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Fluorescence derivatisation of amino acids in short and long-wavelengths

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Abstract—3-(Naphthalen-1-ylamino)propanoic acid was coupled to the amino group of the main and lateral chains of various amino acids in order to evaluate its applicability as a fluorescent derivatising reagent. The resulting amino acid derivatives are strongly fluorescent with a maximum emission of about 415 nm. Condensation of these derivatives with 5-ethylamino-4-methyl-2-nitrosophenol hydrochloride resulted in the corresponding blue benzo[*a*]phenoxazinium conjugates, also revealing strong fluorescence in ethanol and water at physiological pH and good quantum yields, but with emission wavelengths between 644 and 657 nm, which was preferable in biological assays.

Amino acids are an important class of compounds found in a large number of samples, namely, tissues, biological fluids, food and industrial products. Most of them are small aliphatic molecules with neither autofluorescence nor strong absorbance in the ultraviolet/visible region. Thus, derivatisation is a needed to enhance both the selectivity and the sensitivity of amino acid analysis in biological samples. Many fluorophores have been tested to produce highly fluorescent compounds with amino acids.[1–4] Among them are naphthalene reagents, such as naphthalene-2,3-dicarboxaldehyde (NDA)[5–7] or dansyl chloride,[8,9] which form conjugates with absorption and emission maxima in wavelengths below 500 nm in the electromagnetic spectrum.

Despite the importance of short-wavelength fluorophores, the derivatisation and analysis of amino acids and other molecules related with them are sometimes limited in bioapplications, where long-wavelength light-emitting (600–1000 nm) molecules are required because of the minimum interference from the absorption scattering and the intrinsic fluorescence of biological materials.

Bearing this in mind, as well as our actual research interests,[10–12] we decided to use the 3-(naphthalen-1-ylamino)propanoic acid in the derivatisation of several α-amino acids, that have no (or low) intrinsic fluorescence or that are not easily detectable by ultraviolet absorption, thus excluding tryptophan and tyrosine, as representative models of biomolecules. Furthermore, these amino acid derivatives were reacted with 5-ethylamino-4-methyl-2-nitrosophenol hydrochloride to allocate their transformation into the corresponding benzo[*a*]phenoxazinium conjugates. Evaluation of absorption and emission properties of all compounds synthesised were performed in ethanol and water at physiological pH (when soluble).

The synthesis of 3-(naphthalen-1-ylamino)propanoic acid **1a**[10] was undertaken by the alkylation of 1-naphthylamine with methyl-3-bromopropionate, followed by hydrolysis (NaOH/1,4-dioxane) of the isolated ester intermediate methyl 3-(naphthalen-1-ylamino)propanoate (**1b**).[13]

In order to investigate the possibility of using naphthalene carboxylic acid **1a** in the derivatisation of biomolecules, the covalent linkage between this compound and several α-amino acids, as representative models, was studied. Compound **1a** was bonded to the α-amine group of L-amino acid methyl esters, namely, alanine, glycine, phenylalanine and glutamic acid (protected at its side-chain as methyl ester) (**2a**–**d**), by

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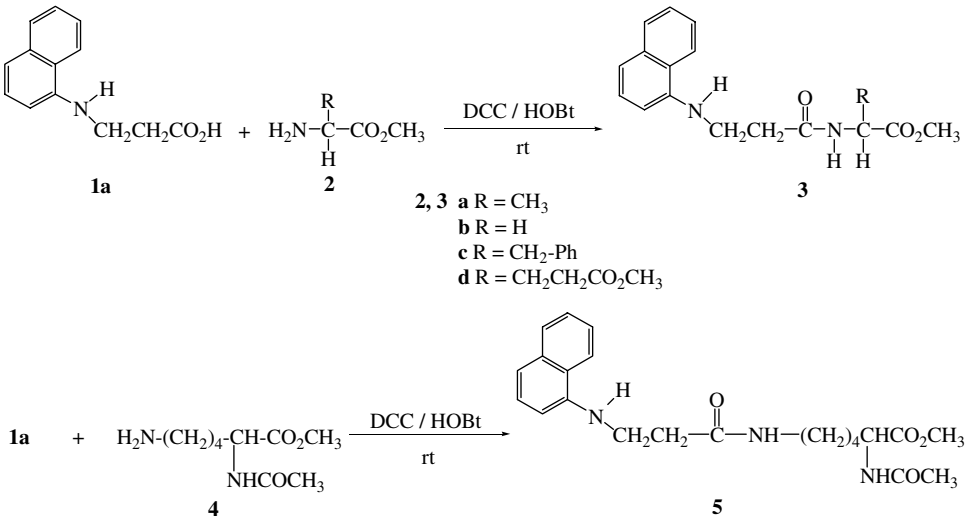


