# An amide-to-chloroalkene substitution improves the peptide permeability

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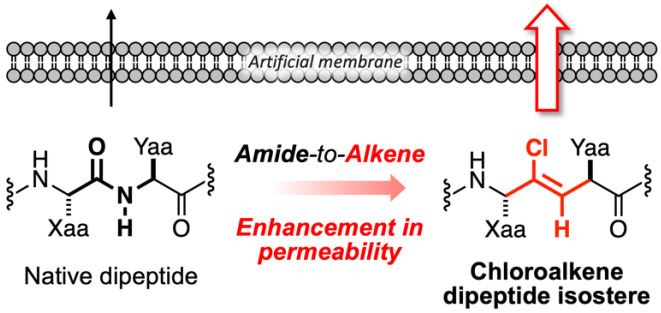



**Abstract:**

This study highlights the novel application of Chloroalkene Dipeptide Isosteres (CADIs) in enhancing peptide membrane permeability. Replacing the peptide bond with CADIs in model dipeptides significantly improved passive permeability. This enhancement is attributed to the increased lipophilicity provided by the CADI substitution, as confirmed by AlogP calculations and HPLC retention times. Molecular dynamics simulations further indicated that CADI substitution reduces water interaction, potentially lowering hydration energy. Our findings demonstrate that CADI incorporation can effectively improve the permeability of peptides, offering a valuable approach for developing bioactive peptidomimetics with enhanced pharmacological properties including permeability and hydrolytic stability.

**Introduction:**

Peptides are emerging as key molecules in drug discovery and development due to their high specificity and potency. However, beyond their susceptibility to rapid enzymatic hydrolysis, their low membrane permeability presents a significant challenge in peptide drug discovery.[1] One of the primary reasons for this low permeability is the highly polar amide bonds forming the backbone of peptides.[2] These bonds, due to their H-bonding character, are prone to hydration, which creates a high desolvation energy barrier during membrane translocation, thereby hindering permeability.[3]

To enhance peptide permeability, various chemical modifications have been explored. A well-studied modification is the N-methylation[4] of the amide bond in cyclic peptides, which reduces the number of hydrogen bond donors (HBDs), lowering desolvation penalty and improving membrane permeability. Another approach involves using peptide bond isosteres, such as esters[5] and thioamides.[6] Ester substitution also reduces the number of HBDs by removing the amide proton, which improves membrane permeability similar to the mechanism of N-methylation. On the other hand, thioamide substitution enhances membrane permeability by reducing the hydrogen bond acceptor (HBA) ability of the carbonyl oxygen, which lowers hydration energy and simultaneously enhances thermostability.[7] Therefore, chemical modifications that control the H-bonding ability of the peptide backbone are effective strategies for improving membrane permeability in peptides.

As part of our research programs on peptidomimetics for peptide drug discovery, we have explored alkene dipeptide isosteres (ADIs) (**Figure 1A**),[8] where the peptide bond is replaced by a structurally similar carbon-carbon double bond. These isosteres, including (*E*)-alkene,[9] (*E*)-methylalkene,[10] (*Z*)-fluoroalkene,[11] and (*Z*)-chloroalkene,[12] have been applied to various bioactive peptides to improve resistance to enzymatic degradation[13] and chemical stability.[14] Since ADIs lack H-bond donors and possess only weak H-bond acceptors derived from halogen atoms (**Figure 1B**),[15]

