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Synthesis of Asparagusic-Acid-Modified Lysine and its Application in Solid-Phase Synthesis of Peptides with Enhanced Cellular Uptake

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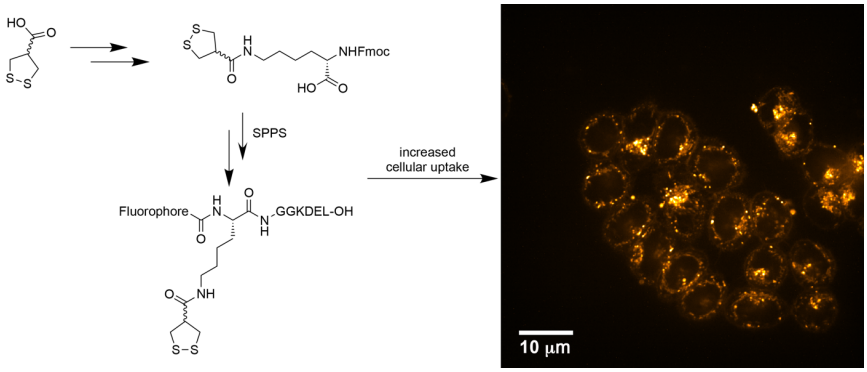

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Abstract Cyclic disulfides, such as asparagusic acid, enhance the uptake of a variety of cargoes into live cells. Here, we report a robust and scalable synthesis of an asparagusic-acid-modified lysine. This amino acid can be used in solid-phase peptide synthesis. We confirmed that incorporation of this building block into the sequence of a peptide increases its cellular uptake substantially.

Key words asparagusic acid, modified amino acid, cellular uptake, solid-phase peptide synthesis, cell-penetrating peptides

The cellular membrane has a highly regulated mechanism for controlling the uptake of compounds, which generally impedes the internalization of large or highly polar molecules, such as peptides, oligonucleotides and nanoparticles.[1] This barrier must be overcome to use biomolecules as therapeutic candidates[2], and several strategies have been developed employing liposomes[3], viral vectors[4], polymers,[5] and cell-penetrating peptides (CPPs)[6] as delivery vectors.

CPPs are short cationic sequences that have been used to deliver small molecules, proteins, nucleic acids, and quantum dots to the intracellular space.[6,7] CPPs can be obtained from natural sources or be synthetically designed, and the most common peptides used for translocation across the plasma membrane are Tat, polyarginine, penetratin and transportan.[7] The cellular uptake usually takes place via an endocytotic mechanism, which can be a drawback of CPPs if they fail to escape the vesicles (endosomes), thus not delivering the cargo to the correct intracellular location.[6,8]

Recently, a new method of intracellular delivery has emerged, which uses the endogenous thiol groups present on the cell surface (exofacial thiols) to enhance cellular uptake.[1] The method of action is believed to be disulfide exchange between exofacial thiols and the thiols attached to the cargo, such as fluorescent probes, peptides, CPPs, proteins, or oligonucleotides.[9] Matile and coworkers have shown that cellular uptake can be further improved by attaching either cell-penetrating poly(disulfide)s (siCPDs)[10] or ring-strained disulfides such as asparagusic acid (AspA) to the cargo.[9] Employing these strategies, small fluorescent molecules[9] as well as larger compounds, such as 20-mer peptides,[11] liposomes and polymersomes,[12] and quantum dots[13] were successfully brought into the cell.

AspA has a very small molecular weight (134 Da) and a simple structure, thus making it an attractive target for further studies (Figure 1a). Specifically, we were interested in its ability to improve the uptake of any peptides, independent of their net charge. For convenience, we envisioned that the AspA fragment could be attached to the side chain of an Fmoc-protected lysine. This building block (Fmoc-Lys(ASpA)-OH, Figure 1b) could be used like any other amino acid in solid-phase peptide synthesis (SPPS), streamlining the generation of peptides with AspA tags in defined positions within the amino acid sequence, even in the presence of other nucleophilic side chains.

