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3,6-Bis-β-Dicarbonylsubstituted Carbazoles Bearing N-Spacers and Their Eu(III) Complexes as Immunofluorescent Labelling Agents

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Abstract

New reagents for immunofluorescence analysis of carbazole series containing fluorinated β-dicarbonyl fragments and carboxylic substituent groups separated by spacers of different lengths from the light-gathering carbazole scaffold have been developed. The markers in complex with Eu^{3+} ions possess stability in the aqueous phase, intense and prolonged luminescence (τ 550 - 570 μs) with characteristic emission maxima in the region of 615 nm and excitation wavelengths in the region of 380 - 390 nm, which distinguishes them from most of the analogs used. In the study of marker conjugation with streptavidin, a reagent containing 4 - 5 europium labeling complexes based on spacer-containing carbazole tetraketone was obtained. The marker-doped silicate nanoparticles exhibit intense and long-lived luminescence in the characteristic region.

Keywords

Fluorescence Immunoassay, Fluorinated β-Diketones, Carbazole, Europium Complexes, Streptavidin, Nanodispersions

1. Introduction

Lanthanide fluorescence immunoassay (LFIA) with delayed fluorescence involving immunofluorescence of lanthanide reagents is one of the primary immunobiological assays for the determination of microbial and viral pathogens as well as genetic mutations [1] [2].

The most intensely luminescent complexes in aqueous media are complexes of aromatic fluorinated β-diketones with Eu(III) ions. A large number of scientific

researches on the luminescence of such complexes and their application in various fields of molecular detection [3] [4] [5], as well as in the field of OLED technologies [6] [7] have been published.

One of the ways to further increase the sensitivity and rapidity of the method is the search for aromatic β-diketones with enhanced spectral properties and improved stability. Previously studied carbazole compounds containing two β-dicarbonyl polyfluorinated fragments have satisfactory luminescence and increased stability of complexes by two to three orders of magnitude in comparison with conventional β-diketones [8] [9]. Earlier, we obtained a biomolecular labelling agent—a carbazole-based tetraketodiester with two methoxycarbonyl groups separated by difluoromethylene spacers from the chelating center [10]. The drawback of this marker is its bifunctionality that leads to "crosslinking" of protein molecules with the subsequent precipitation.

In order to cope with various tasks of molecular diagnostics, it seemed necessary to synthesize and study markers bearing spacers of various lengths that separate the labelling groups from the chromogenic fragment. The present work includes the synthesis of carbazole based long-life fluorescence complexes with long-wavelength absorption, high binding constants with lanthanide ions and reactive groups for the formation of conjugates with biomolecules. The choice of the carbazole moiety is based among other things on the auxochromic effect of the heterocyclic N atom on the fused aromatic fragment as well as N being a reactive site for the introduction of labelling group spacers. Meanwhile, well-established "mild" protocols for the conjugation with proteins have been developed for the carboxyl group, so this group is suitable for labelling. It was also necessary to obtain labelling reagents with a short and with a long spacer separating the carboxylic group from the chromophore. The formation of luminescent nanodispersions including such labelling probes was also of interest, as it can result in the development of corresponding biochips.

2. Results and Discussion

2.1. Preparation of Precursors

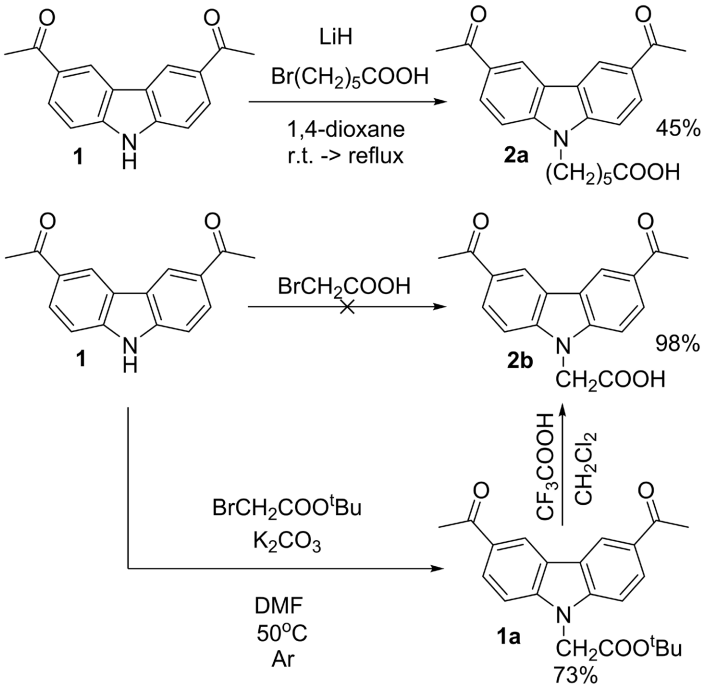
The carboxyl group with C_5 spacer was introduced by the reaction of the diacetyl derivative **1** with 6-bromohexanoic acid in dry 1,4-dioxane in the presence of lithium hydride (Scheme 1). Compound **2**a yields were not less than 45% after crystallization from ethyl acetate. The introduction of a shorter spacer C_1 is more difficult and has to be achieved in 2 steps, as direct alkylation of diacetylcarbazole **1** with bromoacetic acid was unsuccessful under various conditions. Therefore, tert-butyl bromoacetate was used as the alkylating agent, giving the intermediate **1**a. The subsequent cleavage of the tert-butyl group with trifluoroacetic acid yielded 79% of **2**b.