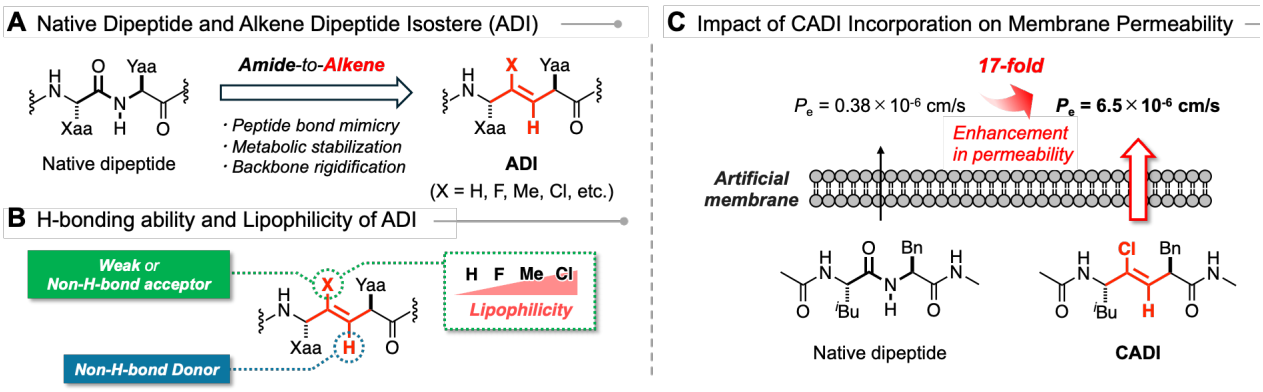



**Figure 1. (A)** Structure and characteristics of ADI. **(B)** Potential of ADI as permeability enhancer to improve the permeability of peptides. **(C)** Membrane permeability of L-Leu-L-Phe-type CADI mimic measured by PAMPA.

their introduction into peptides can significantly alter the H-bonding properties of the peptide backbone. While these modifications have potential for enhancing membrane permeability, its impact remains underexplored.

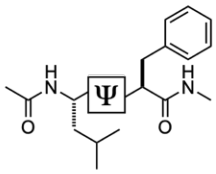
In this study, we sought to substitute a peptide bond with an ADI to probe the effect of alkene substitution on membrane permeability. we directly compare the membrane permeabilities of dipeptides and their corresponding peptidomimetics containing chloroalkene dipeptide isosteres (CADIs) (**Figure 1C**). Our study demonstrates that CADI incorporation into the backbone of dipeptides significantly improves the passive permeability.

**Result and Discussion:**

**Comparison of AlogP value.** To investigate the effect of ADI incorporation on membrane permeability, we employed the Leu-Phe and Phe-Phe models, which were selected due to their hydrophobic side chains, offering sufficient lipophilicity and enabling the detection of the peptides in permeability experiments.[16] At the onset of our studies, we calculated the AlogP values[17] of Ac-Leu-Phe-NHMe, Ac-Phe-Phe-NHMe, and their corresponding isosteres, including N-methyl amide, ester, thioamide, and representative alkenes, to assess their lipophilicities (**Table 1**). AlogP was derived from a regression model based on atomic lipophilicity and is used here to predict how structural modifications may affect the overall hydrophobic character of the peptides. The results indicate that the ADI mimics exhibit higher AlogP values compared to their respective amides and other isosteres. These findings show a clear trend towards higher lipophilicity with ADI incorporation. Notably, the

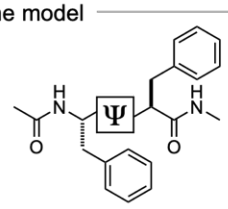
**Table 1.** AlogP values of diepeptides and their peptide bond isosteres. **(A)** Leu-Phe model. **(B)** Phe-Phe model.

**A** Leu-Phe model



| Comp. | Ψ | AlogP |
|---|---|---|
| **1** | -C(=O)-NH- | 1.01 |
| **2** | -C(=O)-NMe- | 1.49 |
| **3** | -C(=O)-O- | 1.68 |
| **4** | -C(=S)-NH- | 2.21 |
| **5** | -[(*E*)CH=CH]- | 2.70 |
| **6** | -[(*Z*)CF=CH]- | 2.89 |
| **7** | -[(*Z*)CCl=CH]- | 3.20 |
| **8** | -[(*E*)CMe=CH]- | 2.93 |

