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Conformationally rigid pyrazoloquinazoline α-amino acids: one- and two-photon induced fluorescence†

Jonathan D. Bell,[a] Alexander H. Harkiss,[a] David Nobis,[a] Eilidh Malcolm,[a] Astrid Knuhtsen,[a] Christopher R. Wellaway,[b] Andrew G. Jamieson,[a] Steven W. Magennis[a] and Andrew Sutherland *[a]

The synthesis and photophysical properties of a new class of α-amino acid bearing a rigid pyrazoloquinazoline chromophore are described. Confromational constraint of the amino acid side-chains resulted in high emission quantum yields, while the demonstration of two-photon-induced fluorescence *via* near-IR excitation signifies their potential for sensitive bioimaging applications.

Fluorescence spectroscopy is a powerful technique for studying biological structure and function and for visualizing molecular interactions and intracellular processes.[1] This is due to its high sensitivity and information content, its multiplexing capability, the ability to probe processes over a wide time range, and the availability of an array of versatile fluorescent probes. The investigation of cellular functions such as enzyme mechanisms and protein–protein interactions have often utilized extrinsic fluorescent labels such as green fluorescent protein (GFP) and its derivatives[2] or intrinsic labels such as the proteinogenic fluorescent α-amino acids, tyrosine or tryptophan.[3] However, the attachment of a fluorescent protein can alter the stability and functionality of the fusion partner, while tyrosine and tryptophan have poor optical properties and the occurrence of multiple residues in different environments can complicate their analysis.[3]

In recent years these limitations have been overcome by the development of unnatural fluorescent α-amino acids.[4] The photophysical properties of such amino acids can be tuned for a particular application and they can be incorporated into proteins and peptides by solid phase peptide synthesis (SPPS) or by unnatural amino acid mutagenesis.[5] Of particular interest are amino acids with side-chain chromophores that can be easily incorporated into peptides and proteins with minimal disruption to structure and function.[4,6] While some of these fluorescent α-amino acids have found application in biological and medicinal imaging, an inherent issue of small chromophores is that ultraviolet (UV) light is required for excitation. Prolonged exposure to UV light causes cell and tissue damage through photo-bleaching. In recent years, this limitation has been overcome by the use of two-photon fluorescence microscopy, which permits excitation of the chromophore at longer wavelength, allowing long-term imaging of biological specimens, deeper tissue penetration, with minimization of photo-bleaching and photo-toxicity.[7] While this technique has been utilized with a wide range of fluorophores and applied to the imaging of various biological processes and diseases,[8] there are relatively few reports with unnatural α-amino acids. Examples include the seminal work of Mély and co-workers who used two-photon excitation of peptides containing 3-hydroxyflavone derived α-amino acids (*e.g.* **1**, Fig. 1) for monitoring interactions with cell membranes and intracellular RNA,[9] while the Vendrell group used two-photon excitation of a cyclic peptide bearing a tryptophan-BODIPY conjugate (**2**) for the visualization of fungal infections.[10]

We previously reported the synthesis of 5-arylpyrazole-derived α-amino acids such as **3** (Fig. 1).[11] These compounds possessed

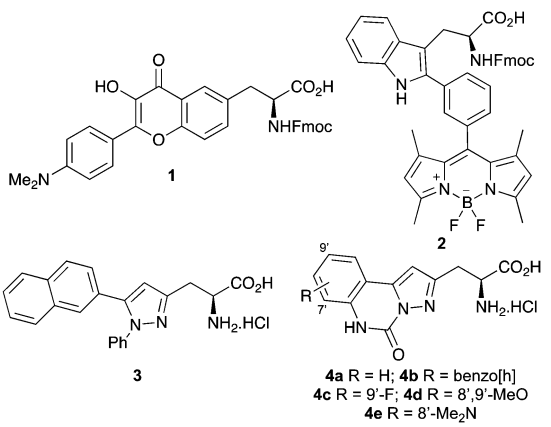

Fig. 1 Selected unnatural fluorescent α-amino acids.

