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## A new amino acid for improving permeability and solubility in macrocyclic peptides through side chain-to-backbone hydrogen bonding

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### Abstract

Despite the notoriously poor membrane permeability of peptides in general, many cyclic peptide natural products show high passive membrane permeability and potently inhibit a variety of “undruggable” intracellular targets. A major impediment to designing cyclic peptides with good permeability is the high desolvation energy associated with the peptide backbone amide NH groups. While several strategies have been proposed to mitigate this deleterious effect, only few studies have used polar side chains to sequester backbone NH groups. We investigated the ability of *N,N*-pyrrolidinyl glutamine (Pye), whose side chain contains a powerful hydrogen bond accepting C=O amide group but no hydrogen bond donors, to sequester exposed backbone NH groups in a series of cyclic hexapeptide diastereomers. Analyses revealed that specific Leu-to-Pye substitutions conferred dramatic improvements in aqueous solubility and permeability in a scaffold- and position-dependent manner. Therefore, this approach offers a complementary tool for improving membrane permeability and solubility in cyclic peptides.

### Graphical Abstract

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#### ASSOCIATED CONTENT

##### Supporting Information

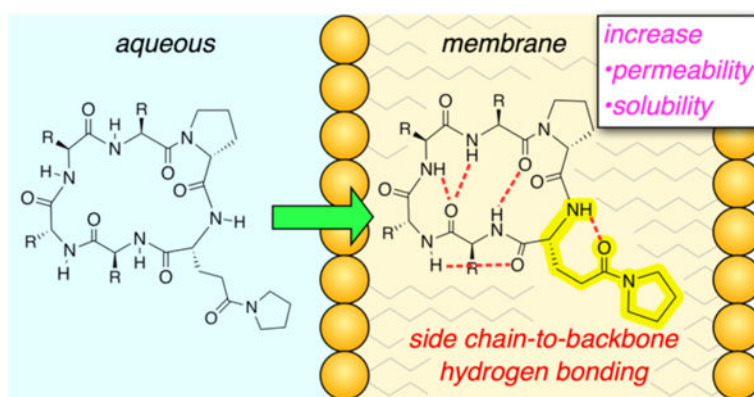
The Supporting Information is available free of charge on the ACS Publications website.

Syntheses of model compounds **4** and amino acid building blocks; Details of solid-phase synthesis, *In silico* SC-BB hydrogen bonding, shake flask partition experiment, PAMPA assay, thermodynamic solubility assay, amide proton temperature coefficient experiment, NOE buildups and interproton distances, IMHB patterns, crystal structure of **3-Pye<sup>2</sup>(Ala<sup>3</sup>Nal<sup>5</sup>)** and **3-Pye<sup>2</sup>(HPhe<sup>5</sup>)** (PDF)

The 20 structures of **3-Pye<sup>2</sup>(Ala<sup>3</sup>Nal<sup>5</sup>)** from the McMD ensemble at T = 300K with the fewest violations from the NOESY-derived distances (PDB)

CCDC 2122986–2122987 contain the supplementary crystallographic data for this paper. These data can be obtained free of charge via [www.ccdc.cam.ac.uk/data\\_request/cif](http://www.ccdc.cam.ac.uk/data_request/cif), or by emailing [data\\_request@ccdc.cam.ac.uk](mailto:data_request@ccdc.cam.ac.uk), or by contacting The Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336033

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## Introduction

Advances in medicine and molecular cell biology continue to generate therapeutic targets whose large, flat binding interfaces make them challenging to drug with traditional, Rule of 5 (Ro5)-compliant small molecules.<sup>1–6</sup> Although large biomolecules can often bind with high affinity to such undruggable sites, due to their inability to cross the cell membrane these drugs are generally restricted to parenteral delivery against extracellular targets.<sup>7–9</sup> Thus the inverse relationship between molecular weight and cell permeability has focused the search for new chemical matter against undruggable intracellular targets within the chemical space that lies between small molecules and biologics, where there are a growing number of drugs, leads, and model systems that can engage classically undruggable sites yet exhibit the favorable membrane permeability and oral absorption typical of small molecule drugs.<sup>10–13</sup> Among the chemotypes that are in the “beyond Rule of 5 (bRo5)” chemical space, cyclic peptides are gaining attention as they can potentially interact with protein targets but still present some of the physicochemical properties of traditional small molecule drugs. The influence of emerging bRo5 scaffolds has already driven novel approved oral drugs toward larger molecular weights over the past two decades.<sup>14, 15</sup>

Cyclic peptides have several favorable pharmacological characteristics as therapeutic agents and chemical probes.<sup>16–18</sup> Cyclization of peptides reduces conformational flexibility, enhances proteolytic stability and increases binding affinity and selectivity.<sup>16–18</sup> The relatively larger surface areas of cyclic peptides, compared to conventional small molecules, allow them to engage large protein surfaces, leading to a high affinity toward targets that lack well-defined binding pockets.<sup>18–20</sup> Moreover, cyclic peptides can be diversified enormously by the addition of non-proteinogenic amino acids (e.g., D-amino acids, *N*-methyl amino acids, peptoids, etc.) and by varying ring size and backbone linkages (e.g., lariats, depsipeptides, inclusion of polyketide fragments).

Lessons learned from the orally bioavailable cyclic peptide cyclosporine A (CsA)<sup>9, 21–23</sup>, along with numerous other model systems<sup>17, 19, 24</sup>, have confirmed the importance of overall lipophilic character, as well as the degree to which hydrogen bond donors can be shielded from solvent, in determining membrane permeability. Several methods have been proposed to minimize exposed amide protons, such as (i) stabilization of intramolecular hydrogen

bonding through stereochemistry of amino acid residues<sup>24–28</sup>, (ii) replacement of polar NH residues that cannot participate in intramolecular hydrogen bonding (IMHB) with *N*-methyl amino acids or peptoids<sup>9, 29–33</sup>, (iii)  $\alpha$ -methylation of amino acid residues to reinforce the IMHB network<sup>34</sup> and (iv) steric shielding with nearby hydrophobic side chains.<sup>1, 30, 35, 36</sup>

While these methods (which have been summarized in a recent review<sup>18</sup>) successfully enhance membrane permeability, some drawbacks impede their application. *N*-methylation removes amide protons that can potentially interact with protein targets, and *N*-methylation at some residues may disrupt IMHB networks leading to a decrease in membrane permeability.<sup>30</sup> Furthermore, steric occlusion of HBD requires the introduction of bulky side chains, which can impede target binding and increase overall lipophilicity beyond acceptable limits. Consequently, new strategies for sequestering polar amide protons that are orthogonal to *N*-methylation and steric occlusion would be useful.

Our interest in the use of side chain hydrogen bond acceptors (HBAs) to sequester exposed amide hydrogen bond donors (HBDs) was motivated in part by the observation that these types of side chain-to-backbone (SC-BB) interactions are present in some crystal structures of natural product cyclic peptides that are known to have intracellular targets and whose structures suggest that they may be passively permeable. For example, argyrin B,<sup>37</sup> which inhibits the prokaryotic and mitochondrial elongation factor G (EF-G), contains an IMHB between the MeO-of its 4'-methoxytryptophan and its backbone amide NH (Figure 1A).<sup>38</sup>

A few synthetic cyclic peptides have been reported that utilize exocyclic HBAs to sequester exposed backbone amides. Thansandote, *et al.*,<sup>34</sup> incorporated a 2-pyridinylalanine side chain into a cyclic hexapeptide, which improved solubility but failed to improve permeability, possibly due to the deleterious effect of the pyridyl side chain on hydrocarbon-water partitioning. Another system reported by Yudin, *et al.*, integrated an exocyclic amide motif into a peptide macrocycle, which formed hydrogen bonds with two backbone NH groups, resulting in an improvement of passive membrane permeability. However, the exocyclic amide introduced an additional hydrogen bond, thus potentially limiting the generality of this approach.<sup>39–42</sup> Although other cyclic peptide systems have been described that show side chain HBAs interacting with backbone amide NH groups, the impact of these interactions on the compounds' drug-like properties were not investigated systematically.<sup>43–45</sup>

Herein, we set out to investigate the interaction between side chain HBAs and backbone amides in a series of cyclic hexapeptide model systems, and, in turn, evaluate their effect on conformation and their ability to improve membrane permeability. By substituting a side chain HBA on each position in three different congeneric series, we found that side chain HBAs can sequester exposed amide protons by forming SC-BB H-bonds, supported by X-ray crystal and NMR solution structures. As a result, we observed an increase in lipophilicity and membrane permeability compared to isomeric scaffolds in which the position and stereochemistry of the polar side chain did not favor SC-BB H-bonding. Moreover, the substitution of a polar, HBA-containing side chain dramatically increased aqueous solubility, thereby offsetting the combined deleterious effect of multiple lipophilic residues.

## Results and Discussion

### Side chain-to-backbone Hydrogen Bonding of Model Structures

In order to identify side chains that could sequester backbone amide NH groups through IMHB interactions, we performed computational studies on a series of model compounds bearing various side-chain HBAs. We performed conformational searches in an implicit, low-dielectric solvent continuum to mimic the membrane dielectric, and plotted the distance between the side chain HBA and backbone amide NH vs. energy, relative to the lowest-energy conformation (Figure S1). The results showed that *N,N*-disubstituted Gln residues had low-energy conformers with short HBA-HBD distances ( $< 2.0 \text{ \AA}$ ; Figure S1a, d, e), while the distances from *N,N*-disubstituted Asn were slightly longer (Figure S1f, h). Similar HBA-HBD distances were observed when replacing an amide functional group with an ester (Figure S1c).

The encouraging *in silico* data prompted us to investigate the degree of SC-BB H-bonding in the set of synthetic model structures **4** shown in Table 1. For commercially available amino acids (**4-Ala**, **4-Val**, **4-Leu**, **4-Ile**, **4-Gln**, **4-Glu(OMe)**), Boc-protected amino acids were esterified with 4-phenyl-1-butanol, deprotected, and acetylated to obtain compounds **4** (Scheme S1a). Substituted amides (**4-Gln(NMe<sub>2</sub>)**, **4-Gln(Pyr)**, **4-Asn(Pyr)**, **4-Asn(NMe<sub>2</sub>)**) were synthesized from corresponding *N*- and *C*-protected Glu or Asp amino acids (Boc-Glu-O<sup>t</sup>Bu and Boc-Asp-O<sup>t</sup>Bu; Scheme S1b). After amide coupling with pyrrolidine (Pyr) or *N,N*-dimethylamine, the intermediates were treated in TFA to deprotect all acid-labile protecting groups, the Boc-protecting group was reintroduced, and the compounds were carried forward as described above.

