Synthesis and fluorescent properties of new derivatives of 4-amino-7-nitrobenzofurazan

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Abstract
The following new compounds were obtained by reacting 4-chloro-7-nitrobenzofurazan (NBD-Cl, 1) with five primary amines: 3b with a benzo-crown ether 18C6; 3c with an N-(α-naphthyl)-ethylenediamine group; 3d, with a 2,2,6,6-tetramethylpiperidin-N-oxyl group; 3e, with an α-picoyl group; and 3f, derived from tris(hydroxymethyl)aminomethanol. Also, from the reaction of 1 with N-methylhydroxylamine an N-hydroxy-N-methyl-NBD derivative (3g) was prepared. All these six new NBD derivatives 3b-g were studied (in comparison with the known compound 3a prepared from 1 and aniline) for their physical and chemical properties, with special emphasis on hydrophobiciticy, UV-Vis, fluorescence, using also structural studies trough QSPR.

Keywords: 4-Amino-7-nitrobenzoxadiazole derivatives, UV-Vis, fluorescence, EPR, hydrophobicity, QSPR

Introduction

Many 4-substituted-7-nitro-2,1,3-benzoxadiazoles (NBD derivatives) have a strong fluorescence which has led to their use in bioanalytical chemistry. The benzoxadiazoole ring system also been called 3,4-benzo-1,2,5-oxadiazoole or benzofurazan. The usual synthesis is based on the
nucleophilic substitution of halogens from 4-halo-7-nitrobenzofurazan, with the halogen being either chlorine (NBD-Cl) or fluorine. Some of these compounds have biological activity as antileukemic, immunosuppressive, or monoamine oxidase inhibiting activity. Previous papers from our laboratories have reported studies of NBD derivatives having 4-aryloxy, 4-formyaryloxy groups or with amino acids. In the present article we describe the synthesis of six new NBD derivatives (3b–3g) prepared from NBD-Cl (1) and five primary amines (2b–2f) or N-methylhydroxylamine (2g). Their properties are described and compared with the known compound 3a obtained from 1 and aniline (2a). Spectral characterization was performed by 1H- and 13C-NMR spectrometry, IR and UV-Vis absorption spectroscopy, electron spin resonance (EPR for 3d), and the hydrophobicity was measured by reverse phase thin-layer chromatography (RP-TLC).

**Results and Discussion**

**Synthesis of compounds 3a–3g**

Starting from 1 and primary amines such as aniline (2a), amino-benzo-crown[18C6] (2b), \( N-(\alpha\text{-naphthyl})\)-ethylenediamine dihydrochloride (2c), 4-amino-2,2,6,6-tetramethylpiperidin-\(N\)-oxyl (4-amino-TEMPO, 2d), 2-(methylamino)pyridine (2e), tris(hydroxymethyl)aminomethane (2f) or from N-methylhydroxylamine hydrochloride (2g), the NBD derivatives 3a–3g displayed in Table 1 were prepared. The reaction was carried out in a convenient solvent (methanol, ethanol, acetonitrile), under heating. For 3b–3e and 3g the addition of sodium hydrogen carbonate was needed. The appearance of a red-brown color and theoretical studies prove the intermediacy of Meisenheimer complex (Scheme 1), according to the literature data; in our case, the red-brown colored reaction medium, by treating with acid, turned to yellow-orange, thus proving the conversion of the Meisenheimer complex into the reaction product which could be isolated. The crystalline 3a was obtained directly, but the other compounds needed purification by preparative TLC.

![Scheme 1. Synthesis of compounds 3a – 3g.](image-url)
Table 1. Structures of the NBD derivatives 3a – g

| Compound | R¹     | R²    |
|----------|--------|-------|
| 3a       | H      | ![Structure 3a](image) |
| 3b       | H      | ![Structure 3b](image) |
| 3c       | H      | ![Structure 3c](image) |
| 3d       | H      | ![Structure 3d](image) |
| 3e       | H      | ![Structure 3e](image) |
| 3f       | H      | ![Structure 3f](image) |
| 3g       | ![8 CH₃](image) | ![OH](image) |

NMR Spectra of compounds 3a–g

The NMR data of compounds 3a-c, 3e-g (Table 2) confirm the proposed structure.
Table 2. $^1$H-NMR and $^{13}$C-NMR data of compounds 3a-g ($\delta$ ppm; $J$ Hz)

