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ScienceDirect
Bioorganic & Medicinal Chemistry 15 (2007) 7318–7324
Bioorganic & Medicinal Chemistry

Molecular probe for enzymatic activity with dual output

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Received 17 April 2007; revised 7 August 2007; accepted 17 August 2007
Available online 29 August 2007

Abstract—A novel molecular probe for enzymatic activity with a dual output detection-mode has been developed. The probe effectively detected the presence of the bacterial protease penicillin-G–amidase; a single cleavage by the enzyme initiated the fragmentation of a self-immolative dendritic platform to release two reporter units. The signals of the free reporters were detected by two different spectroscopic techniques, fluorescence and UV–vis. This is the first reported molecular probe with two different chromogenic reporter units activated by a specific stimulus.

1. Introduction

There is a constant and growing need for the detection of enzymatic activity for imaging and diagnostics purposes.[1–4] Most currently used molecular probes are based on generation of a chromogenic compound through the catalytic activity of a specific enzyme.[5–7] The chromogenic assay is usually monitored by fluorescence or UV–vis spectroscopy and in other examples by generation of luminescence or precipitated dye compounds.[8] Each assay has its own advantages and disadvantages. For example, fluorescence can be detected at very low chromophore concentration (down to nm range). However, it cannot be used in an environment—as in the presence of certain proteins—that quenches the fluorescent signal. A molecular probe with dual output signals offers two detection modes allowing use of the same probe in different environments. Here we report the design, synthesis, and enzymatic activation of a novel molecular probe with UV–vis and fluorescence modes for the detection of a specific catalytic activity.

The molecular probe is illustrated in Figure 1. The central unit of the probe (the molecular adaptor) is linked to an enzymatic substrate that acts as a trigger and to two different reporter molecules. Cleavage of the enzymatic substrate triggers the release of the two reporters and a consequent activation of their signals.

In order to construct a molecular probe with the required activity we have used AB_2 self-immolative dendron **1** (Fig. 2). Self-immolative dendrimers are novel class of molecules that can amplify a single cleavage event received at the focal point into release of multiple tail groups at the periphery.[9–17] In dendron **1**, a phenylacetamide group (red), known to be a substrate for the bacterial enzyme penicillin-G–amidase (PGA), is used as a trigger and the reporter units are 4-nitrophenol and 6-aminoquinoline. The reporter molecules are attached through stable carbamate linkages that maintain the signals in an OFF position. Upon cleavage of the trigger and release of the reporter units, the signal from each is turned ON. The 4-nitrophenol is detected by visible yellow color and 6-aminoquinoline by fluorescence emission. PEG5000 is also attached to the dendritic adaptor in order provide substantial aqueous solubility.[18]

The disassembly pathway of molecular probe **1** is initiated by catalytic cleavage of phenylacetic acid by PGA, elimination of azaquinone-methide, decarboxylation,

Abbreviations: ACN, acetonitrile; Boc, *t*-butoxycarbonyl; DCM, dichloromethane; DIPEA, diisopropyl ethyleneamine; DMAP, dimethylaminopyridine; DMF, dimethyl formamide; EDC, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide; EtOAc, ethylacetate; He, hexanes; HOBT, 1-hydroxybenzotriazole; PEG, polyethylene glycol; PNP, *p*-nitrophenol; RT, retention time; TBSCl, *t*-butyldimethylsillyl chloride; TBTA, tris-(1-benzyl-1*H*-[1,2,3]triazol-4-ylmethyl)-amine; TFA, trifluoroacetic acid; THF, tetrahydrofuran.

Keywords: Molecular probe; Assay; Enzyme; Dendrimer; Self-immolative.

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doi:10.1016/j.bmc.2007.08.046

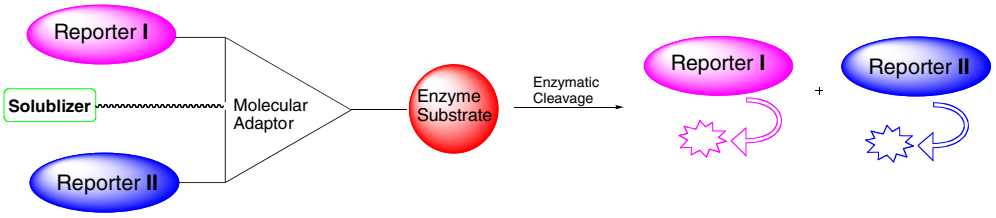

Figure 1. A graphical illustration of a molecular probe for detection of enzymatic activity with dual output.

