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Synthesis and Characterization of 4,4'-Di(*p*-aminophenylethynyl)-6,6'-*bis*(N,N-*bis*(*t*-butoxycarbonylmethyl)amino methyl)-2,2'-bipyridine

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To obtain a new solid phase time-resolved fluorescence immunoassay bifunctional chelate, a new intermediate compound, 4'-di(*p*-aminophenylethynyl)-6,6'-*bis*[N,N-*bis*(*t*-butoxycarbonylmethyl)amino methyl]-2,2'-bipyridine was synthesized using 4,4'-dinitro-2,2'-bipyridine-6,6'-dimethyl *bis*(trifluoroacetate) as the initial material by four steps *viz.*, hydrolyzation, bromination, esterification and substitution. The structure of the compound was charactered by IR, ^{1}H NMR and HRMS.

Keywords: Solid-phase time-resolved fluorescence immunoassay, Bifunctional chelate, Intermediate.

INTRODUCTION

Time-resolved fluorescence immunoassay (TRFIA) analysis method[1] is a ultramicro analytical techniques *in vitro*, which based on lanthanide chelates as labels. There are two different systems in the field of TRFIA:DELFIA (dissociation enhanced lanthanide fluoroimmunoassay, DELFIA) and DSLFIA (direct solid lanthanide fluoroimmunoassay, DSLFIA)[2]. DELFIA system, although highly sensitivie, detection range have some limitatoins.

Direct solid lanthanide fluoroimmunoassay can be measured directly, from which we can get more biological information. For example, DSLFIA can be used in such as in hybrization, immuno histochemsity, homogeous assays and fluoresence imaging[3]. However, its sensitivity is not high due to the lack of chelating agent which fluorescence intensity is not ideal and other factors, The synthesis of an ideal chelating agent has become a challenging research in the field of bio-chemistry and immunology analysis.

According to the chelating agents on the needs of certain functional groups, a newintermediate compound 4,4'-di(*p*-aminophenylethynyl)-6,6'-*bis*(N,N-*bis*(*t*-butoxycarbonyl-methyl)amino methyl)-2,2'-bipyridine on the basis of the chelating agent having been synthesized[4,5] and a variety of other intermediates[6-8].

EXPERIMENTAL

All the reagents and solvents employed were commercially available and used as received without further purification. 4,4'-Dinitro-2,2'-bipyridine-6,6'-dimethylene *bis* trifluoroacetate (compound **1**) was prepared in accordance with reference[9]. 4,4'-Di(*p*-aminophenylethynyl)-6,6'-*bis*(N,N-*bis*(*t*-butoxy-carbonylmethyl)amino methyl)-2,2'-bipyridine (compound **5**) was prepared in accordance with reference[8].

Elemental analysis were performed with a Vario EL analyzer. ^{1}H NMR spectra were obtained from Unity-400 M NMR spectrometer with $CDCl_3$ as a solvent. Fourier transform infrared spectrometer: Vertex 70; fluorescence spectra: F-7000 analyzer; Fourier transform ion cyclotron resonance mass spectrometer: 7.0T superconducting magnet type.

Caution! Although we did not encounter any problems, there are potentially explosive in organic synthesis process. They should be used in small quantities and handled with extreme care. Synthetic routes of 4,4'-di(*p*-aminophenylethynyl)-6,6'-*bis*(N,N-*bis*(*t*-butoxycarbonylmethyl)amino methyl)-2,2'-bipyridine is shown in Fig. 1.