| Compound | NMR-spectra |
|----------|-------------|
| 3a       | $^1$H-NMR (CDCl$_3$, $\delta$ ppm, $J$ Hz): 8.46(d, 1H, H-5, 8.6); 7.9-8.0(br, 1H, NH, deuterable); 7.54(dd, 2H, H-3’-5’, 7.8, 8.5); 7.44(dd, 2H, H-2’-6’, 1.3, 8.5); 7.38(tt, 1H, H-4’, 1.3, 7.8); 6.75(d, 1H, H-6, 8.6). $^1$C-NMR(CDCl$_3$, $\delta$ ppm): 144.73(C-4); 143.84(C-3a); 141.09(C-1’); 136.60(C-7); 125.57(C-1a); 136.03(C-5); 130.13(C-3’-5’); 127.28(C-4’) 123.59(C-2’-6’); 100.93(C-6). |
| 3b       | $^1$H-NMR (CDCl$_3$, $\delta$ ppm, $J$ Hz): 8.41(d, 1H, H-5, 8.7); 8.20-8.30(br, 1H, NH, deuterable); 6.95-6.90(m, 3H, H-2’-5’-6’); 6.55(d, 1H, H-6, 8.7); 4.18(m, 4H, H-41-50); 3.95(m, 4H, CH$_2$); 3.78(m, 4H, CH$_2$); 3.70(s, 4H, H-45-46). $^1$C-NMR(CDCl$_3$, $\delta$ ppm): 149.57(C-3’); 147.96(C-4’); 144.54(C-4); 143.93(C-3a); 142.15(C-7); 129.62(C-1’); 124.79(C-1a); 136.27(C-5); 117.05(C-6’); 113.69(C-5’); 109.92(C-2’); 100.75(C-6); 71.04(CH$_2$); 70.62(CH$_2$); 70.51(CH$_2$); 70.44(CH$_2$); 70.27(CH$_2$); 69.43(CH$_2$); 69.26(CH$_2$); 69.20(CH$_2$); 68.82(CH$_2$). |
| 3c       | $^1$H-NMR (dmso-d$_6$, $\delta$ ppm, $J$ Hz): 9.50(br, 1H, NH, deuterable); 8.42(d, 1H, H-5, 8.9); 8.06(dd, 1H, H-18, 1.0, 7.1); 7.74(dd, 1H, H-15, 1.2, 6.4); 7.40(m, 2H, H-16-17); 7.29(t, 1H, H-12, 7.7); 7.11(d, 1H, H-13, 7.7); 6.62(1H, H-11, 7.7); 6.41(d, H-6, 8.9); 3.80(br, 2H, H-9); 3.60(t, 2H, H-8, 5.8). $^1$H-NMR (CDCl$_3$, $\delta$ ppm, J Hz): 8.39(d, 1H, H-5, 8.5); 7.84(m, 2H, H-13-15); 7.52÷7.35(m, 5H, H-11-12-16-17-18); 6.19(d, 1H, H-6, 8.6); 6.86(bs, 1H, H-8', deuterable); 6.70(bs, 1H, H-9', deuterable); 3.92(bs, 2H, H-9, 6.2). $^{13}$C-NMR (dmso-d$_6$, $\delta$ ppm): 145.32(Cq-10); 144.38(Cq-4); 143.47(Cq-3a); 137.92(Cq-7); 134.08(Cq-14); 123.07(Cq-1a); 120.86(Cq-19); 137.81(C-5’); 99.23(C-6’); 128.03(C-15’); 126.78(C-12); 125.70(C-17); 124.08(C-18); 121.44(C-16); 115.91(C-13); 103.13(C-11); 42.25(C-8 or C-9); 41.41(C-9 or C-8). |
| 3c in TFA | $^1$H-NMR (CDCl$_3$+TFA, $\delta$ ppm, $J$ Hz): 8.39(d, 1H, H-5, 8.7); 8.02(dd, 1H, H-11, 8.4, 0.9); 7.98(m, 2H, H-18-16); 7.70(dd, 1H, H-13, 0.9, 7.6); 7.65(m, 2H, H-15-17); 7.52(dd, 1H, H-12, 7.7, 8.8); 6.26(d, 1H, H-6, 8.7); 4.18(s, 4H, H-8 and H-9). $^{13}$C-NMR(CDCl$_3$+TFA, $\delta$ ppm): 144.13(Cq-10); 143.83(Cq-4); 143.43(Cq-3a); 134.69(Cq-7); 134.68(Cq-14); 124.93(Cq-1a); 124.33(Cq-19); 136.81(CH-5); 131.75(CH); 129.78(CH); 129.08(CH); 128.01(CH); 125.24(CH); 121.44(CH); 118.55(CH); 100.62(CH-6); 51.08(CH-9); 40.00(CH-8). |
| 3e       | $^1$H-NMR (dmso-d$_6$, $\delta$ ppm, J Hz): 9.9(br, 1H, HN); 8.53(dd, 1H, H-13, 1.0, 4.6); 8.5(d, 1H, H-5, 8.9); 7.78(dd, 1H, H-11, 7.8, 1.0); 7.42(d, 1H, H-10, 7.8); 7.31(dd, 1H, H-12, 4.6, 7.8); 6.33(d, 1H, H-6, 8.9); 4.80(br, 2H, H-8). $^{13}$C-NMR (dmso-d$_6$, $\delta$ ppm): 156.26(C-9’); 149.39(C-13); 145.17(C-4’); 144.12(C-3a); |
138.61(C-7); 137.97(C-5); 137.36(C-11); 122.98(C-10); 121.80(C-12); 125.97(C-1a); 100.09(C-6); 48.31(C-8).

$^1$H-NMR (dmsø-d$_6$, δ ppm, J Hz): 8.51(d, 1H, H-5, 9.0); 7.54(br, 1H, NH, deuterable); 6.86(d, 1H, H-6, 9.0); 5.13(t, 3H, HO, deuterable, 5.4); 3.77(d, 6H, H-9-10, 11, 5.4).

$^{13}$C-NMR(dmsø-d$_6$, δ ppm): 145.39(C-4); 144.52(C-3a); 143.88(C-7); 137.93(C-5); 121.13(C-1a); 102.30(C-6); 64.73(C-9-10-11); 64.13(C-8).

Assignments in Table 2 are using the atom numbering indicated in Table 1. No NMR data are reported for the paramagnetic compound 3d. Compound 3c, with two amino groups, is converted into an ammonium salt by protonation of the naphthylamino group (the strongly electron-withdrawing NBD group cancels the basicity of the adjacent amino group). The changes in the NMR spectra are evident – there are significant differences for C-9 and protons H-8 and H-9 in the ethylene group, small changes for H-11 in the naphthyl group, and no changes in the NBD moiety.

