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Environment-sensitive fluorescent tryptophan analogues *via* indole C-2 alkenylation reactions†

Valeria K. Burianova, Liyao Zeng, Abigail W. Thom, Niamh C. Radcliffe-Kennedy, Steven W. Magennis and Andrew Sutherland *

A modular synthetic strategy for the preparation of intrinsically fluorescent unnatural α-amino acids by C-2 oxidation and alkenylation of a tryptophan-derived tetrahydro-β-carboline is described. This approach yielded a novel tryptophan–coumarin hybrid with strong environmental sensitivity and compatibility with near-infrared two-photon excitation.

Unnatural α-amino acids are important targets for a range of applications. In synthesis, modified α-amino acids are widely used as chiral ligands, catalysts and starting materials.[1,2] In medicinal chemistry and the life sciences, unnatural α-amino acids are used as enzyme inhibitors or as probes to study biological structure and function.[3] In this regard, fluorescent unnatural α-amino acids in combination with fluorescence spectroscopy techniques have been used to study biological processes.[4,5] This overcomes the limitations of large extrinsic fluorophores that require attachment by chemical spacers and are restricted to the peptide termini or amino acid sidechains. Fluorescent α-amino acids can be inserted into peptides at key positions using solid phase peptide synthesis (SPPS) or genetic encoding allowing more localised reporting.

One approach for the development of novel fluorescent α-amino acids is the modification of proteinogenic α-amino acids.[5] With the strongest fluorescence, L-tryptophan (**1**) has been widely utilised for this purpose (Fig. 1a). In particular, extended conjugation of the indole sidechain has generated fluorophores with improved photophysical properties that have been used for various applications.[6] For example, L-4-cyanotryptophan (**2**), which differs by only two atoms and has a long fluorescence lifetime and good photostability, has been used to visualise HEK cells and evaluate peptide-membrane interactions.[7] Incorporation of a BODIPY motif at the C-2 position of L-tryptophan such as adduct **3** has produced compounds that have been used for imaging fungal infection in human tissue, as well as tumor-associated macrophages in aggressive carcinomas.[8,9] Pyrazoloquinazoline α-amino acids **4** were designed as rigid, extended analogues of L-tryptophan.[10] These were found to be compatible with two-photon excitation and incorporated into cell-penetrating peptides using SPPS. Carbazole α-amino acids **5** have also been reported as highly fluorescent tryptophan mimics and used as probes to measure protein–protein binding.[11]

As extended conjugation at the C-2 position of the indole ring has led to the discovery of tryptophan fluorophores with enhanced photophysical properties, we were interested in establishing a synthetic approach that would allow the preparation of C-2 alkenyl tryptophan analogues as charge transfer-based fluorophores for biological imaging. Tryptophan compounds bearing a C-2 alkenyl side chain have been reported. Wang and co-workers developed a palladium-catalysed C(sp^2)–H olefination of tryptophan using the peptide backbone as a directing group.[12] This methodology was used for macrocyclisation and the preparation of cyclic peptides with indole-alkene crosslinks. Zhu and co-workers used an *N*-heterocycle within the indole sidechain of tryptophan to direct rhodium- (Fig. 1b) or palladium-catalysed C(sp^2)–H olefination.[13] This reaction was also used as a stapling strategy for the preparation of cyclic peptides. To avoid the use of precious transition metals, the Vendrell and Ackerman groups developed a directed manganese-catalysed C(sp^2)–H olefination of tryptophan (Fig. 1b).[14] This chemistry was used for the incorporation of BODIPY and nitrobenzodiazole moieties, with the subsequent peptidic probes used for real-time imaging of live cells. To complement this previous methodology and avoid the need for directing groups, we proposed that new fluorescent C-2 alkenyl tryptophan analogues could be prepared using a novel two-step strategy from a readily available tryptophan-derived tetrahydro-β-carboline intermediate. Here, we report the synthesis of C-2 alkenyl tryptophan analogues by selenium dioxide oxidation of a tetrahydro-β-carboline intermediate, followed by alkenylation reactions (Fig. 1c). We also describe the preparation of a tryptophan–coumarin hybrid, which possesses bright environment-sensitive fluorescence and is compatible with two-photon excitation in the near-infrared.

