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Journal of Photochemistry and Photobiology A: Chemistry 149 (2002) 217–225

Journal of Photochemistry and Photobiology A:Chemistry

www.elsevier.com/locate/jphotochem

Short communication

Synthesis and spectroscopic characterisation of 2-(2′-hydroxyphenyl)benzazole isothiocyanates as new fluorescent probes for proteins

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Received 12 December 2001; accepted 7 January 2002

Abstract

Three new benzazole isothiocyanate (BzITC) fluorescent dyes, 2-(5′-isothiocyanate-2′-hydroxyphenyl)benzoxazole, 2-(5′-isothiocyanate-2′-hydroxyphenyl)benzimidazole and 2-(5′-isothiocyanate-2′-hydroxyphenyl)oxazole[4,5-b]pyridine, were synthesised, purified until optical purity grade and characterised by elemental analysis, ^{1}H NMR, IR, UV–VIS and fluorescence spectroscopy. These dyes exhibit an intense fluorescence emission with a large Stokes shift due to an excited state intramolecular proton transfer (ESIPT) mechanism. The BzITCs were also studied for labelling three proteins (bovine serum albumin (BSA), concanavalin-A (con-A) and rabbit immunoglobulin G (rabbit IgG)) and the resulting conjugates presented good and stable fluorescence. A simple assay for detection of these proteins was reported here. The method is based on the direct fluorescence detection of protein-labelled with BzITC fluorophores after polyacrylamide gel electrophoresis and present potential use as fluorescent probes for proteins.

Keywords: Fluorescent probes; Benzazole dyes; Excited state intramolecular proton transfer (ESIPT); Protein detection

1. Introduction

Fluorescent probes are useful tools in analytical techniques [1], as immunofluorescence and immunofluorometric assays [1–4], protein conformation studies [5–10], detection of compounds in HPLC [2,11], capillary electrophoresis (CE) [2,12,13] as well as coupling CE with laser-induced fluorescence detection [14–16]. An effective biological fluorophore has to present a good luminescent intensity and an emission spectra free of interferences due to the emitting substances present in the analysed matrix. A high Stokes shift is an interesting characteristic for a fluorescent probe that permits an improved separation of the light inherent to the matrix and the light dispersed by the sample [16].

Several researches have been developed using intrinsic fluorescence of biomolecules [17–19] to investigate biological process through fluorescence measurements. Furthermore, when an organic fluorescent probe covalently binds with the biomolecule, the interaction between them provided a significant enhancement in fluorescence emission spectra as well as an extreme sensitivity at very low concentration of the organic probe (detectabilities in the 10^{-12} to 10^{-13} M range) [1,2,16]. For use as reporter molecules in biological systems many organic dyes have been studied, like coumarin derivatives [20], fluorescein isothiocyanate [20,21], anthracene derivatives [22], 2-naphthol [23] and others [2,16].

2-(2′-Hydroxyphenyl)benzazoles are interesting fluorescent molecules which shows high Stokes shift and present great thermal and photophysical stability due to an excited state intramolecular proton transfer (ESIPT) mechanism (see Fig. 1). These compounds have been studied theoretically [24–30], as laser dyes [31–34], polymer UV-light stabilisers [35–38], for organic NLO materials [29] and have been incorporated in inorganic [39] and organic polymeric materials [34,40].

In this work we present the synthesis and characterisation of three new 2-(2′-hydroxyphenyl)benzazole derivatives

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1010-6030/02/$ – see front matter
PII: S1010-6030(02)00008-4

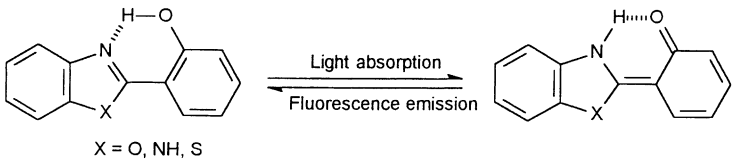

Fig. 1. Chemical structure of 2-(2′-hydroxyphenyl)benzazole dyes and the tautomeric forms involved in the emission of light-ESIPT.

(BzITC) **4a**–**c**, the purification until optical purity grade, and the use to label three model proteins, concanavalin-A (con-A), bovine serum albumin (BSA) and rabbit immunoglobulin G (rabbit IgG).

2. Experimental

2.1. Biological and chemical materials

2-Aminophenol, 1,2-phenylenediamine, 2-amino-3-hydroxypyridine and thiophosgene were purchased from Aldrich. The 5-amino-2-hydroxybenzoic acid was purchased from Aldrich or synthesised from salicylic acid using a methodology described in [41]. All other reagents and solvents were from Merck. BSA, con-A, and rabbit IgG were purchased from Sigma Chemical Co. Commercially available reagents and solvents were used as supplied. Column chromatography were performed using silica gel 60 (Merck).

2.2. General procedure for synthesis of the aminobenzazoles *3a*–*c*

A mixture of 5-amino-2-hydroxybenzoic acid (13 mmol) and the corresponding *ortho*-substituted aniline (13 mmol) in polyphosphoric acid (10 ml) was heated at 200 °C for 4 h with stirring. The reaction mixture was poured into 400 ml of water and the precipitate obtained was filtered, neutralised with a solution of sodium carbonate (10% w/v), washed with water and dried.