**Hydrophobic/hydrophilic balance of compounds 3a–g**

All biological uses of chemical compounds depend on how they interact with biomembranes, and such interactions are governed by the hydrophobic/hydrophilic balance, so that we had to include such effects in the present study. Following previous reports$^{25,26}$ the hydrophobic/hydrophilic balance of compounds 3a–g was studied experimentally by reverse phase TLC (RP-TLC), a simple, efficient, and precise method. The molecular hydrophobicity $R_{M0}$ was determined by means of equations (1) and (2), using the data presented in Table 3.

$$R_M = \log(\frac{1}{R_f} - 1)$$  \hspace{1cm} (1)

$$R_M = R_{M0} + bK$$  \hspace{1cm} (2)

The hydrophobicity of compounds 3a–g decreases in the order 3c > 3a > 3d > 3e > 3g > 3b > 3f (hydrophilicity increasing obviously in the reverse order). The NBD group has log P = 1.69.$^{35}$ The remaining $R^1R^2N$ moiety combines its effect leading to increased hydrophobicity due to the presence of phenyl, naphthyl, pyridine and 2,2,6,6-tetramethylpiperidyl (3a, 3c-e) or to decreased hydrophobicity in the presence of OH groups and the crown ether macrocycle (3b, 3f, 3g).
Table 3. Experimental hydrophobicity (RM0, b) and calculated (log P) for 3a – g

| Comp. | RM in aqueous ethanol, conc.(v/v) | Exp. | Statistical parameters | Calcd. |
|-------|-----------------------------------|------|------------------------|--------|
|       | 80%  | 70%  | 60%  | 50%  | RM0 | b  | R  | F  | SD  | log P |
| 3a    | -0.508 | -0.281 | 0.067 | 0.407 | 1.932 | -0.031 | -0.996 | 238 | 0.045 | 4.39 |
| 3b    | 0.103  | 0.097  | 0.216 | 0.320 | 0.685 | -0.010 | -0.939 | 14  | 0.145 | 3.34 |
| 3c    | -0.447 | -0.165 | 0.301 | 0.733 | 2.708 | -0.040 | -0.995 | 202 | 0.063 | 5.18 |
| 3d    | -0.574 | -0.281 | 0.067 | 0.322 | 1.857 | -0.030 | -0.998 | 641 | 0.020 | 3.92 |
| 3e    | -0.508 | -0.407 | -0.112 | 0.281 | 1.543 | -0.027 | -0.971 | 32  | 0.104 | 2.94 |
| 3f    | -1.255 | -0.985 | -0.740 | -0.619 | 0.497 | -0.021 | -0.987 | 76  | 0.055 | 1.80 |
| 3g    | -0.727 | -0.553 | -0.301 | -0.084 | 1.001 | -0.022 | -0.998 | 436 | 0.023 | 2.68 |

a) Silica gel RP-18 F254 (Merck); RM0 = molecular hydrophobicity (eq. 2); b = change in RM value caused by increasing the concentration (K) of the organic component in the mobile phase (eq. 1); R = correlation coefficient for parameters RM0 and b in eq. 2.31-34

On calculating logP values using fragmental constants, a relatively good correlation ($R^2=0.857$) with experimental data for RM0 was obtained for compounds 3a–g (Figure 1).

![Figure 1. RM0 vs logP for compounds 3a–g.](image)

Electronic absorption spectra and fluorescence of compounds 3a-g

UV-Vis spectra
Compounds 3a–g are reddish or brown in crystalline state, and their solutions in organic solvents are yellow, orange, or red. All are soluble in absolute ethanol, so that one can make comparisons between their electronic absorption bands. As seen from Table 4, all compounds present a strong band in the visible region ($\lambda_{\text{max}} = 457 – 483$ nm) due to the NBD chromophore.3,25 The differences are due to extended conjugation with the acceptor NBD group10,24,36 for aromatic...
substituents at the amino group (3a, 3b, which absorb at higher wavelengths), whereas the remaining compounds having alkyl, hydroxy, or aralkyl groups absorb at lower wavelengths.

Calculated Mulliken net atomic charges on the amino nitrogen (NAC\(_N\)) using the AM1 algorithm for molecular geometries,\(^\text{37}\) and the CODESSA program\(^\text{38}\) are presented in Table 5 together with the values found by a simple linear correlation, eq. (3), where NAC\(_N\) is the net atomic charge for the nitrogen atom, and SD is the standard deviation (calculated and experimental values had two decimals).

\[
\lambda_{\text{max}}(\text{calc.}) = -145.9(\pm 25.72)NAC_N + 434.9 \\
N = 7; R^2 = 0.865; \text{SD} = 3.648; F = 32.2; R^2_{\text{cross-valid.}} = 0.815
\] (3)

### Table 4. UV-Vis spectral data of compounds 3a–g in absolute ethanol

| Comp. | Conc.(M)  | \(\lambda_{\text{max}}\) (nm) | \(\varepsilon \times 10^3\) (L × mole\(^{-1}\) × cm\(^{-1}\)) |
|-------|-----------|------------------------------|--------------------------------------------------|
| 3a    | 1.21×10\(^{-4}\) | 279 (sh)                     | 2.80                                             |
|       |           | 330                          | 6.28                                             |
|       |           | 475                          | 17.60                                            |
| 3b    | 1.20×10\(^{-4}\) | 279                          | 2.16                                             |
|       |           | 334                          | 2.75                                             |
|       |           | 483                          | 6.75                                             |
| 3c    | 4.25×10\(^{-4}\) | 333                          | 1.08                                             |
|       |           | 465                          | 1.43                                             |
| 3d    | 4.25×10\(^{-4}\) | 331                          | 1.08                                             |
|       |           | 464                          | 2.23                                             |
| 3e    | 4.25×10\(^{-4}\) | 261 (sh)                     | 0.611                                            |
|       |           | 326                          | 0.752                                            |
|       |           | 457                          | 1.88                                             |
| 3f    | 1.43×10\(^{-4}\) | 265 (sh)                     | 1.39                                             |
|       |           | 330                          | 3.49                                             |
|       |           | 463                          | 7.83                                             |
| 3g    | 4.25×10\(^{-4}\) | 332                          | 1.41                                             |
|       |           | 462                          | 2.96                                             |
Table 5. Net atomic charges on the amino nitrogen (NAC N), and λ_{max} (exp. in Table 4 and calc. with eq. 3, in nm) for compounds 3a–g in absolute ethanol

| Compound | NAC N | λ_{max}(exp)^a | λ_{max}(calc.) | Residual |
|----------|-------|----------------|----------------|----------|
| 3a       | -0.285 | 475            | 476            | -0.59    |
| 3b       | -0.294 | 483            | 477            | 6.18     |
| 3c       | -0.229 | 465            | 468            | -3.24    |
| 3d       | -0.216 | 464            | 466            | -1.83    |
| 3e       | -0.126 | 457            | 453            | 3.66     |
| 3f       | -0.204 | 463            | 464            | -1.78    |
| 3g       | -0.185 | 462            | 462            | 0.48     |

^a see Table 4

Compound 3b with the 18C6 is able to form complexes with some alkali cations. Indeed, an acetonitrile solution of compound 3b undergoes a slight hypsochromic shift on treatment with potassium perchlorate (molar ratio 1:1) from 480 nm to 477 nm, with an isosbestic point at λ = 514 nm.