[a] *WestCHEM, School of Chemistry, The Joseph Black Building, University of Glasgow, Glasgow, G12 8QQ, UK. E-mail: Andrew.Sutherland@glasgow.ac.uk*
[b] *Medicinal Chemistry, GlaxoSmithKline Medicines Research Centre, Gunnels Wood Road, Stevenage, SG1 2NY, UK*

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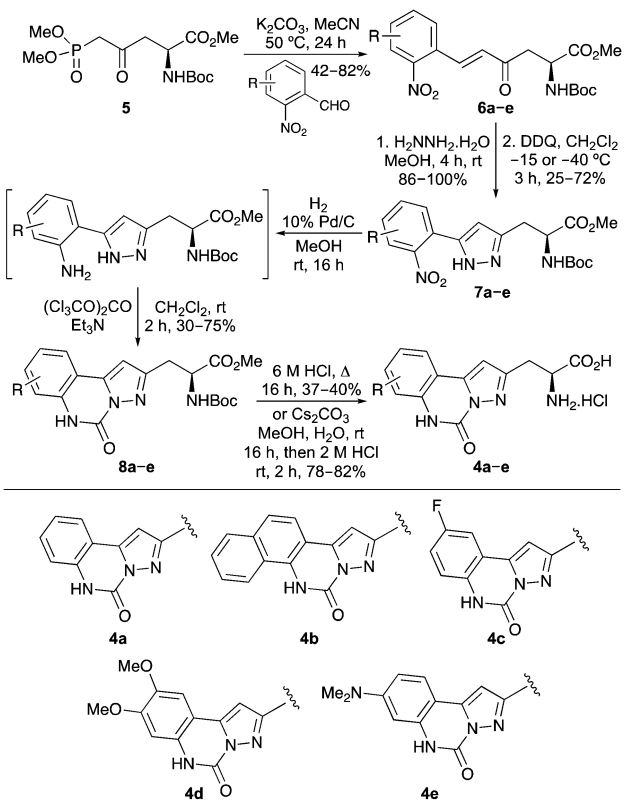
some interesting photophysical properties however, the rotational flexibility between the aryl and pyrazole rings resulted in low quantum yields and brightness. As conformationally rigid, molecular structures permit more efficient π-overlap and subsequently brighter chromophores,[12] we proposed that conformational restriction of these 5-arylpyrazole motifs would lead to α-amino acids with enhanced photophysical properties. Here we describe a new class of α-amino acid (**4**), bearing planar pyrazoloquinazoline chromophores. We also report the photophysical properties of these compounds, demonstrating how conformational restraint leads to fluorophores with high quantum yields and spectroscopic properties that are sensitive to environment polarity. We also show the application of these α-amino acids to two-photon excitation, for potential use in imaging UV sensitive, biological systems.

A synthetic route for the preparation of pyrazoloquinazoline-containing α-amino acids **4** was developed from L-aspartic acid. Initially, L-aspartic acid was converted to phosphononorvaline derivative **5** in five steps and 84% overall yield using previously described methods.[13,14] A range of 2-nitrobenzaldehydes was reacted with phosphononorvaline **5** *via* a Horner–Wadsworth–Emmons reaction, which gave the *E*-isomer of enones **6a–e** in 42–82% yields (Scheme 1).[14,15] The enone moiety was used to construct the pyrazole ring by reaction of **6a–e** with hydrazine *via* a one-pot condensation/aza-Michael process. Low temperature oxidation of the resulting 2-pyrazolines with DDQ gave 5-(2-nitroaryl)pyrazoles **7a–e**.[11] Carbonylation was then achieved by reduction of the nitroaryl moiety, followed by reaction with triphosgene under basic conditions.[16] This gave pyrazoloquinazolines **8a–e** in 30–75% yields over the two steps. Deprotection to the parent amino acids was either conducted in one step under acidic conditions (**4b**, 37%; **4c**, 40%) or more efficiently by a two-step strategy involving ester hydrolysis, followed by removal of the Boc-protecting group under milder acidic conditions (**4a**, 80%; **4d**, 82%; **4e**, 78%).



