SYNTHESIS OF NOVEL COUMARIN-BASED FLUORESCENT PROBES

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Dedicated to Professor Štefan Toma on the occasion of his 70th birthday.

We report on synthesis of new fluorescent probes suitable for site-specific incorporation into oligonucleotides. Coumarin derivatives were used as sensitive fluorescent labels and were attached to glycerol unit by two types of linkers as potential building blocks for oligonucleotide synthesis. Spectral characteristics of the functionalized coumarin building blocks were measured.

Keywords: Coumarin; Fluorescent labeling; Oligonucleotides; Fluorescent spectroscopy; Click chemistry; Triazoles; Azides.

Oligodeoxynucleotides bearing reporter groups, e.g. fluorescent labels, are useful tools in molecular biology, medicine and diagnostics[1–10]. Various nucleosidic as well as non-nucleosidic[11–22] 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 often used as fluorescent labels for molecular studies of nucleic acids and proteins[23–26]. For high quantum yields we used coumarin derivatives **1**–**5** as sensitive labels. 7-Hydroxycoumarin derivatives **4** and **5** are commercially available as fluorescent labels and coumarin-4-acetic acids **1**–**3** were synthesized in our laboratory by the Pechman reaction in the frame of new coumarin studies.

For further applications, functionalization of basic coumarin skeleton was necessary. Two linkers were attached to coumarin derivatives. 3-(3-Aminopropoxy)propane-1,2-diol (**6**) we prepared via a published procedure[12] and 3-azidopropane-1,2-diol unit (**7**) was prepared via method common in azidonucleoside synthesis[27].

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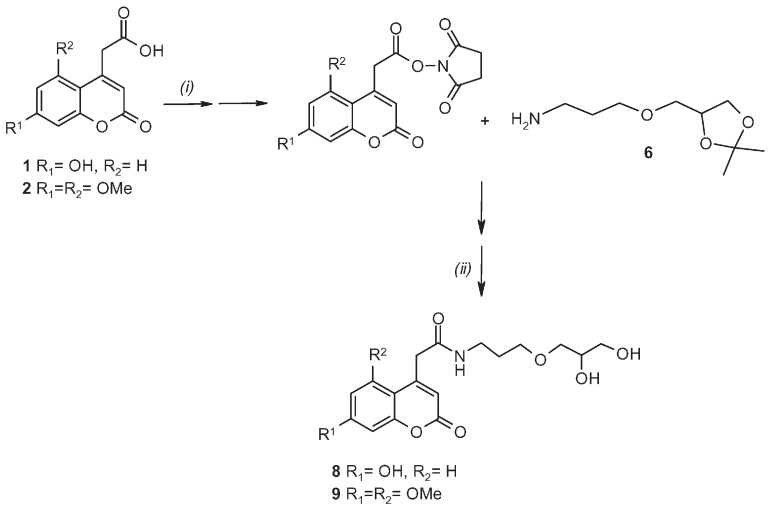
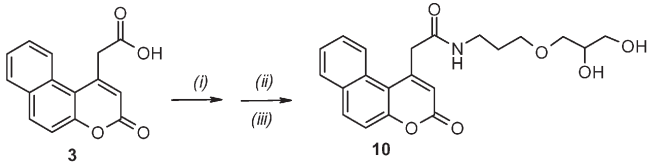
We attached 3-(3-aminopropoxy)propane-1,2-diol unit on coumarin-4-acetic acids **1**–**3** using a method common in peptide synthesis[28] (Schemes 1 and 2). The reaction of coumarin-4-acetic acid with *N*-hydroxysuccinimide (NHS) in the presence of dicyclohexylcarbodiimide (DCC) gave NHS ester, which was coupled with the linker to give protected product in the 57–75% after chromatography. The yields were lowered by lenghty separation of products from contaminating dicyclohexylurea (DCU). Hydrolysis of isopropylidene groups with Dowex WX8 (H^+) gave the desired products **8**–**10** in 93–96% yields.

