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Synthesis of a new tetradentate β-diketonate–europium chelate and its application for time-resolved fluorimetry of albumin[1]

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Abstract

A new tetradentate β-diketone chelator, 1,10-bis(8′-chlorosulfo-dibenzothiophene-2′-yl)-4,4,5,5,6,6,7,7,-octafluorodecane-1,3,8,10-tetraone (BCOT), was synthesized. This compound forms a stable chelate with europium(III) and can be used as a fluorescence label for time-resolved fluorescence spectrometry. BCOT can be covalently bound to a protein under relatively mild conditions and, when complexed with europium(III), both in 0.1 M Tris–HCl solution and 1.0×10^{-5} M tri-*n*-octyl phosphine oxide (TOPO)–0.05% sodium *n*-dodecyl sulfate (SDS)–0.1 M $NaHCO_3$ solution, forms a strongly fluorescent chelate having lifetimes of 225 μs and 240 μs, respectively. As a model reaction, bovine serum albumin (BSA) was labeled with BCOT in a carbonate buffer solution. Fluorescence properties of the labeled BSA solution were studied. A time-resolved fluorescence determination of the $BSA(BCOT)_{40}$–Eu^{3+} solution was carried out. Detection limits of 9.3×10^{-14} M (TOPO–SDS–$NaHCO_3$ solution) and 6.7×10^{-13} M (Tris–HCl solution) for BSA were obtained by the method. © 1997 Elsevier Science B.V.

Keywords: Tetradentate β-diketone–europium chelate; Time-resolved fluorimetry

1. Introduction

The fluorescence of some europium complexes represents four distinct properties: the complex absorb light at wavelengths typical of the ligand absorption usually in the UV region; the lifetimes are very long (> 100 μs); the complexes exhibit a large Stokes shift that is usually larger than 250 nm; and the emission peaks always lie near 615 nm with very sharp peak profiles with a FWHM of approximately 10 nm. These distinct properties of europium complexes have found use as fluorescent labels in the time-resolved fluoroimmunoassay (TR-FIA) of biological materials such as antibodies, antigens or DNA [1–6]. TR-FIA has attained a high sensitivity that surpasses that of radioimmunoassay by reducing the background fluorescence originating from the samples [7]. One of the two commercially available TR-FIA systems is the dissociation-enhanced lanthanide fluoroimmunoassay system (Delfia, LKB Wallac and Pharmacia), in which antibodies labeled with isothiocyanatophenyl–EDTA–Eu(III) are used

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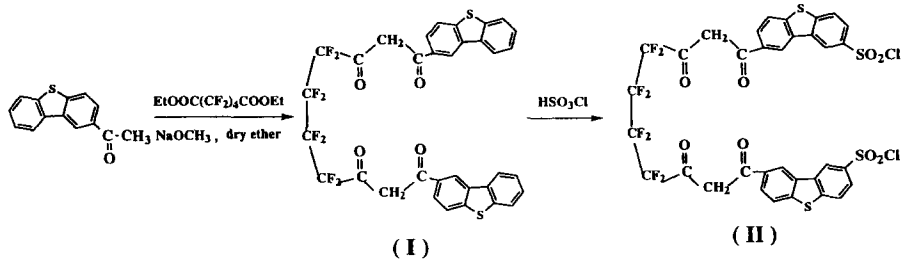


(I) (II)

Fig. 1. Schematic presentation of the synthesis of BCOT.

