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# Fused oxopyrans as fluorescent labels for neurotransmitter amino acids

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**Abstract –** Fluorescent conjugates of *N*-benzyloxycarbonyl protected γ-aminobutyric acid (GABA) were prepared by coupling to its C-*terminus* several polyheteroaromatic labels, based on the pyran ring. Photophysical properties were evaluated and the results showed that these GABA conjugates presented strong fluorescence with maximum emission wavelengths between 393 and 503 nm, as well as high fluorescence quantum yields in ethanol.

**Keywords**: Fluorescent labelling; Benzopyrans; Neurotransmitter amino acids.

## 1. Introduction

The improvement of the sensitivity of analytical methodologies has shifted the research towards the area of fluorescent labelling, as fluorescence is far more sensitive than common UV techniques. The development of novel fluorescent derivatisation reagents and their subsequent evaluation with model compounds has broad application in biology and biochemistry, to investigate the structure and dynamics of living systems.[1,2]

The analysis of neuroactive amino acids provides a means of diagnosis of disease and possible treatment in neuropsychiatric diseases.[3,4] Most of the neurotransmitter amino acids are small aliphatic molecules with neither fluorescence nor strong absorption in the ultraviolet/visible region. Thus, amino acids are derivatized to improve both the selectivity and sensitivity of their detection in biological samples. Amoung the fluorescent labels that have been reported for determination of amino acids are dansyl chloride (Dns), dabsyl chloride, fluorescamine, fluorenylmethyl chloroformate, *o*-phthalaldehydes (OPA), etc.[5-7]

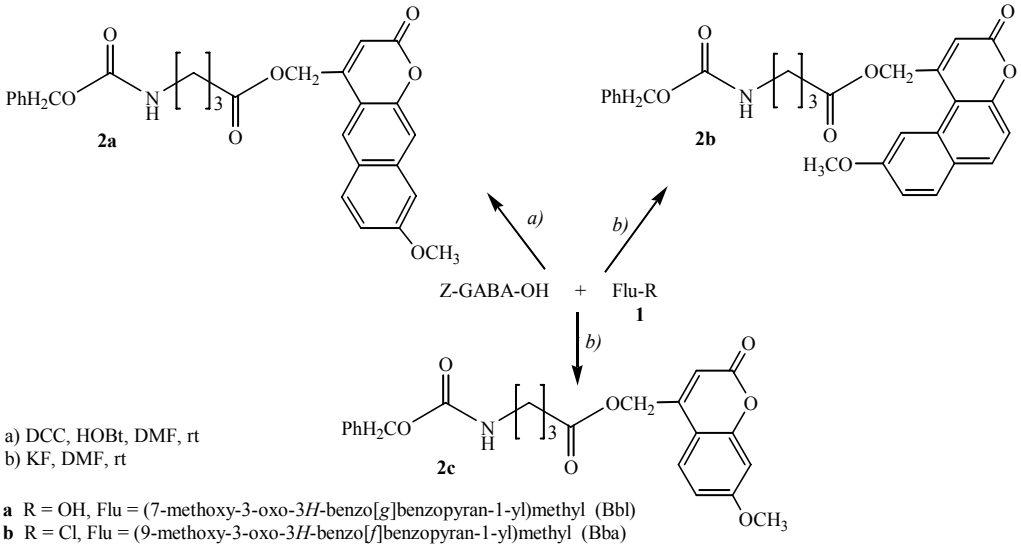
Considering these facts in connection with our current research interests in the development of new fluorescent heterocyclic compounds and their application as labels[8,9] we now report the use of different heteroaromatic fluorophores, such as benzopyran derivatives, in the preparation of fluorescent conjugates of γ-aminobutyric acid (GABA), one of the most important transmitters in the central nervous system, with the aim of undertaking a comparative study of their performance as labelling units.

## 2. Results and Discussion

1-Hydroxymethyl-7-methoxy-3-oxo-3*H*-benzo[*g*]benzopyran **1a** was synthesised through a Pechmann reaction, between 2,7-dihydroxynaphthalene and ethyl acetoacetate, catalysed by sulphuric acid at room temperature,[10] followed by methylation of the resulting 1-methyl-7-hydroxy-3-oxo-3*H*-benzo[*g*]benzopyran with methyl iodide. The methyl group was oxidised to the aldehyde, by reaction with selenium dioxide, which was then reacted with sodium borohydride, affording the hydroxymethyl group.[11] 1-Chloromethyl-9-methoxy-3-oxo-3*H*-benzo[*f*]benzopyran **1b** and 1-chloromethyl-6-methoxy-3-oxo-3*H*-benzopyran **1c** were obtained by a similar procedure from the reaction of 7-methoxy-2-naphthol and 3-methoxyphenol with ethyl chloroacetoacetate.[8] Fluorophores **1a-c** will be designated in this report by a three letter code for simplicity of naming the various fluorescent conjugates, as indicated in Table 1.