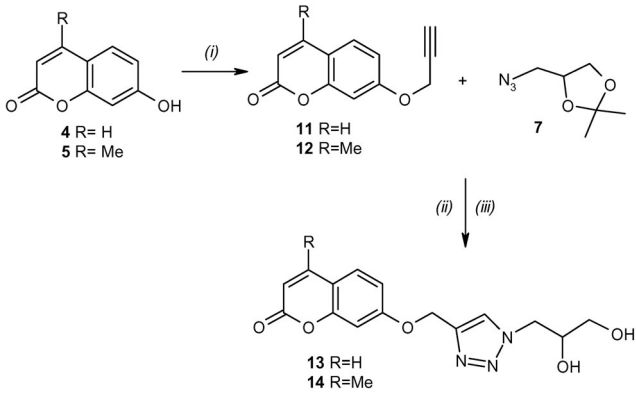


SCHEME 1
(i) 1.0 molar equiv. NHS, 2.0 molar equiv. DCC, dry dioxane; (ii) Dowex WX8 (H^+), aqueous methanol



SCHEME 2
(i) 1.0 molar equiv. NHS, 2.0 molar equiv. DCC, dry dioxane; (ii) 1.0 molar equiv. **6**; (iii) Dowex WX8 (H^+), aqueous methanol

Copper(I)-catalyzed Huisgen 1,3-dipolar cycloaddition was applied to linking protected 3-azidopropane-1,2-diol (**7**) to the coumarin derivatives with terminal alkyne functionality **11** and **12**, which we prepared by the reaction of coumarin **4** and **5** with propargyl bromide[29] (Scheme 3). Huisgen 1,3-dipolar cycloaddition is one of the ideal chemoselective reactions, where two unsaturated reactants fuse together in mild conditions, generating no byproduct[30–39]. The reaction of azides with terminal alkynes is regioselective only in the presence of copper(I) ions. A number of copper(I) sources can be used[32,35], most often Cu(I) prepared by in situ reduction of $CuSO_4 \cdot 5H_2O$. As a catalyst we used 0.15 molar equivalent of $CuSO_4 \cdot 5H_2O$ 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**) were formed along with desired products. The isolated yields of protected products after chromatography were in the range 88–90%. Hydrolysis of isopropylidene group with Dowex WX8 (H^+) gave the desired products in yield 93% for compound **13** and 96% for compound **14**.



SCHEME 3

(i) 1.0 molar equiv. K_2CO_3, 1.0 molar equiv. propargyl bromide, dry acetone; (ii) 0.15 molar equiv. $CuSO_4 \cdot 5H_2O$, 0.30 molar equiv. sodium ascorbate, *tert*-BuOH–H_2O = 1:1 (v/v); (iii) Dowex WX8 (H^+), aqueous methanol

Spectral characteristics of isolated products are summarized in Table I. All newly prepared derivatives display only one peak in the fluorescence spectrum in methanol. The absorption and fluorescence maxima of our compounds are similar to maxima of coumarins **1**–**5**. The fluorescence intensity of the compounds depends on the site of coumarin modification. We ob-

served only slight changes of fluorescence intensity (Fig. 1) and quantum yields in the series of derivatives of coumarin-4-acetic acids (**8**–**10**). On the contrary, the modification of the coumarin C7-hydroxy groups (**13** and **14**) caused decreasing of intensity (Fig. 2) and quantum yields in comparison with that of corresponding unmodified coumarins **4** and **5**.

TABLE I
Total yields and spectral characteristics of fluorescent probes **8**–**10**, **13** and **14**

Compd[a]	Yield, %	λ_{abs}, nm	ε cm^{-1} l mol^{-1}	λ_{ems}, nm	Φ^b
1	–	326	12487	392	0.21
8	73	326	11883	396	0.24
2	–	323	3032	418	0.40
9	75	323	11291	426	0.30
3	–	318/349	7444	417	0.10
10	58	319/350	8195	417	0.10
4	–	325	14509	392	0.08
13	80	320	11301	387	0.02
5	–	322	15552	387	0.15
14	78	319	20338	381	0.05

[a] $c = 10^{-4}$ mol l^{-1} in methanol. [b] Anthracene as a standard (Φ = 0.27 in ethanol), excitation wavelength: 322 nm.