Scheme 1.

coupling them with the aid of *N*,*N*′-dicyclohexylcarbodiimide (DCC) assisted by 1-hydroxybenzotriazole (HOBt) under standard conditions (Scheme 1).[14]

In addition of derivatising amino acids at their N-terminus, the alternative acylation at a lysine ω-amine group was also performed. Thus, the methyl ester of *N*-acetyllysine (**4**) was reacted with **1a** under the conditions reported above, to give the expected fluorescent derivative **5** (Scheme 1).

After purification by dry chromatography on silica gel, derivatives **3a**–**d** and **5** were obtained in yields ranging from 85% to 98% (Table 1).

The structure of compounds **3a**–**d** and **5** was confirmed by high resolution mass spectrometry, IR and NMR (^{1}H and ^{13}C) spectroscopy. In these compounds, the ^{13}C NMR spectra showed signals at δ 170.24–172.17 ppm and from 171.89 to 173.44 ppm due to the presence of the carbonyl carbon of the amide and ester type, respectively. The IR of compounds **3a**–**d** and **5** also presented the expected bands for the carbonyl groups at 1627–1651 cm^{-1} (amide) and 1726–1750 cm^{-1} (ester).

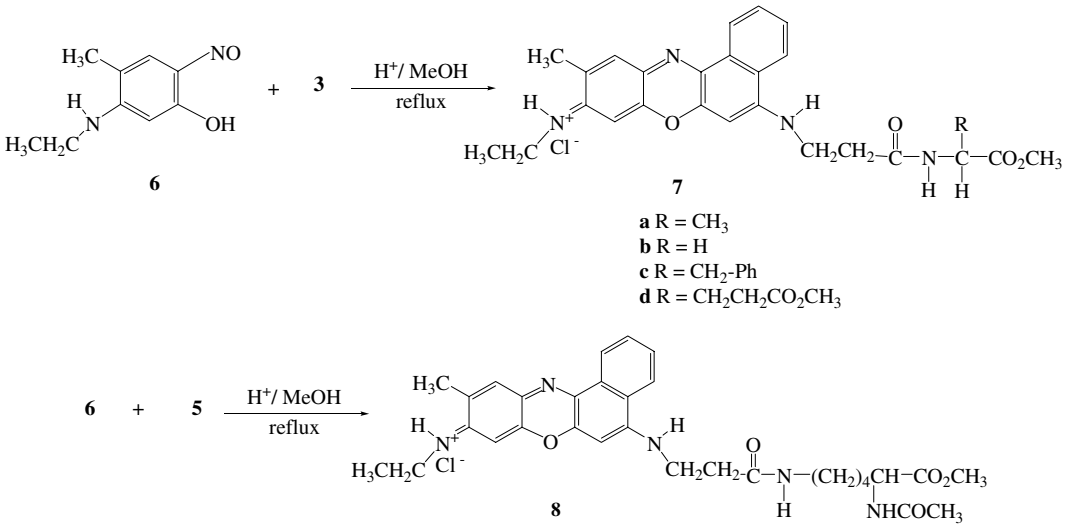
As was expected, and according to the photophysic studies which will be discussed later, derivatives **3a**–**d** and **5** showed maximum absorption in the ultraviolet range (~330 nm) and a strong emission at the beginning of the visible region (~415 nm).