for immunoreactions; after the reaction, Eu(III) is eluted from the EDTA on the antibodies with a 2-naphthoyltrifluoroacetonate (β-NTA)–trioctylphosphine oxide (TOPO)–Triton X-100 solution. The Eu(III)–β-NTA–TOPO ternary complex thus formed in the solution is subjected to fluorimetric measurement. While the LKB system is highly sensitive, it has several drawbacks. These include: (1) the solution measured contains excess β-NTA and TOPO, thus is vulnerable to contamination by Eu in the environment, which leads to background fluorescence; (2) the assay needs conversion of the non-fluorescent europium label complex into a fluorescent β-NTA–Eu–TOPO complex, which makes the whole procedure tedious and time-consuming. Owing to this conversion step, solid-phase measurement is impossible with LKB [2]. In the second system, CyberFluor, the protein to be determined is directly labeled with a fluorescent Eu chelate, 4,7-bis(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid (BCPDA)–Eu(III). In this system, the two main drawbacks of the LKB system are overcome; however the label is not so intensely fluorescent as the β-NTA–Eu–TOPO complex [8]. Recently a new β-diketone–Eu(III) label that can be covalently bonded to proteins, 5-chlorosulfonyl-2-thenoyltrifluoroacetonate (CTTA)–Eu(III), has been reported [9–11]. Although this has overcome several drawbacks of the two systems to some extent, its use has problems since the fluorescence intensity is not very high and the complex is not very stable in solid-state measurement.

The fluorescence intensity of Eu(III)–β-diketonate complexes is reported to depend strongly on the structure of the β-diketonate, ($R_1COCHCOR_2^-$). In order to attain strong fluorescence, both electron donation of R_1 and electron withdrawal of R_2 must be strong. The fluorescence intensity and the fluorescence quantum yield of Eu(III) complexes increases in the order $R_2 = CF_3 < CF_2CF_3 < CF_2CF_2CF_3$ [12].

Based upon the tendency described above, a new tetradentate β-diketone chelating ligand for Eu(III), 1,10-bis(8′-chlorosulfodibenzothiophene-2′-yl)-4,4,5,5,6,6,7,7-octafluorodecane-1,3,8,10-tetraone (BCOT, Fig. 1-II) has been synthesized. BCOT can bind to amino groups of proteins in carbonate buffer at room temperature and forms a stable complex with Eu(III) with its four oxygen atoms of the two β-diketonate groups. BCOT has a further advantage that, since it has two diketone groups, its 1:1 complex corresponds to the 1:2 (Eu(III):β-diketone group) complexes of hitherto used β-diketones such as β-NTA and CTTA. Therefore, stronger fluorescence can be expected. As an example of the usage of BCOT in bioanalysis, bovine serum albumin (BSA) was labeled with BCOT and the Eu^{3+} complex was formed on the protein by addition of Eu^{3+}. The fluorescence characteristics of the labeled BSA was examined and the time-resolved fluorimetric assay of BSA was carried out.

2. Experimental

2.1. Preparation of 1,10-bis(dibenzothiophene-2′-yl)-4,4,5,5,6,6,7,7-octafluorodecane-1,3,8,10-tetraone

The starting material, 2-acetyl-dibenzothio-

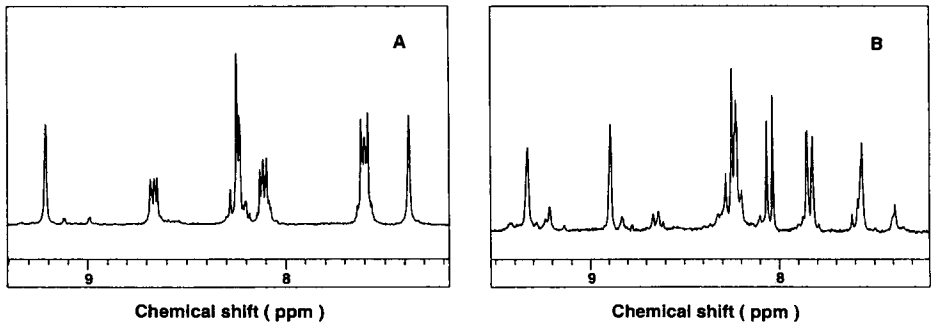


Fig. 2. ^{1}H-NMR spectra of 1,10-bis(dibenzothiophene-2′-yl)-4,4,5,5,6,6,7,7-octafluorodecane-1,3,8,10-tetraone (A, DMSO-d_6/TMS) and BCOT (B, DMSO-d_6/TMS).

phene, was prepared according to the published method [13,14], and was confirmed by the ^{1}H-NMR spectrum and m.p.

