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An image contrast agent selectively activated by prostate specific antigen

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Abstract—A family of image contrast agent conjugates designed to undergo enzymatic activation has been synthesized. The agents underwent activation both with enzymatically active prostate specific antigen (PSA) and α-chymotrypsin, releasing free fluorophore via cleavage of a three-component system. A hexapeptide derivative showed exclusive activation by PSA and constitutes a method for tracking PSA activity in vitro.

1. Introduction

Despite improvements in local therapy and increased awareness, prostate cancer continues to be second only to lung cancer as a cause for cancer deaths in men.[1] Prior investigations show that the presence of prostatectomy Gleason grade $\geqslant 4$ in the radical prostatectomy specimen is the most important predictor of progression following surgery.[2] Unfortunately, the transrectal ultrasound guided sextant sampling of the prostate is subject to sampling error, and therefore biopsy Gleason grade will underestimate prostatectomy Gleason grade 4 or 5 disease in as many as 40% of men with clinically localized disease.[3] Therefore, an imaging method capable of identifying Gleason grade $\geqslant 4$ disease within the prostate gland could provide the basis for patient selection for more aggressive initial therapeutic approaches.[4] A number of image contrast enhancing agents have been studied for use in conjunction with ultrasound methods of detection.[5] However, immunohistochemical studies have also shown that Gleason grade bears an inverse correlation with the concentration of enzymatically active prostate specific antigen (PSA).[6] PSA is a serine protease; however, PSA in serum (but not in the prostatic microenvironment) is rapidly inactivated by binding to serum proteins.[7] An attractive possibility, therefore, would be the design of an imaging system, which exploits the enzymatic efficiency of PSA in the prostatic microenvironment. Our strategy was to conjugate a proteinogenic PSA substrate to a masked fluorophore via an inert spacer/linker group, such that the free fluorescent molecule is liberated on proteolysis (Scheme 1).[8]

1.1. Choice of linker

Our preferred choice for the inert linker was the *p*-aminobenzyl alcohol pioneered by Katzenellenbogen and coworkers.[9] This allows coupling of peptide based enzyme substrates through the N terminus, with the alcohol group incorporated into a carbamate which masks the amino containing molecule targeted for delivery. Selective enzymatic hydrolysis of the amide group results in (a) generation of an exomethylene iminium ion which is then captured by water to regenerate the free linker (b) concomitant expulsion of CO_2, rendering the reactions essentially irreversible and (c) expulsion of the free amine as shown in Scheme 2.[9] The key is to harness amino containing substrates in the system where differences in the chemistry between the carbamate and amino form are pronounced, and we have previously employed this method for enzyme mediated cytotoxin release.[10]

Keywords: Enzyme substrates; PSA; Linker; Imaging agents; Chemical synthesis; Prodrugs; Coumarin; Hexapeptides.
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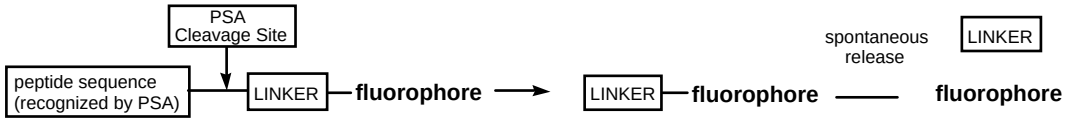
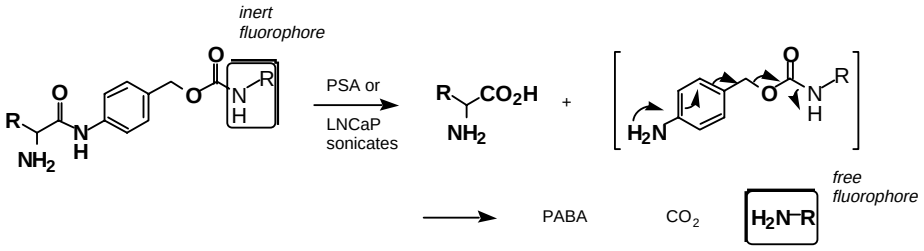


Scheme 1. Three component system for PSA activated image contrast agent.

