ARTICLE

OBC
www.rsc.org/obc

Development of a temporary marker for peptides †

M. Sameiro T. Gonçalves and Hernâni L. S. Maia *
Department of Chemistry, University of Minho, Gualtar, P-4710-057 Braga, Portugal.
E-mail: hlsmaia@quimica.uminho.pt

Received 16th December 2002, Accepted 28th February 2003
First published as an Advance Article on the web 31st March 2003

3-[(*N,N*-Dimethylaminophenyl)-4′-diazenyl]benzoic acid was coupled with several amino acid esters and the product acylated further with Boc. The material thus obtained was then submitted to cleavage by electrolysis and nucleophilic attack in order to evaluate the possibility of using this chromophore as a temporary marker.

Introduction

In recent years, the use of dyes, or dye-like molecules in biomedical applications, has seen a remarkable growth in research interest and technical importance and, at present, it is probably the fastest expanding area of dye chemistry. This can be illustrated by the use of dyes in many diagnostic applications, often to allow qualitative and quantitative determinations to be performed easily and reliably by rapid and economic methods.[1] Such applications range from simple organic reactions for the spectroscopic detection and measurement of body fluid analytes to high definition imaging technology for tumor detection.

Diazo coupling is particularly useful in methods for the identification of proteins and for determining enzyme activity.[2] Although the procedures do not employ dyes as such, the end result is an azo dye chromophore. Diazo coupling has long been employed in protein chemistry, and as early as 1915 Pauly first used diazotised sulfanilic acid (Pauly reagent) for coupling with tyrosine and histidine residues.[3,4] The resulting azo compounds are coloured and several spectrophotometric methods have since been developed for various applications, such as protein labelling, detection of drug abuse, diagnosing diseases, immunological assays and cancer treatment. Later, Kozaki *et al.*[5] improved Pauly's method for quantitative analysis of L-histidine. Meanwhile, new methods and also improvements to existing methods for the determination of enzyme activity have been developed.[6,7] The search for photoresponsive conformational and biological properties led Behrendt *et al.*[8] to design small, cyclic peptides containing azobenzene moieties in the backbone. Sebestyén *et al.*[9] reported the synthesis, and some properties, of free peptides and peptide libraries labelled with chromophores and studied the effect of colour labelling on the biological activity of a model peptide. It is now clear that dye chemistry will continue to attract the biochemist or clinician into becoming involved with such materials and, thus, they should retain an awareness of classical dye chemistry.

With this in mind, we acylated several amino acid derivatives with an azo dye to test its use as a marker for possible application in biological assays. The acylating reagent was a reactive azo dye we had developed for textile applications and that was used with good results in dyeing wool and polyamide fibres.[10] The coloured products were submitted to different cleavage tests in order to investigate their use as temporary markers.

Results and discussion

One equivalent of a carboxyl azo dye (**1**) obtained from 3-aminobenzoic acid and *N,N*-dimethylaniline was reacted with amino acid methyl or ethyl esters in DMF by a DCC–HOBt coupling (Scheme 1). After purification by chromatography (dry or flash) on silica gel followed by recrystallisation, the corresponding orange 3-[(*N,N*-dimethylaminophenyl)-4′-diazenyl]-benzoyl derivatives (**2a**–**h**) were obtained as solid materials in yields ranging from 56 to 99% (Table 1); these were characterised by elemental analysis and by NMR (^{1}H and ^{13}C), FTIR and visible spectroscopy. The visible spectra showed a peak with λ_{max} at 415 nm and ε falling between 17000 (**2a**) and 33145 (**2g**). All products were stable on storage in the air and at room temperature. The *tert*-butyl ester of the dipeptide phenylalanyl-valine was also acylated under identical conditions to yield 78% of the expected stable derivative (**2i**) with λ_{max} 419 nm and ε 27648.

Table 1 Synthesis of coloured compounds **2** and **3**

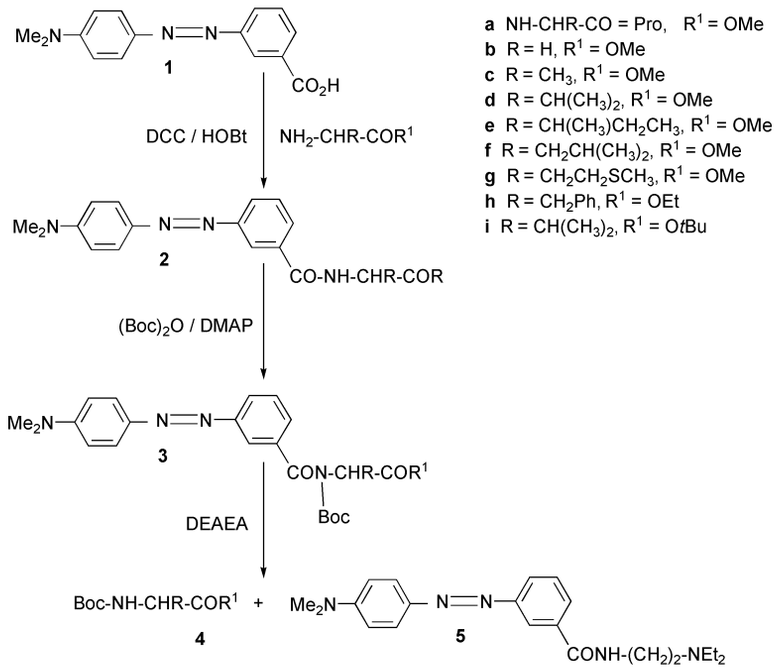
Product (compound no.)	Yield (%)	
	2 (R = H)	**3** (R = Boc)
Dpa-Pro-OMe (**a**)	56	—
Dpa-Gly(*N*-R)-OMe (**b**)	99	90
Dpa-Ala(*N*-R)-OMe (**c**)	91	99
Dpa-Val(*N*-R)-OMe (**d**)	81	85
Dpa-Ile(*N*-R)-OMe (**e**)	79	56
Dpa-Leu(*N*-R)-OMe (**f**)	59	99
Dpa-Met(*N*-R)-OMe (**g**)	70	60
Dpa-Phe(*N*-R)-OEt (**h**)	77	99
Dpa-Phe(*N*-R)-Val-O*t*Bu (**i**)	78	—