The first stage of this project focused on a short, scalable synthesis of a 2-formyl-L-tryptophan analogue that could be used

School of Chemistry, The Joseph Black Building, University of Glasgow, Glasgow G12 8QQ, UK. E-mail: Andrew.Sutherland@glasgow.ac.uk

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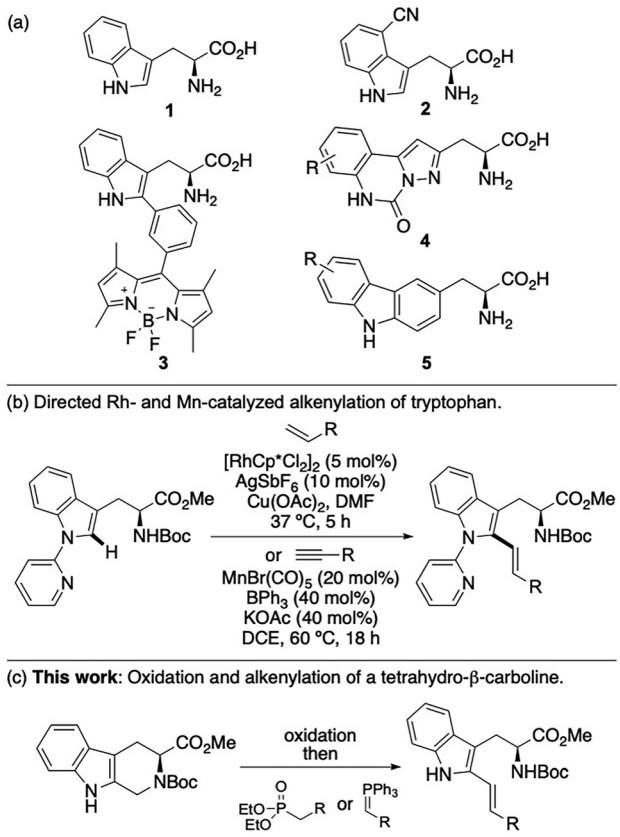
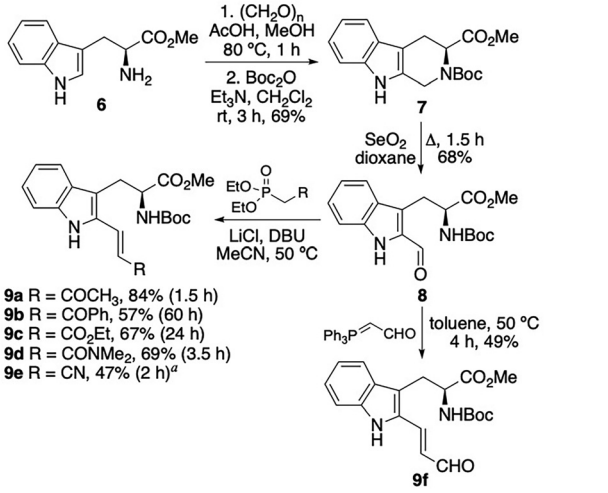


Fig. 1 (a) L-Trp and fluorescent analogues. (b) Directed metal-catalysed alkenylation of Trp and associated peptides. (c) This work: alkenylation of a Trp analogue.



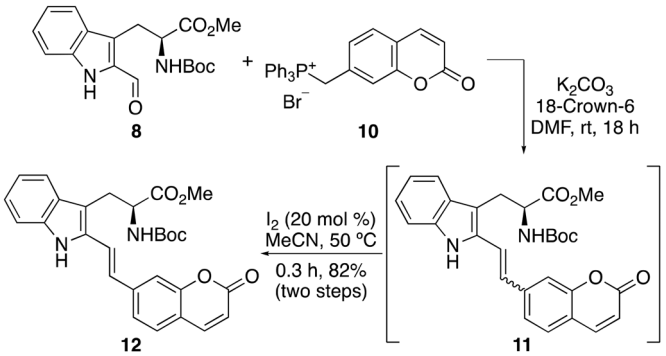
Scheme 1 Synthesis of C-2 alkenyl tryptophans **9a–f**. [a] Reaction conducted at rt.