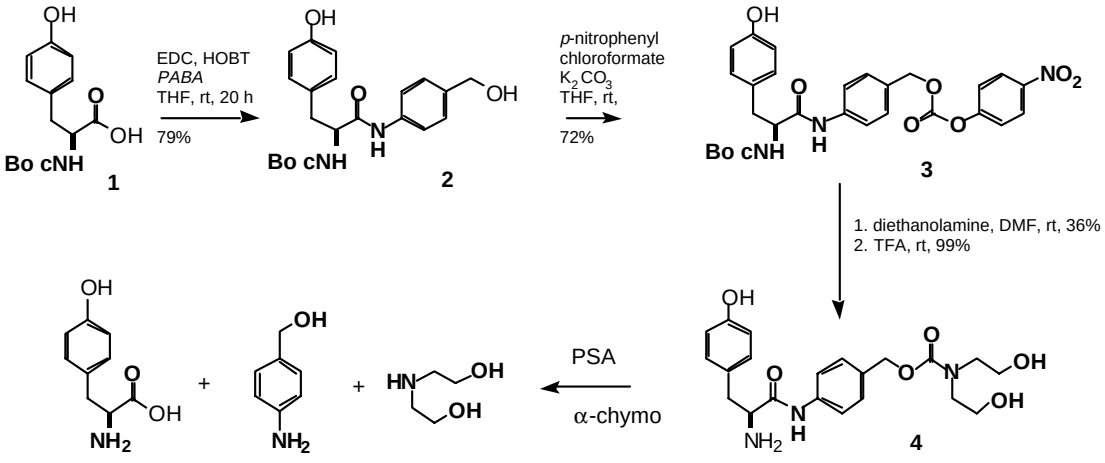


Scheme 2. PSA mediated release of Tyr-linker conjugates.

Anilino containing fluorophores were deemed excellent substrates for this system, as discernible differences in UV and fluorescence characteristics would be expected for the free aniline as opposed to the linked carbamate form.

1.2. Choice of enzyme substrate

Though a number of high-affinity peptide substrates for PSA have been identified, we initially wished to provide proof-of-principle with a minimal substrate and selected tyrosine conjugates for examination. In addition to precedent for PSA mediated hydrolysis of tyrosyl conjugates, such derivatives would also be substrates of α-chymotrypsin, an important and well-studied enzyme.[8] Accordingly, a dummy substrate **4** was prepared to assess proof-of-principle for the release methodology and to perfect coupling chemistry (Scheme 3). Boc tyrosine was converted to amide **2** and then the carbamate precursor was assembled by preparation of the 4-nitrophenyl carbonate **3**. Nucleophilic displacement gave only moderate yields of the Boc carbamate when using close stoichiometry (36% with 4:1 ratio), although near quantitative yields could be obtained when using large excesses of amine (>10 equiv). With the substrate in hand, enzymatic release was tracked using both α-chymotrypsin



Scheme 3. Proof-of-principle: enzymatic release of amine from a *p*-aminobenzyl carbamate-amino acid conjugate.

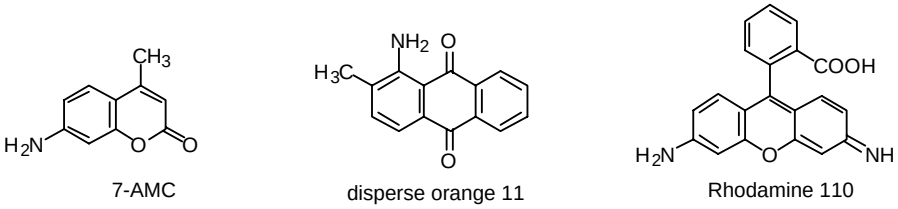


Table 1. Enzyme mediated release of chromophores[d]

Entry	Substrate	UV λ_{max} conj.	UV λ_{max} free	α-Chymotrypsin [a]	PSA[a]	Plasmin	Trypsin
1	**4**	n/a	n/a	15	5	—	—
2	**7**	300	497	9	4	—	—
3	**11**	328[b]	352[c]	24	10	—	—
4	**15**	285	486	18	6	—	—
5	**17**	328[b]	352[c]	0	4.8	0	0

[a] mM/h/mg fluorophore released.
[b] Fluorescence emission λ_{max} 397.
[c] Fluorescence emission λ_{max} 435.
[d] Substrates and controls were incubated for 24–120 h at 37 °C. Specific activity was determined on the basis of mM released fluorophore per unit time per unit mass of enzyme.

