **2a**, and **2b** were approximately 6.7, 6.3, 7.2 and 7.8 min, respectively.

Partition coefficient experiments

Partition coefficients were determined by overnight shaking of pH 7.4 buffer solution of the compounds with a volume of octanol-saturated pH 7.4 buffer. All manipulation was done at room temperature. Phase volumes of the buffer and the organic solvents were 1:25, 1:35, and 1:45 for each compound. After overnight shaking and centrifugation, the buffer layers were collected. The amount of the peptidomimetics in the samples was determined by HPLC. The partition coefficients were calculated from the concentration of the peptidomimetic in the octanol divided by the concentration of the peptidomimetics in the buffer.

CD spectra

CD spectra were recorded on a JASCO J-710 spectropolarimeter using a quartz cell of 0.02-cm path length. Spectra were measured in 10 mM Hepes (pH 7.4 buffer) at a compound concentration of 0.5 mM at 37 °C.

NMR

All spectra were taken at room temperature on a Bruker AM-500 equipped with an inverse detection probe. TPPI phase sensitive, double-quantum filtered COSY and phase sensitive NOESY experiments were performed with standard Bruker pulse programs. Typical two-dimensional conditions were: sweep width 4000 Hz, 2K data points in t_2, 300 increments in t_1, NOESY mixing time 700 ms. The data were transferred to a Silicon Graphics Indigo 2 workstation and processed using Felix 95 (Biosym, San Diego, CA).

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17. The peptide still retains adequate solubility for NMR work in this mixture while avoiding deuterium exchange of the amide resonances as would be the case for pure methanol-d_4; DMSO is not a suitable solvent for CD measurements.

18. The high values of the amide temperature coefficients do not, in our view, accurately reflect the extent of intramolecular hydrogen bonding occurring in these compounds. It has been noted before that the temperature coefficients can be high in the presence of intramolecular hydrogen bonding if there is conformational exchange in an appropriate time scale.[18b,c] Preliminary pulsed field gradient diffusion measurements have indicated these compounds undergo aggregation–deaggregation exchange under the conditions of the NMR experiment. This may have a bearing on the temperature coefficients as well but we do not believe self-association affects the results of the transport experiments, in which the concentrations of the compounds are 5–10 times lower and the various cell membrane constituents and hydrophobic macromolecules in the Caco-2 cells are present. (b) Andersen, N. H.; Chen, C.; Marschner, T. M.; Krystek Jr., S. R.; Bassolino, D. A. *Biochemistry* **1992**, *31*, 1280. (c) Rothemund, S.; Weißhoff, H.; Beyermann, M.; Krause, E.; Bienert, M.; Mügge, C.; Sykes, B. D.; Sönnichsen, F. D. *J. Biomol. NMR* **1996**, *8*, 93.

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