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Development of a new enzyme-responsive self-immolative spacer conjugate applicable to the controlled drug release

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ABSTRACT

A new self-immolative spacer conjugate based on a chemical adaptor unit aiming at controlled releasing drugs was designed and synthesized. It releases a fluorophore which was used as a model drug via a spontaneous cyclization mechanism after cleavage of an enzyme substrate. This system was proved to be stable under physiological conditions and only decomposed triggered by enzyme. It provides a generic linkage allowing connection of a variety of drugs and targeted devices to the chemical adaptor.

1. Introduction

Delivering a parent drug via prodrug or reversible carrier-drug conjugate has been considered as one of the most important and promising therapeutic tool in nanomedicine, especially in cancer therapy and typically being associated with severe side effects of the drugs.[1–5] The free parent drug was bounded to the linker or carrier and could be released at the target site activated by site-specific enzyme under physiological conditions to exert a controlled drug release.[6–17] This way, the use of enzymes to release the drug could be carried out under very mild neutral and aqueous conditions and the side effect or drug toxicity could be avoided.

From the chemical concept, one of the component of the prodrug or reversible carrier-drug bioconjugate is self-immolative spacer which should be designed rationally in order to be applied in the different usages. The spacer can incorporate three parts: target device, site-specific activation trigger and parent drug.[18–22]

In here, we choose an amino acid L-cysteine bearing three different functional groups as central core and design an enzyme-responsive self-immolative spacer conjugate (Fig. 1). Solubilizer, enzyme substrate and model drug are conjugated to the corresponding position of the central core in which tris-TEG as solubilizer, phenylacetyl group as enzyme substrate and fluorescent 7-amino-4-trifluoromethylcoumarin (AFC) as model drug. Cleaving the enzyme substrate can generate an amine group that spontaneously cyclizes to form piperazine-2,5-dione derivative (**13**) and the model drug releases from the spacer conjugate at the same time.

2. Results and discussion

2.1. Synthesis

For the purpose of constructing three-components spacer conjugate with the respective required functions, 2-amino-3-mercaptopropionic acid (L-cysteine) is chosen as the central core. It has three different functional groups, SH, CO_2H and NH_2, suitable for linkage. In here, this conjugate includes enzyme substrate, reporter and solubilizer. A phenylacetamide group, known to be a substrate for the enzyme penicillin-G-amidase from *Escherichia coli* (PGA), is used as trigger and is attached to amino group through glycine moiety. Cleavage of the enzyme substrate triggers the release of the reporter from the spacer conjugate. 7-Amino-3-trifluoromethylcoumarin is selected as the reporter unit and linked to spacer via carbamate bond. With the concept of attaching the molecules bearing different functional groups to the self-immolative spacer conjugate, 4-hydroxymethylphenol is selected between L-cysteine and reporter. Therefore, diverse molecules containing NH_2, CO_2H, or OH moieties can be coupled to 4-hydroxy group through carbamate, ester, and carbonate bonds, respectively. To improve the solubility of the spacer conjugate in aqueous circumstance tris-triethylene glycol chains are introduced in here.

Spacer conjugate **1** was synthesized according to the route shown in Scheme 1. Compound **2** containing enzyme substrate

