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Dendritic chain reaction: Responsive release of hydrogen peroxide upon generation and enzymatic oxidation of methanol

Michal Avital-Shmilovici, Doron Shabat *

Department of Organic Chemistry, School of Chemistry, Raymond and Beverly Sackler Faculty of Exact Sciences, Tel-Aviv University, Tel Aviv 69978, Israel

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ABSTRACT

Signal amplification dramatically increases the sensitivity of diagnostic methods. Recently, we introduced a new technique for signal amplification that uses a distinctive dendritic chain reaction (DCR) to generate exponential evolution of a diagnostic signal. In this report, we demonstrate how the modular design of our DCR probe can be used to improve the detection sensitivity. We synthesized a new probe based on a methyl carbonate linkage, which has superior stability in aqueous media. Triggered release of methanol, which was oxidized by alcohol oxidase present in the solution, produced hydrogen peroxide that used as a reagent in the DCR amplification technique. The new probe exhibited higher sensitivity in detection of hydrogen peroxide than our previously reported probe.

1. Introduction

Molecular probes, used for identification of various analytes, commonly depend on responsive release of a chromogenic molecule.[1] The analyte of interest usually interacts with the probe through a specific reaction that activates a latent chromophore.[2,3] Recently, we introduced a new technique for signal amplification[4,5] that can be used in diagnostic detection of analytes[6–10], which is based on a distinctive dendritic chain reaction (DCR).[11] This technique depends on the disassembly properties of a self-immolative dendrimer[12,13] to generate an exponential evolution of a diagnostic signal. The analyte of interest reacts with the trigger of the DCR probe to release a reporter unit and reagents. Upon release, the reagents acquire the chemical reactivity of the analyte and thus can activate two additional probe molecules (Fig. 1). This process repeats itself to ultimately release all of the reporter units from the DCR probe.

The DCR technique was demonstrated for detection of hydrogen peroxide as the analyte of choice. Hence, the probe was equipped with a phenylboronic acid trigger[14] that can be cleaved by hydrogen peroxide, 4-nitroaniline reporter and two choline molecules as reagent units (compound **2**, Fig. 2). In order to generate hydrogen peroxide upon release of the choline molecules, the assay was performed in the presence of choline oxidase. The modularity of the probe molecule allows replacing structural elements like the choline substrate with other compounds that can be oxidized to produce hydrogen peroxide and thereby to apply other oxidases in the DCR assay. In this report, we demonstrate the advantages of the modular design and characterize a probe with higher sensitivity in detection of hydrogen peroxide than our previously reported probe.

2. Results and discussion

2.1. Design of the DCR probe

The detection limit of the analyte is dependent on the signal-to-noise ratio.[15] The sensitivity of the DCR assay is limited by the spontaneous hydrolysis of the probe. This background signal is likely generated through hydrolysis of the carbonate linkage of the choline in dendron **2**. In general, carbonates are more reactive when they are constructed from alcohols with low pK_a. The pK_a of choline is 13.9, whereas that of methanol is 15.5, more than one order of magnitude higher. Thus, by replacing the choline with methanol, the carbonate linkage in dendron **3** should be less likely to spontaneously hydrolyze and the background signal of the DCR assay is expected to be lower.

The methyl carbonate in dendron **3** is much more hydrophobic than the choline carbonate of dendron **2**. Since the DCR assay is performed under aqueous conditions, we replaced the 4-nitroaniline reporter with 5-amino-2-nitrobenzoic acid. The addition of ionized carboxylic acid group should compensate for the loss of polarity in the reagent units.

The disassembly pathway of dendron **3** is presented in Figure 3. Oxidation of the phenylboronic acid with hydrogen peroxide followed by hydrolysis generates phenol **3a**, which undergoes 1,6 elimination to release phenol **3b**. The latter is known to undergo

* Corresponding author. Tel.: +972 (0) 3 640 8340; fax: +972 (0) 3 640 9293.
E-mail address: chdoron@post.tau.ac.il (D. Shabat).