2.2. Synthesis of Labelling Reagents

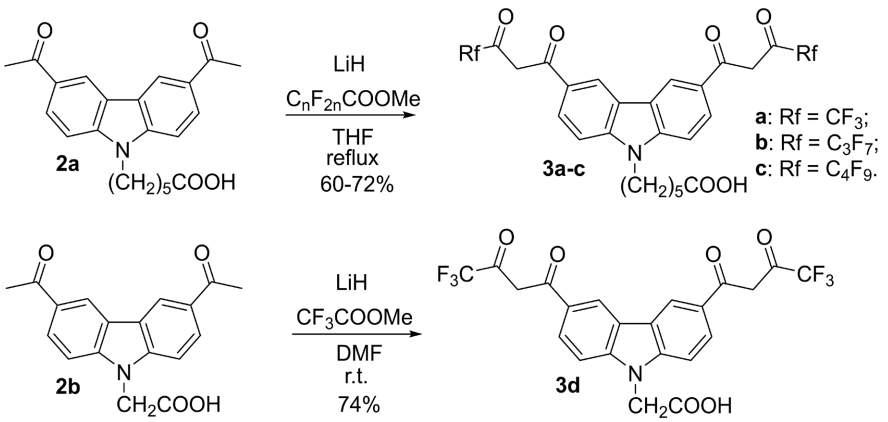
Compounds **3**a-d were obtained by the Claisen condensation of perfluorinated

carboxylic acid esters with diacetylcarbazoles **2**a, b (Scheme 2). Three different perfluorinated fragments were introduced—CF_3, C_3F_7, C_4F_9, as it seemed interesting to evaluate the effect of fluorinated substituents on the luminescent properties of the corresponding complexes. It is known that the introduction of perfluorinated fragments of various lengths can be utilized to regulate the hydrophilic-hydrophobic balance which sometimes has a strong influence on the spectral and luminescent parameters of tetraketones and their Eu^{3+} complexes [11].

Lithium hydride has been successfully applied as a base for the synthesis of fluorinated β-diketones [12]. To obtain tetraketones **3**a-c we used lithium hydride in refluxing THF. Due to the low solubility of compound **2**b, the synthesis of **3**d was carried out in DMF at ambient temperature (Scheme 2). The resulting yields of compounds **3**a-d are satisfactory: 65% - 74%.



Scheme 1. Synthesis of N-carboxyl-containing 3,6-diacetylcarbazoles.



Scheme 2. Synthesis of N-carboxyl-containing fluorinated tetraketones.

2.3. Luminescence Properties and Complex Formation

Two major absorption maxima are observed in the spectra of carbazoles **3**a-d in bidistilled water (Table 1).

A stable bathochromic shift in the area of >400 nm is observed for spectra recorded in Tris (pH 7.8). This effect becomes more distinguished as the length of the perfluorinated fragment increases (Figure 1 for **3**c). When a solution of Eu(III) is added, a hypsochromic shift is induced in the absorption spectra of the complexes of tetraketones **3**a-d, with distinct maxima at about 375 - 380 and 340 nm (Figure 1, e.g. **3**c, and Table 2).

When luminescence parameters of Eu^{3+} mixed complexes with compounds **3**a-d and trioctylphosphine oxide (TOPO) (3:2:6 ratio) were studied in aqueous solutions. It was found that for compounds with a longer perfluorinated fragment—**3**b,c—the intensity of luminescence is by average 15% higher than for **3**a

Table 1. Absorption of carbazoles **3**a-d in bidistilled water (C_3 = 10^{-5} M. (n = 3; P = 0.95)).

