New fluorescent derivatives of oligopropylamines

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Abstract
New fluorescent-tagged amines have been obtained starting from 4-chloro-7-nitro-2,1,3-benzoxadiazole and oligopropylamines containing fragments of N-methylpropylamine close to those found in diatom frustules. Optical properties of new dyes and staining of silica structures in vivo and in vitro are described.

Keywords: 7-Nitro-2,1,3-benzoxadiazole, fluorescent dyes, silica, diatoms

Introduction

Oligopropylamines (OPAs) are intensively studied nowadays for several reasons: (i) these compounds are structurally close to biogenic amines such as spermine, spermidine and polyamines from diatoms, (ii) OPAs are prospective building blocks for more complicated structures, i.e. dendrimers, graft and branched polymers; (iii) OPAs are effective catalysts or initiators of various processes including epoxy-resin synthesis and sol-gel reactions. Weakly basic fluorescent dyes are known as lysotrackers - compounds capable to penetrate into acidic cytoplasmic vesicles of eukaryotic cells. Recently we reported the synthesis of two representatives of fluorescence-tagged methylated OPAs by the reaction between 4-chloro-7-nitro-2,1,3-benzoxadiazole (Cl-NBD) and the corresponding amines.
These compounds were used for in vivo staining of diatom cells and we have found that NBD-N2 and NBD-N3 were not removed from stained diatom frustules by drastic treatment with H$_2$SO$_4$ and H$_2$O$_2$. Fluorescent silica can also be obtained by chemical precipitation from its precursors in the presence of NBD-N2 or NBD-N3. This work was aimed at the synthesis of other tagged OPAs including derivatives bearing two 7-nitro-2,1,3-benzoxadiazole (NBD) groups or having a free NH group. Fluorescence properties of new dyes and their activity during in vivo and in vitro staining of silica structures have been studied too.

Results and Discussion

We have synthesized four new NBD-tagged OPAs and also have studied UV-Vis spectral properties of them and two substances obtained earlier (Table 1). Synthetic procedures were similar to the previously published ones$^4$ and in the case of single-tagged derivatives of di-NH amines a 10-20 fold excess of the amine was used. Purification of the target compounds was done by re-precipitation and flash-chromatography. HPLC and HRMS tools were applied to confirm purity and structure of the tagged amines. NMR signals were assigned based on the data from Ref.$^5-7$ and from Ref.$^8$ for $^{13}$C NMR of NBD ring.

Table 1. Structures and UV-Vis spectral data of the NBD derivatives (0.025 mM solutions)