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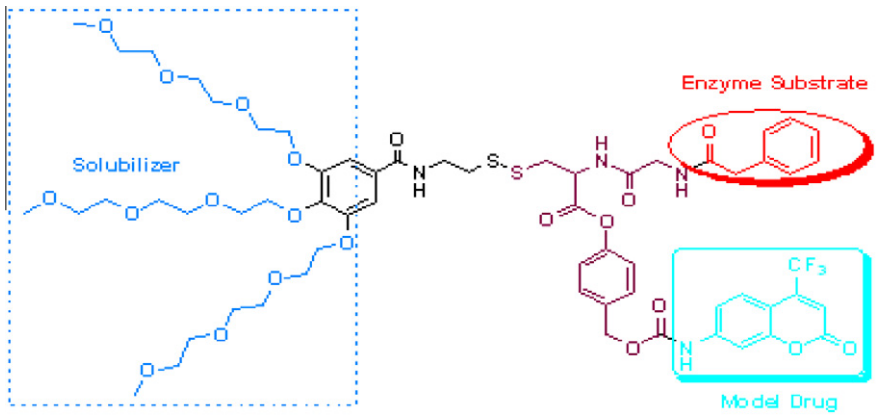
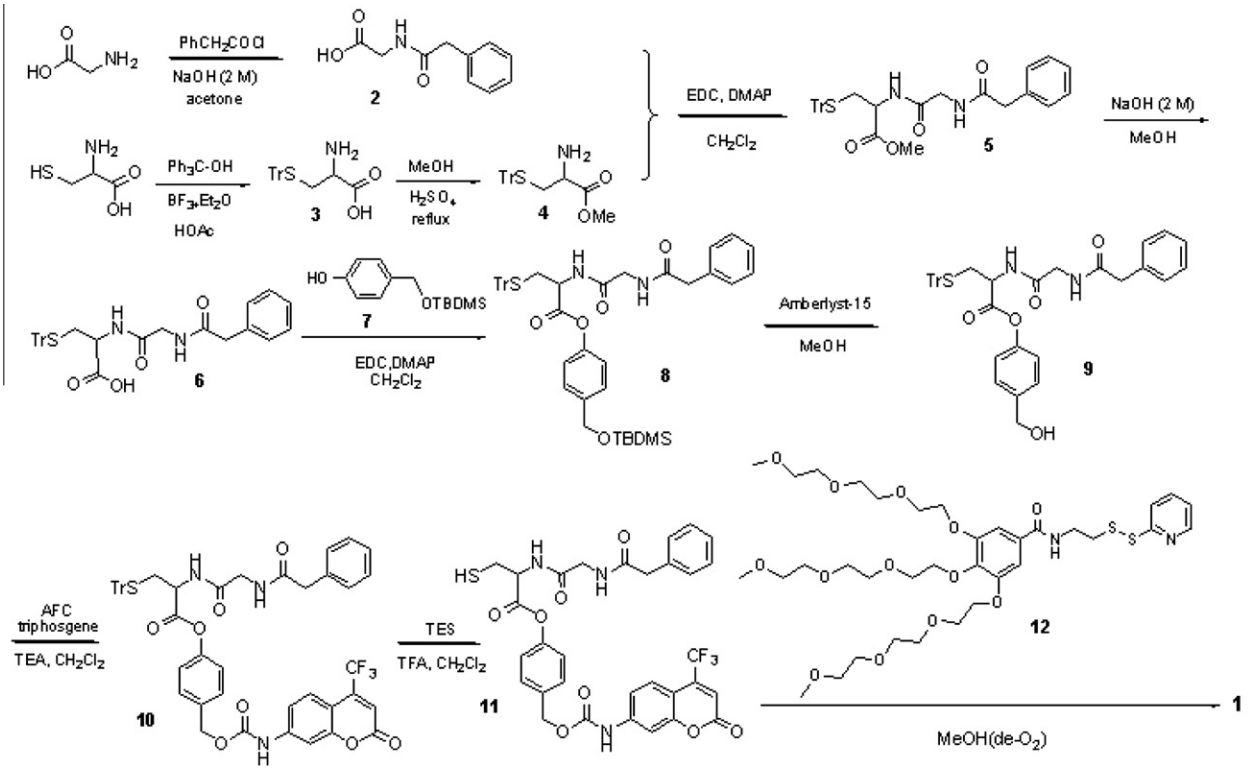


Figure 1. Structure of enzyme-responsive self-immolative conjugate **1**.



Scheme 1. Synthesis of conjugate **1**.

was formed by reacting glycine with phenylacetyl chloride in the basic acetone solution. The functional group SH and CO_2H of L-cysteine were protected with triphenylmethyl and methyl ester successively to afford **4**, which was further coupled with **2** to produce compound **5**. Hydrolysis of **5** gave carboxylic acid **6**, which was coupled with compound **7** (TBDMS-protected 4-hydroxymethylphenol) via EDC/DMAP condition to generate compound **8**. Deprotecting of TBDMS group selectively using acidic amberlyst-15 afforded hydroxyl derivative **9**. The benzylic alcohol was reacted with 7-amino-4-trifluorocoumarin isocyanate to give **10**, in which AFC was attached to the spacer through stable carbamate formation. The trityl group was removed from **10** in 50% TFA/DCM solution catalyzed by triethylsilane (TES) to generate mercapto derivative **11**, which was then reacted with **12** to give the target compound **1**. Compound **12** was prepared from 3,4,5-tris(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)benzoic acid with *S*-(2-pyridylthio)cysteamine via amide formation reaction using EDC/HOBt.