Compound	$\varepsilon \times 10^4$ $M^{-1} \times cm^{-1}$
3a	2.5 ± 0.1
3b	2.8 ± 0.1
3c	2.2 ± 0.2
3d	2.9 ± 0.1

Table 2. Long-life luminescence parameters for the complexes of ligand **3** (10^{-5} M) with Eu^{3+} and TOPO (3:2:6) in Tris (0.05 M, pH 7.8) and Triton X-100 (0.1%) containing Tris (0.05 M, pH 7.8).

Ligand	Medium	λ_{ex}, ±2 nm	$I \times 10^4$, AU	τ, μs
3a	Tris	340 (shoulder) 378	51.1 ± 1.4 60.2 ± 1.4	544 ± 10
3b	Tris	340 (shoulder) 376	32.7 ± 1.1 37.5 ± 1.0	654 ± 12
3c	Tris	340 (shoulder) 378	90.1 ± 2.2 113.3 ± 2.3	566 ± 9
	Tris, Triton X-100	338 (shoulder) 378	58.1 ± 1.3 71.3 ± 2.0	665 ± 13
3d	Tris	338 (shoulder) 376	98.0 ± 1.5 121.2 ± 2.4	570 ± 11
	Tris, Triton X-100	340 (shoulder) 375	61.7 ± 1.4 79.8 ± 1.9	689 ± 11

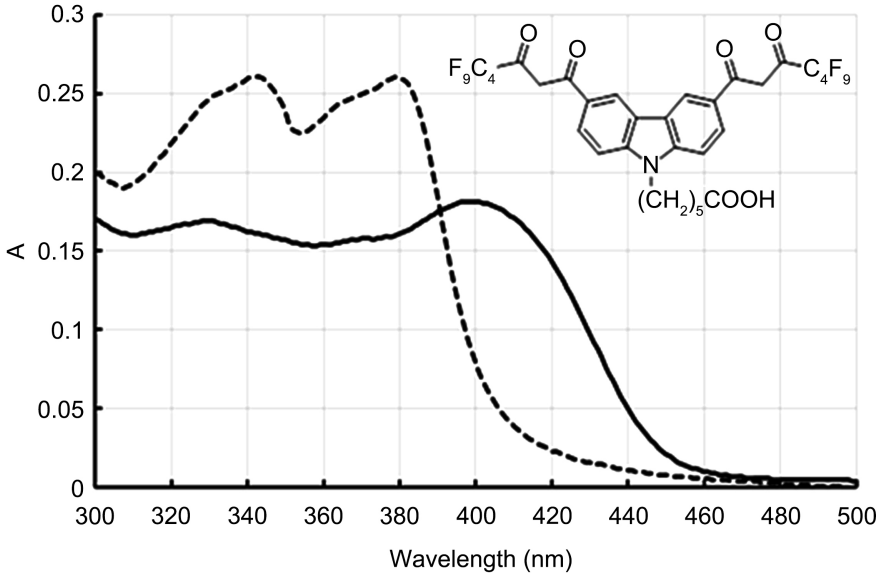


Figure 1. Absorption spectrum of tetraketone **3c** (10^{-5} M) (solid curve) and its complex with Eu^{3+} and TOPO (dashed curve) (ratio 3:2:6) in Tris (0.05 M, pH 7.8).

and **3**d. TOPO is a known synergist of luminescence for europium complexes, as it displaces water from the complexes and thus reduces its fluorescence quenching effect [13]. In a micellar buffer solution, the lifetime of the luminescent state is increased for all the complexes of N-spacer bearing tetraketones **3**a-d, but at the same time the luminescent intensity is decreased, which can be due to the presence of the hydrophilic carboxyl-containing spacers that shift the distribution of Eu complexes between the aqueous and the micellar phase in favor of the former.

Stability of the Eu^{3+} complexes of the synthesized compounds **3** was tested in aqueous buffers for 80 days. It was found that the spacer-bearing tetraketone **3**c with the C_4F_9-fragment possesses the best luminescent performance.

2.4. Synthesis of a Protein Conjugate

The conjugate was obtained using the complex of carbazole **3**c, as its luminescence parameters are the most promising. Conjugation was performed in a model experiment with streptavidin after a preliminary activation of the carboxyl group with dicyclohexylcarbodiimide and hydroxybenzotriazole in DMF. The activated intermediate was not isolated and was added to streptavidin in buffer solution (Scheme 3 and Scheme 4).