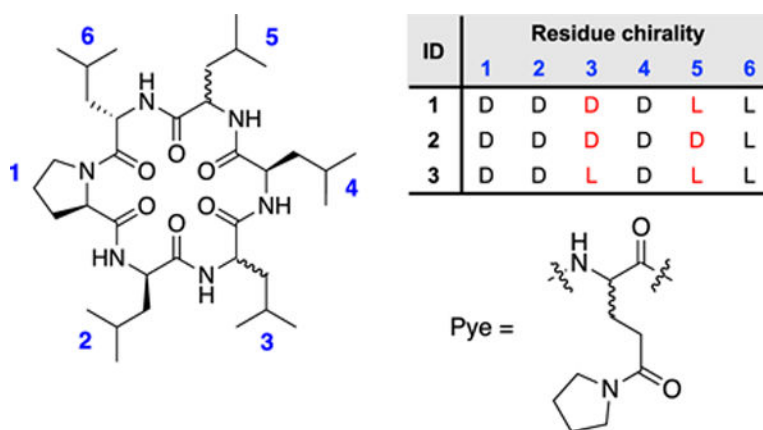
NMR chemical shifts ( $\delta$ ) and temperature coefficients ( $\delta_{\text{NH}}/T$ ) are widely used to gain structural information in peptides and proteins.<sup>26, 46, 47</sup> Temperature coefficients of backbone amide protons have been used to report on their degree of solvent exposure; for well-structured proteins in aqueous solution, amide NH protons with temperature shifts above (more positive than)  $-4.0 \text{ ppb/K}$  are considered to be involved in IMHB, whereas more negative values indicate greater solvent exposure.<sup>46</sup> This relationship has been attributed to the strong ( $1/r^3$ ) dependence of NH chemical shifts on the NH–O distance, where the NH becomes more deshielded and shifts downfield as the H-bond becomes shorter.<sup>48</sup>

In proteins, solvent-exposed amides have relatively large thermal shifts due to the weaker H-bond to water compared to the NH–to–carbonyl H-bond. However, in nonpolar solvents<sup>49, 50</sup>, fully exposed NH groups can have small temperature shifts because they interact with neither solvent nor acceptor atoms within the molecule. For NH groups that are involved in IMHB in nonpolar solvents, temperature shifts can either be large, if the IMHB interaction has a strong negative entropic component, e.g., with multiple rotatable bonds between the donor and acceptor, or small, if the enthalpic gain outweighs the entropic penalty of H-bond formation and the interaction remains stable with increasing temperature. Therefore, interpreting thermal shift data is challenging, especially in small conformationally dynamic peptides, where additional information such as NH chemical shifts derived from model systems can be useful.<sup>51</sup>

To avoid artifacts due to *intermolecular* H-bonding, we determined the NH chemical shifts of **4-Ala** and **4-Gln(Pyr)** as a function of concentration (Figure S4). At concentrations below 50 mM the NH chemical shift remained nearly constant, indicating minimal intermolecular H-bonding. The model compounds with aliphatic side chains (**4-Ala**, **4-Val**, **4-Leu**, **4-Ile**; Table 1 and Figure S3) showed chemical shifts of ~6.0 ppm and temperature shift coefficients between -2.95 and -3.54 ppb/K. These values are consistent with observations from Gellman, *et al.*, who saw comparable chemical shifts and temperature coefficients in CD<sub>2</sub>Cl<sub>2</sub> for similar model systems lacking in IMHB.<sup>50</sup> For the substituted Gln amides (**4-Gln(NMe<sub>2</sub>)**, **4-Gln(Pyr)**), the amide proton chemical shifts appeared approximately 1 ppm downfield of the NH protons in the aliphatic controls and showed significantly larger negative  $\delta_{\text{NH}}/T$  values (-6.48 and -7.84 ppb/K). Both the chemical shifts and temperature coefficients of ester **4-Glu(OMe)** were similar to the aliphatic controls, consistent with the weaker hydrogen bond-accepting ability of esters compared to amides.<sup>50</sup> Taken together, these results are consistent with previous studies finding comparably upfield chemical shifts in nonpolar media for weakly hydrogen-bonding NH groups, and downfield chemical shifts for NH groups that are significantly hydrogen-bonded. Interestingly, while the chemical shifts of substituted Asn amides (**4-Asn(Pyr)**, **4-Asn(NMe<sub>2</sub>)**) were similarly downfield (6.81 and 6.89 ppm) to the Gln compounds, their  $\delta_{\text{NH}}/T$  values (-1.66 and -1.52 ppb/K) were much less negative than either the Gln derivatives or the aliphatic controls. The larger temperature coefficients of the Gln compared to Asn residues are consistent with the larger entropic penalty for H-bond formation of the more flexible Gln side chain. Combined with the computational studies, these NMR data prompted us to investigate the **4-Gln(Pyr)** residue, which we abbreviate as “Pye” (pyrrolidine amide of glutamic acid ‘E’), as the most promising candidate for eliciting permeability-enhancing SC-BB interactions in cyclic peptides.

### NMR Studies of Cyclic Hexapeptides

Both L and D-Pye were synthesized on a multigram scale (Scheme S2) first using EDC and HOBT to couple pyrrolidine to the side chain of Fmoc-Glu-O<sup>t</sup>Bu, followed by removal of the *tert*-butyl ester with TFA and diethyl ether precipitation. Previously we identified a series of diastereomeric cyclic hexapeptide scaffolds whose membrane permeabilities<sup>52</sup> varied significantly depending on their ability to sequester exposed amide NH groups in IMHB.<sup>53</sup> In order to determine the impact of Pye on passive permeability, we selected three of the least permeable diastereomers, **1**, **2** and **3**, which differ in stereochemistry at positions Leu3 and Leu5 (we replaced the Tyr6 residue from the previous study with Leu to avoid the deleterious effect of the phenolic OH on permeability). Herein, compounds are named by the type of polar residue (Asn, Pye, etc) preceded by the scaffold number and followed by a superscript indicating the position of the substitution (e.g., **1-Pye<sup>2</sup>** refers to scaffold **1** in which Leu2 is replaced with Pye). Substituting each Leu with Pye in the three scaffolds yielded a total of 18 compounds, including the three parental compounds **1**, **2** and **3**.



For the series based on **1** and **2**, we were unable to assign all of the resonances due to line broadening and/or peak overlap. However, for the series based on diastereomer **3**, the peaks were sharp and dispersed enough to allow complete assignment of the parental compound as well as all of the Pye positional variants (Figure 2). For **3-Pye<sup>2</sup>** and **3-Pye<sup>5</sup>** (Figure 2), the amide proton of the Pye residue fell significantly downfield of the other NH resonances, even as far as 10 ppm for **3-Pye<sup>2</sup>**. Based on the chemical shifts and temperature coefficients of the model peptides (Table 1), we hypothesized that the Pye side chain in these compounds form IMHB to their own backbone amide NH groups. For many of the other compounds, including **3-Pye<sup>3</sup>**, **3-Pye<sup>4</sup>**, and **3-Pye<sup>6</sup>** (Figure 2), (as well as **1-Pye<sup>4</sup>**, **1-Pye<sup>5</sup>**, and **1-Pye<sup>6</sup>**) (Figure S5b), the chemical shift of the Pye NH was in the same range as that of the other amide NH resonances, between 6 and 8.5 ppm.

The temperature coefficients of **1**, **2** and **3**, showed at least one amide proton exposed to solvent (Figure 2 and S4b, d); for the fully assigned compound **3**, the NH with the high temperature coefficient corresponded to Leu2 (-9.13 ppb/K). For all other **3-Pye** positional variants (excluding **3-Pye<sup>2</sup>**, in which Leu2 is replaced with Pye), the Leu2 NH was the most shielded and also had the largest temperature coefficient compared to the other NH peaks. For **3-Pye<sup>3</sup>**, **3-Pye<sup>4</sup>**, and **3-Pye<sup>6</sup>**, Leu5 also showed a relatively high temperature coefficient. The high variability in both chemical shifts and temperature coefficients indicates that the position of the Pye residue in the scaffold has a significant effect on its ability to participate in IMHB and demonstrates that substitution of a single aliphatic residue with an HBA-containing residue can have a strong position-dependent effect on the scaffold's low-dielectric conformation.

### Lipophilicity, Passive Permeability and Solubility of Pye-scanning Cyclic Hexapeptides

Previously we introduced a metric called lipophilic permeability efficiency (LPE) for assessing the efficiency with which a compound achieves membrane permeability at a given lipophilicity.<sup>53</sup> LPE is defined as  $\log D_{\text{dec/w}} - 1.06(\text{ALogP}) + 5.47$ , where  $\log D_{\text{dec/w}}$  is the experimental partition coefficient between 1,9-decadiene and PBS buffer at pH 7.4, and  $\text{ALogP}$ <sup>54</sup> is a calculated, atomistic 2D octanol/water partition coefficient (a slight variation on the more commonly used  $\text{cLogP}$ ). Hydrocarbons provide a good model of the membrane interior, and thus partitioning between water and 1,9-decadiene reflects the energetic penalty of desolvating exposed polar functionality, in particular HBDs, as they cross the barrier

region of the phospholipid bilayer. Therefore, for compounds that are water-soluble,  $\log D_{\text{dec/w}}$  correlates positively with passive permeability. On the other hand, since octanol is capable of forming hydrogen bonds with solute, ALogP does not penalize exposed HBD but rather reflects a compound's minimum lipophilic character in the aqueous environment. Therefore, for highly lipophilic compounds (with ALogP above  $\sim 4$ ), ALogP shows a good negative correlation with solubility as well as a negative correlation with permeability in the insoluble regime.<sup>55–57</sup> By subtracting ALogP from  $\log D_{\text{dec/w}}$ , LPE thus provides a measure of a scaffold's ability to sequester polar functionality (in particular HBD) and therefore its potential to achieve high permeability at a given ALogP-defined lipophilicity.

