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Title

Novel highly emissive non proteinogenic amino acids: synthesis of 1,3,4-thiadiazolyl asparagines and evaluation as fluorimetric chemosensors for biologically relevant transition metal cations

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Abstract: Highly emissive heterocyclic asparagine derivatives bearing a 1,3,4-thiadiazolyl unit at the side chain, functionalised with electron donor or acceptor groups, were synthesised and evaluated as amino acid based fluorimetric chemosensors for metal cations such as Cu^{2+}, Zn^{2+}, Co^{2+} and Ni^{2+}. The results suggest that there is a strong interaction through the donor heteroatoms at the side chain of the various asparagine derivatives, with high sensitivity towards Cu^{2+} in a ligand-metal complex with 1:2 stoichiometry. Association constants and detection limits for Cu^{2+} were calculated. The photophysical and metal ion sensing properties of these asparagine derivatives confirm their potential as fluorimetric chemosensors and suggest that they can be suitable for incorporation into chemosensory peptidic frameworks.

Keywords: Non proteinogenic amino acids; Asparagine; Thiadiazole; Fluorescence; Chemosensors; Transition metals.

Introduction

Molecular recognition is the basis for most biological functions and, given the high degree of control over biological systems that Nature can get, in recent years the research on molecular recognition has evolved to mimic as much as possible the natural mechanisms of organization (Schneider and Strongin 2009), with the construction of fluorescent synthetic molecules capable of recognizing and binding organic and inorganic molecules involved in biological pathways being a current thrust in molecular recognition (Fan et al. 2010; Martínez-Manêz and Sancenón 2006; Zhao et al. 2004; Lin et al. 2004; Togrul et al. 2005; Wright and Anslyn 2004; Fedorova et al. 2006; Voyer et al. 2001; Mandl and König 2005; Zheng et al. 2003; Heinrichs et al. 2006).

Copper is third in abundance (after iron and zinc) among the essential heavy metals in the human body and plays a fundamental role in the biochemistry of the human nervous system but the accumulation of excess copper ions or their misregulation can cause neurological disorders like Parkinson's and Alzheimer's diseases (Wang et al. 2010). Zinc is recognised as one of the most important cations in catalytic centers and structural cofactors of many zinc-containing enzymes and DNA-binding proteins. It also functions as signalling agent mediating processes such as gene expression, neurotransmission and apoptosis. Several enzymes depend on nickel for activity (i. e. carbon monoxide dehydrogenase, acetyl-coenzyme A synthase and methyl-coenzyme M reductase) but in excess nickel's toxic nature can cause respiratory system diseases as lung and nasal cavity cancer, acute pneumonitis and asthma (Gupta et al. 2007). Like copper, zinc and nickel are also known to play a part in central nervous system disorders as amyotrophic lateral sclerosis and epileptic seizures (Xu et al. 2010). As for cobalt, it is a component of vitamin B-12 and in large doses can cause gastrointestinal disorders and respiratory irritation, pulmonary edema and pneumonia (Kumar and Shim 2009).

Amino acids, and hence peptides, are known for their ability to complex metal ions through the nitrogen, oxygen and sulphur donor atoms at the main and side chain (Shimazaki et al. 2009). Thus, the design of peptides that coordinate metals, by incorporation of modified amino acids, has potential for applications as varied as the study on protein-protein interactions mediated by metals and protein binding to nanoparticles and metal surfaces, and the development of selective chemical sensors for metals for use in vivo and in vitro (Mathews et al. 2008; Joshi et al. 2009). The insertion of the coordination centres into amino acids or peptide enables the construction of supramolecules which may play an innovative role in molecular recognition. Apart from the importance of metal complexation by amino

acids in a protein, the application of amino acids in the detection of metals both in solution (Zheng et al. 2003) and in solid phase through incorporation in polymeric materials (Gooding et al. 2001; Yang et al. 2001) has been encouraged. This requires modified amino acids bearing a metal ion chelating site for stable complex formation for incorporation into a peptide sequence. Fluorescent ligands which are mostly heteroaromatic ring systems often substituted by potentially chelating groups which act as both the recognition and signalling site, can be used in the synthesis of peptide based chemosensors, as reported recently with ligands that are capable to chelate various metal ions and whose complexes possess diversified photophysical properties (Voyer et al. 2001; Mandl and König 2005; Zheng et al. 2003; Heinrichs et al. 2006).

Unnatural amino acids with intrinsic functions have been increasingly studied, to be incorporated into peptides (Ishida et al. 2004; Hodgson and Sanderson 2004). A strategy for the development of such compounds involves the incorporation of multidentate complexing ligands in amino acid residues in the form of heterocyclic moieties. Amino acids bearing 2,2 '-bipyridine and 1,10-phenanthroline have been used in the synthesis of metalopeptides that bind Zn^{2+} (Cheng et al. 1996; Torrado and Imperiali 1996). Histidine participates actively in the coordination of Zn^{2+} in various proteins and imidazole isosters were proposed in order to mimic the side chain of the natural residue, containing a complexing group attached to the β-carbon via a 1,2,3-triazole (Nadler et al. 2009). Also, histidine in the N-terminal position of a peptide binds metals through its terminal nitrogen and the imidazole nitrogen, a process analogous to histamine coordination (Kozlowski et al. 1999). Hydroxyquinolines are also known to complex with metals and thus the synthesis of a (dihydroxyquinolinyl)-alanine from L-tyrosine was proposed (Heinrich and Steglich 2003). The complexes formed between metal ions and amino acids can be considered as models for biochemical interactions such as substrate-enzyme and other metal-mediated interactions with metal ions, with many Cu^{2+} complexes playing a decisive role in biological systems and, as such, heterocyclic nitrogen ligands like benzimidazole and creatinine have been used in the synthesis of ternary complexes of Cu^{2+} with dipeptides such as Gly-Gly, Gly-Ala, Gly-Val, Gly-Tyr, Gly-Trp-Gly and Ala (García-Raso et al. 2003). A cyclopentapeptide was found to display ionophoric properties towards lead cations (Abo-Ghalia and Amr 2004). The indole group of tryptophan has been applied in the area of anion sensors (Pfeffer et al. 2007).

Our current research interests include the synthesis and characterization of unnatural amino acids (Costa et al. 2003, 2008 a, b; Esteves et al. 2009), and innovative heterocyclic colorimetric/fluorimetric chemosensors for anions and cations containing (oligo)thiophene,

benzoxazole, imidazole and amino acid moieties (Costa et al. 2007; Batista et al. 2007, 2008, 2009). Given our previous results with the above mentioned nitrogen, oxygen and sulphur heterocycles, we envisaged the use of a heterocycle that has never been considered for chemosensing applications in combination with an amino acid, namely 1,3,4-thiadiazole. This heterocycle contains N and S heteroatoms and, to the best of our knowledge, has only been used for metal cations in nanoparticle design for the fluorimetric sensing of Ag^{+} and Hg^{2+} (Qu et al. 2008; Li and Yan 2009).

Bearing these facts in mind, we now report the synthesis and evaluation of novel heterocycle-based unnatural amino acids as fluorimetric chemosensors for the recognition of metallic cations (Cu^{2+}, Zn^{2+}, Co^{2+} and Ni^{2+}) of analytical, biological, environmental and medicinal relevance, through the combination of a 1,3,4-thiadiazole as coordinating/reporting unit with an amino acid core in order to obtain new chemosensors. The studied metallic cations can be considered borderline acids by Pearson's Hard Soft Acid Base (HSAB) theory. The novel 1,3,4-thiadiazolyl asparagines possess in its structure a donor atom set consisting of both soft (S) and hard (N and O) bases and should ensure complexation to a variety of metal ions. By using an heterocyclic π-conjugated bridge it is intended to improve the intramolecular electronic delocalisation, for the enhancement of the photophysical properties of the new sensors and the optimization of the recognition process of target analytes, through higher fluorescence and thus higher sensitivity, which represents a challenging goal in biomimetic and supramolecular chemistry.