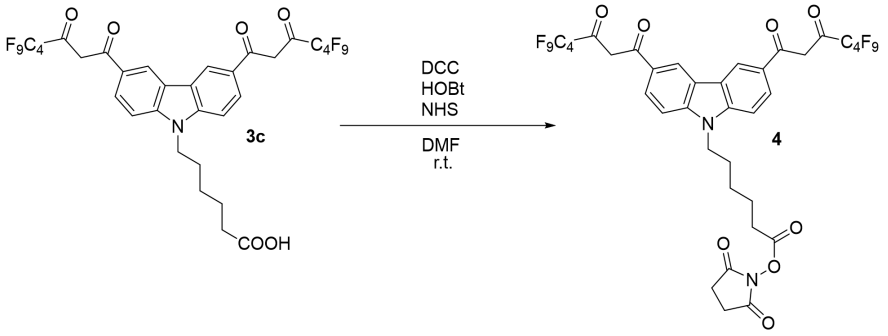
After the conjugation, the reaction mixture was separated on a Sephadex G25 column. Fractions were analyzed by absorption spectroscopy and the degree of conjugation of the complex to the protein was evaluated (Table 3).

The luminescence spectra of the purified conjugates were recorded in buffer solutions (Tris and MES) containing an excess of TOPO. The parameters of luminescence allow the confident detection of labelled proteins at about 10^{-11} M concentration in both buffers. Therefore, we can conclude that in conjugate **5**

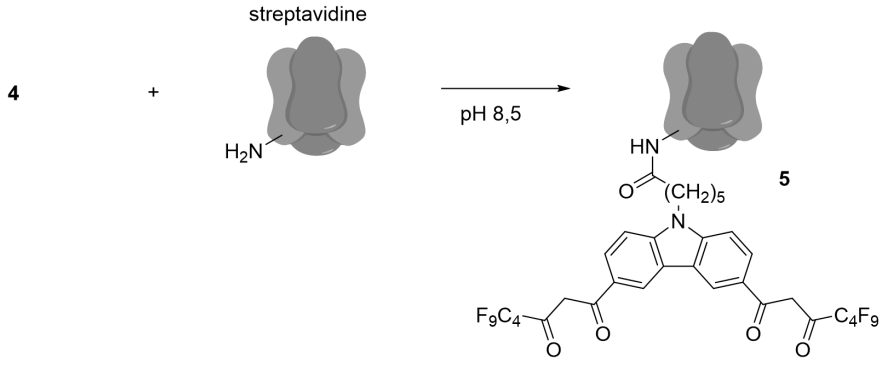
there are about 4 - 5 labelling europium tetraketone **3**c complex molecules.

2.5. Formation of Nanodispersions

Formation of nanodispersions with carbazole based labelling agents was achieved in several ways. The best results were obtained by doping "Aerosil 380" silica based nanomaterial which had been earlier sonicated in order to overcome the formation of aggregates. Thus formed dispersions possess high aggregate stability as well as luminescence stability which is typical for tetraketone complexes of europium (Figure 2).



Scheme 3. Activation of carboxylic group with NHS.



Scheme 4. Preparation of a conjugate 4 with streptavidin.

Table 3. Parameters of the conjugates of compound 3c with streptavidin. F/P is the average number of the bound ligand molecules, detected spectroscopically at 280 and 356 nm.

Fraction №	C_{ligand}, M	$C_{protein}$, M	F/P
1	9.52×10^{-7}	1.48×10^{-7}	6.4
2	1.05×10^{-5}	2.19×10^{-6}	4.8
3	3.70×10^{-5}	8.34×10^{-6}	4.4
4	2.01×10^{-5}	5.62×10^{-6}	3.6
5	5.20×10^{-6}	1.47×10^{-6}	3.5