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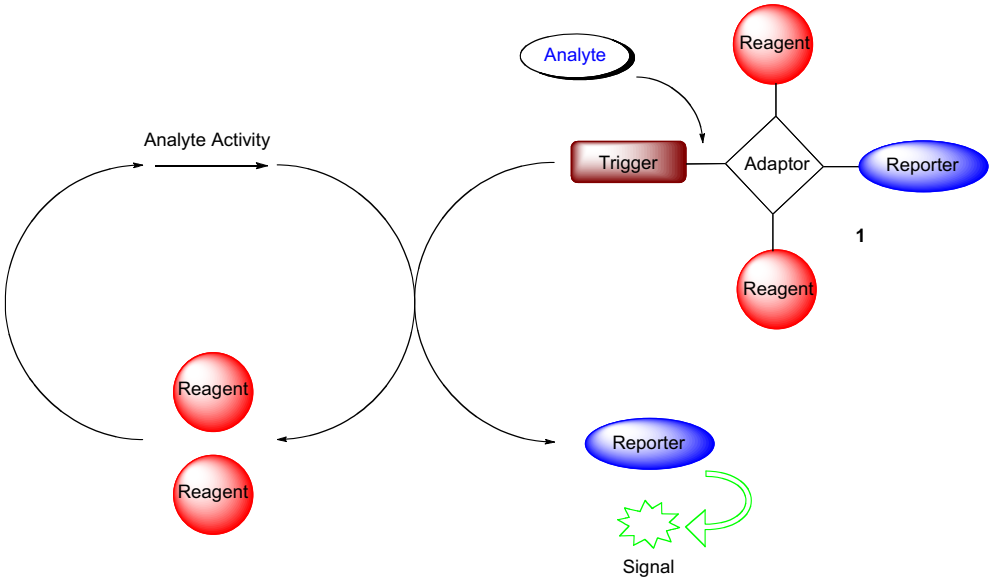

Figure 1. Graphical illustration of the DCR amplification cycle.

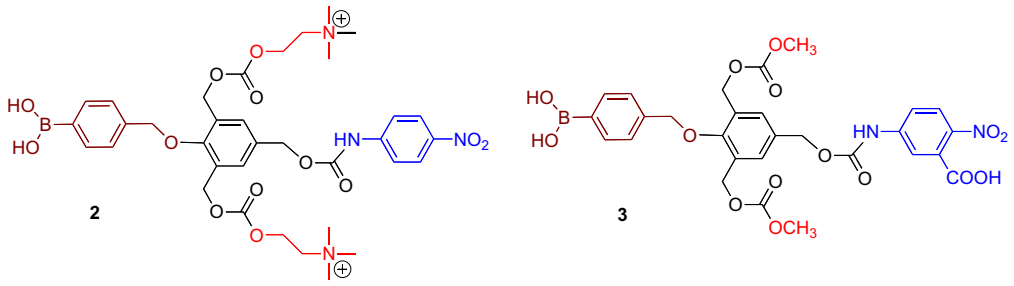

Figure 2. Chemical structure of DCR probes for detection of hydrogen peroxide.

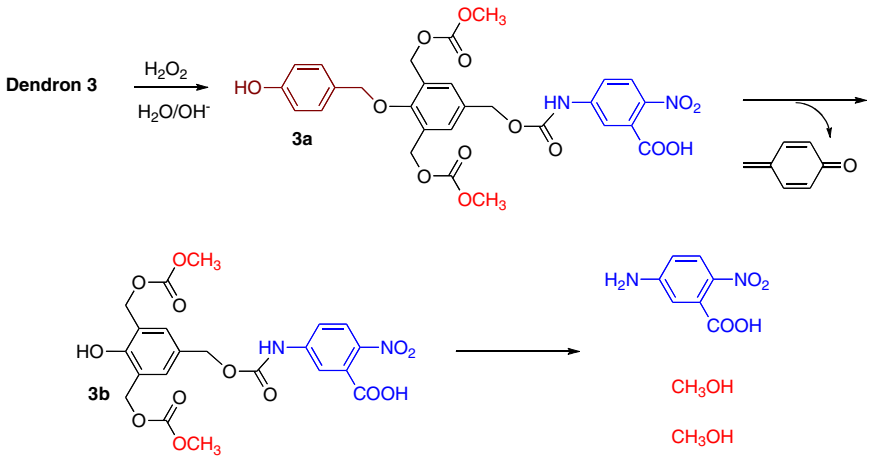

Figure 3. Disassembly pathway of dendron **3** upon reaction with hydrogen peroxide.

three subsequent elimination reactions to release the reporter and two methanol units.