Fluorescent derivatisation is one of the most commonly used methodologies for analysis purposes[15] and labelled biomolecules, revealing fluorescence outside the region where the biological material fluoresces, are desirable for the analytic practice. Considering these facts and with the purpose of red shifting the maximum absorbance and emission of fluorescent amino acid derivatives **3a**–**d** and **5**, which were previously prepared, we studied the possibility of generating the corresponding polycyclic systems of benzo[*a*]phenoxazinium. Thus, condensation of 5-ethylamino-4-methyl-2-nitrosophenol hydrochloride **6**, synthesised according to the known procedure involving treatment of the 3-ethylamino-4-

Table 1. Yields and UV/vis data in ethanol and water (pH 7.4) for compounds **1a**, **3a**–**d**, **5**, **7a**–**d** and **8**

Compound	Yield [%]	λ_{abs} [nm] (ε)[a]	λ_{abs} [nm] (ε)[b]
1a	85[c]/47[d]	330 (7000)	—
3a	87	331 (10,682)	—
3b	90	330 (5960)	—
3c	85	320 (3596)	—
3d	95	330 (7442)	—
5	98	330 (7000)	—
7a	57	630 (23,558)	—
7b	67	630 (32,283)	625 (14,970)
7c	76	630 (40,945)	625 (21,778)
7d	68	630 (30,500)	625 (24,500)
8	88	630 (42,857)	630 (27,439)

[a] Spectra were measured in absolute ethanol.
[b] Spectra were measured in water (pH 7.4).
[c] Yield from compound **1b**.
[d] Yield from 1-naphthylamine.



Scheme 2.

methylphenol with sodium nitrite in an acid solution,[16,11] with compounds **3a–d** and **5** in acidic medium, followed by dry chromatography purification, gave the labelled amino acid derivatives **7a–d** and **8** as blue materials in good yields (57–88%) (Scheme 2, Table 1).[17]

Compounds **7a–d** and **8** were fully characterised by the usual analytical techniques. As in the case of derivatives **3a–d** and **5**, the ^{13}C NMR spectra showed signals at δ 170.65–171.97 ppm and 171.87–173.51 ppm due to the presence of the carbonyl carbon of the amide and ester type, respectively. The IR of these compounds also presented the expected bands for the carbonyl groups at 1633–1659 cm^{-1} (amide) and 1720–1746 cm^{-1} (ester).

Electronic absorption spectra of 10^{-6} M solutions of fluorophore **1a** and amino acid derivatives **3a–d**, **5**, **7a–d** and **8**, in degassed absolute ethanol were measured. Summarised data of this study are presented in Table 1. Compounds **3a–d** and **5** showed absorption in the ultraviolet region, the wavelength of maximum absorption (λ_{abs}) of all compounds being located at 330 or 320 nm (**3c**) with molar absorptivities (ε) between 3596 and 10682 $M^{-1}\,cm^{-1}$. Through the comparison of λ_{abs} of compounds **1a**, **3a–d** and **5**, it was possible to see that the presence of the amino acid residues did not affect its value; or was not significant in its variation (330 nm, **1a**/ 320 nm, **3c**). The chemical transformation of derivatives **3a–d** and **5** into the corresponding polycyclic benzo[*a*]phenoxazinium conjugates **7a–d** and **8**, strongly red shifted the maximum absorption (λ_{abs} 630 nm, **7a–d** and **8**) with a simultaneously increase of the molar absorptivities (ε 23,558–42,857 $M^{-1}\,cm^{-1}$).

Comparison of the wavelengths of maxima absorption of the two series of amino acid derivatives **3a–d** and **5**, **7a–d** and **8**, in ethanol, revealed a bathochromic shift from 299 (**3a/7a** alanine) to 310 nm (**3c/7c** phenylalanine).

