A novel acridone derivative for the fluorescence tagging and mass spectrometric sequencing of peptides

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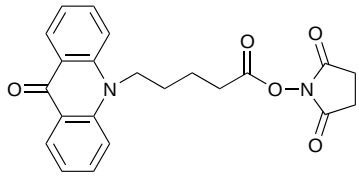
An acridone derivative containing an activated carboxylic group is highly reactive towards primary amine groups, and allows selective analysis either using absorbance at a wavelength of 410 nm or fluorescence at excitation and emission wavelengths of 400 and 418 nm, respectively; MS–MS analysis of peptides tagged with this reagent gives fragmentation patterns which are usually considerably more intense than background.

Peptides tagged with a fluorescent label offer a major advantage in that they can be selectively analysed or localised even in complex biological fluids. Such derivatives have proved to be especially useful in bio- and immuno-assays. Fluorescent labels have included fluorescein, methylanthranylate, a number of cyanine dyes and succinimidyl pyrene-1-butyrate.[1–4]. These molecules can have disadvantages, such as sensitivity to light and fluorescence quenching in acidic or basic conditions. Moreover, the cyanine dyes are bulky molecules (molecular weight *ca*. 1000) which carry three to five charges at neutral pH; such properties can interfere with biological activity.

The acridone moiety is highly fluorescent and is stable to light, heat and oxidation. In earlier studies we have used 2-aminoacridone extensively in the reductive amination of monosaccharides and a variety of complex oligosaccharides.[5,6] A general and highly sensitive assay for antibody catalysis has also been demonstrated recently by using a number of acridone labelled compounds.[7]

We have now synthesised the succinimide ester **1** in three



1

$F^{1}VNQHLC(SO_3H)GSH^{10}LVEALYLVC(SO_3H)G^{20}ERGFFYTPKA^{30}$

2

steps from relatively inexpensive starting materials. Acridone was reacted with methyl 5-bromopentanoate to give methyl 9,10-dihydro-9-oxoacridine-10-pentanoate **3** (31%). Ester **3** was then hydrolysed with 10% aqueous NaOH to give the corresponding pentanoic acid **4** in (80%). Finally, to a solution of **4** and *N*-hydroxysuccinamide was added DCC to give **1** (34%) as a yellow solid (mp 181–182 °C). None of the yields mentioned have been optimised.

Compound **1** absorbs visible light at 410 nm and fluoresces intensely at excitation and emission wavelengths of 400 and 418 nm, respectively. It is neutral over the widest possible pH range and reacts rapidly with primary amines and, to a lesser extent, with phenolic hydroxy groups. To show the diversity in the use of **1** in the labelling and tracking of peptides, we have derivatised insulin chain B (oxidised) **2**, and carried out MS and enzymic digestion studies on material tagged in one or more positions. About 5 nmol of **2** were used in these experiments in 20 μl of solution. In separate experiments we have shown that a 20–50 pmol level of protein could be derivatised especially by performing the derivatisation reaction in volumes as low as 2 μl of solution. We used higher amounts for this study as it was necessary to collect derivatives of **2** for the tryptic digestion experiments which we describe later.

The extent of labelling by **1** can be controlled by its molar ratio to the peptide to be derivatised. Reactions were carried out by mixing the peptide in 10 μl of $NaHCO_3$ buffer (pH 8.5) with a solution (10 μl) of **1** in MeCN. The resulting mixture was left at room temperature for 1 h prior to analysis. For single labelling a 1:1 molar ratio is used, whereas multiple labelling is obtained in the presence of 10 molar excess of **1**. From the preliminary studies carried out on a number of peptides it appears that the ε-amino group in lysine is the first to be derivatised, followed by the amino group at the N-terminal end of the peptide and tyrosine residues. Multiple labelled peptides are easily separated and isolated by HPLC, using a reversed-phase C18 column and a mobile phase consisting of a gradient of 0.1% TFA in water and 0.1% TFA in MeCN.