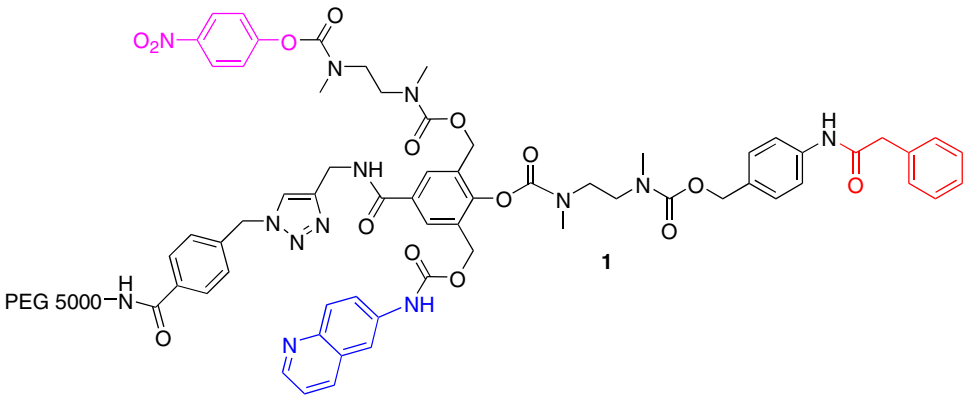

Figure 2. Chemical structure of a molecular probe with UV–vis and fluorescence outputs for penicillin-G–amidase activity. The phenylacetamide group (red) is a substrate for PGA. The reporter units, 4-nitrophenol and 6-aminoquinoline, provide a visible signal and a fluorescence signal, respectively, upon release.

and cyclization to release dimethylurea derivative and phenol **1a** (Fig. 3). The latter rapidly undergoes double elimination[10] to release the two reporter units and byproduct **1b**. The output of these cascade reactions should be expressed by new absorbance and fluorescence signals as a result of the release of 4-nitrophenol and 6-aminoquinoline, respectively.

Dendritic molecule **1** was synthesized according to the scheme shown in Figure 4. 4-Hydroxybenzoic acid was coupled with propargyl amine to form amide **2**. The amide was reacted with formaldehyde to generate dibenzylalchohol **3**, which was then protected with two equivalents of *t*-butyldimethylsilylchloride (TBSCl) to afford phenol derivative **4**. The latter was activated with 4-nitrophenyl-chloroformate to produce carbonate **5**, which was further reacted with mono-Boc-*N*,*N*′-dimethylethylene-diamine to afford compound **6**. Deprotection of **6** with trifluoroacetic acid (TFA) gave an amine salt, which was immediately reacted with the carbonate trigger **7** to give compound **8**. Activation of diol **8** with 4-nitrophenyl-chloroformate afforded double carbonate **9**, which was selectively reacted with one equivalent of mono-Boc-*N*,*N*′-dimethylethylene-diamine to yield compound **10**. Reaction of **10** with 6-aminoquinoline afforded compound **11**, which was deprotected using TFA and in situ reacted with bis(4-nitrophenyl)-carbonate to generate compound **12**. The latter was coupled with PEG5000 azide derivative[18] **13** via the copper(I)-catalyzed Huisgen cycloaddition[19] to give dendritic molecule **1**.

In order to test our molecular probe, we incubated compound **1** in phosphate buffered saline (PBS, pH 7.4) at 37 °C (with and without PGA) and monitored the release of the reporters, 6-aminoquinoline and 4-nitrophenol, by fluorescence and UV–vis spectroscopy. The fluorescence spectrum of **1** exhibited a strong emission band at 390 nm that decreased over time as the probe fragmented (Fig. 5a). The generation of a new band at 460 nm indicated the formation of free 6-aminoquinoline. In order to evaluate the kinetic behavior of the sequential fragmentation, the maximal intensities of the band at 460 nm were plotted as a function of the time (Fig. 5b). The enzymatic cleavage was also monitored in the visible range; upon incubation of **1** in the presence of PGA, the release of 4-nitrophenol resulted in a significant increase in absorbance with a maximum at approximately 405 nm (Fig. 5c). This absorbance gradually increased as a function of time in the presence of PGA (Fig. 5d). Importantly, no release of 6-aminoquinoline or 4-nitrophenol was observed when compound **1** was incubated in the buffer without PGA (data not shown).