General characteristics for the fluorescence of compounds 3a–g

It is known that NBD compounds with a 4-alkylamino substituent are fluorescent, but only weakly fluorescent when they have a 4-aryl amino substituent such as phenyl (3a). Among compounds 3a–g, only compounds 3e–g are strongly fluorescent in solid state and in most solvents. Compounds 3a and 3d are weakly fluorescent in solid state and in most solvents. Compound 3b is not fluorescent either pure or as complex with KClO4. Compound 3c is not fluorescent in solid state, but is weakly fluorescent in some solvents (e.g. dichloromethane, benzene, and toluene); a more detailed account will be seen below.

By choosing the excitation wavelength at λ_{ex} = 450 nm and absolute ethanol as solvent (E_T(30) = 51.9), the characteristic data for the fluorescence of compounds are presented in Table 6. One can observe that the emission wavelength (λ_{em} = 524-545 nm) agrees with the known range for NBD derivatives. The λ_{em} values decrease in the order λ_{em} 3a > λ_{em} 3d = λ_{em} 3f > λ_{em} 3e > λ_{em} 3g; the quantum yields (Φ) decrease in the order Φ 3e > Φ 3f > Φ 3g > Φ 3d >> Φ 3a (the last compound has a very low a value); the natural lifetimes (τ_0) decrease in the order τ_0 3e > τ_0 3d > τ_0 3g > τ_0 3f; and the calculated lifetimes (τ) according to the Strickler-Berg formula (4) which involves the quantum yield (Φ) decrease in the order: τ 3e > τ 3g > τ 3f > τ 3d.

\[
\frac{1}{\tau_0} = 2.88 \times 10^{-9} n^2 \int \frac{\epsilon(v_F) d v_F}{I_F(v_F) W F(v_F) d v_F} \times \int \frac{\epsilon(v_d) d v_d}{v_d} (4)
\]

where: τ_0 is the lifetime, ν is the wavenumber of the maximum of the absorption band, n is the refractive index of the solvent (1.3595 for ethanol), I_F is the fluorescence intensity, ε is the molar absorption coefficient, and τ = τ_0 · Φ.
In the case of the paramagnetic compound 3d one must ascribe the quenching of fluorescence to an intermolecular process, similarly to literature data, due to the 4-amino-TEMPO free radical.44-46

**Table 6.** Fluorescence characteristics $\lambda_{em}$, quantum yield ($\Phi$), natural lifetime ($\tau_0$), and calculated lifetime ($\tau$) in absolute ethanol for compounds 3a, 3d–g for $\lambda_{ex} = 450$ nm

| Compound | $\lambda_{em}$ (nm) | $\Phi$ | $\tau_0$ (ns) | $\tau$ (ns) |
|----------|---------------------|--------|---------------|-------------|
| 3a       | 545                 | Very low $^c$ |               |             |
| 3d       | 531                 | 0.0016 | 79.05         | 0.13        |
| 3e       | 526                 | 0.0587 | 104           | 6.10        |
| 3f       | 531                 | 0.0393 | 24.5          | 0.96        |
| 3g       | 524                 | 0.0298 | 62.6          | 1.86        |

$^a$conc. (3a) = 1.21x10^{-4}$ M, conc. (3d,3e,3g) = 4.25x10^{-4}$ M; conc. (3f) = 1.43x10^{-4}$ M

$^b$ compared to the quinine bisulfate (in 0.1N H$_2$SO$_4$, $\Phi$ = 0.55)

$^c$ 2.03x10^{-5}$ mol/L

As discussed above, the electronic absorption spectra, for the three compounds 3d,3e,3g that have a significant fluorescence, it was possible to correlate the fluorescence lifetime $\tau$ (which involves also the quantum yield) with the calculated net atomic charge for the amino nitrogen atom (NACN) by the equation (5), as seen in Table 7.

$$
\tau = 67.32 \pm 4.791 \text{ NACN} + 14.53 \pm 0.837 \\
N = 3 \quad R^2 = 0.995 \text{ SD} = 0.275 \quad F = 197.4 \quad R^2_{\text{cross-valid.}} = 0.980
$$

**Table 7.** Calculated values of net atomic charge on the amino nitrogen (NACN) by CODESSA program and $\tau$ (exp. in Table 6 and calc. with eq. 5) for compounds 3e-g

| Compounds | NACN | $\tau$(exp.$^a$) | $\tau$(calc.) | Resid. |
|-----------|------|------------------|---------------|--------|
| 3e        | -0.126 | 6.10            | 6.05          | 0.05   |
| 3f        | -0.204 | 0.96            | 0.80          | 0.16   |
| 3g        | -0.185 | 1.86            | 2.07          | -0.21  |

$^a$ See Table 6

**Fluorescence of compound 3d**

The paramagnetic compound 3d is weakly fluorescent due to intermolecular quenching. The EPR spectrum has three lines (Figure 2) due to a hyperfine coupling with $a_N = 14.79$ Gauss (in methylene chloride) in agreement with that of 4-amino-TEMPO.47
Figure 2. EPR spectrum of 3d in dichloromethane.

With an excess of ascorbic acid in absolute ethanol, the solution becomes strongly fluorescent in a few minutes (the intensity of the fluorescence increases about six times, as seen in Figure 3), due to the formation of hydroxylamine 4 (Scheme 2). Compound 4 was detected by TLC ($R_f$ 3d = 0.907, $R_f$ 4 = 0.372, on silica gel with methylene chloride:methanol 9.5:0.5 v/v). The process described in Scheme 2 is reversible, because oxidation of 4 (with PbO$_2$, Ag$_2$O, KMnO$_4$, even with air) produces 3d.