To determine the  $\Delta$ LPE of the Pye residue (relative to an aliphatic control) in the absence of IMHB, we synthesized a simple model compound in which the Pye functional group was introduced as a peptoid side chain (Figure 2c). The Pye substitution decreased LPE by 0.22 units relative to the isobutyl control.<sup>53</sup> Next, LPE and thermodynamic aqueous solubilities were determined for parent compounds **1–3** and their Pye substitutions (Table 2). Replacing Leu with Pye decreases ALogP by 1.28 units. The same substitution also decreased  $\log D_{\text{dec/w}}$ , by a variable amount depending on both the scaffold and the position of the Pye residue within the scaffold. For most compounds, substitution of Leu for Pye caused a significant decrease in LPE (by up to 1 LPE unit); however, for **1-Pye<sup>2</sup>** and **3-Pye<sup>2</sup>**, LPE *increased* upon Pye substitution. This observation is consistent with the upfield chemical shift and large temperature coefficient of Leu2 in parent compound **3**, indicating solvent exposure of the Leu2 NH. Although the NH resonances in parent compound **1** could not be assigned due to peak overlap, there is a single upfield amide peak with a large temperature coefficient that, based on the other similarities to scaffold **1**, likely corresponds to Leu2. The far downfield chemical shifts of the Pye residue in **1-Pye<sup>2</sup>** and **3-Pye<sup>2</sup>** suggest that the Pye side chain's C=O is able to sequester these exposed NH groups via an SC-BB IMHB. The temperature shift of the Pye2 amide in **1-Pye<sup>2</sup>**, however, is small ( $-1.1$  ppb/K), while that of **3-Pye<sup>2</sup>** is much larger ( $-7.0$  ppb/K). Since the Pye2 substitution's effect on LPE is greater for scaffold **1** than scaffold **3** (suggesting that the corresponding SC-BB IMHB is stronger for **1-Pye<sup>2</sup>**), perhaps the SC-BB interaction in **1-Pye<sup>2</sup>** is enthalpically favorable enough to offset the entropic penalty of H-bond formation, thus lowering the temperature coefficient for this amide.

The parallel artificial membrane permeability assay (PAMPA)<sup>58</sup> is a cell-free permeation tool for screening passive permeabilities ( $P_{\text{app}}$ ). PAMPA measures the rate of diffusion of test compounds from donor to acceptor compartments across a solution of 1% lecithin in n-dodecane trapped in a polyvinylidene difluoroethane membrane. The positive correlation between permeation and lipophilicity generally holds for compounds in the soluble regime, generally below ALogP  $\sim 4$ . At higher lipophilicities, effective permeability decreases due to factors such as membrane sequestration and poor aqueous solubility, a trend that has been observed in both cyclic peptides and small molecule drugs.<sup>27, 59, 60</sup> Thus, a plot between  $\log P_{\text{app}}$  and ALogP produces a downward-facing parabola with a vertex near ALogP  $\sim 4$ .<sup>25</sup>

Compounds **1**, **2** and **3** showed excellent PAMPA permeabilities but had modest solubilities, consistent with their relatively high lipophilicity (ALogP = 3.74, Table 2). Most of the

Pye-substituted compounds had  $P_{app}$  values below  $1 \times 10^{-6}$  cm/s except **1-Pye<sup>2</sup>**, **2-Pye<sup>3</sup>**, **2-Pye<sup>4</sup>**, **2-Pye<sup>6</sup>** and **3-Pye<sup>2</sup>** (Figure 3a). Permeabilities and LPEs of **1-Pye<sup>2</sup>** and **3-Pye<sup>2</sup>** were clearly higher than the other Pye-substitutions. In addition, selected compounds in the **3** series were measured for their rate of flux across the human colon carcinoma cell (Caco-2) line, and their permeability rates were similar to those observed in PAMPA (Figure 3b).

Besides their ability to sequester backbone NH groups, another advantage of polar side chains is their potential for increasing a compound's aqueous solubility. Indeed, in all cases Pye substitution led to a significant improvement in solubility over the parental cyclic hexapeptides (Table 2). Interestingly, the degree to which introduction of the Pye residue improved permeability was also dependent on scaffold and position. For example, when Leu6 was replaced with Pye on scaffold **1**, solubility improved 45-fold, whereas replacing Leu3 for Pye on scaffold **2** improved solubility only two-fold. Interestingly, the degree to which Pye substitution improved aqueous solubility did not correlate with the effect on LPE. For example, the two compounds that showed an increase in LPE upon Pye substitution (**1-Pye<sup>2</sup>** and **3-Pye<sup>2</sup>**) also saw substantial improvements in solubility, consistent with a degree to which structural/conformational factors that contribute to solubility and permeability can be considered independently.

### Membrane Permeability of **3-Pye<sup>2</sup>** and **3-Pye<sup>3</sup>** Libraries

To investigate the effect of Pye on lipophilicity and membrane permeability in the context of various amino acid combinations, we synthesized **3-Pye<sup>2</sup>** and **3-Pye<sup>3</sup>** libraries using the split-pool approach in which two residues were mutated to different hydrophobic side chains (Figure 4a). We chose these two scaffolds as they had the largest log  $D_{dec/w}$  difference between Pye positional variants on the same scaffold, and the <sup>1</sup>H NMR spectra in the scaffold **3** series were interpretable. We selected side chain combinations that would cover a broad ALogP range, in this case from 0.5 to 4.7. The experimental hydrocarbon/water coefficients shown in Figure 4b agreed with Pye-scanning data in Table 2, showing that the log  $D_{dec/w}$  values for the compounds based on the **3-Pye<sup>2</sup>** positional variant were greater than those based on **3-Pye<sup>3</sup>**. Matched pairs bearing the same combination of side chains from the **3-Pye<sup>2</sup>** and **3-Pye<sup>3</sup>** libraries had log  $D_{dec/w}$  values that differed on average by one log unit, highlighting the importance of the Pye residue's position in determining its effect on physico-chemical properties.

The plot of PAMPA permeability (log  $P_{app}$ ) versus ALogP for the **3-Pye<sup>2</sup>** and **3-Pye<sup>3</sup>** libraries revealed a similar trend, in which the **3-Pye<sup>2</sup>** derivatives were generally more permeable than the **3-Pye<sup>3</sup>** derivatives of the same ALogP (i.e., comparing pairs of compounds bearing the same side chain combinations; Figure 4c). At ALogP values below 4, the **3-Pye<sup>2</sup>** library was 0.5–1.0 log unit higher in permeability than the **3-Pye<sup>3</sup>** library. Above ALogP = 4, PAMPA permeabilities for both libraries showed the characteristic downward trend as aqueous solubilities fall below the threshold required to sustain permeability (Table S2–3). The two naphthyl-Ala derivatives **3-Pye<sup>2</sup>(Nal<sup>5</sup>)** and **3-Pye<sup>3</sup>(Nal<sup>5</sup>)** have nearly the same ALogP as the parent compound **3**, with the increased lipophilicity introduced by the Nal residue offsetting the decrease in lipophilicity upon substitution of Leu2 or Leu3



with Pye. But whereas the permeability of **3-Pye<sup>2</sup>(Nal<sup>5</sup>)** was comparable to that of **3**, the  $P_{app}$  of **3-Pye<sup>3</sup>(Nal<sup>5</sup>)** was 0.6 log units lower than the  $P_{app}$  of **3**. In addition, two of the **3-Pye<sup>2</sup>** derivatives (**Cha<sup>3</sup>Chg<sup>5</sup>** and **Cha<sup>3</sup>HPhe<sup>5</sup>**; ALogP = 4.1 and 4.2, respectively) achieved permeabilities greater than that of the all-Leu parent scaffold, whereas none of the **3-Pye<sup>3</sup>**-based derivatives matched the permeability of the parent scaffold. Because of the lower LPE of the **3-Pye<sup>3</sup>** scaffold, its derivatives require a higher ALogP to achieve the same of permeability as scaffolds of higher LPE (e.g., **3-Pye<sup>2</sup>**); in this case, the lipophilicity required for the **3-Pye<sup>3</sup>**-based compounds to achieve the permeability of **3** puts them on the descending (low solubility) part of the  $P_{app}$  vs. ALogP curve (Figure 4c and Table S2 and S3).

Overall, the results from scaffold **3** suggest that substitution with Pye can enable SC-BB interactions which preserve the LPE of the parent scaffold, thus allowing for incorporation of larger, more lipophilic side chains without compromising water solubility. For example, **3-Pye<sup>2</sup>(Cha<sup>3</sup>Chg<sup>5</sup>)** (ALogP = 4.1,  $P_{app}$  =  $14 \times 10^{-6}$  cm/s, solubility = 74  $\mu$ M; Table S2) has better solubility and permeability than **3** (ALogP = 3.7,  $P_{app}$  =  $8.0 \times 10^{-6}$  cm/s, solubility = 63  $\mu$ M; Table 2).

### Structural Elucidation of **3-Pye<sup>2</sup>** derivatives

The exceptional permeability of the **3-Pye<sup>2</sup>** library encouraged us to explore the structural basis of the Pye residue's effect on permeability. We obtained structures by single crystal X-ray diffraction of two side chain variants on **3-Pye<sup>2</sup>**, **3-Pye<sup>2</sup>(Ala<sup>3</sup>Nal<sup>5</sup>)** and **3-Pye<sup>2</sup>(HPhe<sup>5</sup>)**, crystallized by vapor-diffusion in THF-pentane (Figure 5). These two crystals showed virtually identical backbone conformations (RMSD = 0.07 Å) and had nearly identical C $_{\alpha}$ -C $_{\beta}$  vectors (RMSD = 0.09 Å). As expected from the NMR studies, the Pye2 side chain C=O showed a strong hydrogen bond to its own backbone amide NH (Figure 5a–b, black arrow). The distance and N–H–O angle were 1.9 Å and 155° for both crystals, respectively, which is an optimal hydrogen bond geometry.<sup>50</sup> Three other backbone amides participated in IMHB as  $\beta$ -turn motifs (i.e., 3<sup>rd</sup> NH to 6<sup>th</sup> CO and 6<sup>th</sup> NH to 3<sup>rd</sup> CO; Figure 5a–b). The NH groups of residues 4 and 5 formed *intermolecular* hydrogen bonds with other molecules in the crystal lattice (Figure S13).