Experimental Section

General

All melting points were measured on a Stuart SMP3 melting point apparatus and are uncorrected. TLC analyses were carried out on 0.25 mm thick precoated silica plates (Merck Fertigplatten Kieselgel $60F_{254}$) and spots were visualised under UV light. Chromatography on silica gel was carried out on Merck Kieselgel (230-240 mesh). IR spectra were determined on a BOMEM MB 104 spectrophotometer. NMR spectra were obtained on a Varian Unity Plus Spectrometer at an operating frequency of 300 MHz for ^{1}H NMR and 75.4 MHz for ^{13}C NMR or a Bruker Avance III 400 at an operating frequency of 400 MHz for ^{1}H NMR and 100.6 MHz for ^{13}C NMR using the solvent peak as internal reference at 25 ºC. All chemical shifts are given in ppm using δ_H Me_4Si = 0 ppm as reference and *J* values are given in Hz. Assignments were made by comparison of chemical shifts, peak multiplicities and *J* values and were supported by spin decoupling-double resonance and bidimensional heteronuclear

HMBC and HMQC correlation techniques. Low and high resolution mass spectrometry analyses were performed at the "C.A.C.T.I. - Unidad de Espectrometria de Masas", at University of Vigo, Spain. Fluorescence spectra were collected using a FluoroMax-4 spectrofluorometer. UV-visible absorption spectra (200 – 800 nm) were obtained using a Shimadzu UV/2501PC spectrophotometer. The linearity of the absorption versus concentration was checked within the used concentration. All commercially available reagents were used as received.

General procedure for the synthesis of 1,3,4-thiadiazolyl asparagines 3a-e

N-t-Butyloxycarbonyl aspartic acid benzyl ester **1** (1 equiv) was dissolved in dry DMF (3 mL/mmol), followed by HOBt (1 equiv), and after stirring for 10 min, *N,N'*-dicyclohexylcarbodiimide (1 equiv) was added. The reaction mixture was placed in an ice bath, stirred for 30 min and the corresponding 2-amino-1,3,4-thiadiazole **2** was added (1 equiv). The mixture was stirred at low temperature for 2 h, and then for 24h at room temperature. After filtering, the solvent was removed under reduced pressure in a rotary evaporator. The residue was dissolved in acetone and placed in the cold overnight to induce separation of by-product *N,N'*-dicyclohexylurea as a precipitate, which was separated by filtration. This procedure was repeated two times. The filtrate was evaporated in a rotary evaporator. The resulting solid was purified by column chromatography, using mixtures of ethyl acetate and light petroleum 40-60 of increasing polarity.

***N-t*-Butyloxycarbonyl (5-phenyl-1,3,4-thiadiazol-2-yl) asparagine benzyl ester (3a)**. Starting from amine **2a** (0.110 g, 6.18×10^{-4} mol) and Boc-Asp-OBzl **1** (0.200 g, 6.18×10^{-4} mol), compound **3a** was obtained as a white solid (0.158 g, 53%); mp = 168.7-169.7 ºC; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.39 (s, 9H, $C(CH_3)_3$), 3.25-3.53 (m, 2H, β-CH_2), 4.87-4.90 (m, 1H, α-H), 5.13-5.28 (m, 2H, CH_2), 5.98 (d, *J* 9.3 Hz, 1H, NH Boc), 7.23-7.29 (m, 5H, 5×Ph-*H*), 7.50-7.52 (m, 3H, H3', H4', H5'), 7.95-7.98 (m, 2H, H2', H6'); ^{13}C NMR (75.4 MHz, $CDCl_3$): δ = 28.19 ($C(CH_3)_3$), 38.58 (β-CH_2), 50.50 (α-C), 67.47 (CH_2), 80.10 ($C(CH_3)_3$), 127.28 (C2' and C6'), 128.19 (C2'' and C6''), 128.30 (C4''), 128.45 (C3'' and C5''), 129.25 (C3' and C5'), 129.96 (C1'), 130.95 (C4'), 135.15 (C1''), 155.64 (C=O urethane), 159.73 (C2), 163.37 (C5) 168.94 (C=O amide), 171.00 (C=O ester); IR (KBr 1%, cm^{-1}): ν = 3359, 3161, 3032, 2977, 2931, 1754, 1746, 1701, 1694, 1562, 1524, 1501, 1454, 1444, 1407, 1382, 1369, 1348, 1324, 1309, 1281, 1249, 1225, 1200, 1165, 1109, 1093, 983,

909, 873, 784, 755, 729, 696, 666; UV/Vis (acetonitrile, nm): λ_{max} (log ε) = 290 (4.25); MS: *m/z* (EI) 482 (M^+, 100); HMRS: *m/z* (EI) calc. for $C_{24}H_{26}N_4O_5S$ 482.16258, found 482.16252.

***N-t*-Butyloxycarbonyl (5-(4'-methoxyphenyl)-1,3,4-thiadiazol-2-yl) asparagine benzyl ester (3b)**. Starting from amine **2b** (0.129 g, 6.22×10^{-4} mol) and Boc-Asp-OBzl **1** (0.201 g, 6.22×10^{-4} mol), compound **3b** was obtained as a white solid (0.159 g, 50%); mp = 131.9-133.0 ºC; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.39 (s, 9H, $C(CH_3)_3$), 3.23-3.50 (m, 2H, β-CH_2), 3.90 (s, 3H, OCH_3), 4.86-4.87 (m, 1H, α-H), 5.14-5.27 (m, 2H, CH_2), 5.97 (d, *J* 8.8 Hz, 1H, NH Boc), 7.01 (d, *J* 8.8, 2H, H3' and H5'), 7.24-7.30 (m, 5H, 5×Ph-*H*), 7.90 (d, *J* 6.8 Hz and 2.0Hz, 1H, H2' and H6'); ^{13}C NMR (100.6 MHz, $CDCl_3$): δ = 28.21 ($C(CH_3)_3$), 38.62 (β-CH_2), 50.53 (α-C), 55.51 (OCH_3), 67.57 (CH_2), 80.11 ($C(CH_3)_3$), 114.69 (C3' and C5'), 122.37 (C1'), 128.21 (C2'' and C6''), 128.31 (C4''), 128.47 (C3'' and C5'') 128.88 (C2' and C6'), 135.12 (C1''), 155.64 (C=O urethane), 159.14 (C2), 161.94 (C4'), 163.23 (C5), 168.84 (C=O amide), 170.96 (C=O ester); IR (KBr 1%, cm^{-1}): ν = 3372, 2972, 2928, 2855, 1748, 1699, 1609, 1581, 1563,1521, 1499, 1456, 1417, 1387, 1368, 1345, 1311, 1256, 1222, 1174, 1113, 1028, 985, 902, 834, 732, 697, 666; UV/Vis (acetonitrile, nm): λ_{max} (log ε) = 291 (4.23); MS: *m/z* (EI) 512 (M^+, 100); HMRS: *m/z* (EI) calc. for $C_{25}H_{28}N_4O_6S$ 512.17314, found 512.17320.

