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Beyond cyclosporine A: conformation-dependent passive membrane permeabilities of cyclic peptide natural products

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Abstract

Many cyclic peptide natural products are larger and structurally more complex than conventional small molecule drugs. Although some molecules in this class are known to possess favorable pharmacokinetic properties, there have been few reports on the membrane permeabilities of cyclic peptide natural products. Here, we present the passive membrane permeabilities of 39 cyclic peptide natural products, and interpret the results using a computational permeability prediction algorithm based on their known or calculated 3D conformations. We found that the permeabilities of these compounds, measured in a parallel artificial membrane permeability assay, spanned a wide range and demonstrated the important influence of conformation on membrane permeability. These results will aid in the development of these compounds as a viable drug paradigm.

Keywords

cyclic peptide; cyclosporine; PAMPA; permeability

The macromolecules that have been successfully modulated by small molecule therapeutics make up only a small fraction of the total number of estimated drug targets [1]. Many other therapeutic target candidates, such as protein–protein interfaces, have binding surfaces that are larger and less pocket-like than traditional target sites [1,2]. While many of these interfaces are considered ‘undruggable’ by small molecules, larger and more structurally complex molecules such as antibodies [3–5] and **cyclic peptides** [6–14] can often achieve high-affinity binding to these more challenging targets. Despite failing to meet common physicochemical guidelines for drug-like cell permeability and bioavailability (such as Lipinski’s ‘Rules of 5′) [15], a number of cyclic peptides have shown activity against intracellular targets [11,16–21].

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An archetypal example is the **natural product cyclosporine** A (CSA), a cyclic undecapeptide immunosuppressant that is passively permeable and has an oral bioavailability of 29% [22]. While many other cyclic peptide natural products have structures that also suggest bioavailability [21], there have been no reports that systematically examine the **passive membrane permeabilities** of other naturally occurring cyclic peptides. Given the recent surge of interest in cyclic peptides and other macrocycles [20,23] and other 'beyond-Ro5′ (bRo5) compounds [24], we set out to investigate the membrane permeabilities of a set of diverse cyclic peptide natural products, including a number of CSA derivatives, in order to uncover structure/property relationships in this chemical space. We found that while their permeabilities vary widely, CSA is not an outlier among bRo5 cyclic peptide natural products with favorable passive permeability. We interpret these results using a computational method that predicts permeability based on 3D structure [25]; the computational results correspond well with the experimental data and provide insights into the chemical and structural features that promote passive permeability in this class of molecules.

Results

We obtained 39 cyclic peptide natural products from a variety of sources, with molecular weights ranging from 414 Da to 1619 Da (Figure 1). The majority of the 39 cyclic peptides included in this study fall into the category that we have defined as 'nonpolar', characterized by mostly aliphatic or lipophilic side chains and, in many cases, by N-methylated backbones and the ability to form intramolecular hydrogen bonds in nonpolar media [21]. We quantified the membrane permeabilities of the 39 cyclic peptides using the parallel artificial membrane permeability assay (PAMPA), a cell-free permeability assay that has been widely used to predict cell permeability and even oral bioavailability for small molecules [26] as well as cyclic peptides [27]. The PAMPA permeabilities spanned four log units, from compounds with high permeability that equilibrated over the 16 h PAMPA time scale ($\log P_e \geq -4.1$), to nonpermeable compounds that were undetected in the acceptor well over 16 h ($\log P_e \leq -8.4$).

Previously, our laboratories developed a physics-based model for predicting **passive membrane permeability**, which worked well for comparing the permeabilities of compounds within a similar scaffold class [25]. This model ($\mathrm{LogPm_{c/w}}$) was used to calculate the expected permeability for each compound, based on available crystal or NMR structure data, prior to obtaining the PAMPA data (full methods available in Supporting Information). We examined the ability of the calculated permeability values to distinguish between 'permeable' ($\log P_e$ equal or greater than −6.4, which corresponds to the permeability of cyclosporin E, a compound known to have intracellular activity [28]), versus poorly permeable ($\log P_e$ less than −6.4) compounds. The area under the curve of an ROC plot illustrates the ability to discriminate true positives from true negatives using the given metric. The $\mathrm{LogPm_{c/w}}$ demonstrated a strong capacity for discriminating between permeable and nonpermeable compounds (Figure 2A). The AUC of the $\mathrm{LogPm_{c/w}}$ ROC plot was 0.94, with SCH-218157 as the first false positive, followed by destruxin E chlorohydrin and desmethyldestruxin B. The $\mathrm{LogPm_{c/w}}$ model outperformed standard descriptors of passive

permeability (see Supplementary Figure 1 online at http://www.future-science.com/doi/full/10.4155/FMC.15.78).