As illustrated in Figure 4, the released methanol units are oxidized in presence of alcohol oxidase (AOX) to produce two molecules of hydrogen peroxide, which can then activate two additional molecules of DCR probe **3**. The amplification cycle repeats itself until all reporter molecules are released.

2.2. Chemical synthesis of the DCR probe

Dendron **3** was synthesized as shown in Figure 5. Amine **3c**[16] was treated with triphosgene to generate isocyanate **3d**, which was coupled in situ with alcohol **3e** (prepared as previously reported[11]) to afford carbamate **3f**. Selective deprotection of the *t*-butyldimethyl-silyl groups of compound **3f** in the presence of *p*-

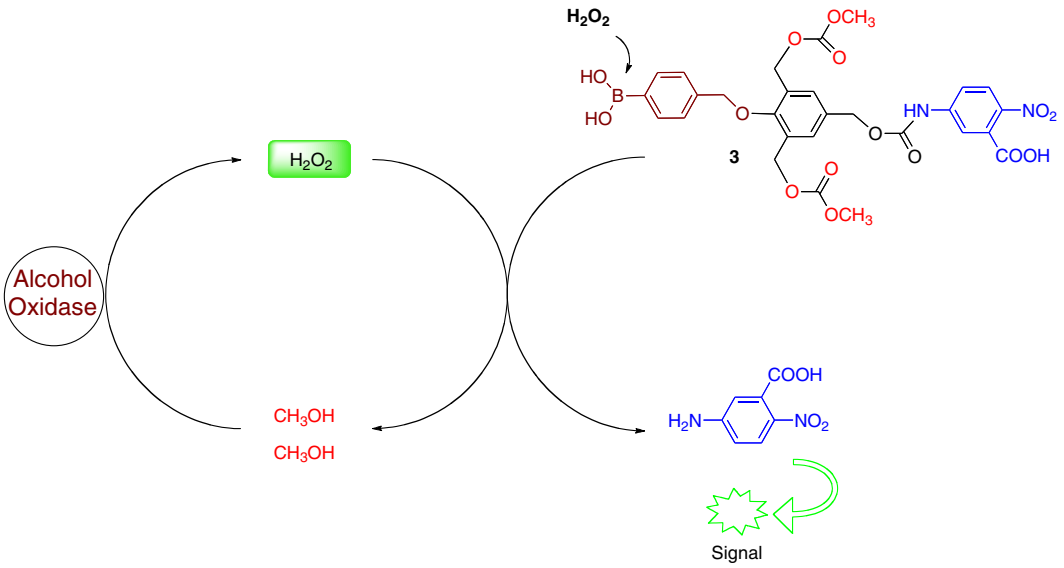

Figure 4. The amplification cycle of DCR probe **3** for detection of hydrogen peroxide.