to investigate subsequent alkenylation reactions. 2-Formyl-tryptophan analogues have been reported *via* oxidation of the corresponding tetrahydro-β-carboline and thus, an efficient and scalable synthesis of compound **7** was developed (Scheme 1).[15–17] Initially, a Pictet–Spengler reaction of L-tryptophan methyl ester (**6**) using paraformaldehyde and AcOH was performed.[18] Following Boc-protection under standard conditions, this gave tetrahydro-β-carboline **7** in 69% overall yield. Oxidation of **7** was initially investigated using persulfate as the oxidant.[16] At 40 °C and an overnight reaction, this gave 2-formyl-L-tryptophan **8** in 53% yield. Alternatively, oxidation of **7** using selenium oxide gave 2-formyl-L-tryptophan **8** after 1.5 h, and in an improved 68% yield.[15]

Following the scalable synthesis of 2-formyl-L-tryptophan **8** (> 2.5 mmol), this compound was examined as a substrate for C-2 olefination reactions (Scheme 1). Our aim was to prepare alkenes with electron-withdrawing groups, which in direct conjugation with the π-excessive indole ring would result in charge transfer-based fluorophores. To access a range of targets using an amino acid-derived aldehyde, the mild conditions of the Masamune–Roush version of the Horner–Wadsworth–Emmons (HWE) reaction was utilised.[19] Reaction of **8** with ketone, ester and amide-substituted phosphonate esters, using lithium chloride and DBU gave the corresponding *E*-alkenes in 57–84% yield. NMR spectroscopy of the reaction mixtures confirmed the presence of only the *E*-isomers. Only the nitrile-substituted phosphonate ester gave a mixture of *E* and *Z*-isomers (3 : 2), respectively. These were separable by column chromatography, which afforded the major *E*-isomer **9e** in 47% yield. 2-Formyl-L-tryptophan **8** was also compatible with the conditions of a Wittig reaction. Treatment of 2-formyl-L-tryptophan **8** with (triphenylphosphoranylidene)acetaldehyde at 50 °C gave **9f** as the sole isomer in 49% yield.[20]

On successful alkenylation of **8**, it was proposed that incorporation of a more conjugated group *via* an olefination reaction would result in a target with enhanced fluorescent properties. The coumarin motif is widely utilised as a key component of fluorescent chemosensors, finding application in analytical and materials chemistry, as well as for biological imaging.[21] Furthermore, coumarin analogues of tryptophan have been reported. In 2024, Ackermann and co-workers used their pyridine-directed manganese-catalysed C–H alkenylation for the preparation of tryptophan–coumarin conjugates.[22] Although the coupling reactions were generally efficient, the alkene products were isolated as *E*/*Z* mixtures (4 : 1) and attempted removal of the indole *N*-pyridine directing group led to compound decomposition. It was proposed that a Wittig reaction with 2-formyl-L-tryptophan **8** may overcome these issues, generating a single adduct that could be fully characterised as a fluorescent probe. Coumarin–tryptophan hybrid **12** was designed and subsequently prepared in two steps from **8** and the known coumarin phosphonium salt **10** (Scheme 2).[23] Room temperature Wittig reaction of **8** with phosphonium salt **10** using K_2CO_3 and 18-crown-6 gave alkene **11**.[24] NMR spectroscopy of the crude reaction mixture showed that this process had formed a 1.5 : 1 mixture of *E*- and *Z*-isomers, respectively. Without isolation, the mixture was treated with iodine (20 mol%) and this gave *E*-isomer **12** as the sole product in 82% yield over the two steps.[25] Thus, the use of **8** and the combined olefination-isomerisation process allowed the efficient preparation of a single isomer, without requiring the use or removal of an indole directing group.

The photophysical properties of α-amino acids **9a–9f** and **12** were then measured.[26,27] The tryptophan analogues with ketone, aldehyde and ester alkenyl-substituents were found to possess weak emission and therefore not investigated further. Alkenyl tryptophan analogues with amide **9d** and nitrile **9e** groups were found to have red-shifted absorption and emission maxima compared to L-tryptophan (**1**), with large molar absorption coefficients and Stokes shifts (Table 1).