***N-t*-Butyloxycarbonyl (5-(4'-fluorophenyl)-1,3,4-thiadiazol-2-yl) asparagine benzyl ester (3c)**. Starting from amine **2c** (0.121 g, 6.22×10^{-4} mol) and Boc-Asp-OBzl **1** (0.201 g, 6.22×10^{-4} mol), compound **3c** was obtained as a white solid (0.204 g, 66%); mp = 193.3-194.0 ºC; ^{1}H NMR (400 MHz, $CDCl_3$): δ =1.38 (s, 9H, $C(CH_3)_3$), 3.26-3.49 (m, 2H, β-CH_2), 4.87-4.88 (m, 1H, α-H), 5.14-5.26 (m, 2H, CH_2), 5.96 (d, *J* 8.8 Hz, 1H, NH Boc), 7.17-7.21 (m, 2H, H3' and H5'), 7.25-7.30 (m, 5H, 5×Ph-*H*), 7.94-7.97 (m, 2H, H2' and H6'), 13.40 (br s, 1H, NH amide); ^{13}C NMR (100.6 MHz, $CDCl_3$): δ = 28.19 ($C(CH_3)_3$), 38.57 (β-CH_2), 50.52 (α-C), 67.47 (CH_2), 80.11 ($C(CH_3)_3$), 116.44 (d, *J* 22.1 Hz, C3' e C5'), 126.32 (d, *J* 3.0 Hz, C1'), 128.15 (C2'' and C6''), 128.31 (C4''), 128.46 (C3'' and C5''), 129.26 (d, *J* 9.1 Hz, C2' and C6'), 135.16 (C1''), 155.61 (C=O urethane), 159.67 (C2), 162.22 (C5), 164.24 (d, *J* 252.5 Hz, C4'), 168.95 (C=O amide), 170.99 (C=O ester); IR (KBr 1%, cm^{-1}): ν = 3359, 2979, 2930, 2852, 1749, 1699, 1609, 1596, 1566,1518, 1455, 1407, 1368, 1349, 1310, 1232, 1161, 1052, 973, 839; UV/Vis (acetonitrile, nm): λ_{max} (log ε) = 289 (4.33); MS: *m/z* (EI) 500 (M^+, 100); HMRS: *m/z* (EI) calc. for $C_{24}H_{25}N_4O_5SF$ 500.15315, found 500.15309.

***N*-*t*-Butyloxycarbonyl (5-(4'-nitrophenyl)-1,3,4-thiadiazol-2-yl) asparagine benzyl ester (3d)**. Starting from amine **2d** (0.137 g, 6.19×10^{-4} mol) and Boc-Asp-OBzl **1** (0.200 g, 6.19×10^{-4} mol), compound **3d** was obtained as a white solid (0.097 g, 30%); mp = 171.3-173.5 ºC; ^{1}H NMR (300 MHz, $CDCl_3$): δ =1.40 (s, 9H, $C(CH_3)_3$), 3.31-3.50 (m, 2H, β-CH_2), 4.89 (br s, 1H, α-H), 5.16-5.27 (m, 2H, CH_2), 5.83 (d, *J* 6.9Hz, 1H, NH Boc), 7.26-7.30 (m, 5H, 5×Ph-*H*), 8.15 (d, *J* 8.7 Hz, 2H, H2' and H6'), 8.37 (d, *J* 9.0 Hz, 2H, H3' and H5') ppm; ^{13}C NMR (75.4 MHz, $CDCl_3$): δ = 28.21 ($C(CH_3)_3$), 38.76 (β-CH_2), 50.45 (α-C), 67.66 (CH_2), 80.40 ($C(CH_3)_3$), 124.58 (C3' and C5'), 128.01 (C2'' and C6''), 128.25 (C4''), 128.43 (C3'' and C5''), 128.52 (C2' and C6'), 135.03 (C1''), 135.49 (C1'), 149.06 (C4'), 155.52 (C=O urethane), 159.52 (C2), 160.73 (C5), 169.02 (C=O amide), 170.78 (C=O ester); IR (KBr 1%, cm^{-1}): ν = 3348, 3160, 2977, 2933, 1738, 1700, 1600, 1557, 1524, 1500, 1455, 1442, 1406, 1368, 1348, 1310, 1250, 1215, 1164, 1106, 1052, 1027, 1012, 992 970, 913, 853, 820, 782, 753, 736, 692; UV/Vis (acetonitrile, nm): λ_{max} (log ε) = 324 (4.34); MS: *m/z* (EI) 527 (M^+, 100); HMRS: *m/z* (EI) calc. for $C_{24}H_{25}N_5O_7S$ 527.14765, found 527.14754.

***N*-*t*-Butyloxycarbonyl (5-(4'-pyridyl)-1,3,4-thiadiazol-2-yl) asparagine benzyl ester (3e)**. Starting from amine **2e** (0.109 g, 6.13×10^{-4} mol) and Boc-Asp-OBzl **1** (0.198 g, 6.13×10^{-4} mol), compound **3e** was obtained as a white solid (0.056 g, 19%); mp = 194.3-195.5 ºC; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.40 (s, 9H, $C(CH_3)_3$), 3.31-3.48 (m, 2H, β-CH_2), 4.83-4.90 (m, 1H, α-H), 5.16-5.27 (s, 2H, CH_2), 5.82 (d, *J* 8.7 Hz, 1H, NH Boc), 7.25-7.30 (m, 5H, 5×Ph-*H*), 7.89 (d, *J* 5.4 Hz, 2H, H3' and H5'), 8.81 (d, *J* 5.4 Hz, 2H, H2' and H6'), 13.12 (br s, 1H, NH amide); ^{13}C NMR (75.4 MHz, $CDCl_3$): δ = 28.21 ($C(CH_3)_3$), 38.70 (β-CH_2), 50.43 (α-C), 67.63 (CH_2), 80.37 ($C(CH_3)_3$), 121.05 (C3' and C5'), 128.24 (C2'' and C6''), 128.41 (C4''), 128.51 (C3'' and C5''), 135.05 (C1''), 137.54 (C4'), 149.43 (C2' and C6'), 155.55 (C=O urethane), 159.37 (C2), 160.51 (C5), 160.81 (C=O amide), 170.81 (C=O ester); IR (KBr 1%, cm^{-1}): ν = 3366, 2985, 2920, 2859, 1744, 1686, 1626, 1607 1580, 1504, 1449, 1434, 1390, 1367, 1353, 1305, 1281, 1253, 1220, 1163, 1110, 1063, 1020, 997, 965, 913, 856, 824, 786, 747, 701; UV/Vis (acetonitrile, nm): λ_{max} (log ε) = 288 (4.15); MS: *m/z* (EI) 483 (M^+, 100); HMRS: *m/z* (EI) calc. for $C_{23}H_{25}N_5O_5S$ 483.15785, found 483.15772.