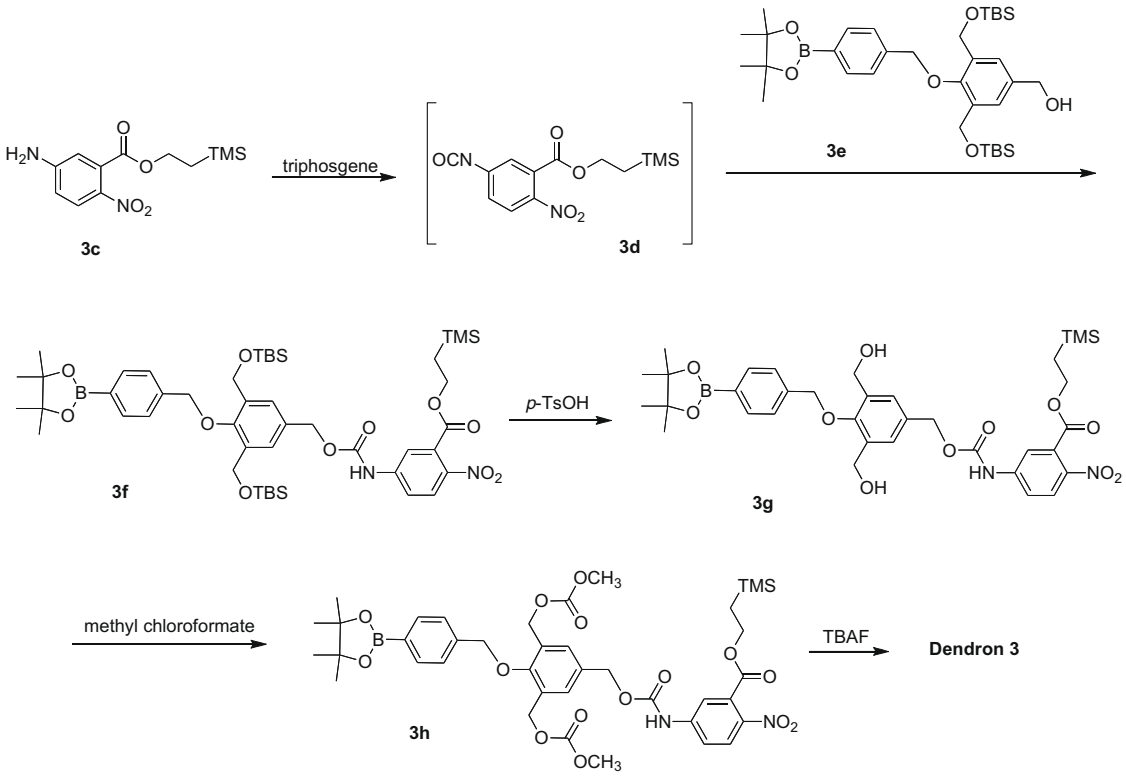

Figure 5. Chemical synthesis of dendron **3**.

toluenesulfonic acid generated diol **3g**, which was acylated with methyl-chloroformate to afford dicarbonate **3h**. Deprotection of the trimethylsilylethanol ester and the pinacol ester groups with tetrabutylammonium-fluoride afforded dendron **3**.

2.3. Evaluation of the signal amplification obtained by the DCR probe upon activation with hydrogen peroxide

In order to evaluate the amplification activity of DCR probe **3**, it was incubated in phosphate buffered saline, pH 7.0 (PBS) with hydrogen peroxide in the presence of AOX and the release of 5-amino-2-nitrobenzoic acid was monitored at a wavelength of 405 nm (Fig. 6). When 1.0 equiv of hydrogen peroxide was added, the system reached complete disassembly within 2 h. Under these conditions, there was no amplification since the analyte interacted with the probe through a stoichiometric reaction. The signal generated from 1.0 equiv of hydrogen peroxide was used as a reference when analyzing smaller amounts of analyte. As expected, the system reached complete disassembly after a longer period of time when less hydrogen peroxide was used. The sigmoidal plots obtained when the reaction was analyzed using various equivalents of H_2O_2 are characteristic of the exponential progress of the