Scheme 1 Synthesis of pyrazoloquinazoline α-amino acid **4a–e**.

The UV/Vis absorption and emission spectra of the amino acids were recorded in methanol at a concentration of 0.5 × 10^{-5} M (Fig. 2 and Table 1).[17] As expected for chromophores that are planar in the ground state, the amino acids, particularly **4a–d**, showed structured absorption spectra with well-resolved vibrational bands (Fig. 2a).[18] The emission spectra of amino acids **4a–e**, following one-photon excitation at the longest wavelength absorption band, showed strong fluorescence ranging from 342–414 nm, with a vibrational progression which mirrored that of the absorption spectra (Fig. 2b).[19] Amino acid **4e** bearing the electron rich dimethylamino substituent gave a red-shifted, structureless emission maximum at 414 nm, which is indicative of a combination of locally excited and internal charge transfer states.[20] More importantly, the quantum yields of **4a–e** were characteristic of conformational restriction and showed a ten-fold increase in comparison to the non-rigid pyrazole-derived amino acid **3**. As a result, these α-amino acids are substantially brighter than the corresponding non-rigid systems.

As amino acid **4e** showed the most interesting photophysical properties, this compound was further investigated. The fluorescence decay was measured at an emission wavelength of 430 nm in methanol using time-correlated single-photon counting, following excitation at 390 nm (see ESI†). Three decay components were fitted with lifetimes of 0.79, 2.17 and 7.90 ns, and *A*-factors of 0.60, 0.36 and 0.04, respectively. This suggests the molecule exists

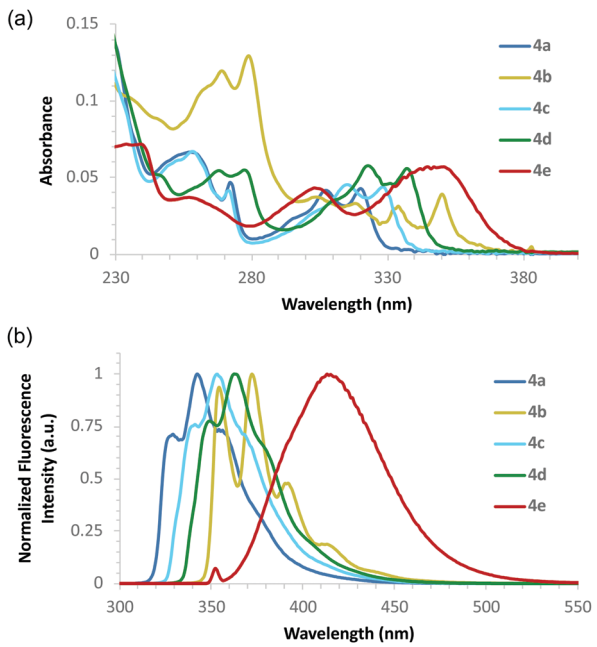


Fig. 2 (a) Absorption spectra of amino acids **4a–4e** recorded at 0.5 × 10^{-5} M in MeOH. (b) Emission spectra of **4a–4e** at 0.5 × 10^{-5} M in MeOH.

Table 1 Photophysical data for 5′-(naphthylen-2″-yl)-1′-phenylpyrazole amino acid **3** and pyrazoloquinazoline-derived α-amino acids **4a–e**[a]

Amino acid	λ_{Abs} (nm)	ε (cm^{-1} M^{-1})	λ_{Em} (nm)	Φ_F[b]	Brightness[c] (cm^{-1} M^{-1})
3	249	23 700	356	0.04	950
4a	321	8300	342	0.42	3500
4b	350 (279)	7700 (24 800)	372 (373)	0.47 (0.27)	3600 (6700)
4c	328	10 000	353	0.47	4700
4d	337	11 200	363	0.56	6300
4e	349	12 200	414	0.48	5900

