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A Scalable Synthesis of the Blue Fluorescent Amino Acid 4-Cyanotryptophan and the Fmoc Derivative: Utility Demonstrated with the Influenza M2 Peptide Tetramer

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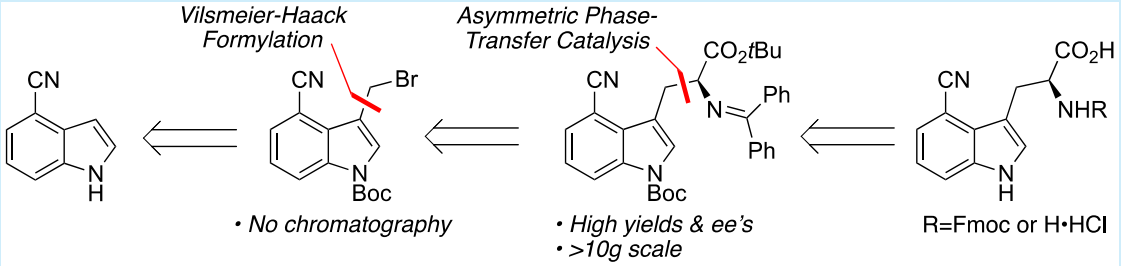

ABSTRACT: A scalable synthesis of the Fmoc-protected blue fluorescent amino acid, L-4-cyanotryptophan (W_{4CN}), that exploits an enantioselective phase transfer-catalyzed alkylation is reported. The red-shifted emission of water-exposed W_{4CN} residues was leveraged to investigate the solvation state of tryptophan (Trp) residues within the influenza M2 proton channel. The correlation of the channel's conformation (i.e., open or closed) with the fluorescence spectrum of a mutated W_{4CN} residue suggests that the channel's conformational state does not impact the hydration status of the Trp residues.

Recently, the tryptophan (Trp) derivative L-4-cyanotryptophan (W_{4CN}, **1**) has emerged as a novel fluorescent reporter for biological spectroscopy and microscopy due to the following features:[1a] (1) the absorption spectrum of W_{4CN} extends beyond 360 nm, permitting selective excitation when naturally occurring fluorescent amino acids are present; (2) W_{4CN} emits readily observable visible (blue) fluorescence; (3) it has both a large fluorescent quantum yield (>0.8 in water) and a long fluorescence lifetime (ca. 12 ns in water); (4) it is chemically and photophysically stable; and most importantly, (5) it is compact in size, which is expected to induce minimal perturbation to the native peptide structure, dynamics, or function.[1b] Indeed, the utility of W_{4CN} as a nonperturbing spectroscopic probe has recently been demonstrated in the study of membrane-associated peptide dynamics,[2a] the folding rates of peptides susceptible to artificial intramolecular interactions,[1b] and the cellular trafficking of peptides with specific localization sequences.[1]

In line with the increased interest in W_{4CN}, several syntheses have been reported. The Arnold laboratory disclosed a remarkable preparative biosynthetic route using a genetically engineered tryptophan synthase derived from *Thermotoga maritima* (Figure 1A).[3a] A second report, also demonstrating the utility of biosynthetic transformations, was recorded by Gai and Jo wherein *rac*-N^{α}-acetyl-4-cyanotryptophan, which was obtained from the alkylation of ethyl 2-nitroacetate by 4-cyanogramine,[3b] was resolved enzymatically by employing amano acylase to provide enantiomerically enriched (ee > 99%) W_{4CN} (Figure 1B).[2a] To highlight the utility of the newly developed N^{α}-acetyl-4-Bpin-tryptophan methyl ester as a surrogate to construct various 4-substituted tryptophans, Bartocinni and co-workers prepared *rac*-N^{α}-acetyl-4-cyanotryptophan methyl ester through the cross-coupling of the boronic ester with copper cyanide.[3c] To date, however, only one asymmetric route utilizing entirely organic synthetic tactics has been reported; Hilaire and co-workers successfully installed the nitrile functionality via a Pd-catalyzed cross-coupling of L-N^{α}-Boc-4-bromotryptophan with $Zn(CN)_2$, albeit in low yield.[1a]

Pursuant to our desire to use W_{4CN} as a nonperturbing fluorophore in a number of peptides and proteins, we recognized that we would require multigram quantities of this Fmoc-protected derivative for use in solid-phase peptide synthesis. Herein, we report an effective 10 g scalable asymmetric synthesis of L-N^{α}-Fmoc-4-cyanotryptophan (−)-**2** that employs the asymmetric phase-transfer alkylation originally developed by Lygo[4c] and Corey[4b] and later employed by the Park group for their asymmetric synthesis