Scheme 2. Reduction of 3d (i = ascorbic acid, molar ratio 3d: ascorbic acid = 1:6).

Figure 3. Variation of the fluorescence intensity ($I_F$) during the reduction of 3d (in absolute ethanol) with an excess of ascorbic acid.
These fluorescence and paramagnetic properties of compound 3d may lead to applications as a molecular probe for biological redox processes.

**Fluorescence of compound 3c**

In absolute ethanol, compound 3c is not fluorescent, but in less polar solvents (benzene, toluene) a weak fluorescence (Table 8) due to the NBD group was detected ($\lambda_{ex}=450$ nm, $\lambda_{em}=505 – 512$ nm).

In acetic acid which has the same polarity as absolute ethanol, a weak fluorescence has also been observed. However, the fluorescence increases significantly in the presence of strong acids such as trifluoroacetic acid and 4-toluenesulfonic acid (Table 9), when the $\alpha$-naphthylamino group becomes protonated affording cation 5 (Scheme 3). Trifluoroacetic acid introduces a significant hypsochromic shift (16 nm) in the visible spectrum, and the protonated compound 5 has the highest value for $\Phi$ (Table 9).

**Table 8.** The effect of solvent polarity on the absorption and fluorescence spectra of compound 3c (using $\lambda_{ex} = 450$ nm)

| Solvent and $E_{T}(30)^{42}$ | Conc. of compound 3c (M) | $\lambda_{max}$ (nm) | $\varepsilon \times 10^{3}$ (L×mol⁻¹×cm⁻¹) | $\lambda_{em}$ (nm) | $\Phi^a$ |
|-------------------------------|--------------------------|----------------------|-------------------------------------------|------------------|--------|
| Ethanol (51.9)                | 4.25×10⁻⁴                | 465                  | 1.43                                      | none             | none   |
|                               |                          | 333                  | 1.08                                      |                  |        |
| Dichloromethane (41.1)        | 1×10⁻⁴                   | 452                  | 15.2                                      | 513              | very low $^b$ |
|                               |                          | 326                  | 11.2                                      |                  |        |
| Benzene (34.5)                | 1×10⁻⁴                   | 447                  | 9.8                                       | 506              | 0.00100 |
|                               |                          | 322                  | 8.3                                       |                  |        |
| Toluene (33.9)                | 1×10⁻⁴                   | 445                  | 9.7                                       | 508              | 0.00112 |
|                               |                          | 320                  | 8.4                                       |                  |        |

$^a$compared to quinine bisulfate (in 0.1N H₂SO₄, $\Phi=0.55$); $^b$5.035×10⁻⁴ M

In compound 3c there is an electron-acceptor NBD group (A) and a $\pi$-electron-donor moiety (D) represented by the $\alpha$-naphthylamino group, linked together by a flexible ethylenediamino chain. An intramolecular D–A interaction will quench the fluorescence, but the protonation cancels the donor effect of the donor group.

By simulating the molecular geometry using the Hyperchem force field MM+, it was possible to simulate the closed-sandwich geometry of 3c as a consequence of the intramolecular D–A interaction. As seen in an earlier Section, NMR data (Table 2) confirm the structure of the salt 5, and its geometry appears as an open structure without such an intramolecular D–A interaction (Scheme 3 and Fig. 4).
Table 9. The fluorescence of 3c (1×10⁻⁴M) in absolute ethanol with acids, λₜₐₓ = 450 nm

| Acid                          | λₘₐₓ (nm) | E×10⁻³ (L×mol⁻¹×cm⁻¹) | λₜₑₘ (nm) | Φₑ |
|-------------------------------|-----------|-------------------------|-----------|----|
| TFA² : EtOH⁴ 1:1 v/v         | 449       | 10                      | 521       | 0.00983   |
| 5×10⁻⁴ M pTSAᵉ in ethanol     | 465       | 17.0                    | 528       | 0.00222   |
| CH₃COOH : EtOHᵇ 1:1 v/v      | 465       | 20.2                    | 534       | 0.00116   |
| 5×10⁻⁴ for N-Ph-Glyᵈ in EtOHᵇ | 393 (sh)  | 25.3                    | 534       | Very low⁷ |

ᵃ TFA = trifluoroacetic acid; ᵇ Absolute EtOH; ⁷ pTSA = 4-toluenesulfonic acid, monohydrate; ᵈ N-Ph-Gly = N-phenylglycine; ᵉ compared to quinine bisulfate (in 0.1N H₂SO₄, Φ=0.55); ⁷ 6.51×10⁻⁴ M.

Scheme 3. The reversible protonation of 3c.

Figure 4. Optimized geometries (with the MM+ program from Hyperchem) for the non-fluorescent compound 3c and its conjugate acid 5 (Scheme 3).
Qualitative experiments with compound 3c evidenced the fluorescence-enhancing effect of inorganic acids (e.g. HCl, H₂SO₄, H₃PO₄, HPO₃, H₄[Si(W₃O₁₀)₄]) or organic acids (e.g. bile acids, nicotinic acid, sulfanilic acid, salicylic acid, tannic acid). Compound 3c does not become fluorescent in the presence of benzoic, ascorbic, or caprilic acids, as well as α-amino acids (i.e. leucine, alanine, phenylalanine, glycine, thyrosine, glutamic acid, arginine, ornitine).

**Fluorescence of compound 3f**

It was shown earlier that compounds 3e, 3f, and 3g have the highest fluorescence in the series examined in this report. The hydrophobicity of these compounds decreases in the order 3e > 3g > 3f. The last compound is actually amphiphilic due to the presence of the hydrophobic NBD moiety, and the hydrophilic tris(hydroxymethyl) group. We examined the behavior of the fluorescence of 3f in aqueous ethanol as a function of the ethanol concentration. As shown in Fig. 5, the fluorescence intensity raises markedly with an increasingly higher ethanol content (about 20 times from 20% to 96% ethanol).

