SYNTHESIS OF NOVEL COUMARIN BASED FLUORESCENT PROBES

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Abstract:

We report on synthesis of new fluorescent probes suitable for site specific incorporation into oligonucleotides. Coumarin derivatives (1-5) were used as sensitive fluorescent labels and two linkers (6-7) were attached to the basic coumarin skeleton for further applications. Spectral characteristic of functionalized coumarin derivatives (8-10, 13-14) were measured and derivative with the best fluorescent properties was chosen. Phosphoramidite derivative 17 was prepared from functionalized coumarin derivative 9.

Keywords: coumarin, labeling, phosphoramidite, oligonucleotide

Oligodeoxynucleotides bearing reporter groups, e.g. fluorescent labels, are useful tools in molecular biology, medicine and diagnostics. Various nucleosidic as well as non-nucleosidic fluorescent labeled phosphoramidites have been successfully incorporated into oligomers into any predetermined position of a nucleic acid chain.

We report on synthesis of new fluorescent probes suitable for site specific incorporation into oligonucleotides. Coumarins are widely used as fluorescent labels for molecular studies of nucleic acids and proteins. For high quantum yields we used coumarin derivatives 1-5 as sensitive labels (Figure 1). 7-hydroxy coumarin derivatives 4-5 are commercially available as fluorescent labels and coumarin-4-acetic acids 1-3 were synthetised in our laboratory via Pechman reaction in the scope of new coumarins studies.

For further applications the functionalization of basic coumarin skeleton was neccessary, two linkers were attached to coumarin derivatives (Figure 1). 3-(3-Aminopropyloxy)propane-1,2-diol unit 6 we prepared via published procedure2b and 3-azidopropane-1,2-diol unit 7 we prepared via method common for azidonucleoside synthesis5.
We attached 3-(3-aminopropyl oxy)propane-1,2-diol unit 6 on coumarin-4-acetic acids 1-3 using method common for peptide synthesis\(^6\) (Scheme 1). Reaction of coumarin-4-acetic acid with N-hydroxysuccinimide (NHS) in the presence of dicyclohexylcarbodiimide (DCC) gave NHS ester, which was coupled with linker moiety to give protected product in yield 57-75% after chromatography. The yields were lowered by lengthy separation of products from contaminating dicyclohexylurea (DCU). Hydrolysis of isopropylidine group with Dowex WX 8 (H\(^+\)) gave desired products 8-10 in yield 93-96%.

Copper(I) catalysed Huisgen 1,3-dipolar cycloaddition was applied for linking blocked 3-azidopropyl-1,2-diol unit (7) to the coumarin derivatives with terminal alkyne functionality 11-12, which we prepared by reaction of coumarin 4-5 with propargyl bromide\(^7\) (Scheme 2). Huisgen 1,3-dipolar cycloaddition is one of ideal chemoselective reactions, when two unsaturated reactants fuse together in mild conditions, generating no byproduct.\(^8\)-\(^10\) Reaction of azides with terminal alkynes is regioselective only in the presence of copper(I) ions. Number of copper(I) sources can be used,\(^8\)\(^b\),\(^9\)\(^b\) most often Cu(I) prepared by in situ reduction of CuSO\(_4\).5H\(_2\)O. As a catalyst we used 0.15 molar equivalent of CuSO\(_4\).5H\(_2\)O and 0.30 molar equivalent of sodium ascorbate in aqueous tert-BuOH and 1,4-substituted triazole bridged products were obtained. The conversion of all reactions was almost quantitative and minor byproduct 7-hydroxycoumarin 4 and 7-hydroxy-4-methylcoumarin 5 was formed along with desired products. Isolated yields of protected products after chromatography were in the range 88-90%. Hydrolysis of isopropylidine group with Dowex WX 8 (H\(^+\)) gave desired products in yield 93% for derivative 13 and 96% for derivative 14.
Spectral characteristic of isolated products are summarized in Table 1. We found that all newly prepared conjugates display only one peak in the fluorescence spectrum in methanol. The process of conjugation did not cause the shift of absorption and fluorescence maxima of our compounds in comparison with maxima of 1-5. The fluorescence intensity of conjugates depends on position of coumarin modification. We observed only slight changes of fluorescence intensity in the serie of coumarin-4-acetic acid derivatives (8-10). On the contrary the coumarin C7-hydroxy group modification (13-14) caused decreasing of intensity in comparison with intensity of corresponding unmodified coumarins 4-5.