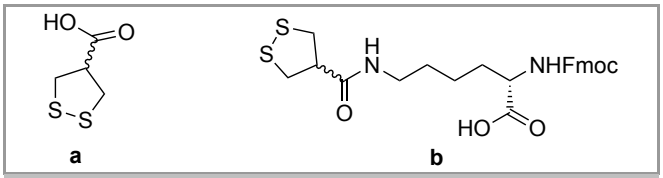


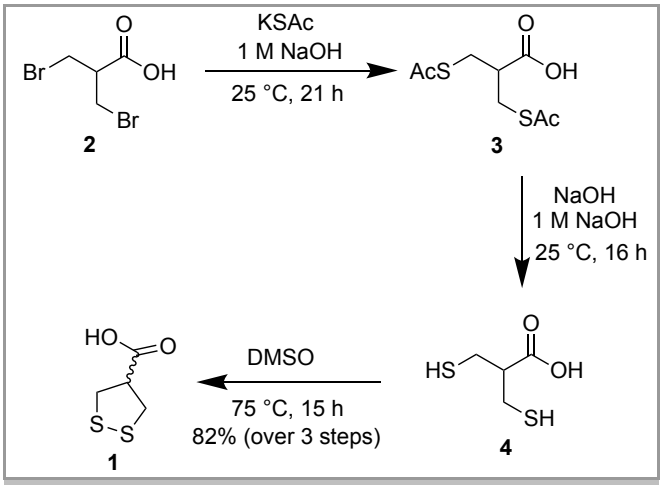
Figure 1 a) AspA; b) Fmoc-Lys(AspA)-OH.

Even though AspA clearly enhances the uptake of peptides into cells, there are few reports concerning the synthesis and stability of this molecule and its derivatives. Herein, we report a

robust synthesis of AspA, an AspA-tagged lysine building block and its application in SPPS.

The synthesis starts following a modified procedure from Venditti et al.[14] Commercially available 3-bromo-2-(bromomethyl)propanoic acid **2** was dissolved in 1 M NaOH and reacted with potassium thioacetate (KSAc) at room temperature for 21 h (Scheme 1). The progress of the reaction can be followed both by thin-layer chromatography (TLC) and liquid chromatography-mass spectrometry (LC-MS, in negative ion mode), and it is crucial that the reaction is allowed to reach completion.

Next, crude thioacetate **3** was hydrolyzed with NaOH to obtain dithiol **4**, which was then oxidized in DMSO, at 75 °C under aerobic conditions, to obtain the desired AspA **1**.[15] When the reaction reaches full conversion, the thiol triplet peak at around 1.53 ppm disappears from the ^{1}H NMR spectrum and the obtained product is pure enough for use in further modifications.

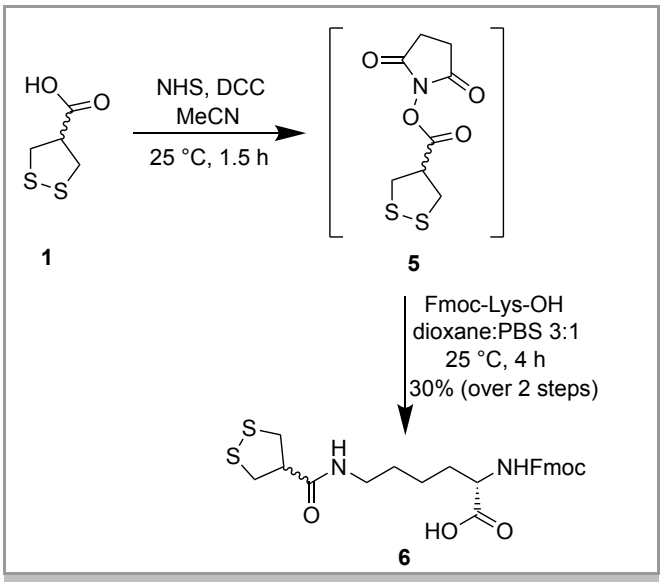


Scheme 1 Synthesis of AspA **1.**

It is noteworthy to mention that pure AspA is prone to polymerization under aerobic atmosphere, even when stored as a solid at −20 °C. Once polymerized, the solid is insoluble in most organic solvents. The polymer can be reversed back to dithiol **4** by reaction with Zn dust in a 2 M ammonium hydroxide suspension for 1 h at room temperature.[15]

To modify commercially available Fmoc-Lys-OH, AspA **1** was converted to the *N*-hydroxysuccinimide (NHS) ester intermediate **5** by reacting **1** with NHS in presence of *N*-*N*'-dicyclohexylcarbodiimide (DCC, Scheme 2).[16] After filtering the resulting urea and solvent removal, Fmoc-Lys-OH was added to the reaction mixture. The starting amino acid is relatively insoluble, so the reaction needs to be carried out in a mixture of 1,4-dioxane and phosphate-buffered saline (PBS, 0.05 M), 3:1.

