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A preorganized β-amino acid bearing a guanidinium side chain and its use in cell-penetrating peptides†

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A cyclic β-amino acid (APC^{Gu}) bearing a side-chain guanidinium group has been developed. The APC^{Gu} residue was incorporated into an α/β-peptide based on the Tat(47–57) fragment, leading to an oligomer with substantial helicity in methanol that enters HeLa cells much more readily than does the corresponding Tat α-peptide.

Cell-penetrating peptides (CPPs) can deliver various molecules, including drugs, proteins, and DNA, into cells.[1–5] To date, a variety of CPPs has been developed, many of which are rich in arginine, including oligo-arginines $(Arg)_n$ and the HIV Tat-(47–57) peptide.[6–11] The guanidino moiety of Arg appears to play a crucial role in the cell entry process,[6–9] and the internalization of these CPPs occurs *via* endocytosis as one of the major routes of the endocytic uptake. However, in the presence of counterions such as pyrenebutyrate, the CPPs such as $(Arg)_8$ were internalized by direct translocation through plasma membranes.[12] Indeed, many unnatural oligomers that possess multiple guanidino side chains, including peptoids,[13] α-peptides,[14] β-peptides,[15–18] and oligocarbamates[19] can enter cells. Previously it was shown that the efficiency of cell entry by β-peptides can be enhanced by increasing helicity.[20] This conformational stabilization was achieved by using cyclic β residues. Here we extend this concept to oligomers with a heterogeneous backbone, containing both α- and β-amino acid residues ("α/β-peptides").

α/β-Peptides containing 25–33% β residues that are evenly distributed among the α residues have been shown to adopt a helical conformation that is very similar to the well-known α-helix formed by pure α-peptide backbones.[21–24] This type of α/β-peptide helix can be stabilized by the use of β residues with a five-membered ring constraint and *trans* disposition of the amino and carboxyl groups. Two specific examples of this type of constrained β residue have been widely explored, *trans*-2-aminocyclopentane-1-carboxylic acid (ACPC) and *trans*-4-aminopyrrolidine-3-carboxylic acid (APC). The pyrrolidine ring amino group of APC can be used to attach other peripheral units.[25] Here we introduce a new derivative of APC in which the pyrrolidine nitrogen forms part of a guanidino group (APC^{Gu}). We describe the preparation of a protected β-amino acid that can be used to incorporate APC^{Gu} residues *via* solid-phase synthesis. In addition, we evaluate the cell-penetrating ability of an APC^{Gu}-containing α/β-peptide based on Tat-(47–57),[26,27] and show that the helicity provided by the new β residue correlates with enhanced activity (Fig. 1).

The synthetic route to Fmoc-(3*S*,4*R*)-APC^{Gu}-OH (**3**) is shown in Scheme 1. Fmoc-(3*S*,4*R*)-APC^{Boc}-OH (**1**) was prepared by the reported method.[28] The side-chain Boc group was removed under acidic conditions to give Fmoc-(3*S*,4*R*)-APC-OH (**2**) in quantitative yield. Guanidinylation of the side-chain amino group in **2** was accomplished by treatment with 3 equivalents of *N*,*N*′-di-Boc-*N*″-triflylguanidine under basic conditions.[29]

Building block **3** was used to prepare an α/β-peptide analogue of Tat(47–57) containing APC^{Gu} residues at positions 3, 7 and 10, to generate **Tat-1**. Two analogous α/β-peptides were prepared as well, **Tat-2**, which has β-Arg at positions 3, 7 and 10, and **Tat-3**, which has APC at these three positions. Two versions of the α-peptide Tat and the three α/β-peptides were synthesized, one set bearing N-terminal fluorescein (6FAM) labels