These crystal contacts suggested that these amide NH groups might be exposed in low-dielectric solvent; however, this hypothesis was inconsistent with the small  $\delta_{NH} / T$  NMR temperature shifts of these NH groups (Figure S6) as well as the high LPE and PAMPA permeabilities of these compounds (Table S2). We hypothesized that crystal packing forces may impact their conformation, prompting us to investigate the solution structure of **3-Pye<sup>2</sup>(Ala<sup>3</sup>Nal<sup>5</sup>)** in CDCl<sub>3</sub>. By collecting NOESY spectra at various mixing times and performing a careful analysis of each crosspeak volume, we obtained a total of 20 crosspeaks that complied with the initial rate approximation.<sup>61–63</sup> The distance between the  $\delta$ -proline geminal protons (1.78 Å) was used as a reference to calculate other interproton distances (Table S5). After applying 10% random noise to the calculated distances, 17 out of the 19 distances matched the crystal structure, and the two remaining distances were close to the predicted distance which suggested the overall structural similarity between the crystallographic and solution structures.

We did not detect a C<sup>α</sup>H-to-C<sup>α</sup>H NOESY cross peak between Pro1 and Leu6, indicating that the Pro1 amide is most likely in the *trans* conformation. To obtain the solution NMR ensembles from the NOESY-derived distances, we performed an unrestrained conformational search using multicanonical molecular dynamics (McMD) simulations.<sup>64</sup> The 20 structures from the ensemble obtained by McMD at T = 300 K with the fewest violations from the NOESY-derived distances closely resembled the crystal structure (RMSD = 0.27 – 0.64 Å; Figure 6a–c) and were in a cluster that made up 9% of the unrestrained ensemble, although NOE violations averaged over the entire unrestrained McMD ensemble were also relatively low, indicating that the McMD simulation accurately captured the solution conformation in CDCl<sub>3</sub> (Table S5). The side chains were flexible except for the Pye2 side chain, which uniformly converged to form the expected SC-BB hydrogen bond.

The backbone NH groups of Ala3 and Leu6 formed the same transannular hydrogen bonding pattern as found in the crystal structure (Figure S14). The amides of Leu4 and Nal5, however, which formed crystal contacts in the X-ray structure, rotated inward in the solution ensemble to orientate toward the center of the macrocycle (Figure 6c). Specifically, the Leu4 NH reoriented to form a hydrogen bond with the C=O of Pye2, and the Nal5 NH reoriented to form a hydrogen bond with the C=O of Ala3 (Figure 6d–e), interactions that were absent in the crystal structure (Figure 6f). These reorientations around the 4<sup>th</sup> and 5<sup>th</sup> amide protons between the solid state and solution conformations amounted to a relatively subtle shift in overall backbone geometry, which is perhaps surprising given the net change in hydrogen bonding between the two structures. In solution all of the amide NH groups of **3-Pye<sup>2</sup>(Ala<sup>3</sup>Nal<sup>5</sup>)** are involved in IMHB, consistent with the high LPE and PAMPA permeability of **3-Pye<sup>2</sup>** and its derivatives. The large discrepancy in overall 3D polar surface area between the solid state and solution conformations despite similar backbone geometries suggests that dramatic shifts in 3D polar surface area can be modulated by relatively subtle shifts in amide rotamers.

### Effect of Different HBAs on Lipophilicity and Permeability

Data from various experiments above confirmed the lipophilicity and permeability improvements are due to the hydrogen bond from side chain HBA to the exposed HBD. To further expand the scope of HBAs that can be employed, we replaced Pye2 with four other HBAs, including *N,N*-dimethylamine substituent {**3-Gln(NMe<sub>2</sub>)<sup>2</sup>**}, methyl ester functional group {**3-Glu(OMe)<sup>2</sup>**} and two short side chain amides {**3-Asn(Pyr)<sup>2</sup>** and **3-Asn(NMe<sub>2</sub>)<sup>2</sup>**} (Figure 7a). Amide temperature coefficient experiments (Figure 7b) showed that changing substituent from pyrrolidine to *N,N*-dimethylamine {**3-Pye<sup>2</sup>** to **3-Gln(NMe<sub>2</sub>)<sup>2</sup>**} did not significantly change the chemical shift or the temperature coefficient of its backbone NH. In contrast, replacing the amide side chain with an ester {**3-Pye<sup>2</sup>** to **3-Glu(OMe)<sup>2</sup>**} caused a significant upfield shift and much more negative  $\delta_{\text{NH}} / T$  (-12.8 ppb/K), consistent with the decreased electron density at the ester carbonyl and the enthalpically weaker nature of the NH-to-ester hydrogen bond.

Shortening the Pye side chain by one methylene group {**3-Pye<sup>2</sup>** to **3-Asn(Pyr)<sup>2</sup>** or **3-Asn(NMe<sub>2</sub>)<sup>2</sup>**} shifted the backbone NH upfield and resulted in a much smaller (less

negative)  $\delta_{\text{NH}} / T$ . These shifts relative to the longer-chain Gln derivatives is consistent with the pattern observed in the simple model compounds, with the decreased temperature shift resulting from the lower entropic penalty associated with the shorter Asn chain and the upfield shift resulting from the differences in the H-bond geometry between the two sets. However, McMD simulations of **3-Asn(Pyr)**<sup>2</sup> in CHCl<sub>3</sub> offer another explanation: The shorter Asn side chain cannot reach its own backbone amide, although its close proximity to the NH groups of both residues 4 and 5 result in a bidentate H-bond with these two HBDS. Although this results in a completely new SC-BB H-bonding pattern, the overall backbone geometry is similar to that of **3-Pye**<sup>2</sup> (Figure S12). The McMD ensemble of **3-Asn(Pyr)**<sup>2</sup> shows no hydrogen bond to the amide NH of residue 2, consistent with its upfield shift and small temperature shift compared to the Gln derivatives (Figure S11). Testing these hypotheses will require further structural analysis.

Lipophilicity and permeability of all four chemotypes were similar to the **3-Pye**<sup>2</sup> scaffold (Figure 7a). LPEs of amide HBAs were within 0.2 log unit difference, while the LPE of the ester was slightly lower. Moreover, all compounds had PAMPA permeabilities within the same trend of **3-Pye**<sup>2</sup> library (Figure S2). Notably, *N,N*-dimethylamine substituent improved cyclic hexapeptide's solubility as expected from higher intrinsic hydrophilicity than pyrrolidine {**3-Pye**<sup>2</sup> vs **3-Gln(NMe<sub>2</sub>)**<sup>2</sup> and **3-Asn(Pyr)**<sup>2</sup> vs **3-Asn(NMe<sub>2</sub>)**<sup>2</sup>}. More surprisingly, considering amides with same substituents {**3-Pye**<sup>2</sup> vs **3-Asn(Pyr)**<sup>2</sup> and **3-Gln(NMe<sub>2</sub>)**<sup>2</sup> vs **3-Asn(NMe<sub>2</sub>)**<sup>2</sup>}, cyclic hexapeptides bearing Gln side chain, despite having higher ALogP, were more soluble than Asn side chain. A short Asn side chain may form IMHB even in the polar solvent unlike a flexible Gln side chain that may have an equilibrium between IMHB and solvent-exposed formations. Alternatively, the alternate low-dielectric conformation calculated for **3-Asn(Pyr)**<sup>2</sup> may be slightly less chameleonic, i.e., less able to access more polar conformations in aqueous solution. These experimental results broaden the opportunity to fine-tune lipophilicity of cyclic hexapeptide, for instance, by introducing a small amide substituent to enhance solubility of a highly hydrophobic cyclic hexapeptide, or a large amide substituent to slightly ameliorate solubility penalty while preserving overall permeability.

## Conclusion

The inability of traditional small molecules to target large protein interfaces steers medicinal chemists to search for novel scaffolds in the bRo5 chemical space. Cyclic peptides have the potential to bind previously “undruggable” protein targets, but poor membrane permeability due to their bRo5 and peptidic nature has impeded the application toward intracellular targets. In this work, we introduce a potentially general approach to mask the amide HBD using SC-BB from simple *N,N*-dialkylated derivatives of Gln and Asn. Our studies in cyclic hexapeptide models showed that *N,N*-disubstituted Gln derivatives can serve as a side chain HBA to sequester the exposed backbone NH group in scaffold **1** and **3** when these units are substituted next to the proline on the C-terminus. Moreover, the SC-BB hydrogen bonding helps promote the IMHB network within the cyclic hexapeptides by restricting backbone conformation based on the McMD calculations. As a result, we observed an improvement in permeability in both artificial and cell-based assays. This method can be applied to

any side chain combinations, and the HBA can be modified extensively by varying the functional group, amide substituents, or length of side chain. While polar and/or charged residues are often used to improve solubility in peptides, every polar proteinogenic residue also contains HBD, which are intrinsically deleterious to passive permeability. As a polar, non-proteinogenic residue that contains a strong HBA moiety but that lacks HBD, the Pye residue may allow medicinal chemists to modulate the chemical properties of cyclic peptides to balance their passive permeabilities, solubilities in the context of maintaining favorable target binding.

Masking exposed HBD by SC-BB hydrogen bonding could be used in conjunction with other methods to improve physico-chemical properties cyclic peptides. HBA-containing side chains can be introduced as pro-drugs, for

example, masking both the side chain functional group as well as the backbone amide in cases where SC-BB interactions are favorable.<sup>65–67</sup> However, since the Pye residue itself dramatically improves solubility while only slightly diminishing LPE, it may be generally useful for improving the properties of lipophilic cyclic peptide leads even in the absence of favorable SC-BB interactions. Therefore, this approach opens an opportunity to deliver cyclic peptides containing polar amino acids and backbone amide NH groups through the passive permeability pathway. Future studies will be directed toward evaluating the scope of Pye and similar residues in the context of improving the properties of bioactive compounds without abrogating their biochemical potency.