2.2.1. 2-(5′-Amino-2′-hydroxyphenyl)benzoxazole (*3a*)

The product obtained by the general procedure can be used without further purification for the synthesis of **4a** or purified by column chromatography using dichloromethane as the solvent. Yield: 74%; mp 174–175 °C. Anal. calc. for $C_{13}H_{10}N_2O_2$: C, 69.02; H, 4.46; N, 12.38. Found: C, 69.06; H, 4.56; N, 12.06.

IR (KBr, cm^{-1}): 3410–3330 (NH_2), 1630, 1545, 1234.

^{1}H NMR (200 MHz, $CDCl_3$): δ 10.94 (s, 1H, OH), 7.74–7.41 (m, H_4, H_5, H_6 and H_7), 7.36 (d, $H_{6'}$, J_{meta} = 2.6 Hz), 6.97 (d, $H_{3'}$, J_{ortho} = 8.8 Hz), 6.85 (dd, $H_{4'}$, J_{meta} = 2.6 Hz and J_{ortho} = 8.8 Hz), 3.52 (broad s, 2H, NH_2).

2.2.2. 2-(5′-Amino-2′-hydroxyphenyl)benzimidazole (*3b*)

The product obtained by the general procedure can be used without further purification for the synthesis of **4b** or purified by column chromatography using dichloromethane as the solvent. Yield: 94%; mp >260 °C (decomposition). Anal. calc. for $C_{13}H_{11}N_3O$: C, 69.32; H, 4.92; N, 18.65. Found: C, 69.03; H, 4.79; N, 18.26.

IR (KBr, cm^{-1}): 3400–3310 (NH_2 and NH), 1630, 1504, 1257.

^{1}H NMR (200 MHz, DMSO-d_6): δ 13.12 and 10.28 (two broad s, 2H, OH and NH), 7.67–7.24 (m, H_4, H_5, H_6, H_7 and $H_{6'}$), 6.80 (d, $H_{3'}$, J_{ortho} = 8.7 Hz), 6.74 (dd, $H_{4'}$, J_{meta} = 2.4 Hz and J_{ortho} = 8.7 Hz), 4.80 (broad s, 2H, NH_2).

2.2.3. 2-(5′-Amino-2′-hydroxyphenyl)oxazole[4,5-b] pyridine (*3c*)

The product obtained by the general procedure can be used without further purification for the synthesis of **4c** or purified by recrystallisation from toluene. Yield: 81%; mp 181–183 °C. Anal. calc. for $C_{12}H_9N_3O_2$: C, 63.43; H, 3.99; N, 18.49. Found: C, 62.89; H, 3.69; N, 18.11.

IR (KBr, cm^{-1}): 3425–3305 (NH_2), 1630, 1543, 1250.

^{1}H NMR (200 MHz, $CDCl_3$): δ 10.56 (s, 1H, OH), 8.60 (dd, H_7, J_{meta} = 1.4 Hz and J_{ortho} = 5.0 Hz), 7.90 (dd, H_5, J_{meta} = 1.4 Hz and J_{ortho} = 8.1 Hz), 7.37 (d, $H_{6'}$, J_{meta} = 2.7 Hz), 7.35 (dd, H_6, J_{ortho} = 8.1 Hz and J_{ortho} = 5.0 Hz), 7.02 (d, $H_{3'}$, J_{ortho} = 8.7 Hz), 6.93 (dd, $H_{4'}$, J_{meta} = 2.7 Hz and J_{ortho} = 8.7 Hz), 3.54 (broad s, 2H, NH_2).

2.3. General procedure for synthesis of the isothiocyanates BzITCs *4a*–*c*

The isothiocyanate **4a** was prepared by the Kaluza synthesis (method A) and the thiophosgene synthesis (method C). The isothiocyanates **4b**–**c** were prepared by the Jochims synthesis (method B) and the thiophosgene synthesis (method C).

- Method A: Kaluza synthesis

  The aminobenzoxazole **3a** (0.5 g) was dissolved in 2 ml of toluene. Triethylamine (0.5 ml) and carbon disulfide (0.5 ml) were added to this solution. The reaction mixture was kept at −18 °C for 7 days, with occasional stirring. The dithiocarbamate salt precipitated was filtered, washed with toluene and dried. It was dissolved in 2 ml of chloroform and the resulting solution was cooled in an ice–salt bath, and an equimolar amount plus a 10% excess of ethyl chloroformate was added, with stirring. After 10 min, an equimolar amount of triethylamine was added, and the stirring was continued at the same temperature for 10 min, and at room temperature for another 60 min. The solution

was washed three times with 10 ml of 3 M HCl, dried with Na_2SO_4, and the solvent was evaporated under reduced pressure. The residue was purified by column chromatography (chloroform) yielding the isothiocyanate **4a**.

- Method B: Jochims synthesis

  To an ice-cooled solution of dicyclohexylcarbodiimide (0.5 g) in 10 ml of CS_2, a solution of the corresponding aminobenzazoles **3b–c** (0.5 g) in 10 ml pyridine was added slowly, with stirring. The resulting mixture was allowed to stand at −18 °C for 24 h with occasional stirring. The mixture was poured into 400 ml of water; the precipitate was filtered, washed with water and dried.
- Method C: thiophosgene synthesis

  The corresponding aminobenzazoles **3a–c** (0.650 g) was dissolved in dry acetone (50 ml) and transferred to a drip funnel equipped with a drying tube. This solution was added dropwise to a thiophosgene solution (1 ml thiophosgene in 5 ml of dry acetone). The isothiocyanate formed was immediately precipitated. After the amine solution was completely added the reaction mixture was stirred for an additional 2 h. The isothiocyanate was filtered, washed with 10 ml of cold acetone and then dried.