As far as we know, this is the first reported molecular probe that includes two different types of reporter units activated upon on a specific stimulus. The only examples in the literature for a molecular probe with dual mode of detection employ generation of a single reporter molecule with UV and fluorescence signals.[20] The other option to achieve dual detection would be to use two separate probes. However, in this case there could be a problem of competitive catalysis (circumstances in which the km of the two substrate is not identical). In our probe, 6-aminoquinoline and 4-nitrophenol, detected by fluorescence and absorbance spectroscopy, respectively, were used as reporter units. Due to the synthetic flexibility of our approach, other reporter mole-

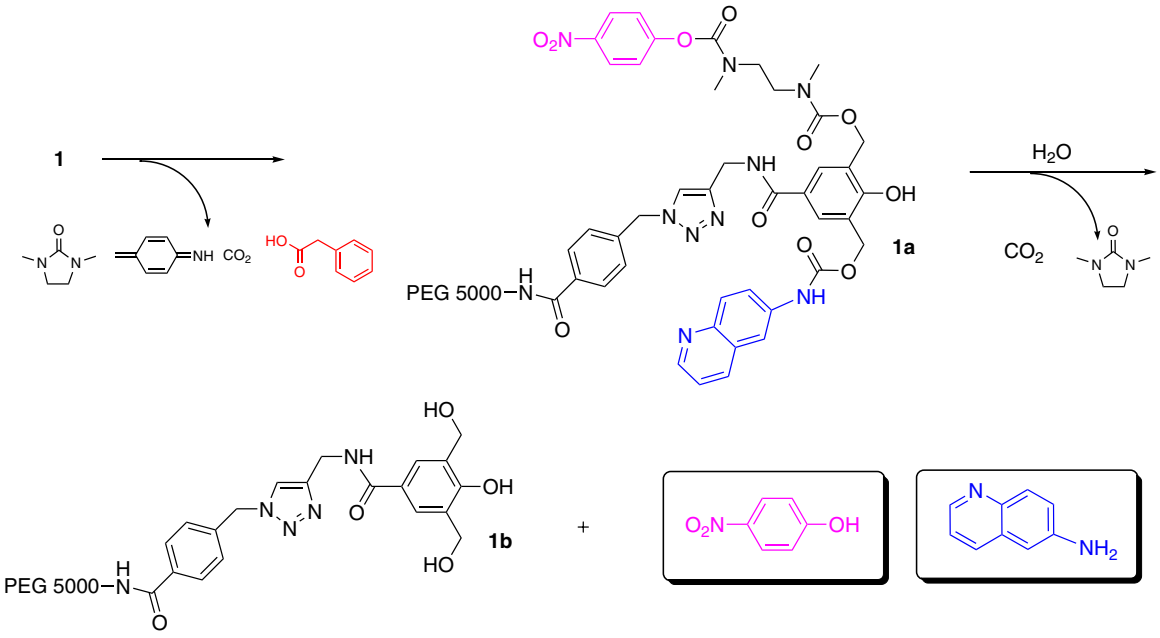

Figure 3. Disassembly pathway of AB_2 self-immolative dendritic molecule **1**.

cules with different types of functional groups, like amine or hydroxyl, can be linked to our molecular probe. The two assays must be orthogonal each to other, in order to prevent disturbances in the detection measurement. Another advantage of the probe is the aqueous solubility provided by the PEG5000 tail; this tail should guarantee sufficient solubility for most organic reporter molecules in assay media. As demonstrated, this probe functioned under physiological conditions and this design should allow detection of any number of specific bioactivities. We have used phenylacetamide as a triggering substrate for PGA, however, many other substrates can be applied instead. Specifically, this system could be used for detection of proteolytic activity by PGA, which is expressed in bacteria.[22] The disassembly of the probe occurred according to Figure 3. Recent studies of our self-immolative dendritic systems have shown that the rate-limiting step of the disassembly takes place after the enzymatic cleavage and is dependent on the nature of the substituent on the aromatic ring.[23]

In summary, we have synthesized and demonstrated the utility under physiological conditions of a novel molecular probe, based on an AB_2 self-immolative dendron, that has a dual output detection-mode for enzymatic activity. The probe was designed for detection of the bacterial protease PGA, such that a single cleavage by the enzyme initiated the fragmentation of the dendritic platform to release two different types of reporter units. Each free reporter was visualized by a different spectroscopic technique, fluorescence and UV–vis. The reporter units can be readily replaced by other chromogenic molecules thereby the type of output signal desired in the probe system. Similarly, substrates other than the phenylacetamide trigger can be used to allow detection of different enzymatic activities.