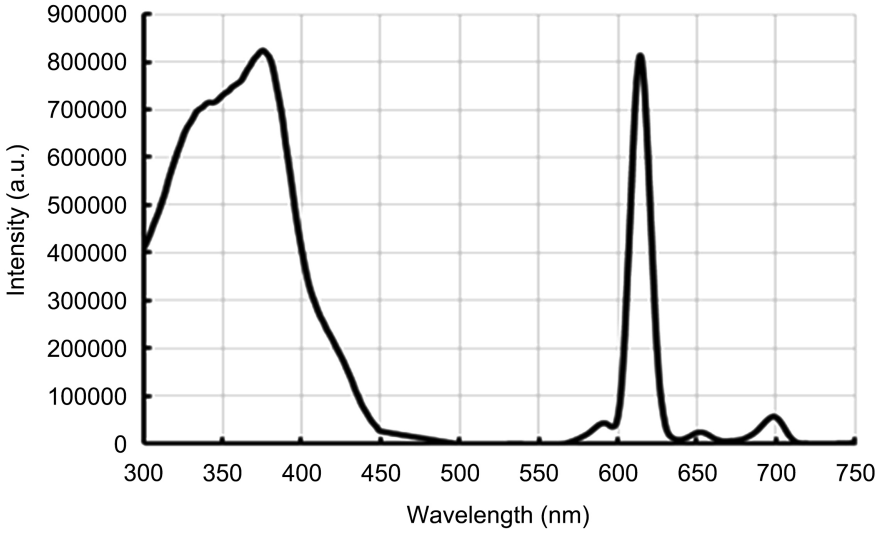


Figure 2. Excitation (left) and emission (right) spectra of the phosphorescent nanoparticles doped with Eu^{3+} chelate **3a** (total Eu^{3+} content in the dispersion is 1.6×10^{-5} mol/L).

3. Conclusion

In this study, 3,6-bis-β-dicarbonylsubstituted carbazoles bearing N-spacers of different lengths were obtained in satisfactory yields. Under conditions of complexation with Eu^{3+}, these compounds exhibit high luminescence intensity, which allowed to consider the successful possibility of their use for streptavidin conjugation. In addition, formation of luminescent nanodispersions based on the studied carbazoles is perspective. The experimental results show good possibility of the synthesized luminescence probes in applications such as immunoassay protocols.

4. Experimental

The ^{1}H, ^{19}F and ^{13}C NMR spectra were recorded on JEOL JNM-ECX400 (400, 376 and 100 MHz, respectively) and Bruker Avance III 500 (500, 470 and 125 МГц, respectively) instruments in DMSO-d_6, with the internal standards being TMS for ^{1}H and ^{13}C NMR and $CFCl_3$ for ^{19}F NMR. Elemental analysis was conducted on a Perkin Elmer CHN PE 2400 SII analyzer. Melting points were determined in glass capillaries on a Gallenkamp MPD350.BM3.5 device. The UV-vis spectra were recorded on Shimadzu UV-1650PC in a 1 cm quartz cell and on an in-house Arduino based photometric device. The luminescence parameters of Eu^{3+} complexes were studied in the wavelength range 200 ÷ 800 nm on a Perkin Elmer LS-5B time-resolved luminescence spectrophotometer and a Thermo Fischer Varioscan Flash microplate reader with a 100 microsecond time delay (td) and 1000 microsecond integration time (tg) in the presence of air oxygen. Sonication was performed in Elmasonic S10/H ultrasound bath at 37 kHz and 30 W power willed with bidistilled deionized water. The reagents used in this work are either industrially produced or supplied by Sigma-Aldrich. Carbazole **1** was synthesized by a modified procedure [14]. Dialysis was conducted

in dialysis tubes (cellulose tubular membrane, CelluSep 4, MWCO 12,000 - 14,000 g/mol, 30 mm tube width). Commercial reagents and solvents were purified and prepared according to known guidelines [15].

Synthesis of tert-butyl 2-(3,6-diacetyl-9H-carbazol-9-yl)acetate 1a:

To a solution of carbazole **1** (0.51 g, 2.03 mmol) in 18 mL of dry DMF, potassium *tert*-butoxide was added (0.76 g, 6.80 mmol) and the mixture was stirred for 20 min. Subsequently, bromoacetic acid *tert*-butyrate was introduced (0.44 mL, 3.00 mmol) and stirring continued for 18 h. The reaction mixture was poured to 80 mL of ethyl acetate and the organic layer was washed with saturated brine (4 × 20 mL) and then water (2 × 20 mL). The organic layer was dried with sodium sulfate and evaporated in vacuo (~20 torr). The residue was crystallized from ethanol. The yield is 0.54 g (73%) of colorless powder, m.p. 179˚C - 182˚C (dec.).

