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Application of 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole in analysis: Fluorescent dyes and unexpected reaction with tertiary amines

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ABSTRACT

4-Chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) is widely applied as a fluorescent tagging reagent in biochemistry, as a derivatization agent in analytical chemistry, and as a component for design of fluorescent nanoparticles. Four new 7-nitrobenzo-2-oxa-1,3-diazole (NBD)-tagged polyamines containing two to four amine moieties were synthesized and used as an effective tool for staining of siliceous frustules of the diatom algae and spicules of the siliceous sponges, including fossilized samples. An unexpected reaction between NBD-Cl and tertiary amine groups was found, giving rise to NBD-tagged amines with elimination of an alkyl group. The reaction proceeds through the Meisenheimer complex and quaternary salt, which transform to the product by Hofmann reaction (alkene elimination) or nucleophilic substitution (halogenated compound formation). In the case of polyamines, NBD-Cl causes chain scissoring, giving a set of NBD-tagged amines. The found NBD-Cl reaction with tertiary amines must be taken into account when using NBD-Cl and similar activated aromatic systems for amine derivatization in analytical and biochemistry applications. The reaction with polyamines opens the way to libraries of NBD-tagged compounds.

4-Chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl)[1] is a well-known substance for amine derivatization. It was obtained by Ghosh and Whitehouse [1] as a reagent capable of reacting with primary and secondary amines, giving rise to stable fluorescence products. NBD-Cl is a relatively cheap reagent, and it is widely used in biochemical and analytical applications:

(i) Fluorescent-tagging of the lipids, including phospholipids and cholesterol, for visualization of membrane processes in living cells and model systems [2–5].
(ii) Introduction of 7-nitrobenzo-2-oxa-1,3-diazole (NBD) fragments into various biological active compounds with the aim of tracking these compounds in organisms, for example, polyamine [6] and anion [7] transmembrane transport, phospholipidosis study [8], wortmannin action as an inhibitor of PI3 kinase [9], fluorescent tagging of proteins [10], and design of the actin probe [11].
(iii) Monitoring of the gene transfection [12].
(iv) Fluorescent tagging of sugars for visualization of cells showing high uptake of the corresponding sugar [13–15]; and
(v) Derivatization of various compounds in spectrofluorimetry, chromatography, and electrophoresis [16–22].

NBD fragments are applied in the synthesis of fluorescent nanoparticles [23–26] as a tool of nanoparticle investigation and also for the design of fluorescent nanoprobes, thermal sensors, and optical devices.

NBD-Cl reacts with amines through formation of an intermediate Meisenheimer complex (Scheme 1) [27]. The formation of Meisenheimer complex is a reversible reaction, but in the presence of basics (e.g., amines, sodium bicarbonate) the adduct transforms into the NBD-containing product [28–30]. We have previously studied short-chain polyamines with trimethylene fragments (oligopropylamines; Scheme 2) that were obtained as synthetic analogs of biogenic amines from diatom algae [31,32]. NBD derivatives of the oligopropylamines **2a–d**, **3a**, and **3b** are useful instruments to study bio-silicification in diatoms and sponges [33–35]

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[1] *Abbreviations used:* NBD-Cl, 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole; NBD, 7-nitrobenzo-2-oxa-1,3-diazole; SEM, scanning electron microscopy; HPLC, high-performance liquid chromatography; TOF, time-of-flight; LC, liquid chromatography; MS, mass spectrometry; ESI, electrospray ionization; UV, ultraviolet; HRMS, high-resolution MS.

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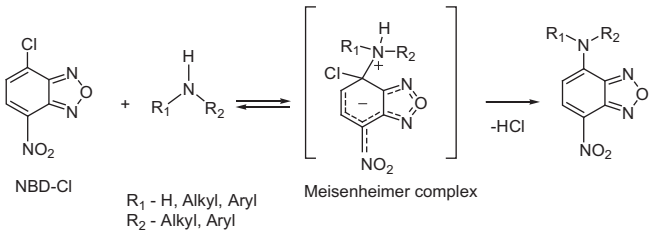


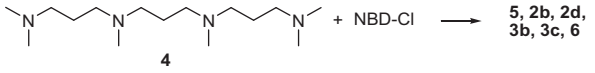
Scheme 1. Reaction of NBD-Cl with primary and secondary amines.

because they stain new siliceous structures during cultivation in the presence of the dye. The NBD-tagged polyamines also stain siliceous and composite nanoparticles obtained by controllable condensation of silicic acid during bio-inspired in vitro experiments [33].