![Figure 5](image)

**Figure 5.** Change of the fluorescence intensity for compound 3f (conc. = 1.4×10⁻³ M, λ_ex = 450 nm) in aqueous ethanol: a = 20% ethanol–water; b = 40% ethanol–water; c = 60% ethanol–water; d = 96% ethanol–water.

One can explain this behavior by the solvent polarity and/or by assuming that 3f may form molecular aggregates like „multivalent molecules“. Thus, compound 3f may be useful as a fluorescent probe for exploring how the stronger non-covalent interactions (hydrogen bonds, hydrophobic interactions, donor-acceptor or charge transfer interactions) behave for biomolecules such as glycoproteins, glycolipids, lectins. More generally, all strongly fluorescent compounds 3e, 3f, 3g may be useful as molecular fluorescent probes for antibody-antigen biochemical species that manifest affinity for 2,4-dinitrophenyl groups, which are similar to the NBD moiety.
Conclusions

The present study was undertaken in order to obtain new 4-amino-7-nitro-NBD derivatives 3b–3g by reacting NBD-Cl with corresponding amines. The known 4-anilino derivative 3a, which is weakly fluorescent, was the reference compound. With a benzo-crown structure, 3b has ionophoric character. The weakly fluorescent N-α-naphthyl-N’-NBD-ethylenediamino derivative 3c becomes intensely fluorescent on treatment with strong acids, as the result of a change in geometry that cancels the intramolecular fluorescence quenching. The weakly fluorescent paramagnetic derivative 3d with an amino-TEMPO nitrooxide group becomes intensely fluorescent on reduction with ascorbic acid yielding the corresponding hydroxylamine derivative. Compounds 3e with an α-picoly group and 3g with a hydroxylamino group are strongly fluorescent. Derivative 3f with a tris(hydroxymethyl) group has an amphiphilic character and may be useful as a molecular probe for studying emulsions and micelles. Other derivatives (3b–3d) may be useful as biochemical fluorescent probes.

Experimental Section

General Procedures. Chemicals (amines 2a–2g) and NBD-Cl (1) were Aldrich commercial products. The 1H-NMR and 13C-NMR spectra were recorded with a Varian Gemini 300BB spectrometer at 300 MHz for protons and 75 MHz for 13C. Electronic absorption spectra were recorded with a Perkin-Elmer Lambda UV-Vis spectrophotometer, and fluorescence with a Perkin-Elmer 204 spectrofluorimeter using an excitation lamp (Xe, 150 W) interfaced with the computer, allowing a pre-established data reading time of 0.5 s. EPR spectra were recorded using a Jeol JES FA100 spectrometer. IR spectra were recorded with a Bruker FTIR spectrophotometer Model Vertex 70, using ATR technique. Melting points have been recorded in open capillary with Electrothermal’s IA 9000 Series of digital melting point instruments.

Synthesis of compounds 3a-g. General procedure

The 4-chloro-7-nitro-benzofurazan 1 was treated with amines 2a–2g in the following molar ratio: 1:1 for 3b, 3d, 3e; 2:1 for 3f, 3g; 1:2 for 3c and a large excess (about 11:1) for 3a. The reaction medium (about 10 mL/gram of 1) was: acetonitrile for 3a, methanol for 3b, 3c, 3e, ethanol for 3d, 3f, and methanol:water 1:1,v/v for 3g. An excess (about 3 mol/1 mol of 1) of sodium hydrogen carbonate was used for 3b-e and 3g. The mixture was stirred for one hour for 3c, 3g, two hours for 3d, 3f, and 24 hrs for 3a, 3b, and 3e (at room temperature for 3a, 3b, 3e, or at 50°C for 3c, 3d, 3f, 3g). The products 3a-g were isolated from the reaction mixture as follows:

(i) For 3a – 3d and 3g after filtration through a G3 glass filter, the solution was shaken with a tenfold volume of 1N hydrochloric acid and extracted with methylene chloride. The organic phase was dried over anhydrous sodium sulfate, and the solution was concentrated under reduced pressure. Compound 3a was obtained in pure state (confirmed by TLC, silica gel Merck GF254,
Compounds 3b, 3c, 3d, and 3g were isolated from the concentrated solution similarly by repeated preparative TLC, and was purified TLC using silica gel Merck GF254 and the following elution solvents: for 3b, CH$_2$Cl$_2$:MeOH 9:1 (v/v), once; for 3c and 3g, CH$_2$Cl$_2$:twice; for 3d, CH$_2$Cl$_2$:MeOH 9.9:0.1 (v/v), once.

(ii) For 3e, the precipitate retained after filtration through a G3 glass filter was purified by preparative TLC using silica gel Merck GF254 with CH$_2$Cl$_2$ (three times).

(iii) For 3f, two consecutive extractions were performed: first with CH$_2$Cl$_2$ till the organic phase remained colorless, then with ethyl acetate till the organic phase was no longer fluorescent. The organic phase was dried over anhydrous sodium sulphate, the solution was concentrated under reduced pressure and the product was obtained in pure state by preparative TLC using silica gel Merck GF254 with CH$_2$Cl$_2$:MeOH 9:1 (v/v), three times.

4-Amino-7-nitro-N-phenyl-2,1,3-benzoxadiazole (3a). 95% yield, red solid, m.p. 151-152°C (lit. m.p. 150°C $^1$ and 152-153°C $^{12}$); Anal.: Calcd. for C$_{12}$H$_8$N$_4$O$_3$: C 56.26; H 3.15; N 21.87; found C 56.24; H 3.10; N 21.81; IR (ATR), cm$^{-1}$: 1554 (NO$_2$), 3289 (NH).