1,10-Bis(dibenzothiophene-2′-yl)-4,4,5,5,6,6,7,7-octa-fluorodecane-1,3,8,10-tetraone was prepared by the Claisen condensation [15,16] of 2-acetyl-dibenzothiophene with diethyl perfluoroadipate (Kanto Chem., > 99%) in dry ether with a catalytic amount of $NaOCH_3$, as follows. Dry ether (50 g) containing 3.0 g $NaOCH_3$ and 3.46 g (10 mmol) diethyl perfluoroadipate and 4.52 g (20 mmol) 2-acetyl-dibenzothiophene in a stoppered flask was stirred at room temperature for 24 h. Ether was removed by evaporation, and the residue was vacuum-dried. The residue was neutralized with 100 ml 15% H_2SO_4, filtered and washed thoroughly with water. After vacuum-drying, the solid was recrystallized from 1,4-dioxane; 3.32 g of the compound was obtained after final vacuum drying (yield 47%), Calculated analysis for $C_{34}H_{18}F_8O_4S_2$: C, 57.79; H, 2.57. Found: C, 57.62; H, 2.51. The product was confirmed by ^{1}H-NMR spectrum (TMS as a reference) measured on a JEOL 270 (Fig. 2A).

2.2. Preparation of BCOT

While stirring 5 ml of $ClSO_3H$ in a ice-water bath, 1.41 g (2 mmol) 1,10-bis(dibenzothiophene-2′-yl)-4,4,5,5,6,6,7,7-octafluorodecane-1,3,8,10-tetraone was gradually added to the solution. After stirring for 4 h at 0°C, the solution was added dropwise to 150 ml H_2O–ice, which was cooled with an ice-water bath. The precipitate formed was separated by centrifugation, washed twice with cold water and then twice centrifuged. The precipitate was transferred to a glass filter with the addition of a small amount of cold water and then vacuum-filtered. After vacuum-drying, 1.65 g of BCOT was obtained (yield 86%). Calculated analysis for $C_{34}H_{22}F_8O_9S_4Cl_2$ (BCOT·$3H_2O$): C, 42.64; H, 2.31; S, 13.39; Cl, 7.40. Found C, 42.38; H, 1.91; S, 14.10; Cl, 6.36. The experimental value for Cl is slightly less and that for S is slightly larger than the corresponding calculated values. This would have been caused by partial hydrolysis of the chlorosulfonyl group of BCOT to sulfonic acid during the analytical procedure. The product was confirmed by ^{1}H-NMR spectrum (Fig. 2B), and FAB-MS (*m/e*, 903 $[M]^+$, 804 $[M-SO_2Cl]^+$). The hydrolysis product of BCOT was not observed in FAM-MS. BCOT was kept in a desiccator under a light shield.

2.3. Labeling of BSA with BCOT

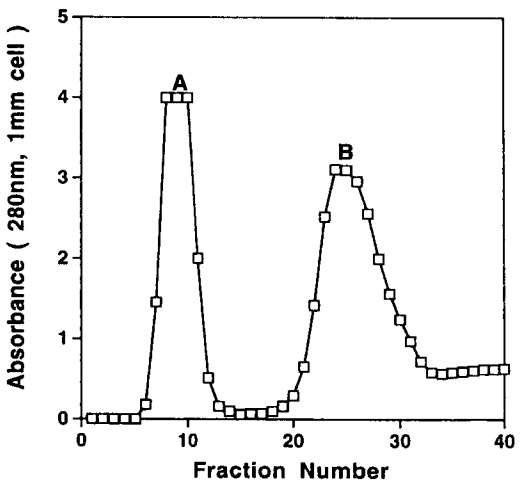
While stirring 82.7 mg BCOT (0.0864 mmol) in 1.0 ml dry DMF, 1.0 ml DMF containing 0.128 M $NH_2CH(CH_3)_2$ and 0.150 M $N(C_2H_5)_3$ was added dropwise. After stirring for 20 min at room temperature, the DMF solution was added dropwise to 10 ml 0.1 M carbonate buffer (pH 9.3) containing 50 mg BSA, with stirring. The mixture was further stirred for 1 h at room temperature.