Preparation of compound 2: Compound **1** (2 g) was dissolved in 40.8 mL THF and 40.8 mL deionized water in a flask. The reaction mixture was stirred for 5 min at room temperature, then added $NaHCO_3$ (1.14 g, 0.136 mmol) and reacted for 20 h. After completion of the reaction, the solution was filtrated. The filtrate was precipitated with water and filtrated again, the precipitate got through the twice filtration mentioned above was dried to obtain a pale yellow solid of compounds **2**. Yield: 1.19 g, (97 %). m.p. 241.58 ºC. Elemental analysis (%) calcd for compound **2**: C, 47.06, H, 3.268, N, 18.30; found: C, 46.47, H, 2.966, N, 17.55. IR (KBr, ν_{max}, cm^{-1}):

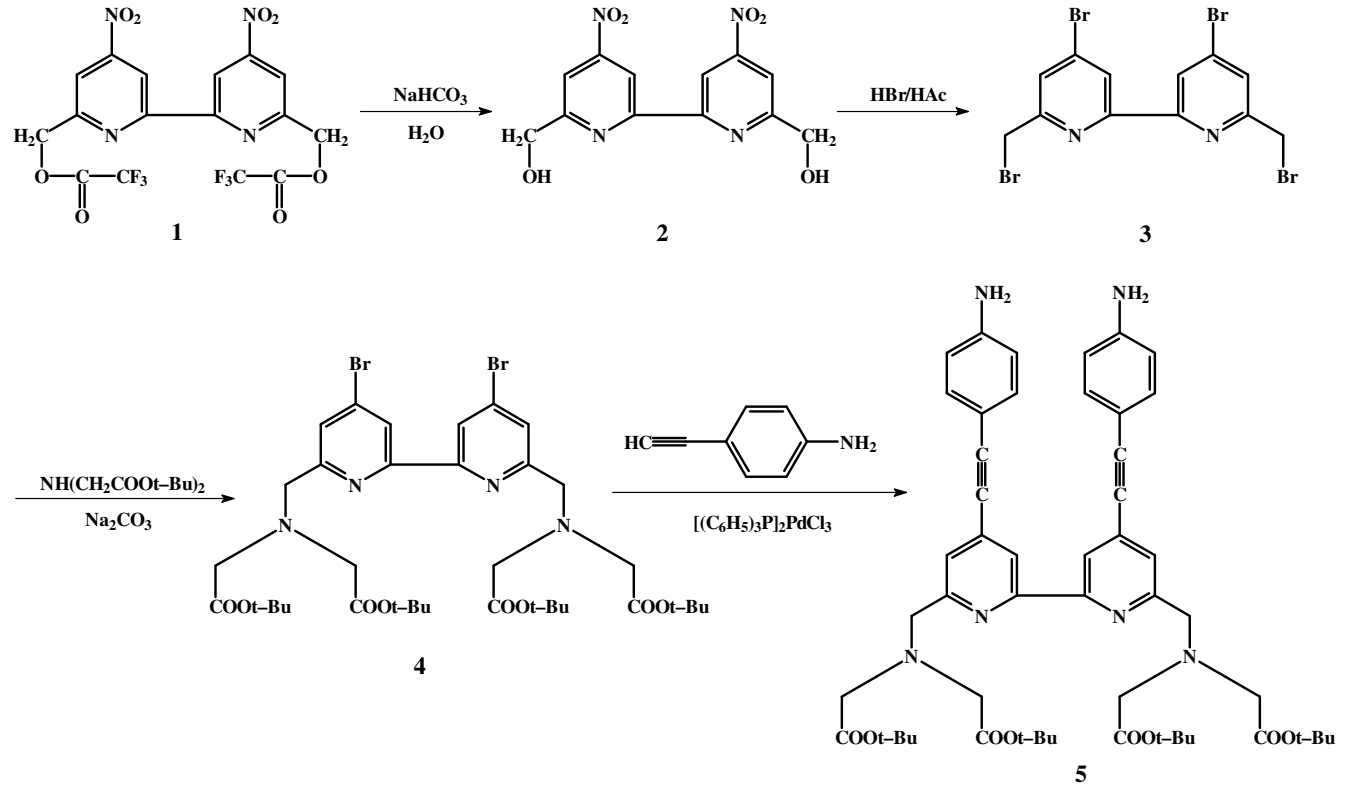

Fig. 1. Synthetic route of target compound

3183 (OH), 1533 and 1355 (NO_2), 3069(C-H). ^{1}H NMR ($CDCl_3$): 4.85 (d, 4H); 5.95 (t, 2H); 8.23 (d, 2H); 8.82 (d, 2H).