| Substance | Structure | $\lambda_{max}$, nm / $\varepsilon \times 10^3$, L × mole$^{-1} \times$ cm$^{-1}$ at various pH values |
|-----------|-----------|------------------------------------------------------------------|
|           |           | 2.0   | 5.5   | 7.0   | 10.0  |
| NBD-N2H   | Me $\text{N}^+$ NBD $\text{Me}$ | 497/30.7 | 497/32.4 | 497/31.4 | 501/16.5 |
|           |           | 354/7.45 | 354/8.09 | 353/7.58 | 349/8.78 |
| NBD-N2    | Me $\text{N}^+$ NBD $\text{Me}$ | 496/32.7 | 496/33.7 | 496/32.6 | 501/37.1 |
|           |           | 354/8.07 | 354/8.48 | 352/7.68 | 358/8.32 |
| 2NBD-N2   | NBD $\text{Me}^+$ NBD $\text{Me}$ | 509/9.95 | 471/17.3 | 471/18.2 | 471/19.7 |
|           |           | 472/12.5 | 349/8.18 | 348/8.92 | 348/9.84 |
|           |           | 350/6.75 |          |            |            |
| NBD-N3H   | Me $\text{N}^+$ NBD $\text{Me}$ | 493/32.5 | 493/32.0 | 497/32.5 | 502/37.1 |
|           |           | 352/8.72 | 352/8.79 | 353/8.70 | 358/8.32 |
| NBD-N3    | Me $\text{N}^+$ NBD $\text{Me}$ | 496/34.2 | 496/32.1 | 499/34.1 | 502/36.1 |
|           |           | 353/8.73 | 354/8.48 | 355/8.54 | 358/8.29 |
| 2NBD-N3   | NBD $\text{Me}^+$ NBD $\text{Me}$ | 475/41.6 | 474/41.1 | 475/27.0 | 530/8.80 |
|           |           | 350/15.6 | 350/15.4 | 347/12.2 | 500/10.9 |
|           |           | 323/9.96 | 475/10.3 | 437/12.5 | 322/18.1 |
The NBD-tagged OPAs show basic properties and titration of NBD-N3 hydrochloride from acidic medium (an excess of HCl was added to adjust pH = 2) proceeds with three inflections which correspond to removal of the corresponding proton from the amine. A fourth inflection was expected at the beginning of the first proton removal but the low value of the corresponding pK$_{BH+}$ (2.8) does not allow this inflection to be observed. The other protonation constants are 7.1 and 9.6. Titration of the NBD derivative of diethylamine gives pK$_{BH+}$ = 2.6, so the value 2.8 obtained for NBD-N3 corresponds to the nitrogen attached to the heterocycle. These pK values allow estimation of protonation degrees at various pH, which are 100, 50, 35 and near 0% at pH 2.0, 5.5, 7.0 and 10.0 correspondingly. NBD-N3H shows similar basic properties with pK$_{BH+}$ values of 9.5, 6.6 and 2.8.

Methylated OPA aggregates in water medium at neutral and alkali pH values due to hydrophobic interactions. The introduction of NBD moieties must increase the aggregation and we have found 2NBD-N2 to give unstable emulsion at pH 7-10. 2NBD-N3 solutions are stable at high pH values but dynamic light scattering (DLS) data show a presence of 1300-1500 nm particles at pH 7 and 10. DLS of the solutions of singly substituted OPAs gives a relatively noisy autocorrelation function at pH 10 corresponding to a small amount of 400-600 nm particles.

Self-association of the dye molecules is often accompanied by a hypochromic effect in UV-Vis spectra. Spectra of mono-NBD derivatives of OPAs are similar to the known NBD- amines and contain two main bands near 350 and 500 nm (Table 1, Figure 1) irrespective of pH values. A decrease of absorbance at 500 nm was observed for NBD-N2H at pH 10 only. 2NBD-N3 spectra at pH 2.0 and 5.5 (Figure 2) are similar to mono-NBD amines but the 500 nm band is moved to 475 nm and its absorption coefficient is lower than

Figure 1. UV-Vis spectra of the tagged OPA at pH 10. Spectra of NBD-N3 and NBD-N3H are similar with NBD-N2 spectrum. The solutions concentration is 0.025 mM.
expected for two NBD units. The pH increase in 2NBD-N3 solution results in new bands in the 430-530 nm range. 2NBD-N2 spectra do not show pH-dependence in 5.5-10 interval and decrease intensity at pH 2 only (Figure 2). The maximum of 2NBD-N2 absorption is at 475 nm with a shoulder near 500 nm, similar to 2NBD-N3 spectra. The observed hypsochromic shift value under addition of second NBD group does not depend on pH and probably it is connected with intramolecular interactions between NBD rings. This conclusion is confirmed by satisfaction of the Beer–Lambert–Bouguer law for 2NBD-N2 and 2NBD-N3 at pH 7.0 and concentrations from 0.0032 to 0.128 mM.

