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L-(6,7-dimethoxy-4-coumaryl) alanine: an Intrinsic Probe for the Labelling of Peptides

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Abstract: The asymmetric synthesis, spectral properties and incorporation into solid phase peptide synthesis are described for L-(6,7-dimethoxy-4-coumaryl)alanine (Dca). Dca has great utility as a specific and highly sensitive intrinsic probe for fluorescence labelling and quantitation of peptides and proteins.
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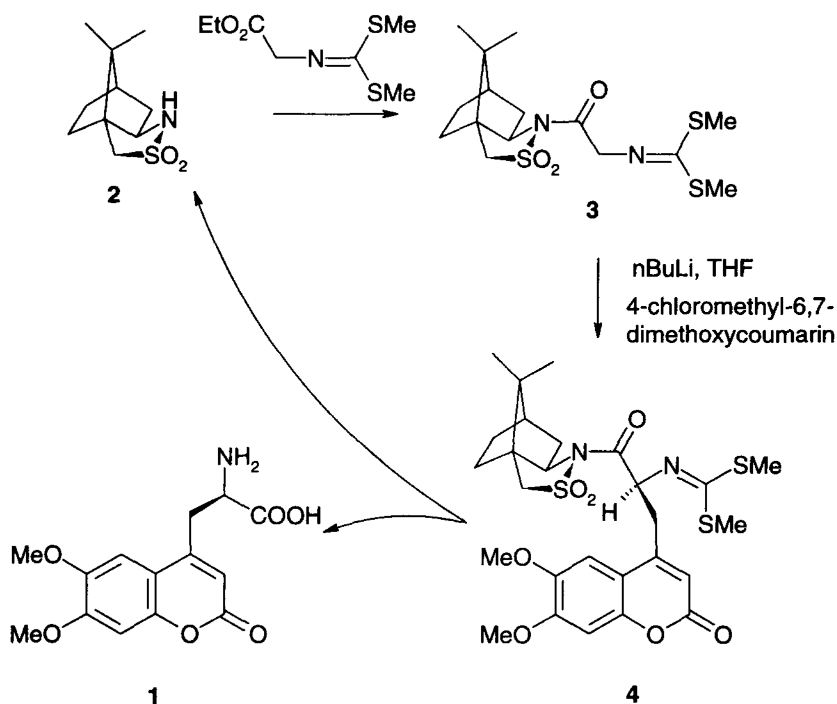
The most commonly adopted general-purpose method for the detection of polypeptides utilises the characteristic UV absorbance of the peptide bond in the 205-230 nm region. Such detection is entirely non-selective however, and does not allow quantitative discrimination of specific peptides in complex mixtures. Fluorescence detection on the otherhand is inherently more selective (because of the use of excitation and emission wavelengths which discriminate against fluorescence from species other than peptide) and also offers the advantage of increased sensitivity (an order of magnitude better than UV detection).[1] For fluorescence detection to be of use in peptide analysis, however, the peptide of interest must either include a tryptophan residue, which may not be possible or desirable, or else it must be labelled using extrinsic probes such as dansyl[2], fluorescamine[3] or OPA[4] which are often intrusive and can lead to changes in the functional and structural properties of the labelled peptide. It would clearly be to great advantage therefore to have a chemically and biologically stable, non-intrusive fluorophore which could be incorporated in a peptide, which would be structurally distinct from tryptophan, and give none of the disadvantages of extrinsic probes. In our own work on pulmonary peptide drug delivery we required a highly sensitive probe of this sort for the labelling and detection of peptides in biological fluid without any complications from background interference and chose to develop a fluorescent amino acid to meet these requirements.

Coumarins, because of their good spectral properties have been used to label amines[5], thiols[6] and carboxyl groups.[7] Although coumarin itself is not fluorescent the addition of various electron donating substituents particularly in the 6 and 7 positions results in a red shift and increased fluorescence intensity.[8] We decided to incorporate the coumarin moiety into an amino acid to yield (6,7-dimethoxy-4-coumaryl) alanine Dca **1**. This amino acid was initially synthesised as the racemate using standard synthetic methods.[9] 3,4-Dimethoxyphenol was condensed with ethyl-4-chloroacetoacetate in the presence of Amberlyst 15 to afford 4-chloromethyl-6,7-dimethoxy coumarin. Diethylacetamidomalonate was treated with sodium hydride followed by the 4-chloromethyl-6,7-dimethoxycoumarin, and subsequent acid hydrolysis afforded the novel amino acid, DL-Dca **1**.[10] For the synthesis of the chiral amino acid, we originally planned to use the Williams method[11] but our efforts here gave only low alkylation yields with high recovery of starting material. We therefore used the Oppolzer[12] sultam which has been recently utilised by Ragnarsson's group[13] for the synthesis of isotopically labelled amino acids.