When we calculated the linear correlation between the logPe and the $LogPm_{c/w}$, we saw that our model performed better than typical 2D descriptors of permeability such as molecular weight and polar surface area (see Supplementary Figure 2); however, the correlation was relatively weak, with an r^2 of 0.38 (Figure 2B). Interestingly, a positive trend between the $logP_e$ and the $LogPm_{c/w}$ was observed for the low permeability compounds, while a negative trend was seen for the highly permeable compounds. To investigate this behavior further, we performed linear regression to identify key physiochemical and structural properties contributing to passive permeability. A stepwise regression model for experimental permeability, with all molecular descriptors calculated by QikProp [29] and $LogPm_{c/w}$ accessible as initial variables, was built using 80% of the data and bootstrapping 1000-times in the program R [30]. The optimal model contained only a single descriptor in addition to $LogPm_{c/w}$: FOSA, or the hydrophobic component of the solvent-accessible surface area. FOSA was not correlated with $LogPm_{c/w}$ or %T or $logP_e$ independently and each variable was significant to the model (*Pm Model*), with p-values less than 0.0001. The resulting linear model had an r^2 of 0.66 (Figure 2D):

$$\mathrm{PAMPA}(\%T) = 100.17 + 11.54\ \mathrm{LogPm}_{c/w} - 0.09\ \mathrm{FOSA}$$

This relationship can be explained by the combined impact of size and hydrophobicity upon passive permeability as well as solubility. Large, hydrophobic compounds cannot readily pass through the lipid bilayer, as these compounds will tend to be rate-limited by resistance within the water layer itself [31]. The linear model captures this additional resistance as size and hydrophobicity increase. The relationship between FOSA and experimental permeability may be further explained by the low aqueous solubility of large, hydrophobic compounds, which can hinder experimental testing. The results observed for this set of diverse macrocycles highlight the importance of considering the impact of aqueous solubility on passive permeation, particularly for large, lipophilic compounds.

Characteristics of permeable cyclic peptides

Of the 39 cyclic peptides tested, a crystal or solution structure of the compound or a closely-related analog existed for 32 of them. From this set of compounds for which an experimental structure was available, 23 were classified as passively permeable, with a PAMPA $logP_e$ of greater than −6.4. Several conformational and structural features were present in this series that may contribute to passive permeability, including depsipeptide linkages, *N*-methylation, thiazole/oxazole rings and intramolecular hydrogen bonding, all of which either eliminate or sequester backbone amide NH groups (Figure 3). Eight of the permeable compounds include at least one depsipeptide linkage in their peptide backbone, reflecting the prominence of this linkage among cyclic peptide natural products and the largely untapped potential of depsipeptides as cell permeable scaffolds. Patellamide C, one of the most permeable compounds with a $logP_e$ of −4.7, demonstrates the power of thiazole/oxazole rings as a mechanism for eliminating hydrogen bond donors and modulating flexibility with half its backbone amides involved in these rings. In contrast, the destruxins contain only a single

pyrrolidine ring and thus have lower passive permeabilities, with $\log P_e$ values ranging from −7.3 to −5.3.

The importance of *N*-methylation and intramolecular hydrogen bonding in the passive permeability of macrocycles is also highlighted in this group of compounds. Only one compound with measurable permeability had no amide *N*-methylation, tentoxin, which is the smallest compound tested (414.5 MW). Moreover, tentoxin is the only compound among those containing free amide protons that does not exhibit intramolecular hydrogen bonding. The remaining permeable compounds that are not permethylated contain at least one intramolecular hydrogen bond in their experimental structures. Finally, three out of the five most permeable compounds (enniatin B, CSA and patellamide C) adopt solution **conformations** in which every amide NH is either N-methylated (enniatin B) or is involved in an intramolecular hydrogen bond (patellamide C and CSA). Not only does backbone *N*-methylation cap polar N-H groups that would otherwise require desolvation upon diffusion into the membrane [21,32–38], but it can also stabilize ‘ membranephilic’ conformations [27,37,39–41].