Fluorescence properties of the NBD-tagged OPAs are similar to the known NBD-amine derivatives: excitation and emission maxima near 500 and 550 nm correspondingly, independently on the number of NBD moieties in the molecule (Figures 3, 4). Quantum yields of the NBD-tagged OPAs are low and decrease slightly when pH increases (Table 2). 2NBD-N2 and 2NBD-N3 show several times lower fluorescence activity compared with mono-substituted derivatives. The decrease of fluorescence is understandable at neutral and high pH values because aggregation often results in fluorescence quenching. On the other hand, there is now aggregation at pH 2 where 2NBD-N2 and 2NBD-N3 have at least one or two protonated nitrogens correspondingly. As mentioned above, the absorption spectra of twice-substituted molecules have maximum near 475 nm and a shoulder near 500 nm. We supposed that this band is a superposition of two bands related to isolated NBD units (500 nm) and to NBD rings involved in some kind of intramolecular interaction. 2NBD-N3 spectrum decomposition into Lorentz curves allowed us to extract 500 nm band (Figure 4). Recalculation of the quantum yield based on this band gives a value of 0.022 which is equal to mono-substituted OPAs.
Table 2. Fluorescence characteristics $\lambda_{em}$ and quantum yield ($\Phi$, relative to sodium fluorescein [16]) for NBD-tagged triamines depending on pH (excitation $\lambda = 500$ nm)

| Compound   | pH 2.0 | pH 5.5 | pH 7.0 | pH 10.0 |
|------------|--------|--------|--------|---------|
|            | $\lambda_{em}$, nm | $\Phi$ | $\lambda_{em}$, nm | $\Phi$ | $\lambda_{em}$, nm | $\Phi$ | $\lambda_{em}$, nm | $\Phi$ |
| NBD-N3     | 550    | 0.022  | 550    | 0.022  | 551    | 0.015  | 550    | 0.016  |
| NBD-N3H    | 550    | 0.022  | 550    | 0.023  | 550    | 0.015  | 549    | 0.0015 |
| 2NBD-N3    | 549    | 0.0054 | 549    | 0.0050 | 548    | 0.0049 | 545    | 0.0036 |
| 2NBD-N2    | 548    | 0.0068 | 549    | 0.0057 | 545    | 0.0023 | -      | -      |

Thus, mono-NBD derivatives of OPAs show similar absorbance and fluorescence properties at pH 2-7 and the corresponding spectra are similar with spectra of diethylamino-NBD (data not shown). Di-NBD amines show anomalies which can be attributed to an intramolecular interaction between NBD rings.

Figure 3. Emission spectra of 0.005 mM solutions of NBD-tagged OPAs normalized to 0.1 absorption at 500 nm, excitation $\lambda = 500$ nm.

Recently fluorescence quenching has been found for a compound containing NBD and naphthyl ring capable of donor-acceptor interactions. In the case of two NBD groups the interaction is possible due to protonation of nitrogen attached to one ring. This protonation must increase acceptor properties of the corresponding ring thus providing non-equivalence of the NBD moieties. Quantum-chemical calculations (Figure 5) confirm this hypothesis: the most stable conformation of protonated NBD-N2 contains closely located rings. The other kind of
interaction is possible for singly protonated 2NBD-N3 (at pH 5.5-7.0): hydrogen bond between NH\(^+\) and nitrogen in one of the heterocycles gives rise to a stable conformation with coplanar rings. It is known\(^\text{17}\) that absorbance near 500 nm and the corresponding fluorescence are connected with a charge transfer, the amino group acting as the electron donor.

**Figure 4.** Absorbance and emission spectra of 2NBD-N3 and NBD-N3H. Spectra of other single-substituted OPAs are similar to NBD-N3H. Dotted lines are Lorentz decomposition of 2NBD-N3 spectra.

**Figure 5.** Optimized structures of protonated 2NBD-N2 (left) and 2NBD-N3 (right) molecules. Numbers near structures - heat of formation, kcal/mol.
donor and the nitro group as the acceptor. So, any donor-acceptor interaction involving NBD ring can obstruct the charge transfer responsible for the fluorescence. We recognize that these speculations are preliminary and thorough quantum-chemical and spectroscopic investigations are necessary to understand the behavior of the OPAs bearing two NBD rings.