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Table 1: Total yields and spectral characteristics of fluorescent probes 8-10, 13-14

We chose derivative with the best fluorescent properties for preparation of phospohoramidite 17. Primary hydroxyl group of derivative 9 was protected by reaction with dimethoxytrityl chloride. Subsequent phosphitylation of protected derivative 9 was carried with (2-cyanoethyl)-N,N'-diisopropyl chlorophosphite in tetrahydrofuran in the presence of diisopropylethylamine (DIPEA, Scheme 3). Under these conditions, the predominant product was the desired phosphitylated product 17 and isolated yield after chromatography was 49%.

![Scheme 3](image)

**Scheme 3:** (i) 1.3 mol. equiv. DMTCl, dry pyridine; (ii) 1.77 mol. equiv. (2-cyanoethyl)-N,N'-diisopropyl chlorophosphite, 4 mol. equiv. DIPEA, dry THF

**Conclusion**

Novel coumarin based fluorescent probes were prepared by reaction of coumarin-4-acetic acid esters and 7-propargyloxycoumarins with appropriate linker. Products were obtained in good yields and their spectral characteristics were determined. Derivative with the best fluorescent properties was chosen for synthesis of coumarinyl phospohoramidite linker suitable for direct incorporation to oligonucleotide. Work is in progress to extend this flexible approach for the preparation of different types of labeled oligonucleotides.
Experimental

General methods:

Solvents were distilled and dried via established methods. Melting points were measured on a Koffler hot stage and are uncorrected. NMR spectra were recorded in CDCl₃ and DMSO with Varian VX UNITY spectrometer (300 MHz/75 MHz for ¹H/¹³C). Chemical shifts are referenced to Me₄Si (¹H) or the residual solvent signal (¹³C). All measurements were run at room temperature. The ¹H and ¹³C assignments were based on ¹H-¹H COSY, ¹³C-¹H HSQC and ¹³C-¹H HMBC experiments. Absorption spectra were recorded using UV-VIS spectrophotometer Agilen 8453, cuvette length 1 cm. Fluorescence spectra were recorded using Hitachi F-2000. TLC were performed on precoated plates of silica gel 60 F₂₅₄ (Merck) using the chloroform/methanol (9/1) as eluent. The crude products were purified using column chromatography on silica gel using the chloroform/methanol (9/1) as eluent. 7-Hydroxy-2H-chromen-2-one (7-hydroxycoumarin) and 7-hydroxy-4-methyl-2H-chromen-2-one (7-hydroxy-4-methylcoumarin) were obtained from Aldrich, 2-(2-oxo-2H-chromen-4-yl)acetic acids were prepared as described in the literature.

General Procedure A:

To a solution of 2-(2-oxo-2H-chromen-4-yl)acetic acid and NHS (1.0 molar equiv.) in dry dioxane, DCC (2.0 molar equiv.) in dry dioxane was added. The resultant mixture was stirred at room temperature for 4 hours. Then linker 6 (1.0 molar equiv.) was added and the mixture was stirred at room temperature for 5 h. The DCU byproduct was filtered off, the solvent was removed under reduced pressure and the crude product was purified by column chromatography. Protected products were diluted in aqueous methanol and Dowex WX 8 (H⁺) was added. Reaction mixture was stirred at room temperature until starting material consumed, as judged by TLC. Dowex WX 8 (H⁺) was filtered off, the solvent was removed under reduced pressure and the crude product was purified by crystallisation from ethyl acetate – hexane mixture.

N-[3-(2,3-dihydroxypropoxy)propyl]-2-(7-hydroxy-2-oxo-2H-chromen-4-yl)acetamide (8)

White solid product (280 mg, 0.8 mmol, 93%), m.p. = 123-130°C, ¹H NMR (DMSO): 10.59 (1H, s, HO-7), 8.18 (1H, m, NH-CO), 7.60 (1H, d, J₅,₆= 8.8 Hz, H-5), 6.79 (1H, dd, J₅,₆= 8.8 Hz, J₆,₈= 2.5 Hz, H-6), 6.72 (1H, d, J₆,₈= 2.5 Hz, H-8), 6.16 (1H, s, H-3), 4.63 (1H, d, JHO,1₅= 4.9 Hz, HO-1₅), 4.49 (1H, t, JHO,1₆a= 4.9 Hz, JHO,1₆b= 4.7 Hz, HO-1₆), 3.63 (2H, s, H-9), 3.55 (1H, m, H-1₅), 3.35-3.30 (2H, m, H-1₆), 3.30-3.21 (2+1H, m, H-1₃), 3.08-3.19 (2+1H, m, H-1₁, H₡-1₄), 1.62 (2H, m, H-1₂); ¹³C NMR (DMSO): 161.16 (2), 160.26 (7), 155.00 (4), 151.27 (8a) 126.69 (5), 112.88 (6), 111.49 (3), 111.50 (4a), 102.29 (8), 72.34 (14), 70.49 (15), 68.18 (13), 63.12 (16), 39.20 (12).