Synthesis of *N*-*t*-butyloxycarbonyl (5-(4'-fluorophenyl)-1,3,4-thiadiazol-2-yl) asparagine (4). Asparagine derivative **3c** (0.120 g, 2.40×10^{-4} mol) was dissolved in 1,4-dioxane (3 mL / equiv) in an ice bath, followed by the addition of aqueous NaOH 6 M (1.5 equiv). After stirring at r.t. for 3h, the pH was adjusted to 2-3 by adding aq. $KHSO_4$ 1M and the solution

extracted with ethyl acetate (3 × 10 mL). The organic extract was dried with anhydrous $MgSO_4$, filtered and the solvent removed in a rotary evaporator. The residue was triturated with diethyl ether and the resulting solid was submitted to silica gel chromatography using a mixture of dichloromethane/methanol (5:1), to afford **4** as a white solid (0.083 g, 85%); mp = 212.3-214.0 ºC; ^{1}H NMR (400 MHz, DMSO-d_6): δ =1.35 (s, 9H, C(CH_3)$_3$), 2.66-2.75 (m, 1H, β-CH_2), 2.88-2.95 (m, 1H, β-CH_2), 4.19 (d, *J* 6.0 Hz, 1H, α-H), 6.72 (br s, 1H, NH Boc), 7.35 (t, *J* 9.2 Hz, 2H, H3' and H5'), 7.98 (t, *J* 9.2 Hz, 2H, H2' and H6') ppm; ^{13}C NMR (100.6 MHz, DMSO-d_6): δ = 28.18 (C(*C*H_3)$_3$), 35.87 (β-CH_2), 50.60 (α-C), 78.13 (*C*(CH_3)$_3$), 116.44 (d, *J* 22.1 Hz, C3' e C5'), 127.00 (d, *J* 3.0 Hz, C1'), 129.20 (d, *J* 9.1 Hz, C2' and C6'), 154.97 (C=O urethane), 158.71 (C2), 160.50 (C5), 163.29 (d, *J* 249.5 Hz, C4'), 169.52 (C=O amide), 172.95 (C=O acid); IR (KBr 1%, cm^{-1}): ν = 3097, 2981, 2930, 2850, 1744, 1700, 1610, 1546, 1509, 1463, 1403, 1367, 1329, 1295, 1234, 1170, 1062, 1052, 996, 954, 915, 852, 823, 782, 728, 706 cm^{-1}; UV/Vis (acetonitrile, nm): λ_{max} (log ε) = 290 (4.20); MS: *m/z* (EI) 410 (M^+, 100); HMRS: *m/z* (EI) calc. for $C_{17}H_{19}N_4O_5SF$ 410.10617, found 410.10643.

Synthesis of (5-(4'-fluorophenyl)-1,3,4-thiadiazol-2-yl) asparagine (5). Asparagine derivative **4** (0.070 g, 1.71 × 10^{-4} mol) was stirred in a trifluoroacetic acid/dichloromethane solution (1:1, 1 mL) at r.t. for 2h. The solvent was evaporated, the residue dissolved in pH 8 aqueous buffer solution and extracted with ethyl acetate (3 × 10 mL). After drying with anhydrous magnesium sulphate and evaporation of the solvent, **5** was isolated as a white solid (0.041 g, 77%); mp = 239.8-242.1 ºC; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 2.66-2.71 (dd, *J* 16.4 and 6.0 Hz, 1H, β-CH_2), 3.06-3.12 (dd, *J* 16.4 and 6.0 Hz, 1H, β-CH_2), 3.68 (t, *J* 6.4 Hz, 1H, α-H), 7.38 (t, *J* 8.7 Hz, 2H, H3' and H5'), 7.99 (t, *J* 8.7 Hz, 2H, H2' and H6') ppm; ^{13}C NMR (75.4 MHz, DMSO-d_6): δ = 37.45 (β-CH_2), 49.61 (α-C), 116.20 (d, *J* 22.2 Hz, C3' e C5'), 126.10 (d, *J* 3.0 Hz, C1'), 129.62 (d, *J* 9.5 Hz, C2' and C6'), 160.10 (C2), 162.40 (C5), 164.32 (d, *J* 251.7 Hz, C4'), 169.10 (C=O amide), 169.93 (C=O acid); IR (KBr 1%, cm^{-1}): ν = 3071, 2923, 2850, 1675, 1611, 1563, 1495, 1462, 1435, 1390, 1355, 1303, 1260, 1260, 1169, 1060, 971, 857, 827, 815, 733, 710 cm^{-1}; UV/Vis (acetonitrile, nm): λ_{max} (log ε) = 289 (4.19); MS: *m/z* (EI) 310 (M^+, 100); HMRS: *m/z* (EI) calc. for $C_{12}H_{11}N_4O_3SF$ 310.05373, found 310.05401.

Spectrophotometric titrations and chemosensing studies of 1,3,4-thiadiazolyl asparagine derivatives 3a-e, 4 and 5

Solutions of asparagine derivatives **3a-e**, **4** and **5** (ca. 1.0×10^{-5} to 1.0×10^{-6} M) and of the metallic cations under study (ca. 1.0×10^{-1} to 1.0×10^{-3} M) were prepared in UV-grade acetonitrile (in the form of hexahidrated tetrafluorborate salts for Cu^{2+}, Co^{2+} and Ni^{2+} and perchlorate salt for Zn^{2+}). Titration of the compounds with the several metallic cations was performed by the sequential addition of equivalents of metal cation to the asparagine derivative solution, in a 10 mm path length quartz cuvette and emission spectra were measured by excitation at the wavelength of maximum absorption for each compound, indicated in Table 1.

The binding stoichiometry of the asparagine derivatives with the metal cations was determined by using Job's plots, by varying the molar fraction of the cation while maintaining constant the total asparagine derivative and metal cation concentration. The association constants were obtained from Benesi–Hildebrand plots in the form of straigth lines with good correlation coefficients by using a equation reported elsewhere (Azab et al. 2010).

Results and Discussion

Synthesis

The new asparagine derivatives **3a-e** with 1,3,4-thiadiazole at its side chain were synthesized by a standard coupling procedure involving DCC and HOBt, between the side chain carboxylic acid group of *N*-*t*-butyloxycarbonyl aspartic acid benzyl ester **1** and 2-amino-1,3,4-thiadiazoles **2a-e**, bearing a phenyl ring with substituents of different electronic character (electron donor or acceptor, such as methoxy, fluor and nitro) or a electron deficient pyridyl ring (Scheme 1, Table 1). From the results in Table 1, it can be seen that the yield for thiadiazolyl asparagine derivatives **3a-e** was influenced by the electronic nature of the substituent at position 5 of the thiadiazole, being low for derivatives **3d-e** with electron acceptor nitro and pyridyl groups. In addition, in order to assess the influence of the presence of additional heteroatoms at the N- and C-termini blocking groups (in the form of Boc and Bzl ester), the selective deprotection at the carboxylic and amino group of fluoro asparagine derivative **3c**, was undertaken by standard deprotection procedures, yielding novel derivatives **4** (with a free carboxylic acid terminal) and **5** (with free amino and carboxylic acid terminals) in good yields. All the heterocyclic asparagine derivatives were fully characterised by the usual spectroscopic techniques.

< SCHEME 1>

< TABLE 1>

The absorption and emission spectra of asparagine derivatives **3a-e**, **4** and **5** were measured in degassed acetonitrile solution (10^{-6}-10^{-5} M) (Table 1). The asparagine derivatives showed similar absorption and emission wavelengths with the exception of **3d**, which displayed a bathochromic shift for the maximum wavelength of absorption and emission when compared to **3a**, accordingly to the electronic character of the nitro substituent. The nature of the ring at position 5 of the thiadiazole did not influence the overall photophysical properties of derivatives **3a** and **3e**, bearing a phenyl and a pyridyl ring, respectively. The relative fluorescence quantum yields (Φ_F) were determined using a 10^{-6} M solution of 9,10-diphenylanthracene in ethanol as standard (Φ_F = 0.95) (Morris et al. 1976). For the Φ_F determination, the fluorescence standard was excited at the wavelengths of maximum absorption found for each of the compounds to be tested and in all fluorimetric measurements the absorbance of the solution did not exceed 0.1. The 1,3,4-thiadiazolyl asparagine derivatives exhibited good to excellent fluorescence quantum yields in acetonitrile, with the exception of the nitro derivative **3d** (0.01) which is a known strong fluorescence quencher. The highest Φ_F value was obtained for the fluoro derivative **3c** (0.71), which is in agreement with previous results reported by us for benzothiazolyl asparagine derivatives bearing different substituents including fluorine (Esteves et al. 2009). *N*-Protected fluoro asparagine **4** and fully deprotected fluoro asparagine derivative **5** displayed similar Φ_F (*ca*. 0.2), which was lower than that of the parent asparagine derivative **3c**, a fact that could be attributed to the possibility for formation of intra and intermolecular H-bonds between the carboxylic acid proton and the heteroatoms at the side chain. All compounds showed large Stokes' shifts (from 6800 to 8500 cm^{-1}), except for the nitro derivative **3d** which was *ca*. 5200 cm^{-1}. Stokes' shifts directly relate to energy differences between the ground and excited states and this large value is an interesting feature for biological fluorescent probes that allows an improved separation of the light inherent to the matrix and the light dispersed by the sample (Holler et al. 2002).