The new NBD-tagged OPAs were used in synthesis of fluorescent-tagged silica (Figure 6) and for *in vivo* staining of diatom cells (Figure 7). The OPAs inclusion in silica obtained by *in vitro* precipitation proceeds through hydrogen and/or ionic bonds between amine nitrogens and silanol groups. This explains the observed more intensive fluorescence of silica samples obtained using NBD derivatives of OPAs with three nitrogen atoms. But it does not explain the higher activity of NBD-N3 comparing with NBD-N3H. Staining of diatom frustules with fluorescence-tagged OPAs is realized by non-specific penetration of amines into the cell followed by its concentration in acidic vesicles, including SDV (Silica Deposition Vesicle) – a special cell organelle in which parts of new silica frustules are synthesized. The data obtained with new NBD-tagged OPAs (Figure 7) confirm our previous observation about the ability of N3

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**Figure 6.** Fluorescent microscopic images of the synthetic silica particles obtained in the presence of NBD-N2 (A), NBD-N3 (B), NBD-N2H (C), NBD-N3H (D), 2NBD-N2 (E) and 2NBD-N3 (F). The scale bar represents 10 µm. The conditions of microphotography (power of the lamp, diaphragm and exposure) were identical in all cases.
fluorescent dyes to stain not only biogenic silica but also other intracellular vesicles in contrast to N2 derivatives which stain silica frustules only. This fact had been explained\(^4\) by the low affinity of NBD-N2 to acidic functions in comparison with NBD-N3 because NBD-N2 has only one nitrogen atom capable of durable interaction with weak acids. The low activity of 2NBD-N2 and 2NBD-N3 in diatom staining is not unexpected taking into account their reduced fluorescent activity, one or even absence of free amine nitrogens and the inclination to aggregation giving large particles which have no any possibilities to penetrate into the cell. NBD-N3 is unexpectedly more active as compared with NBD-N3H in \textit{in vivo} staining similarly to the \textit{in vitro} experiments. We have done also a chromatography experiment (Figure 8) which confirms the higher affinity of NBD-N3 to silica comparing with NBD-N3H. A possible reason for this difference is the lower value of the second protonation constant of NBD-N3H (6.6) comparing with NBD-N3 (7.1) which must decrease the ability of NBD-N3H to interact with low-acidic silica structures. NBD-tagged OPAs are non-toxic for the diatom cells in long-term cultivation experiments (up to one month) in 0.5-4 µM concentration.

**Figure 7.** Optical and fluorescent microphotographs of \textit{S. acus} cells growing in the presence of NBD-N2 (A), NBD-N2H (B), 2NBD-N2 (C), NBD-N3 (D), NBD-N3H (E) and 2NBD-N3 (F) during 24 h. The scale bar represents 10 µm. Red fluorescence – chloroplasts, yellow – new silica valves forming in SDV and acidic cell vesicles.

The oxidative treatment of diatom biomass cultivated in the presence of NBD-tagged OPAs results in fluorescent silica particles which retain the structure of the silica valves (Figure 9). Thus, the new fluorescent amines open way to biotechnological synthesis of fluorescent nano-
and micro-ordered materials, especially taking into account the high variety of diatom species and shapes of silica valves.\textsuperscript{21}

**Figure 8.** Fluorescent image of thin-layer chromatography plates (silica gel) for NBD-N3H (left), NBD-N3 (right) and their mixture (centre). Water buffer solutions of pH 5.5 and 7.0 were used as eluents.

**Figure 9.** SEM and fluorescent image (confocal microscope) of the silica valve of \textit{S. acus} cultivated with the addition of 1 \textmu M NBD-N2H. The scale bar represents 10 \textmu m.

**Conclusions**

Thus, we have elaborated synthetic procedures for several new NBD-tagged oligopropylamines. Two of these substances bear terminal NH groups which allow their use in the design of more complicated fluorescent-tagged structures including polymers and dendrimers. Absorption and fluorescence properties of twice-substituted amines depend on the pH of the water solution. Aggregation at high pH values results in hypochromic effect. Protonation in the acidic area facilitates donor-acceptor interactions between rings which give a new band in the visible spectra but this transition is not active in fluorescence. Fluorescent silica materials can be obtained by \textit{in}
vitro precipitation and also in vivo by cultivation of diatoms in the presence of NBD-tagged oligopropylamines.