Preparation of compound 3: Compound **3** was synthesized by the condensation of the compound **2** in solution of hydrobromic acid and CH_3CH_2OH in a 33 % (w/w) molar ratio under stirring at 101 ºC for 5 h, then the mixture was poured on crushed ice and the precipitated solid complex was separated from the solution by filtration, purified by washing several times with deionized water until pH = 7, then dried to obtain 1.39 g solid powder. The solid powder was washed with ethyl ether, then purified through column chromatography (dichloromethane: *n*-hexane = 5:4, silica gel particle size 50-75 μm) and tracked the received liquid by TLC, after collecting and drying, 1.14 g solid was obtained. Yield: 1.14 g, (47 %). m.p. 220.5 ºC. Elemental analysis (%) calcd. for compound **3**: C, 28.84, H, 1.163, N, 5.16, Br, 63.95; found: C, 28.70, H, 2.087, N, 5.57, Br, 63.48. IR (KBr, ν_{max}, cm^{-1}):1556 (C=N). ^{1}H NMR ($CDCl_3$): 4.56 (d, 4H); 8.54(d, 2H); 7.66 (d, 2H).

Preparation of compound 4: 1.5 g compound **3**, 1.6 g of anhydrous sodium carbonate and 1.6 g iminodiacetic acid di-*tert*-butyl ester were successively added to a three-necked flask, then 150 mL anhydrous acetonitrile was added under stirring. The mixture was refluxed for 24 h at 80 ºC. After completion of the reaction, filtrated the solution and concentrated the filtrate to get a pale yellow solid. Then it was recrystallizated with *n*-hexane, obtain 1.86 g white solid, yield 75 %, m.p. 132.67 ºC, elemental analysis (%) calcd. for compound **4**: C, 52.17, H, 6.28, N, 6.76, O, 15.46, Br, 19.32. Found: C, 52.15, H, 6.14, N, 6.82. O, 15.54, Br, 19.34. IR (KBr, ν_{max}, cm^{-1}): 1550 (C=N), 1750 (C=O), 1146 (CH_3). ^{1}H NMR ($CDCl_3$): 1.47(s, 36H); 3.50(s, 8H); 4.17 (s, 4H); 7.88 (d, 2H);8.46 (d, 2H).

Preparation of compound 5: 1.5 g compound **4**, 0.050 g *bis*(triphenylphosphine) dichloride palladium(II) and 0.027 g cuprous iodide were successively added to a flask, then tetrahydrofuran (45 mL), triethylamine (45 mL) and *p*-aminophenyl acetylene (0.47 g) were added under nitrogen atmosphere. The mixture was stirred for 24 h in the temperature range of 50-52 ºC. After completion of the reaction, the solution was cooled, filtered. Then the filtrate which was concentrated was dissolved in chloroform (86 mL) and dried with anhydrous sodium sulfate after washing. 0.90 g compound **5** was obtained through column chromatography (petroleum ether: ethyl acetate = 1:1, silica gel particle size 50-75 μm), yield 55 %, decomposition temperature: 221.62 ºC, elemental analysis (%) calcd. for compound **5**: C, 69.33, H, 7.11, N, 9.33; found: C, 69.31, H, 7.14, N, 9.28. IR (KBr, ν_{max}, cm^{-1}): 3374 (N-H), 1729 (C=O), 2200 (Ca≡C). ^{1}H NMR ($CDCl_3$): 8.36(s, 2H); 7.69(s, 2H); 7.36 (d, 2H); 6.65 (d, 2H); 4.13 (s, 4H); 3.88 (s, 4H); 3.54 (s, 8H); 1.48 (s, 36H); EI-MS: 901.54837 (M + H), 923.53365 (M + Na), 939.50632 (M + K).