2.2. Release studies

Conjugate **1** was incubated in phosphate buffer solution (PBS, pH 7.4) at 37 °C (with or without PGA) and tested whether the reporter (AFC) can be released after activated by PGA which process was monitored by fluorescence and UV-vis spectroscopy. According to our design (Fig. 2), the phenylacetamide group can be recognized by enzyme PGA and the amide bond will be broken firstly. The generated amine will attack ester bond and occurs tandem cyclization reaction to generate piperazine-2,5-dione derivative (**13**) and the ester bond breaks. Then 1,6-elimination will occur within phenyl ring, and do expulsion of CO_2 concomitantly. Following this, the reporter AFC can be released from the spacer conjugate immediately.

The fluorescence spectrum of conjugate **1** exhibited as strong emission band at 391 nm and no significant fluorescence emission at 494 nm (AFC fluorescence band, shown in Figure 3A, black line).

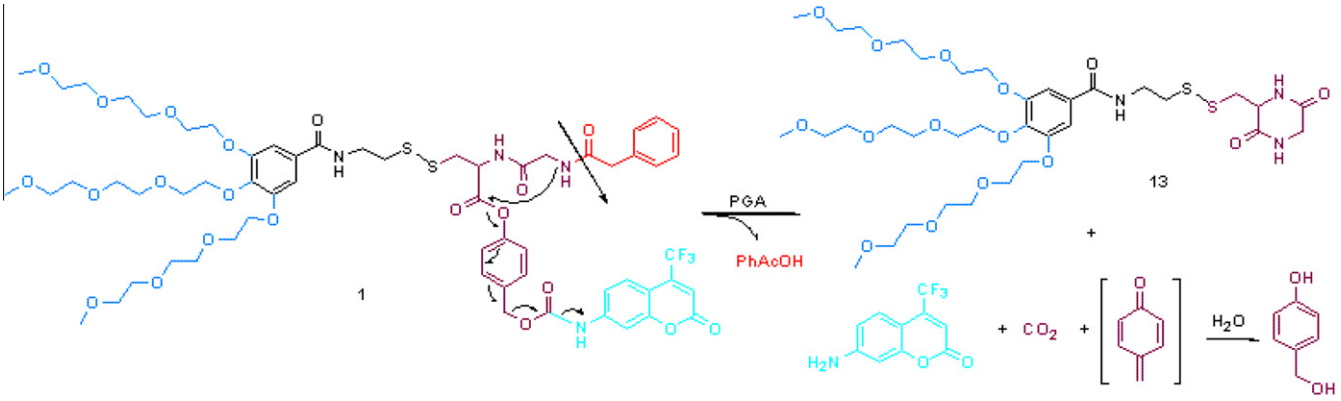


Figure 2. Enzyme-responsive self-immolative mechanism of conjugate **1**.

In order to test whether the AFC can be released from conjugate **1** by the catalytic activity of PGA, we incubated conjugate **1** in PBS (pH 7.4) at 37 °C in the presence of PGA and followed the process. As shown in Figure 3A, along with the time consuming the emission band of 391 nm decreased and a new strong band generated at 494 nm which indicated that the catalytic cleavage of the phenylacetamide bond and the release of free AFC do occurred. For further evaluating the kinetic behavior of conjugate **1** releasing reporter, the maximum intensities of the band at 494 nm were plotted as a function of the time (Fig. 3B). The maximum fluorescence intensity was reached after about 4 h which showed that the most reporters can be released during 4 h. Importantly, no spontaneous AFC release was observed in the absence of the enzyme.