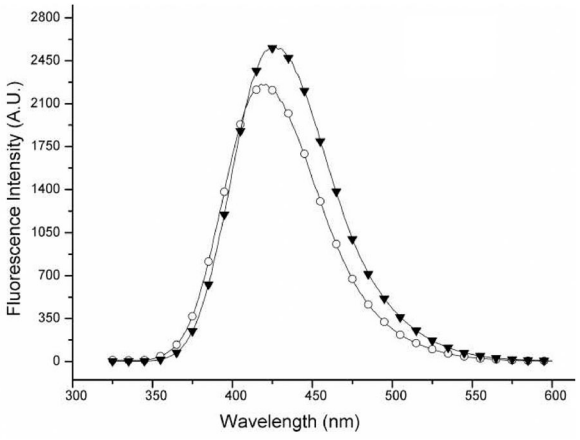


FIG. 1
Fluorescent intensities of 5,7-dimethoxycoumarin-4-acetic acid (**2**) and its derivative **9**, functionalization at carboxyl group causes slight changes in fluorescence; ○ **2**, ▼ **9**

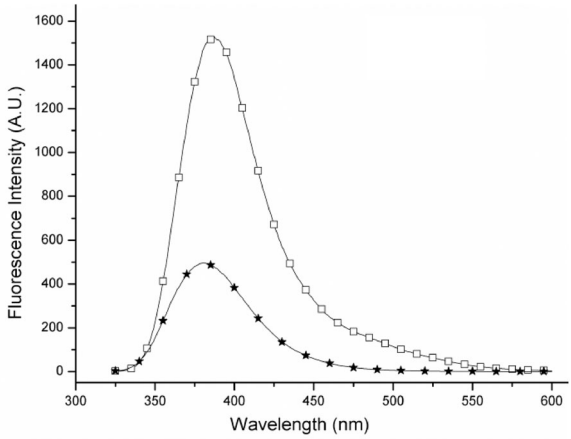


FIG. 2
Fluorescent intensities of 7-hydroxy-4-methylcoumarin (**5**) and its derivative **14**, functionalization at hydroxyl group causes large decreasing of fluorescence; □ **5**, ★ **14**

CONCLUSION

Novel coumarin-based fluorescent probes were prepared by the reaction of coumarin-4-acetic acid esters and 7-(propargyloxy)coumarins with appropriate linker. The products were obtained in good yields and their spectral characteristics were determined. Work is in progress to extend this flexible approach to the preparation of different types of labeled oligonucleotides.

EXPERIMENTAL

Solvents were distilled and dried by the established methods[40]. Melting points were measured on a Koffler hot stage and are uncorrected. NMR spectra were recorded in $CDCl_3$ and DMSO-d_6 with a Varian VX Unity spectrometer (300 MHz for ^{1}H and 75 MHz for ^{13}C). Chemical shifts are referenced to Me_4Si (^{1}H) or to the residual solvent signal (^{13}C) and are given in ppm (δ-scale), coupling constants (*J*) are given in Hz. All measurements were run at room temperature. The ^{1}H and ^{13}C assignments were based on ^{1}H-^{1}H COSY, ^{13}C-^{1}H HSQC and ^{13}C-^{1}H HMBC experiments. Elemental analysis was performed on an analyzer model 1106 (Carlo Erba Strumentacione). Infrared spectra were recorded on a Bruker IFS 55 Equinox FTIR in KBr, wavenumbers are given in cm^{-1}. Absorption spectra were recorded using a 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 F_{254} (Merck) with chloroform–methanol (9:1) as eluent. The crude products were purified by column chromatography on silica gel with chloroform–methanol (9:1) as eluent. 7-Hydroxy-2*H*-chromen-2-one (7-hydroxycoumarin) and 7-hydroxy-4-methyl-2*H*-chromen-2-one (7-hydroxy-4-methylcoumarin) were obtained from Aldrich, 2-(2-oxo-2*H*-chromen-4-yl)acetic acids were prepared as described in the literature[41].