Characteristics of nonpermeable cyclic peptides

Most of the cyclic peptides that displayed poor passive permeability either contained one or more ionizable groups, such as mirabamide C and daptomycin, or highly polar side chains, such as SCH-218157 and verrucamide B. The two destruxin chlorohydrin derivatives also showed relatively poor permeability, especially compared with the other destruxins included in this study. No crystallographic structure was available for a chlorohydrin derivative of destruxin. Although their low-dielectric backbone conformations are predicted to be similar to that of the parent destruxins, with both amide NH groups involved in transannular hydrogen bonds, the deleterious effect of the chlorohydrin substitution was anticipated by our computational predictions of $\text{LogPm}_{c/w}$, reflected in the significantly higher desolvation penalty associated with the chlorohydrin moiety. In other cases such as the enniatins, the $\text{LogPm}_{c/w}$ calculation overpredicted the permeability values of the more weakly permeable analogs. Enniatin B was the most permeable analog with $\log P_e$ of −4.7 and $\text{LogPm}_{c/w}$ of 3.31, indicating moderate permeability, while the least permeable Enniatin H has a higher predicted permeability with $\text{LogPm}_{c/w}$ of 4.02. The majority of the 12 least permeable compounds did not have an available experimental structure, which may have contributed to the differences between their predicted permeabilities and PAMPA data. For example, PC1026 is predicted to be modestly permeable, with an expected $\log P_e$ of −5.6, while its observed value was −7.2. Also, despite its expected $\log P_e$ of −5.75, the measured permeability of sclerotiotide F was below the PAMPA assay’s detection limit. These results may highlight the difficulty in accurately predicting the solution conformations of cyclic peptide natural products with nonproteinogenic side chains and backbone elements.

Permeability trends within related analogs

The destruxin analogs possessed low permeability overall, ranging from a $\log P_e$ of −7.35 for destruxin E2 chlorohydrin up to −5.29 for destruxin B, with only destruxins A, B and B2 displaying moderate permeability in the PAMPA system. The most striking observation was the significant decrease in permeability caused by removal of one *N*-methyl group from

destruxin B to yield desmethyldestruxin B (NMeVal->Val), in which the $\log P_e$ dropped from −5.29 to −6.67. NMR studies coupled with computational modeling predicted that desmethyldestruxin B is conformationally homogenous and contains the same intramolecular hydrogen bonds as destruxin B [42,43]. However, the proposed structure also contains longer and weaker transannular hydrogen bonds [43], which can oppose passive permeability.

CSA is a well-studied 11-residue cyclic peptide with seven N-methylated amino acids and it is passively permeable in both PAMPA and cell-based permeability assays. In the X-ray structure [44], CSA contains four intramolecular hydrogen bonds between amide protons and carbonyls; the solution structure of CSA in $CDCl_3$ contains an additional hydrogen bond between the Bmt –OH and Bmt carbonyl [44–49]. We tested the passive permeabilities of CSA ($\log P_e$ = −5.01) in addition to seven natural and synthetic CSA analogs with structural variations from the parent ranging from very conservative side chain substitutions (e.g., CSB; Abu^2 -> Ala^2), to those with more dramatic backbone modifications (e.g., CSE; $MeVal^{11}$ -> Val^{11}). The cyclosporine analogs exhibited a range of permeability values in the PAMPA assay, with most analogs having moderate-to-good permeability.