Spectrophotometric and spectrofluorimetric titrations of 3a-e, 4 and 5 with metallic cations

The modification of asparagine through the introduction of a UV-active and highly fluorescent heterocycle at its side chain is expected to provide additional binding sites for a

variety of metal ions through the heterocycle donor atoms, as well as improved photophysical properties for the chemosensing studies. With heterocyclic asparagine derivatives **3a-e** it was intended to assess the influence in the chemosensing ability of metallic cations of the type of the substituent at the 1,3,4-thiadiazolyl system. Considering the biological, environmental and analytical relevance of transition metals such as Cu^{2+}, Zn^{2+}, Co^{2+} and Ni^{2+}, the interaction of asparagine derivatives **3a-e** with these cations was evaluated through UV-vis and fluorescence spectroscopies in spectrophotometric and spectrofluorimetric titrations in acetonitrile. In the spectrophotometric titrations, no changes were seen in the bands corresponding to the maximum wavelength of absorption of asparagine derivatives **3a-e** after addition of up to 200 equiv of each metal cation. In the presence of Cu^{2+}, two new absorption bands appeared at shorter wavelengths (212 and 235 nm) and increased with the addition of up to 10 equiv of Cu^{2+}. Increase of the metal concentration to 15 equiv lead to the decrease of the band at 212 nm, accompanied by a hipsochromic shift and the disappearance of the band at 235 nm, resulting in a new band centered at 206 nm. This band increased with further addition of Cu^{2+}. These observations may indicate the formation of a new species, a asparagine-metal complex, absorbing at shorter wavelengths. In the presence of Ni^{2+}, a similar behaviour was observed for a new band visible at 266 nm, which increased upon addition of up to 10 equiv of metal and decreased at higher metal concentration (up to 30 equiv). In the presence of Zn^{2+} and Co^{2+}, the formation of asparagine-metal complexes was not detected by UV-vis spectroscopy.

In the spectrofluorimetric titrations with Cu^{2+}, a strong decrease of the fluorescence intensity (a chelation-enhanced quenching, CHEQ effect) was observed for all the asparagine derivatives, with an almost complete fluorescence quenching. In Figure 1A is shown the spectrofluorimetric titration of asparagine derivative **3c** with Cu^{2+}, where the drastic effect of ion complexation is evident in the band centred at the wavelength of maximum emission at 361 nm. This Figure is representative of the Cu^{2+} titrations of asparagine derivatives **3a-e**, the only difference between them being the number of metal equivalents necessary to quench at least 90% of the initial fluorescence intensity (before complexation) of the heterocyclic amino acid (20 equiv for **3a**, 30 equiv for **3b**, 40 equiv for **3c**, 25 equiv for **3d** and 70 equiv for **3e**) (see Figure 2 for spectrofluorimetric titrations of **3a-b**,**d-e** with Cu^{2+}).

In order to assess the influence on the chemosensing ability of the presence of additional heteroatoms from the urethane and ester protecting groups at the N- and C-terminals, spectrofluorimetric titrations with Cu^{2+} of the fluoro asparagine derivatives **4**, having a free carboxylic acid group, and **5**, having free amino and carboxylic acid groups, were also carried

out in acetonitrile (Figure 2). It was found that both derivatives required similar amounts of Cu^{2+} (15 equiv) for a 90% quench of the initial fluorescence, revealing that the free amino terminal was not essential for coordination (comparing **4** and **5**) and that free carboxylic acid group had a positive effect on the coordination ability (when comparing **3c**, 40 equiv, and **4**), as less metal equivalents were necessary for the same decrease in fluorescence. Considering these findings, it can be suggested that the coordination process should occur through the heteroatoms at the side chain of the amino acid, aided by the carbonyl oxygen at the C-terminal of the amino acid (either in ester or carboxylic acid form).

< Figure 1>

< Figure 2>

With regard to the other cations Zn^{2+}, Co^{2+} and Ni^{2+}, a less pronounced CHEQ effect was also observed after metal addition, without complete quenching of fluorescence. In Figure 1 B, C and D are shown the spectrofluorimetric titrations of asparagine derivative **3c** with Zn^{2+}, Co^{2+} and Ni^{2+}, and, as already stated for Figure 1A, these are also representative of the titrations of asparagine derivatives **3a-e** with Zn^{2+}, Co^{2+} and Ni^{2+}. For these metallic cations, the complete quenching of fluorescence was not achieved even after addition of up to 200 equiv of metal, with a plateau being reached depending on the metal and the asparagine derivative: for Zn^{2+}, there was a maximum decrease of 70% in fluorescence of asparagine derivatives **3a-d** with the addition of 35 to 60 equiv of cation, while for asparagine derivative **3e** a decrease of less than 30% was achieved with 30 equiv of cation; as for Co^{2+}, a fluorescence quenching of 70% was visible with the addition of 20 to 40 equiv to asparagine derivatives **3a-c**,**e** and a 90% quenching was achieved upon the addition of 35 equiv to **3d**; and finally, asparagine derivatives **3a-e** responded weakly to titration with Ni^{2+} with a 25 to 35% quenching after 20 to 30 equiv of metal were added.

For each 1,3,4-thiadiazolyl asparagine derivative, the variation in fluorescence intensity was plotted against the concentration of the metal cation, which gave a linear correlation (until it reached a plateau). Stern-Volmer plots for the titration of Cu^{2+} with asparagine derivatives **3a-e** indicated that the linear dependence of the fluorescence quenching and the metal concentration is of dynamic nature (Figure 3). The spectrofluorimetric titration results indicated that all the heterocyclic asparagine derivatives were sensitive to Cu^{2+}, whereas the sensing of Zn^{2+}, Co^{2+} and Ni^{2+} was non selective with lower sensitivity, especially for nitro

asparagine derivative **3d**. From these plots, and with regard to the sensing of Cu^{2+}, it appeared that the presence of an extra nitrogen atom at the pyridyl ring at position 5 of the thiadiazole (asparagine derivative **3e**) had no additional positive effect on metal complexation, when compared to asparagine derivative **3a** (bearing a phenyl ring). A similar conclusion could be drawn for asparagine derivatives **3a-d**, with comparable results, which can also indicate that the type of substituent (electron donor or acceptor) present at the phenyl ring did not influence significantly the coordination process. Nevertheless, considering the photophysical properties in acetonitrile of asparagine derivatives **3a-d** (presented in Table 1), fluoro asparagine derivative **3c** would be the more interesting candidate as chemosensor due to the higher fluorescence quantum yield, which is important for maximization of response to analyte in the analysis of very dilute samples.

The comparative fluorimetric response of asparagine derivatives **3a-e** to Cu^{2+}, Zn^{2+}, Co^{2+} and Ni^{2+} is summarised in Figure 4, considering the variation of the fluorescence intensity (I/I_0) upon the addition of 100 equiv of each metal (the shorter the bar, the higher the change in fluorescence and higher sensitivity).