With the aim of testing the possibility of recovering the initial amino acid esters by removal of the chromophore, (considering that formyl, acetyl and benzoyl groups can be cleaved by *N,N*-diethylaminoethylamine (DEAEA) from the amide bond of Boc-acylamides under very mild conditions[11]) compounds **2b**–**h** were converted into the corresponding equally coloured *tert*-butoxycarbonyl derivatives. For this purpose they were reacted at room temperature with di-*tert*-butyl pyrocarbonate in dry acetonitrile and in the presence of a catalytic amount of 4-(*N,N*-dimethylamino)pyridine (DMAP). After purification by dry chromatography, the coloured reaction products **3b**–**h** were obtained in yields ranging from 56 to 99% (Table 1) and characterised as above. Their visible spectra showed λ_{max} falling within 418 nm (**3b**) and 470 nm (**3e**), with ε values varying between 7723 (**3d**) and 24589 (**3g**).

Deacylation of the coloured Boc-acylamides (**3b**–**h**) was then carried out by aminolysis with DEAEA in dry acetonitrile at room temperature; the expected Boc-amino acids (**4b**–**h**) were isolated as colourless and usually non-crystalline materials in yields within the range 40–78% (Scheme 1, Table 2). TLC showed that a coloured by-product was also formed in all cleavages of compounds **3b**–**h** with DEAEA; in a few cases (**3b**, **3f**, **3g** and **3h**) it was isolated in yields within the range 74–100% and characterised. Suspecting that this was the transamination product resulting from transfer of the dye moiety to DEAEA, a genuine sample of this compound was prepared by direct

† Electronic supplementary information (ESI) available: IR, UV, ^{1}H NMR and ^{13}C NMR spectra of compounds **2a**–**i**, **3b**–**h**, **4b**–**h**, **5**, **6c** and **h**, **7h**, **8**–**12**, **13c** and **h**, and **14**. See http://www.rsc.org/suppdata/ob/b2/b212470j/

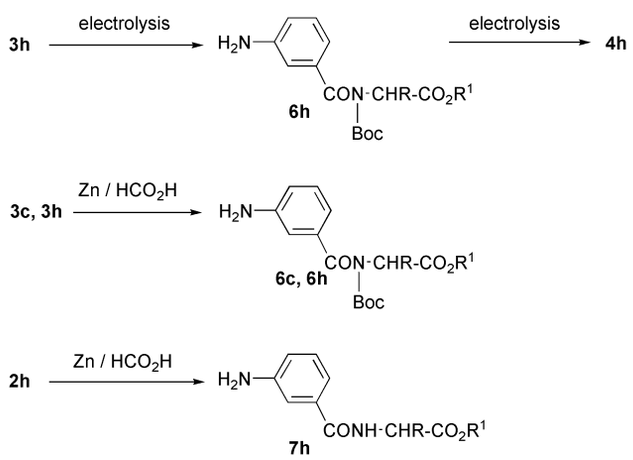
DOI: 10.1039/b212470j



Scheme 1

Table 2 Selective cleavage of the chromophores

Product	Starting material	Deprotection method	Yield (%)
4b	**3b**	DEAEA	56
4c	**3c**	DEAEA	63
4d	**3d**	DEAEA	40
4e	**3e**	DEAEA	72
4f	**3f**	DEAEA	49
4g	**3g**	DEAEA	78
4h	**3h**	DEAEA	71
11	**9**	DEAEA	84
4h + **6h**	**3h**	Electrolysis at −2.03 V	32 + 32
4h	**3h**	Electrolysis at −2.5 V	48
4h	**6h**	Electrolysis at −2.31 V	30
7h	**2h**	Zn–HCO_2H	30
6c	**3c**	Zn–HCO_2H	39
6h	**3h**	Zn–HCO_2H	45



Scheme 2

acylation of DEAEA with the dye. The product (**5**) was characterised by IR and NMR (^1H and ^{13}C) spectroscopy and by high resolution mass spectrometry and compared well with the by-product referred to above. As the formation of this by-product consumed DEAEA, at least two equivalents of this reagent had to be used in the cleavage reactions.

In order to investigate the electrochemical behaviour of the dye (**1**) with the aim of testing deacylation by electrolysis, a cyclic voltammogram of its methyl ester (**14**) in dimethylformamide (DMF) containing tetrabutylammonium tetrafluoroborate (TBAB) as the supporting electrolyte was obtained, showing peaks at −1.16 and −2.22 V. The compound was then electrolysed[12] at a constant potential 50 mV more negative than that of the second peak, as no reaction was observed when electrolysis was attempted at the first peak; the material was completely consumed after a few hours, which we assigned to reduction of the azo group.[13–15] Compound **3h** behaved similarly to the dye, showing reduction peaks at −1.19 and −2.03 V and no reaction at the first peak. Thus, it was electrolysed at a constant potential 50 mV more negative than that of the second peak. The reaction was monitored by HPLC, showing that 88% of the initial material was consumed 6 hours after the starting point; work up of the reaction mixture at this stage yielded 32% of the expected cleavage product (**4h**) together with a material that corresponded to 32% of *N*-(3-aminobenzoyl)-*N*-*tert*-butyloxycarbonylphenylalanine ethyl ester (**6h**) (Scheme 2, Table 2). The latter must have resulted from reductive cleavage of the azo group and its cyclic voltammogram showed a single peak at −2.31 V, which falls within the region of potentials that would be expected for cleavage of the 3-aminobenzoyl group. When **6h** was electrolysed at a potential 50 mV more negative than that corresponding to this peak, the expected cleavage product (**4h**) was obtained in a yield of 30%. These results suggest that reductive cleavage at the azo group and at the amide nitrogen atom occur at similar potentials, below −2 V, with compound **6h** as a possible intermediate. Following this result, **3h** was electrolysed at −2.5 V to give **4h** in a yield of 48% of pure product.

