10th International Electronic Conference on Synthetic Organic Chemistry (ECSOC-10). 1-30 November 2006. http://www.usc.es/congresos/ecsoc/10/ECSOC10.htm & http://www.mdpi.org/ecsoc-10/

[a027]

SYNTHESIS OF NOVEL COUMARIN BASED FLUORESCENT PROBES

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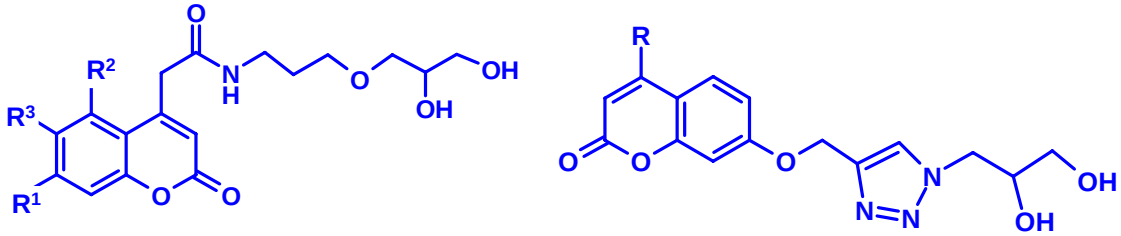
Abstract:



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. Phosporamidite 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.[1] Various nucleosidic as well as non-nucleosidic[2-3] 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.[4] 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 procedure[2b] and 3-azidopropane-1,2-diol unit **7** we prepared via method common for azidonucleoside synthesis.[5]

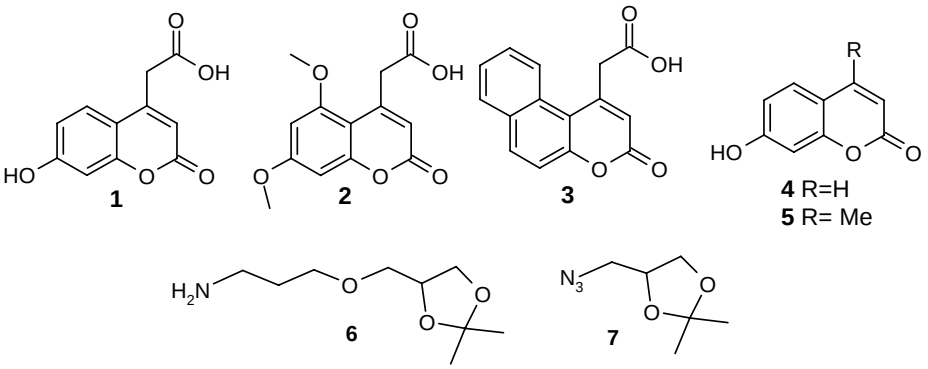
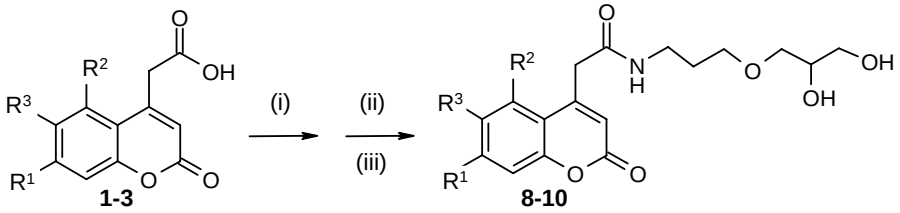


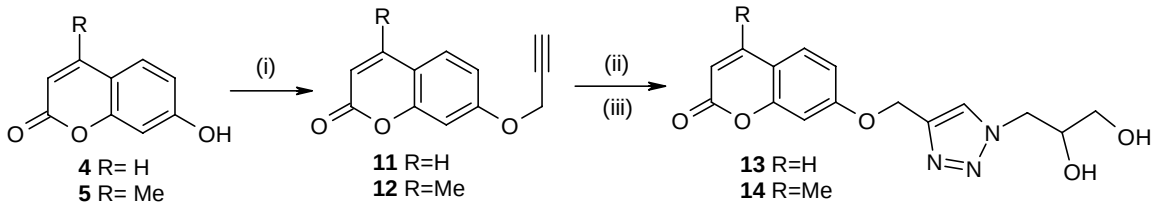
Figure 1: Substrates for synthesis of fluorescent labels

We attached 3-(3-aminopropyloxy)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 lenghty 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%.



Scheme 1: *(i)* 1.0 mol. equiv. NHS, 2.0 mol. equiv. DCC, dry dioxane; *(ii)* 1.0 mol. equiv. **6**; *(iii)* Dowex WX 8 (H^+), aqueous methanol

Copper(I) catalysed Huisgen 1,3-dipolar cycloaddition was applied for linking blocked 3-azidopropane-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,[8b,9b] most often Cu(I) prepared by in situ reduction of $CuSO_4.5H_2O$. As a catalyst we used 0.15 molar equivalent of $CuSO_4.5H_2O$ 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** 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**.



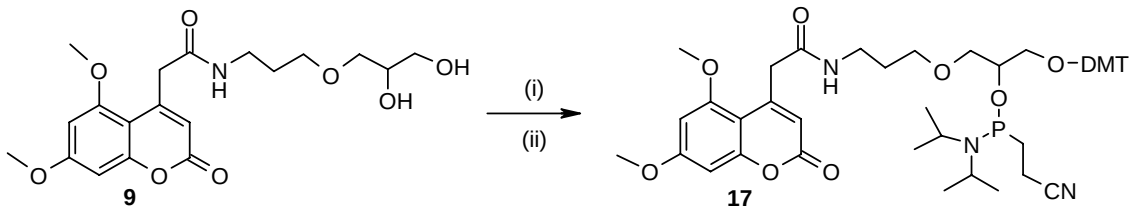
Scheme 2: *(i)* 1.0 molar equiv. K_2CO_3, 1.0 molar equiv. propargylbromide, dry acetone; *(ii)* 0.15 molar equiv. $CuSO_4.5H_2O$, 0.30 molar equiv. sodium ascorbate, *tert*-BuOH/ H_2O = 1/1 (v/v); *(iii)* Dowex WX 8 (H^+), aqueous methanol

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**.

Compd	*%*	λ_{abs} *(nm)*	ε *($cm^{-1}M^{-1}$)*	λ_{ems} *(nm)*	*FI (A.U.)*	Φ
1	-	326	12487	392	1955	0.21
8	73	326	11883	396	2138	0.24
2	-	323	3032	418	2260	**0.47**
9	75	323	11291	426	2567	0.30
3	-	318/349	7444	417	719/930	0.10
10	58	319/350	8195	417	870/975	0.10
4	-	325	14509	392	748	0.08
13	80	320	11301	387	169	0.02
5	-	322	15552	387	1530	0.15
14	78	319	20338	381	496	0.05

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: *(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 distiled and dried *via* established methods.[11] Melting points were measured on a Koffler hot stage and are uncorrected. NMR spectra were recorded in $CDCl_3$ and DMSO with Varian VX UNITY spectrometer (300 MHz/75 MHz for $^1H/^{13}C$). Chemical shifts are referenced to Me_4Si (^1H) or the residual solvent signal (^{13}C). All measurements were run at room temperature. The ^1H and ^{13}C assignments were based on ^1H-^1H COSY, ^{13}C-^1H HSQC and ^{13}C-^1H HMBC experiments. Absorption spectra were recorded using UV-VIS spectrophotometer Agilen 8453, cuvette lenght 1 cm. Fluorescence spectra were recorded using Hitachi F-2000. TLC were performed on precoated plates of silica gel 60 F_{254} (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-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.[12]

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 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 dilluted 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-2*H*-chromen-4-yl)acetamide (8)

White solid product (280 mg, 0.8 mmol, 93%), m.p. = 123-130°C, **^{1}H NMR (DMSO):** 10.59 (1H, s, HO-7), 8.18 (1H, m, NH-CO), 7.60 (1H, d, $J_{5,6}$= 8.8 Hz, H-5), 6.79 (1H, dd, $J_{5,6}$= 8.8 Hz, $J_{6,8}$= 2.5 Hz, H-6), 6.72 (1H, d, $J_{6,8}$= 2.5 Hz, H-8), 6.16 (1H, s, H-3), 4.63 (1H, d, $J_{HO,15}$= 4.9 Hz, HO-15), 4.49 (1H, t, $J_{OH,16a}$= 4.9 Hz, $J_{HO,16b}$= 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), H*a*-14), 3.08-3.19 (2+1H, m, H-11, H*b*-14), 1.62 (2H, m, H-12); **^{13}C NMR (DMSO):** 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).

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

White solid product (100 mg, 0.3 mmol, 95%), m.p.= 72-75°C, **^{1}H 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, $J_{HO,15}$= 4.9 Hz, HO-15), 4.48 (t, 1H, $J_{HO,\ 16a}$= 5.8 Hz, $J_{HO,16b}$= 5.6 Hz, HO-16), 3.84 (3H, s, CH_3-7), 3.79 (3H, s, CH_3-5), 3.69 (2H, s, H-9), 3.58-3.49 (1H, m, H-15), 3.41-3.20 (5H, m, H-13), H-14), H-16), 3.10 (2H, m, H-11), 1.63 (2H, m, H-12); **^{13}C NMR (DMSO):** 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).

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

White solid product (252 mg, 0.7 mmol, 96%), m.p.= 144-148˚C, **^{1}H NMR (DMSO):** 8.43 (1H, d, $J_{10,9}$= 8.2 Hz, H-10), 8.25 (1H, d, $J_{9,10}$= 9.1 Hz, H-9), 8.07 (1H, d, $J_{8,7}$= 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, $J_{HO,17}$= 5.2 Hz, HO-17), 4.48 (1H, t, $J_{HO,18a}$= 5.6 Hz, $J_{HO,18b}$= 5.8 Hz, HO-18), 4.18 (2H, s, H-11), 3.58-3.49 (1H, m, H-17),

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 crystalisation from ethyl acetate-hexane mixture.

7-(Prop-2-yn-1-yloxy)-2*H*-chromen-2-one (11)

Light yellow solid product (586 mg, 2.93 mmol, 95%), m.p.= 118-120 ˚C (lit. 119 ˚C), **^{1}H NMR (DMSO):** 8.01 (1H, d, $J_{3\text{-}4}$= 9.4 Hz, H-4), 7.66 (1H, d, $J_{5\text{-}6}$= 8.6 Hz, H-5), 7.06 (1H, d, $J_{6\text{-}8}$= 2.5 Hz, H-8), 7.00 (1H, dd, $J_{5\text{-}6}$= 8.6 Hz, $J_{6\text{-}8}$= 2.5 Hz, H-6), 6.33 (1H, d, $J_{3\text{-}4}$= 9.4 Hz, H-3), 4.94 (2H, d, $J_{9\text{-}10}$= 2.3 Hz, H-9), 3.67 (1H, $J_{9\text{-}10}$= 2.3 Hz, H-10); **^{13}C NMR (DMSO):** 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)-2*H*-chromen-2-one (12)

Light yellow solid product (565 mg, 2.64 mmol, 93%), m.p.= 130-134 ˚C (lit. 134 ˚C), **^{1}H NMR ($CDCl_3$):** 7.51 (1H, dd, $J_{4\text{-}5}$= 7.5 Hz, H-4), 6.93 (1H, d, $J_{5\text{-}6}$= 2.6 Hz, H-6), 6.91 (1H, dd, $J_{4\text{-}5}$= 7.5 Hz, $J_{5\text{-}6}$= 2.6 Hz, H-5), 6.15 (1H, d, $J_{3\text{-}Me}$= 1.2 Hz, H-3), 4.75 (2H, d, $J_{9\text{-}10}$= 2.3 Hz, H-9), 2.56 (1H, t, $J_{9\text{-}10}$= 2.5 Hz, H-10), 2.39 (3H, d, $J_{3\text{-}Me}$= 1.1 Hz, 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 **11-12** in *tert*-BuOH/H_2O 1/1 (v/v) $CuSO_4.5H_2O$ (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 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.

Protected products were dilluted 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.

7-{[1-(2,3-dihydroxypropyl)-1*H*-1,2,3-triazol-4-yl]methoxy}-2*H*-chromen-2-one (13)

White solid product (133 mg, 0.4 mmol, 93%), m.p. = 123-125°C, **^{1}H NMR (DMSO):** 8.19 (1H, s, H-10), 8.01 (1H, d, $J_{4,3}$= 9.6 Hz, H-4), 7.66 (1H, d, $J_{5,6}$= 8.8 Hz, H-5), 7.17 (1H, d, $J_{8,6}$= 2.5 Hz, H-8), 7.05 (1H, dd, $J_{6,8}$= 2.5 Hz, $J_{6,5}$= 8.5 Hz, H-6), 6.32 (1H, d, $J_{3,4}$= 9.3 Hz, H-3), 5.26 (2H, s, H-9), 5.15 (d, 1H, $J_{HO,12}$= 5.5 Hz, HO-12), 4.86 (1H, t, $J_{HO,13a}$= 5.8 Hz, $J_{HO,13b}$= 5.5 Hz, HO-13), 4.49 (1H, m, H*b*-13), 3.30-3.42 (2H, m, CH_2-11), 4.26 (1H, m, H*a*-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)-1*H*-1,2,3-triazol-4-yl]methoxy}-4-methyl-2*H*-chromen-2-one (14)

White solid product (130 mg, 0.4 mmol, 96%) m.p. = 108-113°C, **^{1}H NMR (DMSO):** 8.19 (1H, s, H-10), 7.71 (1H, d, $J_{5,6}$= 8.7 Hz, H-5), 7.17 (1H, d, $J_{8,6}$= 2.5 Hz, H-8), 7.00 (1H, dd, $J_{6,5}$= 8.7 Hz, $J_{6,8}$= 2.5 Hz, H-6), 6.22 (1H, s, H-3), 5.26 (2H, s, H-9), 5.12 (1H, m, H*b*-13), 4.85 (1H, m, H*a*-13), 4.68 (1H, m, H-12), 4.50 (1H, dd, $J_{11b,11a}$= 13.9 Hz, $J_{11b,10}$= 3.4 Hz, H*b*-11), 4.22 (dd, 1H, $J_{11a,12}$= 8.1 Hz, $J_{11a,11b}$= 13.9 Hz, H*a*-11), 3.80 (1H, s, HO), 3.61 (1H, s, OH), 2.40 (3H, s, CH_3); **APT (NMR):** 161.08 (2), 160.13 (7), 154.66 (8a), 1153.42 (4), 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 (CH_3).

Acknowledgement:

The present work has been supported by the Slovak Grant Agency VEGA 1/3559/06 and Science and Technology Assistance Agency under the contract No. APVV-51-046505.

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