< Figure 3>

< Figure 4>

The binding stoichiometry of asparagine derivatives **3a-e**, **4** and **5** with Cu^{2+} were determined from Job's plots and the binding affinity was calculated from a Benesi–Hildebrand plot by using an equation reported elsewhere (Azab et al. 2010). The results suggest the formation of a ligand-metal complex with 1:2 stoichiometry and the association constants (K_a) were calculated (Table 2). Also, the detection limit (DL) was calculated taking into account the fluorimetric titrations in the presence of metal cations and the standard deviation of a set of ten fluorescence measurements of a blank asparagine derivatives solution according to a previously reported expression (Miller 1993). The results presented in Table 2 indicate that there was a strong interaction between 1,3,4-thiadiazolyl asparagine derivatives **3-5** and Cu^{2+}, with low detection limits which constitute an important feature considering their potential application as chemosensors for transition metals with biological, environmental and analytical relevance.

< Table 2>

With the aim of further elucidating the metal binding mode and to complement the findings of the spectrofluorimetric titrations, ^{1}H NMR titrations of fluoro asparagine derivative **3c** (as representative of asparagines **3a-e**) with Cu^{2+} were carried out in $CDCl_3$ at 25 ºC. It was found that after addition of increasing amounts of metal cation, there was a displacement to lower chemical shift of several signals suggesting that the metallic cation is coordinated by the thiadiazole heteroatoms at the side chain and the oxygens at the amide and ester carbonyl groups. Upon the addition of 10 equiv of Cu^{2+}, a change was visible for the signals corresponding to the protons of the phenyl group attached to the thiadiazole and the α-CH and β-CH_2 and a very slight shift of the benzylic CH_2 group; after the addition of 60 equiv of Cu^{2+}, along with a more pronounced shift of the previous signals, it was also seen the dislocation of the benzylic phenyl group signal and in the presence of 160 equiv of Cu^{2+}, the signals of the previously mentioned protons were further displaced. The chemical shifts of the Boc and the urethane NH were not affected by the presence of the metal cation, thus indicating that this part of the amino acid derivative did not participate in the coordination process (Figure 5). These observations are in agreement with the data collected from the spectrofluorimetric titrations of asparagine derivatives **3c**, **4** and **5** with Cu^{2+} in acetonitrile.

< Figure 5>

Conclusions

A family of novel 1,3,4-thiadiazolyl asparagine derivatives **3a-e**, **4** and **5** were synthesised and evaluated as fluorescent chemosensors based on an amino acid core for a series of transition metal cations, namely Cu^{2+}, Zn^{2+}, Co^{2+} and Ni^{2+}. From the spectrofluorimetric titrations in acetonitrile, it was found that asparagine derivatives **3-5** were suitable chemosensors for Cu^{2+}, showing higher sensitivity for this cation when compared to Zn^{2+}, Co^{2+} and Ni^{2+}, as a pronounced fluorescence quenching was achieved with the addition of a low number of metal equivalents. The results suggested that there was a strong interaction with Cu^{2+} through the donor heteroatoms at the side chain of the various asparagine derivatives, aided by the carbonyl oxygen at the amino acid C-terminal (both in ester and carboxylic acid forms). Considering the electronic nature of nitrogen, one could foresee a similar behaviour for C-terminal amide derivatives. Bearing in mind the photophysical properties and the chemosensing ability, the novel highly emissive 1,3,4-thiadiazolyl asparagine derivatives **3a-e**, **4** and **5** can be considered very promising candidates as amino

acid based fluorescent probes for chemosensing applications within a peptidic framework (where the *N*-and *C*-terminals will be blocked with amide links).

Acknowledgements

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CAPTIONS

Scheme 1. Synthesis of 1,3,4-thiadiazolyl asparagine derivatives **3-5**. Reagents and conditions: (*i*) 1,4-dioxane, aq. NaOH 6M, rt; (*ii*) trifluoroacetic acid/dichloromethane (1:1), rt.

Table 1. Yields, UV-visible absorption and fluorescence data for 1,3,4-thiadiazolyl asparagine derivatives **3-5** in acetonitrile.

Table 2. Association constants (K_a) and detection limits (DL) for the interaction of asparagine derivatives **3-5** with Cu^{2+} in acetonitrile.

Figure 1. Fluorimetric titrations of asparagine derivative **3c** with Cu^{2+} (A), Zn^{2+} (B), Co^{2+} (C) and Ni^{2+} (D) in acetonitrile [λ_{exc} **3c** = 289 nm]. Inset: normalised emission at 361 nm as a function of added metal equivalents.

Figure 2. Fluorimetric titrations of asparagine derivatives **3a** (A), **3b** (B), **3d** (C), **3e** (D), **4** (E) and **5** (F) with Cu^{2+} in acetonitrile [λ_{exc} **3a** = 290 nm, λ_{exc} **3b** = 291 nm, λ_{exc} **3d** = 324 nm, λ_{exc} **3e** = 288 nm, λ_{exc} **4** = 290 nm, λ_{exc} **5** = 289 nm]. Inset: normalised emission at the wavelength of maximum emission as a function of added metal equivalents.

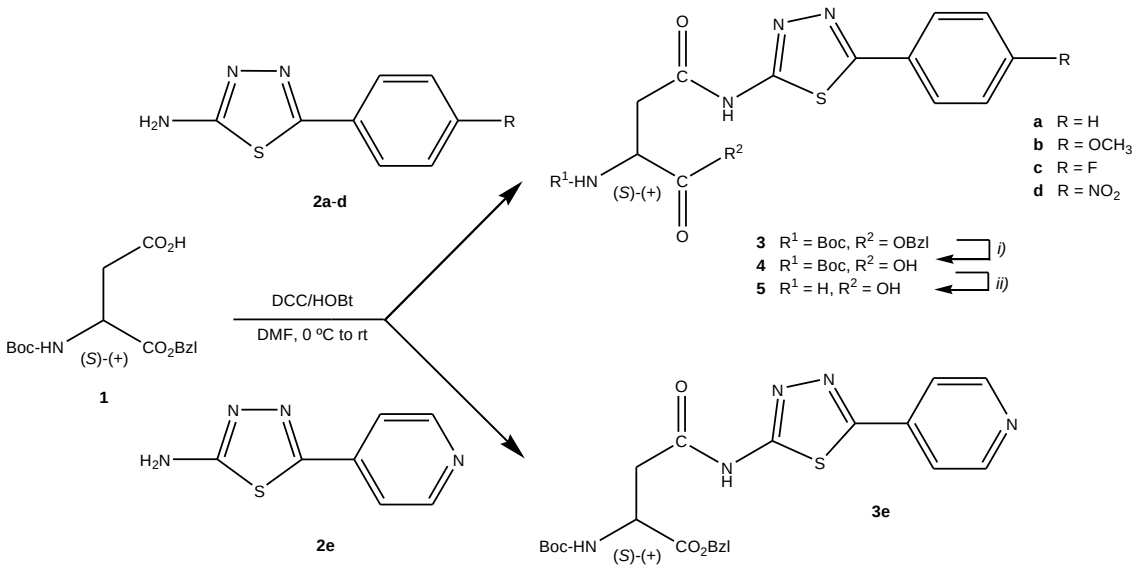
Figure 3. Stern-Volmer plots for the titration of Cu^{2+} with asparagine derivatives **3** (**a** ◊, **b** □, **c** Δ, **d** ×, **e** ○) in acetonitrile.