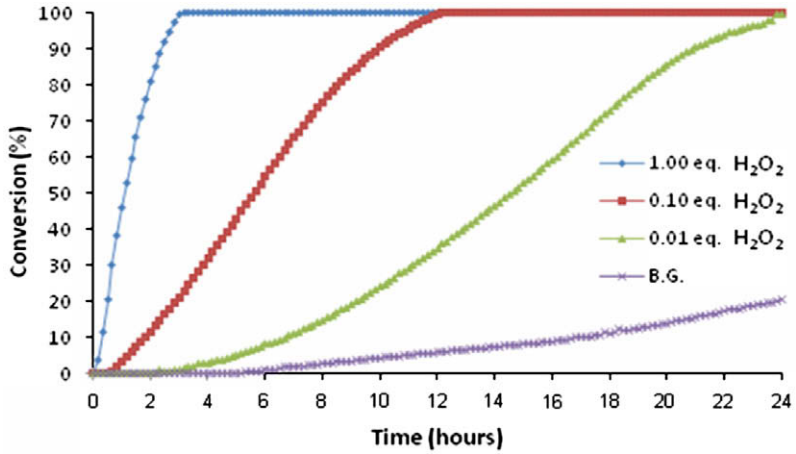

Figure 6. The release of 5-amino-2-nitrobenzoic acid from 500 μM dendron **3** in the presence of 0.1 mg/mL alcohol oxidase in PBS 7.0 upon addition of buffer only (B.G.) or the indicated equivalents of H_2O_2. The reaction was monitored at 405 nm.

reaction (Fig. 6). In the absence of H_2O_2, DCR probe **3** exhibited background signal that was significantly lower than the signal observed in the presence of 0.01 equiv of H_2O_2.

The increased sensitivity of the DCR technique is clearly demonstrated when signals obtained with and without the addition of AOX are compared (Fig. 7). When 1.0 equiv of the analyte was applied and no AOX was added, dendron **3** exhibited the behavior of a classic probe, a molecule constructed from a triggering protecting group attached to a reporter. Signal levels at 1 equiv hydrogen peroxide were identical with and without enzyme. However, when less hydrogen peroxide was evaluated, the signal measured in the presence of AOX was significantly larger than that obtained in the absence of enzyme. In fact, the ratio between the signal with and without enzyme was increased as analyte concentration decreased. When 0.01 equiv of hydrogen peroxide was evaluated, the signal of the DCR technique is 70-fold stronger than that obtained by a classic probe.

The self-immolative dendritic platform used in the DCR technique has a modular design. Thus, some structural elements can be replaced in order to optimize the probe sensitivity. By replacing the choline unit in dendron **2** with methanol, a carbonate linkage was generated (dendron **3**) that was less likely to undergo spontaneous hydrolysis. This adjustment reduced the background signal of the DCR assay and to some extent improved the signal-to-noise ratio in comparison to DCR probe **2**. When 0.01 equiv of hydrogen peroxide (vs. the concentration of the probe) were evaluated with DCR probe **3**, the observed signal was 70-fold stronger than that obtained by a classic probe. When the same experiment was performed with DCR probe **2**, the observed signal was only 53-fold stronger than that obtained by a classic probe.[11]

In order to obtain an ideal DCR probe, one should design a molecule that is highly stable to spontaneous hydrolysis but yet can undergo rapid disassembly upon relation with the analyte. The higher stability of the current probe reduces to some extent its rate of disassembly. In this case, the obtained increase in sensitivity was accompanied with a longer response time. In principle numerous analytes that have cleavage reactivity toward a specific trigger could be incorporated into the dendritic platform. Thus, the DCR amplification technique could be used to detect a variety of clinically or environmentally important analytes.

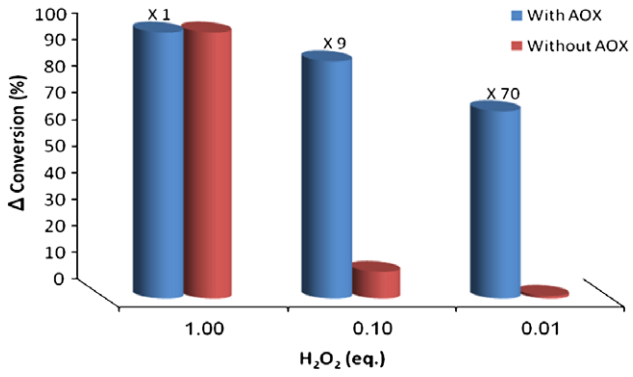

Figure 7. Comparison of signals measured after complete disassembly (1.00 equiv H_2O_2—2 h, 0.10 equiv H_2O_2—12 h, 0.01 equiv H_2O_2—24 h) in the presence of AOX by the DCR amplification technique (blue) versus signals measured using dendron **3** as a classic probe (i.e., without AOX) (red). The background signal (in the absence of the analyte) was subtracted from the values shown.