2. Experimental

2.1. General

All reactions requiring anhydrous conditions were performed under argon or N_2 atmosphere. 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 25 g phosphomolybdic acid, 10 g $Ce(SO_4)_2$*-H_2O, 60 ml conc. H_2SO_4, and 940 ml H_2O, followed by heating. Flash chromatography (FC): silica gel Merck 60 (partical size 0.040–0.063 mm), eluent given in parentheses. ^{H} NMR: Bruker AMX 200 or 400. The chemical shifts are expressed in δ relative to TMS ($\delta = 0$ ppm) and the coupling constants J in Hz. The spectra were recorded in $CDCl_3$ or CD_3OD as a solvent at room temperature. All reagents, including salts and solvents, were purchased from Sigma–Aldrich. TBTA was kindly obtained from the Sharpless laboratory (Scripps, CA).

2.1.1. 4-Hydroxy-*N*-(prop-2-ynyl)benzamide (compound 2). Commercially available 4-hydroxybenzoic acid (2.0 g, 14.5 mmol) was dissolved in DMF. Then, EDC (3.3 g, 17.4 mmol), HOBT (1.0 g, 7.3 mmol), and propargyl amine (1.0 ml, 14.5 mmol) were added. The mixture was stirred overnight and monitored by TLC (EtOAc/Hex = 2:3). After completion of the reaction the solvent was removed under reduced pressure. The crude product was purified by column chromatography on silica gel (EtOAc/Hex = 2:3) to give compound **2** (1.8 g, 70%) in the form of yellowish oil.

^{1}H NMR (200 MHz, $CDCl_3$)$\delta = 7.70$ (d, $J = 6.8$ Hz, 2H); 6.81 (d, $J = 6.8$ Hz, 2H); 4.11 (d, $J = 2.5$ Hz, 2H); 2.71 (t, $J = 2.5$ Hz, 1H). ^{13}C(400 MHz,