Derivatisation of **2** with excess **1** yields four major products, one of which is single-tagged and the remainder of which are double tagged. We carried out LC–MS–MS analysis of triply charged parent ions using a Finnigan MAT LCQ ion trap mass spectrometer. Fig. 1 shows the total ion current for all product ions from an MS–MS experiment after derivatisation of **2** using excess **1**. Electrospray mass spectrometric and MS–MS analysis showed that peak 1 was related to **2** containing a single acridone moiety at the lysine residue in position 29. As expected this molecule elutes first as it is less hydrophobic than the double-labelled peptides. The latter peptides are again labelled at the same lysine and in addition contain a tag at the N-terminal phenylalanine (peak 2) or at a tyrosine in positions 26 (peak 3) and 16 (peak 4). It is interesting that these double labelled peptides also differ in hydrophobicity, and this may be partly due to conformational changes.

The product ion spectra from the fragmentation of the triply charged underivatised (*m/z* 1166) and double tagged **2** (*m/z*

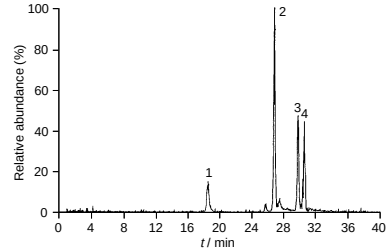


Fig. 1 Reversed-phase HPLC–MS–MS analysis of insulin chain B derivatised with excess **1**. Detection of peaks was by monitoring total ion current for all product ions produced from triply charged parent ions. Peak 1 is related to a derivative of **2** labelled only at ^{29}Lys, whereas peaks 2–4 are double tagged derivatives, again labelled at ^{29}Lys and at ^{1}Phe, ^{26}Tyr and ^{16}Tyr, respectively.

Table 1 Fragment ions from triple protonated **2**, single tagged

Fragment	Mass	*m/z* observed	Charge
FVNQHLC(SO_3H)GSHLVEALYLVC(SO_3H)GERGFFYTPK(tag)A[a]	3772	1259	3+
FVNQHLC(SO_3H)GSHLVEALYLVC(SO_3H)GERGFFYTPK(tag)	3684	1229	3+
FVNQHLC(SO_3H)GSHLVEALYLVC(SO_3H)GERGFFYT	3182	1592	2+
FVNQHLC(SO_3H)GSHLVEALYLVC(SO_3H)GERGFFY	3080	1541	2+
FVNQHLC(SO_3H)GSHLVEALYLVC(SO_3H)GERGFF	2917	1459	2+
FVNQHLC(SO_3H)GSHLVEALYLVC(SO_3H)GERGF	2770	1386	2+
FVNQHLC(SO_3H)GSHLVEALYLVC(SO_3H)GER	2566	1284	2+
FVNQHLC(SO_3H)GSHLVEALYLV	2073	1038	2+
FVNQHLC(SO_3H)GSHLVEALYL	1973	988	2+
FVNQHLC(SO_3H)GSHLVEALY	1860	931	2+
FVNQHLC(SO_3H)GSHLVEAL	1696	849/1697	2+/1+
FVNQHLC(SO_3H)GSHLVEA	1583	793/1584	2+/1+
FVNQHLC(SO_3H)GSHLV	1383	1384	1+
VC(SO_3H)GERGFFYTPK(tag)A	1799	1800	1+
FYTPK(tag)A	1002	1003	1+
TPK(tag)A	692	693	1+
PK(tag)A	591	592	1+

[a] Parent ion (single tag, peak 1).

1351) are shown in Fig. 2(*a*) and (*b*), respectively. Signals from the single and multiple tagged peptides are more intense than those from intact **2**. Product ions obtained from the fragmentation of the single tagged derivative of **2** are collected in Table 1. It is clear that besides confirming the positions of the acridone labels on **2** these data provide an abundance of sequencing information. Moreover, covalent tagging of the acridone derivative to **2** *via* a stable amide bond appears to have simplified the distribution of fragment ions compared to those obtained from **2** itself, most probably due to a decrease of the number of basic sites.