CSA analogs with conservative substitutions adopt similar conformations in solution to that of the parent compound [47] and we found these analogs had only small decreases in permeability relative to CSA. The CSH (L-$MeVal^{11}$ -> D-$MeVal^{11}$) analog demonstrated similar passive permeability to CSA ($\Delta\log P_e$ = −0.35). This was expected based on previous modeling indicating that, despite the stereochemical inversion at $MeVal^{11}$, CSH adopts a low-dielectric structure very close to that of CSA in $CDCl_3$ [47,48]. Similarly, a minor impact on passive permeability ($\Delta\log P_e$ = −0.21) was observed for iso-CSA, which is derived from the acid-catalyzed *N*-to-*O* acyl shift of the $MeVal^{11}$ carbonyl onto the Bmt–OH [50]. In addition to the basic amine resulting from the rearrangement, the crystal structure of iso-CSA shows two exposed amide NH groups that are involved in intermolecular hydrogen bonds in the unit cell [51]. The crystal structure of iso-CSA also suggests that the Bmt side chain is capable of folding over the secondary amine, possibly lowering the desolvation energy by steric shielding [52,53]. However, the low-dielectric solution conformation of iso-CSA may be closer to that of the parent compound, and the 2° amine may not have a large negative impact on permeability since its calculated pK_a of 7.8 [54] suggests that a significant portion of the neutral species exists at pH 7.4.

CSC (Abu^2 -> Thr^2) [55] has somewhat reduced permeability ($\Delta\log P_e$ = −0.55) relative to CSA, presumably due to the addition of a polar –OH substituent. The difference in permeability is predicted by the computational model, which calculates a substantially lower permeability for CSC compared with CSA (3.18 vs 5.59). CSA acetate, which is acetylated on the Bmt – OH group [56] also displayed a decrease in permeability compared with CSA ($\Delta\log P_e$ = −0.47). A crystal structure of this nonimmunosuppressive analog has been published, and the compound exhibits a backbone conformation highly similar to that of CSA (RMSD = 0.27 Å) [57].

CSE ($MeVal^{11}$ -> Val^{11}) was the least permeable of any of the CSA derivatives ($\Delta\log P_e$ = −1.37). In the crystal structure of CSE, the unmethylated-NH group is hydrogen bonded to

the carbonyl of D-Ala[8], and the Bmt –OH is hydrogen bonded to the carbonyl group of the Sar residue [58]. The higher polarity and altered hydrogen bonding pattern relative to that of CSA suggests that the additional hydrogen bond donor present in CSE has the potential to allow for alternative networks of intramolecular hydrogen bonds in solution. These might be strong enough to sufficiently penalize adoption of the low-dielectric conformation such that passive diffusion through the membrane occurs, but is less favored.

In general, the destruxin and CSA derivatives with conservative side chain substitutions had permeabilities that were close to that of the parent compound. Our findings across these related analogs illustrate that within a given scaffold, permeability can be modulated by the polarity of the side chains [59]. Importantly, alterations in backbone stereochemistry and *N*-methylation patterns are known to be major determinants of the conformation of any given cyclic peptide, which in turn can have a significant impact on passive permeability [27]. Therefore, it is critical that substitution of these features be carefully examined within the context of a molecular scaffold to ensure optimal placement.

Conclusion

CSA has long been considered a prototypical ‘rule breaker’: a cell permeable and orally bioavailable drug that grossly violates common rules-of-thumb for drug-likeness. However, we have shown here that many other cyclic peptides with different scaffolds are also passively permeable. Using PAMPA as a model membrane avoided potential active transport effects that could confuse the mechanism through which permeation occurs. Further, we derived a model that allows us to predict passive membrane permeability among a diverse set of cyclic peptide natural products. This model was most successful for cyclic peptides for which solution conformations could be inferred from NMR or crystal structure data, highlighting the difficulty of predicting structures of complex macrocyclic natural products. Our results indicate that the permeability of cyclic peptide natural products, even relatively lipophilic ones, varies widely. We also identify a number of non-CSA scaffolds that possess drug-like passive permeability. In addition to clarifying known mechanisms that contribute to good permeability of these compounds, we have elucidated new ones, such as depsi-linkages and heterocycles incorporated into the peptide backbone. Cyclosporine A is clearly not a ‘lone island’ of oral bioavailability among bio active macrocycles, but in fact is representative of many macro cycles with promising permeability properties, which have been heretofore largely uncharacterized.