Experimental Section

General. $^1$H and $^{13}$C NMR spectra were recorded using DPX 400 Bruker spectrometer in DMSO-d$_6$. Absorption, excitation and emission spectra were obtained with a Cintra 20 UV/VIS spectrophotometer (Sangji, Korea) and Shimadzu RF-5301 PC spectrofluorimeter with a 10 mm quartz cell. The following 10 mM buffer solutions were used: HCl, acetic acid/NaOH, 1-methylimidazole/HCl and ammonia/HCl at pH values 2.0, 5.5, 7.0 and 10.0 correspondingly. Semiempirical quantum-chemical calculations (PM3 method) were performed with the HyperChem program.$^{22}$

Epifluorescence microscopy was performed using an inverted microscope Axiovert 200 with a mercury lamp HBO 50W/AC ASRAM. To obtain fluorescent images, a ×100 immersion objective was used. Excitation was performed at 450-490 nm (Ex), emission was observed at 515 nm (Em) and beam splitting at 510 nm. Zeiss LSM710 microscope was used to obtain confocal images with the following parameters: excitation 488 nm, detector slit – 501–572 nm. Scanning electron microscopy (SEM) was performed using an FEI Quanta 200 instrument. An acetone suspension of silica frustules was placed on aluminium sample holders and then sputter coated with gold using an SDC 004 (BALZERS) device.

DLS experiments were performed using a LAD-079 instrument built in the Institute of Thermophysics (Novosibirsk, Russia). All solutions were purified from dust using filter units with 0.45 µm pore size (Sartorius 16555-Q Minisart syringe filters). The experiments were performed at 20 °C ± 0.02 °C. Measurements were done with 650 nm solid-state laser at 90º scattering angle. Autocorrelation functions were analyzed with a polynodal model using random-centroid optimization method.$^{23}$

Cl-NBD was purchased from Alfa Aesar (99% purity). $N^1,N^3$-dimethylpropane-1,3-diamine (N2) and $N^1,N^3$-dimethyl-$N^1$-[3-(methylamino)propyl]-propane-1,3-diamine (N3) were synthesized according to Ref.$^6$; $N,N$-diethyl-7-nitro-2,1,3-benzoxadiazol-4-amine (diethylamino-NBD) was obtained according to Ref.$^{17}$, NBD-N3 and NBD-N2 were synthesized according to Ref.$^4$. Other inorganic salts and chemicals were purchased from Sigma Aldrich, Fisher or Acros Chemicals and used without further treatment.

Purity estimation. Mass spectrometric analysis. The apparatus used was an Agilent 6210 TOF LC/MS (time-of-flight liquid chromatography/mass spectrometry) System. The samples were dissolved in deionized water at concentrations of 10 mg/L. Water and acetonitrile with 0.1% (v/v) formic acid were used as solvents A and B, respectively. A solvent gradient of 50% B to 100% B in 5 min was applied. The flow rate of the mobile phase was set at 0.2 ml/min, whereas the injection volume of sample solution was 20 µL. The conditions for TOF MS were as follows. The mass range was m/z 100 to 1000, and the scan time was 1 s with an interscan delay of 0.1 s.
Mass spectra were recorded under electrospray ionization (ESI)+, V mode, centroid, normal dynamic range, capillary voltage 3500 V, desolvation temperature 350 °C, and nitrogen flow 10 L/min. Under these conditions, peaks of the amine derivatives of 4-nitrobenzofurazane appeared as protonated ions. 2NBD-N2 was analysed with MALDI-TOF "Ultraflex" (BrukerDaltonikGmbH) instrument, laser wavelength was 355 nm, pulse width - 8 ns. α-Cyano-4-hydroxycinnamic acid (CHCA) was used as matrix, the device was calibrated with the standard peptide mixture (m/z = 757-3147).