Figure 4. Relative fluorimetric response (I/I_0) of asparagine derivatives **3a-e** in the presence of 100 equiv of Cu^{2+}, Zn^{2+}, Co^{2+} and Ni^{2+} as a function of metal concentration in acetonitrile.

Figure 5. ^{1}H NMR titration of asparagine derivative **3c** with Cu^{2+} in $CDCl_3$: a) in the absence of Cu^{2+}; and upon addition of b) 10 equiv, c) 60 equiv and d) 160 equiv of Cu^{2+}.

SCHEMES

Scheme 1



TABLES

Table 1

Cpd.	R	Yield (%)	UV/Vis		Fluorescence		
			λ_{max}	log ε	λ_{em}	Stokes' shift (cm^{-1})	Φ_F
3a	H	53	290	4.25	364	7010	0.27
3b	OCH_3	50	291	4.23	363	6816	0.43
3c	F	66	289	4.33	361	6901	0.71
3d	NO_2	30	324	4.34	390	5223	0.01
3e	---	19	288	4.15	362	7098	0.34
4	F	85	290	4.20	385	8509	0.23
5	F	77	289	4.19	369	7502	0.21

Table 2

Cpd.	K_a (mol^{-1} L)	DL (mol L^{-1})
3a	3.3×10^3	2.0×10^{-6}
3b	2.4×10^3	8.0×10^{-6}
3c	1.4×10^3	1.2×10^{-6}
3d	5.4×10^3	4.4×10^{-5}
3e	6.5×10^3	8.2×10^{-6}
4	1.4×10^5	9.8×10^{-7}
5	4.5×10^4	1.7×10^{-7}

FIGURES

Figure 1

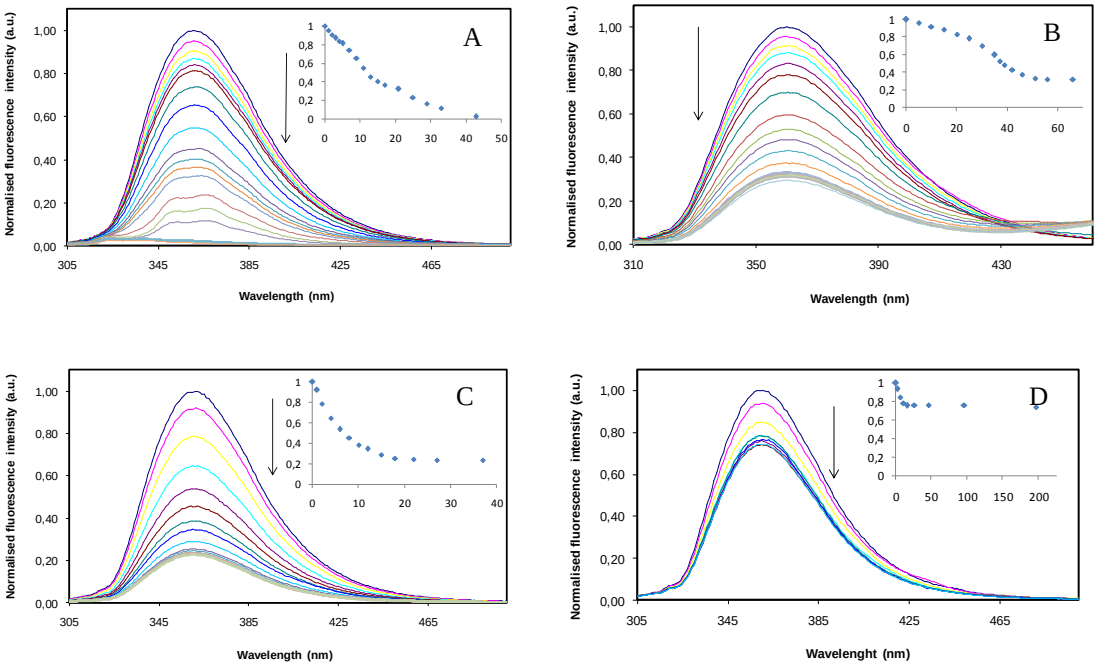


Figure 2

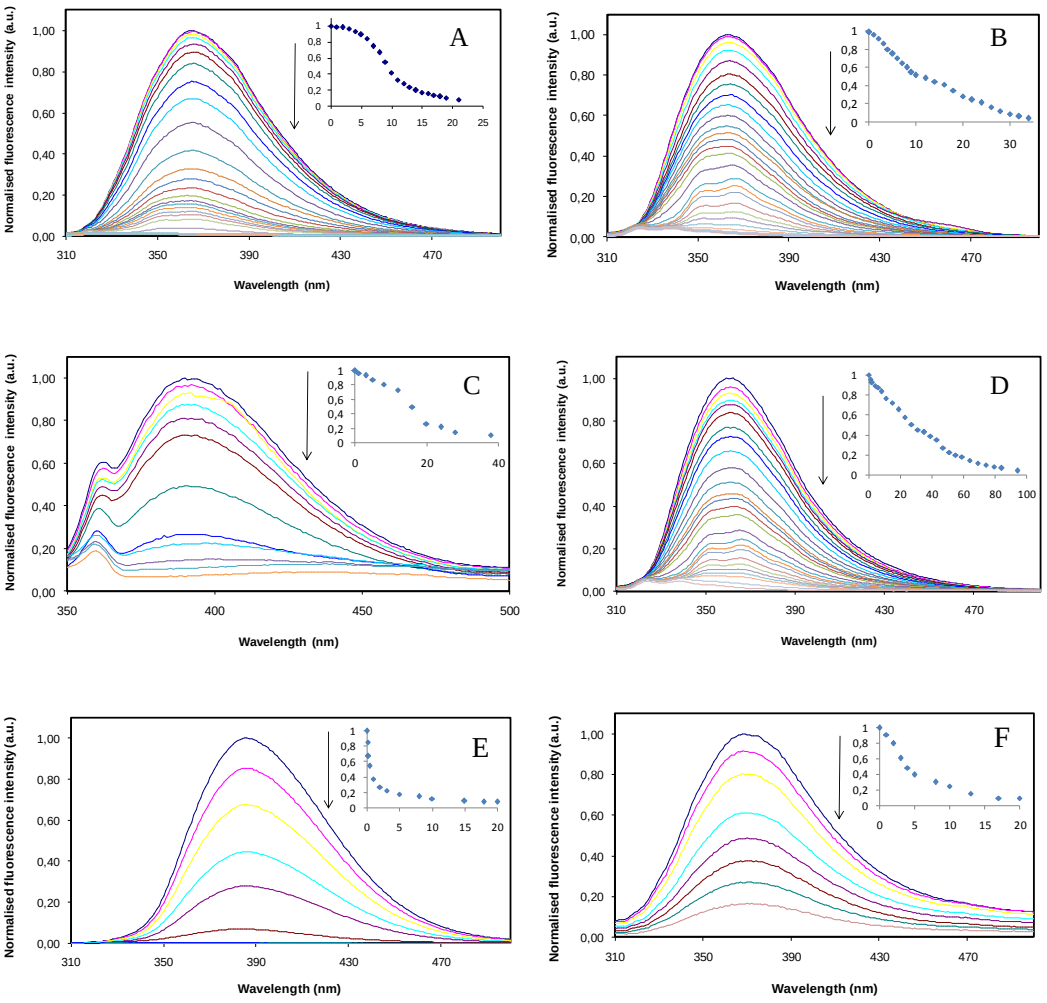

A
B
C
D
E
F
Normalised fluorescence intensity (a.u.)
Wavelength (nm)