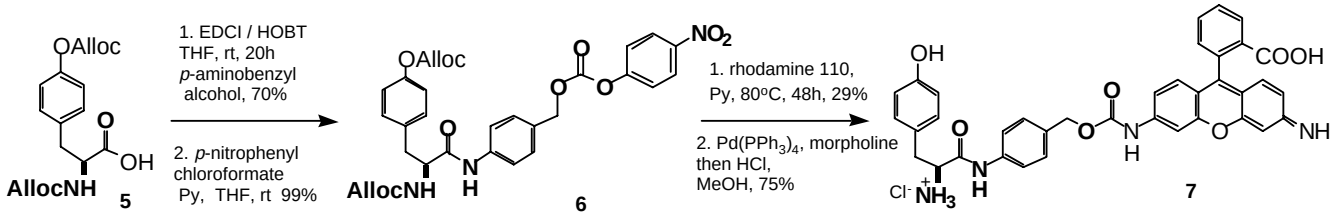
and PSA. Substantial release was observed within 12 h in both cases (Table 1) and despite numerous attempts only tyrosine, 4-aminobenzyl alcohol, and diethanolamine were detected, suggesting the intermediate anilino carbamate has a short half-life.

1.3. Choice of fluorophores

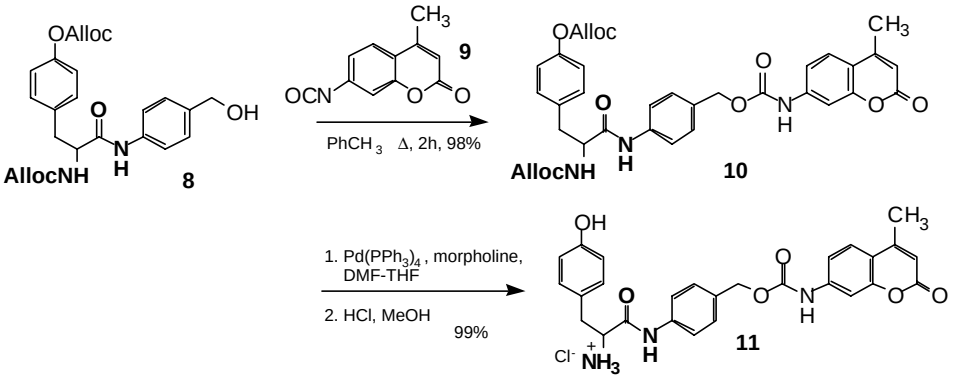
With proof-of-concept for release of amino containing prodrugs established, we turned our attention to selection of appropriate fluorophores. For our initial studies three readily available fluorophore dyes were selected—aminomethyl coumarin (7-AMC), disperse orange 11, and rhodamine 110—on the basis that their UV characteristics are all greatly influenced by the electron-donating capacity of the anilino nitrogen group.

Commencing with the previously available carbonate **3**, coupling with free rhodamine gave the corresponding adduct cleanly, albeit in low yield. However, all attempts to unmask the Boc group resulted in decomposition of the molecule, rendering desired target **7** unisolable. Remedy was found using the alternate bis-alloc substrate **6**, which was prepared from commercially available building block **5** under analogous conditions (Scheme 4). Nucleophilic displacement followed by unmasking using Pd chemistry[11] allowed isolation of the hydrochloride salt **7** in good yield, and the product was freely soluble in assay buffer media.