**Cultivation of the diatom cells in the presence of NBD-tagged OPAs and synthesis of fluorescent stained silica in vitro**

These experiments were done according to Ref.4,24 Briefly, a clonal culture of the diatom *Synedra acus* subsp. radians (Kützing) Skabichevsky was cultivated in DM medium.25 After three day starvation in silicon-free medium, NBD-tagged OPAs were added as 1 mM stock solutions to give 0.5 µM final concentration and sodium silicate was added at a concentration of 100 µM. The cells were observed with epifluorescent microscope after 24 h. Silica valves were cleaned from organic components by the successive treatment with CH2Cl2 – 2-propanol mixture (1:1 v/v) and H2O2 (30%) - concentrated sulfuric acid mixture (1:1 v/v).

To obtain silica in vitro, we prepared solution of 10 mM sodium silicate and N1,N3-dimethyl-N1,N3-bis(7-nitro-2,1,3-benzoxadiazol-4-yl)propane-1,3-diamine as a catalyst of silicic acid condensation.6 The concentration of the NBD-tagged OPAs in these solutions was 1 mM, pH was adjusted to 7.0 using 1 M HCl and precipitate was collected after 24 h.

**Synthetic procedures**

*N1,N3-Dimethyl-N1,N3-bis(7-nitro-2,1,3-benzoxadiazol-4-yl)propane-1,3-diamine (2NBD-N2)*

A solution of 157 mg (0.8 mmol) of NBD-Cl in 8 ml of ethanol cooled to 4 °C was mixed with a stirred ice-cooled suspension of 67.04 mg (0.8 mmol) of sodium hydrocarbonate NaHCO3 and 32.38 mg (0.32 mmol) of N2 in 4 ml of ethanol in an argon atmosphere. The resulting dark brown mixture was stirred in an ice bath for 1 h, then 2 h at room temperature, and finally 7.5 h at 40-45 °C. After that the volatiles were removed under aspirator vacuum while keeping the bath temperature at ~ 25 °C. The residue was thoroughly mixed with 1 ml of an aqueous solution of K2CO3 (50%, w/w) and extracted with dichloromethane (6×5 ml). The combined extracts...
were dried with anhydrous potassium carbonate and filtered. The filtrate was evaporated to dryness under aspirator vacuum followed by keeping under oil pump vacuum for 2 h at room temperature. The product was a carmine red powder whose yield was 45.9 mg (34%), mp 210-215 °C (with destruction).

$^1$H NMR (400.1 MHz, DMSO-$d_6$): $\delta$H 2.17 (2H, H$_a$, q, $^3$J$_{HH}$, 6.4 Hz, CH$_2$CH$_2$CH$_2$), 2.66 (6H, H$_c$, t, $^4$J$_{HH}$, 1.7 Hz, CH$_3$NCH$_2$), 4.20 (4H H$_b$, m), 6.42 (2H, H$_d$, d, $^3$J$_{HH}$, 4.6 Hz C-CH-CH-N), 8.42 (2H, H$_e$, d, $^3$J$_{HH}$, 4.6 Hz C-CH-CH-N). $^{13}$C NMR (100.6 MHz, DMSO-$d_6$): $\delta$C 23.10 (1C, C$_a$), 41.52 (2C, C$_c$), 53.98 (2C, C$_b$), 102.42 (2C, C$_d$), 120.12 (2C, C$_f$), 136.95 (2C, C$_e$), 143.25 (2C, C$_i$), 144.39 (2C, C$_g$), 147.15 (2C, C$_h$). MALDI-TOF: found 429.18, calcd 429.13 (for [M+H]$^+$, C$_{17}$H$_{17}$N$_8$O$_6$).