^{1}H NMR ($CDCl_3$), δ: 1.42 (s, 9H, $-C(CH_3)_3$), 2.71 (s, 6H, $2CH_3$), 4.94 (s, 2H, CH_2), 7.36 (d, 2H, carb., J = 8.0 Hz), 8.16 (dd, 2H, carb., J = 8.0 Hz, J = 2.0 Hz), 8.76 (d, 2H, carb., J = 2.0 Hz). Found, %: C 72.53; H 6.42; N 3.71. $C_{22}H_{23}NO_4$. Calculated, %: C 72.31; H 6.34; N 3.83.

6-(3,6-diacetyl-9H-carbazol-9-yl)hexanoic acid (2a)

To a mixture of carbazole **1** (0.20 g, 0.79 mmol) and 6-bromohexanoic acid (0.33 g, 1.69 mmol) in 30 mL of dry dioxane, 60% sodium hydride suspension was added (0.12 g, 3.00 mmol) and the mixture was refluxed for 2 days. After cooling to ambient temperature, the mixture was poured into a mixture of 20 g of ice and 100 mL of 2% HCl. The separated precipitate was filtered, washed with 2 × 50 mL water, dried in a dessicator over P_2O_5 and then crystallized from 18 mL of ethyl acetate. The yield is 0.13 g (45%) of light-beige powder, m.p. 122-123°C.

^{1}H NMR (DMSO-d_6), δ: 1.25 (m, 2H, CH_2), 1.44 (m, 2H, CH_2), 1.75 (m, 2H, CH_2), 2.12 (m, 2H, CH_2), 2.69 s (6H, $2CH_3$), 4.44 (m, 2H, CH_2), 7.69 (d, 2H, carb., J = 8.0 Hz), 8.05 (dd, 2H, carb., J = 8.0 Hz, J = 2.0 Hz), 9.01 (d, 2H, carb., J = 2.0 Hz). Found, %: C 72.68; H 6.71; N 3.55. $C_{22}H_{23}NO_4$. Calculated, %: C 72.31; H 6.34; N 3.83.

2-(3,6-diacetyl-9H-carbazol-9-yl)acetic acid (2b)

To compound **1**a (0.3 g, 0.82 mmol) in 10 mL of methylene chloride, trifluoroacetic acid (0.61 mL, 8.2 mmol) was added and the mixture was stirred for 12 h. The mixture was then evaporated (~20 torr) and 3 times co-evaporated with 20 mL portions of methanol. The yield is 0.25 g (98%) of beige powder, m.p. 284˚C - 288˚C (dec.).

^{1}H NMR (DMSO-d_6), δ: 2.68 (s, 6H, $2CH_3$), 5.35 (s, 2H, CH_2), 7.68 (d, 2H, carb., J = 8.0 Hz); 8.06 (dd, 2H, carb., J = 8.0 Hz, J = 2.0 Hz), 9.01 (d, 2H, carb., J = 2.0 Hz). Found, %: C 69.95; H 4.74; N 4.61. $C_{18}H_{15}NO_4$. Calculated, %: C 69.89; H 4.89; N 4.53.

6-(3,6-bis(4,4,4-trifluoro-3-oxobutanoyl)-9H-carbazol-9-yl)hexanoic acid (3a)

To a chilled (5˚C) suspension of lithium hydride (0.050 g, 6.3 mmol) in 30 mL of dry dioxane, methyl trifluoroacetate (0.35 g, 2.7 mmol) was added, followed by **3**a (0.40 g, 1.1 mmol) in 10 mL of dry dioxane. The obtained mixture was maintained at 80˚C for 96 h. The reaction mixture was cooled to ambient temperature and poured into a mixture of ice and 4% sulfuric acid. The aqueous layer was extracted with 3 × 15 mL of chloroform, washed with 2 × 10 mL of distilled water, the organic extract was dried with sodium sulfate and evaporated to dryness under reduced pressure. The residue was dissolved in 8 mL of chloroform and precipitated with 15 mL of heptane. Yield 0.26 g (43%) of yellow powder, m. p. 108˚C - 112˚C.

^{1}H NMR (DMSO-d_6), δ: 1.25 (m, 2H, CH_2), 1.44 (m, 2H, CH_2), 1.75 (m, 2H, CH_2), 2.12 (m, 2H, CH_2), 2.69 (s, 6H, $2CH_3$), 4.48 (m, 2H, CH_2), 7.13 (s, 2H, =CH), 7.76 (d, 2H, carb., J = 8.0 Hz), 8.20 (dd, 2H, carb., J = 8.0 Hz, J = 2.0 Hz), 9.18 d (d, 2H, carb., J = 2.0 Hz). ^{19}F NMR (DMSO-d_6), δ: −74.55 s (6F, $2CF_3$). Found, %: C 56.33; H 4.01; N 2.19. $C_{26}H_{21}F_6NO_6$. Calculated, %: C 56.02; H 3.80; N 2.51.