Scheme 1

Commercially available (2R)-bornane-10,2-sultam auxiliary **2**, was acylated with methyl [bis(methylsulfamyl)methylene]glycinate, to afford (2R)-N-[bis(methylsulfamyl)methylene] glycyl bornane-10,2-sultam **3**. Successive treatment of **3** with nBuLi and the 4-chloromethyl-6,7-dimethoxycoumarin provided the alkylation product **4**. Selective deprotection of the nitrogen by acid hydrolysis followed by saponification with LiOH gave the recovered sultam **2** (85%), Scheme 1. Acidification of the aqueous phase provided the enantiomerically pure L-Dca **1** in 80% yield from **4**. The enantiomeric purity of L-Dca (>99.5% ee) was determined by comparison with the racemate by micellar electrokinetic chromatography using γ-cyclodextrin and sodium dodecyl sulphate.[14] Dca, was protected with the Fmoc group and used in synthesis of a wide range of model peptides.[15] Peptide synthesis was carried out using Fmoc chemistry on a PerSeptive Biosystems 9050 Pepsynthesiser. A PEG-polystyrene resin was used with a modified Rink amide linker. Fmoc amino acids were used in four fold excess and activation carried out using 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate in the presence of diisopropylethylamine. Since the coumarin is less reactive to carbocations than tryptophan the peptides were cleanly cleaved from the resin using trifluoroacetic acid, water and triisopropylsilane (95:4.5:0.5). This is illustrated for the case of the model peptide,[15] H-Arg-Pro-Gly-DPhe-Ser-Gly-Ala-Ser-Dca-OH in Fig. 1.

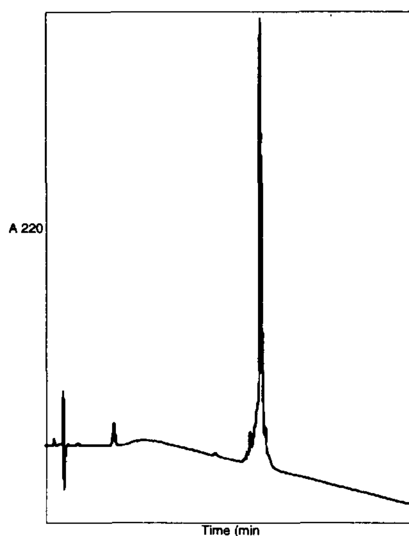


Fig.1. HPLC chromatogram of crude H-Arg Pro-Gly-DPhe-Ser-Gly-Ala-Ser-Dca-OH

The spectral properties of Dca were characterised using the model compound Ac-Dca-NH_2. The molar absorptivity of Ac-Dca-NH_2 at 345 nm was 11,000; its fluorescence excitation spectrum has an excitation at 345 nm and an emission centred at 445 nm (Fig. 2). The HPLC detection sensitivity for peptides containing Dca was at least 5 pmole. Peptides containing Dca were found to be completely stable to non-enzymatic degradation for a period of 10 days in a buffer used for biological transport studies at both 25 °C and 37 °C.

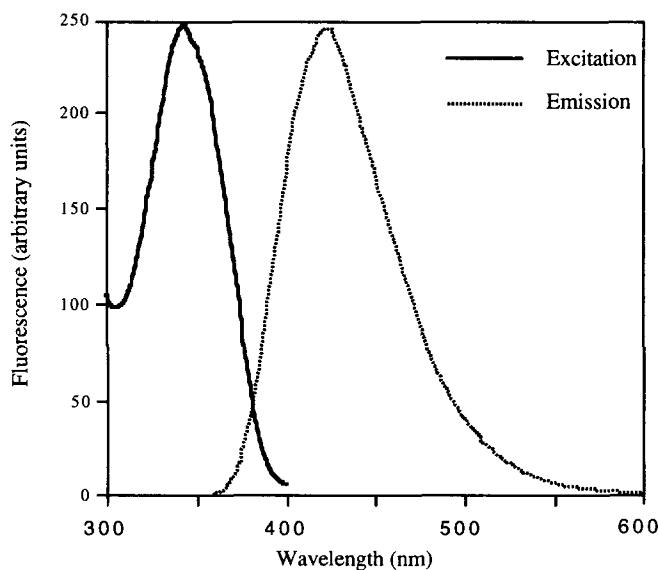


Fig.2. Fluorescence excitation (solid line) and emission (dotted line) spectra of a 0.4 mM solution of Ac-Dca-NH2

In conclusion we note that we have successfully completed the chiral synthesis of a novel amino acid, Dca **1**, using the methods developed by Oppolzer. Dca has been successfully incorporated in synthetic peptides and is found to be stable to repetitive treatment with piperidine and cleavage with TFA, and hence can be incorporated at any position within a peptide sequence. Dca is less susceptible to attack by reactive carbocations as compared to tryptophan. Dca absorbs at longer wavelengths than the naturally occurring amino acids. Dca is relatively small compared with other fluorescent probes, being only slightly larger than tryptophan. The partition coefficient of Ac-Dca-NH_2 was found to be essentially the same as Ac-Trp-NH_2.[16] Dca is both chemically and biologically stable. The novel amino acid Dca is an excellent intrinsic probe for the labelling of peptides and may also find other uses in the labelling of amines, carboxylic acids, and alcohols.

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16. Data to be published.

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