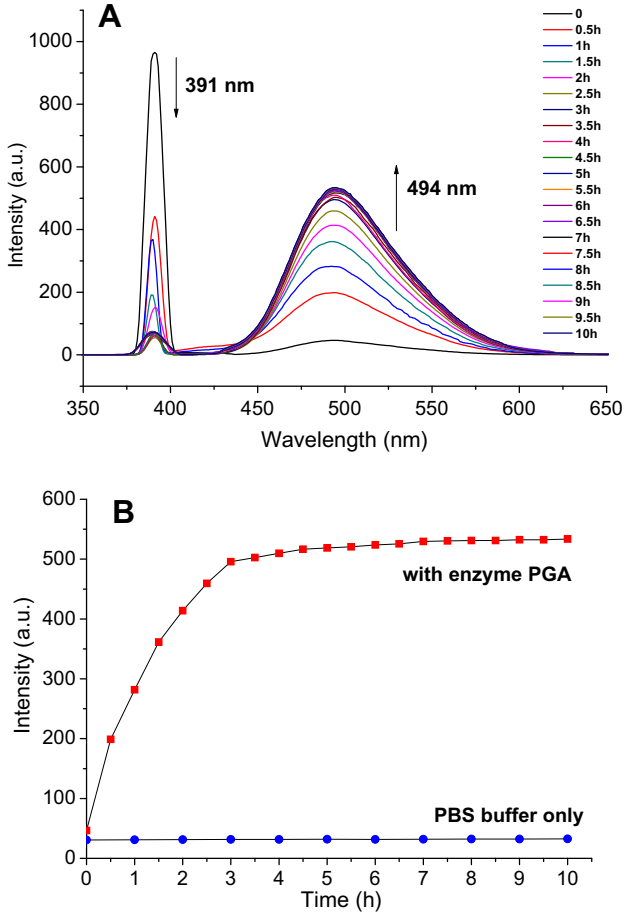


Figure 3. (A) Fluorescence emission spectrum of conjugate **1** with enzyme PGA incubated at 37 °C in 10% DMSO–PBS buffer. (B) Fluorescence emission time-course of conjugate **1** with or without enzyme.

In addition, GC–MS analysis of the enzyme catalytic hydrolysis mixture of **1** was performed to confirm the enzyme-responsive released mechanism. The results proved the existence of the decomposition fragments, phenylacetic acid (t_R = 7.46 min, [M] = 136.05), 4-hydroxymethylphenol (t_R = 10.21 min, [M] = 124.05), and AFC (t_R = 13.57 min, [M] = 229.04) and the efficacy of this spontaneous release process.

3. Conclusion

In conclusion, a new three-component enzyme-responsive self-immolative cyclization spacer conjugate based on L-cysteine as the central core that allowed the release of fluorophore AFC triggered by enzyme has been designed and synthesized. This spacer system was proved to be stable under physiological conditions and only decomposed trigger by enzyme. It provides a generic linkage allowing connection of a variety of drugs and target devices to the chemical adaptor. Now the solubilizer was displaced by *m*PEG and further to form amphiphilic molecular and some continuous works about amphiphilic drug carriers have been carried out in our lab.

4. Experimental

All commercial available chemical reagents were purchased from Aladdin, Alfa, GL (Shanghai, China) companies. Enzyme PGA was purchased from Zhejiang Shunfeng Haider Company. ^{1}H NMR and ^{13}C NMR spectra were recorded using a AV300 (300 MHz, Bruker) spectrometer. The chemical shifts (ppm) were expressed in δ relative to TMS and the coupling constants *J* in Hz. The MALDI-TOF MASS data were recorded using a MALDI AXIMA-CFR (PLUS, Japan; Bruker, Germany). Anhydrous solvent were distilled as follows: tetrahydrofuran (THF) from sodium/benzophenone; dichloromethane (DCM) from calcium hydride; methanol from magnesium.

4.1. Chemistry

4.1.1. 2-(2-Phenylacetamido)acetic acid (2)

L-Glycin (100 mg, 1.33 mmol) was dissolved in 2 M sodium hydroxide solution (2 mL), then acetone (2 mL) was added and the mixture was cooled under ice-bath. Phenyl acetyl chloride (161 μL, 1.21 mmol) was added dropwise at the same temperature, followed by warming to room temperature. After 6 h, the solvent was evaporated off, and the residue was adjust to pH = 2–3 using 1 M HCl and extracted with ethyl acetate. The combined organic

layer was dried over anhydrous magnesium sulfate, filtered, and concentrated to give **2** (190 mg, 81%) as a white solid. ^{1}H NMR (300 MHz, acetone-d_6): δ = 7.41–7.20 (m, 5H), 3.95 (d, J = 5.74 Hz, 1H), 3.59 (s, 2H). ^{13}C NMR (75 MHz, DMSO-d_6): δ = 717.53, 170.81, 136.32, 129.25, 128.36, 126.55, 42.15, 40.95. MALDI-TOF-MS: Calcd for $C_{10}H_{12}NO_3$ 194.1. Found 194.1 $[M+H]^+$.