2.3.1. 2-(5′-Isothiocyanate-2′-hydroxyphenyl)benzoxazole (4a)

Kaluza synthesis (method A): yield 20%. Thiophosgene synthesis (method C): yield 86%. The solid was purified by column chromatography (chloroform as the eluent) and recrystallised from acetone; mp 169–171 °C. Anal. calc. for $C_{14}H_8N_2O_2S$: C, 62.68; H, 3.00; N, 10.44. Found: C, 62.74; H, 3.10; N, 10.18.

IR (KBr, cm^{-1}): 2137 (N=C=S), 1625, 1492, 1265.

^{1}H NMR (200 MHz, $CDCl_3$): δ 11.60 (s, 1H, OH), 7.85 (d, $H_{6'}$, $J_{meta} = 2.5$ Hz), 7.77–7.41 (m, H_4, H_5, H_6 and H_7), 7.27 (dd, $H_{4'}$, $J_{meta} = 2.5$ Hz and $J_{ortho} = 9.0$ Hz), 7.07 (d, $H_{3'}$, $J_{ortho} = 9.0$ Hz), 3.54 (broad, 2H, NH_2).

2.3.2. 2-(5′-Isothiocyanate-2′-hydroxyphenyl) benzimidazole (4b)

Jochims synthesis (method B): yield 13%. Thiophosgene synthesis (method C): yield 86%. The solid was purified by column chromatography (chloroform as the eluent) and recrystallised from acetone; mp 298–301 °C. Anal. calc. for $C_{14}H_9N_3OS$: C, 62.91; H, 3.39; N, 15.72. Found: C, 62.91; H, 3.30; N, 15.56.

IR (KBr, cm^{-1}): 3317 (NH), 2143 (N=C=S), 1620, 1496, 1265.

^{1}H NMR (200 MHz, DMSO-d_6): δ 13.4 (broad s, 2H, OH and NH), 8.2 (d, $H_{6'}$, $J_{meta} = 2.4$ Hz), 7.8–7.6 (m, H_4, H_5, H_6 or H_7), 7.4–7.3 (m, H_4, H_5, H_6 or H_7), 7.45 (dd, $J_{meta} = 2.6$ Hz and $J_{ortho} = 8.8$ Hz), 7.1 (d, $H_{3'}$, $J_{ortho} = 8.8$ Hz).

2.3.3. 2-(5′-Isothiocyanate-2′-hydroxyphenyl)oxazole [4,5-b]pyridine (4c)

Jochims synthesis (method B): yield 22%. Thiophosgene synthesis (method C): yield 80%. The solid was purified by column chromatography (toluene as the eluent) and recrystallised from acetone; mp 181–183 °C. Anal. calc. for $C_{13}H_7N_3O_2S$: C, 57.99; H, 2.62; N, 15.60. Found: C, 58.36; H, 2.48; N, 15.65.

IR (KBr, cm^{-1}): 2111 (N=C=S), 1625, 1489, 1223.

^{1}H NMR (200 MHz, $CDCl_3$): δ 11.30 (s, 1H, OH), 8.65 (dd, H_7, $J_{meta} = 1.4$ Hz and $J_{ortho} = 5.0$ Hz), 8.00 (dd, H_5, $J_{meta} = 1.4$ Hz and $J_{ortho} = 8.2$ Hz), 7.92 (d, $H_{6'}$, $J_{meta} = 2.6$ Hz), 7.43 (dd, H_6, $J_{ortho} = 8.2$ Hz and $J_{ortho} = 5.0$ Hz), 7.37 (dd, $H_{4'}$, $J_{meta} = 2.6$ Hz and $J_{ortho} = 9.0$ Hz), 7.14 (d, $H_{3'}$, $J_{meta} = 2.6$ Hz).

2.4. General procedure for conversion of the isothiocyanates 4a–c to thioureas 5a–c

To a solution of 50 mg of the corresponding BzITCs **4a–c** in chloroform (5 ml) was added an excess (0.2 ml) of *tert*-butylamine. After 15 min at room temperature, the excess of the solvent and *tert*-butylamine were removed under reduced pressure yielding the thiourea derivatives **5a–c** in quantitative yield.

2.4.1. 2-[5′-(N-tert-Butylthiourea)-2′-hydroxyphenyl] benzoxazole (5a)

Melting point (mp) 166–167 °C. Anal. calc. for $C_{18}H_{19}N_3O_2S$: C, 63.32; H, 5.61; N, 12.31. Found: C, 63.36; H, 5.46; N, 11.71.

IR (KBr, cm^{-1}): 3256 (NH), 3047 (OH), 1625, 1538, 1370 (N–CS–N), 1247.