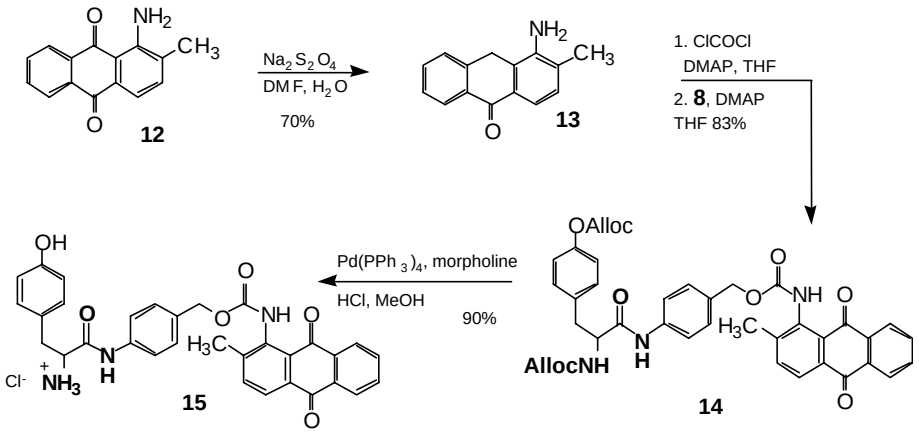
With the alloc route in hand, the coumarin analog **11** was next prepared. This involved coupling of the *p*-aminobeznyl amide prepared in Scheme 4 (**8**) with isocyanate **9** to give the masked analog **10** (Scheme 5). The alloc derivative underwent similarly clean deprotection to give **11** on workup, the product also soluble in buffer media. Finally, hoping to exploit the benefits of intramolecular hydrogen bonding, the anthraquinone conjugate **15** was assembled (Scheme 6). This necessitated selective removal of the quinone carbonyl group of aminomethylanthraquinone **12** with dithionite to form



Scheme 4. Preparation of rhodamine conjugate via alloc protected building block.



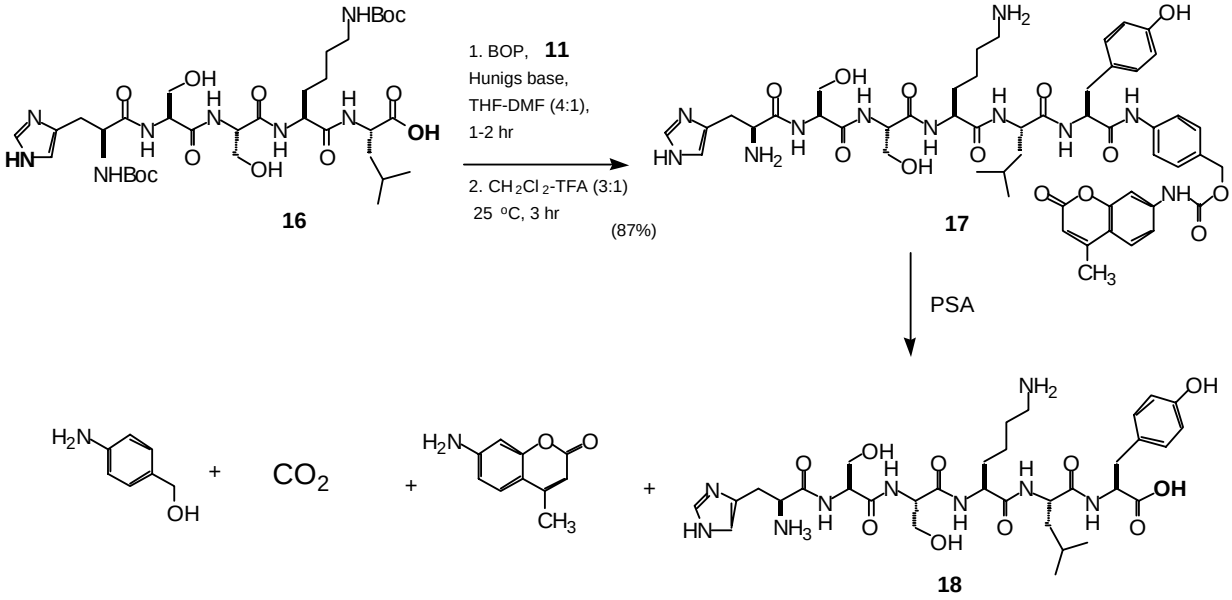
Scheme 5. Preparation of tyrosyl aminomethylcoumarin conjugate.



Scheme 6. Preparation of tyrosyl aminomethylanthraquinone conjugate.

required carbamoyl building block **13**. Conversion to the intermediate *p*-nitrophenyl carbamate was inefficient, giving a complex mixture which allowed only low recovered yields of the subsequently coupled product **14**. However, reaction with phosgene followed by coupling with **8** gave alloc derivative **14** directly, reoxidation taking place during workup and with yields of up to 80% attainable on scale-up. Finally, unmasking allowed isolation of the hydrochloride salt of anthraquinone substrate **15** in good yield.