## Experimental Section

All reactions were carried out under an inert atmosphere (nitrogen, or argon where stated) with dry solvents under anhydrous conditions. Glassware for anhydrous reactions was dried in an oven at 140 °C for minimum 6 h prior to use. Dry solvents were obtained by passing the previously degassed solvents through activated alumina columns. Reagents were purchased at a high commercial quality (typically 97 % or higher) and used without further purification, unless otherwise stated. Amino acids and amines were purchased from Combi-Blocks, Oakwood, Sigma-Aldrich, or Chem-Impex. COMU and HATU were purchased from Combi-Blocks or Chem-Impex. Piperidine was purchased from Spectrum Chemical. 1,9-Decadiene was purchased from TCI Chemicals. 2-Chlorotrityl chloride polystyrene resin was purchased from Rapp-Polymere. High field NMR spectra were recorded with Bruker Avance III HD at 500 MHz for <sup>1</sup>H and 126 MHz for <sup>13</sup>C or Bruker Ascend™ with cryoprobe at 800 MHz for <sup>1</sup>H and 201 MHz for <sup>13</sup>C. NMR spectra were calibrated using residual non-deuterated solvent as an internal reference (CDCl<sub>3</sub>: <sup>1</sup>H NMR = 7.27, <sup>13</sup>C NMR = 77.0). The following abbreviations were used to explain the multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, quint = quintet, dd = double doublet, dt = double triplet, dq = double quartet, m = multiplet, br = broad. Purity of synthesized compounds were determined on an Advion AVANT HPLC-expression® CMS system using a C18 Kinetex® colum (30 x 2.1 mm, 2.6 μm 100 Å) at a flow rate of 0.4 mL/min. The mobile phase was composed of 0.1% (v/v) formic acid in Milli-Q water (solvent A) and 0.1% formic acid in CH<sub>3</sub>CN (solvent B). The gradient elution was ramped from 20 to 100% B over 6 minutes and held at 100% B for 1 minute. The detection was performed at 200 nm and 254 nm and the column

temperature in an oven was 50 °C. All individual synthesized compounds are 95% pure by HPLC or NMR analysis. **3-Pye<sup>2</sup>** and **3-Pye<sup>3</sup>** libraries were synthesized as purified mixtures and quantified via UPLC-MS and selected-mass monitoring.

### Loading Pye on 2-Chlorotrityl Resin.

2-Chlorotrityl chloride resin was swelled in dried CH<sub>2</sub>Cl<sub>2</sub> for 45 minutes. The solution of Pye and 2 eq of DIPEA in CH<sub>2</sub>Cl<sub>2</sub> (0.16 M) was prepared 10 minutes prior adding to the resin. The resin was shaken in the solution for 3 h and drained. The mixture of 1:2:17 <sup>i</sup>Pr<sub>2</sub>NEt:MeOH:CH<sub>2</sub>Cl<sub>2</sub> was added to cap any remaining active site. Resin was washed with CH<sub>2</sub>Cl<sub>2</sub> (3x), DMF (3x) and CH<sub>2</sub>Cl<sub>2</sub> (3x) and dried under vacuum overnight. The loading capacity was quantified by UV absorbance of the dibenzofulvene byproduct at 301 nm in ethanol by microcleavage of weighted resin.

### Automatic Linear Peptide Synthesis.

Linear peptides were synthesized using an automated peptide synthesizer (Prelude X, Protein Technologies). Commercial pre-loaded 2-chlorotrityl resin was used in 0.05 mmol scale. Fmoc deprotection was carried out with 20% piperidine in DMF for 3 minutes at 90 °C twice. Couplings were performed using Fmoc-protected amino acids (4 eq), HATU (3.8 eq), and <sup>i</sup>Pr<sub>2</sub>NEt (6 eq) in DMF (0.4 M with respect to amino acid) for 10 minutes at 90 °C. A capping step was performed after each amide coupling with a 1:1 mixture of acetic anhydride and <sup>i</sup>Pr<sub>2</sub>NEt in DMF. Each coupling, deprotection, and capping step was followed by a wash with DMF (6x), CH<sub>2</sub>Cl<sub>2</sub> (3x) and DMF (6x). Complete linear peptides were cleaved off resin with 30% HFIP in CH<sub>2</sub>Cl<sub>2</sub> for 1 h three times with a CH<sub>2</sub>Cl<sub>2</sub> wash equivalent to 5 resin volumes in between each step. Solvent was removed with Biotage V10 evaporator to obtain linear peptides as solid.

### Peptide Cyclization.

Crude linear peptides were dissolved in dry THF with 4 eq of <sup>i</sup>Pr<sub>2</sub>NEt and added dropwise to a solution of 1:1 THF/MeCN containing 2 eq of COMU for a final concentration of 2 mM under argon atmosphere. Reactions were stirred for 12–24 h until complete cyclization was achieved as monitored by LC-MS. The reaction was reduced in vacuo and purified via reverse-phased column chromatography using a Biotage Isolera Prime.

### Purification of Cyclic Hexapeptides.

Purification of cyclic peptides were carried by Biotage Isolera Prime equipped with Biotage Sfär Bio C18 D 25 g column eluting with the 20–100% MeCN/H<sub>2</sub>O gradient modified with 0.1% TFA at a flow rate of 40 mL/min.

### Synthesis for 3-Pye<sup>2</sup> and 3-Pye<sup>3</sup> Libraries.

Cyclic hexapeptide libraries were synthesized manually by split-pool approach (Scheme S3 and S4). Linear peptide sequences were synthesized from commercial pre-loaded 2-chlorotrityl-L-leucine resin starting with 200 mg for each R<sup>1</sup> amino acids (1 g total; 1.13 mmol). Fmoc deprotections were carried out with 20% piperidine in DMF for 20 minutes twice. Couplings were performed using Fmoc-protected amino acids (4 eq), HATU (4 eq),

HOAt (4 eq) and <sup>i</sup>Pr<sub>2</sub>NEt (8 eq) in DMF (0.3 M with respect to amino acid) for 1 h. After each coupling and deprotection step, the resin was washed with DMF (5x), CH<sub>2</sub>Cl<sub>2</sub> (3x) and DMF (5x), and monitored the completeness of reaction by Kaiser test with small amount of resin. After coupling the R<sup>2</sup> amino acids, the resin was kept separately as the sub library. Linear peptides were cleaved in 30% HFIP / CH<sub>2</sub>Cl<sub>2</sub> solution. Cyclization was performed same as mentioned above.

### Synthesis of Pye peptoid monomer.

2-Chlorotriptyl chloride resin was swelled in CH<sub>2</sub>Cl<sub>2</sub> 1 h before charging with 1M bromoacetic acid, 1M DIPEA in CH<sub>2</sub>Cl<sub>2</sub>. Resin was shaken for 1 h, then rinsed with DMF (3x) and CH<sub>2</sub>Cl<sub>2</sub> (3x). 3-Amino-1-(pyrrolidin-1-yl)propan-1-one (4 eq) was first free-base by shaking in NMP in the presence of powdered KOH (12 eq). The KOH powder was separated by centrifuging at 16,000 x g for 5 minutes. Resin was shaken in this supernatant for 2 h and washed with DMF (3x) and CH<sub>2</sub>Cl<sub>2</sub> (3x). Phenylacetic acid (3 eq), COMU (2.8 eq) and DIPEA (6 eq) were added to cap the resin. After successively wash with DMF (3x) and CH<sub>2</sub>Cl<sub>2</sub> (3x), 3M HCl generated by mixing acetyl chloride in anhydrous MeOH.<sup>68</sup> was added and the resin was shaken overnight to cleave the peptoid off the resin as well as undergoing methyl esterification simultaneously. The crude was dried under nitrogen stream and used for the experiment without further purification (scheme S5).

### General Protocol of UPLC-MS Analysis.

UPLC-MS analyses of cyclic peptides were performed via a Thermo Scientific Ultimate 3000 UPLC system, using a Thermo Hypersil GOLD C18 30 x 2.1 (1.9u) column (#25002-032130). Flow rate was set to 1 mL/min and a gradient method was as followed: 0.0–0.5 min, 5% MeCN; 0.5–0.75 min, ramp to 95% MeCN; 0.75–3.0 min, 95% MeCN; 3.0–3.5 min, 5% MeCN. Mass identification and quantification used an inline Thermo Scientific Orbitrap VelosPro (FTMS mode), tuned for maximum ionization of cyclosporin A, background ion locking on octyl phthalate, 200–2000 AMU mass windows, using +/- 0.02 AMU windows for integration.

### Shake Flask Partition Experiment.

Test compounds (400 μM, 8 μL) in DMSO were mixed in the solution of 800 μL 1,9-decadiene and 800 μL PBS buffer in Eppendorf tubes and agitated by vortex (30 min) and sonication (30 min). Tubes were centrifuged for 10 min at 16,000 x g to separate two layers. 150 μL of each layer was transferred to the 96-well plate in quadruplicate and evaporated overnight in a Genevac centrifugal evaporator (60 °C). Test compounds were resuspended in 150 μL of 1:1 MeCN/H<sub>2</sub>O and analyzed by UPLC-MS as described above.

### LPE calculation:

LPE was calculated from the equation published previously.<sup>53</sup>

$$\text{LPE} = \log D_{\text{dec/w}} - 1.06A\text{LogP} + 5.47$$

A<sub>LogP</sub> is a 2D molecular descriptor to represent the octanol/water partition coefficient determined from atoms in a molecule, which was calculated from Discovery Studio software.

#### **PAMPA Assays:**

A 96-well donor plate with 0.45  $\mu\text{m}$  hydrophobic Immobilon-P membrane supports (Millipore MAIPNTR10) was loaded with 5  $\mu\text{L}$  of 1% lecithin in *n*-dodecane. Cyclic peptides in PBS solution containing 5% DMSO were loaded into donor plate (150  $\mu\text{L}$ ) and attached to the acceptor plate having 300  $\mu\text{L}$  of 5% DMSO in PBS buffer. Each sample was run in quadruplicate for  $\sim$ 15 h at 20  $^{\circ}\text{C}$ . The concentration of each compound in the donor and acceptor wells was quantified by UPLC-MS to calculate  $P_{\text{app}}$ .

#### **Thermodynamic Solubility Assay:**

20  $\mu\text{L}$  of 10 mM stock solutions were dispensed into a 96-well conical plate and evaporated overnight in a Genevac centrifugal evaporator (60  $^{\circ}\text{C}$ ). PBS (100  $\mu\text{L}$ ) was reintroduced to the plate to yield a maximum 2 mM concentration, then the plate was sealed and sonicated for 1 h. The plate was then gently agitated at 37  $^{\circ}\text{C}$  for  $\sim$ 24 h. The mixtures were filtered through a 0.7  $\mu\text{m}$  glass fiber filter plate (Agilent 200965–100) into a 96-well conical plate. The filtrate was further diluted up to 40 fold with MeCN in a new 96-well plate for quantification via UPLC-MS. Standard curves of each compound were acquired from serial dilution of stock solution with DMSO (50  $\mu\text{M}$  to 0.1  $\mu\text{M}$ ) and used to calculate concentrations of analytes. All standards and analytes were performed in triplicate and averaged.