Scheme 2 Synthesis of tryptophan–coumarin conjugate **12**.

However, with relatively low quantum yields (0.036–0.063), these compounds were only marginally brighter than tryptophan. In contrast, coumarin analogue **12** was found to possess significantly enhanced photophysical properties. In THF, absorption and emission maxima were found at 400 and 505 nm, respectively and with a quantum yield of 0.52, **12** was found to have a near 17-fold increase in brightness compared to tryptophan. Comparison of the fluorescence properties of **12** with amino acids bearing only coumarin side-chains highlights the advantage of using the coumarin motif in conjugation with the indole ring.[28]

As these compounds were designed as charge transfer-based fluorophores, a solvatochromic study was conducted using amino acid **12**. Across a range of solvents, the absorption spectra showed minimal variation, indicating negligible ground-state charge transfer between the indole and coumarin moieties. In contrast, the emission maxima were highly solvent dependent, with more polar environments inducing red-shifted emission. For example, the emission maximum was found at 505 nm in ethyl acetate, shifting to 560 nm in DMSO (Table 1 and Fig. 2a) and 585 nm in methanol (Fig. 2b). This pronounced solvatochromism confirms the charge-transfer character of the indole-coumarin system in the excited state, which is stabilised in polar solvents.

To further characterise the solvatochromic behaviour of amino acid **12**, photophysical studies were performed in water. Interestingly, a significant suppression of emission was observed. This is noteworthy as amino acids that exhibit strong emission in lipophilic media but weak fluorescence in aqueous solutions have been developed as probes for imaging biological membranes.[8,9,14a] To evaluate the potential of **12** as a lipophilic probe, its emission in phosphate-buffered saline (PBS) was compared to phospholipid bilayer membranes designed to mimic biological environments

Table 1 Photophysical data of α-amino acids[a]

Amino acid	λ_{Abs} (nm)	ε ($\times 10^4$ cm^{-1} M^{-1})	λ_{Em} (nm)	Φ_F	Brightness ($\times 10^3$ cm^{-1} M^{-1})
1	279	0.56	348	0.20	1.1
9d	346	2.91	410	0.036	1.0
9e	340	3.32	425	0.063	2.1
12	400	3.71	505	0.52	19
12[b]	406	4.06	560	0.48	19

[a] Spectra were recorded in THF (5 μM). Quantum yields (Φ_F) were determined using anthracene (**9d** and **9e**) or diphenylanthracene (**12**) as the standard. [b] DMSO was used as the solvent (5 μM).