3. Conclusions

In summary, we designed and synthesized a DCR probe for amplification of a diagnostic signal that is generated by hydrogen peroxide. The probe was triggered by hydrogen peroxide that was produced through release and oxidation of methanol molecules by alcohol oxidase. The switch from choline-based carbonate to a methyl carbonate derivative generated a linkage with higher stability, which reduced the noise of the reaction. The new DCR probe exhibited slightly higher sensitivity in detection of hydrogen peroxide than our previously reported probe. Other modifications in the modular structure could yield even more sensitive DCR probes for detection of hydrogen peroxide or other analytes.

4. Experimental

4.1. General

All reactions requiring anhydrous conditions were performed under an Argon atmosphere. All reactions were carried out at room temperature unless stated otherwise. Chemicals and solvents were either A.R. grade or purified by standard techniques. Thin layer chromatography (TLC): Silica Gel plates Merck 60 F_{254}; compounds were visualized by irradiation with UV light and/or by treatment with a solution of phosphomolybdic acid (20 wt % in ethanol), followed by heating. Flash chromatography (FC): Silica Gel Merck 60 (partical size 0.040–0.063 mm), eluent given in parentheses. ^{1}H NMR: Bruker AMX 200 or 400 instrument. ^{13}C NMR: Bruker AMX 200 or 400 instrument. The chemical shifts are expressed in δ rel-

ative to TMS (δ = 0 ppm) and the coupling constants J in Hz. The spectra were recorded in $CDCl_3$ as a solvent at room temp unless stated otherwise. All reagents, including alcohol oxidase, salts and solvents, were purchased from Sigma–Aldrich.

4.2. Compound 3c

Compound **3c** was synthesized as previously reported.[16]

4.3. Compound 3e

Compound **3e** was synthesized as previously reported.[11]

4.4. Compound 3f

Toluene (15 mL) was heated to reflux (110 °C) under Argon atmosphere and triphosgene (705 mg, 2.37 mmol) was added. Then, a solution of compound **3c** (560 mg, 1.98 mmol) in 5 mL toluene was slowly added dropwise with a syringe. The reaction was stirred for 30 min at reflux and monitored by ^{1}H NMR (200 MHz, $CDCl_3$). After the isocyanate derivative (compound **3d**) was observed, the solvent was removed under reduced pressure. A solution of compound **3e** (950 mg, 1.52 mmol) in 9 mL dry THF under Argon, followed by the addition of 25 μL DBTL, was added to the isocyanate residue. The reaction mixture was allowed to warm to 55 °C and was stirred for 1 h. The reaction was monitored by TLC (EtOAc/Hex 15:85). The solvent was removed under reduced pressure and the crude product was purified by using column chromatography on silica gel (EtOAc/Hex 1:9) to give compound **3f** (1.03 gr, 60%) as a yellow oil.

^{1}H NMR (200 MHz, $CDCl_3$): δ = 8.01 (1H, d, J = 10.3 Hz); 7.80 (2H, d, J = 8 Hz); 7.65–7.59 (2H, m); 7.38–7.34 (4H, m); 7.20 (1H, br s); 5.19 (2H, s); 4.86 (2H, s); 4.68 (4H, s); 4.44–4.35 (2H, m); 1.32 (12H, s); 1.14–1.03 (2H, m); 0.87 (18H, s); 0.04 (21H, s). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 179.2, 152.7, 142.9, 141.4, 139.4, 135.1, 134.4, 131.7, 130.7, 129.7, 128.5, 127.1, 125.8, 119.0, 117.7, 115.3, 83.9, 67.1, 65.2, 60.4, 25.5, 24.7, 24.6, 22.6, −1.6, −3.7. MS (FAB): m/z calcd for $C_{47}H_{73}BN_2O_{11}Si_3$: 936.4; found: 959 $[M+Na]^+$.