**B** Phe-Phe model

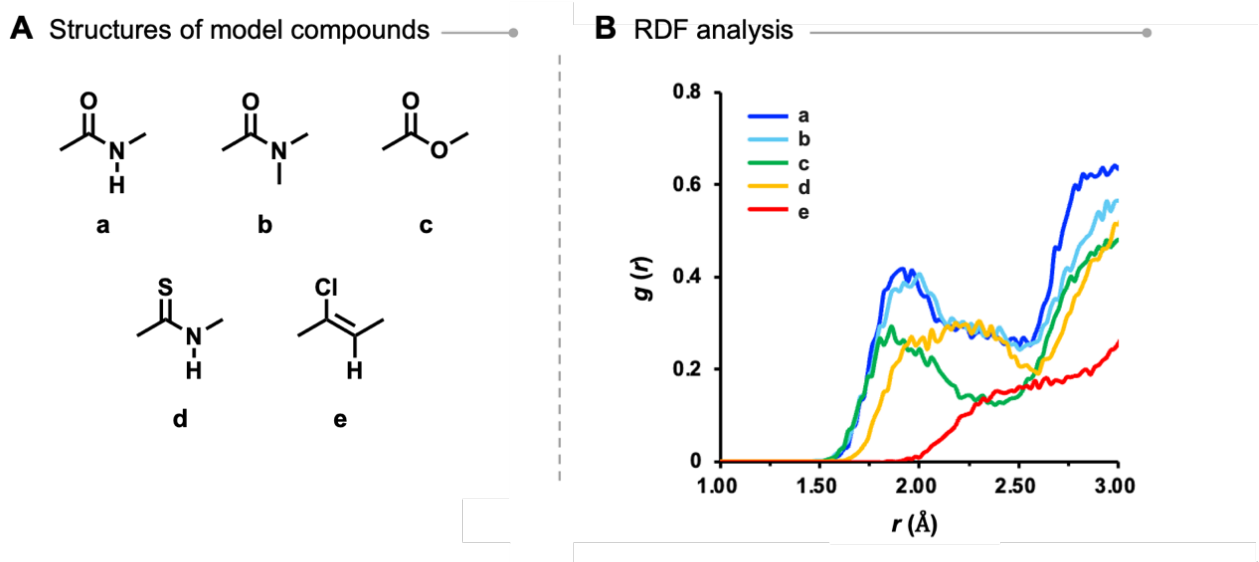


| Comp. | Ψ | AlogP |
|---|---|---|
| **9** | -C(=O)-NH- | 1.44 |
| **10** | -C(=O)-NMe- | 1.89 |
| **11** | -C(=O)-O- | 2.02 |
| **12** | -C(=S)-NH- | 2.76 |
| **13** | -[(*E*)CH=CH]- | 3.06 |
| **14** | -[(*Z*)CF=CH]- | 3.21 |
| **15** | -[(*Z*)CCl=CH]- | 3.48 |
| **16** | -[(*E*)CMe=CH]- | 3.40 |