N-[3-(2,3-dihydroxypropoxy)propyl]-2-(5,7-dimethoxy-2-oxo-2H-chromen-4-yl)acetamide (9)

White solid product (100 mg, 0.3 mmol, 95%), m.p. = 72-75°C, ¹H NMR (DMSO): 7.83 (1H, t, NH-CO), 6.60 (1H, d, H-5), 6.46 (1H, d, H-7), 6.06 (1H, s, H-3), 4.60 (1H, d, JHO,1₅= 4.9 Hz, HO-1₅), 4.48 (t, 1H, JHO,1₆a= 5.8 Hz, JHO,1₆b= 5.6 Hz, HO-1₆), 3.84 (3H, s, CH₃-7), 3.79 (3H, s, CH₃-5), 3.69 (2H, s, H-9), 3.58-3.49 (1H, m, H-1₃), 3.41-3.20 (5H, m, H-1₂), 3.10 (2H, m, H-1₁, H₡-1₄); ¹³C NMR (DMSO): 168.38 (10), 161.16 (2), 156.25 (7), 155.00 (4), 151.27 (8a) 126.69 (5), 112.88 (6), 111.49 (3), 111.50 (4a), 102.29 (8), 72.34 (14), 70.49 (15), 65.81 (13), 63.12 (16), 39.20 (12).

N-[3-(2,3-dihydroxypropoxy)propyl]-2-(3-oxo-3H-benzo[f]chromen-1-yl)acetamide (10)

White solid product (252 mg, 0.7 mmol, 96%), m.p. = 144-148°C, ¹H NMR (DMSO): 8.43 (1H, d, J1₀,₁₀= 8.2 Hz, H-1₀), 8.25 (1H, d, J₉,₁₀= 9.1 Hz, H-9), 8.07 (1H, d, J₈,₇= 7.4 Hz, H-8), 7.68-7.57 (3H, m, H-5, H-6, H-7), 6.58 (1H, s, H-3), 4.60 (1H, d, JHO,1₇= 5.2 Hz, HO-1₇), 4.48 (1H, t, JHO,1₈a= 5.6 Hz, JHO,1₈b= 5.8 Hz, HO-1₈), 4.18 (2H, s, H-1₁, H₡-1₄), 3.58-3.49 (1H, m, H-1₁, H₡-1₄), 3.10 (2H, m, H-1₂); ¹³C NMR (DMSO): 158.21 (5), 156.20 (4), 150.98 (8a), 113.15 (3), 103.67 (4a), 95.28 (8), 93.51 (6), 72.22 (14), 70.35 (15), 63.01 (13), 62.25 (16), 55.81 (CH₃-7), 42.83 (9), 39.20 (12).
3.26-3.18 (4H, m, H-15, H-16), 3.12 (2H, m, H-13), 1.58 (2H, m, H-14); APT (DMSO): 167.92 (12), 159.31 (2), 154.25 (4), 151.73 (10a), 133.86 (9), 130.89 (5a), 129.62 (8), 129.17 (8a), 127.91 (6), 125.54 (7), 124.74 (5), 118.19 (10), 117.57 (3), 113.92 (4a), 72.34 (16), 70.48 (17), 68.16 (15), 63.14 (18), 43.83 (11), 36.14 (13), 29.23 (14).

General Procedure B:
To a solution of coumarin 4-5 in dry acetone, anhydrous potassium carbonate (1.0 molar equiv.) and propargyl bromide (1.0 molar equiv.) were added. The resultant mixture was stirred at 50 °C for 18 h, then the mixture was cooled and the solvent was removed under reduced pressure. The residue was treated with 15 mL of water and extracted with ethyl acetate. The combined organic phases were washed with water, dried over anhydrous sodium sulfate and evaporated in vacuum. The crude product was purified by crystallisation from ethyl acetate-hexane mixture.

7-(Prop-2-yn-1-yloxy)-2H-chromen-2-one (11)
Light yellow solid product (586 mg, 2.93 mmol, 95%), m.p.= 118-120 °C (lit. 119 °C), 1H NMR (DMSO): 8.01 (1H, d, J3-4 = 9.4 Hz, H-4), 7.66 (1H, d, J5-6 = 8.6 Hz, H-5), 7.06 (1H, d, J6-8 = 2.5 Hz, H-8), 7.00 (1H, dd, J5-6 = 8.6 Hz, J6-8 = 2.5 Hz, H-6), 6.33 (1H, d, J3-4 = 9.4 Hz, H-3), 4.94 (2H, d, J9-10 = 2.3 Hz, H-9), 3.67 (1H, J9-10 = 2.3 Hz, H-10); 13C NMR (DMSO): 160.19 (2), 160.18 (7), 155.13 (8a), 144.24 (4), 112.98 (3), 112.86 (4a), 112.83 (6), 101.78 (8), 78.94 (10), 78.52 (11), 56.11 (9).