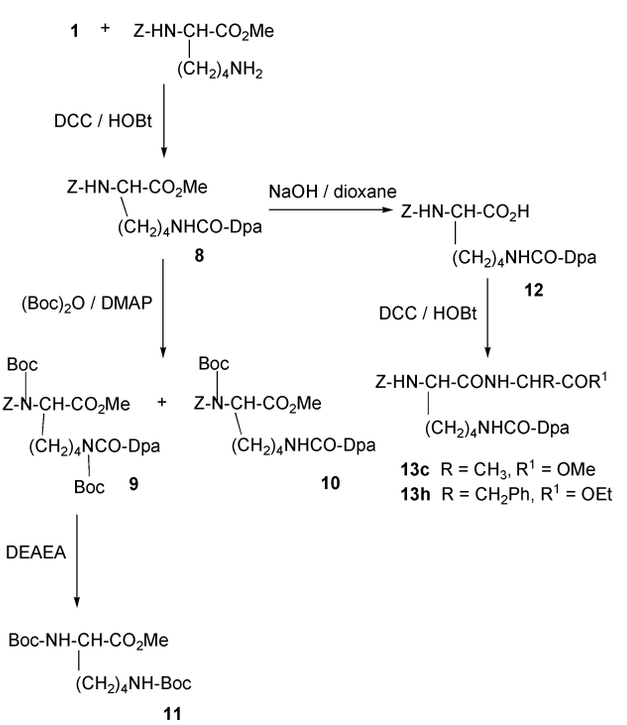
Colourless products were also obtained by reductive cleavage of the azo bond of compounds **3c** and **3h** by zinc powder,[16] in the presence of formic acid to give the corresponding *N*-(3-aminobenzoyl)amino acid esters **6c** and **6h** in yields of 39 and 45% as pure materials (Scheme 2, Table 2). As it would be expected, for this reduction to occur there would be no need for the aid of a Boc group, which was confirmed when **2h** was reacted under the same conditions as above to give phenylalanine ethyl ester (**7h**) in a yield of 30%. The low yields of pure

Table 3 Results obtained in the synthesis of compounds **8**–**12**

Product (compound no.)	Yield (%)
Z-Lys(ω-Dpa)-OMe (**8**)	66
Z-Lys(*N*-Boc,ω-Boc,ω-Dpa)-OMe (**9**)	90
Z-Lys(ω-Dpa)-OH (**12**)	98
Z-Lys(ω-Dpa)-Ala-OMe (**13c**)	95
Z-Lys(ω-Dpa)-Phe-OEt (**13h**)	97

products obtained in the reactions with zinc were assigned to difficulties met during purification. In fact, the reactions reached completion within ten minutes and no sign of side products was observed, which supports our belief that the initial coloured materials were completely converted into the expected colourless derivatives.

In addition to labelling amino acids or peptides at their *N*-terminus, an alternative acylation at a lysine ω-amine group was also investigated (Scheme 3, Table 3). Thus, the methyl ester of *N*-benzyloxycarbonyl lysine was reacted with **1** under the conditions reported above, to give the expected coloured derivative (**8**) in a yield of 66%. The product was then reacted with di-*tert*-butyl pyrocarbonate; when only a slight excess of the reagent was used (2.4 equivalents) and a mixture of the required diacylation product (**9**, 45%) was obtained together with the monoacylated product (**10**, 35%). Thus, the reaction was repeated with a larger excess of pyrocarbonate (6 equivalents), which yielded 90% of **9**. This was treated with DEAEA as above, giving the product of cleavage (**11**) of both the ω-acyl group and the initial *N*-protecting group (Z) in a yield of 92%. The product (**12**) of saponification of **8** was obtained in a yield of 98% and then coupled with alanine methyl ester and phenylalanine ethyl ester to produce**13c** and **13h** in high yields (95 and 97%, respectively).



Scheme 3

In conclusion, acylation with the dye can be performed at the *N*-terminus of either amino acid esters or peptide esters to give coloured products in high yields. As the compounds explored in our work are acceptable models for larger peptides or even proteins, our results suggest that the dye is suitable for marking materials of biological interest. Alternatively, marking can be performed at a lysine residue, when this is appropriate, either before or after the peptide is made. Moreover, if required, the colour can be eliminated *in situ* either by removing the chromophore with base or by electrolysis, the efficiency of the latter being not as satisfactory as that of the former. However, our best approach seems to lie in breaking the chromophore by reducing the azo group with zinc.