#### **Amide Proton Temperature Coefficient Experiment:**

Approximately 3 mg of model compounds or cyclic peptides samples were dissolved in 550  $\mu\text{L}$   $\text{CDCl}_3$  and  $^1\text{H}$  NMR spectra were recorded at temperatures 300, 305, 310, 315, 320 and 323 K. Chloroform residue was used for calibration, and temperature coefficients of amide protons were calculated from best fit of experimental data points.

#### **Caco-2 Cell Permeability:**

The Caco-2 assay was performed by Axcelead Drug Discovery Partners, Inc. (Kanagawa, Japan).

#### **Conformational Search McMD Simulations:**

The procedure of the conformational search was same as previous reports.<sup>52</sup> Briefly, flat potential energy was obtained corresponding to the temperature between at  $T = 280$  and 1505 K, and after production run, resampling method was used to obtain the ensemble at  $T = 300$  K. From this ensemble, 5,000 conformers were used to further analysis.

#### **Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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## ABBREVIATIONS USED

<b>AA</b>	arbitrary amino acid
<b>A<sub>LogP</sub></b>	atomistic calculated octanol/water partition coefficient
<b>Boc<sub>2</sub>O</b>	di-tert-butyl decarbonate
<b>bRo5</b>	beyond-Rule of 5
<b>Caco-2</b>	human colorectal adenocarcinoma cells
<b>COMU</b>	(1-cyano-2-ethoxy-2-oxoethylideneaminoxy)dimethylaminomorpholino-carbenium hexafluorophosphate
<b>DBU</b>	1,8-diazabicyclo[5.4.0]undec-7-ene
<b>DCM</b>	dichloromethane
<b>DMAP</b>	4-dimethylaminopyridine
<b>DMF</b>	<i>N,N</i> -dimethylformamide
<b>DMSO</b>	dimethylsulfoxide
<b>EDC</b>	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
<b>Fmoc</b>	9-fluorenylmethoxycarbonyl
<b>HATU</b>	1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate
<b>HB</b>	hydrogen bond
<b>HBA</b>	hydrogen bond acceptor
<b>HBD</b>	hydrogen bond donor
<b>HFIP</b>	1,1,1,3,3,3-hexafluoro-2-propanol
<b>HOBt</b>	hydroxybenzotriazole
<b>IMHB</b>	intramolecular hydrogen bond
<b>log <i>D</i></b>	shake flask distribution coefficient at pH 7.4
<b>LPE</b>	Lipophilic Permeability Efficiency
<b>MeCN</b>	acetonitrile



<b>MeOH</b>	methanol
<b>MW</b>	molecular weight
<b>NMM</b>	<i>N</i> -methylmorpholine
<b>PAMPA</b>	cell-free parallel artificial membrane permeability assay
<b>P<sub>app</sub></b>	apparent permeation rate (A-to-B, x10 <sup>-6</sup> cm/s)
<b>PBS</b>	phosphate-buffered saline
<b>Ro5</b>	Rule of 5
<b>SPPS</b>	solid phase peptide synthesis
<b>TFA</b>	trifluoroacetic acid
<b>THF</b>	tetrahydrofuran
<b>UPLC-MS</b>	ultra-high performance liquid chromatography mass spectrometry

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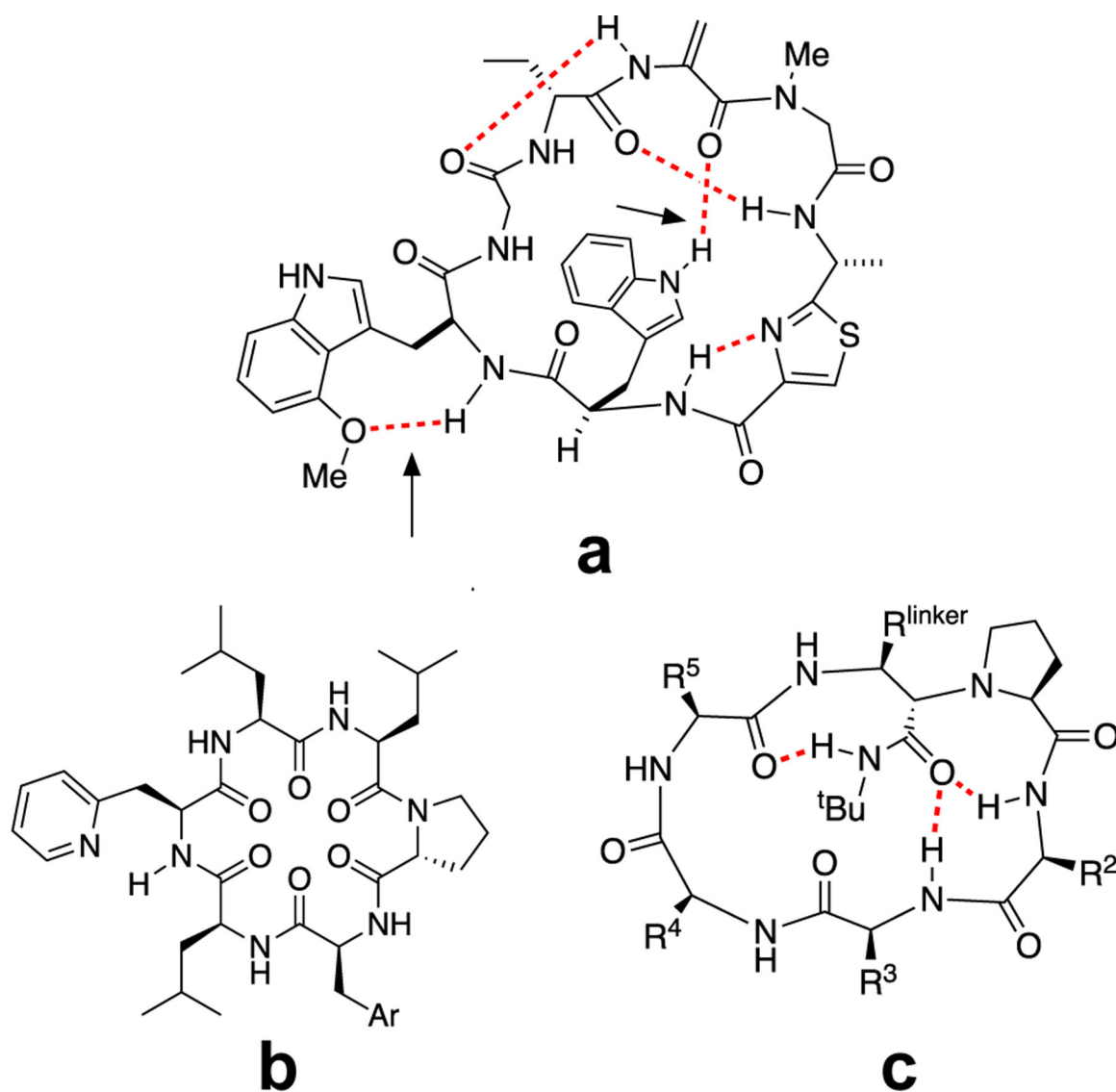
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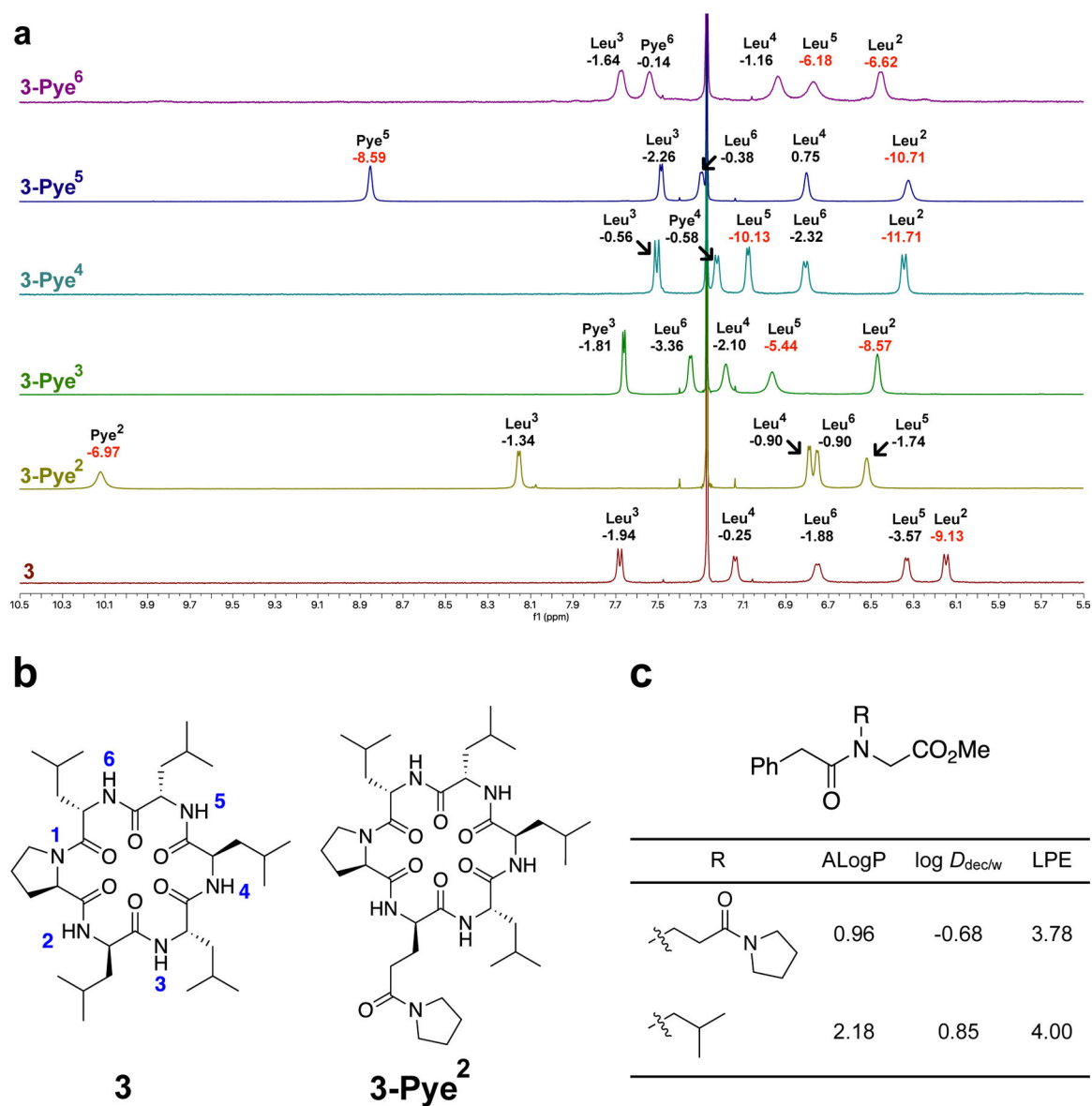
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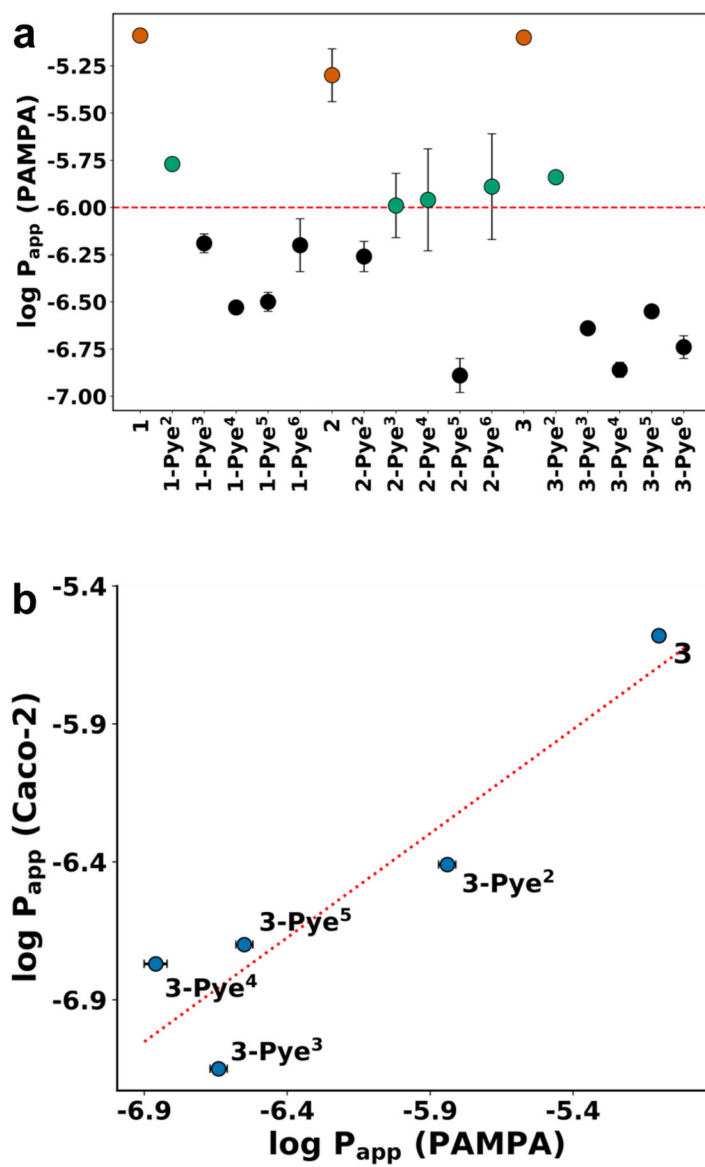
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**Figure 1.** Structures of **(a)** argyrin B, **(b)** Simpson's 2-pyridinylalanine cyclic hexapeptide and **(c)** Yudin's 18-membered ring macrocycle with exocyclic amide. Hydrogen bonds are indicated with dashed red lines.