General Procedure *A*

To a solution of 2-(2-oxo-2*H*-chromen-4-yl)acetic acid **1**–**3** 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 h. 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.

The protected products were dissolved in aqueous methanol and Dowex WX8 (H^+) was added. The reaction mixture was stirred at room temperature until starting material was consumed (TLC). Dowex WX8 (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 (yield 280 mg, 0.8 mmol, 93%), R_F 0.1, m.p. 123–130 °C. For $C_{17}H_{21}NO_7$ (351.4) calculated: 58.11% C, 6.02% H, 3.99% N; found: 58.32% C, 6.12% H, 4.06% N. ^{1}H NMR (DMSO-d_6): 10.59 s, 1 H (HO-7); 8.18 m, 1 H (NH-CO); 7.60 d, 1 H, $J_{5,6}$ = 8.8 (H-5); 6.79 dd, 1 H, $J_{5,6}$ = 8.8, $J_{6,8}$ = 2.5 (H-6); 6.72 d, 1 H, $J_{6,8}$ = 2.5 (H-8); 6.16 s, 1 H (H-3); 4.63 d, 1 H, $J_{HO,15}$ = 4.9 (HO-15); 4.49 t, 1 H, $J_{OH,16a}$ = 4.9, $J_{HO,16b}$ = 4.7 (HO-16); 3.63 s, 2 H (H-9); 3.55 m, 1 H (H-15); 3.35–3.30 m, 2 H (H-16); 3.30–3.21 m, 2 + 1 H (H-13, H*a*-14); 3.08–3.19 m, 2 + 1 H (H-11, H*b*-14); 1.62 m, 2 H (H-12). ^{13}C NMR (DMSO-d_6): 167.54 (10), 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), 36.19 (11), 29.20 (12). IR (KBr): 3540, 3331, 3300, 3160, 3112, 1719, 1687, 1663, 1624, 1610, 1569, 1519, 1399, 1122, 1106, 1074, 1089, 1055.

N-[3-(2,3-Dihydroxypropoxy)propyl]-2-(5,7-dimethoxy-2-oxo-2H-chromen-4-yl)acetamide (**9**). White solid product (yield 100 mg, 0.3 mmol, 95%), R_F 0.26, m.p. 72–75 °C. For $C_{19}H_{25}NO_8$ (395.4) calculated: 57.71% C, 6.37% H, 3.54% N; found: 57.93% C, 6.28% H, 3.36% N. ^{1}H NMR (DMSO-d_6): 7.83 t, 1 H (NH-CO); 6.60 d, 1 H (H-5); 6.46 d, 1 H (H-7); 6.06 s, 1 H (H-3); 4.60 d, 1 H, $J_{HO,15}$ = 4.9 (HO-15); 4.48 t, 1 H, $J_{HO,16a}$ = 5.8, $J_{HO,16b}$ = 5.6 (HO-16); 3.84 s, 3 H (CH_3-7); 3.79 s, 3 H (CH_3-5); 3.69 s, 2 H (H-9); 3.58–3.49 m, 1 H (H-15); 3.41–3.20 m, 5 H (H-13, H-14, H-16); 3.10 m, 2 H (H-11); 1.63 m, 2 H (H-12). ^{13}C NMR (DMSO-d_6): 168.38 (10), 162.52 (2), 159.96 (7), 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 (CH_3-7), 42.83 (9), 35.93 (11), 29.35 (12). IR (KBr): 3417, 3290, 1729, 1700, 1645, 1616, 1607, 1560, 1550, 1496, 1386, 1119, 1068, 1043.