Experimental

All melting points are uncorrected and they were measured on a Gallenkamp melting point apparatus. TLC analyses were carried out on 0.25 mm thick precoated silica plates (Merck Fertigplatten Kieselgel 60 F_{254}) and spots were visualised under UV or by exposure to vaporised iodine. Dry and column chromatography were carried out on Merck Kieselgel (230–240 mesh). Light petroleum refers to the fraction boiling within the range (40–60) °C. IR spectra were determined on a Perkin-Elmer FTIR-1600 and UV spectra were determined on a Hitachi U-2000 spectrophotometer. ^{1}H NMR spectra were recorded on a Varian 300 spectrometer in 5% $CDCl_3$ solution at 25 °C. All chemical shifts are given in δ ppm using δ_H $Me_4Si = 0$ as a reference and J values are given in Hz. Assignments were made by comparison of chemical shifts, peak multiplicities and J values. ^{13}C NMR spectra were run on the same instrument but at 75.4 MHz using the solvent peak as an internal reference. Spectrometric analyses were performed at the "Unidad de Espectrometria de Masas" of the University of Vigo, Spain. Elemental analyses were carried out on a Leco CHNS 932 instrument. For controlled potential electrolysis experiments, a Hi-Tek potentiostat DT 2101, and a Hi-Tek wave generator PP RI, connected to a Philips recorder PM 8043 were used. The electrolysis cell was a conventional two-compartment, three-electrode, home-built batch cell of the type illustrated elsewhere.[17] HPLC experiments were run on a Shimadzu instrument, type 6A, connected to a Merck pre-packed column, type Hibar RT 250-4, filled with LiChrospher 100 CH-18/2 (5 μm) and the eluent was a mixture of acetonitrile and water. The peaks were measured with a Shimadzu integrator, type C-R6A Chromatopack. Phenylalanine methyl ester and *N*-benzyloxycarbonyllysine were commercial products. Other amino acid methyl esters were prepared with thionyl chloride by the usual procedure and compound **1** was synthesised according to a procedure described elsewhere.[10]

General method for acylation with the dye

3-[(*N,N*-Dimethylaminophenyl)-4′-diazenyl]benzoic acid (**1**) in DMF was reacted with an amino acid methyl or ethyl ester hydrochloride (or peptide *tert*-butyl ester) in DMF by a standard DCC–HOBt coupling. After dry chromatography on silica and recrystallisation from ethyl acetate–hexane, the required product was obtained as an orange solid.

***N*-{3-[(*N,N*-Dimethylaminophenyl)-4′-diazenyl]benzoyl}-proline methyl ester 2a.** The product of reaction of **1** with proline methyl ester hydrochloride (185 mg, 1.12 mmol) was chromatographed using chloroform–methanol 6.5 : 0.5 as the eluent to give the ester **2a** (237 mg, 56%), mp 119.4–121.3 °C, R_f 0.94 (chloroform–methanol 6.5 : 0.5) (Found: C, 66.38; H, 6.45; N, 14.70. $C_{21}H_{24}N_4O_3$ requires C, 66.30; H, 6.36; N, 14.73%).

***N*-{3-[(*N,N*-Dimethylaminophenyl)-4′-diazenyl]benzoyl}-glycine methyl ester 2b.** The product of reaction of **1** with glycine methyl ester hydrochloride (141 mg, 1.12 mmol) was chromatographed using diethyl ether–hexane (mixtures of increasing polarity) as the eluent to give the ester **2b** (375 mg, 99%), mp 122.2–124.6 °C, R_f 0.20 (ethyl ether–hexane 9 : 1) (Found: C, 63.72; H, 6.15; N, 16.26. $C_{18}H_{20}N_4O_3$ requires C, 63.51; H, 5.92; N, 16.46%).

***N*-{3-[(*N,N*-Dimethylaminophenyl)-4′-diazenyl]benzoyl}-alanine methyl ester 2c.** The product of reaction of **1** with

alanine methyl ester hydrochloride (156 mg, 1.12 mmol) was chromatographed using diethyl ether–hexane 9 : 1 as the eluent to give the ester **2c** (360 mg, 91%), mp 132.4–134.1 °C, R_f 0.56 (diethyl ether–hexane 9 : 1) (Found: C, 64.65; H, 6.21; N, 15.68. $C_{19}H_{22}N_4O_3$ requires C, 64.39; H, 6.26; N, 15.81%).

***N*-{3-[(*N,N*-Dimethylaminophenyl)-4′-diazenyl]benzoyl}valine methyl ester 2d.** The product of reaction of **1** with valine methyl ester hydrochloride (188 mg, 1.12 mmol) was chromatographed using diethyl ether–hexane 9 : 1 as the eluent to give the ester **2d** (346 mg, 81%), mp 114.3–115.9 °C, R_f 0.70 (diethyl ether–hexane 9 : 1) (Found: C, 65.74; H, 6.88; N, 14.74. $C_{21}H_{26}N_4O_3$ requires C, 65.95; H, 6.85; N, 14.65%).

***N*-{3-[(*N,N*-Dimethylaminophenyl)-4′-diazenyl]benzoyl}-isoleucine methyl ester 2e.** The product of reaction of **1** with isoleucine methyl ester hydrochloride (203 mg, 1.12 mmol) was chromatographed using diethyl ether–hexane 9 : 1 as the eluent to give the ester **2e** (351 mg, 79%), mp 117.3–118.1 °C, R_f 0.78 (diethyl ether–hexane 9 : 1) (Found: C, 66.68; H, 7.16; N, 14.21. $C_{22}H_{28}N_4O_3$ requires C, 66.64; H, 7.12; N, 14.13%).

***N*-{3-[(*N,N*-Dimethylaminophenyl)-4′-diazenyl]benzoyl}l-eucine methyl ester 2f.** The product of reaction of **1** with leucine methyl ester hydrochloride (162 mg, 1.12 mmol) was chromatographed using diethyl ether–light petroleum 9.5 : 0.5 as the eluent to give the ester **2f** (260 mg, 59%), mp 136.4–137.5 °C, R_f 0.71 (diethyl ether–light petroleum 9.5 : 0.5) (Found: C, 66.69; H, 7.11; N, 14.10. $C_{22}H_{28}N_4O_3$ requires C, 66.64; H, 7.12; N, 14.13%).

***N*-{3-[(*N,N*-Dimethylaminophenyl)-4′-diazenyl]benzoyl}-methionine methyl ester 2g.** The product of reaction of **1** with methionine methyl ester hydrochloride (223 mg, 1.12 mmol) was chromatographed using diethyl ether–light petroleum 9.5 : 0.5 as the eluent to give the ester **2g** (323 mg, 70%), mp 120.5–121.3 °C, R_f 0.68 (diethyl ether–light petroleum 9.5 : 0.5) (Found: C, 60.81; H, 6.32; N, 13.56; S, 7.53. $C_{21}H_{26}N_4O_3S$ requires C, 60.84; H, 6.32; N, 13.52; S, 7.74%).