After completion of the labeling, 3 ml of the reaction solution was chromatographed on a Sephadex G-50 column (1.0 cm × 29.1 cm), which

separated the labeled BSA from the unreacted BCOT and its hydrolyzed products. The eluent was 0.05 M NH_4HCO_3 (pH 8.0) and 40 fractions each of 1 ml were collected. The chromatogram with the absorption measured at 280 nm (1 mm cell) is shown in Fig. 3. The BSA fractions were collected and dialyzed at 4°C against water for 24 h to remove inorganic salts. After dialysis, the solution was transferred to a flask and kept at 4°C. The molar extinction coefficient (ε) of BCOT at 330 nm was measured by using the reaction solution before the gel-chromatographic separation. Assuming that the ε of BCOT does not change on labeling, the concentration of BCOT in the labeled BSA solution and the BCOT to BSA binding ratio was determined.

2.4. Time-resolved fluorescence measurement of the labeled BSA–Eu^{3+} solution

Standard solutions of BSA(BCOT)$_n$–Eu^{3+} for calibration were prepared by adding a BSA (BCOT)n solution to either 0.1 M Tris–HCl (pH 9.1) or 0.1 M $NaHCO_3$ (pH 8.4) containing 1.0×10^{-5} M TOPO and 0.05% SDS, and a 1.0×10^{-5} M $EuCl_3$ aqueous solution was added to all the solutions. The Eu^{3+} concentration was fixed at 1.0×10^{-6} M for all the solutions while the concentration of BSA(BCOT)$_n$ was varied. The prepared standard solutions were left at room temperature for 2 h and were then used for measurement. Each solution was pipetted into four polystyrene wells (300 µl per well) and the average fluorescence intensity (I) of the four wells was used. Likewise, the solvent containing 1.0×10^{-6} M Eu^{3+} was pipetted into 4 wells and the average measurement value (I_0) was used as the background intensity. The values ($I - I_0$) were used in the calibration.



ig. 3. Separation of labeled BSA (peak A) from unreacted BCOT derivatives (hydrolyzed product and amidation product, peak B) by gel filtration chromatography. Mobile phase, 0.05 M NH_4HCO_3 at pH 8.0, 1.0 × 29.1 cm Sephadex G-50 column, flow-rate 1.0 ml per 90s. Fractions collected were 1 ml each.

2.5. Instruments and method of measurement

The UV spectra were measured on a Shimadzu UV 260 spectrometer. The fluorescence spectra were recorded on a Hitachi F-4500 fluorescence spectrophotometer with a 150 W Xenon lamp. The fluorescence lifetime (τ) was measured using a Lambda Physik LPX 100 Excimer Laser pulse light source and a SPEX HR320 monochromator. I was measured as a function of delay time (t) and τ was determined according to the equation, $\ln I(t) = \ln I(0) - t/\tau$ [2]. The lifetime measurement was carried out with a 0.1 M Tris–HCl buffer solution at pH 9.0 containing $[BSA(BCOT)_{40}] = 1.8 \times 10^{-7}$ M and $[Eu^{3+}] = 5.0 \times 10^{-5}$ M. Time-resolved fluorescence measurements were carried out on a CyberFluor 615 fluorimeter with a nitrogen laser at 337 nm excitation. The fluorescence of 615 nm was measured with a delay time of 200 µs and a counting time of 200–600 µs.