Future perspective

This work confirms the potential of drug-like macrocyclic peptides as membrane permeable scaffolds. We expect to see an increase in the pharmaceutical development of cyclic peptides as potential drugs, as well as an increase in the number of available, clinically relevant targets for macrocycles to modulate. Our data challenges conventional models for passive membrane permeability and supports the possibility of increased numbers of orally bioavailable, bioactive cyclic peptide drugs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Key terms

Cyclic peptide
A molecule composed of sequential amino acids, typically connected through amide linkages, with the N- and C-terminus connected via an amide bond. Linkages can also be depsi bonds, which are ester bonds instead of amides. Many cyclic peptides are produced endogenously by bacteria and fungi and have bioactivity

Natural product
A compound produced endogenously by an organism, typically bacteria, fungi or lesser eukaryotes. Natural products have been the source or inspiration of many clinically relevant drugs

Cyclosporine A
A large (MW=1202) cyclic peptide natural product that is clinically used as an immunosuppressant. Cyclosporin A is highly orally bioavailable and passive permeable. It has been believed to be a canonical molecule in its ability to cross a cell membrane despite its large size

Passive membrane permeability
The process of a compound diffusing through a cell membrane directly through the hydrophobic layer, without the aid of active transport mechanisms. Most drugs are believed to be absorbed in the intestinal lumen through passive diffusion

Conformation
The 3D shape adopted by a molecule. Conformations can change depending on the chemical environment, and have been shown to be important considerations for the passive membrane permeability of a molecule

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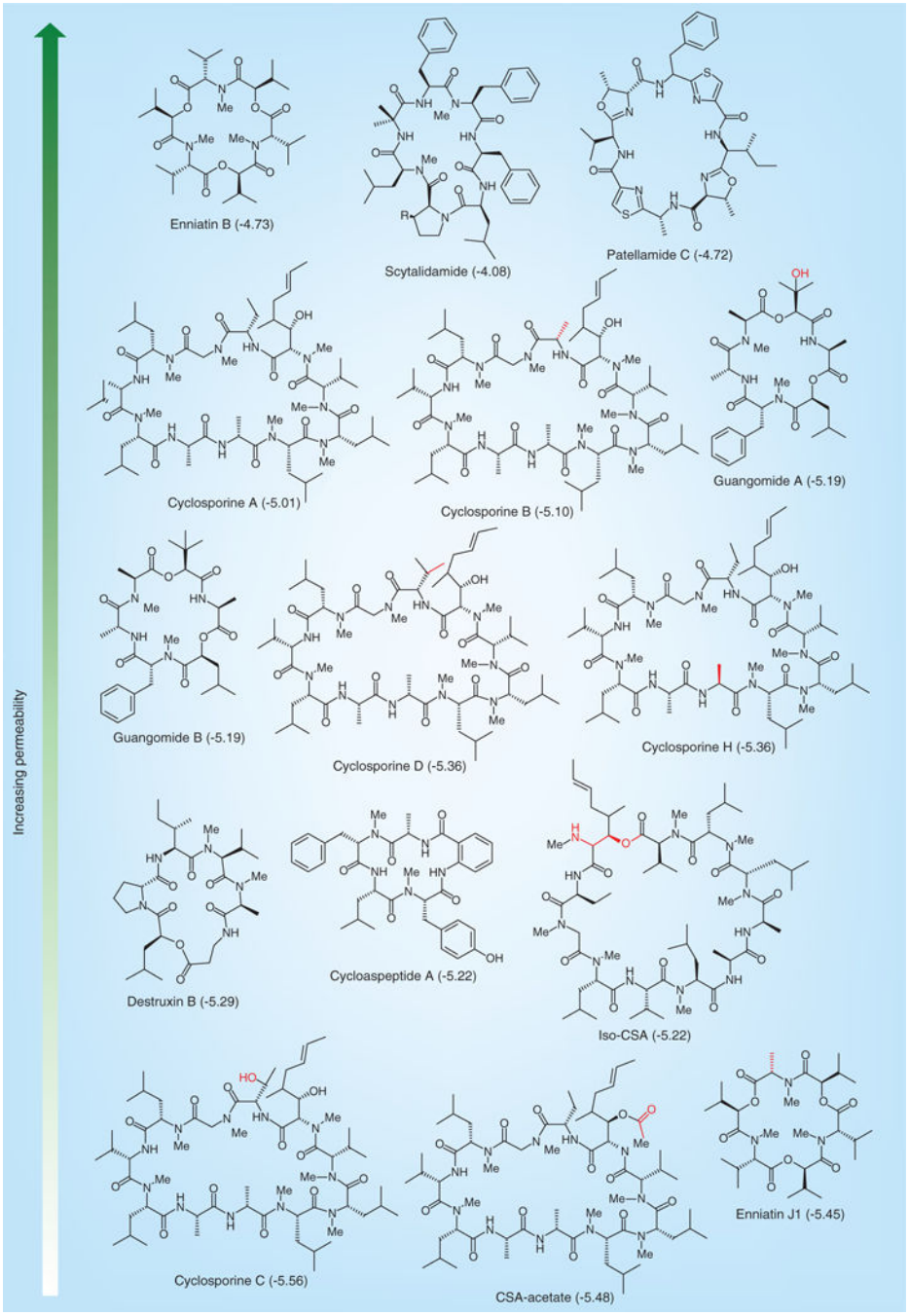
Executive summary