**Figure 2.**

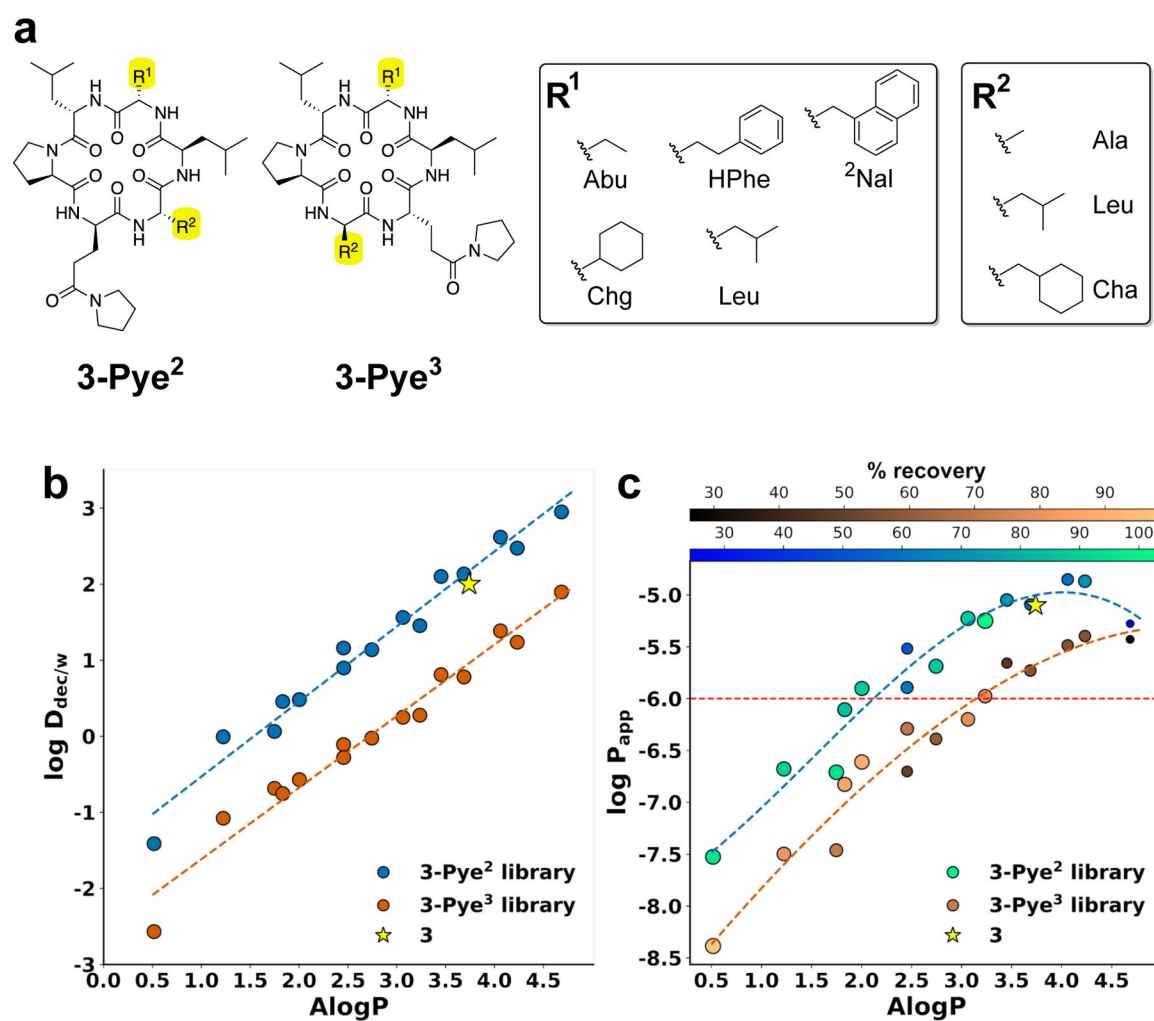
(a)  $^1\text{H}$  NMR of amide protons of **3** series at a concentration of  $\sim 10$  mM. Values under labels indicate the  $\delta_{\text{NH}}/T$  in ppb/K. Amide temperature coefficients less than  $-4.0$  are highlighted in red. (b) Structure of **3** and **3-Pye<sup>2</sup>**. (c) The intrinsic effect of Pye and isobutyl side chains on LPE in the absence of HBD.