^{1}H NMR (200 MHz, $CDCl_3$): δ 11.60 (s, 1H, OH), 7.96 (d, $H_{6'}$, $J_{meta} = 2.5$ Hz), 7.83–7.45 (m, H_4, H_5, H_6 and H_7), 7.35 (dd, $H_{4'}$, $J_{meta} = 2.5$ Hz and $J_{ortho} = 8.8$ Hz), 7.23 (d, $H_{3'}$, $J_{ortho} = 8.8$ Hz), 5.88 (s, 1H, NH), 1.52 (s, 9H, CH_3).

2.4.2. 2-[5′-(N-tert-Butylthiourea)-2′-hydroxyphenyl] benzimidazole (5b)

Melting point (mp) >350 °C. Anal. calc. for $C_{17}H_{17}N_3O_2S$: C, 61.98; H, 5.20; N, 12.76. Found: C, 62.27; H, 5.38; N, 12.25.

IR (KBr, cm^{-1}): 3381 (NH), 3303 (NH), 3153 (OH), 1625, 1502, 1370 (N–CS–N), 1227.

^{1}H NMR (200 MHz, DMSO-d_6): δ 13.30 (broad s, 1H, OH or NH), 13.18 (broad s, 1H, NH or OH), 9.20 (s, 1H, NH), 8.09–7.06 (m, 7H aromatic), 1.59 (s, 9H, CH_3).

2.4.3. 2-[5′-(N-tert-Butylthiourea)-2′-hydroxyphenyl] oxazole[4,5-b]pyridine (5c)

Melting point (mp) 150 °C (decomposition), 186–189 °C (melt). Anal. calc. for $C_{17}H_{18}N_4O_2S$: C, 59.63; H, 5.30; N, 16.36. Found: C, 59.43; H, 5.16; N, 16.40.

IR (KBr, cm^{-1}): 3370 (NH), 3171 (OH), 1625, 1532, 1375 (N–CS–N), 1200.

^{1}H NMR (200 MHz, $CDCl_3$): δ 11.25 (broad s, 1H, OH), 8.64 (dd, H_5, $J_{meta} = 1.4$ Hz and $J_{ortho} = 8.2$ Hz), 7.98 (dd, H_7, $J_{meta} = 1.4$ Hz and $J_{ortho} = 5.0$ Hz), 7.96 (d, $H_{6'}$, $J_{meta} = 2.6$ Hz), 7.42 (dd, H_6, $J_{ortho} = 8.2$ Hz and

J_{ortho} = 5.0 Hz), 7.37 (dd, $H_{4'}$, J_{meta} = 2.6 Hz and J_{ortho} = 9.0 Hz), 7.22 (d, $H_{3'}$, J_{ortho} = 9.0 Hz), 5.85 (broad s, 1H, NH), 1.52 (s, 9H, CH_3).

2.5. General procedure for protein labelling with the isothiocyanates BzITCs **4a–c**

Proteins con-A, BSA and rabbit IgG, were labelled with different concentrations of BzITC fluorophores **4a–c**. The procedure was similar to standard procedures previously described for other fluorescent probes [42]. BzITC fluorophores **4a–c** were dissolved in DMSO/dioxan (2:1) to a final concentration of 10 mg/ml. Small aliquots of this solution were added, slowly and with gentle stirring, to 1 ml of protein solution (5 mg/ml in 0.1 M sodium carbonate, pH 9). Doses of 10, 20, 50, 100 and 200 μg fluorophores/ml protein solution were used. The mixture was incubated for 60 min at room temperature under gentle stirring, and 20 μl of 1 M ammonium chloride was added. Labelled protein was separated of free BzITC fluorophores by gel filtration chromatography on Sephadex G-50. Column was equilibrated and eluted with phosphate buffered saline (PBS, 10 mM phosphate, 150 mM NaCl, pH 7.4).

2.6. Polyacrylamide gel electrophoresis

SDS-PAGE was performed essentially as described by Laemmli [43]. After labelling with BzITC fluorophores **4a–c**, protein suspension was diluted in an equal volume of sample buffer (125 mM Tris, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol). The suspension was boiled for 5 min, and loaded (5 μg per lane) onto 10% polyacrylamide gels. After SDS-PAGE, gels were stored in 40% methanol, 10% acetic acid until observed under UV-light and photographed.

3. Instruments

Melting points were measured with a Thermolyne and are uncorrected. IR spectra were recorded on a Mattson Galaxy Series FT-IR3000 in KBr. NMR spectra measurements were recorded on a Varian VXR-200 spectrometer at 200.1 MHz using tetramethylsilane (TMS) as the internal standard in deuterium chloroform or DMSO-d_6 as the solvent at room temperature. Absorption spectra were taken on a Shimadzu UV-160A spectrophotometer. Fluorescence spectra were measured with a Hitachi spectrophotometer, model F-4500. Elemental analyses were recorded on a Perkin-Elmer model 2400.

4. Results and discussion

4.1. Synthesis of the 2(2′-hydroxyphenyl)benzazole heterocycles

The BzITC fluorophores **4a–c** were synthesised in a two-step procedure: (a) synthesis of the aminobenzazoles **3a–c** (see Fig. 2), and (b) the conversion to the desired isothiocyanate derivatives **4a–c** (see Fig. 3).