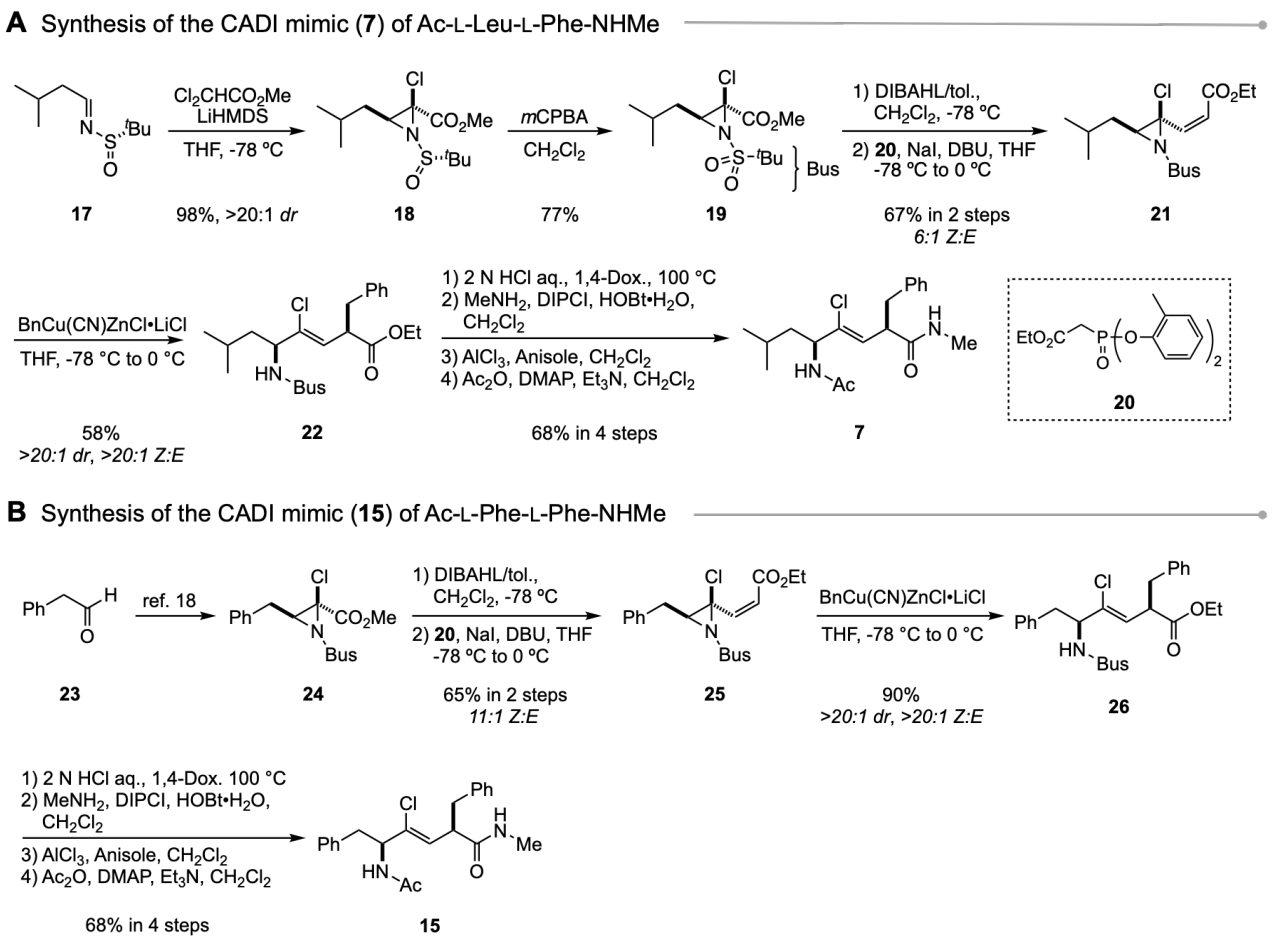
chloroalkene isostere, where chlorine substitutes for the carbonyl oxygen, displayed the highest lipophilicity among the ADI mimics.