4.5. Compound 3g

Compound **3f** (300 mg, 0.32 mmol) was dissolved in 3 mL MeOH. Catalytic amount of p-TsOH was added to the suspension and the reaction mixture was stirred at room temperature for 20 min, and monitored by TLC (EtOAc/Hex 1:1). After completion, the solvent was removed under reduced pressure and the crude product was purified by using column chromatography on silica gel (EtOAc/Hex 1:1) to give compound **3g** (143 mg, 63%) as a white powder.

^{1}H NMR (200 MHz, MeOD): δ = 7.99 (1H, d, J = 10.3 Hz); 7.76 (2H, d, J = 8 Hz); 7.64–7.59 (2H, m); 7.38–7.34 (4H, m); 5.13 (2H, s); 4.88 (2H, s); 4.61 (4H, s); 4.41–4.29 (2H, m); 1.29 (12H, s); 1.08–0.99 (2H, m); 0.03 (9H, s). ^{13}C NMR (100 MHz, MeOD): δ = 176.3, 150.7, 144.6, 140.4, 135.8, 135.3, 132.8, 131.5, 128.9, 127.9, 126.6, 119.7, 118.4, 84.7, 65.9, 60.7, 30.4, 25.5, 17.7, -0.9. MS (FAB): m/z calcd for $C_{35}H_{45}BN_2O_{11}Si$: 708.3; found: 731 $[M+Na]^+$.

4.6. Compound 3

To a solution of compound **3g (**103 mg, 0.15 mmol) in dry THF (2 mL) and pyridine (153 μL, 1.90 mmol) was added methyl-chloroformate (138 μL, 1.78 mmol) dropwise with a syringe at 0 °C. The reaction mixture was stirred for 1 h, while warming to room temperature and monitored by TLC (EtOAc/Hex 2:3). Upon completion, the reaction mixture was diluted with EtOAc, and washed with saturated solution of NH_4Cl. The organic layer was separated, dried over $MgSO_4$, and evaporated under reduced pressure. The crude product was purified by column chromatography on silica gel (EtOAc/Hex 2:3) to give compound **3h** (92 mg, 77%) as a yellow oil. The compound was (92 mg, 0.11 mmol) directly dissolved in dry THF (2 mL) under Argon atmosphere and TBAF (340 μL, 1 M in THF) was added. The reaction was stirred in room temperature for 1 h and was monitored by C-18 RP-HPLC (gradient: 30–100% ACN in water over 20 min). Upon completion of the reaction, the solvent was removed under reduced pressure and the crude product was purified by using preparative RP-HPLC (gradient: 30–100% ACN in water over 20 min) to give compound **3** (40 mg, 50%) as a white powder.

^{1}H NMR (400 MHz, CD_3CN): δ = 8.48 (1H, br s); 7.99 (1H, d, J = 8.8 Hz); 7.84–7.82 (3H, m); 7.75 (1H, dd, J = 8.8, 2.2 Hz); 7.54 (2H, s); 7.49 (2H, d, J = 8 Hz); 5.24 (4H, s); 5.21 (2H, s); 4.98 (2H, s); 3.74 (6H, s). ^{13}C NMR (50 MHz, CD_3OD): δ = 164.3, 155.6, 152.3, 144.1, 142.8, 142.3, 141.8, 133.3, 132.7, 130.4, 129.7, 128.7, 126.8, 125.1, 118.6, 117.2, 77.0, 65.8, 64.2, 53.9. MS (ESI): m/z calcd for $C_{28}H_{27}BN_2O_{15}$: 642.1; found: 641.0 $[M-H]^-$.

5. Spectroscopic assay conditions

The signal developed by the DCR amplification technique was monitored with a spectrophotometer using a 96-well plate reader. A total volume of 100 μM incubation mixture was used for each well. The absorbance was measured upon addition of various amounts of H_2O_2 [1.00, 0.10 and 0.01 equiv] at 405 nm.

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