We found that extraction with CH_2Cl_2 is an efficient way to obtain the product from the reaction mixture. Although a stable emulsion is formed under these conditions, it can be easily separated by centrifugation at 1750 × G (see SI). Compound **6** (Fmoc-Lys(AspA)-OH) was obtained by evaporation of the organic phase and trituration of the solid with pentane. The obtained solid can be purified further by flash column chromatography if needed (see SI). This synthesis is reproducible and has been performed on a gram scale.



Scheme 2 Synthesis of Fmoc-Lys(AspA)-OH **6.**

Next, we tested the suitability of the building block for SPPS. Using Wang resin, we synthesized the peptide Lys(AspA)-Gly-Gly-Lys-Asp-Glu-Leu (K(AspA)GGKDEL, see SI), and the deprotected *N*-terminus was capped with 5-carboxyrhodamine B. Cleavage and global deprotection resulted in peptide **7** (Figure 2). HPLC purification and ESI-MS analysis confirmed the purity and identity of the peptide (see SI). This result confirms that the modified amino acid can be incorporated into a peptide using SPPS. Moreover, this peptide contains two lysine residues, but only one bears an AspA tag. The selective synthesis of this peptide is greatly simplified by the availability of amino acid **6**.

To investigate the ability of AspA to improve cellular uptake, we also synthesized peptide **8,** which has the same amino acid sequence and fluorophore as **7**, but no AspA tag (Figure 2)

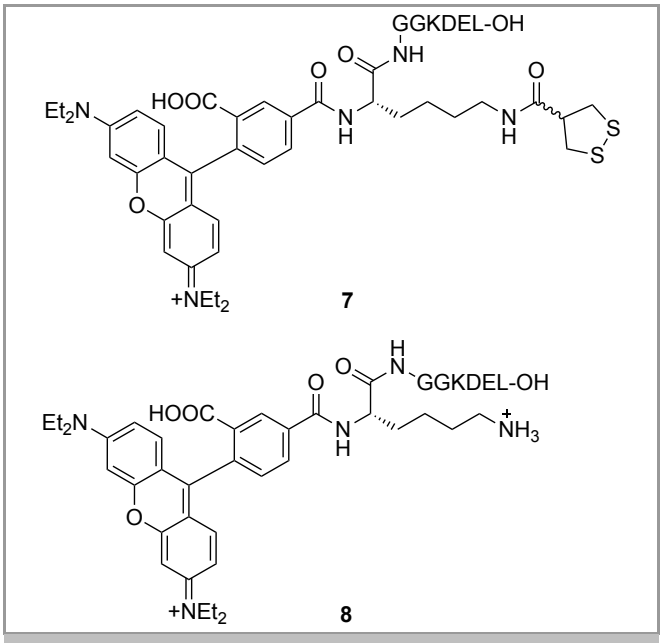


Figure 2 Structures of peptides **7** and **8.**

The peptides were tested for uptake in live human cervical cancer (HeLa) cells. The cells were incubated for 2 h at 37 °C with a 10 μM solution of peptide in Dulbecco's Modified Eagle's medium (DMEM), in the absence of serum. The cells were washed, the medium was exchanged and the cells were imaged after 2 h using a confocal fluorescence microscope (Figure 3).

As it can be seen in Figures 3C and 3F, the uptake of peptide **7** is superior to that of **8**. By comparing fluorescence intensity of cells treated with the two peptides, we can conclude that the AspA moiety improves the cellular uptake by at least a factor of four (Figure 4).

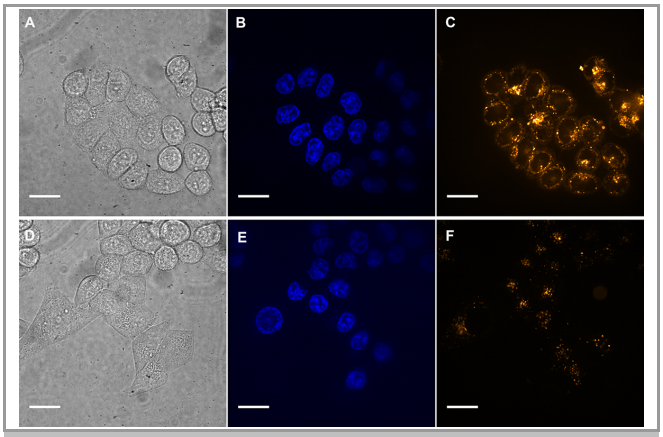


Figure 3 Live HeLa cell imaging. A) Brightfield image; B) same cells as in (A), blue channel image of nuclei labeled with dye Hoechst 33342; C) same cells as in (A), red channel image of cells incubated with 10 μM **7**; D) Brightfield image; E) same cells as in (D), blue channel image of nuclei labeled with dye Hoechst 33342; F) same cells as in (D), red channel image of cells incubated with 10 μM **8**. Scale bar = 10 μm