Figure 3

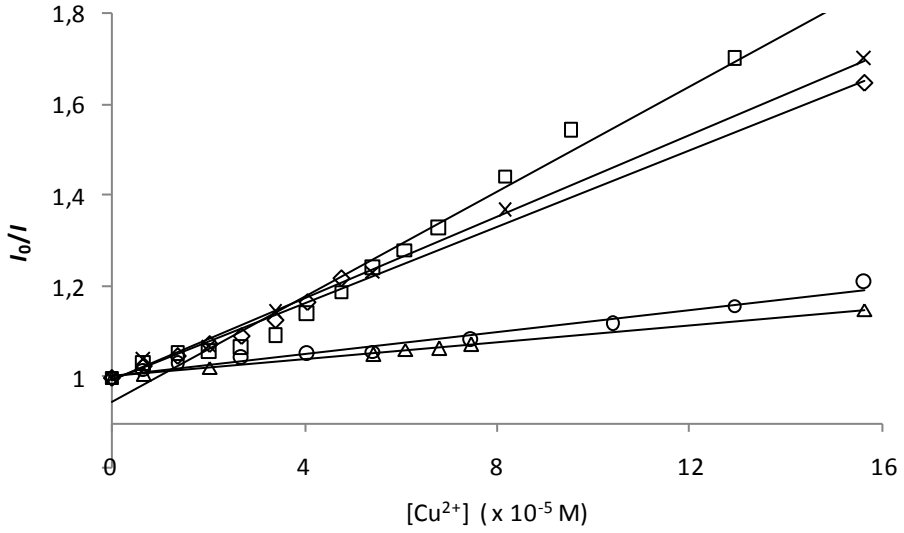


Figure 4

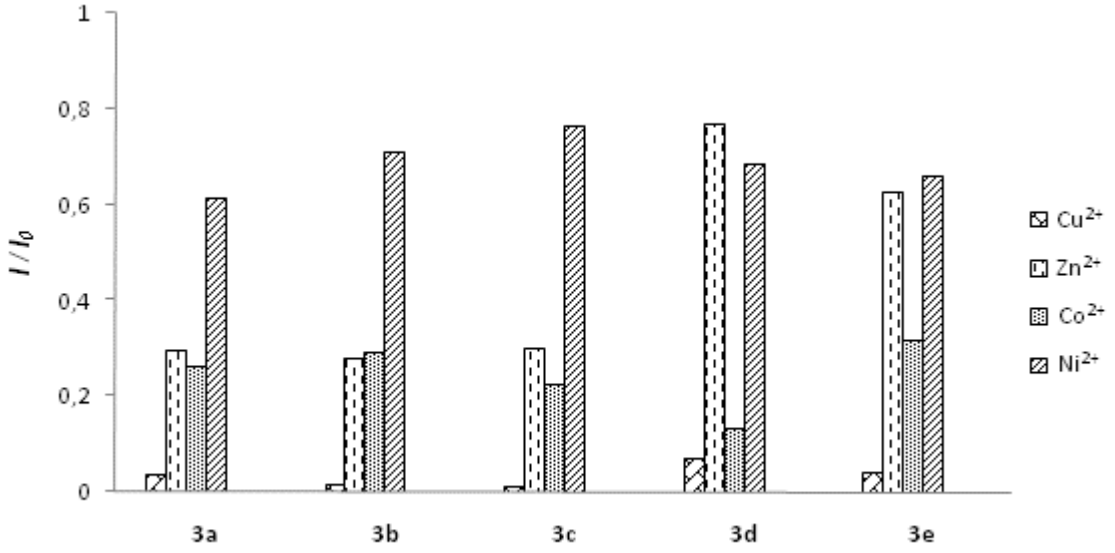
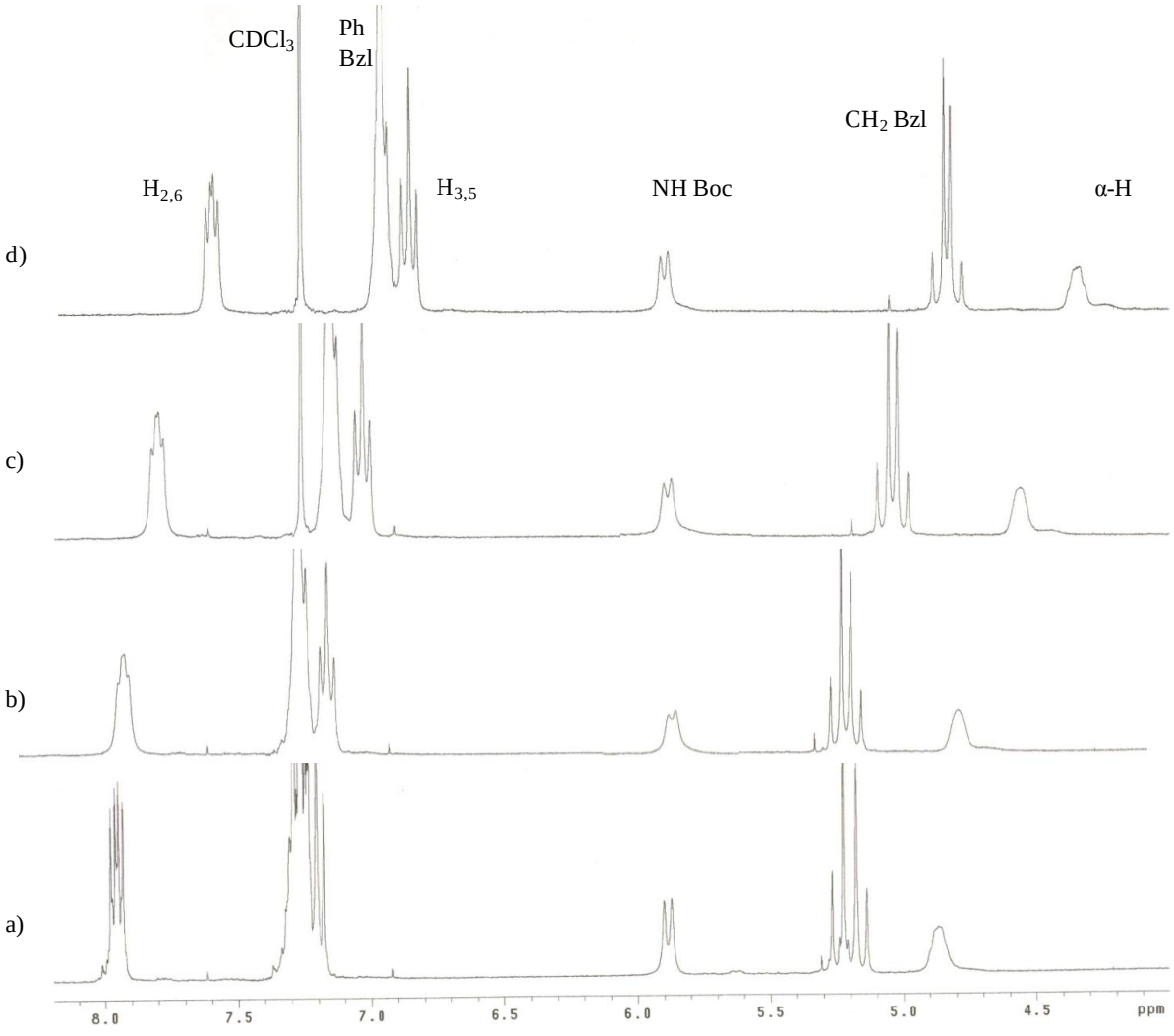


Figure 5


CDCl3
Ph
Bzl
CH2 Bzl
H2,6
H3,5
NH Boc
α-H
d)
c)
b)
a)
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
ppm