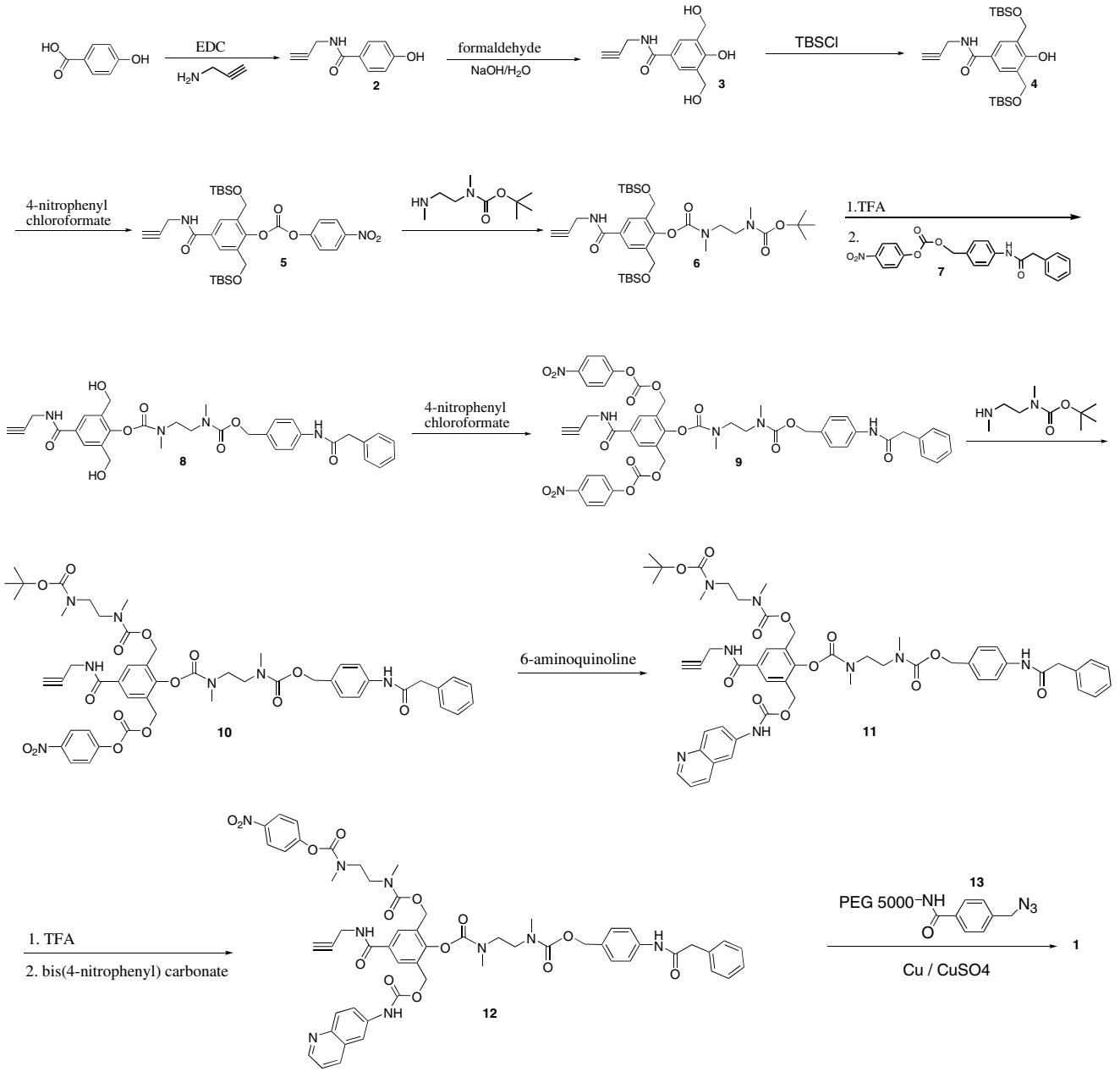

Figure 4. Chemical synthesis of molecular probe **1**.

$CDCl_3$)δ = 167.9, 160.6, 128.8, 124.4, 114.5, 79.5, 70.3, 28.3. MS (FAB): calculated for $C_{10}H_9NO_2$176.0 [M+H$^+$], found 176.0.

2.1.2. 4-Hydroxy-3,5-bis(hydroxymethyl)-*N*-(prop-2-ynyl)-benzamide (compound 3). To a cool 12% solution of NaOH in water (12 ml) compound **2** (1.8 g, 10.2 mmol) was added while maintaining the temperature at 0 °C. Formaldehyde 37% in water (10 ml) was added. The reaction mixture was stirred at 55 °C for 3 days and monitored by TLC (EtOAc/ MeOH = 95:5). After completion, the reaction mixture was diluted with EtOAc and washed with saturated ammonium chloride solution. The aqueous layer was washed twice with EtOAc. The combined organic layer was dried over magnesium sulfate and the solvent was removed under reduced pressure. The crude product was purified by column chromatography on silica gel (EtOAc/MeOH = 19:1) to give compound **3** (1.9 g, 80%) in the form of a white solid.

^{1}H NMR (200 MHz, CD_3OD) δ = 7.80 (s, 2H); 4.91 (s, 4H); 4.26 (d, J = 2.5 Hz, 2H); 2.70 (t, J = 2.5 Hz, 1H). ^{13}C(400 MHz, CD3OD) δ = 168.1, 156.7, 126.8, 126.0, 124.4, 79.4, 70.2, 60.3, 28.3. MS (FAB): calculated for $C_{12}H_{13}NO_4$ 236.0 [M+H$^+$], found 236.0.

2.1.3. 3,5-Bis((*tert*-butyldimethylsilyloxy)methyl)-4-hydroxy-*N*-(prop-2- ynyl)benzamide (compound 4). Compound **3** (713 mg, 3.0 mmol) was dissolved in DMF at 0 °C. Imidazole (408 mg, 6.0 mmol) and TBSCl (910 mg, 6.0 mmol) were added. The reaction mixture