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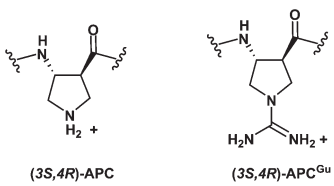
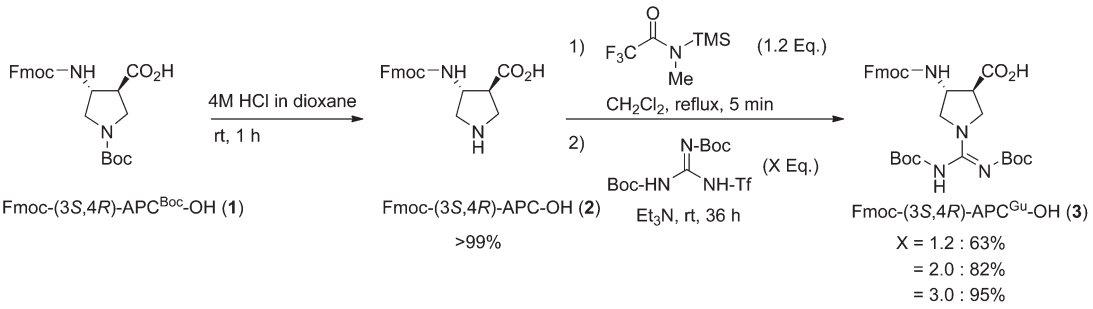


Fig. 1 Chemical structures of (3*S*,4*R*)-APC and (3*S*,4*R*)-APC^{Gu}.



Scheme 1 Synthesis of Fmoc-(3*S*,4*R*)-APC^{Gu}-OH (**3**).

(**F-Tat**, **F-Tat-1**, **F-Tat-2** and **F-Tat-3**), and another set bearing acetyl groups at the N-termini (**Ac-Tat**, **Ac-Tat-1**, **Ac-Tat-2** and **Ac-Tat-3**). Microwave-assisted solid-phase methods were used in each case, and each peptide was purified by reverse-phase HPLC and characterized by MALDI mass spectrometry (Fig. 2).[30]

We examined the abilities of **F-Tat** and the three analogous α/β-peptides **F-Tat-1–3** to enter HeLa cells. After incubation of cells with each compound for 2 h at 37 °C, the cells were lysed, and the fluorescence intensity of the lysate was measured. The α/β-peptides entered cells more efficiently than the α-peptide **F-Tat**. In particular, **F-Tat-1**, containing (3*S*,4*R*)-APC^{Gu} (Z^g) residues, showed a 65-fold higher extent of entry relative to **F-Tat** (Fig. 3).

Fig. 4 shows the effects of three endocytosis pathway inhibitors on entry of **F-Tat** and **F-Tat-1–3** into HeLa cells.[31–34] Amiloride is an inhibitor of macropinocytosis,[33,34] chlorpromazine is an inhibitor of clathrin-mediated endocytosis,[32] and filipin is an inhibitor of caveolae-mediated endocytosis.[32–34] None of these agents hindered the uptake of α-peptide **F-Tat** or **F-Tat-2** (β residues = β^3-hArg) which suggests that cell entry by these two molecules involves pathways other than the three inhibitors. In contrast, amiloride inhibited cell entry by **F-Tat-3** (β residues = APC), which suggests that macropinocytosis is at least partially responsible for entry in this case. The uptake level of **F-Tat-1** (β residues = APC^{Gu}) was inhibited by both amiloride and chlorpromazine, which suggests that both macropinocytosis and clathrin-mediated endocytosis contribute to

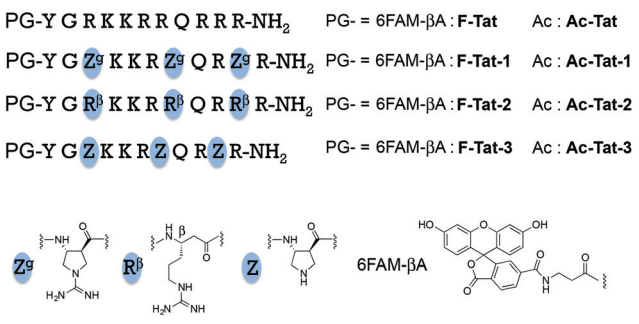


Fig. 2 Sequences of Tat derivatives **Tat** and **Tat-1–3**.