- Cyclic peptides and other macrocycles have the potential to access chemical space to interact with targets (e.g., protein–protein interactions) that are typically inaccessible to small molecule screening collections. However, cyclic peptides in particular have often been overlooked by medicinal chemists due to their violation of common predictors of ‘drug-likeness’ like the Rule of 5.
- We tested 39 natural product cyclic peptides in a passive membrane permeability assay (PAMPA). There was significant structural variation (in terms of MW, side chains, stereochemistry, and so on) in this set, as well as a 4-log-unit range in their ability to penetrate the PAMPA membrane.
- Using a physics-based computational algorithm, the majority of permeabilities were successfully classified as ‘permeable’ or ‘not permeable.’ Further computational analysis elucidated the effects of solubility and size on permeability.
- Most of the compounds had previously published conformations (either by NMR or x-ray crystallography), with many of the permeable compounds showing different ways of hiding polar N-H groups (depsilinkages, intramolecular hydrogen bonds, *N*-methylation).
- Nonpermeable compounds were either extremely large (MW >1300), or had ionizable side chains.
- The CSA analogs varied in permeability, demonstrating the powerful effect of conformation on the ability of a molecule to cross a hydrophobic membrane.



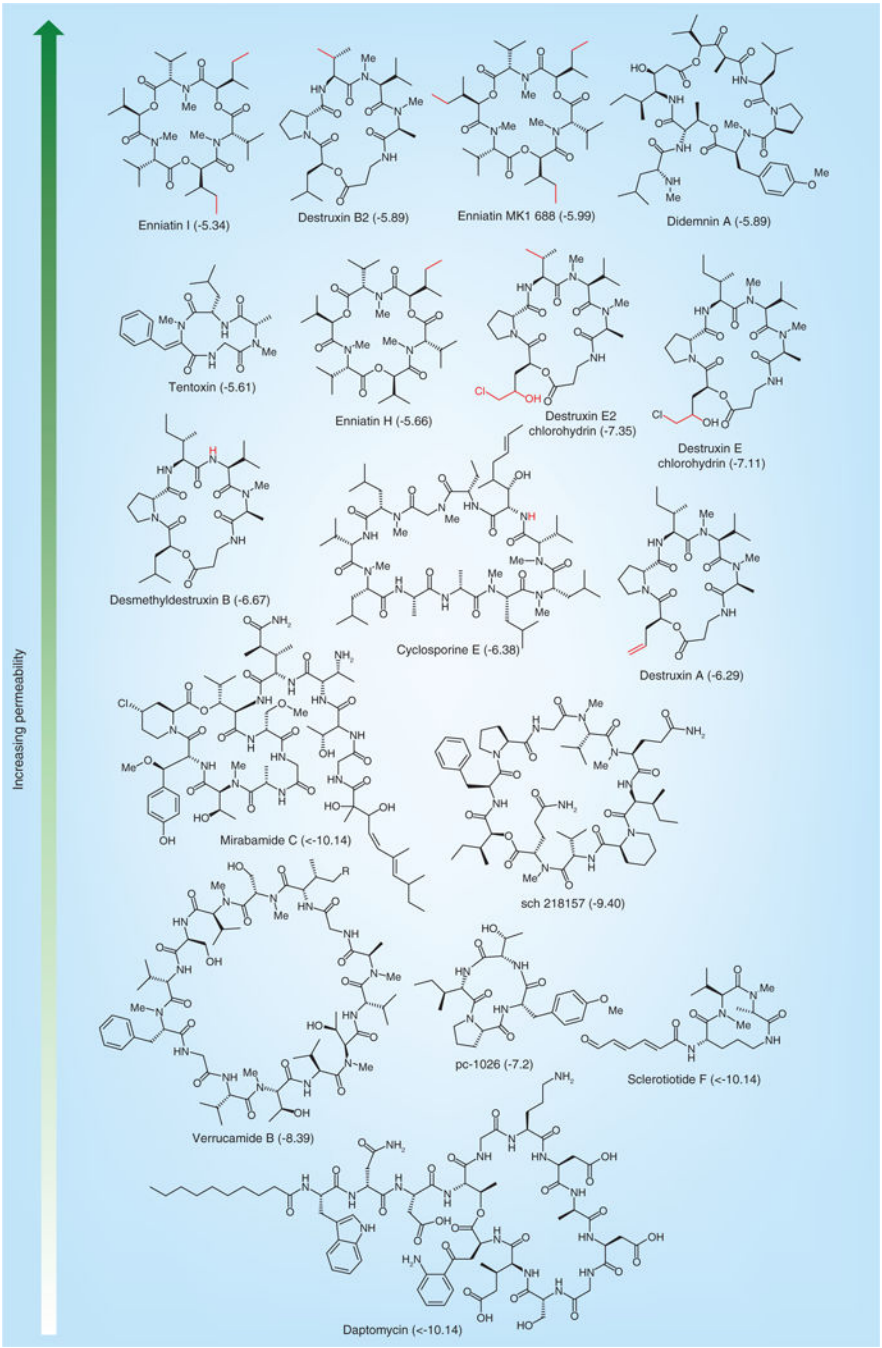


Figure 1. 2D structures of 32 of the compounds tested with log(P_e) permeability values
Differences between analogs and the related parent compounds highlighted in red.