$N^1,N^3$-Dimethyl-$N^1$-[3-[methyl-(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]propyl]-$N^3$-(7-nitro-2,1,3-benzoxadiazol-4-yl)propane-1,3-diamine (2NBD-N3)

A solution of 205.84 mg (1.03 mmol) of NBD-Cl in 10 ml of ethanol cooled to 4 °C was mixed with a stirred ice-cooled suspension of 86.88 mg (1.03 mmol) of sodium hydrocarbonate Na$_2$CO$_3$ and 46.38 mg (0.27 mmol) of N3 in 5 ml of ethanol in an argon atmosphere. The resulting dark brown mixture was stirred in an ice bath for 1 h and then for 2 h at room temperature. After that the solvent was removed under aspirator vacuum while keeping the bath temperature at ~ 25 °C. The residue was thoroughly mixed with 1 ml of an aqueous solution of K$_2$CO$_3$ (50%, w/w) and extracted with dichloromethane (5×2 ml). The combined extracts were dried with anhydrous potassium carbonate and filtered. The filtrate was concentrated to a volume of 3 ml under reduced pressure and saturated with gaseous hydrogen chloride. Then 3 ml of ether were added to the solution to obtain an orange precipitate which was filtered off, washed with ether (3×2 ml), and kept in oil pump vacuum at room temperature for 2 h. The product was a carmine red powder whose yield was 48.42 mg (34%), mp 145-150 °C (with destruction). Hydrogen chloride content was determined by potentiometry titration.

$^1$H NMR (400.1 MHz, D$_2$O): $\delta$H 2.06 (3H, H$_a$, s), 2.14 (4H, H$_b$, bs), 3.33 (6H, H$_d$, s), 2.80 (4H H$_a$, s), 3.12 (4H, H$_c$, t, $^3$J$_{HH}$, 8.1 Hz, N-CH$_2$-CH$_2$), 6.09 (4H, H$_g$, d, $^3$J$_{HH}$, 9.4 Hz C-CH-CH-N), 8.05 (4H, H$_f$, d, $^3$J$_{HH}$, 9.4 Hz C-CH-CH-N). $^{13}$C NMR (100.6 MHz, D$_2$O): $\delta$C 22.82 (2C, C$_b$), 40.84 (2C, C$_d$), 43.77 (1C, C$_e$), 53.39 (2C, C$_c$), 54.16 (2C, C$_a$), 104.24 (2C, C$_g$), 120.01 (2C, C$_k$), 138.11 (2C, C$_f$), 145.08 (2C, C$_h$), 145.20 (2C, C$_j$), 147.90 (2C, C$_i$). TOF MS (m/z): found
500.2019, calcd 500.2001 (for [M+H]^+, C_{21}H_{26}N_9O_6); found 999.3949, calcd 999.3928 (for [2M+H]^+, C_{42}H_{51}N_{18}O_{12}).

\[
N^1,N^3\text{-dimethyl-}N^1\text{-(7-nitro-2,1,3-benzoxadiazol-4-yl)propane-1,3-diamine (NBD-N2-NH)}
\]

A solution of 301.6 mg (1.51 mmol) of NBD-Cl in 50 ml of ether cooled to 4 °C was added dropwise to an ice-cooled solution of 3.08 g (30.14 mmol) of N2 in 50 ml of ether in an argon atmosphere during 2 h. The resulting mixture was stirred in an ice bath for 1 h and then for 2 h at room temperature. Then the ether was evaporated to dryness under reduced pressure. The residue was thoroughly mixed with 1 ml of an aqueous solution of K_2CO_3 (50%, w/w) and extracted with dichloromethane (4×2 ml). The combined extracts were dried with anhydrous potassium carbonate and filtered. The filtrate was evaporated to dryness under reduced pressure to give sticky mass which was triturated with n-hexane (3×6 ml). After every trituration the solution over the mass was discarded. The residue was dried under reduced pressure and dissolved in 2.5 ml CH_2Cl_2. Then this solution was poured to 15 ml of n-hexane. The precipitated product was filtered off and kept in oil pump vacuum at room temperature for 2 h. The product yield was 0.2154g (53.7 %).