***N*-{3-[(*N,N*-Dimethylaminophenyl)-4′-diazenyl]benzoyl}-phenylalanine ethyl ester 2h.** The product of reaction of **1** with phenylalanine ethyl ester hydrochloride (257 mg, 1.12 mmol) was chromatographed using chloroform–methanol 6 : 1 as the eluent to give the ester **2h** (385 mg, 77%), mp 152.7–153.8 °C, R_f 0.70 (diethyl ether–light petroleum 9.5 : 0.5) (Found: C, 70.28; H, 6.37; N, 12.68. $C_{26}H_{28}N_4O_3$ requires C, 70.25; H, 6.35; N, 12.61%).

***N*-{3-[(*N,N*-Dimethylaminophenyl)-4′-diazenyl]benzoyl}-phenylalanylvaline *tert*-butyl ester 2i.** The product of reaction of **1** with phenylalanyl-valine *tert*-butyl ester (161 mg, 0.60 mmol) was chromatographed using chloroform–methanol (mixtures of increasing polarity) as the eluent to give ester **2i** (267 mg, 78%), mp 164.8–166.9 °C, R_f 0.71 (ethyl acetate–hexane 6 : 4) (Found C, 69.02; H, 7.00; N, 12.30. $C_{33}H_{41}N_5O_4$ requires C, 69.33; H, 7.23; N 12.25%).

General method for preparation of Boc-acylamides

To a solution of the required substrate in dry acetonitrile (47 mmol dm^{-3}) 0.3 eq. of DMAP was added followed by 3.6 eq. of di-*tert*-butyl pyrocarbonate under rapid stirring overnight at room temperature, the reaction being monitored by TLC. Evaporation under reduced pressure followed by dry chromatography on silica gel and recrystallisation gave the required Boc-acylamide as an orange residue.

***N*-{3-[(*N,N*-Dimethylaminophenyl)-4′-diazenyl]benzoyl}-*N*-*tert*-butyloxycarbonylglycine methyl ester 3b.** The product of reaction of **2b** (247 mg, 0.73 mmol) was chromatographed with diethyl ether–hexane 6 : 4 as the eluent; the solid material thus obtained was recrystallised from ethyl acetate–hexane to give ester **3b** (288 mg, 90%), mp 114.8–116.0 °C, R_f 0.40 (diethyl ether–hexane 6 : 4) (Found: C, 62.61; H, 6.38; N, 12.55. $C_{23}H_{28}N_4O_5$ requires C, 62.71; H, 6.41; N, 12.72%).

***N*-{3-[(*N,N*-Dimethylaminophenyl)-4′-diazenyl]benzoyl}-*N*-*tert*-butyloxycarbonylalanine methyl ester 3c.** The product of reaction of **2c** (50 mg, 0.14 mmol) was chromatographed with diethyl ether–light petroleum 9 : 1 as the eluent to give ester **3c** (63 mg, 99%), mp 101.8–103.4 °C, R_f 0.74 (diethyl ether–hexane 8 : 2) (Found: C, 63.52; H, 6.55; N, 12.21. $C_{24}H_{30}N_4O_5$ requires C, 63.42; H, 6.65; N, 12.33%).

***N*-{3-[(*N,N*-Dimethylaminophenyl)-4′-diazenyl]benzoyl}-*N*-*tert*-butyloxycarbonylvaline methyl ester 3d.** The product of reaction of **2d** (189 mg, 0.50 mmol) was chromatographed using diethyl ether–hexane 2 : 8 as the eluent to give ester **3d** (203 mg, 85%), mp 147.6–149.0 °C, R_f 0.62 (diethyl ether–hexane 6 : 4) (Found: C, 64.94; H, 6.82; N, 11.63. $C_{26}H_{34}N_6O_5$ requires C, 64.71; H, 7.10; N, 11.61%).

***N*-{3-[(*N,N*-Dimethylaminophenyl)-4′-diazenyl]benzoyl}-*N*-*tert*-butyloxycarbonylisoleucine methyl ester 3e.** The product of reaction of **2e** (220 mg, 0.56 mmol) was chromatographed with diethyl ether–hexane 4 : 6 as the eluent to give ester **3e** (154 mg, 56%), mp 89.2–91.0 °C, R_f 0.56 (diethyl ether–hexane 4 : 6) (Found: C, 65.20; H, 7.39; N, 11.04. $C_{27}H_{36}N_4O_5$ requires C, 65.30; H, 7.31; N, 11.28%).

***N*-{3-[(*N,N*-Dimethylaminophenyl)-4′-diazenyl]benzoyl}-*N*-*tert*-butyloxycarbonylleucine methyl ester 3f.** The product of reaction of **2f** (130 mg, 0.33 mmol) was chromatographed with ethyl acetate–hexane 6 : 4 as the eluent to give ester **3f** (161 mg, 99%), R_f 0.59 (diethyl ether–hexane 6 : 4); *m*/*z* (EI) 496.268479 (M^+. $C_{27}H_{36}N_4O_5$ requires 496.268571).

***N*-{3-[(*N,N*-Dimethylaminophenyl)-4′-diazenyl]benzoyl}-*N*-*tert*-butyloxycarbonylmethionine methyl ester 3g.** The product of reaction of **2g** (210 mg, 0.51 mmol) was chromatographed with diethyl ether–hexane 3 : 7 as the eluent to give ester **3g** (155 mg, 60%), R_f 0.47 (diethyl ether–hexane 6 : 4); *m*/*z* (EI) 514.225675 (M^+. $C_{26}H_{34}N_4O_5S$ requires 514.224992).