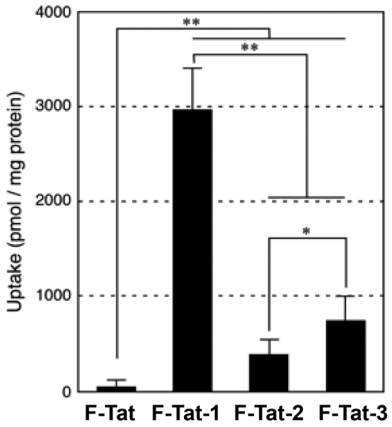


Fig. 3 Cellular uptake of **F-Tat** and **F-Tat-1–3** at a concentration of 1 μM (incubation time: 2 h). Error bars represent standard deviation, n = 5. *p < 0.05, **p < 0.01.

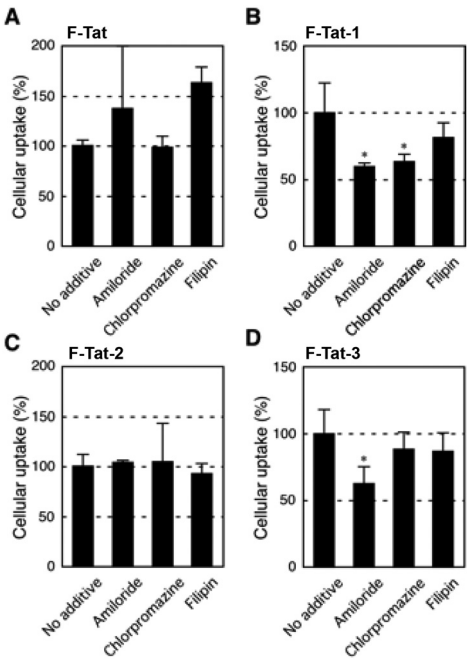


Fig. 4 Effects of inhibitors on internalization of **F-Tat** and **F-Tat-1–3** at a concentration of 1 μM (incubation time: 2 h). Error bars represent standard deviation, n = 5. *p < 0.05, **p < 0.01.

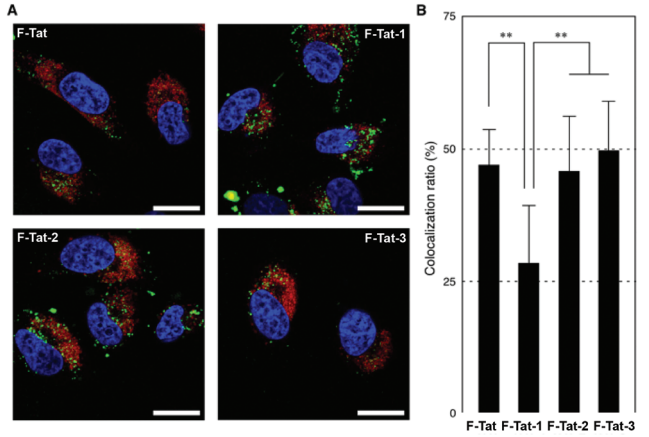


Fig. 5 Intracellular distribution of **F-Tat** and **F-Tat-1–3** (green) at a concentration of 1 μM (incubation time: 2 h). The acidic late endosomes/lysosomes were stained with LysoTracker Red (red) and the nuclei were stained with Hoechst 33342 (blue). (A) CLSM images of HeLa cells treated with Tat peptide derivatives. The scale bars represent 20 μm. (B) Quantification of Tat peptide derivatives colocalized with LysoTracker Red. Error bars in the graph represent standard deviation, n = 15. $^{**}p < 0.01$.