3. Results and discussion

3.1. Labeling of BSA

Since BCOT has two sulfonyl chloride groups, direct labeling of BSA with BCOT may cause an undesirable protein–protein crosslinking reaction leading to polymer formation of the protein. In order to avoid this reaction, one of the sulfonyl chloride groups was protected by reaction with $NH_2CH(CH_3)_2$ in DMF in the presence of

$N(C_2H_5)_3$. In order to protect at least one of the sulfonyl chloride groups, an excess amount of $NH_2CH(CH_3)_2$ was used. Therefore, after the reaction, either one or both sulfonyl chloride groups are protected, and there is no or very little unprotected BCOT in the DMF solution. For BCPDA labeling, which also has two $ClSO_2^-$ groups, no polymer formation was detected without the protection reaction [17]. In the present β-diketone system, the protection is carried out to avoid polymer formation and increase the fluorescence intensity [9,11].

While the labeling of BSA with BCOT proceeds easily in an aqueous carbonate buffer solution (pH 9.0–9.5) [9,17] and a higher DMF concentration is advantageous to dissolve BCOT, the labeling reaction may be hampered in a mixture of DMF and H_2O. Therefore, the concentration of DMF was kept as low as possible, i.e. less than 20%. Since hydrolysis of sulfonylchloride of BCOT occurs in parallel with the labeling reaction, several times the amount of BCOT in excess of the amino-group of BSA (59 NH_2 groups per molecule [17]) is necessary to obtain a solution of BSA(BCOT)$_n$ with a high labeling ratio n. The unreacted labeling reagents (hydrolysis products of BCOT and the BCOT with both of the two sulfonyl groups amidated) were separated from the labeled BSA by gel filtration chromatography (Fig. 3). The labeling ratio n was determined by measuring the absorbance of the labeled BSA solution (ε of BCOT $= 3.2 \times 10^4$ cm^{-1} M^{-1}), assuming that all the BSA added had been recovered from the chromatography. By varying the amount of BCOT relative to BSA in the labeling reaction, batches of BSA(BCOT)$_n$ ($n \leq 40$) with various n's were obtained. For instance, BSA(BCOT)$_{40}$ was prepared by using 247.7 mg (0.258 mmol) of BCOT, 0.300 M of $NH_2CH(CH_3)_2$, and 0.450 M of $N(C_2H_5)_3$ in DMF.

Although BCOT is very gradually hydrolyzed, it can be stored for a year in a desiccator. The labeling procedure described above gives a highly reproducible labeling ratio to BSA. The R.S.D. for 5 times labeling was less than 5% as determined by ε measurement of the labeled BSA.

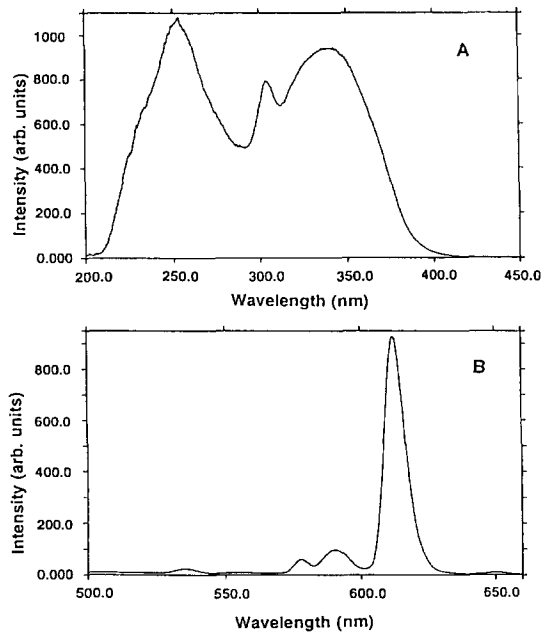


Fig. 4. Excitation (A) and emission (B) spectra of BCOT-labeled BSA in the presence of Eu^{3+}, [BSA(BCOT)$_{40}$] $= 2.2 \times 10^{-8}$ M, [Eu^{3+}] $= 2.5 \times 10^{-6}$ M. The buffer solution was 0.1 M Tris–HCl at pH 9.1.