N-[3-(2,3-Dihydroxypropoxy)propyl]-2-(3-oxo-3H-benzo[f]chromen-1-yl)acetamide (**10**). White solid product (yield 252 mg, 0.7 mmol, 96%), R_F 0.28, m.p. 144–148 °C. For $C_{21}H_{23}NO_6$ (385.4) calculated: 65.44% C, 6.02% H, 3.63% N; found: 65.22% C, 6.18% H, 3.95% N. ^{1}H NMR (DMSO-d_6): 8.43 d, 1 H, $J_{10,9}$ = 8.2 (H-10); 8.25 d, 1 H, $J_{9,10}$ = 9.1 (H-9); 8.07 d, 1 H, $J_{8,7}$ = 7.4 (H-8); 7.68–7.57 m, 3 H (H-5, H-6, H-7); 6.58 s, 1 H (H-3); 4.60 d, 1 H, $J_{HO,17}$ = 5.2 (HO-17); 4.48 t, 1 H, $J_{HO,18a}$ = 5.6, $J_{HO,18b}$ = 5.8 (HO-18); 4.18 s, 2 H (H-11); 3.58–3.49 m, 1 H (H-17); 3.26–3.18 m, 4 H (H-15, H-16); 3.12 m, 2 H (H-13); 1.58 m, 2 H (H-14). ^{13}C NMR (DMSO-d_6): 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). IR (KBr): 3045, 3289, 1726, 1703, 1645, 1589, 1552, 1521, 1122, 1065, 1045.

General Procedure *B*

To a solution of coumarin **4** or **5** in dry acetone, anhydrous potassium carbonate (1.0 molar equiv.) and propargyl bromide (1.0 molar equiv.) were added. The resulting 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 crystalisation from an ethyl acetate–hexane mixture.

7-(Prop-2-yn-1-yloxy)-2H-chromen-2-one (**11**). Light yellow solid product (yield 586 mg, 2.93 mmol, 95%), m.p. 118–120 °C (lit. 119 °C). ^{1}H NMR (DMSO-d_6): 8.01 d, 1 H, J_{3-4} = 9.4 (H-4); 7.66 d, 1 H, J_{5-6} = 8.6 (H-5); 7.06 d, 1 H, J_{6-8} = 2.5 (H-8); 7.00 dd, 1 H, J_{5-6} = 8.6, J_{6-8} = 2.5 (H-6); 6.33 d, 1 H, J_{3-4} = 9.4 (H-3); 4.94 d, 2 H, J_{9-10} = 2.3 (H-9); 3.67 t, 1 H, J_{9-10} = 2.3 (H-10). ^{13}C NMR (DMSO-d_6): 160.19 (2), 160.18 (7), 155.13 (8a), 144.24 (4), 129.53 (5), 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 (yield 565 mg, 2.64 mmol, 93%), m.p. 130–134 °C (lit. 134 °C). ^{1}H NMR ($CDCl_3$): 7.51 dd, 1 H, J_{4-5} = 7.5 (H-4); 6.93 d, 1 H, J_{5-6} = 2.6 (H-6); 6.91 dd, 1 H, J_{4-5} = 7.5, J_{5-6} = 2.6 (H-5); 6.15 d, 1 H, $J_{3\text{-Me}}$ = 1.2 (H-3); 4.75 d, 2 H, J_{9-10} = 2.3 (H-9); 2.56 t, 1 H, J_{9-10} = 2.5 (H-10); 2.39 d, 3 H, $J_{3\text{-Me}}$ = 1.1 (CH_3-4). ^{13}C NMR ($CDCl_3$): 161.34 (2), 160.36 (7), 155.08 (4), 152.40 (8a), 125.62 (5), 114.29 (4a), 112.75 (6), 112.47 (3), 102.17 (8), 76.50 (11), 56.17 (9), 18.70 (CH_3-4).

General Procedure *C*

To a solution of compounds **11** or **12** in *tert*-BuOH–H_2O 1:1 (v/v) $CuSO_4 \cdot 5H_2O$ (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 the resulting mixture was stirred at room temperature until starting material **11** or **12** was consumed (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 an ethyl acetate–hexane mixture.

The protected products were dissolved in aqueous methanol and Dowex WX8 (H^+) was added. Reaction mixture was stirred at room temperature until the starting material was consumed (TLC). Dowex WX8 (H^+) was filtered off, the solvent was removed under reduced pressure and the crude product was purified by crystallisation from an ethyl acetate–hexane mixture.