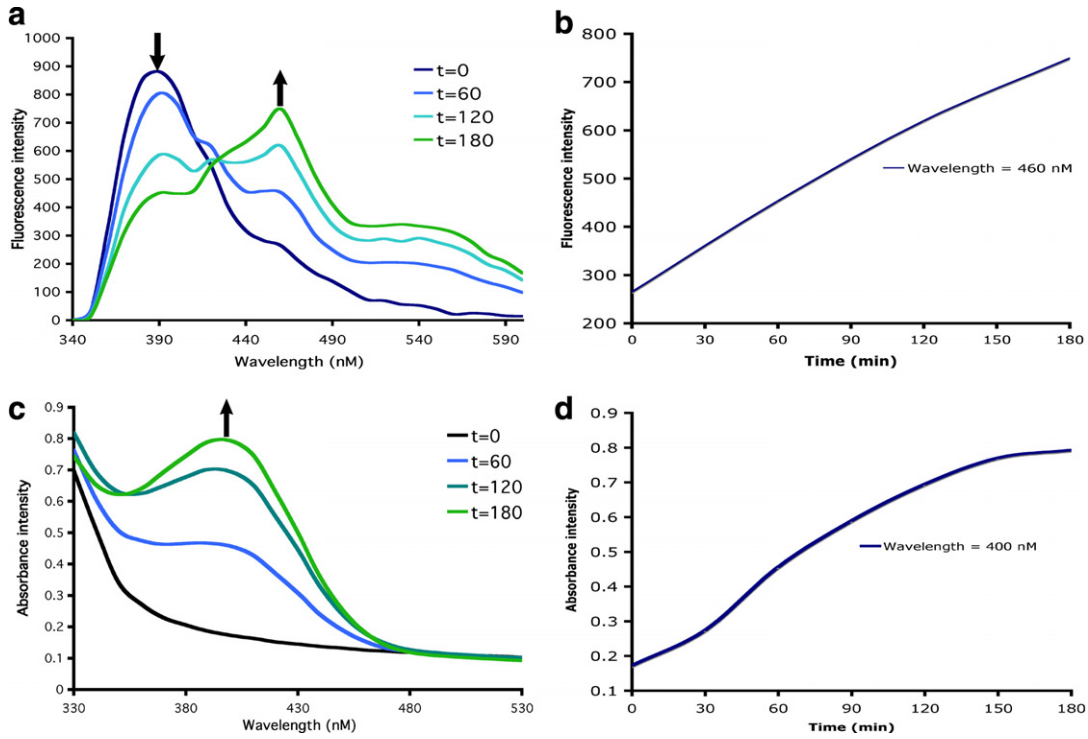

Figure 5. (a) Emission fluorescence (λ_{ex} = 250 nm) of **1** [500 μM] upon addition of PGA (1.0 mg/mL). (b) Fluorescence of **1** at 460 nm in the presence of PGA (1.0 mg/mL) as a function of time. (c) UV–vis absorbance spectra of **1** [500 μM] in the presence of PGA (1.0 mg/mL). (d) Absorbance of **1** at 400 nm after addition of PGA (1.0 mg/mL) as a function of time.

was stirred at room temperature for 2 hours and monitored by TLC (EtOAc/He = 2:8). After completion, the reaction mixture was diluted with ether and washed with saturated ammonium chloride solution. The organic layer was dried over magnesium sulfate and the solvent was removed under reduced pressure. The crude product was purified by column chromatography on silica gel (EtOAc/Hex = 15:85) to give compound **4** (1.12 g, 80%) in the form of a colorless oil.

^{1}H NMR (400 MHz, $CDCl_3$)δ = 7.57 (s, 2 H); 4.87 (s, 4H); 4.23 (dd, J = 5.0, 2.6 Hz, 2H); 2.17 (t, J = 2.5 Hz, 1H); 0.95 (s, 18H); 0.13 (s, 12H). ^{13}C(400 MHz, $CDCl_3$)δ = 166.7, 156.4, 126.1, 124.5, 79.6, 71.7, 62.7, 29.6, 25.8, 25.6, 18.2, −5.5. MS (FAB): calculated for $C_{24}H_{41}NO_4Si_2$ 464.2 [M+H$^+$], found 464.2.

2.1.4. 2,6-Bis((*tert*-butyldimethylsilyloxy)methyl)-4-(prop-2-ynylcarbamoyl)phenyl 4-nitrophenyl carbonate (compound 5). Compound **4** (1.12 g, 2.4 mmol) was dissolved in dry THF. Et_3N (1.0 ml, 7.2 mmol) was added and the mixture was cooled to 0 °C. Then, *p*-nitrophenyl chloroformate (581 mg, 2.9 mmol) dissolved in dry THF (10 ml) was added dropwise and the reaction mixture was stirred for 1 h at room temperature and monitored by TLC (EtOAc/He = 2:8). After completion the reaction mixture was filtered, the solvent was evaporated, and the crude product was purified by column chromatography on silica gel (EtOAc/Hex = 15:85) to give compound **5**(1.35 g, 90%) in the form of a colorless oil.

^{1}H NMR (200 MHz, $CDCl_3$)δ = 8.43 (d, J = 8.1 Hz, 2H); 8.02 (s, 2H); 7.63 (d, J = 8.1 Hz, 2H); 7.01 (m, 1H); 4.91 (s, 4H); 4.38 (dd, J = 5.0, 2.6, 2H); 2.41 (t, J = 2.5 Hz, 1H); 1.08 (s, 18H); 0.29 (s, 12H). ^{13}C(400 MHz, $CDCl_3$) δ = 166.4, 155.2, 149.4, 147.7, 145.5, 133.9, 132.2, 126.3, 125.3, 121.5, 79.2, 71.8, 60.3, 31.5, 25.8, 18.2, −5.5. HRMS (MALDI-TOF): calculated for $C_{31}H_{44}N_2O_8Si_2$ 651.2528 [M+Na$^+$], found 651.2562.