4-(4'-Aminobenzo-18-crown-6)-7-nitro-2,1,3-benzoxadiazole (3b). 72% yield, red solid, m.p. 147-148°C; Anal.: Calcd. for C$_{22}$H$_{26}$N$_4$O$_9$: C 53.88; H 5.34; N 11.42; found C 53.85; H 5.33; N 11.38; IR (ATR), cm$^{-1}$: 1566 (NO$_2$), 2912 (CH$_2$), 3520 (NH).

N-1-Naphthyl-N’-(7-nitro-2,1,3-benzoxadiazole-4-yl)ethane-1,2-diamine (3c). 68% yield, red-brown solid, m.p. 195-196°C; Anal.: Calcd. for C$_{18}$H$_{15}$N$_5$O$_3$: C 61.88; H 4.32; N 20.04; found C 61.85; H 4.30; N 20.00; IR (ATR), cm$^{-1}$: 1574 (NO$_2$), 2924 (CH$_2$), 3327 (NH). On treatment with the acids mentioned in the text and Table 2, a strong fluorescence due to the salt 5 is observed.

4-(Amino-2’,2’,6’,6’-tetramethylpiperidinyloxy)-7-nitro-2,1,3-benzoxadiazole (3d). 16% yield, red-brown solid, m.p. 235-236°C; Anal.: Calcd. for C$_{15}$H$_{20}$N$_5$O$_4$: C 53.88; H 6.02; N 20.94; found C 53.85; H 6.00; N 20.88; IR (ATR), cm$^{-1}$: 1313 (N-O), 1577 (NO$_2$), 2932, 2980 (CH$_2$, CH$_3$), 3215 (OH).

7-Nitro-N-(pyridine-2-yl-methyl)-2,1,3-benzoxadiazole (3e). 52% yield, yellow-reddish solid, m.p. 194-195°C; Anal.: Calcd. for C$_{12}$H$_9$N$_5$O$_3$: C 53.14; H 3.34; N 25.82; found C 53.11; H 3.33; N 25.77; IR (ATR), cm$^{-1}$: 1577 (NO$_2$), 2920 (CH$_2$), 3293 (NH).

2-(Hydroxymethyl)-2-[(7-nitro-2,1,3-benzoxadiazole-4-yl)amino]propane-1,3-diol (3f). 22% yield, dark brown solid, m.p. 216-217°C; Anal.: Calcd. for C$_{10}$H$_{12}$N$_4$O$_6$: C 42.26; H 4.25; N 19.71; found C 42.23; H 4.21; N 19.67; IR (ATR), cm$^{-1}$: 1576 (NO$_2$), 2923 (CH$_2$), 3277, 3354 (OH).

4-Amino-N-hydroxy-N-methyl-7-nitro-2,1,3-benzoxadiazole (3g). 17% yield, brown-reddish solid, m.p. 235-236°C; Anal.: Calcd. for C$_7$H$_6$N$_4$O$_4$: C 40.00; H 2.87; N 26.66; found C 39.96; H 2.84; N 26.60; IR (ATR), cm$^{-1}$: 1579 (NO$_2$), 2920 (CH$_3$), 3292 (OH).

Reduction of compound 3d to 4 (Scheme 2)

A six-fold molar excess of ascorbic acid was added to the solution of 3d in absolute ethanol under stirring at room temperature till TLC shows the disappearance of 3d and the complete
formation of 4 (Rf 3d = 0.907, Rf 4 =0.372, silica gel, CH2Cl2:MeOH 9.5:0.5 (v/v), detection by UV at 254 nm and 360 nm, Figure 3.