6-(3,6-Bis(6,6,6,5,5,4,4-heptafluoro-3-oxohexanoyl)-9H-carbazol-9-yl)hexanoic acid (3b)

To a chilled (5˚C) suspension of lithium hydride (0.09 g, 11.3 mmol) in 30 mL of dry dioxane, methyl perfluorobutanoate (1.25 g, 5.50 mmol) was added, followed by **3**a (0.40 g, 1.1 mmol) in 10 mL of dry dioxane. The resulting mixture was refluxed for 84 h. The reaction mixture was then cooled to ambient temperature and poured into a mixture of ice and 4% sulfuric acid. The aqueous layer was extracted with 3 × 15 mL of chloroform, washed with 2 × 10 mL of distilled water, the organic extract was dried with sodium sulfate and evaporated under reduced pressure to a volume of 15 mL. Heptane (5 mL) was added and the mixture was crystallized at −20˚C. The small crystals formed were filtered and the filtrate was evaporated under reduced pressure. The yield was 0.30 g (36%) of a yellow-green powder, m.p. 110˚C - 114˚C.

^{1}H NMR (DMSO-d_6), δ: 1.25 (m, 2H, CH_2), 1.44 (m, 2H, CH_2), 1.75 (m, 2H, CH_2), 2.12 (m, 2H, CH_2), 2.69 (s, 6H, $2CH_3$), 4.43 (m, 2H, CH_2), 7.15 (s, 2H, =CH), 7.79 (d, 2H, carb., J = 8.0 Hz), 8.22 (dd, 2H, carb., J = 8.0 Hz, J = 2.0 Hz), 9.21 (d, 2H, carb., J = 2.0 Hz). ^{19}F NMR (DMSO-d_6), δ, ppm: −83.4 (wid.t, 6F, CF_3, J = 12.0 Hz), −127.1 (m, 4F, CF_2); −129.4 (m, 4F, CF_2). Found, %: C 47.09; H 2.29; N 1.98. $C_{30}H_{21}F_{14}NO_6$. Calculated, %: C 47.57; H 2.79; N 1.85.

6-(3,6-Bis(7,7,7,6,6,5,5,4,4-nonafluoro-3-oxoheptanoyl)-9H-carbazol-9-yl)hexanoic acid (3c)

To a chilled (5˚C) suspension of lithium hydride (0.09 g, 11.3 mmol) in 30 mL of dry dioxane, methyl perfluoropentanoate (1.52 g, 5.50 mmol) was added, followed by **3**a (0.40 g, 1.1 mmol) in 10 mL of dry dioxane. The obtained mixture was refluxed for 84 h. The reaction mixture was then cooled to ambient temperature and poured into a mixture of ice and 4% sulfuric acid, followed by extraction with 3 × 20 mL of chloroform, washing with 2 × 10 mL of distilled water,

drying with sodium sulfate and evaporation to dryness under reduced pressure. The residue was dissolved in 30 mL of chloroform and refluxed for 1 h with activated charcoal. On filtering the charcoal, half of the solvent was evaporated and the product was precipitated with 20 mL of heptane. The yield was 0.55 g (58%) of a greed powder, m. p. 140˚C - 146˚C (dec.).

^{1}H NMR (DMSO-d_6), δ: 1.25 (m, 2H, CH_2), 1.44 (m, 2H, CH_2), 1.75 (m, 2H, CH_2), 2.12 (m, 2H, CH_2), 2.69 (s, 6H, $2CH_3$), 4.44 (m, 2H, CH_2), 7.14 (s, 2H, =CH), 7.80 (d, 2H, carb., J = 8.0 Hz), 8.24 (dd, 2H, carb., J = 8.0 Hz, J = 2.0 Hz), 9.19 (d, 2H, carb., J = 2.0 Hz). ^{19}F NMR (DMSO-d_6), δ: −82.2 (t, 6F, CF_3, J = 6.0 Hz), −121.3 (m, 4F, CF_2), −124.3 (m, 4F, CF_2), −127.0 (m, 4F, CF_2). Found, %: C 44.67; H 2.86; N 1.81. $C_{32}H_{21}F_{18}NO_6$. Calculated, %: C 44.82; H 2.47; N 1.63.