***N*-{3-[(*N,N*-Dimethylaminophenyl)-4′-diazenyl]benzoyl}-*N*-*tert*-butyloxycarbonylphenylalanine ethyl ester 3h.** The product of reaction of **2h** (62.6 mg, 0.14 mmol) was chromatographed with diethyl ether–hexane 1 : 1 as the eluent to give ester **3h** (76 mg, 99%), R_f 0.40 (diethyl ether–hexane 1 : 1) (Found: C, 68.48; H, 6.75; N, 9.88. $C_{31}H_{36}N_4O_5$ requires C, 68.36; H, 6.66; N, 10.29%).

General method of aminolysis of the coloured amino acid esters

The coloured substrates **3b**–**h** in acetonitrile were treated with a given amount of DEAEA for one or more days according to the procedure of Grehn *et al.*[11] The products were purified by flash chromatography to give the corresponding Boc-amino acid esters **4b**–**h** together with the transamination product **5**.

***N*-*tert*-Butyloxycarbonylglycine methyl ester 4b, by aminolysis of 3b.** The product of a 1 day reaction of **3b** (100 mg, 0.23 mmol) with DEAEA (0.16 cm^3, 0.11 mmol) was chromatographed with diethyl ether–hexane 4 : 6 as the eluent to give ester **4b** (24 mg, 56%), R_f 0.54 (diethyl ether–hexane 6 : 1) (Found: C, 50.57; H, 7.82; N, 7.09. $C_8H_{15}NO_4$ requires C, 50.78; H, 7.99; N, 7.40%).

***N*-*tert*-Butyloxycarbonylalanine methyl ester 4c, by aminolysis of 3c.** The product of a 2 day reaction of **3c** (75 mg, 0.17 mmol)

with DEAEA, (47×10^{-3} cm^3, 0.33 mmol) was chromatographed with diethyl ether–hexane 8 : 26 as the eluent to give ester **4c** (21 mg, 63%), R_f 0.67 (diethyl ether–hexane 6 : 4) (Found: C, 53.47; H, 8.28; N, 6.59. $C_9H_{17}NO_4$ requires C, 53.18; H, 8.43; N, 6.89%).

***N-tert*-Butyloxycarbonylvaline methyl ester 4d, by aminolysis 3d.** The product of a 2 day reaction of **3d** (79.5 mg, 0.17 mmol) with DEAEA, (93×10^{-3} cm^3, 0.66 mmol) was chromatographed with diethyl ether–hexane 4 : 6 to give ester **4d** (15 mg, 40%), R_f 0.79 (diethyl ether–hexane; 6 : 4) (Found: C, 57.36; H, 8.92; N, 5.65. $C_{11}H_{21}NO_4$ requires C, 57.12; H, 9.15; N, 6.06%).

***N-tert*-Butyloxycarbonylisoleucine methyl ester 4e, by aminolysis of 3e.** The product of a 1 day reaction of **3e** (81.8 mg, 0.17 mmol) with DEAEA (93×10^{-3} cm^3, 0.66 mmol) was chromatographed with diethyl ether–hexane 2 : 8 to give ester **4e** (29 mg, 72%), R_f 0.78 (diethyl ether–hexane 6 : 4) (Found: C, 58.89; H, 9.43; N, 5.32. $C_{12}H_{23}NO_4$ requires C, 58.75; H, 9.45; N, 5.71%).

***N-tert*-Butyloxycarbonylleucine methyl ester 4f, by aminolysis of 3f.** The product of a 1 day reaction of **3f** (79 mg, 0.16 mmol) with DEAEA (93×10^{-3} cm^3, 0.66 mmol) was chromatographed with diethyl ether–light petroleum 2 : 8 to give ester **4f** (19 mg, 49%), R_f 0.76 (diethyl ether–hexane 6 : 4) (Found: C, 58.96; H, 9.55; N, 5.43. $C_{12}H_{23}NO_4$ requires C, 58.75; H, 9.45; N, 5.71%).

***N-tert*-Butyloxycarbonylmethionine methyl ester 4g, by aminolysis of 3g.** The product of a 1 day reaction of **3g** (83 mg, 0.16 mmol) with DEAEA (93×10^{-3} cm^3, 0.66 mmol) was chromatographed with diethyl ether–light petroleum 2 : 8 to give ester **4g** (31 mg, 78%), R_f 0.66 (diethyl ether–light petroleum 6 : 4) (Found: C, 50.13; H, 7.80; N, 5.28; S, 12.59. $C_{11}H_{21}NO_4S$ requires C, 50.17; H, 8.04; N, 5.32; S, 12.18%).

***N-tert*-Butyloxycarbonylphenylalanine ethyl ester 4h, by aminolysis of 3h.** The product of a 1 day reaction of **3h** (60 mg, 0.11 mmol) with DEAEA (31×10^{-3} cm^3, 0.22 mmol) was chromatographed with diethyl ether–hexane 2 : 8 to give ester **4h** (23 mg, 71%), R_f 0.90 (chloroform–methanol 5.8 : 0.2) (Found: C, 65.38; H, 7.77; N, 4.86. $C_{16}H_{23}NO_4$ requires C, 65.51; H, 7.90; N, 4.78%).

General method of controlled-potential electrolysis of the coloured amino acid esters

Both compartments of a two-compartment cell for controlled-potential electrolysis were filled with acetonitrile containing Et_4NHCl (0.1 mol dm^{-3}) as supporting electrolyte and Et_3NHCl (0.12 mol dm^{-3}) as a proton donor.[12] At this stage the substrate (0.31 mmol) was added to the cathodic compartment and a cyclic voltammogram recorded at a sweep rate of 100 mV s^{-1}. Then, the potential was adjusted to a value 50 mV more negative than that corresponding to the peak chosen for electrolysis and the apparatus switched on. When the intensity of the current was almost zero, the reaction mixture (catholyte) was transferred to a round-bottomed flask and the solvent evaporated under reduced pressure. The residue was dissolved in water and extracted with ethyl acetate, dried over $MgSO_4$ and after concentration of the organic layer under reduced pressure the residue was chromatographed on silica gel (diethyl ether–hexane, mixtures of increasing polarity).