4.2.2. 2-Amino-3-(tritylthio)propanoic acid (3)

To a solution of L-cysteine (1.0 g, 5.69 mmol) in acetic acid (15 mL) was added triphenyl methanol (1.64 g, 6.30 mmol), followed by adding trifluoroboron ethylether (720 μL, 5.69 mmol) dropwise and the reaction was stirred at room temperature. After 5 h, the reaction mixture was neutralized with saturated sodium acetate. The resulting precipitate was washed with ethylether and collected to give the desired compound **3** (1.87 g, 90%) as a white solid. ^{1}H NMR (300 MHz, acetone-d_6): δ = 7.46–7.23 (m, 15H), 3.64 (dd, J = 8.10 Hz, 4.20 Hz, 1H), 2.67–2.52 (m, 2H). ^{13}C NMR (75 MHz, DMSO-d_6): δ = 169.89, 144.42, 129.25, 128.11, 126.78, 65.95, 53.64, 34.39. MALDI-TOF-MS: Calcd for $C_{22}H_{20}NO_2S$ 362.1. Found 362.1 $[M-H]^-$.

4.2.3. Methyl 2-amino-3-(tritylthio)propanoate (4)

Compound **3** (500 mg, 1.38 mmol) was dissolved in well-dried methanol (8 mL) and was stirred under reflux catalyzed by concentrated sulfuric acid (0.5 mL). After 4 h, the solvent was removed under reduced pressure and the crude product was extracted with ethyl acetate and washed with saturated sodium bicarbonate for several times. The organic layer was dried over anhydrous magnesium sulfate, filtered and concentrated to give ester **4** (420 mg, 81%) as a yellow viscous oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.45–7.18 (m, 15H), 3.66 (s, 3H), 3.20 (q, J = 7.61 Hz, 4.94 Hz, 1H), 2.59 (dd, J = 12.5 Hz, 4.76 Hz, 1H), 2.47 (dd, J = 12.4 Hz, 7.76 Hz, 1H), 1.65 (s, 2H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 173.61, 144.15, 129.11, 127.53, 126.34, 66.31, 53.28, 51.58, 36.46. MALDI-TOF-MS: Calcd for $C_{23}H_{23}KNO_2S$ 416.1. Found 416.1 $[M+K]^+$.

4.2.4. Methyl 2-(2-(2-phenylacetamido)acetamido)-3-(tritylthio)propanoate (5)

Compound **4** was dissolved in DCM (5 mL). EDC (183 mg, 0.95 mmol) and DMAP (97 mg, 0.79 mmol) were added. The reaction was stirred at room temperature for 1 h. Then compound **2** (153 mg, 0.79 mmol) was added and the mixture was stirred for further 26 h. After completion, the mixture was washed with saturated sodium carbonate. The organic layer was dried over anhydrous magnesium sulfate, filtered and concentrated. The residue was purified by column chromatography on silica gel (V(EA)/V(Hexane) = 1:1) to give **5** (420.3 mg, 82%) as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.39–7.18 (m, 20H), 6.22 (d, J = 7.62 Hz, 1H), 6.06 (s, 1H), 4.46 (dd, J = 12.0 Hz, 5.49 Hz, 1H), 3.84 (d, J = 4.80 Hz, 2H), 3.69 (s, 3H), 3.60 (s, 2H), 2.70 (dd, J = 12.6 Hz, 6.12 Hz, 1H), 2.58 (dd, J = 12.5 Hz, 4.53 Hz, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 171.47, 170.57, 168.51, 144.29, 134.54, 129.56, 129.10, 128.12, 127.47, 127.02, 107.84, 67.14, 52.75, 51.36, 43.46, 43.04, 33.59. MALDI-TOF-MS: Calcd for $C_{33}H_{32}N_2NaO_4S$ 575.2. Found 575.2 $[M+Na]^+$.

4.2.5. 2-(2-(2-Phenylacetamido)acetamido)-3-(tritylthio)propanoic acid (6)