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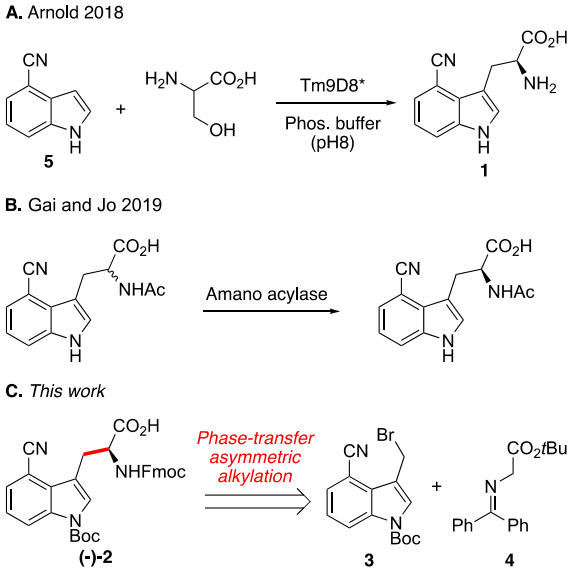
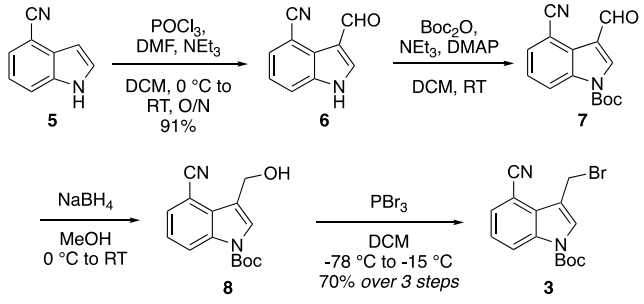


Figure 1. (A and B) Recent syntheses of W_{4CN} employing biosynthetic transformations and (C) the proposed asymmetric alkylation to access (−)-**2**.

of (−)-*cis*-clavicipitic acid (Figure 1C).[4a] In addition, we validated the utility of the Fmoc-protected W_{4CN} (−)-**2** by incorporating an unnatural amino acid into a peptide−lipid vesicle model of the influenza M2 proton channel and then leveraged the solvent sensitivity of W_{4CN} to monitor changes in the hydration state of the membrane-bound peptide (*vide infra*).

For the synthesis, we envisioned bromide **3** as a viable electrophile to incorporate the requisite side chain of W_{4CN}. To this end, **3** was constructed from commercially available 4-cyanoindole (**5**; Scheme 1). First, a Vilsmeier−Haack

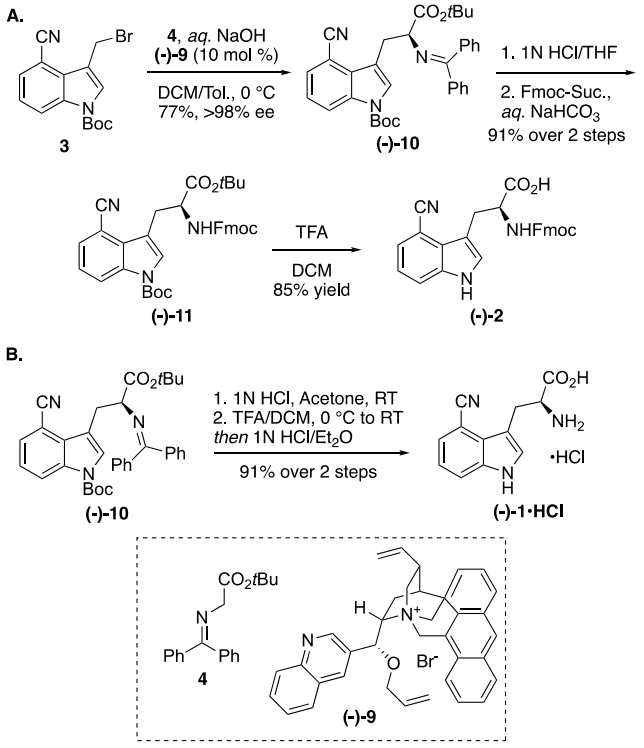
Scheme 1. Synthesis of Bromide 3 from Commercially Available 4-Cyanoindole 5



formylation of **5** yielded indole-3-carbox-aldehyde (**6**) in a 91% yield. the installation of N^{indole}-Boc and benzylic bromide was then accomplished over three steps in a good overall yield (70%). Notably, the four-step sequence required no column chromatography to obtain **3** in an excellent purity (>98%).