***N-tert*-Butyloxycarbonylphenylalanine ethyl ester 4h, by electrolysis of 3h.** Electrolysis of **3h** (54 mg, 0.10 mmol) at a potential of −2.50 V gave ester **4h** (14 mg, 48%). When **3h** (167 mg, 0.31 mmol) was electrolysed at a potential of −2.03 V, **4h** was obtained (18 mg, 32%) together with the corresponding aminobenzoyl derivative **6h** (25 mg, 32%); *m*/*z* (EI) 412.198227 (M^+. $C_{23}H_{28}N_2O_5$ requires 412.199822).

***N-tert*-Butyloxycarbonylphenylalanine ethyl ester 4h, by electrolysis of 6h.** Electrolysis of **6h** (90 mg, 0.22 mmol) at a potential of −2.31 V gave ester **4h** (19 mg, 30%).

General method of reductive cleavage of the coloured amino acid esters with zinc dust

Reductive cleavage by zinc dust in methanol in the presence of formic acid was carried out according to the procedure described by Gowda *et al*.[16] The required product was isolated by flash chromatography (silica: ethyl acetate–hexane, mixtures of increasing polarity) and then characterised.

***N-tert*-Butyloxycarbonyl-*N*-(3-aminobenzoyl)alanine methyl ester 6c, by chemical reduction of 3c.** Reduction of **3c** (357 mg, 0.79 mmol) with zinc gave the corresponding aminobenzoyl derivative **6c** (99 mg, 39%) as an oil; *m*/*z* 322.152869. (M^+. $C_{16}H_{22}N_2O_5$ requires 322.152872).

***N-tert*-Butyloxycarbonyl-*N*-(3-aminobenzoyl)phenylalanine ethyl ester 6h, by chemical reduction of 3h.** Reduction of **3h** (280 mg, 0.52 mmol) with zinc gave the corresponding aminobenzoyl derivative **6h** (96 mg, 45%), which compared well with a sample obtained by electrolysis.

***N*-(3-Aminobenzoyl)phenylalanine ethyl ester 7h, by chemical reduction of 2h.** Reduction of **2h** (198 mg, 0.45 mmol) with zinc gave the amino acid ester **7h** (41 mg, 30%), mp 121.0–123.4 °C, R_f 0.55 (ethyl acetate–hexane 8 : 2); *m*/*z* (EI) 312.147630 (M^+. $C_{18}H_{20}N_2O_5$ requires 321.147393).

***N*-Benzyloxycarbonyl-ω-{3-[(*N*,*N*-dimethylaminophenyl)-4′-diazenyl]benzoyl}lysine methyl ester 8.** The product of reaction of **1** with *N*-benzyloxycarbonyl-lysine methyl ester hydrochloride (329 mg, 1.12 mmol) carried out according to the general method described above for acylation with the dye was chromatographed using chloroform–methanol 5.8 : 0.2 as the eluent to give ester **8** (401 mg, 66%), mp 114.0–115.9 °C, R_f 0.75 (chloroform–methanol 5 : 1) (Found: C, 65.76; H, 6.56; N, 12.49. $C_{30}H_{35}N_5O_5$ requires C, 66.04; H, 6.47; N, 12.84%).

***N*-Benzyloxycarbonyl-*N*,ω-bis(*tert*-butyloxycarbonyl)-ω-{3-[(*N*,*N*-dimethylaminophenyl)-4′-diazenyl]benzoyl}lysine methyl ester 9.** The product of a 2 day reaction of **8** (100 mg, 0.18 mmol) with di-*tert*-butyl pyrocarbonate (240 mg, 1.10 mmol) carried out according to the general method described above for preparation of Boc-acylamides was chromatographed with ethyl acetate–hexane 2 : 8 as the eluent to give ester **9** (127 mg, 90%), R_f 0.88 (diethyl ether–hexane 6 : 4); *m*/*z* (EI) 745.369343 (M^+. $C_{40}H_{51}N_5O_9$ requires 745.368679).

***N*-Benzyloxycarbonyl-*N*-*tert*-butyloxycarbonyl-ω-{3-[(*N*,*N*-dimethylaminophenyl)-4′-diazenyl]benzoyl}lysine methyl ester 10.** The product of a 3-day reaction of **9** (234 mg, 0.43 mmol) with di-*tert*-butyl pyrocarbonate (224 mg, 1.03 mmol) was chromatographed with ethyl acetate–hexane 2 : 8 to give ester **10** (100 mg, 35%), R_f 0.77 (diethyl ether–hexane 6 : 4); *m*/*z* (EI) 645.315487 (M^+. $C_{35}H_{43}N_5O_7$ requires 645.316249) together with **9** (148 mg, 45%).

***N*,ω-Bis(*tert*-butyloxycarbonyl)lysine methyl ester 11 by aminolysis of 9.** The fully acylated ester **9** (100 mg, 0.13 mmol) was reacted for 3 days with DEAEA (0.148 cm^3, 1.04 mmol) according to the general method described above for aminolysis of the coloured amino acid esters and the product chromatographed with diethyl ether–hexane (mixtures of increasing polarity) to give ester **11** (43 mg, 92%) (Found: C, 56.88; H, 8.93; N, 7.43. $C_{17}H_{32}N_2O_6$ requires C, 56.64; H, 8.95; N, 7.76%).

***N*-Benzyloxycarbonyl-ω-{3-[(*N,N*-dimethylaminophenyl)-4′-diazenyl]benzoyl}lysine 12.** To the fully protected amino acid **8** (151 mg, 0.28 mmol) in 1,4-dioxane (1.39 cm^3) 1 M NaOH (0.28 cm^3, 0.28 mmol) was added. The solution was stirred at room temperature for 4 h and acidified to pH 2–3 with 1 M $KHSO_4$. The red precipitate thus formed was filtered off to give the amino acid derivative **12** (144 mg, 98.0%), mp 112.5–114.5 °C.