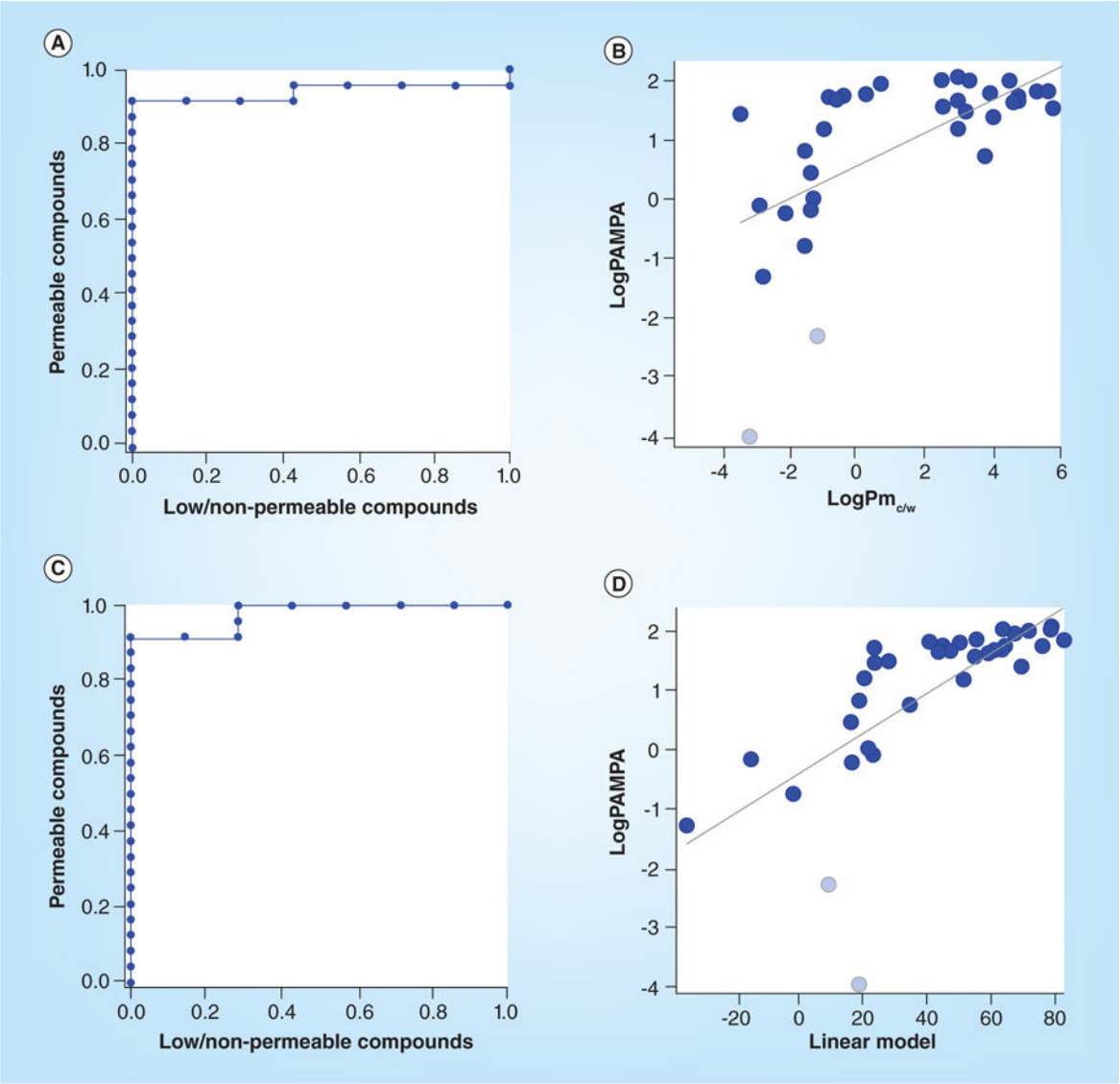


Figure 2. Relationship between predicted and experimental permeabilities

(A) The ROC plot for permeable and nonpermeable compounds based on $LogPm_{c/w}$ (AUC = 0.94) demonstrating good discrimination of true positives from true negatives; **(B)** the correlation between the experimental permeability ($logP_e$) and the predicted permeability $LogPm_{c/w}$ was fairly weak ($r^2 = 0.38$); **(C)** the linear model combines $LogPm_{c/w}$ and