^1H NMR (400.1 MHz, D_2O): δ_H 2.16 (2H, H_a, bs), 3.23 (3H, H_d, bs), 2.83 (3H H_c, s), 3.36 (4H, H_b, H_e, bs), 6.10 (2H, H_f, d, ^3J_{HH}, 8.6 Hz C-CH-CH-N), 8.04 (2H, H_g, d, ^3J_{HH}, 8.6 Hz C-CH-CH-N). ^13C NMR (100.6 MHz, D_2O): δ_C 22.75 (1C, C_a), 40.92 (1C, C_e), 43.55 (1C, C_d), 53.48 (1C, C_b), 54.02 (1C, C_c), 104.18 (1C, C_f), 120.19 (1C, C_g), 137.95 (1C, C_h), 145.22 (1C, C_k), 145.36 (1C, C_l), 147.82 (1C, C_j). TOF MS (m/z): found 266.1291, calcd 266.1248 (for [M+H]^+, C_{11}H_{16}N_5O_3).

\[
N^1,N^3\text{-dimethyl-}N^1\text{-[3-(methylamino)propyl]-}N^3\text{-(7-nitro-2,1,3-benzoxadiazol-4-yl)propane-1,3-diamine (NBD-N3-NH)}
\]
A solution of 0.1508 g (0.7517 mmol) of NBD-Cl in 25 ml of ether cooled to 4 °C was added dropwise to an ice-cooled solution of 1.33 g (7.52 mmol) of N3 in 25 ml of ether under argon atmosphere during 2 h. The resulting mixture was stirred in an ice bath for 1 h and then for 2 h at room temperature. Then the ether was evaporated under reduced pressure. The residue was thoroughly mixed with 1 ml of an aqueous solution of K$_2$CO$_3$ (50%, w/w) and extracted with dichloromethane (4×2 ml). The combined extracts were dried with anhydrous potassium carbonate and filtered. The filtrate was evaporated to dryness under reduced pressure to give sticky mass which was triturated with hexane (3×10 ml). After every trituration the solution over the mass was discarded. The residue was dissolved in 2.5 ml CH$_2$Cl$_2$ and poured to 12 ml of hexane. The precipitate was filtered off, washed with 2 ml of hexane and dried under reduced pressure. The product was obtained in pure state by preparative chromatography on silica gel Panreac 60, 63-200 microns RE using a solvent system composed of CH$_2$Cl$_2$:EtOH:NH$_4$OH 9:4:1 (v/v). The product was a brown oil whose yield was 0.130 mg (51.2%).

$^1$H NMR (400.1 MHz, D$_2$O): $\delta_H$ 2.13 (7H, H$_a$, H$_b$, H$_c$, bs), 2.80 (3H, H$_f$, s), 2.84 (4H H$_c$, H$_d$, s), 3.12 (2H, H$_g$, d, $^3$J$_{HH}$, 8.2 Hz, N-CH$_2$-CH$_2$), 3.24 (3H, H$_h$, s), 3.34 (2H, H$_e$, bs), 6.09 (2H, H$_j$, d, $^3$J$_{HH}$, 8.7 Hz C-CH-CH-N), 8.05 (2H, H$_k$, d, $^3$J$_{HH}$, 8.7 Hz C-CH-CH-N). $^{13}$C NMR (100.6 MHz, D$_2$O): $\delta_C$ 20.41 (1C, C$_a$), 22.82 (1C, C$_b$), 40.84 (1C, C$_i$), 43.77 (1C, C$_j$), 53.39 (1C, C$_c$), 53.71 (1C, C$_g$), 54.16 (1C, C$_d$), 55.00 (1C, C$_h$), 104.24 (1C, C$_j$), 120.00 (1C, C$_l$), 138.11 (1C, C$_k$), 145.07 (1C, C$_o$), 145.20 (1C, C$_m$), 147.91 (1C, C$_n$). TOF MS (m/z): found 337.2054, calcld 337.1983 (for [M+H]$^+$, C$_{15}$H$_{25}$N$_6$O$_3$); found 673.3882, calcld 673.3893 (for [2M+H]$^{2+}$, C$_{30}$H$_{49}$N$_{12}$O$_6$).

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