The absorption properties of compounds **7b–d** and **8** were also studied in water at physiological pH (pH 7.4, adjusted with HCl and NaOH); other compounds were not soluble in water. Comparison of λ_{abs} values

Table 2. Fluorescence data in ethanol and water (pH 7.4) for compounds **1a**, **3a–d**, **5**, **7a–d** and **8**

Compound	Fluorescence[a]			Stokes' shift [nm]	Fluorescence[b]			Stokes' shift [nm]
	λ_{exc} [nm]	λ_{em} [nm]	Φ_F		λ_{exc} [nm]	λ_{em} [nm]	Φ_F	
1a	330	417	0.54	87	—	—	—	—
3a	331	415	0.66	84	—	—	—	—
3b	330	417	0.55	87	—	—	—	—
3c	330	415	0.55	95	—	—	—	—
3d	330	414	0.57	84	—	—	—	—
5	330	417	0.54	87	—	—	—	—
7a	590	644	0.39	14	—	—	—	—
7b	600	644	0.37	14	590	652	0.45	27
7c	590	644	0.45	14	590	657	0.40	32
7d	600	644	0.48	14	600	652	0.16	27
8	590	645	0.32	15	590	654	0.46	24

[a] Spectra were measured in absolute ethanol.
[b] Spectra were measured in water (pH 7.4).

in ethanol and water showed only a slight hypsochromic shift (~5 nm) or were equal (**8**).

Evaluation of the fluorescent properties of amino acid derivatives **3a–d**, **5**, **7a–d** and **8** in ethanol and water at physiological pH (**7b–d** and **8**), using 9,10-diphenylanthracene ($\Phi_F = 0.95$ in ethanol,[18] **3a–d** and **5**) or oxazine 1 ($\Phi_F = 0.11$ in ethanol,[19] **7a–d** and **8**) as standards was performed (Table 2). In ethanol, the labelled amino acids **3a–d** and **5** exhibited good fluorescence with wavelengths of maximum emission (λ_{em}) at 414–417 nm, high Stokes's shifts (84–95 nm) and quantum yields ranging from 0.54 to 0.66. Considering these results, and as in the case of maximum absorption, the presence of the amino acid residues did not practically affect the λ_{em} of derivatising reagent **1a**. However, fluorescent quantum yields increased (except for compound **5**), the highest value being that of compound **3a** (Φ_F 0.66).

Regarding blue amino acid derivatives **7a–d** and **8** in the two solvents studied, they fluoresced with wavelengths of maximum emission in the 644–657 nm region and showed quantum yields ranging from 0.16 to 0.48 (Table 2). Through the comparison of λ_{em} values in ethanol and water (pH 7.4), it was notorious that a red shift (8 or 13 nm, **7c**) occurred for the four derivatives. Despite the low Stokes's shifts exhibited for the compounds, their values are superior in water (being the highest 32 nm, **7c**).

Following the same tendency as the λ_{abs}, wavelengths of maximum emission obtained for two series **3a–d**, **5** and **7a–d**, **8** presented a separation from 227 to 230 nm in their values. As a result, for example, it was possible to obtain alanine derivatives showing maximum fluorescence at 415 nm and at 644 nm, in ethanol.

Figure 1 shows the comparison between the emission spectra of compounds **3b** in ethanol and **7b** in ethanol and water (pH 7.4).

In this work, efficient fluorescent amino acid derivatisation was achieved in the short wavelength region (λ_{em} at about 414–417 nm) using the naphthalene functionalised, 3-(naphthalen-1-ylamino)propanoic acid. Transformation of the fluorescent derivatives into the corresponding benzo[*a*]phenoxazinium based conjugates following a straightforward procedure was possible in good yields. The latter amino acid derivatives revealed a maximum emission of 645 nm (in ethanol) and 652 or 657 nm (in water at physiological pH), which corresponds to a red shift from 227 to 230 nm in relation to the previously derivatising procedure. Considering the photophysical properties of the all obtained conjugates, both possibilities are interesting, depending on the specific application. However, the last one represents an example of long-wavelength fluorescent labelling of biomolecules.