Compound **5** (420 mg, 0.76 mmol) was dissolved in methanol (5 mL) and 2 M sodium hydroxide (1 mL) was added. The reaction was stirred at room temperature for 1.5 h. After completion, methanol was removed under reduced pressure, and the basic aqueous layer was washed with ethyl acetate followed by acidified with 1 M HCl. The acidic aqueous layer was extracted with ethyl acetate and the organic layer was dried over anhydrous magnesium sulfate, filtered and concentrated to give the desired compound **6** (371 mg, 91%) as a yellow solid. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.37–7.14 (m, 20H), 6.74 (d, J = 7.08 Hz, 1H), 6.53 (s, 1H), 4.29 (t, J = 12.6 Hz, 6.30 Hz, 1H), 3.82 (d, J = 5.34 Hz, 1H), 3.77 (d, J = 5.25 Hz, 1H), 3.54 (s, 2H), 2.68 (dd, J = 12.8 Hz, 5.94 Hz, 1H), 2.59 (dd, J = 12.8 Hz, 8.04 Hz, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 172.76, 172.55, 169.34, 144.20, 134.30, 129.45, 129.35, 128.80, 128.00, 127.21, 126.85, 67.01, 60.52, 51.58, 42.80, 33.26. MALDI-TOF-MS: Calcd for $C_{32}H_{30}N_2NaO_4S$ 561.2. Found 561.2 $[M+Na]^+$.

4.2.6. 4-Tributyldimethylsilylhydroxymethylphenol (7)

4-Hydroxymethylphenol (600 mg, 4.83 mmol) was dissolved in well-dried THF (5 mL) and cooled to 0 °C in ice-bath. Then imidazole (658 mg, 9.67 mmol) was added. After stirring for 10 min, *tert*-butylchlorodimethylsilane (TBDMSCl, 729 mg, 4.83 mmol) was added. The reaction was stirred at room temperature for 3 h. The mixture was quenched with saturated NH_4Cl and extracted with ethyl acetate. The combined organic layer was dried over anhydrous magnesium sulfate, filtered and concentrated. The residue was purified by column chromatography on silica gel (V(EA)/V(Hexane) = 1:15) to give the desired compound **7** (860 mg, 75%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.08 (d, J = 8.08 Hz, 2H), 6.66 (d, J = 8.10 Hz, 2H), 5.18 (s, 1H), 4.57 (s, 1H), 0.84 (s, 9H), 0.7 (s, 6H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 155.01, 132.89, 128.13, 115.37, 65.16, 26.11, 18.58, −5.03. ESI-MS: Calcd for $C_{13}H_{21}O_2Si$ 237.1. Found 237.1 $[M-H]^-$.

4.2.7. 4-((*Tert*-butyldimethylsilyloxy)methyl)phenyl 2-(2-(2-phenylacetamido)acetamido)-3-(tritylthio)propanoate (8)

A mixture of compound **6** (500 mg, 0.93 mmol), **7** (222 mg, 0.93 mmol), EDC (268 mg, 1.40 mmol), HOBt (190 mg, 1.40 mmol) and TEA (260 μL, 1.86 mmol) in DCM (6 mL) was stirred at room temperature for 5 h. After completion, the organic layer was washed with saturated sodium bicarbonate, then dried over anhydrous magnesium sulfate, filtered and concentrated. The residue was purified by column chromatography on silica gel (V(EA)/V(Hexane) = 1:2) to give compound **8** (530 mg, 75%) as a light pink solid. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.24 (m, 24H), 6.50 (d, J = 7.52 Hz, 1H), 6.14 (s, 1H), 4.72 (s, 2H), 4.66 (t, J = 11.5Hz, 6.34 Hz, 1H), 4.12 (d, J = 4.91 Hz, 2H), 3.59 (s, 2H), 2.88 (dd, J = 12.6 Hz, 6.13 Hz, 1H), 2.65 (dd, J = 12.49 Hz, 4.21 Hz, 1H), 0.94 (s, 9H), 0.10 (s, 6H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 171.62, 168.98, 168.79, 149.10, 148.87, 144.09, 139.20, 134.59, 129.39, 129.27, 128.71, 127.97, 127.04, 126.84, 120.89, 67.03, 64.23, 60.29, 51.67, 43.01, 33.49, 25.87, 18.28, -5.32. MALDI-TOF-MS: Calcd for $C_{45}H_{50}N_2NaO_5SSi$ 781.3. Found 781.3 $[M+Na]^+$.

4.2.8. 4-(Hydroxymethyl)phenyl 2-(2-(2-phenylacetamido)-acetamido)-3-(tritylthio) propanoate (9)

To a solution of compound **8** (530 mg, 0.70 mmol) in methanol (10 mL) was added amberlyst-15 (79.5 mg, 15% weight to compound **8**). The reaction was stirred at room temperature for 2 h. The solid was filtered off and the solvent was removed under reduced pressure. The residue was purified by column chromatography on silica gel (V(EA)/V(Hexane) = 1:2) to give **9** (396 mg, 88%) as a white foamy solid. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.20 (m, 24H), 6.41 (d, J = 4.83 Hz, 1H), 4.59 (d, 3H), 3.80 (d, J = 4.98 Hz, 2H), 3.52 (s, 2H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 171.80, 168.97, 168.83, 149.57, 148.26, 144.17, 139.20, 134.43, 129.53, 129.07, 128.18, 128.08, 67.29, 64.45, 51.65, 43.34, 42.98, 33.56. MALDI-TOF-MS: Calcd for $C_{39}H_{36}N_2NaO_5S$ 667.2. Found 667.2 $[M+Na]^+$.