The investigation of organisms that form siliceous elements of the skeleton requires thorough study of these siliceous materials with the up-to-date microscopy techniques. Confocal microscopy allows investigating internal surfaces of the complex constructions, which is impossible with scanning electron microscopy (SEM) and atomic force microscopy (AFM). The high surface area of most artificial siliceous materials (e.g., silica gels) allows staining by direct sorption of various dyes. On the contrary, biogenic silica (e.g., diatom frustules, sponge spicules) shows low surface area, and special methods for its staining needs to be elaborated. Recently, a procedure for fluorescent staining of the diatom frustules was proposed with the use of (3-aminopropyl)trimethoxysi lane and isothiocyanate derivative of a fluorescent dye [36]. The obtained fluorescent samples were investigated with confocal microscopy, giving rise to exact three-dimensional reconstruction of the frustule geometry, which is important for taxonomy and bioproductivity studies. The dye immobilization proceeds by the reaction between methoxysilane and silanol moieties on the silica surface. This is a complicated method that requires special reagents and skills on the last stage of the specimen preparation. We supposed that dyes attached to a polyamine chain with three or more amine groups will be able to stain diatom and similar silica samples by means of hydrogen/ionic bonding with ≡Si—OH surface groups and that the staining procedure will consist simply in the immersion of the siliceous material into aqueous solution of the dye.

In this article, we present synthesis of new NBD-tagged polyamines (Scheme 2) that were effective in in vitro staining of the diatom frustules and spicules of the siliceous sponges,

Table 1
Major products of reaction between NBD-Cl and amine **4**.



Product	Yield (%)[a]
5	12.7
2b	3.9
2d	3.6
3b	2.2
3c	13.2
6	1.5

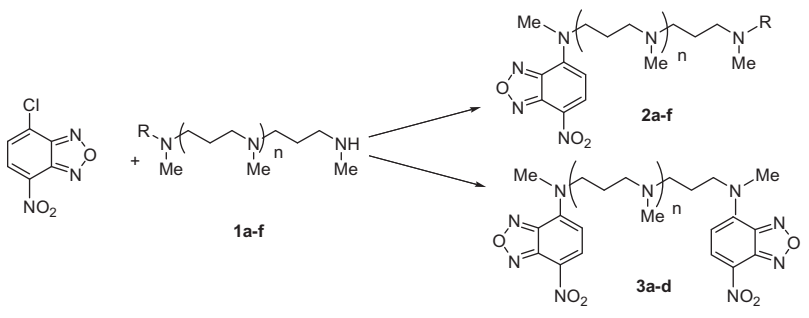
[a] The yield values (HPLC) were calculated per initial NBD-Cl amount.

including fossilized samples. NBD-Cl reaction with tetra- and pentaamines unexpectedly results in side products containing shorter polyamines. We found that NBD-Cl readily reacts with tertiary amines, and this reaction must be taken into account in the analytical applications of NBD-Cl and other activated aromatic systems.

Materials and methods

Materials

The initial amines **1e**, **4**, and **1f** were prepared according to the previously published method [31,32]. Synthesis of NBD-tagged amines based on di- and triamines was described previously: **2b** and **2d** [33], **3b** [37], and **6** [38]. NBD-Cl was purchased from Alfa Aesar (99% purity). Silica gel Panreac 60 (63–200 μm) was used for flash chromatography. Acetonitrile was of high-performance liquid chromatography (HPLC) grade (Cryochrom, St. Petersburg, Russia). Methanol, diethyl ether, acetone, dichloromethane, potassium carbonate, 1,4-dioxane, ammonium hydroxide, hexane, and formic acid were of reagent grade (Sigma–Aldrich, Fisher, or Acros).



1a n = 0, R = H; **1b** n = 0, R = Me; **1c** n = 1, R = H; **1d** n = 1, R = Me; **1e** n = 2, R = H; **1f** n = 3, R = H

2a n = 0, R = H; **2b** n = 0, R = Me; **2c** n = 1, R = H; **2d** n = 1, R = Me; **2e** n = 2, R = H; **2f** n = 3, R = H

3a n = 0, **3b** n = 1, **3c** n = 2, **3d** n = 3

Scheme 2. Structure of polyamines and synthesis of NBD-tagged polyamines.