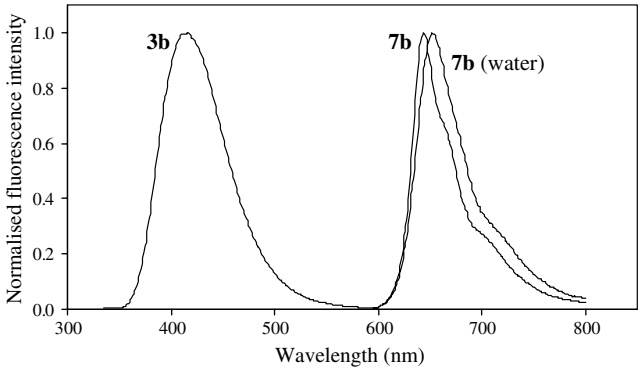


Figure 1. Normalised fluorescence spectra of compounds **3b** and **7b** in ethanol and **7b** in water (pH 7.4).

Acknowledgment

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13. Synthesis of compound **1a**: To a suspension of methyl 3-(naphthalen-1-ylamino)propanoate (**1b**)[10] (0.470 g; 2.05 mmol) in 1,4-dioxane (4.0 mL) 1 M NaOH (3.08 mL; 3.08 mmol) was added. The solution was stirred at room temperature for 6 hours and acidified to pH 2–3 with 1 M $KHSO_4$. The mixture was extracted with chloroform (4 × 15 mL) and the organic extracts were dried ($MgSO_4$) and evaporated to dryness giving 3-(naphthalen-1-ylamino)propanoic acid **1a** as a white solid (0.375 g, 85%). The spectroscopic data confirmed the structure of compound **1a** and were in accordance with the previously reported characterisation of this compound obtained by another procedure.[10]
14. Typical procedure for the synthesis of compounds **3a–d** and **5** (described for **3a**): 3-(Naphthalen-1-ylamino)propanoic acid **1a** (0.224 g; 1.04 mmol) was reacted with

alanine methyl ester hydrochloride **2a** (0.208 g; 1.50 mmol) in DMF (3 mL) by a standard DCC/HOBt coupling. After dry chromatography on silica gel (chloroform/methanol, 5.9:0.1), *N*-[3-(naphthalen-1-ylamino)propanoyl] alanine methyl ester **3a** was obtained as an off-white oil (0.270 g, 87%). $R_f = 0.69$ (chloroform/methanol, 5.8:0.2). FTIR (KBr): ν_{max} 3393, 3310, 3049, 2929, 2852, 1750, 1641, 1587, 1532, 1490, 1467, 1412, 1351, 1287, 1216, 1120, 983 cm^{-1}. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.38 (3H, d, *J* 7.2 Hz, β-CH_3 Ala), 2.67 (2H, t, *J* 6.2 Hz, NCH_2CH_2), 3.66 (2H, t, *J* 5.7 Hz, NCH_2CH_2), 3.74 (3H, s, OMe), 4.56–4.67 (1H, m, α-CH Ala), 5.14–5.18 (1H, br s, NH), 6.24 (1H, d, *J* 6.6 Hz, α-NH Ala), 6.66 (1H, d, *J* 7.5 Hz, 4-H), 7.27 (1H, d, *J* 8.1 Hz, 2-H), 7.36 (1H, t, *J* 7.5 Hz, 3-H), 7.40–7.50 (2H, m, 6-H and 7-H), 7.76–7.82 (1H, m, 5-H), 7.84–7.92 (1H, m, 8-H) ppm. ^{13}C NMR ($CDCl_3$, 75.4 MHz): δ 18.20 (β-CH_3 Ala), 35.04 (NCH_2CH_2), 40.26 (NCH_2CH_2), 48.03 (α-CH Ala), 52.46 (OMe), 104.56 (C-4), 117.79 (C-2), 120.18 (C-5), 123.80 (C-4a), 124.79 (C-6), 125.75 (C-7), 126.41 (C-3), 128.48 (C-8), 134.31 (C-8a), 142.85 (C-1), 171.49 (CONH), 173.44 (CO_2Me). The assignments were supported by HMBC and HMQC techniques. HRMS: *m*/*z* (FAB): calcd for $C_{17}H_{20}N_2O_3[M^+]$ 300.1474; found 300.1484.