4.2.9. 4-((2-Oxo-4-(trifluoromethyl)-2H-chromen-7-ylcarbamoyloxy)methyl)phenyl 2-(2-(2-phenylacetamido)acetamido)-3-(tritylthio)propanoate (10)

7-Amino-4-trifluoromethylcoumarin (36 mg, 0.16 mmol) was dissolved in well-dried DCM (2 mL) under nitrogen, then cooled

in ice-bath. TEA (43 μL, 0.31 mmol) was added followed by triphosgene (55 mg, 0.19 mmol). The yellow solution was stirred at the same temperature for 1.5 h. Then a solution of **9** (100 mg, 0.16 mmol) in DCM (1 mL) was added and the resulting mixture was stirred for further 2 h at room temperature. The reaction mixture was washed with saturated sodium bicarbonate. The organic layer was dried over anhydrous magnesium sulfate, filtered and concentrated. The residue was purified by column chromatography on silica gel (*V*(EA)/*V*(Hexane) = 1:2) to give **10** (85 mg, 61%) as a blue solid. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.40 (m, 24H), 7.05 (d, *J* = 8.31 Hz, 2H), 6.67 (s, 1H), 6.40 (d, *J* = 7.53 Hz, 1H), 6.08 (s, 1H), 5.20 (s, 2H), 4.61 (d, *J* = 6.03 Hz, 2H), 3.84 (d, *J* = 5.13 Hz, 2H), 3.59 (s, 2H), 2.88 (dd, *J* = 12.8 Hz, 6.24 Hz, 1H), 2.65 (dd, *J* = 12.6 Hz, 4.47 Hz, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 171.95, 171.39, 168.98, 168.93, 159.54, 155.45, 150.40, 144.13, 141.66, 141.23, 134.40, 133.68, 129.80, 129.52, 129.47, 129.07, 128.21, 127.49, 127.13, 126.00, 119.78, 113.36, 108.68, 106.25, 67.34, 66.75, 60.54, 51.73, 43.32, 43.04, 33.48. MALDI-TOF-MASS Calcd for $C_{50}H_{40}F_3N_3NaO_8S$ 922.3. Found 922.5 $[M+Na]^+$.

4.2.10. 4-((2-Oxo-4-(trifluoromethyl)-2H-chromen-7-ylcarbamoyloxy)methyl)phenyl- 3-mercapto-2-(2-(2-phenylacetamido)acetamido)propanoate (11)

To a solution of **10** (20 mg, 0.018 mmol) in DCM (1 mL) was added trifluoroacetic acid (1 mL), followed by catalytic amount of triethylsilane. The reaction was stirred at room temperature for 30 min. After completion, the reaction mixture was concentrated and the residue was purified rapidly by column chromatography on silica gel (*V*(EA)/*V*(Hexane) = 1:2) to give **11** (12 mg, 80%) as a light blue solid. ^{1}H NMR (300 MHz, DMSO): δ = 8.60 (d, *J* = 6.99 Hz, 1H), 8.37 (t, *J* = 5.35 Hz, 1H), 7.71–7.61 (m,overlap, 2H), 7.53–7.47 (m, overlap, 3H), 7.31–7.24 (m, 5H), 7.16 (d, *J* = 6.87 Hz, 2H), 6.88 (s, 1H), 5.21 (s, 2H), 4.70 (dt, *J* = 6.45 Hz, 5.37 Hz, 1H), 3.82 (s, br, 2H), 3.49 (s, 2H), 2.97 (dd, *J* = 8.34 Hz, 5.10 Hz, 2H), 2.70 (t, *J* = 8.10 Hz, 1H), MALDI-TOF-MASS: Calcd for $C_{31}H_{26}F_3N_3NaO_8S$ 680.1. Found 680.1 $[M+Na]^+$.