General method for coupling the coloured amino acid

Compound **12** was reacted with one equivalent of an amino acid methyl or ethyl ester hydrochloride in DMF by a standard DCC–HOBt coupling. After dry chromatography on silica and recrystallisation from ethyl acetate–hexane, the required product was obtained as an orange solid.

***N*-Benzyloxycarbonyl-ω-{3-[(*N,N*-dimethylaminophenyl)-4′-diazenyl]benzoyl}lysylalanine methyl ester 13c.** The product of reaction of **12** (144 mg, 0.27 mmol) with alanine methyl ester hydrochloride (38 mg, 0.27 mmol) was chromatographed with chloroform–methanol 5.8 : 0.2 as the eluent to give ester **13c** (159 mg, 95%), mp 179.9–181.7 °C, R_f 0.5 (chloroform–methanol 5.5 : 0.5) (Found: C, 63.99; H, 6.58; N, 13.58. $C_{33}H_{40}N_6O_6$ requires C, 64.27; H, 6.54; N, 13.63%).

***N*-Benzyloxycarbonyl-ω-{3-[(*N,N*-dimethylaminophenyl)-4′-diazenyl]benzoyl}lysylphenylalanine ethyl ester 13h.** The product of reaction of **12** (163 mg, 0.31 mmol) with phenylalanine ethyl ester hydrochloride (71 mg, 0.31 mmol) was chromatographed with chloroform–methanol 5.8 : 0.2 as the eluent to give ester **13h** (266 mg, 97%), mp 146.9–148.9 °C, R_f 0.68 (ethyl acetate–hexane 8 : 2) (Found: C, 67.94; H, 6.45; N, 11.87. $C_{40}H_{46}N_6O_6$ requires C, 68.00; H, 6.56; N, 11.89%).

2-(*N,N*-Diethylamino)-*N*′-{3-[(*N,N*-dimethylaminophenyl)-4′-diazenyl]benzoyl}ethylamine 5. The product of reaction of **1** with DEAEA (0.15 cm^3, 1.04 mmol), carried out according to the general method described above for acylation with the dye, was chromatographed using ethyl acetate–methanol (mixtures of increasing polarity) as the eluent to give compound **5** as an orange solid (374 mg, 98%), mp 86.0–88.2 °C, R_f 0.77 (chloroform–methanol 6 : 4); *m*/*z* (EI) 367.238227 (M^+. $C_{21}H_{29}N_5O$ requires 367.237211).

Methyl 3-[(*N,N*-dimethylaminophenyl)-4′-diazenyl]benzoate 14. Compound **1** (269 mg, 1.0 mmol) was reacted with thionyl chloride (73×10^{-3} cm^3, 2.0 mmol) by the usual procedure. Recrystallisation of the product from acetone–hexane gave the required ester **14** (283 mg, 100%) as a dark red solid, mp 82.8–84.7 °C, R_f 0.77 (chloroform).

Acknowledgements

We thank the Foundation for Science and Technology (Portugal) for financial support to the Institute of Biotechnology and Fine Chemistry (University of Minho).

References

1 J. C. V. P. Moura, in *Colorants for Non-Textile Applications*, eds. H. S. Freeman and A. T. Peters, Elsevier Science B. V., New York, 2000, p. 189.
2 T. Imai, *Med. Technol. (Tokyo)*, 1985, **13**, 1403.
3 J. Kapuscinski., *J. Histochem. Cytochem.*, 1990, **38**, 1323.
4 W. Beisker and W. G. Eisert, *J. Histochem. Cytochem.*, 1989, **37**, 1699.
5 Y. Kozaki, S. Kozai and H. Matsumoto, *Tokushima Bunri Daigaku Kenkyu Kiyo*, 1995, **50**, 135.
6 R. Schmidt, *Ger. Offen. DE,* 4 117 619, 1992.
7 J. Backhaus, D. Mangold and W. R. Knappe, *Ger. Offen. DE*, 3 813 503, 1989.
8 R. Behrendt, M. Schenk, H. Musiol and L. Moroder, *J. Peptide Sci.*, 1999, **5**, 519.
9 F. Sebestyén, G. Szendrei, M. Mák, M. Dóda, E. Illyés, G. Szókán, K. Kindla, W. Rapp, P. Szegó, E. Câmpian and À. Furka, *J. Peptide Sci.*, 1998, **4**, 294.
10 J. I. N. R. Gomes, J. Griffiths, H. L. S. Maia, J. C. V. P. Moura and A. M. F. Oliveira-Campos, *Dyes Pigm.*, 1991, **17**, 269.
11 L. Grehn, K. Gunnarsson and U. Ragnarsson, *Acta Chem. Scand. Ser. B*, 1987, **41**, 18.
12 L. Grehn, H. L. S. Maia, L. S. Monteiro, M. I. Montenegro and U. Ragnarsson *J. Chem. Res.*, 1991, (S), **144**; (M), 1501.
13 L. Horner and H. Lung, in *Organic Electrochemistry*, eds. M. M. Baizer, H. Lung, Marcel Dekker, New York, 1991.
14 T. M. Florence, D. A. Johnson and G. E. Batley, *Electroanalytical Chemistry and Interfacial Electrochemistry*, 1974, **50**, 113.
15 T. M. Florence, *Aust. J. Chem.*, 1965, **18**, 609.
16 S. Gowda, K. Abiraj and D. C. Gowda, *Tetrahedron Lett.*, 2002, **43**, 1329.
17 L. Grehn, K. Gunnarsson, H. L. S. Maia, M. I. Montenegro, L. Pedro and U. Ragnarsson, *J. Chem. Res.*, 1988, (S)**399**; (M), 3081.