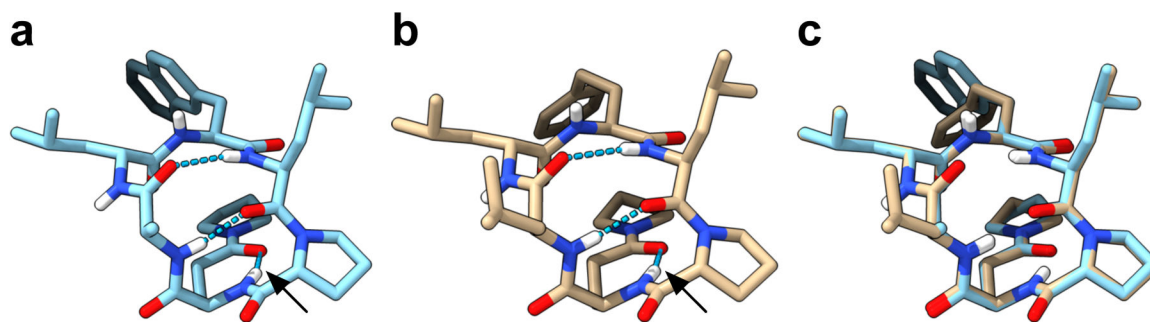


**Figure 3.**

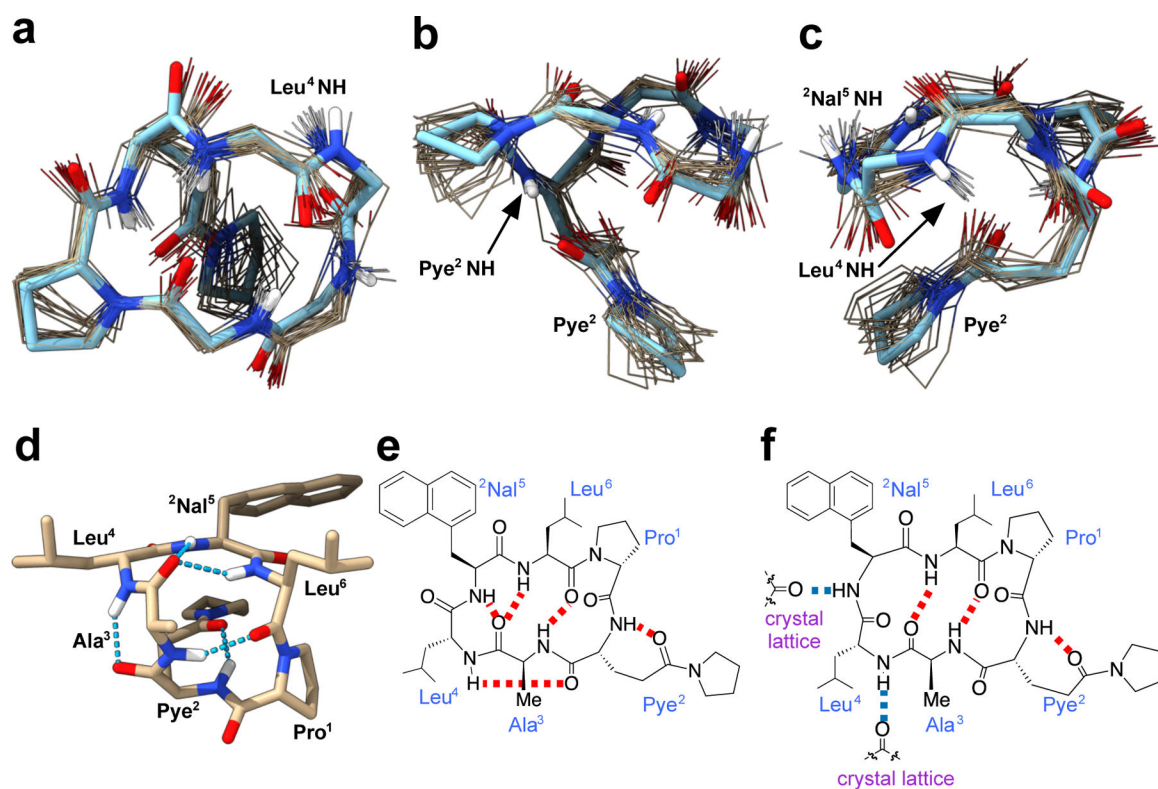
(a) PAMPA permeabilities of cyclic hexapeptides **1**, **2**, **3** and their derivatives. Minimum acceptable oral permeability is shown in dashed line. (b) PAMPA and Caco-2 experiments of compounds **3** and their derivatives show similar permeability rate ( $R^2 = 0.86$ ). High and low permeability standards are measured along with test compounds in Caco-2 experiment to ensure the accuracy;  $N=4$  for PAMPA experiment;  $N=1$  for Caco-2 experiment.



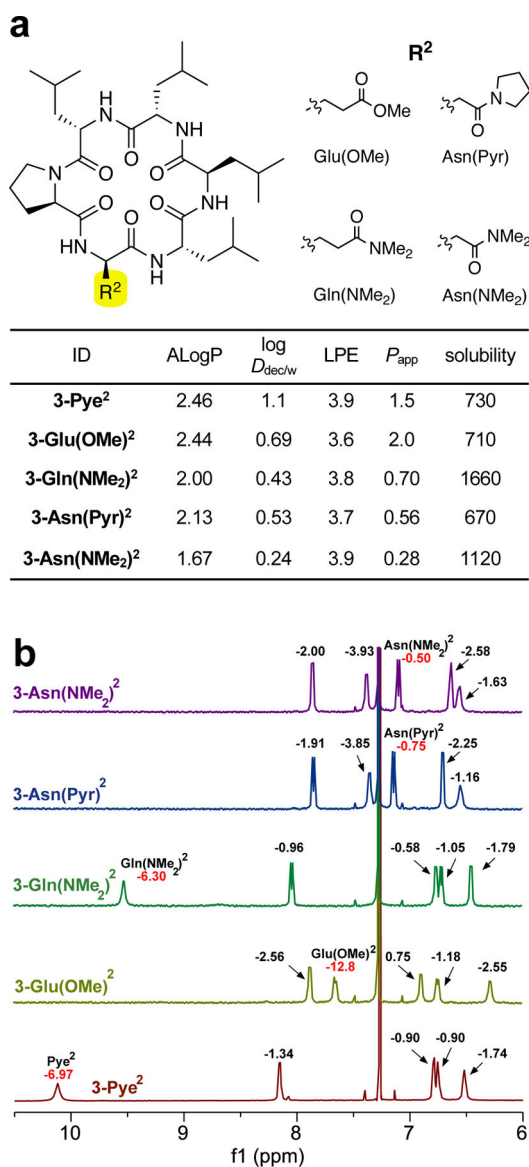




**Figure 5.** X-ray crystal structures of (a) **3-Pye<sup>2</sup>(Ala<sup>3</sup>Nal<sup>5</sup>)** and (b) **3-Pye<sup>2</sup>(HPhe<sup>5</sup>)**. Hydrogen bond between Pye and 2<sup>nd</sup> amide is indicated with black arrow. Putative hydrogen bonds are indicated with dashed blue lines. (c) Overlay between **3-Pye<sup>2</sup>(Ala<sup>3</sup>Nal<sup>5</sup>)** and **3-Pye<sup>2</sup>(HPhe<sup>5</sup>)**.

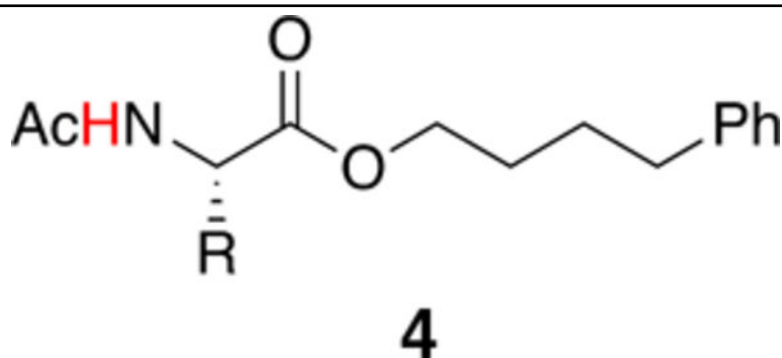


**Figure 6.**  
 (a-c) Overlays of  $3\text{-Pye}^2(\text{Ala}^3\text{Nal}^5)$  crystal structure (blue) and 20 simulated McMD structures (gold) derived from NOESY information. Only the backbone and Pye side chain are shown for clarity. (d) Structure of simulated  $3\text{-Pye}^2(\text{Ala}^3\text{Nal}^5)$  having 5 IMHBs. (e) IMHB pattern of (d). (f) IMHB pattern of  $3\text{-Pye}^2(\text{Ala}^3\text{Nal}^5)$  crystallographic structure.

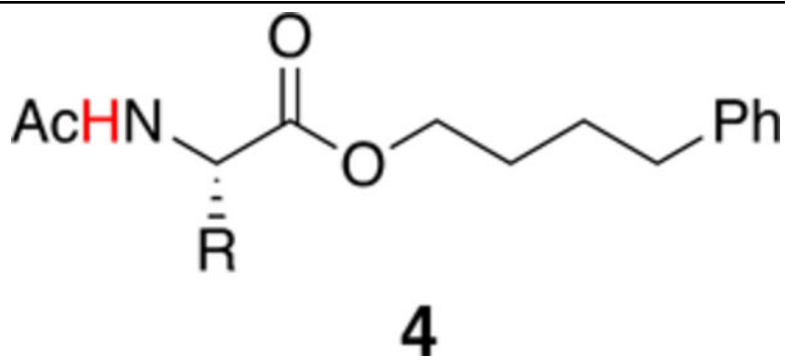
**Figure 7.**

(a) Compound 3 derivatives bearing different HBAs on 2<sup>nd</sup> position and their experimental data.  $P_{app} \times 10^{-6}$  cm/s, solubility in  $\mu\text{M}$ . (b) Amide-proton NMR spectra of compounds shown in (a). Amide NHs corresponding to the side-chain HBA residues are labeled, and the values indicate the  $\delta_{\text{NH}} / T$  in ppb/K.

Table 1.

Chemical Shifts and Amide Temperature Coefficients of Model Structures in CDCl<sub>3</sub><sup>a</sup>.

ID	R	$\delta_{\text{NH}}$ (300 K)	$\delta_{\text{NH}} / T$ (ppb/K)
4-Ala	Me	6.08	-3.54
4-Val		5.97	-3.31
4-Leu		5.86	-3.02
4-Ile		5.96	-2.95
4-Gln		6.59	-5.37
4-Glu(OMe)		6.18	-3.51
4-Gln(NMe <sub>2</sub> )		6.88	-6.48



ID	R	$\delta_{\text{NH}}$ (300 K)	$\delta_{\text{NH}} / T$ (ppb/K)
4-Gln(Pyr) (Pye)		7.11	-7.84
4-Asn(Pyr)		6.81	-1.66
4-Asn(NMe <sub>2</sub> )		6.89	-1.52

<sup>a</sup>All experiments were performed at a concentration of 50 mM or below.

**Table 2.**

## Pye-scanning Cyclic Hexapeptides Experimental Data

ID	$\log D_{dec/w}$	LPE <sup>a</sup>	$P_{app}$ <sup>b</sup>	solubility <sup>c</sup>
<b>1</b>	2.0	3.5	8.1	16
<b>1-Pye</b> <sup>2</sup>	1.3	4.1	1.7	640
<b>1-Pye</b> <sup>3</sup>	-0.41	2.5	0.66	550
<b>1-Pye</b> <sup>4</sup>	0.02	2.9	0.30	580
<b>1-Pye</b> <sup>5</sup>	-0.42	2.5	0.32	510
<b>1-Pye</b> <sup>6</sup>	-0.30	2.6	0.66	720
<b>2</b>	2.0	3.5	5.3	240
<b>2-Pye</b> <sup>2</sup>	-0.29	2.6	0.56	610
<b>2-Pye</b> <sup>3</sup>	-0.29	2.6	1.1	450
<b>2-Pye</b> <sup>4</sup>	0.040	2.9	1.3	460
<b>2-Pye</b> <sup>5</sup>	-0.82	2.0	0.13	550
<b>2-Pye</b> <sup>6</sup>	-0.29	2.6	1.5	560
<b>3</b>	2.0	3.6	8.0	63
<b>3-Pye</b> <sup>2</sup>	1.1	3.9	1.5	730
<b>3-Pye</b> <sup>3</sup>	-0.22	2.7	0.23	140
<b>3-Pye</b> <sup>4</sup>	0.21	3.1	0.14	580
<b>3-Pye</b> <sup>5</sup>	0.14	3.0	0.28	550
<b>3-Pye</b> <sup>6</sup>	0.16	3.0	0.18	600

<sup>a</sup>LPE =  $\log D_{dec/w} - 1.06 \times ALogP + 5.47^{53}$ ; ALogP = 3.74 for **1**, **2** and **3**; 2.46 for Pye-containing cyclic hexapeptides.

<sup>b</sup>PAMPA  $\times 10^{-6}$  cm/s, N=4.

<sup>c</sup> $\mu$ M, pH 7.4, N=3.