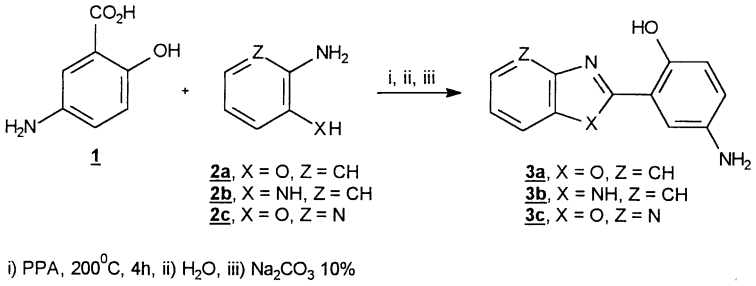

Fig. 2. Synthetic process for the synthesis of the aminobenzazoles **3a–c**.

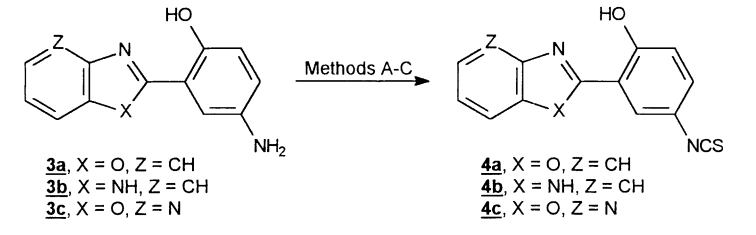

Fig. 3. Synthesis of the BzITC dyes **4a–c** by the Kaluza (method A), Jochims (method B) and thiophosgene (method C) methods.

For the synthesis of **3a–c** we use a standard methodology which consists of a condensation reaction between the 5-amino-2-hydroxybenzoic acid (**1**) and *ortho*-substituted anilines (**2a**, X = O and Z = H; **2b**, X = NH and Z = H; **2c**, X = O and Z = N), in polyphosphoric acid at 200 °C [44,45] to give the aminobenzazoles compounds **3a–c** with good yields [40].

The conversion of aminobenzazoles **3a–c** to the desired isothiocyanate derivatives **4a–c** was carried out by three different synthetic procedures, depending on the synthesised product (see Fig. 3). It is well known that a general methodology for the synthesis of an isothiocyanate compound is the reaction of an aromatic primary amine with thiophosgene [21,46–48]. This procedure is easy and results in very good yields. However, the high toxicity of thiophosgene [49] led us to try other methodologies for the synthesis. The Kaluza synthesis was the first one [46,50]. In this process, a primary amine is dissolved in an organic solvent and reacts with carbon disulfide and triethylamine, at low temperatures, to form a triethylammonium dithiocarbamate. This salt reacts with ethyl chloroformate, and then, with an alkali to yield the desired isothiocyanate. Using this method we could synthesise only the isothiocyanate **4a** and in low yield (20%).

Another synthetic approach was used, described by Jochims [51], which consists of the reaction of a primary amine, dicyclohexylcarbodiimide (DCC) and CS_2, in pyridine, to form an isothiocyanate and dicyclohexylthiourea (see Fig. 3). This process occurs in one step. The use of this procedure led us to obtain only the isothiocyanate derivatives **4b** and **4c** in very low yields (13 and 22%, respectively).

The use of thiophosgene, however, results in very good yields of the three desired isothiocyanate derivatives **4a–c** (>80%) and this process is easier and simpler than the others are.

We decide to study the reaction of these compounds with a model amine before to test the conjugation of the BzITCs **4a–c** with proteins in order to obtain some spectroscopic data. The reaction with *tert*-butylamine in organic solvents is easy and results in the thiourea derivatives **5a–c** in almost quantitative yields (see Fig. 4).

4.2. UV–VIS and fluorescence emission data

The new aminobenzazoles **3a–c**, BzITCs **4a–c** and thioureas **5a–c** heterocyclic dyes synthesised are fluorescent in solution and in the solid state when irradiated with UV-light. The thioureas **5a–c** present a green emission of

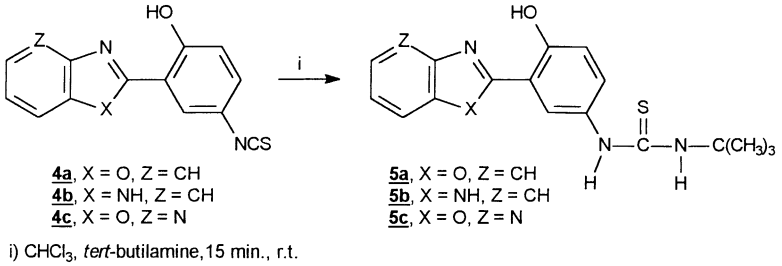

Fig. 4. Synthetic process for the synthesis of the thioureas **5a–c**.