References

1. Ghosh, P. B.; Whitehouse; M. W. J. Med. Chem. 1968, 11, 305.
2. Ghosh, P. B.; Whitehouse, M. W. Biochem. J. 1968, 108, 155.
3. Birkett, D. J.; Price, N. C.; Radda, G. K.; Salmon, A. G. FEBS Lett. 1970, 6, 346.
4. Kenner, R. A.; Aboderin, A. A. Biochemistry 1971, 10, 4433.
5. Lawrence, J. F.; Frei, R. W. Anal. Chem. 1972, 44, 2046.
6. Klimisch, H.-J.; Stadler, L. J. Chromatogr. 1974, 90, 141.
7. Hoff, F. V.; Heyndrickx, A. Anal. Chem. 1974, 46, 286.
8. Imai, K.; Toyo’oka, T.; Miyano, H. Analyst 1984, 109, 1365.
9. Matsumoto, K.; Ichitani, Y.; Ogasawara, N.; Yuki, H.; Imai, K. J. Chromatogr. A, 1994, 678, 241.
10. Halle, J.-C.; Mokhtari, M.; Soulie, P.; Pouet, M.-J. Can. J. Chem., 1997, 75, 1240.
11. Santa, T.; Takeda, A.; Uchiyama, S.; Fukushima, T.; Homma, H.; Suzuki, S.; Yokosu, H.; Lim, C.K.; Imai, K. J. Pharm. Biomed. Anal., 1998, 17, 1065.
12. Uchiyama, S.; Santa, T.; Fukushima, T.; Homma, H.; Imai, K. J. Chem. Soc., Perkin Trans. 2, 1998, 2165.
13. Al-Kindy, S.; Santa, T.; Fukushima, T.; Homma, H.; Imai, K. Biomed. Chromatogr., 1998, 12, 276.
14. Oe, T.; Morita, M.; Toyo’oka T. Anal. Sci., 1999, 15, 1021.
15. Uchiyama, S.; Santa, T.; Imai, K. J. Chem. Soc., Perkin Trans. 2, 1999, 569.
16. Uchiyama, S.; Santa, T.; Imai, K. J. Chem. Soc., Perkin Trans. 2, 1999, 2525.
17. Uchiyama, S.; Santa, T.; Imai, K. Analyst, 2000, 125, 1839.
18. Uchiyama, S.; Santa, T.; Okiyama, N.; Fukushima, T.; Imai, K. Biomed. Chromatogr., 2001, 15, 295.
19. Onoda, M.; Uchiyama, S.; Santa, T.; Imai, K. Luminiscence, 2002, 17, 11.
20. Onoda, M.; Uchiyama, S.; Endo, A.; Tokuyama, H.; Santa, T.; Imai K. Org. Lett., 2003, 5, 1459.
21. Bem, M.; Caproiu, M.T.; Vasilescu, M.; Tudose, M.; Socoteanu, R.; Nicolae, A.; Constantinescu, T.; Banciu, M.D. Rev. Roum. Chim., 2003, 48, 709.
22. Lakshmi, C.; Hanshaw, R.G.; Smith, B.D. Tetrahedron, 2004, 60, 11307.
23. Toyo’oka, T. Curr. Pharm. Anal., 2005, 1, 57.
24. Crampton, M.R.; Delaney, J.; Rabbitt, L.C. J. Chem. Soc., Perkin Trans. 2, 1999, 2473.
25. Bem, M.; Caproiu, M.T.; Stoicescu, D.; Constantinescu, T.; Balaban, A.T. Central Eur. J. Chem., 2003, 3, 260.
26. Bem, M.; Culita, D.C.; Caproiu, M.T; Constantinescu, T.; Banciu, M.D. Rev. Roum. Chim., 2003, 48, 387.
27. Bem, M.; Vasilescu, M.; Caproiu, M.T.; Draghi ci, C.; Beteringhe A.; Constantinescu, T.; Banciu, M.D.; Balaban, A.T. Central Eur. J. Chem. 2004, 2, 672.
28. Moutires, G.; Pinson, J.; Terrier, F.; Goumont, R. Chem. Eur. J., 2001, 7, 1712.
29. Makosza, M.; Winiarski, J. Acc. Chem. Res., 1987, 20, 282.
30. Hansch, C.; Leo, A. Substituent Constants for Correlation Analysis in Chemistry and Biology, Wiley, New York, 1979.
31. Cserhati, T. Anal. Chim. Acta, 1994, 292, 17.
32. Cserhati, T.; Forgacs, E. J. Chromatogr. A, 1994, 660, 313.
33. Kossoy, A.D.; Risley, D.S.; Kleyle, R.M.; Nurok, D. Anal. Chem., 1992, 64, 1345.
34. Soczewinski, E. Anal. Chem., 1969, 41, 179.
35. Calvino, R.; Gasco, A.; Leo, A. J. Chem. Soc., Perkin Trans. 2, 1992, 1643.
36. Terrier, F.; Chatrousse, A.-P.; Millot, F. J. Org. Chem., 1980, 45, 2666.
37. Mulliken R. S. J. Chem. Phys., 1955, 23, 1833.
38. Katritzky, A. R.; Lobanov V. S.; Karelson, A. CODESSA: A Reference Manual (Version 2.0), Gainesville, Florida, 1994.
39. Weber, E.; Toner, J.L.; Goldberg, I.; Vögtle, F.; Laydler, D.A.; Stoddart, J.F.; Bartsch, R.A.; Liotta, C.L. Crown Ethers and Analogs, Wiley, Chichester, 1989, p. 7.
40. Vögtle, F. Supramolecular Chemistry, Wiley, 1991, p.27.
41. Haugland, R.P. The Handbook. A Guide to Fluorescent Probes and Labeling Technologies, 10th edition, Molecular Probes, 2005, pp. 87, 105, 127, 272, 591, 610, 618, 713, 794, 873.
42. Reichardt, C. Solvents and Solvent Effects in Organic Chemistry, 3rd ed., Wiley-VCH, 2003, p. 352.
43. Strickler, S.J.; Berg, R. J. Chem. Phys., 1962, 37, 814.
44. Suzuki, T.; Obi, K. Chem. Phys. Letters, 1995, 246, 130.
45. Szajdzinska-Pietek, E.; Wolszczak, M. Chem. Phys. Letters, 1997, 270, 527.
46. Mischie, A.; Maior, O.; Badea, F.; Vasilescu, M.; Caragheorgheopol, A.; Caldararu, H.; Socoteanu, R.; Pencu, G.; Constantinescu, T. Rev. Roum. Chim., 2001, 46, 107.
47. Forrester, A.R.; Hay, J.M.; Thomson, R.H. Organic Chemistry of Stable Free Radicals, Academic Press, London, 1968, p. 180; Tudose, M.; Ionita, P.; Dumitrascu, F.; Draghici, C.; Caproiu, M. T.; Covaci, I. C.; Constantinescu, T.; Banciu, M. D.; Balaban, A. T., Arkivoc 2005 (iv), 225.
48. www.hyper.com/products/evaluation/hyper75/default.html.
49. Buncel, E.; McKe rrow, A.J.; Kazmaier, P.M. J. Chem. Soc., Chem. Commun., 1992, 1242.
50. Das, S.; Thanulingam, T.L ; Thomas, K.A.; Kamat, P.V.; George, M.V. J. Phys. Chem., 1993, 97, 13620.
51. Das, S.; Thomas, K.G.; Ramanathan, R.; George, M.V.; Kamat, P.V. J. Phys. Chem., 1993, 97, 13625.
52. Song, Q.; Evans, C.E.; Bohn, P.W. J. Phys. Chem., 1993, 97, 13736.
53. Das, S.; Thomas, K.G.; Thomas, K.J.; Kamat, P.V.; George, M.V. *J. Phys. Chem.*, **1994**, *98*, 9291.
54. Baldini, L.; Casnati, A.; Sansone, F.; Ungaro, R. *Chem. Soc. Rev.*, **2007**, *36*, 254.
55. Mammen, M.; Choi, S.-K.; Whitesides, G.M. *Angew. Chem., Int. Ed.*, **1998**, *37*, 2755.
56. Varki, A. *Glycobiology*, **1993**, *3*, 97.
57. Ercolani, G. *J. Am. Chem. Soc.*, **2003**, *125*, 16097.
58. Kitov, P.I.; Bundle D.R. *J. Am. Chem. Soc.*, **2003**, *125*, 16271.
59. Lancet, D.; Pecht, I. *Biochemistry*, **1977**, *16*, 5150.