4-Methyl-7-(prop-2-yn-1-yloxy)-2H-chromen-2-one (12)
Light yellow solid product (565 mg, 2.64 mmol, 93%), m.p.= 130-134 °C (lit. 134 °C), 1H NMR (CDCl3): 7.51 (1H, dd, J4-5 = 7.5 Hz, H-4), 6.93 (1H, d, J5-6 = 2.6 Hz, H-6), 6.91 (1H, dd, J4-5 = 7.5 Hz, J5-6 = 2.6 Hz, H-5), 6.15 (1H, d, J3-Me = 1.2 Hz, H-3), 4.75 (2H, d, J9-10 = 2.3 Hz, H-9), 2.56 (1H, t, J9-10 = 2.5 Hz, H-10), 2.39 (3H, d, J3-Me = 1.1 Hz, CH3-4); 13C NMR (CDCl3): 161.34 (2), 160.36 (7), 155.08 (4), 152.40 (8a), 125.62 (5), 112.75 (4a), 112.47 (3), 102.17 (8), 76.50 (11), 56.17 (9), 18.70 (CH3-4).

General Procedure C:
To a solution of compounds 11-12 in tert-BuOH/H2O 1/1 (v/v) CuSO4.5H2O (0.15 molar equiv.) and sodium ascorbate (0.30 molar equiv.) were added. The mixture was stirred at room temperature for 15 min. Then linker 7 (1.0 molar equiv.) was added and resulting reaction mixture was stirred at room temperature until starting material consumed as judged by TLC. Then the reaction mixture was washed with ethyl acetate, the combined organic phases were washed with water, dried over anhydrous sodium sulfate and evaporated in vacuum. The crude product was purified by column chromatography and crystallized from ethyl acetate-hexane mixture.

7-[[1-(2,3-dihydroxypropyl)-1H-1,2,3-triazol-4-ylmethoxy]-2H-chromen-2-one (13)
White solid product (133 mg, 0.4 mmol, 93%), m.p. = 123-125°C, 1H NMR (DMSO): 8.19 (1H, s, H-10), 8.01 (1H, d, J3,3 = 9.6 Hz, H-4), 7.66 (1H, d, J5,6 = 8.8 Hz, H-5), 7.17 (1H, d, J8,6 = 2.5 Hz, H-8), 7.05 (1H, dd, J6,8 = 2.5 Hz, J6,5 = 8.5 Hz, H-6), 6.32 (1H, d, J3,4 = 9.3 Hz, H-3), 5.26 (2H, s, H-9), 5.15 (d, 1H, JH0,12 = 5.5 Hz, HO-12), 4.86 (1H, t, JH0,13a = 5.8 Hz, JH0,13b = 5.5 Hz, HO-13), 4.49 (1H, m, Hb-13), 3.30-3.42 (2H, m, CH2-11), 4.26 (1H, m, Ha-13), APT NMR:
161.19 (2), 160.29 (7), 155.32 (8a), 144.32 (4), 141.51 (10), 129.52 (5), 125.82 (11), 112.95 (3), 112.67 (6), 112.56 (4a), 101.53 (8), 70.41 (13), 63.27 (9), 61.67 (14), 52.91 (12).

7-[(1-(2,3-dihydroxypropyl)-1H,1,2,3-triazol-4-yl)methoxy]-4-methyl-2H-chromen-2-one (14)
White solid product (130 mg, 0.4 mmol, 96%) m.p. = 108-113°C, ¹H NMR (DMSO): 8.19 (1H, s, H-10), 7.71 (1H, d, J₅₆= 8.7 Hz, H-5), 7.17 (1H, dd, J₆₅= 2.5 Hz, H-8), 7.00 (1H, dd, J₆₅= 8.7 Hz, J₆₈= 2.5 Hz, H-6), 6.22 (1H, s, H-3), 5.26 (2H, s, H-9), 5.12 (1H, m, Hₐ₁₃), 4.85 (1H, m, Hₐ₃), 4.68 (1H, m, Hₐ₁₂), 4.22 (dd, 1H, J₈₇= 8.1 Hz, J₈₉= 13.9 Hz, H₉₈), 3.80 (1H, s, HO), 3.61 (1H, s, OH), 2.40 (3H, s, CH₃); APT (NMR): 161.08 (2), 160.13 (7), 154.66 (8a), 141.53 (10), 126.50 (5), 125.75 (11), 113.32 (3), 112.61 (6), 111.27 (4a), 101.55 (8), 70.38 (13), 63.25 (9), 63.02 (14), 58.86 (12), 18.14 (CH₃).

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References:

