Scientific Paper

SYNTHESIS AND CHEMILUMINESCENT PROPERTIES OF NOVEL BIOTINYLATED ACRIDINIUM ESTERS

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Abstract

Biotinylated acridinium ester, 9-(3-biotinyloxypropyl)-10-methylacridinium-9-carboxylate trifluoromethane sulphonate, **5** and its precursors, 9-(3-hydroxypropyl)-acridine-9-carboxylate **3** and 9-(3-biotinyloxypropyl)-acridine-9-carboxylate **4** were synthesized from acridine-9-carboxylic acid and were tested for their chemiluminescent properties. In contrast to aqueous solutions, in which the chemiluminescence is very low, in polar aprotic solvents such as *N,N*-dimethylformamide, it increases by a few orders of magnitude. The acridinium ester 5 as well as the precursors **3** and **4** could be detected down to few picomoles.

Key words: Chemiluminescence, acridinium salts, biotin

Introduction

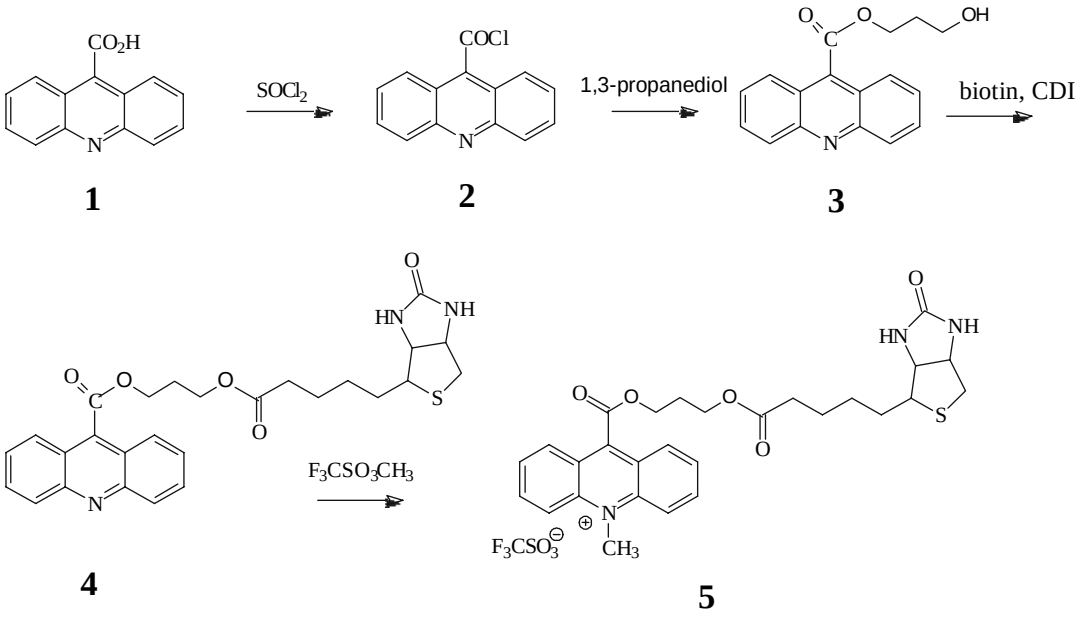
Chemiluminescent (CL) compounds have found wide utility in tests developed for medical diagnosis providing at least comparable sensitivity to radio immunoassay (RIA) and enzyme immunoassay (EIA) procedures.[1,2] Although the technique offers improvements in terms of reagent stability over the use of radioisotopes, it also presents certain disadvantages. The major drawback is their slight solubility in water, which is necessary for coupling the chemiluminescent compound to biomolecules. In case of hydrazides or oxalates the system is more complicated due to the use of external additives, like fluorescent compounds or catalysts. Using acridinium esters as chemiluminescent conjugates all the above mentioned drawbacks can be reduced. As acridinium esters and their chemiluminescent products are strongly fluorescent, they do not need any external fluorophor or any additional catalyst for chemiluminescence measurements. The chemiluminescent reaction can simply be triggered by alkaline hydrogen peroxide.[3-5] In immunoassays, usually, the acridinium esters are firstly transformed into active *N*-hydroxysuccinimide esters (NHS-esters), which can directly

be coupled to biomolecules under very mild conditions.[6-14] The drawback of these activated esters is their instability in aqueous solutions and their difficult chromatographic purification after their coupling to large biomolecules. To avoid the last two mentioned drawbacks, we decided to synthesise biotinylated acridinium esters, which are more stable in water and can be purified by simple column chromatography. The major advantage of these chemiluminescent conjugates is their very high affinity to avidin or streptavidin, which can be utilized in non-competitive immunoassay techniques (sandwich method).[15] The aim of this work was to prepare novel chemiluminescent biotinylated acridinium esters and to determine their chemiluminescence efficiency. As far as we know, with the exception of isoluminol derivatives[16] little is known about biotinylated chemiluminescent conjugates. In this paper we present the synthesis and chemiluminescent properties of biotinylated acridinium ester **5** with 1,3-propanediol as the binding molecule.

Results and discussion

Synthesis: The biotinylated acridinium ester **5** was prepared from the parent acid **1** as shown in Scheme 1. Acridine-9-carboxylic acid was converted to the acid chloride **2** by refluxing with thionylchloride for 4 hours.[17] Reaction of the acid chloride with excess of 1,3-propanediol in the presence of triethylamine gave the monoester **3** in high yields. Initial attempts to biotinylate the monoester using dicyclohexylcarbodiimide (DCC) or *N*-hydroxysuccinimide activated biotin ester (NHS-ester) in a variety of solvents gave inconsistent yields of the desired product. Using carbonyldiimidazole (CDI) as activator, excellent chemical yields of the biotinylated acridine ester **4** were obtained.

Finally, the acridine ester **4** was transformed to acridinium ester **5** by treatment with a 1:1 molar ratio of methyl trifluoromethanesulphonate ($F_3CSO_3CH_3$) in dry *N*,*N*-dimethylformamide (DMF) to avoid byproducts. At this point it should be noted that the latter compound is not stable at room temperature in organic solvents and decomposes very slowly (few weeks) to an unknown product with a characteristic intense UV-band at 290 nm and a fluorescence band at 440 nm. This compound could not be isolated in pure form and was not fully characterised.



Scheme 1. Synthesis of the biotinylated acridinium ester **5**.

Chemiluminescence: When biotinylated acridine compounds **3**, **4** and **5** were dissolved in aqueous solutions, they suffered a dramatic loss in chemiluminescence, which is difficult to explain. Contrarily, using polar aprotic solvents, such as *N*,*N*-dimethylformamide either as pure solvent or as a mixture with water, they exhibit intense light signals. The kinetics of the light reactions depends strongly on the solvent as well as on the concentrations of the alkaline hydrogen peroxide used. Optimisation experiments showed that addition of 50 μL of sample in DMF to a mixture containing 250 μL aqueous solution hydrogen peroxide (1.0 M) and 250 μL sodium hydroxide (1.0 M) gave the most intense light signals and these conditions were employed in all measurements (Figure 1). At this point it should be noted that derivatives **3**, **4**, and **5** also produced light of comparable intensities upon oxidation with saturated solution of potassium superoxide (KO_2) in dimethylsulphoxide (DMSO).

The duration of the light signals of all derivatives is less than two seconds. Maximal light output is reached after ca. 0.06 sec by compound **3** and after 0.10 sec for compounds **4** and **5**. After 0.4 sec, 100% of the total light output has already been emitted. Typical chemiluminescence signals of equimolar amounts of all three compounds are shown in Figure 2.

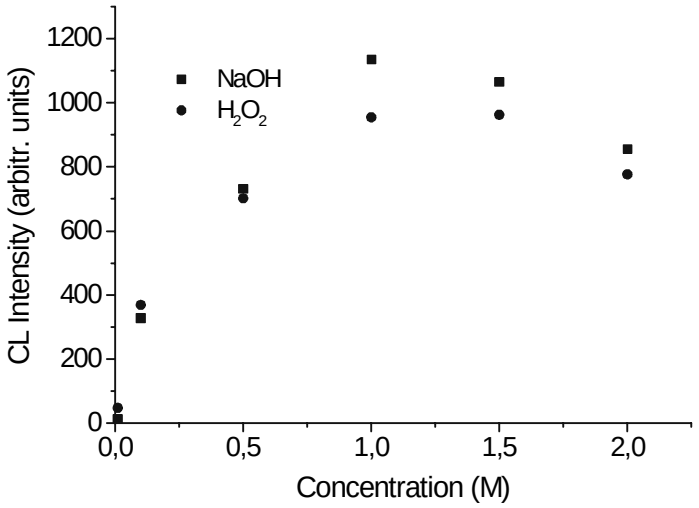


Figure 1. Diagrams of CL intensity of biotinylated acridinium ester **5** vs. concentration of sodium hydroxide or hydrogen peroxide.

From Figure 2, it is obvious that the biotin moiety in compound **4** affects positively the chemiluminescence intensity. This fact makes derivative **4** important for chemiluminescent immunoassays in which avidin or streptavidin should be used as binding moieties between the biomolecule and the chemiluminescent conjugate.

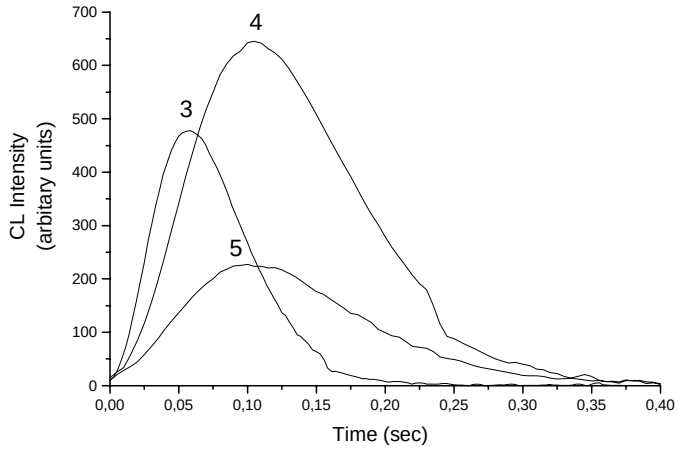


Figure 2. Typical chemiluminescence light intensity-time diagrams of equimolar amounts of compounds **3**, **4** and **5**.

Furthermore it is also obvious from Figure 2, that the chemiluminescence intensity of biotinylated acridinium derivative **5** is almost three times less than that of the non-alkylated biotinylated acridine derivative **4**. This decrease of the signal can be attributed

to the non-chemiluminescent product (9-hydroxy-9-biotinyloxypropylcarbonyl-10-methyl-9,10-dihydroacridine, **6**) produced in a competition reaction during the CL reaction[18] (Scheme 2). Although the light efficiency of acridinium salt **5** is less than that of derivatives **3** and **4**, this compound could be detected down to few picomoles (Figure 3).

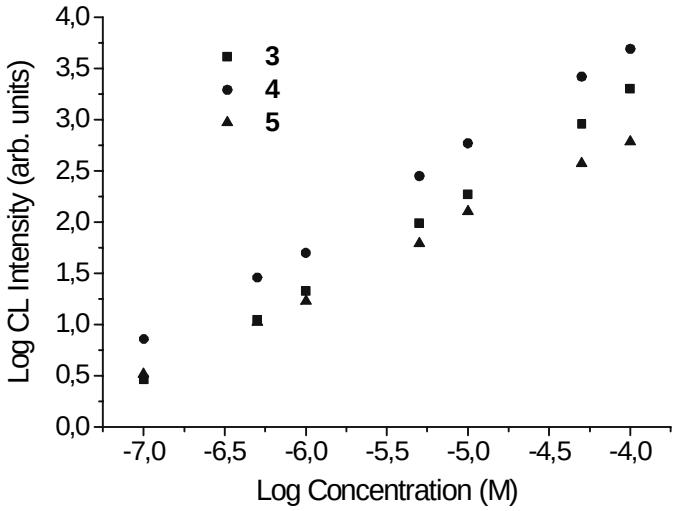
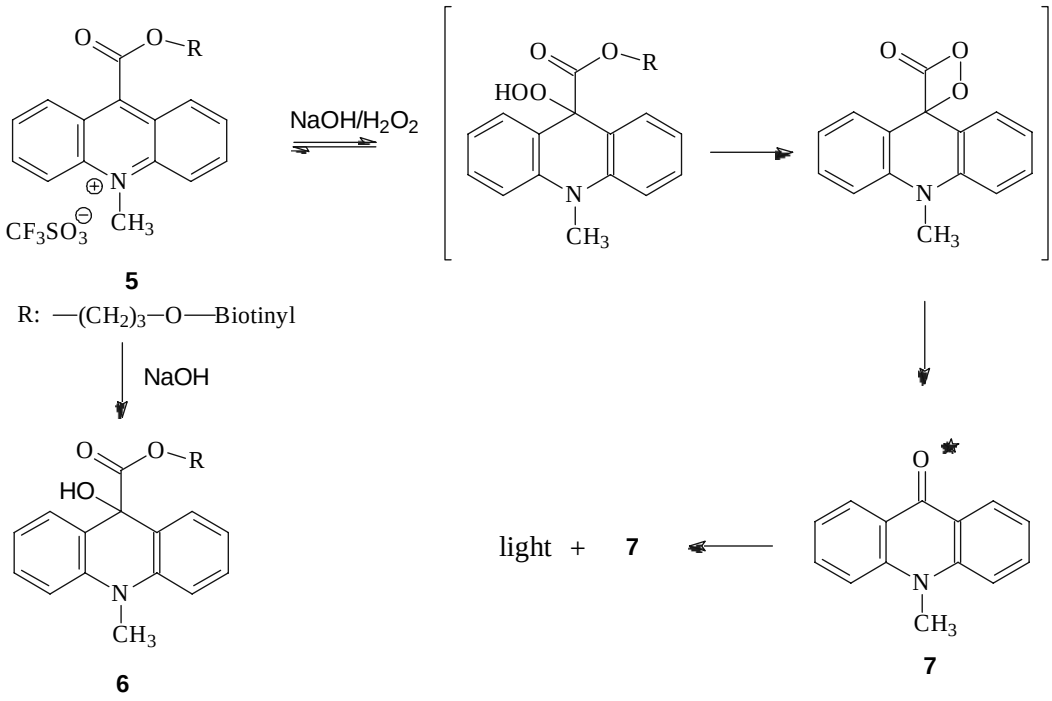


Figure 3. Chemiluminescence intensities vs. concentration of acridine derivatives **3**, **4**, and **5.** Concentrations of the chemiluminescent compounds are those in the 50 μL sample injected. Peak light intensities were averaged from triplicate measurements.

As shown in Figure 3, the peak light intensities increase linearly with concentration of acridine derivatives **3**, **4** and **5** and the lower limit for quantitative measurement was 10^{-7} M or 5 picomoles in 50 μL sample. This sensitivity is comparable to that of biotinylated isoluminols[16] but has the advantage of better reproducibility of the light signals. An additional advantage of the biotinylated acridine derivatives presented in this paper is their strong fuorescence quantum yields in aqueous solutions. Measuring in some preliminary experiments the fluorescence of compound **4** in the presence and absence of avidin, it was shown that this compound could be detected down to 10^{-10} M (unpublished results of the present authors).

The chemical reactions taking place during the chemiluminescence of acridinium ester **5** are shown in Scheme 2. Similar reactions take place also with the acridine derivatives **3** and **4** with the difference that in these cases acridone is produced. The first step of these light reactions is believed to be the nucleophilic attack at C-9 on the

acridinium ring by the hydrogen peroxide anion. As mentioned above, a side reaction in this step is also the competitive addition of hydroxide anion to **5**, which leads to a non-chemiluminescent product, **6**. The rate determining step involves cleavage of the acridinecarboxylate group.[19,20] The light emitting species are *N*-methylacridone, **7** for acridinium ester **5** and acridone for acridine derivatives **3** and **4**. These products are characterised spectroscopically (UV, fluorescence) by comparison of their spectra with those of authentic samples.



Scheme 2. Reaction sequence in the chemiluminescence of acridinium ester **5**.

Conclusions

In this paper, we present a novel synthesis of biotinylated acridines and acridinium derivatives in good overall chemical yields, having a binding bifunctional molecule, without using any protecting/deprotecting techniques. As mentioned above, the chemiluminescence of biotinylated acridinium derivative **5** presented in this paper is comparable to that of biotinylated isoluminol derivatives (detection limit 10^{-7} M). The major advantage of compounds **4** and **5** over biotinylated isoluminols is the improved

reproducibility of their chemiluminescent measurements, probably due to the absence of added catalyst. Moreover, as these compounds show strong fluorescence efficiencies in aqueous solution, especially compound **4**, this property can be probably utilized in fluorogenic immunoassays. Despite the competitive reaction of the CL step leading to **6**, the light intensities are adequate for employment of this reaction in CL assays.

Experimental

Equipment / reagents: Chemiluminescence measurements were performed on a 1250 Bio-Orbit luminometer. The luminometer (output range 1.0 mV–10 V) is equipped with a photomultiplier tube (HAM 105-21) with side window and spectral range from 300 to 620 nm, connected to a potentiometer chart recorder (GOW-MAC Instrument CO., Model 70–150) or personal computer equipped with a home made software program which allows the continuous monitoring and analysis of the output signal. Absorption spectra were run on a JASCO V-560 spectrophotometer. Fluorescence spectra were recorded on a JASCO FP-777 Spectrofluorimeter (Scan speed 200 nm min^{-1}, emission band 5 nm, photomultiplayer sensitivity, medium). ^{1}H- and ^{13}C nmr spectra were measured on a Bruker AC 250 spectrometer. Chemiluminescence spectra were run on the JASCO FP-777 Spectrofluorimeter with the excitation source off, employing wide slits (20 nm) and a scanning rate of 2000 nm min^{-1}. Melting points were recorded on a Gallenkamp melting point apparatus and are uncorrected. Infrared spectra were obtained using a Perkin Elmer 283 FT-IR spectrometer. ^{1}H NMR (250 MHz) and ^{13}C NMR (62.5 MHz) spectra were measured on a Bruker AC 250 spectrometer with tetramethylsilane as an internal standard. Chemical shifts (δ) are expressed in ppm and coupling constants (J) are in Hz. ESI Mass spectra were recorded on a Finnigan spectrometer, AQA Navigator (products **4** and **5**) and on an Agilent 5973N mass spectrometer equipped with an Agilent 6890 gas chromatograph (product **3**). Elemental analyses were obtained with a Perkin Elmer CHN 2004 instrument. Column chromatography was carried out with silica gel 60 (0.063-0.2 mm, Merck). Acridine-9-carboxylic acid, 1,3-propanediol, carbonyldiimidazol (CDI), *N*,*N*-dimethylformamide (DMF) (spectroscopy grade) were purchased from Aldrich and used without further purification. Biotin was purchased from Fluka.

Chemiluminescence measurements: The stock solutions were prepared in DMF at concentrations 10^{-3} M and diluted in the same solvent down to 10^{-10} M. The light reactions were started by adding 50 μL of biotinylated acridinium salt **5** or acridine derivatives **3** or **4** into a mixture of 500 μL containing hydrogen peroxide (1.0 M) and sodium hydroxide (1.0 M) in a ratio of 1:1.

Synthesis of acridine-9-carbonylchloride hydrochloride (2):

This compound was prepared following known procedure.[17] A sample of 9-acridinecarboxylic acid hydrate (5 g) was heated in 50 mL of thionylchloride in the presence of three drops of N,N-dimethylformamide until total dissolution. The solution was maintained at the boiling temperature of thionylchloride for 4 hours. The solvent was evaporated under reduced pressure and the product was used without any further purification. After evaporation to dryness, the product was collected and used without any further purification.

Synthesis of 9-(3-hydroxypropyl)acridine-9-carboxylate (3):

Acridinecarbonylchloride hydrochloride (920 mg, 3.31 mmol) was dissolved in a large excess of 1,3-propanediol (20 mL) containing 4 mL triethylamin. The mixture was first stirred for 12 hours at room temperature and then heated at 100 °C for 4 hours. After cooling, the residue was dissolved in 100 mL chloroform and 100 mL saturated $NaHCO_3$, the organic phase was separated and dried over $MgSO_4$. The solvent was evaporated under reduced pressure and the product was purified by column chromatography (silica gel, chloroform-methanol, 15:1, Rf 0.25). Chemical yield (650 mg, 70%), mp 133–135 °C, UV (DMF) λ_{max}, 363 nm, ε=10070 L $mol^{-1}cm^{-1}$, fluorescence (DMF): λ_{max}, 440 nm, λ_{exc}, 363 nm, fluorescence intensity 375 arb. units. IR (KBr) ν 3163, 3061, 2947, 2920, 2856, 1722 (CO), 1612, 1517 (C=C), 1461, 1419, 1299, 1101 (C-O), 1049 (C-O), 769 (cm^{-1}). ^{1}H NMR (250 MHz, DMSO-d_6) δ 8.24 (d, 2H, *J* 8.66 Hz), 8.05 (d, 2H, *J* 8.25 Hz), 7.93 (m, 2H), 7.73 (m, 2H), 4.72 (m, 1H), 3.57 (m, 2H), 1.97 (m, 2H). ^{13}C NMR (62.5 MHz, DMSO-d_6) δ 166.82 (C=O), 148.03, 136.99, 130.90, 129.51, 127.70, 124.98, 121.36, 63.82, 57.16, 31.35. MS *m/z* (relative intensity): 281 (M^+, 100), 223 (71.4), 206 (57), 178 (85.7), 167 (23.6), 151 (28.6), 75 (5.7). Anal. Calcd for $C_{17}H_{15}NO_3$, C 72.6%, H 5.34%, N 4.98%, found: C 72.83%, H 5.84%, N 5.01%.

Synthesis of 9-(3-biotinyloxypropyl)-acridine-9-carboxylate (4):

In a 100 mL round flask, 370 mg biotin (1.5 mmol) were added under argon in 10 mL *N*,*N*-dimethylformamide containing 50 mg molecular sieves (4 Å) and was heated at 80 °C until the biotin was dissolved. Then 245 mg carbonyldiimidazole (CDI) (1.5 mmol) was added and the mixture was stirred at room temperature for 2 hours. Finally, a DMF solution containing of 9-(3-hydroxypropyl)acridine-9-carboxylate **3** (422 mg, 1.5 mmol) was added at room temperature and stirred at this temperature for 18 hours. The solvents were evaporated under reduced pressure and the product purified by column chromatography (silica gel, chloroform-methanol, 30:1, Rf 0.2). Chemical yield, (305 mg, 40%). UV (DMF): λ_{max} 362 nm, ε = 9770 L mol^{-1} cm^{-1}; fluorescence (DMF), λ_{max}, 440 nm, λ_{exc}, 363 nm, fluorescence intensity 360 arb. units. IR (KBr) ν 3222, 3065, 2929, 2866, 1728, 1705 (CO), 1516 (C=C), 1460, 1438, 1288 (C-N), 1263, 1170 (C-O), 1207 (C-O), 1031, 752, 637 (cm^{-1}). ^{1}H NMR (250 MHz, DMSO-d_6) δ 8.24 (d, 2H, *J* 8.56 Hz), 7.98 (d, 2H, *J* 8.93 Hz), 7.78 (m, 2H), 7.58 (m, 2H), 6.23 (s, NH), 5.78 (s, NH), 4.70 (m, 2H), 4.40 (m, 1H), 4.22 (m, 3H), 3.03–2.63 (m, 3H), 2.21 (m, 4H), 1.69–1.29 (m, 6H). ^{13}C NMR (62.5 MHz, $CDCl_3$) δ 173.4 (C=O), 167.3 (C=O), 163.8 (C=O) 148.4, 136.6, 130.3, 129.7, 127.1, 124.9, 122.1, 63.0, 61.8, 60.7, 59.9, 55.4, 40.4, 33.7, 28.2, 28.0, 27.9, 24.6. MS (ESI) *m/z* (relative intensity): 510.2 (M^+ + 3, 12.5), 509.2 (M^+ + 2, 37.5), 508.3 (M^+ + 1, 69), 285.2 (21), 227.2 (75.5), 224.1 (100), 206.2 (86), 196.2 (14). Anal. Calcd for $C_{27}H_{29}N_3O_5S$, C 63.87%, H 5.72%, N 8.28%, found: C 63.50%, H 5.62%, N 8.12%.

Synthesis of 9-(3-biotinyloxypropyl)-10-methylacridinium-9-carboxylate trifluoromethane sulphonate (5):

Dry dichloromethane (5 mL) containing 9-(3-biotinyloxypropyl)acridine-9-carboxylate **4** (250 mg, 0.5 mmol) were mixed with 82 mg methyl trifluoromethanesulphonate (0.5 mmol) and stirred at room temperature for 24 hours. The solvent was removed under reduced pressure and the residue washed thoroughly with dry diethyl ether. The isolated product is highly viscous. Chemical yield: 207 mg (62%). UV-VIS (DMF), λ_{max}, 367 nm; Fluorescence (DMF) λ_{max} 445 nm. IR (KBr) ν 3433, 3030, 2966, 2941, 1732 (C=O), 1710 (C=O), 1650, 1464, 1370, 1280, 1255, 1170, 1030, 762, 640, 577 cm^{-1}. ^{1}H NMR (250 MHz, D_2O) δ (ppm) 8.51 (m, 2H), 8.22 (m, 4H), 7.83 (m, 2H), 5.22–4.44 (m, 4H), 4.40–3.50 (m, 6H), 3.22–2.63 (m, 4H), 2.4–2.21 (m, 2H), 1.69–1.29 (m, 8H). ^{13}C NMR (62.5 MHz, D_2O) δ 178.6 (C=O), 168.3 (C=O),

161.8 (C=O) 149.6, 144.4, 141.9, 131.7, 130.3, 128.7, 122.5, 65.3, 61.9, 60.7, 53.7, 49.1, 41.5, 40.4, 33.7, 27.3, 26.7, 24.6, 24.3. MS (ESI) *m/z* (relative intensity): 522.3 (M^+, 28); $C_{29}H_{32}F_3N_3O_8S_2$ (671.1).

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Povzetek

9-(3-Biotiniloksipropil)-10-metilakridinijev-9-karboksilat trifluorometan sulfonat **5** in njegova prekurzorja, 9-(3-hidroksipropil)-akridin-9-karboksilat **3** in 9-(3-biotiniloksipropil)-akridin-9-karboksilat **4**. smo sintetizirali iz akridin-9-karbksilne kisline in testirali njihove kemoluminiscentne lastnosti. Za razliko od vodnih raztopin, v katerih je kemoluminiscenca zelo nizka, v polarnih aprotičnih topilih, kot je *N*,*N*-dimetilformamid, naraste za nekaj velikostnih redov. Akridinijev ester **5** in prekurzorja **3** in **4** je mogoče detektirati v pikomolih.