2-(3,6-Bis(4,4,4-trifluoro-3-oxobutanoyl)-9H-carbazol-9-yl)acetic acid (3d)

To a solution of **3**b (0.20 g, 0.65 mmol) in 8 mL of dry DMF at ambient temperature, lithium hydride (0.025 g, 3.23 mmol) was added and the mixture was stirred for 5 min. Methyl trifluoroacetate (0.21 g, 1.62 mmol) was subsequently added and the mixture was stirred for 20 hours without heating. The reaction mixture was poured into 50 mL of 5% HCl and extracted with 2 × 20 mL of ethyl acetate. The organic layer was washed with 2 × 10 mL of saturated brine, dried with sodium sulfate and evaporated under reduced pressure. The product was crystallized from 75 mL of chloroform. The yield was 0.24 g (74%) of a yellow powder, m. p. 263˚C - 268˚C (dec.).

^{1}H NMR (DMSO-d_6), δ: 1.25 (m, 2H, CH_2), 1.44 (m, 2H, CH_2), 1.75 (m, 2H, CH_2), 2.12 (m, 2H, CH_2), 2.69 (s, 6H, $2CH_3$), 4.44 (m, 2H, CH_2), 7.69 (d, 2H, carb., J = 8.0 Hz), 8.05 (dd, 2H, carb., J = 8.0 Hz, J = 2.0 Hz), 9.01 (d, 2H, carb., J = 2.0 Hz). ^{19}F NMR (DMSO-d_6), δ: −74.57 (s, 6F, $2CF_3$). Found, %: C 52.98; H 2.81; N 2.55. $C_{22}H_{13}F_6NO_6$. Calculated, %: C 52.71; H 2.61; N 2.79.

A general procedure for the preparation of Eu^{3+} complexes with carbazoles 3 and TOPO in Tris

A required amount of ligand **3** was dissolved in 2 mL of DMF to give a solution with a concentration of 5×10^{-3} M. An aliquot was taken and diluted with 0.05 M Tris (pH 7.8) to 10^{-5} M. Freshly prepared solutions of 10^{-4} M of Eu^{3+} in 10^{-5} M HCI and 2×10^{-4} M of TOPO in ethanol was added, subsequently diluted with the buffer to yield the final solution with the ligand:Eu:TOPO ratio of $(1.00:0.67:5.00) \times 10^{-6}$ M. The solution was left in the dark for 2 h. In order to prepare a micellar solution, 10% Triton X-100 in ethanol was added that the final concentration of the surfactant was 0.1%. For the study of luminescence properties, 100 μL portions were taken from the solution.

Conjugation of compound 3c with streptavidin

To a 6×10^{-3} M solution of compound **3**c in 875 μL of DMF, a 0.4 M solution of HOBt in 13.25 μL DMF and a 0.2 M solution of DCC in 26.5 μL DMF were added. The mixture was stirred for 15 min. Then, a 0.2 M solution of NHS in 26.5 μL DMF was added and the stirring continued for 19 hours at ambient temperature (20˚C). To perform the conjugation with streptavidin, to 436 μL of

the solution of the protein with a concentration of 1.55 mg/mL in 0.05 M phosphate buffer (pH 8.0), 41 μL of the activated ligand solution was added, followed by 3.5 h of stirring at ambient temperature. The conjugate **5** was purified on a column with Sephadex G25, eluting with 0.05 M tris buffer (pH 7.8), flow rate 14 mL/h. The volume of the fractions collected was 0.5 mL.

Synthesis of luminescent silica nanoparticles

Aqueous dispersion of "Aerosil 380" (0.1%, 10 mL) was preliminary sonicated for 4 hours in order to break aggregates, then diluted to 100 mL. While the mixture was stirred at ambient temperature, gradual (30 - 40 min) addition of 1 mL of earlier prepared DMF solution of Eu^{3+} - tetraketone **3**a complex in the presence of luminescence synergist trioctylphosphine oxide (TOPO) was performed (C_{Eu}^{3+}:C_{3a}:C_{TOPO}; 1.6×10^{-2}:2.4×10^{-2}:4.8×10^{-2} mol/L). The formed dispersion was subjected to purification via 3 cycles of dialysis, 10 h each. The procedure was conducted in dialysis tubes, 10 mL of dispersion against 1 L of distilled and deionized water. The intensity of luminescence of the obtained dispersion is comparable to the one of the separately prepared tetraketone 3a complex, but the stability of luminescence is significantly greater (>6 months).

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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