Pleasingly, the asymmetric phase-transfer alkylation of glycine-derived imine **4** with **3** proceeded uneventfully, employing (−)-(*N*-(9-anthracenylmethyl)cinchonindinium (−)-**9** (Scheme 2A). The imine (−)-**10** was obtained in a good yield (77%) and excellent enantioselectivity (>98%) on a 10 g scale. The selective hydrolysis of the benzophenone imine was affected using dilute aqueous HCl, and the crude hydrochloride salt was immediately subjected to Fmoc

Scheme 2. Completion of the Synthesis of (A) L-N^{α}-Fmoc-W_{4CN} (−)-2 and (B) W_{4CN} Hydrochloride (−)-1·HCl



protection (98% over 2 steps). The TFA-mediated hydrolysis of the *t*-butyl ester and *t*-butyl carbamate furnished L-N^{α}-Fmoc-4-cyano-tryptophan (−)-**2** in an 85% yield. The fully deprotected W_{4CN} hydrochloride salt (−)-**1·HCl** was also synthesized from (−)-**10** via a two-step deprotection sequence in an excellent yield (91%, Scheme 2B).

To show the utility of the synthesized W_{4CN}, especially the Fmoc-protected form, we employed W_{4CN} to probe the effect of pH on the local hydration status of the tryptophan gate in the M2 protein of the influenza A virus,[5a] which was constructed by solid-phase synthesis (Figure 2A). By forming

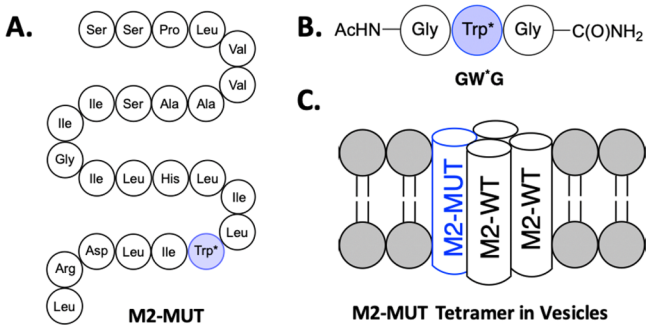


Figure 2. Primary sequences of (A) the M2 mutant peptide M2-MUT and (B) the tripeptide GW*G. (C) Cartoon of the 3:1 (wild-type/mutant) M2 tetrameric channel within the vesicle membrane.

an α-helical homotetrameric channel spanning the viral envelope, M2 enables acidification of the viral interior in the low-pH environment of endosomes, a process that is crucial for viral replication.[5b]

The proton-conduction mechanism of the M2 proton channel has been extensively studied;[6−11] a major finding is

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that the two channel-lining aromatic residues, His37 and Trp41, are largely responsible for the pH-gated and asymmetric (i.e., from the exterior to the interior of the virus) proton conduction function of M2.[6b] More specifically, the His37 tetrad acts as a proton collector and trigger for channel opening via the protonation of the imidazole rings, whereas the Trp41 tetrad permits protons to pass through the channel only when the pH is low on the outside of the virus. In addition, a recent study by Markiewicz et al.[9] indicated that the Trp41 side chain is situated in an environment that is not fully hydrated even under low pH conditions where the channel is open, suggesting that the Trp41 gate may also play an important role in controlling the proton conduction rate of M2.[9,10] To substantiate this notion further, we employed W_{4CN} fluorescence to assess the effect of pH on the hydration status of the Trp41 gate of M2.

Following the work of Markiewicz et al.,[9] we employed the transmembrane (TM) domain of the M2 protein (i.e., **M2-TM**; sequence SSDPLVVAASIIGILH37LILW41I-LDRL) as our model system by mutating the Trp residue to W_{4CN} (the resultant peptide is hereafter referred to as **M2-MUT**). The **M2-TM** retains the ability to form a tetrameric channel in lipid membranes and conduct protons in a similar fashion as the full-length M2 proton channel[2a] and has hence served as a functional surrogate in various mechanistic and structural studies.[12] Accordingly, the results of Markiewicz et al. indicated that, by the mutation of Trp41 to 5-cyanotryptophan (W_{5CN}) in **M2-TM**, the corresponding channel maintains the proton-conduction property of the wild-type **M2-TM** channel in lipid bilayers. Considering the size similarity of the two isomeric cyanotryptophans W_{5CN} and W_{4CN}, we anticipated that the proton channel formed by **M2-MUT** would also behave similarly to that of **M2-TM**. Indeed, the circular dichroism spectra of **M2-TM** in a model lipid membrane indicate that it is folded under both neutral and acidic pH conditions (Figure S1 in the Supporting Information).

As shown in Figure 3, the fluorescence spectrum of a short peptide, Gly-W_{4CN}-Gly (GW*G), in water where the W_{4CN} side chain is expected to be fully hydrated peaks at ca. 420 nm. On the other hand, the fluorescence spectrum of GW*G in tetrahydrofuran (THF), a hydrophobic solvent, is shifted to ca. 400 nm. To exclude the possibility that peptide aggregation is responsible for the red-shift, we assessed the effect of concentration on fluorescence. A serial dilution had no effect on the emission spectra of GW*G in either THF or water (Figure S2 in the Supporting Information), thus ruling out aggregation as the source of the red shift.