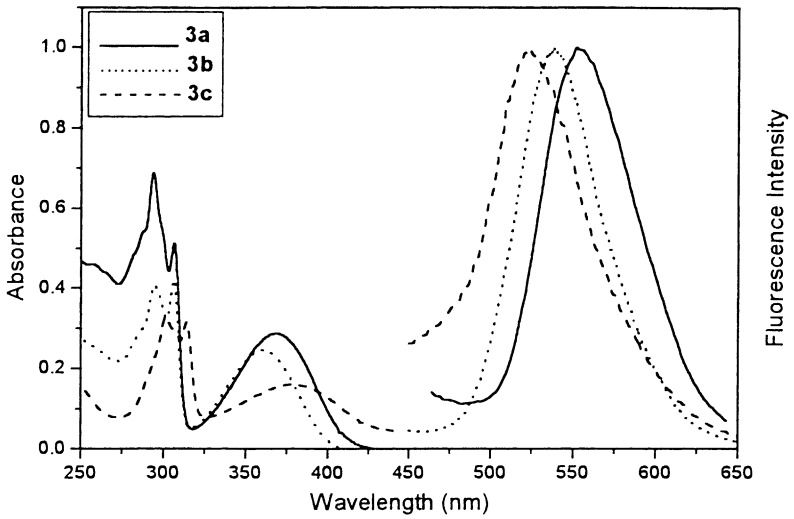

Fig. 5. Normalised absorption and fluorescence spectra of aminobenzazoles **3a–c**. The dye concentrations are 10^{-6} M in CH_2Cl_2. The excitation wavelength was 355 nm at room temperature.

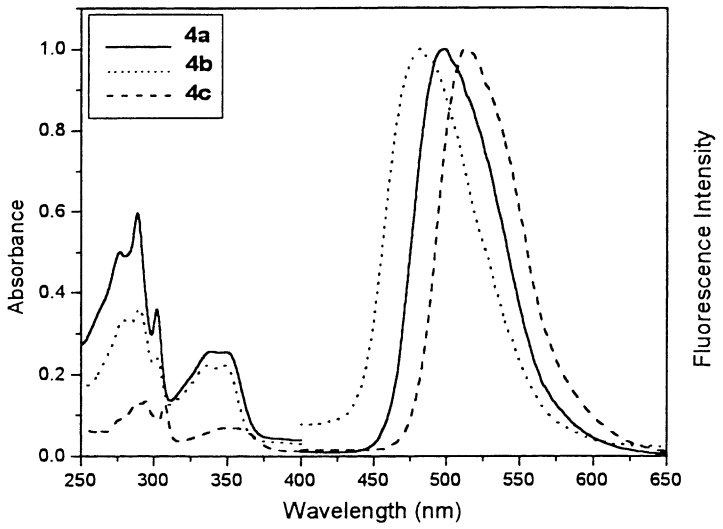

Fig. 6. Normalised absorption and fluorescence spectra of BzITCs **4a–c**. The dye concentrations are 10^{-6} M in CH_2Cl_2. The excitation wavelength was 355 nm at room temperature.

fluorescence (see Fig. 7) adequate for the analytical applications in medicine and biology. Fluorochromes with emission in the blue–purple range are less useful, since the emission in this range is inherent to some biological systems [52]. The UV–VIS and fluorescence emission spectra of **3a–c**, **4a–c** and **5a–c** are shown in Figs. 5–7, respectively. All experiments were performed at room temperature in CH_2Cl_2 as the solvent and the dye concentration are 10^{-6} M.

Table 1 shows the maximum absorption and emission values of wavelength and the corresponding Stokes shift for **3a–c**, **4a–c** and **5a–c**. The Stokes shift is in agreement with those presented by fluorochromes that exhibit ESIPT. The Stokes displacement for molecules that do not suffer structural changes in the excited state is generally found between 50 and 70 nm [53]. In contrast, the heterocycles **3a–c**, **4a–c** and **5a–c**, that exhibit ESIPT, present a Stokes shift between 134 and 189 nm.

4.3. Protein labelling with BzITC fluorophores

BzITCs **4a–c** are insoluble in water. However, using the methodology described in this work these molecules can be used as protein probes in aqueous solutions. They should be dissolved in DMSO/dioxan and added slowly, in small aliquots, to the protein solution in order to warrant an efficacious label. The unbound dye was efficiently removed by gel filtration chromatography on Sephadex G-50.

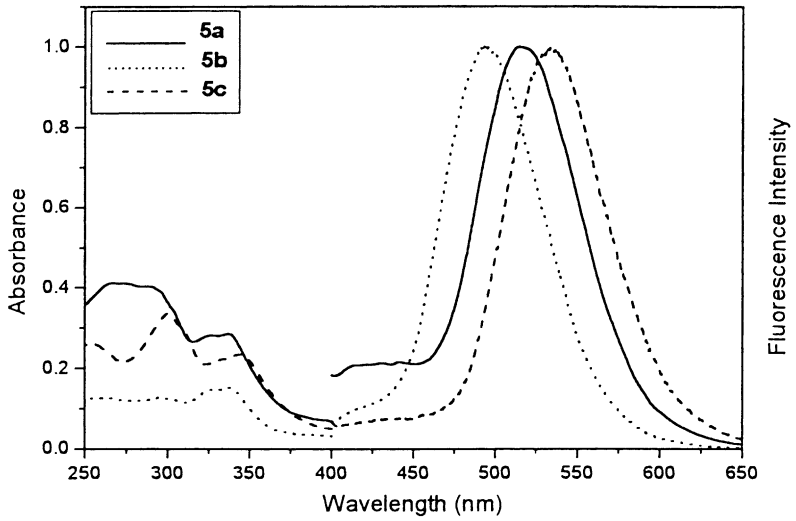

Fig. 7. Normalised absorption and fluorescence spectra of thioureas **5a–c**. The dye concentrations are 10^{-6} M in CH_2Cl_2. The excitation wavelength was 355 nm at room temperature.