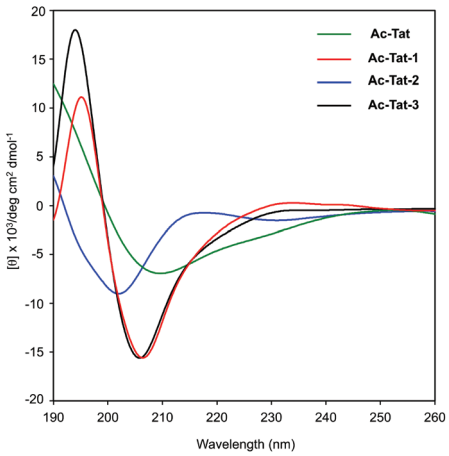


Fig. 6 CD spectra in the 190–260 nm region of **Ac-Tat** (green), **Ac-Tat-1** (red), **Ac-Tat-2** (blue), and **Ac-Tat-3** (black). Peptide concentration: 30 μM in MeOH solution.

cell entry in this case. Low temperature (4 °C) incubation almost completely inhibited the internalization of **F-Tat** and **F-Tat-1–3** (Fig. S2†), suggesting that these peptides were mainly internalized into the cells *via* endocytosis.

The intracellular distribution of **F-Tat** and α/β-peptides **F-Tat-1–3** (green) in HeLa cells was assessed by confocal laser scanning microscopy (CLSM) after staining late endosomes/lysosomes with LysoTracker Red (red) and nuclei with Hoechst 33342 (blue). All four peptides co-localized with the late endosomes/lysosomes, as shown in Fig. 5.[35,36] Roughly 50% of **F-Tat**, **F-Tat-2** and **F-Tat-3** were localized in the late endosomes/lysosomes, while <30% of **F-Tat-1** was localized in this way. This difference could arise if the distinctive entry mechanism of **F-Tat-1**, involving both macropinocytosis and clathrin-mediated endocytosis, causes this molecule to be delivered to a different sub-cellular region relative to the other three. An alternative possibility is that **F-Tat-1** is better able to move out of vesicles than the other three peptides, and therefore most of the **F-Tat-1** fluorescence is diffusely distributed throughout the cytoplasm.[37,38] Research to clarify the underlying mechanism of **F-Tat-1** is now under way in our group, and the results will be reported elsewhere in the near future.

The conformations of **Ac-Tat** and **Ac-Tat-1–3** were analyzed *via* circular dichroism (CD) in MeOH (Fig. 6). The α/β-peptides containing cyclic β residues (**Ac-Tat-1** and **Ac-Tat-3**) showed a minimum at around 207 nm, which is similar to the minima observed in CD spectra of α/β-peptides that form right-handed (*P*) helices (Fig. 6).[39,40] Furthermore, addition of 10 mM SDS has little effect on the helical structures of **Tat-1** and **Tat-3** (Fig. S3†). On the other hand, **Ac-Tat** and **Ac-Tat-2**, which contain exclusively acyclic residues, show weaker CD signatures that suggest little or no folding.

Conclusions

Tat-1 with (3*S*,4*R*)-APC^{Gu} (Z^g), **Tat-2** with β^3-Arg (R^β), and **Tat-3** with (3*S*,4*R*)-APC (Z) showed higher cell penetrating activities than the α-peptide Tat in HeLa cells. In particular, **Tat-1** showed a 65-fold higher permeability than **Tat**. These results indicate that stabilization of a helical secondary structure is synergistic with the presence of guanidino groups in promoting cell entry. It is noteworthy that **Tat-3** which contains (3*S*,4*R*)-APC residues and only three guanidino groups (on the three Arg residues) enters cells relatively efficiently. The new cyclic β-amino acid residue APC^{Gu}, which combines the advantages of conformational preorganization and a side-chain guanidino group should prove to be a useful tool for further development of biologically active β-peptides and α/β-peptides.

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