**MD simulations.** To further investigate the influence of ADI incorporation on peptide hydration properties, molecular dynamics (MD) simulations were carried out to assess the interactions between water molecules and *N*-methylacetamide (NMA, **a**) and the corresponding isosteres, including *N*,*N*-dimethyl acetamide (**b**), ester (**c**), thioamide (**d**), and CADI (**e**) (**Figure 2**). Radial distribution function (RDF) analysis was used to measure the probability distribution of water molecules around the amide moiety or its equivalent in each isostere. The results demonstrated that NMA (**a**) and *N*,*N*-dimethyl acetamide (**b**) showed a significant peak at 1.8–1.9 Å, suggesting strong and structured H-bonding interactions with water molecules. The peak for ester (**c**) shifted slightly to longer distances compared to **a** and **b**. This shift can be attributed to the replacement of the amide carbonyl oxygen with an ester carbonyl, which results in a different H-bonding environment and a reduction in the strength of the interactions with water molecules. For thioamide (**d**), the RDF peak was broader and shifted to longer distances, indicating weaker and more dispersed H-bonding. The RDF peak for CADI (**e**) exhibited a more pronounced shift to longer distances compared to the other compounds, with the distinct peak disappearing. This suggests that water molecules around CADI are less structured and engage in weaker hydrogen bonding interactions, indicating a significant reduction in the ability of CADI to form hydrogen bonds, which may contribute to a lower hydration energy. These results suggest that CADI substitution can reduce the interactions between the peptide backbone and water, thereby facilitating membrane permeability.

**Figure 2.** Molecular dynamics simulation results. **(A)** Structures of *N*-methylacetamide (NMA, **a**) and the corresponding isosteres, including *N*,*N*-dimethyl acetamide (**b**), ester (**c**), thioamide (**d**), and CADI (**e**). **(B)** Radial distribution function (RDF) between water molecules and **a**-**e**.



**CADI Synthesis.** Based on both the highest lipophilicity observed in AlogP calculations and the reduced water interaction indicated by MD simulations, we selected the CADI for further investigation. The synthetic schemes of CADI mimics are depicted in **Scheme 1**. For the synthesis of Leu-Phe-type CADI mimic (**7**), the *N*-*tert*-butylsulfonyl (Bus)-protected 2-chloroaziridine (**18**), prepared from aldimine (**17**), was treated with DIBAL-H followed by a *Z*-selective Horner-Wadsworth-Emmons olefination with [bis(*o*-tolylphosphono)phosphono]acetates (**20**),[19] affording the corresponding (*Z*)-enoate (**21**). Anti-$S_N2'$-type allylic alkylation of **21** with organocuprate (BnCu(CN)ZnCl) provided the α-benzylated ester (**22**),[20] which was converted to the desired amide (**7**) by acidic hydrolysis of the ester followed by coupling with methylamine and conversion of the *N*-Bus group to the *N*-Ac group. The Phe-Phe-type CADI mimic (**15**) was synthesized by a procedure similar to that used for **7** as shown in **Scheme 1B**. The corresponding dipeptides were synthesized according to standard procedures (see Supporting Information Section IV and V for the details).




**Scheme 1.** Synthetic scheme of CADI mimics of dipeptides.

**Lipophilicity Comparison.** With these compounds in hand, we investigated the lipophilicities of CADI mimics by comparing their retention times on a hydrophobic C18 column using

HPLC (**Figure 3**). As expected, the CADI mimics showed a significant increase in retention time, consistent with the values of AlogP, confirming that CADI substitution enhances lipophilicity.