Table 1
Spectral characteristics of the studied compounds[a]

Structure	Absorption λ_{max} (nm)	ε_{max} ($\times 10^3$)[b]	Emission λ_{max} (nm)	Stokes shift (nm)
3a	367	10.5	551	184
3b	359	11.7	539	180
3c	376	10.7	522	146
4a	338	16.0	498	160
4b	348	20.5	482	134
4c	344	16.5	513	169
5a	336	20.6	514	178
5b	338	13.4	493	155
5c	344	17.2	533	189

[a] Absorption and fluorescence spectra were measured in dichloromethane at 10^{-6} M.

[b] The values of molar extinction coefficients are in l mol^{-1} cm^{-1} and in the maximum of the first band.

Bovine serum albumin labelled with BzITCs **4a–c** presented a deep green fluorescence, in both aqueous solution and solid after freeze-drying. Fig. 8, for example, shows the fluorescence spectra of BzITC **4a** and BSA-labelled BzITC **4a**. The excellent overlap of the emission spectrum of free BzITC **4a** and BSA-labelled BzITC **4a** indicates that the BzITC **4a** was really bounded to protein BSA. The emission maximum of BzITC **4a** and BSA-labelled BzITC **4a** lies at 500 nm, similar to fluorescein isothiocyanate [53], which lies at 520 nm. The con-A and rabbit IgG labelled with BzITCs **4a–c** also presented fluorescence, however, with less intensity than BSA.

The conjugation extent of BSA with BzITCs at various dye/protein ratios is shown in Fig. 9. The amount of BzITC bound to protein increased gradually as the dye/protein (w/w) increased from 0.001 to 0.1, considering the increased

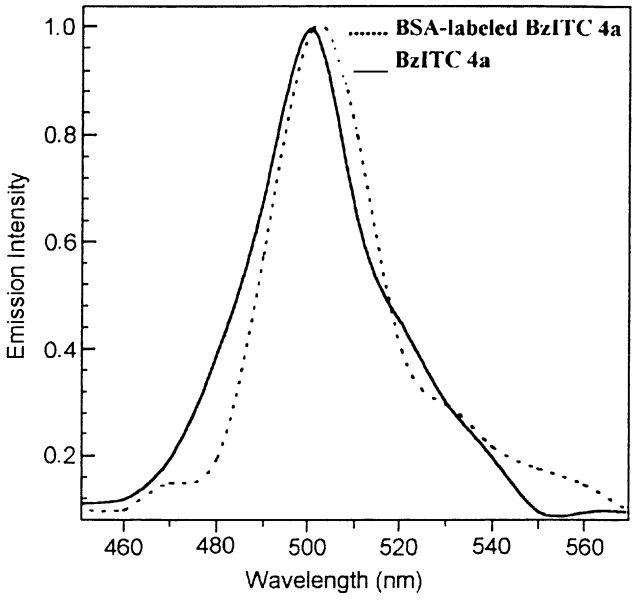

Fig. 8. Normalised fluorescence spectra of BzITC **4a** itself and BSA-labelled with BzITC fluorophore **4a**.

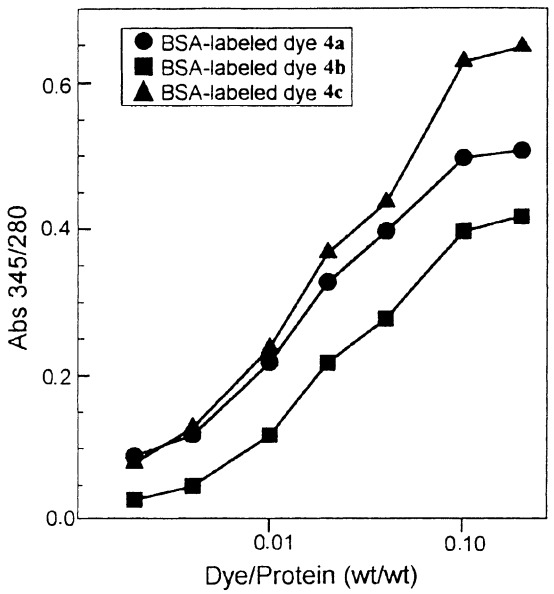

Fig. 9. BSA labelled with BzITCs **4a–c**. Conjugation of **4a–c** with BSA was obtained as a function of dye/protein ratio. The degree of conjugation was estimated from the absorption ratios at 345/280 nm.

ratios of absorption at 345/280 nm. At higher dye concentrations the recovery of protein decreased sharply, probably due to protein precipitation.

Table 2 presents the ratios of absorption at 345/280 nm of labelled BSA, rabbit IgG and con-A at a dye/protein ratio of 0.04.

The stability versus photodecomposition of BzITCs **4a–c** is depicted in Fig. 10. BzITCs were stored at room temperature and light exposed in DMSO/dioxan solution. Aliquots were taken at different times to label proteins. The ratios of absorption at 345/280 nm remains constant for almost 3 weeks (Fig. 9). Fluorescein isothiocyanate, which is widely used as fluorescent probe to label proteins [53], lacks the fluorescence quickly when exposed to light source. Other protein probes like 7-hydroxy-4-methylcoumarin and rhodamine retain full fluorescence more than fluorescein isothiocyanate when exposed to light source [20,42,54], however, BzITCs **4a–c** described in this work showed higher photostability than these compounds and do not need to be stored in the dark.