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17. Typical procedure for the synthesis of compounds **7a**–**d** and **8** (described for **7a**): To a cold solution (ice bath) of 5-ethylamino-4-methyl-2-nitrosophenol hydrochloride **6** (0.092 g; 0.51 mmol) in methanol (2 mL), *N*-[3-(naphthalen-1-ylamino)propanoyl] alanine methyl ester (**3a**) (0.300 g; 0.51 mmol) and concentrated hydrochloride acid (5.0×10^{-2} mL) were added. The mixture was refluxed for 1 h and 30 min and monitored by TLC (silica: chloroform–methanol, 5.7:0.3). The solvent was removed under reduced pressure and the crude mixture was purified by dry chromatography (silica: dichloromethane–methanol, 5.4:0.6). *N*-{5-[3-(1-methoxy-1-oxopropan-2-ylamino)-3-oxopropylamino]-10-methyl-9*H*-benzo[*a*]phenoxazin-9-ylidene}ethanaminium chloride **7a** was obtained as a blue solid (0.133 g, 57%). Mp above 300 °C. R_f 0.30 (silica: chloroform–methanol, 5.5:0.5). FTIR (KBr, 1%): ν_{max} 3400, 2954, 2924, 2854, 1726, 1651, 1633, 1587, 1556, 1504, 1463, 1377, 1311 cm^{-1}. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.41 (3H, br s, NCH_2CH_3), 1.48 (3H, d, *J* 7.2 Hz, β-CH_3 Ala), 2.39 (3H, s, CH_3), 3.05 (2H, br s, NCH_2CH_2), 3.28 (2H, br s, NCH_2CH_3), 3.68 (3H, s, OMe), 3.65–3.82 (2H, m, NCH_2CH_2), 4.42–4.56 (1H, m, α-CH Ala), 6.18 (1H, s, 8-H), 6.35 (1H, s, 6-H), 6.74 (1H, br s, α-NH Ala), 7.37 (1H, s, 11-H), 7.82 (2H, br s, 2-H and 3-H), 8.32 (1H, d, *J* 6.6 Hz, NH), 8.75 (1H, br s, 1-H), 9.04 (1H, br s, 4-H), 10.16 (1H, br s, NH) ppm. ^{13}C NMR ($CDCl_3$, 75.4 MHz): δ 13.87 (NCH_2CH_3), 17.42 (β-CH_3 Ala), 18.05 (CH_3), 34.21 (NCH_2CH_2), 38.79 (NCH_2CH_3), 41.98 (NCH_2CH_2), 48.46 (α-CH Ala), 52.21 (OMe), 92.83 (C-6), 93.27 (C-8), 123.40 (Ar-C), 124.03 (C-1), 125.31 (C-4), 127.22 (C-10), 129.98 (C-3), 130.18 (Ar-C), 130.54 (Ar-C), 131.11 (C-11), 131.67 (C-2), 133.42 (Ar-C), 146.92 (Ar-C), 150.51 (Ar-C), 154.29 (C-9), 157.24 (C-5), 170.65 (CONH), 173.17 (CO_2Me) ppm. The assignments were supported by HMBC and HMQC techniques. HRMS: *m*/*z* (FAB): calcd for $C_{26}H_{29}N_4O_4[M^+]$ 461.2189; found 461.2203.
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