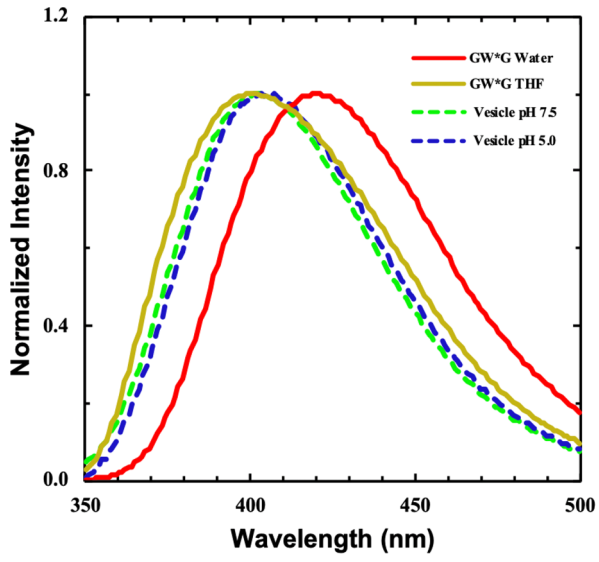

Figure 3. Normalized fluorescence spectra of the 3:1 **M2-TM**/**M2-MUT** channel obtained in lipid vesicles with different pH values and **GW*G** in water and THF, as indicated. The excitation wavelength was 310 nm.

The dependence of W_{4CN}'s fluorescence on the solvent is not unprecedented; van Wilderen and co-workers[1d] reported a similar red-shift for *N*-acetyl-4-cyanotryptophan in water. Although similar hydration effects have been reported for other isomeric cyanoindole-based fluorophores (e.g., 5-cyanotryptophan),[1d] the quantum yield of W_{4CN} has been reported to increase upon hydration, while the fluorescence of W_{5CN} is effectively quenched in water.[1d] Taken together, these results indicate that the fluorescence of W_{4CN} is sensitive to the interactions with water molecules and hence can be used as a local protein hydration reporter.

We next synthesized the two individual peptides, **M2-TM** and **M2-MUT**, that would comprise the mutant M2 proton channel possessing the W_{4CN} fluorophore via an automated solid-phase synthesis. The 3:1 (**M2-TM**/**M2-WT**) mutant channel was chosen for our fluorescence study to avoid the self-quenching and spectral complexity that arise from channels possessing multiple W_{4CN} fluorophores. The 3:1 mutant channel was prepared according to known procedures[9] and embedded within single unilamellar vesicles at either pH 7.5 or pH 5.0 to mimic endosomal conditions.

The fluorescence spectrum of the membrane-bound mutant channel at pH 7.5 peaks at ca. 402 nm (Figure 3), which is close to the fluorescence wavelength maximum (ca. 400 nm) of GW*G in THF. These results thus indicate that the W_{4CN} residues in the mutant channel are situated in a significantly dehydrated environment. This finding is consistent with the notion that the **M2-TM** proton channel is in a closed state at pH 7.5 where the Trp41 tetrad is accessible to only a few, if any, water molecules according to a molecular dynamics (MD) simulation study.[13] What is more interesting, however, is that when the pH was lowered to 5.0, where the channel was anticipated to adopt an open state with more water molecules filling the Trp41 region to assist with proton conduction,[14] the fluorescence spectrum of the mutant channel was only red-shifted by ca. 1 nm. This result thus corroborates the study of Markiewicz et al.,[9] which revealed that the degree of hydration of the Trp41 gate does not significantly increase upon channel acidification and that a transient conformational or hydration event is required to permit the proton released from the His37 tetrad to pass through the lower region of the channel.[10]

In summary, we have achieved a concise, scalable (10 g), and efficient synthetic route to the blue fluorescent amino acid, 4-cyanotryptophan, and the corresponding Fmoc derivative. Solvent studies of various forms of the fluorescent amino acid demonstrate that W_{4CN} can be used as a reporter for local hydration in biological systems. The application of W_{4CN} to inspect the hydration state of tryptophan residues within the influenza M2 proton channel further supports the utility of W_{4CN} as both a structurally unperturbing fluorescent amino acid and a reporter of hydration.

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.orglett.0c04055.

Experimental procedures, product identification data, copies of NMR and UPLC spectra, and fluorescence raw data (PDF)

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#R.J.M. and A.A. contributed equally.

Notes

The authors declare no competing financial interest.

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