FOSA, demonstrating a similar ROC plot (AUC = 0.98); **(D)** improved quantitative correlation ($r^2 = 0.66$) for all natural product compounds. The two outlying light blue dots represent compounds lacking any form of structural information.

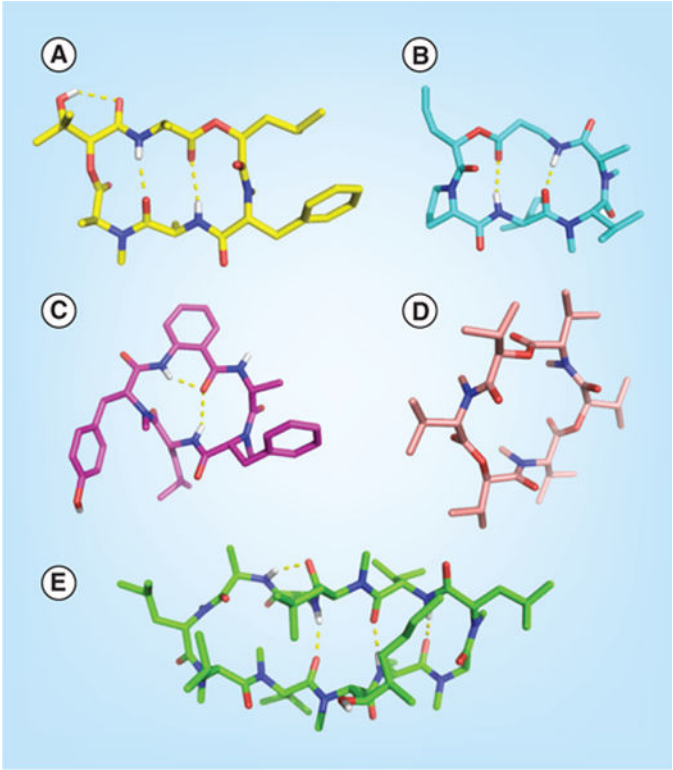


Figure 3. Crystal structures of selected permeable compounds

Hydrogen bonds are shown as yellow dashes. **(A)** Guangomide A; **(B)** destruxin A; **(C)** cycloaspeptide A; **(D)** enni-atian B; **(E)** cyclosporine A.