4.2.11. 3,4,5-Tris(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)-N-(2-(pyridin-2-yldisulfanyl)ethyl)benzamide (12)

To a solution of 3,4,5-tris(2-(2-(2-methoxyethoxy)ethoxy)-ethoxy)benzoic acid in DCM (2 mL) were added *S*-(2-pyridylthio)-cysteamine[23] (100 mg, 0.16 mmol), EDC (38 mg, 0.20 mmol), HOBt (27 mg, 0.20 mmol) and TEA (55 μL, 0.39 mmol). The reaction was stirred at room temperature for 5 h. After completion, the reaction mixture was washed with sodium bicarbonate for twice and the organic layer was dried over anhydrous magnesium sulfate, filtered and concentrated. The residue was purified by column chromatography on silica gel (*V*(EA)/*V*(MeOH) = 10:1) to give compound **12** (62 mg, 58%) as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 8.40 (d, *J* = 0.72 Hz, 1H), 8.38 (t, *J* = 4.02 Hz, 2.40 Hz, 1H), 7.58 (t, *J* = 7.38 Hz, 5.58 Hz, 1H), 7.50 (d, *J* = 8.04 Hz, 1H), 7.17 (s, 2H), 7.13 (t, *J* = 4.89 Hz, 1.08 Hz, 1H), 4.19 (m, 6H), 3.82 (m, 6H), 3.71 (m, 8H), 3.65 (m, 12H), 3.54 (m, 6H), 3.38 (s, 3H), 3.36 (s, 5H), 3.01 (t, *J* = 5.32 Hz, 2.53Hz, 2H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 166.86, 159.03, 152.18, 149.42, 140.98, 136.89, 129.63, 121.10, 120.70, 106.81, 72.11, 71.63, 70.36, 70.22, 69.40, 68.68, 58.71, 53.40, 38.37, 37.71. MALDI-TOF-MASS: Calcd for $C_{35}H_{57}N_2O_{13}S_2$ 777.3. Found 777.3 $[M+H]^+$.

4.2.12. Conjugate 1

A mixture of compound **11** (12 mg, 0.018 mmol) and compound **12** (12 mg, 0.015 mmol) was stirred in de-oxygen methanol (4 mL) under nitrogen atmosphere for 24 h. After completion, the solvent was evaporated off under reduced pressure. The crude product was purified by column chromatography on silica gel (*V*(EA)/*V*(MeOH) = 5:1) to give the conjugate **1** (12 mg, 52%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.59 (d, *J* = 7.76 Hz, 2H), 7.50 (d, *J* = 7.87 Hz, 2H), 7.42 (d, *J* = 8.21 Hz, 2H), 7.34 (s, 2H), 7.15 (d, *J* = 9.56 Hz, 2H), 7.03 (s, 1H), 6.66 (s, 1H), 5.17 (s, 2H), 5.09 (d, *J* = 6.03 Hz, 1H), 4.19 (m, 6H), 4.17 (d, *J* = 5.13 Hz, 2H), 3.50-3.85 (m, 29H), 3.37 (s, 10H), 3.33 (dd, *J* = 6.31 Hz, 1.63 Hz, 1H), 3.00 (q, 2H). ^{13}C NMR (75 MHz, DMSO-d_6): δ = 170.58, 169.56, 169.35, 165.71, 159.13, 158.68, 154.93, 153.11, 151.83, 149.67, 143.76, 140.01, 137.86, 136.26, 129.73, 129.58, 129.13, 128.19, 126.36, 121.75, 121.25, 119.26, 115.16, 107.68, 106.29, 105.08, 71.88, 71.31, 70.00, 69.88, 69.79, 69.64, 68.96, 68.40, 58.07, 54.83, 51.72, 42.08, 41.73, 37.29, 36.93. MALDI-TOF-MS: Calcd for $C_{61}H_{77}F_3N_4O_{21}S_2$ 1322.5. Found 1323.0 $[M]^+$.

4.2. Enzyme activity release

PGA (penicillin-G-amidase, 1 U/μL) was purchased from Zhejiang Shunfeng Haider Co. Ltd. Compound **1** was dissolved in DMSO (not more than 10%, V/V) and further diluted with 0.1 M PBS (pH 7.4) to give the final stock solution, followed by treating with PGA. The incubation was kept at 37 °C and the fluorescence spectra were measured every 30 min by RF-5301PC spectroscopy.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.04.012. These data include MOL files and InChiKeys of the most important compounds described in this article.

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