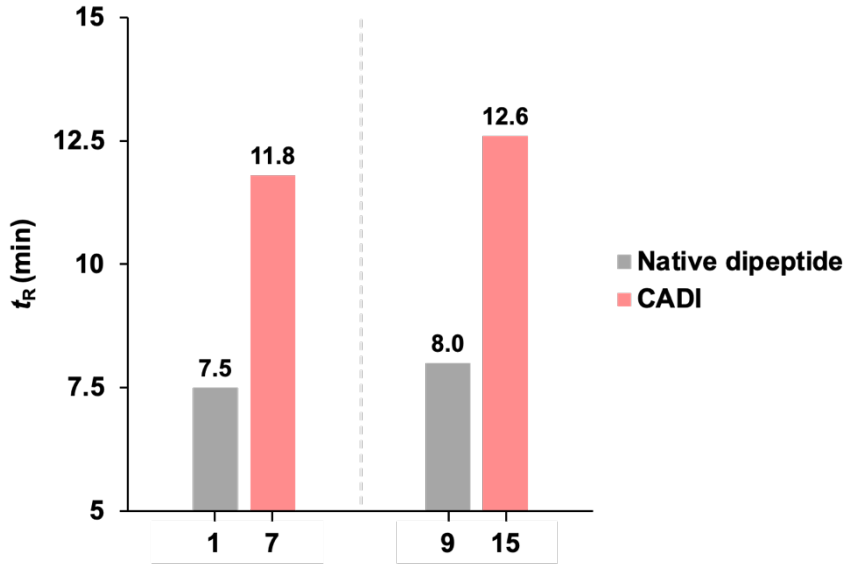



**Figure 3.** HPLC retention time in C18 column of peptides (**1** and **9**) and their CADI mimics (**7** and **15**). (Solvent A: 0.1% TFA in water, solvent B: 0.1% TFA in acetonitrile, gradient 30-75% solvent B over 15 minutes).

**Membrane Permeability Evaluation.** Next, we investigated the membrane permeabilities of CADI mimics using the parallel artificial membrane permeability assay (PAMPA) (**Figure 4**).[19] PAMPA was conducted with 200 mM compounds in 10% methanol solution containing 0.5% DMSO and 5 h incubation at room temperature. Each bar represents the mean value, and the error bars the standard deviation from experiments carried out in quintuplicate. In both sequences, the CADI-containing mimics demonstrated a significant enhancement in permeability compared to their respective dipeptides. Specifically, the Leu-Phe-type CADI mimic (**7**) exhibited a 17-fold increase in permeability ($P_e$ value: 6.5 × $10^{-6}$ cm/s vs. 0.38 × $10^{-6}$ cm/s), while the Phe-Phe-type mimic (**15**)

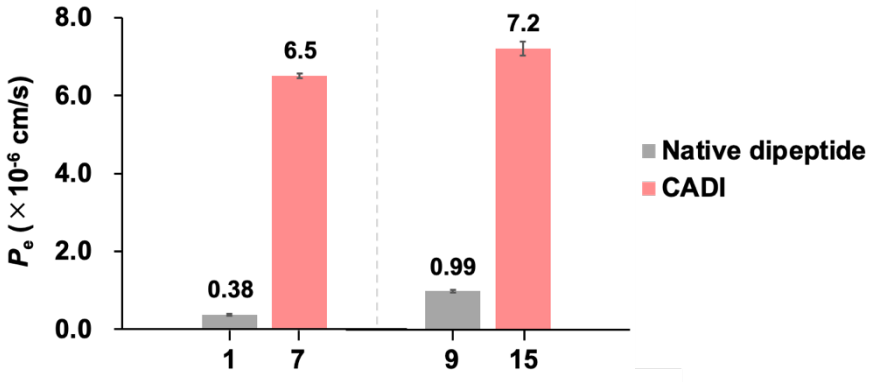



**Figure 4.** Membrane permeability of the peptides determined by the PAMPA.

showed a 7-fold increase ($P_e$ value: $7.2 \times 10^{-6}$ cm/s vs. $0.99 \times 10^{-6}$ cm/s). These results confirm that CADI substitution enhances passive permeability through the membrane, likely due to the increased lipophilicity conferred by the CADI moiety.

**Conclusions:**

In summary, we have demonstrated that CADI incorporation into dipeptides significantly enhances membrane permeability, with Leu-Phe and Phe-Phe models showing 17-fold and 7-fold increases in passive permeability, respectively. This significant improvement is attributed to the increased lipophilicity provided by the CADI substitution. The ADI offers a straightforward modification to the peptide backbone, significantly improving both hydrolytic stability and membrane permeability, which are crucial factors in peptide drug discovery. Further studies will explore the application of ADI approach to cyclic peptides and longer linear peptides, as well as comparing the effects of ADI substitution with ester and thioamide modifications to further optimize peptide pharmacological properties.

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