2.1.5. Compound 6. Compound **5** (1.5 g, 2.3 mmol) was dissolved in DMF. *N*,*N*′-Dimethylethylenediamine-mono-Boc[10] (541 mg, 2.9 mmol) was added. The reaction mixture was stirred at room temperature for 1 h and monitored by TLC (EtOAc/He = 1:1). After completion, the solvent was removed under reduced pressure and the crude product was purified by column chromatography on silica gel (EtOAc/Hex = 2:8) to give compound **6** (1.45 g, 90%) in the form of a colorless oil.

^{1}H NMR (400 MHz, $CDCl_3$)δ = 7.79 (s, 2H); 6.32 (m, 1H); 4.68–4.67 (m, 4H); 4.27–4.25 (m, 2H); 3.61–3.43 (m, 4H); 3.24 (s, 2H); 3.12 (s, 1H); 2.96 (s, 3H); 2.32 (bs, 1H); 1.51–1.46 (m, 9H); 0.92 (s, 18H); 0.08 (s, 12H). ^{13}C(400 MHz, $CDCl_3$) δ = 167.2, 153.1, 153.0, 134.6, 130.8, 125.1, 80.2, 78.8, 72.0, 59.9, 46.4, 46.1, 36.4, 35.9, 35.1, 29.8, 28.3, 25.7, 18.2, −5.5. MS (FAB): calculated for $C_{34}H_{59}N_3O_7Si_2$ 700.4 [M+Na$^+$], found 700.3.

2.1.6. Compound 8. Compound **6** (1.4 g, 2.1 mmol) was dissolved in TFA and the reaction mixture was stirred for a few minutes. The excess of acid was removed under reduced pressure and the crude amine salt was dissolved in DMF (1.0 ml). Then, previously reported compound

7[21] (4-nitrophenyl 4-(2-phenylacetamido)benzyl carbonate) (1.1 g, 2.7 mmol) and Et_3N (1.0 ml) were added and the reaction mixture was monitored by TLC (EtOAc). After completion the solvent was removed under reduced pressure. The crude product was purified by column chromatography on silica gel (MeOH/EtOAc = 5:95) to give compound **8** (870 mg, 67%) as a white solid.

^{1}H NMR (400 MHz, CD_3OD) δ = 7.88 (s, 2 H); 7.52–7.19 (m, 9H); 5.09–5.02 (m, 2H); 4.54–4.43 (m, 4H); 4.12 (s, 2H); 3.64 (s, 2H); 3.60–3.48 (s, 4H); 3.00–2.83 (s, 6H); 2.57 (s, 1H). HRMS (MALDI-TOF): calculated for $C_{33}H_{36}N_4O_8$ 639.2425 [M+Na$^+$], found 639.2483.

2.1.7. Compound 9. Compound **8** (400 mg, 0.64 mmol) was dissolved in dry THF and the reaction mixture was cooled to 0 °C. Then, DIPEA (950 μl, 5.12 mmol) was added followed by PNP-chloroformate (780 mg, 3.90 mmol) and pyridine (25 μl, 0.32 mmol). The reaction mixture was allowed to warm up to room temperature and monitored by TLC (EtOAc/Hex = 3:1). After completion the reaction mixture was diluted with EtOAc and washed with saturated NH_4Cl and with saturated $NaHCO_3$ solutions. The organic layer was dried over magnesium sulfate. The solvent was removed under reduced pressure. The crude product was purified by column chromatography on silica gel (EtOAc/Hex = 7:3) to give compound **9** (300 mg, 50%) in the form of a white solid.

^{1}H NMR (200 MHz, $CDCl_3$)δ = 8.18 (d, J = 8 Hz, 4H); 7.85 (m, 2H); 7.30–6.98 (m, 13H); 5.15–4.93 (m, 6H); 4.19 (dd, J = 5.0, 2.6 Hz, 2H); 3.72 (s, 2H); 3.51–3.46 (m, 4H); 3.14–2.87 (m, 6H); 2.24 (t, J = 2.5 Hz, 1H). HRMS (MALDI-TOF): calculated for $C_{47}H_{42}N_6O_{16}$ 969.2545 [M+Na$^+$], found 969.2594.

2.1.8. Compound 10. Compound **9** (200 mg, 0.21 mmol) was dissolved in dry DMF and the reaction mixture was cooled to 0 °C. *N*,*N*′-Dimethylethylenediamine-mono-Boc (541 mg, 2.9 mmol) dissolved in 2 ml of DMF was added dropwise. The reaction mixture was allowed to warm up to room temperature and monitored by TLC (EtOAc). After completion the solvent was removed under reduced pressure. The crude product was purified by column chromatography on silica gel (EtOAc) to give compound **10** (90 mg, 45%) in the form of a white solid.