3.2. Fluorescent properties of the labeled BSA in the presence of Eu(III)

Since BCOT has 4 oxygen atoms of the two β-diketone groups, two six-membered chelate rings are formed when $EuCl_3$ is added to the solution of BSA(BCOT)$_n$ and a stable, highly fluorescent Eu^{3+} complex is produced.

The fluorescence spectra of BSA(BCOT)$_n$–Eu^{3+} complex are shown in Fig. 4. BSA(BCOT)$_n$–Eu^{3+} has excitation maxima at 253 and 339 nm and a fluorescent maximum at 612 nm, which corresponds to $^5D_0$–$^7F_2$ transition of Eu^{3+} and has a FWHM of approximately 9 nm in Tris–HCl buffer. In phosphate buffer, excitation maxima of 253 and 332 nm and a fluorescent maximum of 611.6 nm were observed. In TOPO–SDS–carbonate buffer, the excitation maxima were 253 and 341 nm, whereas the fluorescent maximum was 613.5 nm. These spectral features did not change on varying the labeling ratio (n) of BSA(BCOT)$_n$. The above slight spectral depen-

Table 1
Effects of pH and buffer on the fluorescent intensity[a] of BSA(BCOT)$_n$–Eu^{3+}

Buffer solution	0.1 M Tris–HCl					0.1 M carbonate	0.1 M phosphate	1.0×10^{-5} M TOPO–0.05% SDS–0.1 M $NaHCO_3$
pH	7.2	7.8	8.5	9.1	9.9	9.3	9.0	8.4
Relative intensity	54	72	92	100	89	76	37	621

[a] The excitation wavelength was 337 nm and the solution was $[BSA(BCOT)_{40}] = 2.2\times10^{-8}$ M and $[Eu^{3+}] = 2.5\times10^{-6}$ M.

dence on the solution composition may be due to the additional coordination of Tris or TOPO to Eu^{3+}.

The effect of pH and buffer on the fluorescent intensity is shown in Table 1. The intensity becomes maximum at pH about 9 in Tris–HCl. Similar dependence on pH and buffer solution was observed for other Eu^{3+}-labeling reagents [11,17] and might be due to the diacidic nature of BCOT. The fluorescence intensity in various buffers was in the order, TOPO–SDS—$NaHCO_3 \gg$ Tris–HCl > carbonate > phosphate. The marked intensity increase in TOPO–SDS–$NaHCO_3$ is due to the 'synergic effect' of TOPO, which forms a ternary complex Eu(III)–β-diketone-TOPO [18,19]. The relatively high intensity in Tris–HCl might be due to the coordination of Tris to Eu^{3+}. In respect of coordination, the Eu^{3+}–β-diketone–glyme complex is reported to be highly stable [20].

It was confirmed that dissolved oxygen does not affect the fluorescent intensity. The fluorescence lifetimes of BSA(BCOT)$_{40}$–Eu^{3+} in Tris–HCl and TOPO–SDS–$NaHCO_3$ were 225 and 240 μs, respectively.

3.3. Time-resolved fluorimetric measurement of BSA(BCOT)$_n$–Eu^{3+}

The calibration curves for BSA(BCOT)$_{40}$–Eu^{3+} obtained by time-resolved fluorometric measurements in 0.1 M Tris–HCl (pH 9.1) and 1.0×10^{-5} M TOPO–0.05% SDS–0.1 M $NaHCO_3$ (pH 8.4) are shown in Fig. 5, from which detection limits (3σ) of BSA were obtained as 6.7×10^{-13} and 9.5×10^{-14} M, respectively. These values correspond to BCOT concentrations of 2.7×10^{-11} and 3.7×10^{-12} M, respectively. Since the detection limit of Eu^{3+} in the Eu^{3+}–β-NTA–TOPO system obtained on the same fluorimeter was 5.6×10^{-11} M, the new chelate has a higher sensitivity than that of the Eu^{3+}–β-NTA–TOPO system. It is also expected to give excellent sensitivity in immunoassays if used as a label in combination with the methods proposed in the literature [3,8,10,21,22].