[a] All spectra were recorded at 0.5 × 10^{-5} M in MeOH. [b] Quantum yields (Φ_F) were determined in MeOH using anthracene and L-tryptophan as standards. [c] Brightness was calculated as the product of the QY and the molar absorptivity at the wavelength maximum specified.

predominantly in two states, as has been observed previously for dimethylamino-substituted fluorophores.[21] The amplitude-weighted average lifetime is 1.57 ns, which is comparable to fluorophores typically used in fluorescence lifetime imaging microscopy (FLIM).[22]

To assess the environmental sensitivity of amino acid **4e**, its photophysical properties were examined in a range of solvents. The absorption maxima (342–348 nm) were found to be independent of polarity (see ESI†), indicating negligible intramolecular interaction between the electron-rich dimethylamino and electron-deficient *N*-acylpyrazole moieties in the ground state. In contrast, the emission spectra were found to be highly sensitive to the solvent used, with increasing polarity leading to concomitantly broadened, structureless emission spectra at longer wavelengths (Fig. 3a).[23,24] For example, in THF, an emission maximum at 374 nm was observed, while in phosphate-buffered saline (PBS), the emission maximum was found at 424 nm. These spectral features and strong solvatochromism are likely due to increasing solvent stabilization of both the locally excited and intramolecular charge transfer states.

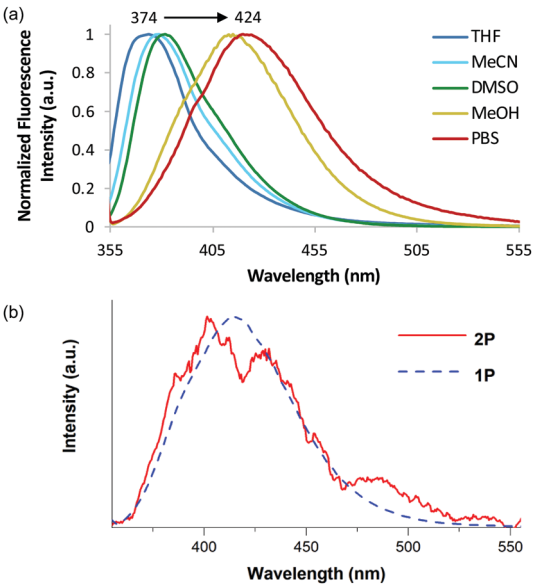
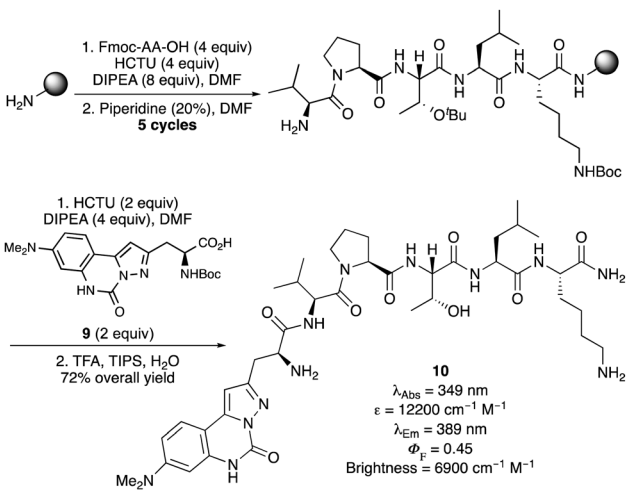


Fig. 3 (a) Solvatochromic study of **4e**. Spectra were recorded using a concentration of 0.5 × 10^{-5} M. (b) Emission of **4e** after two-photon absorption (red) and one-photon (blue) absorption in MeOH. For the two-photon measurement, a solution of 200 μM in MeOH and an excitation centred around 700 nm with FWHM of 10 nm was used.