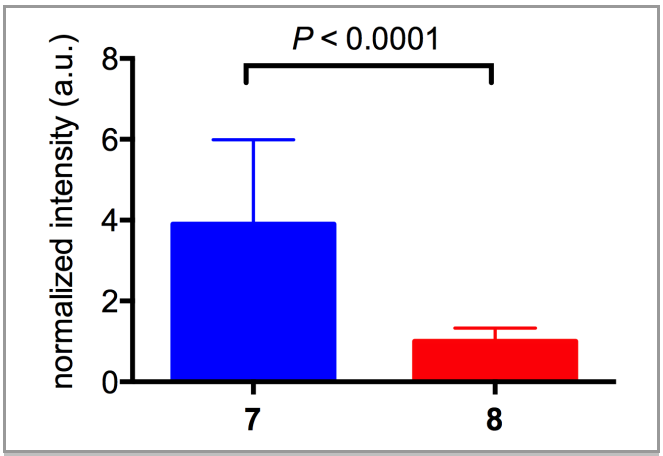


Figure 4 Quantification of intracellular fluorescence intensity of cells treated with peptides **7** or **8**.

In summary, we report the synthesis of both AspA and an AspA-tagged lysine. This amino acid can be obtained in five steps, with only one flash column chromatography required in the last step of the synthesis. Furthermore, the modified amino acid is stable under standard SPPS conditions and its incorporation into a peptide sequence increases the cellular uptake of the attached cargo.

The availability of this building block will enable the rapid generation of libraries of peptides with AspA tags in defined positions. We envision that this modified amino acid will facilitate the discovery of peptides with enhanced cellular uptake and potential applications in chemical biology and medicinal chemistry.

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Supporting Information

Supporting information for this letter is available online.

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(16) ***N*²-(((9*H*-Fluoren-9-yl)methoxy)carbonyl)-*N*⁶-(1,2-dithiolane-4-carbonyl)-*L*-lysine (Fmoc-Lys(AspA)-OH, 6)**

Crude NHS ester **5** (328 mg, 1.32 mmol) was dissolved in dioxane-PBS, 3:1 (28 mL) at room temperature (25 °C) and Fmoc-Lys-OH (538 mg, 1.46 mmol, 1.1 eq.) was added in one portion. After 4 h, the solution was acidified by slow addition of 1 M HCl (~2 mL) and CH_2Cl_2 (~25 mL) added until phase separation occurred. The aqueous phase was extracted again with CH_2Cl_2 (2 × 25 mL). A white emulsion formed, which could be separated by centrifugation (3 min at 1750 × G). The combined organic phases were dried

over $MgSO_4$, filtered, and concentrated under reduced pressure to give a very viscous light-yellow oil. The product was precipitated from pentane. The obtained solid was purified by flash column chromatography (SiO_2; CH_2Cl_2-MeOH, 100 to 90:10) to give a white solid (198 mg, 30% yield). ^{1}H NMR (400 MHz, DMSO-d_6): δ = 1.24 – 1.46 (m, 4H, H-5 and H-6), 1.54 – 1.76 (m, 2H, H-7), 2.98 – 3.22 (m, 5H, H-4, H-2, two of H-1), 3.37 (m, 2H, two of H-1), 3.86 – 3.95 (m, 1H, H-8), 4.19 – 4.26 (m, 1H, H-11), 4.25 – 4.30 (m, 2H, H-10), 7.33 (td, *J* = 7.4, 1.1 Hz, 2H, H-13), 7.42 (td, *J* = 7.5, 1.6 Hz, 2H, H-14), 7.59 (d, *J* = 8.0 Hz, 1H, H-9), 7.73 (*d*, J = 7.5 Hz, 2H, H-12), 7.89 (d, *J* = 7.5 Hz, 2H, H-15), 8.11 (t, *J* = 5.6 Hz, 1H, H-3) ppm. ^{13}C NMR (101 MHz, DMSO-d_6): δ = 23.5, 28.9, 30.8, 39.0, 42.5, 42.6, 47.1, 52.0, 54.1, 66.0, 120.5, 120.6, 125.7, 125.8, 127.5 (2C), 128.1(2C), 141.1, 141.2, 144.2, 144.3, 156.6, 170.6, 174.4 ppm. HRMS (ESI): *m/z* calcd for $[C_{25}H_{28}N_2O_5S_2Na]^+$: 523.1332, found: 523.1340.