Due to the intense fluorescence of the acridone moiety we also investigated the ease of adaptation of this fluorophore as a label useful in assaying the bioactivity of an enzyme. We were especially interested to find out whether labelling hinders biological activity. For this purpose we followed the hydrolysis of single and double labelled **2** by the proteolytic enzyme trypsin. The single (peak 1) and double labelled (peak 2) insulin derivatives were isolated by HPLC and about half of the solvent was removed *in vacuo*. The volume of the remaining solution was doubled by adding 50 mM NH_4HCO_3 buffer (pH 7.8). Trypsin in water was added to give a derivatised peptide to enzyme ratio of 200 : 1. The hydrolysis was monitored with time

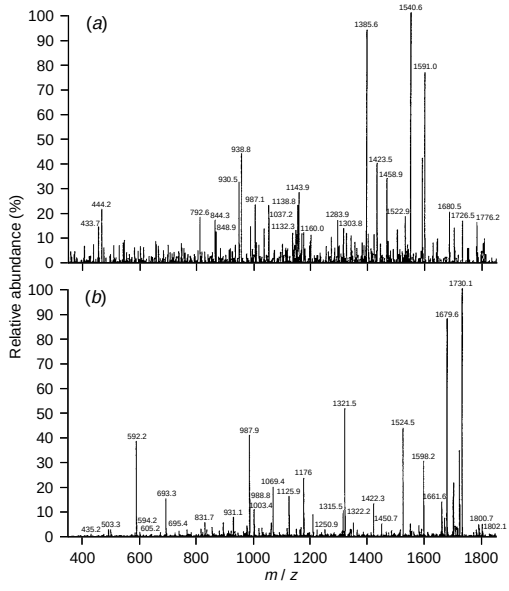


Fig. 2 Influence of the presence of label on the product ion spectrum from the fragmentation of triply charged (*a*) original insulin chain B and (*b*) peptide labelled in positions 1 and 29

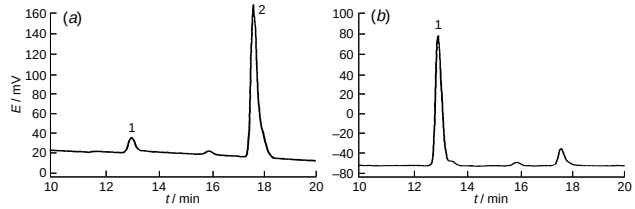


Fig. 3 HPLC analysis of the mixture from the enzymic hydrolysis of single tagged insulin chain B. Starting material (peak 2) disappears leading to the formation of one product (peak 1) with time: (*a*) 0 and (*b*) 60 min.

by direct HPLC analysis of the reaction mixture and fluorescence detection, and was found to be complete in about 60 min.

As expected, in the case of single tagged **2** only one fluorescent product was obtained (Fig. 3). MS confirmed that the fluorescence signal was related to the peptide GFFYTPK-(tag)A (*m/z* 1206), as a result of hydrolysis at the arginine residue in position 22. The remainder of **2**, that is FVNQ...GER (*m/z* 2584), had a retention time shorter than that containing the acridone moiety, which emphasises the hydrophobic character imparted by this tag.

The tryptic digestion of double labelled **2** showed the simultaneous formation of two fluorescent signals. This confirmed that the arginine residue was located in between the two tags in the sequence. One of the signals again corresponded to the peptide GFFYTPK(tag)A. The other peptide, now with a longer retention time, corresponded to F(tag)V NQ...GER (*m/z* 2860). From these studies it is clear that derivatised lysine residues are not susceptible to tryptic digestion. However, hydrolysis on the carbonyl end of the arginine residue was not affected by this labelling procedure.

Footnote and References

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