^{1}H NMR (200 MHz, $CDCl_3$)δ = 8.18 (d, J = 8 Hz, 2H); 7.95 (m, 2H); 7.30–7.00 (m, 11H); 5.23–4.92 (m, 6H); 4.18 (bs, 2H); 3.71 (s, 2H); 3.45–3.32 (m, 8H); 2.95–2.80 (m, 12H); 2.15 (s, 1H); 1.40–1.34 (m, 9H). HRMS (MALDI-TOF): calculated for $C_{50}H_{57}N_7O_{15}$ 1018.3805 [M+Na$^+$], found 1018.3770.

2.1.9. Compound 11. Compound **10** (85 mg, 0.09 mmol) was dissolved in DMF. Then, 6-aminoquinoline (50 mg, 0.32 mmol) and a catalytic amount of HOBT were added, followed by the addition of DIPEA (40 μl, 0.12 mmol). The reaction mixture was heated to 50 °C, stirred overnight, and monitored by TLC (MeOH/EtOAc = 1:9). After completion, the solvent was removed under reduced pressure. The crude product was purified by column chromatography on silica gel (MeOH/EtOAc = 1:9) to give compound **11** (50 mg, 62%) in the form of a white solid.

^{1}H NMR (200 MHz, $CDCl_3$)δ = 8.75 (s, 1H), 8.05–7.55 (m, 7H), 7.39–6.97 (m, 9H); 5.04 (bs, 6H), 4.20–4.19 (m, 2H); 3.79–3.29 (m, 10H); 3.10–2.85 (m, 12H); 2.30–2.22 (m, 1H); 1.43–1.36 (m, 9H). HRMS (MALDI-TOF): calculated for $C_{53}H_{60}N_8O_{12}$ 1023.4223 [M+Na$^+$], found 1023.4139.

2.1.10. Compound 12. Compound **11** (50 mg, 0.05 mmol) was dissolved in TFA and stirred for few minutes, the excess of acid was removed under reduced pressure and the crude amine salt was dissolved in DMF (0.5 ml). Then, bis-(4-nitrophenyl)carbonate (50 mg, 0.16 mmol) and Et_3N (0.1 ml) were added and the reaction was monitored by TLC (MeOH/EtOAc = 1:9). After completion, the solvent was removed under reduced pressure. The crude product was purified by column chromatography on silica gel (MeOH/EtOAc = 1:9) to give compound **12** (45 mg, 90%) as a white solid.

^{1}H NMR (200 MHz, $CDCl_3$)δ = 8.60 (s, 1 H); 8.03–7.67 (m, 9H); 7.38–7.00(m, 11H); 5.13–4.94 (m, 6H); 4.00 (bs, 2H); 3.56–3.46 (m, 10H); 3.00–2.79 (m, 12H); 2.54 (s, 1H). HRMS (MALDI-TOF): calculated for $C_{55}H_{55}N_9O_{14}$ 1088.3761 [M+Na$^+$], found 1088.3818.

2.1.11. Compound 1. Compound **11** (25 mg, 0.025 μmol) was dissolved in DMF. Then, previously reported compound **13**[18]) (150 mg, 0.027 μmol) was added followed by addition of copper sulfate (3.0 mg, 19.0 μmol), TBTA (10.0 mg, 19 μmol) and a few copper turnings. The reaction mixture was stirred for overnight at room temperature. It was monitored by RP-HPLC and after completion, the mixture was filtered and the solvent was removed under reduced pressure. The crude product was purified by column chromatography on silica gel (MeOH/DCM = 2:8) to give compound **1** (130 mg, 85%) in the form of a white solid.

2.2. Reporters' release analysis—general protocol

PGA (56 mg/ml) was purchased from sigma and was diluted with PBS 7.4 to give a 1.0 mg/ml solution. Stock solution (10 mM) of compound **17** was prepared in DMSO. The stock solution (10 μL) was diluted either with 190 μL PBS 7.4 (control) or with 190 μL of the 1.0 mg/ml PGA solution to give final concentration of 500 μM for compound **17**. All solutions were kept at 37 °C and their fluorescence and absorbance spectra were measured by SpectraMax M2 spectrophotometer (molecular devices). Standard coaster 96-well plates were used with sample volumes of 200 μL. The fluorescence spectra were performed by excitation at 250 nm. The emission fluorescence was recorded between 330 nm and 600 nm. The absorbance spectra were recorded between 330 nm and 530 nm.

Acknowledgments

D.S. thanks the Israel Science Foundation and the Israel Ministry of Science 'Tashtiot' program for financial support.

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