Our purpose being the investigation of compounds **1a-c** as fluorescent labels for neurotransmitter amino acids, namely γ-aminobutyric acid (GABA), we synthesised the corresponding conjugates in order to perform a comparative study of their photophysical properties.

Derivatisation at the C-*terminus* of *N*-benzyloxycarbonyl-protected GABA with fluorophores **1a-c** was carried out in DMF, at room temperature, with the aid of *N,N*'-dicyclohexylcarbodiimide (DCC) assisted by 1-hydroxybenzotriazole (HOBt) under standard conditions[12] (for **1a**) or by using potassium fluoride[13] (for **1b** and **1c**), yielding fluorescent GABA conjugates **2a-c** (Scheme 1, Table 1). All conjugates were characterised by IR, $^{1}$H and $^{13}$C NMR spectroscopy and elemental analyses or high resolution mass spectrometry.




**a** R = OH, Flu = (7-methoxy-3-oxo-3*H*-benzo[*g*]benzopyran-1-yl)methyl (Bbl)
**b** R = Cl, Flu = (9-methoxy-3-oxo-3*H*-benzo[*f*]benzopyran-1-yl)methyl (Bba)
**c** R = Cl, Flu = (6-methoxy-3-oxo-3*H*-benzopyran-1-yl)methyl (Bpm)

**Scheme 1.**

The IR spectra of labelled GABA **2a-c** showed bands due to stretching vibrations of the carbonyl group of the fluorophore-amino acid ester linkage from 1716 to 1739 $cm^{-1}$. The spectra also showed the carbonyl bands of the *N*-benzyloxycarbonyl protecting group (1688 to 1691 $cm^{-1}$), as well as the oxo group at the pyran ring (1622 to 1691 $cm^{-1}$).

$^{1}H$ NMR spectra showed signals of the amino acid residue, in the form of multiplets for the GABA methylene groups centered at about $\delta$ 1.90, 2.55 and 3.30 ppm, and at $\delta$ 5.11-5.24 ppm the signal for the ester methylene group, as well as the characteristic protons of the heterocyclic moiety at $\delta$ 6.31-6.65 ppm for the H-2. The confirmation of the presence of the newly formed ester linkage was also supported by $^{13}C$ NMR spectra signals of the ester carbonyl group, which were found at about $\delta$ 172 ppm. Signals of the oxo group of the heterocycle (at about $\delta$ 160 ppm) and the urethane carbonyl of the Z protecting group (at about $\delta$ 155 ppm) were also visualised.

**Table 1.** Yields, UV/Vis and fluorescence data for GABA ester conjugates **2a-c** in absolute ethanol.

| Compound | | Yield (%) | UV/Vis | | Fluorescence | | |
|---|---|---|---|---|---|---|---|
| | | | $\lambda_{max}$ (nm) | log ε | $\lambda_{max}$ (nm) | Stokes' shift (nm) | $\Phi_F$ |
| **2a** | Z-GABA-OBbl | 27 | 345 | 3.91 | 503 | 158 | 0.21 ± 0.03 |
| **2b** | Z-GABA-OBba | 81 | 345 | 3.95 | 472 | 127 | 0.76 ± 0.02 |
| **2c** | Z-GABA-OBpm | 85 | 320 | 4.22 | 393 | 73 | 0.27 ± 0.03 |

The UV/Vis absorption and emission spectra of degassed $10^{-5}$-$10^{-6}$ M solutions in absolute ethanol of compounds **2a-c** were measured, absorption and emission maxima, molar absorptivities and fluorescence quantum yields are also reported (Table 1). Fluorescence quantum yields were calculated using 9,10-diphenylanthracene as standard ($\Phi_F$ = 0.95 in ethanol).[14] The wavelength of maximum absorption and emission was red-shifted for conjugates bearing the labels with more fused rings (compare compounds **2a**,**2b** with **2c**) (Figure 1). Labelled GABA **2a-c** exhibited moderate to excellent quantum yields ($0.21 < \Phi_F < 0.76$), and Stokes' shift from 73 to 158 nm, the highest values being associated with the linear benzopyran in compound **2a**.