7-{[1-(2,3-Dihydroxypropyl)-1H-1,2,3-triazol-4-yl]methoxy}-2H-chromen-2-one (**13**). White solid product (yield 133 mg, 0.4 mmol, 93%), R_F 0.33, m.p. 123–125 °C. For $C_{15}H_{15}N_3O_5$ (317.3) calculated: 56.78% C, 4.76% H, 13.24% N; found: 56.52% C, 4.90% H, 13.01% N. ^{1}H NMR (DMSO-d_6): 8.19 s, 1 H (H-10); 8.01 d, 1 H, $J_{4,3}$ = 9.6 (H-4); 7.66 d, 1 H, $J_{5,6}$ = 8.8 (H-5); 7.17 d, 1 H, $J_{8,6}$ = 2.5 (H-8); 7.05 dd, 1 H, $J_{6,8}$ = 2.5, $J_{6,5}$ = 8.5 (H-6); 6.32 d, 1 H, $J_{3,4}$ = 9.3 (H-3); 5.26 s, 2 H (H-9); 5.15 d, 1 H, $J_{HO,16}$ = 5.5 (HO-16); 4.86 t, 1 H, $J_{HO,17a}$ = 5.8, $J_{HO,17b}$ = 5.5 (HO-17); 4.49 dd, 1 H, $J_{15a,15b}$ = 13.9, $J_{16,15a}$ = 3.4 (H*a*-15); 4.26 dd, 1 H, $J_{15a,16}$ = 8.1, $J_{15a,15b}$ = 13.9 (H*b*-15); 3.82 m, 1 H (H-16); 3.42–3.30 m, 2 H (H-17). ^{13}C NMR (DMSO-d_6): 161.19 (2), 160.29 (7), 155.32 (8a), 144.32 (4), 141.51 (10), 129.52 (5), 125.82 (14), 112.95 (3), 112.67 (6), 112.56 (4a), 101.53 (8), 70.41 (16), 63.27 (9), 61.67 (17), 52.91

(15). IR (KBr): 3540, 3416, 3344, 3287, 1741, 1724, 1708, 1697, 1622, 1560, 1509, 1474, 1399, 1107, 1073.

7-{[1-(2,3-Dihydroxypropyl)-1H-1,2,3-triazol-4-yl]methoxy}-4-methyl-2H-chromen-2-one (**14**). White solid product (yield 130 mg, 0.4 mmol, 96%), R_F 0.33, m.p. 108–113 °C. For $C_{16}H_{17}N_3O_5$ (331.3) calculated: 58.00% C, 5.17% H, 12.68% N; found: 58.14% C, 5.32% H, 12.47% N. ^{1}H NMR (DMSO-d_6): 8.19 s, 1 H (H-14); 7.71 d, 1 H, $J_{5,6}$ = 8.7 (H-5); 7.17 d, 1 H, $J_{6,8}$ = 2.5 (H-8); 7.00 dd, 1 H, $J_{6,5}$ = 8.7, $J_{6,8}$ = 2.5 (H-6); 6.22 s, 1 H (H-3); 5.26 s, 2 H (H-9); 5.16 d, 1 H (HO-16); 4.86 t, 1 H (HO-17); 4.52 dd, 1 H, $J_{15a,15b}$ = 13.9, $J_{16,15a}$ = 3.4 (H*a*-15); 4.25 dd, 1 H, $J_{15a,16}$ = 8.1, $J_{15a,15b}$ = 13.9 (H*b*-15); 3.80 m, 1 H (H-16); 3.61 m, 2 H (H-17); 2.40 s, 3 H (CH_3-4). ^{13}C NMR (DMSO-d_6): 161.08 (2), 160.13 (7), 154.66 (8a), 1153.42 (4), 141.53 (10), 126.50 (5), 125.75 (14), 113.32 (3), 112.61 (6), 111.27 (4a), 101.55 (8), 70.38 (16), 63.25 (9), 63.02 (17), 58.86 (15), 18.14 (CH_3). IR (KBr): 3447, 3415, 1730, 1713, 1688, 1614, 1562, 1511, 1466, 1393, 1098, 1074.

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