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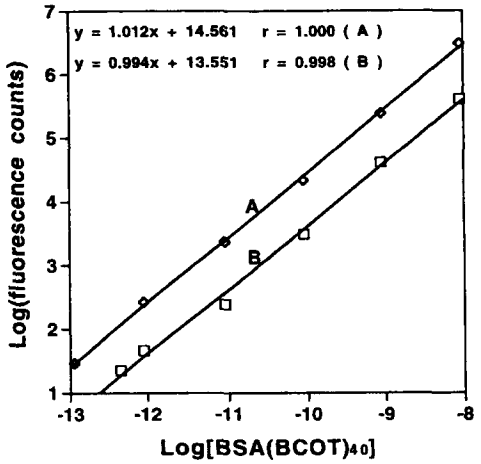

Fig. 5. Log (fluorescence counts) vs. log[BSA(BCOT)$_{40}$] (M) in the presence of 1.0×10^{-6} M of Eu^{3+}. A, 1.0×10^{-5} M TOPO–0.05% SDS–0.1 M $NaHCO_3$, B; 0.1 M Tris–HCl of pH 9.1.

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References

[1] E. Soini, T. Lövgren, CRC Crit. Rev. Anal. Chem. 18 (1987) 105–154.
[2] E.P. Diamandis, Clin. Biochem. 21 (1988) 139–150.
[3] E.P. Diamandis, T.K. Christopoulos, Anal. Chem. 62 (1990) 1149A–1157A.
[4] I. Hemmilä, Appl. Fluoresc. Technol. 1 (1989) 1–8.
[5] A. Mayer, S. Neuenhofer, Angew. Chem. Int. Ed. Engl. 33 (1994) 1044–1072.
[6] E. Lopex, C. Chypre, B. Alpha, G. Mathis, Clin. Chem. 39 (1993) 196–201.
[7] Y.-Y. Xu, I. Hemmilä, T. Lövgren, Analyst 117 (1992) 1061–1069.
[8] R.C. Morton, E.P. Diamandis, Anal. Chem. 62 (1990) 1841–1845.
[9] Y.X. Ci, X.D. Yang, Chin. Chem. Lett. 3 (1992) 1007–1010.
[10] X.D. Yang, Y.X. Ci, W.B. Chang, Anal. Chem. 66 (1994) 2590–2594.
[11] Y.X. Ci, X.D. Yang, W.B. Chang, J. Immunol. Methods 179 (1995) 233–241.
[12] J. Yuan, K. Matsumoto, Anal. Sci. 12 (1996) 31–36.
[13] A. Burger, W.B. Wartman Jr., R.E. Lutz, J. Am. Chem. Soc. 60 (1938) 2628–2630.
[14] H. Gilman, A.L. Jacoby, J. Org. Chem. 3 (1938) 108–119.
[15] J.C. Reid, M. Calvin, J. Am. Chem. Soc. 72 (1950) 2948–2952.
[16] M. Das, Inorg. Chim. Acta 48 (1981) 33–36.
[17] R.A. Evangelista, A. Pollak, B. Allore, E.F. Templeton, R.C. Morton, E.P. Diamandis, Clin. Biochem. 21 (1988) 173–178.
[18] F. Halverson, J.S. Brinen, J.R. Leto, J. Chem. Phys. 41 (1964) 157–163.
[19] F. Halverson, J.S. Brinen, J.R. Leto, J. Chem. Phys. 41 (1964) 2752–2760.
[20] S.R. Drake, A. Lyons, D.J. Otway, A.M.Z. Slawin, D.J. Williams, J. Chem. Soc., Dalton Trans. (1993) 2379–2386.
[21] E.P. Diamandis, R.C. Morton, E. Reichstein, M.J. Khosravi, Anal. Chem. 61 (1989) 48–53.
[22] J. Kropf, E. Quitte, A.M. Gressner, Anal. Biochem. 197 (1991) 258–265.