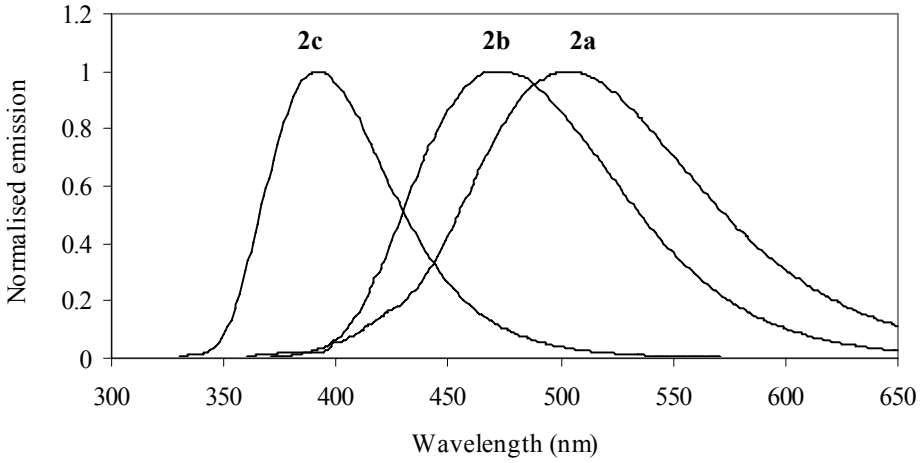



**Figure 1.** Normalised emission spectra of GABA conjugates **2a-c** in absolute ethanol ([**2a**] = $1.0 \times 10^{-5}$ M, $\lambda_{exc}$= 345 nm; [**2b**] = $1.0 \times 10^{-5}$ M, $\lambda_{exc}$= 345 nm; [**2c**] = $5.2 \times 10^{-6}$ M, $\lambda_{exc}$= 320 nm.

Fluorescent γ-aminobutyric acid ester conjugates **2a-c** were prepared in low to excellent yields by using general synthetic methods, involving chloromethyl or hydroxymethyl benzopyran precursors and the C-*terminus* of *N*-benzyloxycarbonyl-protected GABA. The photophysical studies showed that all labels are appropriate fluorogenic reagents for the derivatisation of non-fluorescent molecules due to the high Stokes' shifts and good fluorescence quantum yields, being the 1-chloromethyl-9-methoxy-3-oxo-3*H*-benzo[*f*]benzopyran (**1b**) the most interesting fluorophore.

## 3. Experimental

**3.1.** Synthesis of fluorescent GABA conjugate **2a**: *N*-Benzyloxycarbonyl-L-γ-aminobutyric acid, Z-GABA-OH (0.100 g; 0.42 mmol) was reacted with 1-hydroxymethyl-7-methoxy-3-oxo-3*H*-benzo[*g*]benzopyran, Bbl-OH (**1a**) (0.050 g; 0.197 mmol) in DMF (2 mL) using a standard DCC/HOBt coupling. After chromatography on silica gel (chloroform/methanol, 100:1), *N*-benzyloxycarbonyl-L-γ-aminobutyric acid (7-methoxy-3-oxo-3*H*-benzo[*g*]benzopyran-1-yl)methyl ester, Z-GABA-OBbl (**2a**) was obtained as a yellow solid (0.025 g, 27 %). Mp = 194.0-195.6 ºC. IR (KBr, 1%): $\upsilon_{max}$ 3327, 2928, 2851, 1732, 1721, 1688, 1627, 1575, 1486, 1455, 1312, 1262, 1232, 1171, 1089, 1028 $cm^{-1}$. $^1H$ NMR ($CDCl_3$, 300 MHz): $\delta$ = 1.92-1.96 (2H, m, β-$CH_2$), 2.56 (2H, t, *J* 7.2 Hz, α-$CH_2$), 3.28-3.35 (2H, m, γ-$CH_2$), 3.97 (3H, s, $OCH_3$), 4.90 (1H, broad s, NH), 5.11 (2H, s, $CH_2$ Bbl), 5.41 (2H, s, $CH_2$ Z), 6.49 (1H, s, H-2), 7.13 (1H, d, *J* 2.4 Hz, H-6), 7.17 (1H, dd, *J* 9.3 and 2.7 Hz, H-8), 7.31-7.36 (5H, m, 5 × Ph-*H*), 7.64 (1H, s, H-5), 7.81 (1H, d, *J* 9.0 Hz, H-9), 7.91 (1H, s, H-10)