RESULTS AND DISCUSSION

Design of intermediate molecule of the bifunctional chelator: Bipyridine and some derivatives of the macrocyclic compounds in combination with some rare earth ions can form a stable chelate cryptand[10], where the rare earth ions can not be easily substituted, also with high fluorescence stability[11] and the low influence of solvent. Therefore, 4,4'-di(*p*-aminophenylethynyl)-6,6'-*bis*(N,N-*bis*(*t*-butoxycarbonyl-methyl)-amino methyl)-2,2'-bipyridine was synthesized as the intermediate. Isothiocyano groups as the activated moieties for coupling to protein could be introduced through isothiocyanation of the amino- phenylethynyl.

Comparison of synthetic routes and methods: The yield of complex **4** was increased to 75 % by the reaction of compound **3** and iminodiacetic acid di-*t*-butyl ester instead of iminodiacetic acid diethyl ester. The yield of compound **5** was increased to 55 %. The complex **4** was purified through the processing step of recrystallization instead of column chromatography, which not only simplified the process of purification but also avoided the use of methanol which has higher toxicity.

Structure and features of 4,4'-di(*p*-aminophenylethynyl)-6,6'-*bis*(N,N-*bis*(*t*-butoxycarbonylmethyl)amino methyl)-2,2'-bipyridine: Due to the super conjugation effect of tertiary butyl carbon cations, the hydrolysis reaction rate and yield of complex **5** are all higher than 4,4'-di(*p*-aminophenylethynyl)-6,6'-*bis*(N,N-*bis*(ethoxycarbonylmethyl)amino methyl)-2,2'-bipyridine[7].

Fluorescence characteristics of compound 5: 4,4'-Di(*p*-aminophenylethynyl)-6,6'-*bis*(N,N-*bis*(carboxymethyl)amino methyl)-2,2'-bipyridine, generated by hydrolysis of compound **5**, could chelate with europium ions to form a stable rare earth eight coordination compound, which inhibit fluorescence quenching of rare earth ions[12]. Under UV excitation, the compound emits the characteristic fluorescence of europium ions from the energy level transition of 5D0-7F1 and 5D0-7F2, the main emission wavelength is 596 and 620 nm (Fig. 2). Fluorescence lifetime is longer than 710 μs. The strongest fluorescence peak can be used for fluorescence analysis of protein.

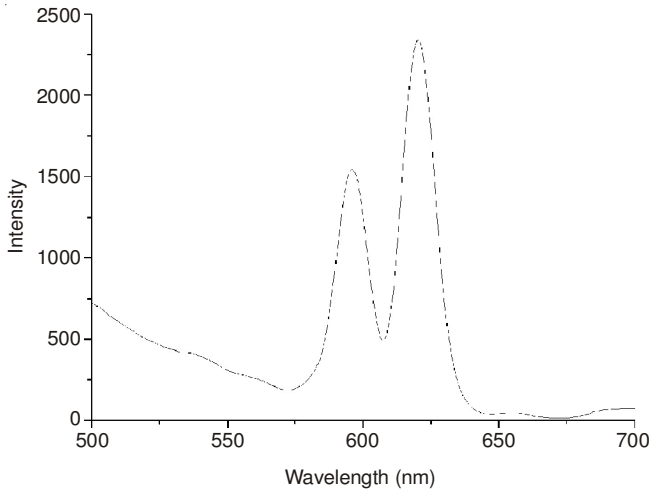

Fig. 2. Fluorescence spectrum of 4,4'-di(*p*-aminophenylethynyl)-6,6'-*bis*(N,N-*bis*(carboxymethyl)amino methyl)-2,2'-bipyridine-Eu^{3+}

Conclusion

Synthesis of the present compound will be conducive to the improvement of the sensitivity of DSLFIA. So this experiment provides an important intermediate compound reference for the final synthesis of solid-phase time-resolved fluorescence immunoassay chelate.

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