Application of amino acid **4e** for two-photon spectroscopy was next investigated. Two- and three-photon excitation has recently been shown to be a promising approach for the ultra-sensitive analysis of fluorescent nucleobases, suggesting a similar approach may be applicable to α-amino acids.[25] Two-photon absorption was confirmed with a quadratic dependence of the emitted intensity on the excitation intensity (see ESI†). Furthermore, the molecule has no one-photon absorption in the near IR, precluding linear excitation. The emission spectra of amino acid **4e** in methanol after one- and two-photon excitation have a similar profile (Fig. 3b). The two-photon cross section was measured to be 0.38 ± 0.06 GM using rhodamine B as a reference compound (see ESI†). This value is comparable to other molecules of similar size.[8d,e] Thus, **4e** can undergo two-photon absorption using near-IR excitation, thereby avoiding the use of UV excitation and the issues of photobleaching.

The potential of these amino acids for incorporation into peptides *via* SPPS methodology was also investigated. A model pentapeptide (Val-Pro-Thr-Leu-Lys), based on the Bax-binding domain of Ku70, a multifunctional protein involved in DNA repair and cell-death regulation was chosen.[26] This pentapeptide and similar analogues have been shown to have low cytotoxicity and are effective at penetrating living cells.[26b] The pentapeptide was prepared in an automated, peptide synthesizer using TentaGel™ S Rink Amide (RAM) resin as the polymer support[27] and routine SPPS methodology (Scheme 2). Coupling of each Fmoc-protected amino acid was performed by HCTU[28] activation, followed by piperidine-mediated *N*-deprotection, which gave the N-terminal unprotected pentapeptide. Boc-Protected pyrazoloquinazoline-derived amino acid **9** (see ESI† for preparation) was then coupled manually onto the polymer-supported pentapeptide. A TFA cleavage cocktail was used to remove the protecting groups and release the hexapeptide from the polymer support. Purification by reverse phase-HPLC gave hexapeptide **10** in 72% yield with >95% purity. Analysis of the photophysical properties of hexapeptide **10** showed that apart from a small hypsochromic shift in the emission spectra



Scheme 2 SPPS and photophysical data of hexapeptide **10**.

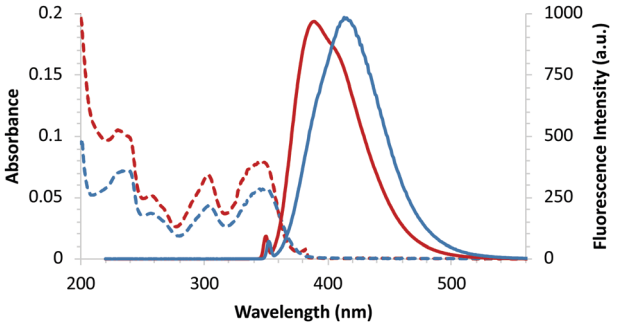


Fig. 4 Absorption (dotted lines) and emission (solid lines) spectra of amino acid **4e** (blue) and hexapeptide **10** (red), recorded at 0.5×10^{-5} M in MeOH.

(Fig. 4), the key photophysical properties of α-amino acid **4e** were retained, including a high quantum yield (0.45) and brightness value (6900 cm^{-1} M^{-1}).

In summary, a new class of conformationally rigid, unnatural α-amino acid has been synthesized using a highly regioselective olefination reaction to introduce side-chain diversity, followed by a one-pot condensation/aza-Michael reaction and carbonylation process to form the key pyrazoloquinazoline ring system. Although containing relatively small side-chains, these compounds were found to be strongly fluorescent with high quantum yields (42–56%). The potential of amino acid **4e** as a sensitive bio-imaging tool was demonstrated with successful excitation of this compound at 700 nm using two-photon spectroscopy. Furthermore, amino acid **4e** was efficiently incorporated into a biologically relevant peptide as part of a SPPS process. Based on the exciting optical properties of this new class of α-amino acid, current work is investigating the introduction of these minimally invasive compounds into other biologically relevant peptides for *in vitro* and *in vivo* spectroscopic and microscopic applications.

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Conflicts of interest

There are no conflicts to declare.

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