ppm. $^{13}$C NMR ($CDCl_3$, 75.4 MHz): $\delta$ = 25.17 (β-$CH_2$), 31.18 (α-$CH_2$), 40.23 (γ-$CH_2$), 55.49 ($OCH_3$), 61.22 ($CH_2$ Z), 66.77 ($CH_2$ Bbl), 104.61 (C-6), 112.06 (C-5), 112.66 (C-2), 114.81 (C-10a), 119.85 (C-8), 123.59 (C-10), 125.55 (C-4a), 128.14 (2 × Ph-*C*), 128.51 (3 × Ph-*C*), 130.23 (C-9), 136.48 (C-5a), 136.51 (C-1 Ph), 148.76 (C-1), 150.71 (C-9a), 156.46 (C=O urethane), 159.83 (C-7), 160.61 (C-3), 172.41 (C=O ester) ppm. MS: *m/z* (FAB, %): 476 ($[M+H]^+$, 3), 475 ($M^+$, 2), 307 (12), 226 (16), 225 (100), 155 (15), 154 (50). HRMS: *m/z* (FAB): calcd. for $C_{27}H_{26}NO_7$ $[M+H]^+$ 476.1709; found 476.1711.

**3.2.** General experimental procedure for the synthesis of fluorescent GABA conjugates **2b** and **2c**, (described for **2b**): 1-Chloromethyl-9-methoxy-3-oxo-3*H*-benzo[*f*]benzopyran, Bba-Cl (**1b**) (0.110 g; 0.40 mmol) was reacted with *N*-benzyloxycarbonyl-L-γ-aminobutyric acid (0.095 g; 0.40 mmol) and potassium fluoride (0.07 g; 1.2 mmol) in dry DMF (2 mL). The reaction mixture was stirred at room temperature for 3 days. The solvent was removed by rotary evaporation under reduced pressure and the crude residue was purified by column chromatography with chloroform. *N*-benzyloxycarbonyl-L-γ-aminobutyric acid (9-methoxy-3-oxo-3*H*-benzo[*f*]benzopyran-1-yl)methyl ester, Z-GABA-OBba (**2b**) was obtained as a light brown solid (0.154 g, 81 %). Mp = 120.7-121.9 ºC. IR (KBr, 1%): $\upsilon_{max}$ 3329, 2927, 2851, 1739, 1721, 1691, 1626, 1553, 1456, 1365, 1265, 1229, 1182, 1100, 942, 837 $cm^{-1}$. $^1$H NMR ($CDCl_3$, 300 MHz): $\delta$ = 1.91-1.96 (2H, m, β-$CH_2$), 2.57 (2H, t, *J* 7.2 Hz, α-$CH_2$), 3.28-3.34 (2H, m, γ-$CH_2$), 3.95 (3H, s, $OCH_3$), 5.00 (1H, broad s, NH), 5.10 (2H, s, $CH_2$ Z), 5.66 (2H, s, $CH_2$ Bba), 6.65 (1H, s, H-2), 7.22 (1H, dd, *J* 8.7 and 2.1 Hz, H-8), 7.31-7.35 (6H, m, 5 × Ph-*H* and H-5), 7.42 (1H, d, *J* 2.1 Hz, H-10), 7.83 (1H, d, *J* 9.3 Hz, H-7), 7.91 (1H, d, *J* 8.7 Hz, H-6) ppm. $^{13}$C NMR ($CDCl_3$, 75.4 MHz): $\delta$ = 25.04 (β-$CH_2$), 31.12 (α-$CH_2$), 40.16 (γ-$CH_2$), 55.43 ($OCH_3$), 64.21 ($CH_2$ Bba), 66.76 ($CH_2$ Z), 105.79 (C-10), 111.88 (C-4b), 112.61 (C-2), 115.26 (C-5), 116.53 (C-8), 126.34 (C-6a), 128.10 (2 × Ph-*C*), 128.47 (3 × Ph-*C*), 130.55 (C-6b), 131.31 (C-7), 133.80 (C-6), 136.35 (C-1 Ph), 151.02 (C-1), 155.49 (C-4a), 156.54 (C=O urethane), 159.62 (C-9), 160.39 (C-3), 172.33 (C=O ester) ppm. MS (FAB, %): *m/z* = 476 ($[M+H]^+$, 22), 475 ($M^+$, 5), 432 (10), 314 (28), 276 (14), 240 (10), 230 (14), 193 (12), 192 (61), 175 (22), 174 (42), 154 (25). HRMS: *m/z* (FAB): calcd. for $C_{27}H_{26}NO_7$ $[M+H]^+$: 476.1709, found: 476.1705.

**Acknowledgements**

Thanks are due to the Foundation for Science and Technology (Portugal) for financial support through Centro de Química (UM).

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