Table 2
Ratios of absorption 345/280 nm of proteins labelled with BzITCs **4a–c**[a]

Protein	Absorption 345/280 nm		
	4a	**4b**	**4c**
BSA	0.335	0.220	0.370
con-A	0.275	0.333	0.381
Rabbit IgG	0.346	0.302	0.390

[a] Proteins at 5 mg/ml in pH 9.0 bicarbonate buffer were incubated with BzITCs **4a–c** for 30 min at room temperature. Unbound dye was removed by gel filtration on Sephadex G-50.

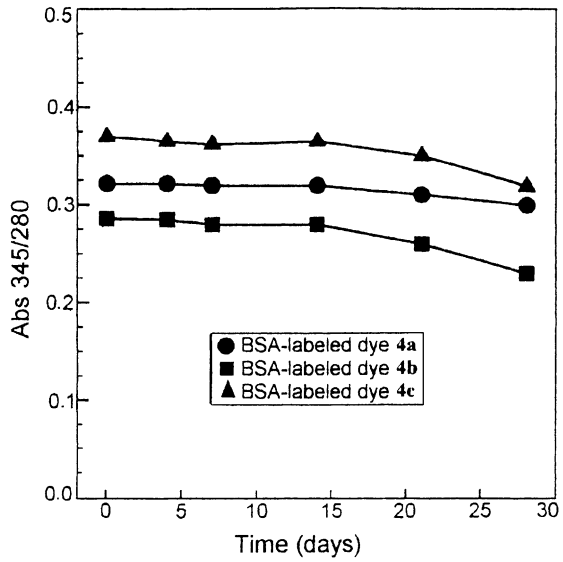

Fig. 10. Stability of BzITCs vs. light exposition. BzITCs **4a–c** were dissolved in DMSO/dioxan and stored at room temperature and light exposition. Small portions were taken at different times to label BSA and the ratios of absorption at 345/280 nm were estimated.

4.4. Polyacrylamide gel electrophoresis

Fluorogenic substrates have been used to detect specific enzymatic activities (generally hydrolysis) in polyacrylamide gels, by means of zymogram assays [55,56]. However, in this work the experiment was developed to direct protein detection in polyacrylamide gel. For this purpose, the BSA-labelled BzITC **4a** was submitted to polyacrylamide gel electrophoresis. After electrophoresis, the gel was submitted to UV-light excitation and direct detection of conjugated BSA-labelled is performed and can be seen only a fluorescent signal which lies at 500 nm (see Fig. 11). At low dye/protein ratios (0.004, lane A), the fluorescence was very low. However, at ratios of 0.01 (lane B) and 0.04 (lane C) the band corresponding to BSA was clearly observed, which indicates an important application for these compounds as fluorophores to protein detection.

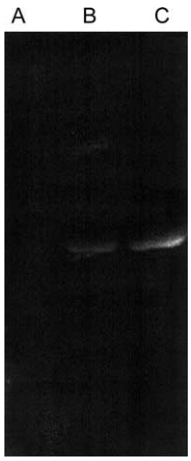

Fig. 11. SDS-PAGE of BSA-labelled (5 μg per lane) onto 10% polyacrylamide gels. BSA was labelled with the BzITC fluorophore **4a**, then submitted to polyacrylamide gel electrophoresis and revealed by UV-light excitation.

5. Conclusion

Three new BzITCs present ESIPT, i.e. **4a–c**, were synthesised, purified until optical purity grade and characterised by elemental analysis, ^{1}H NMR, IR, UV–VIS and fluorescence emission. These BzITCs-ESIPT compounds are extremely fluorescent when irradiated with UV-light and have a potential use as fluorescent probes for protein labelling. To test the reactivity of the BzITCs fluorophores with amines before the conjugation with the proteins, the BzITCs were reacted with *tert*-butylamine. The BzITCs-thioureas obtained show large Stokes shift and wavelength emission maxima between 493 and 533 nm.

The BzITCs were also studied for labelling three proteins (BSA, con-A and rabbit IgG) and the resulting conjugates presented good and stable fluorescence. A simple and highly sensitive assay for detection of these proteins was reported here. The method is based on the direct fluorescence detection of protein-labelled with BzITC fluorophores after polyacrylamide gel electrophoresis. The more evident fluorescent signal was obtained at dye/protein ratio of 0.04 and could be detected as only one band in SDS-PAGE. This band presents a fluorescent signal that lies at 500 nm and corresponds to BSA-labelled with BzITC **4a**, which indicates an important application for these compounds as fluorophores to protein detection. A number of other uses can be inferred, since BSA, rabbit IgG and con-A were effectively labelled with BzITCs **4a–c**. These applications may include labelling of antibodies for immunofluorescence assays and labelling of lectins for detection of specific carbohydrate moieties [57].

The synthesised organic fluorescent dyes present potential use as fluorescent probes for proteins, although additional studies are necessary to characterise the photophysic behaviour of these dyes when bounded to specific substrates in a better way.

Acknowledgements

Authors thanks the Brazilian agencies Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundaçãe de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS) and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for grants and financial support.

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