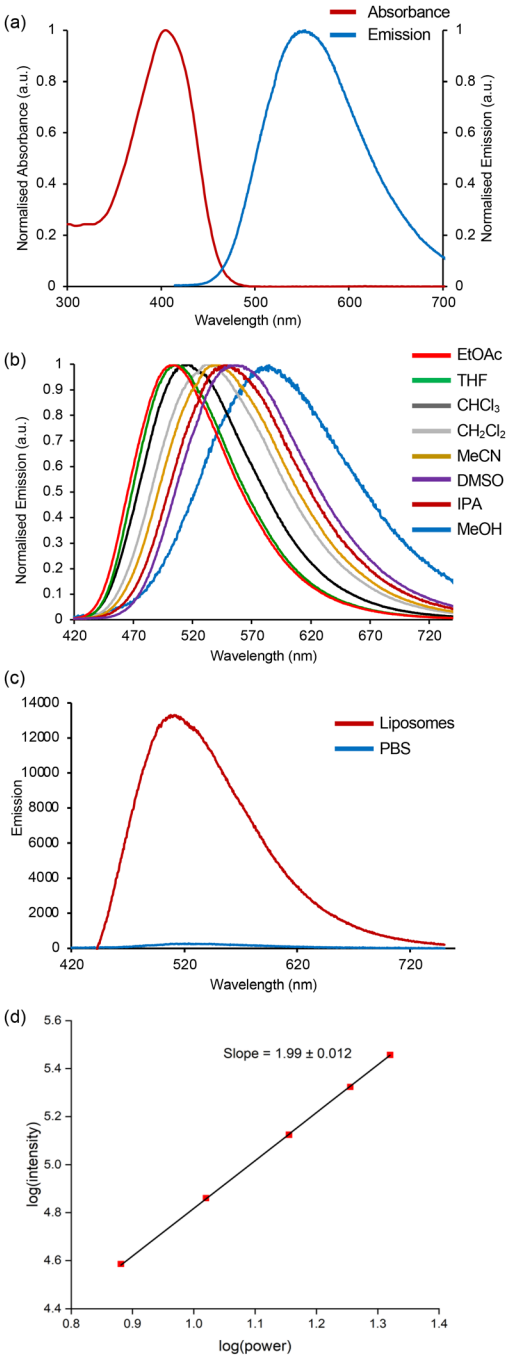


Fig. 2 (a) Normalised absorbance and emission spectra of **12** (5 μM in DMSO). (b) Normalised emission spectra of **12** in various solvents (5 μM). (c) Emission spectrum of **12** in liposomes (PC : cholesterol, 7 : 1) *versus* PBS (5 μM). (d) Log–log plot of fluorescence intensity *versus* laser power for **12** in DMSO (λ_{Ex} = 800 nm).

(Fig. 2c). In PBS, **12** displayed weak emission, likely due to aggregate-induced quenching. In contrast, strong fluorescence was observed in liposomes (phosphatidylcholine/cholesterol, 7 : 1),[8] with a 48-fold increase in intensity. These findings suggest that upon accumulation in lipid environments, **12** adopts a membrane-bound form that is highly emissive.

Based on these interesting results, the photophysical properties of amino acid **12** were further investigated. A pH study demonstrated

that **12** is relatively insensitive to pH changes with only a 20% reduction in emission intensity between neutral and basic pH.[26] The time-resolved fluorescence in both THF and DMSO after one-photon excitation followed mono-exponential kinetics with lifetimes of 4.05 ns and 4.02 ns, respectively, mirroring the similar quantum yields in the same solvents (Table 1).[26] The application of **12** in DMSO for two-photon excited fluorescence was then assessed. Two-photon fluorescence spectroscopy with near-infrared laser excitation has emerged as an important technique for biomedical research as it allows deeper tissue penetration, 3D control of excitation and a reduction in photobleaching.[29] Two-photon excitation of amino acid **12** at 800 nm using a Ti:sapphire laser produced an emission spectrum with the same profile as obtained from one-photon excitation at 405 nm (see ESI†). Two-photon excitation was confirmed *via* a log–log plot of fluorescence intensity *versus* power, which exhibited a slope of 1.99 (Fig. 2d). The two-photon cross section of **12** was then measured at 800 nm and found to be 81 GM. This was used to calculate the two-photon brightness ($\sigma_2\Phi_F$) for **12**, which was determined as 39 GM. This represents a substantial increase in two-photon brightness compared to the recently reported thiazoloindole amino acids ($\sigma_2\Phi_F$ = 14–15 GM).[6d] This study represents a comprehensive analysis of the photophysical properties of amino acid **12** demonstrating its potential as a lipophilic sensitive, one- and two-photon fluorescent probe for biological imaging. Although, amino acids and peptides have previously been labelled with coumarin for imaging applications,[30] **12** represents a rare example of the direct conjugation of a coumarin motif to an existing fluorescent natural side chain, with the combination producing enhanced optical properties.

In summary, we have developed a scalable synthesis of a 2-formyl-L-tryptophan intermediate, enabling the novel preparation of C-2 alkenyl tryptophans *via* olefination reactions. This modular strategy facilitated the discovery of new fluorescent probes, including a directly conjugated coumarin–tryptophan adduct. One- and two-photon fluorescence studies demonstrated the potential of amino acid **12** as a bright, solvatochromic probe suitable for biological imaging applications. Ongoing work is focused on assessing **12** for peptide synthesis and evaluating the resulting peptide-based probes for applications in analysing lipid-rich environments.

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Conflicts of interest

There are no conflicts to declare.

Data availability

Experimental procedures, characterisation and photophysical data, as well as NMR spectra are available in the ESI.†

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