With three substrates in hand, spectroscopic and enzymatic studies were conducted to establish proof-of-concept for use as image contrast agents. Enzymatic release of the fluorophores was probed using fresh, enzymatically active PSA and chymotrypsin, using UV detection to quantitate (and fluorescence in the case of **11**). Release of fluorophore correlated with release of *p*-aminobenzyl alcohol and tyrosine, confirming the function of the self-immolative linker. As can be seen (Table 1), though proof-of-concept is established, in the present examples, chymotrypsin is more effective than PSA at cleavage. Though this is unsurprising, more complex oligopeptide substrates are known whose specificity for PSA outranks chymotrypsin significantly, and it was decided to elaborate the substrate with the most selectivity and activity (**11**) to incorporate these recognition elements. Based on the work of Denmeade, the HSSKL motif was selected and a synthetic route to a chimera of **11** investigated.[8] After numerous iterations, the most successful (and economical) route was to couple bis-Boc-protected HSSKL (**16**) with **11** via the BOP route, which, following deprotection gave hexapeptide substrate **17** in good yield (Scheme 7). Enzymatic studies confirmed the hypothesis, with the substrate completely selective for PSA, even against an extended panel of enzymes (Table 1). Though less active than **11**, the selectivity demonstrated by **17** is noteworthy and is now the



Scheme 7. Preparation and activation of HSSKLY aminomethylcoumarin conjugates.

basis for ongoing development of appropriate in vitro and in vivo studies. Several improvements to the basic system can be envisioned, tailored to the desired application, based on the synthetic chemistry platform outlined. For example, linker architecture has been shown to have a marked impact on substrate half-life in three component systems,[9,11] suggesting that enhanced activity may be attainable, e.g., with halogen containing linker moieties. For application with in vivo analysis it will be necessary to employ fluorophores with spectral characteristics tailored to match imaging devices. Contrast agents in the near IR range (e.g., the Cy dye family) may prove desirable,[12] in that the conjugated amino function has a profound influence on its quantum yield.[13] The coupling chemistries described herein for amino substituted fluorophores offer flexibility toward this goal, providing the potential for in situ CCD based near-IR imaging of systemic agents that are locally activated under in vivo conditions.[12] Besides image contrast agents, the system may also prove promising for the slow release of prostate specific chemotherapeutics, where a systemic drug conjugate could be degraded by PSA in the prostatic microenvironment.[14] The results of these studies will be reported in due course.

In summary, a three-component system comprised of enzyme substrate, inert linker, and fluorophore has been designed and activation by chymotrypsin and PSA demonstrated. A hexapeptide chimera of one of these is selectively activated by PSA, and the results support application in the in vitro and in vivo evaluation of more complex functional substrates, for use as image contrast agents and chemotherapeutics.

2. Experimental procedures

HSSKL conjugates and protected Y derivatives were supplied by BACHEM. Unless otherwise stated, all other reagents were purchased from the Aldrich Chemical Company and used as supplied. ^{1}H and ^{13}C NMR spectra were obtained either on a 300 MHz Varian Mercury, 300 MHz Bruker AC 300, or 500 MHz Varian Unity machine ($CDCl_3$ unless otherwise stated). Combustion analyses were performed on a Carlo-Erba EA1108 system at the Northeastern University Microanalytical Facility. Mass spectra were conducted at the University of Illinois, Urbana Champaign. Chromatographic separations were made using E Merck 230–400 mesh 60H silica gel or using a Harrison Research Inc. radial chromatotron unit.

2.1. Boc-L-tyrosyl 4-(hydroxymethyl)anilide (2)

To a solution of *p*-aminobenzyl alcohol (88 mg, 0.712 mmol) in anhydrous THF (10 ml) cooled to 0 °C were added HOBt (96 mg, 0.712 mmol), Boc tyrosine (200 mg 0.712 mmol), and then EDC (150 mg, 0.783 mmol). The mixture was stirred at 0 °C for 2 h and at rt for 12 h. The solution was washed with HCl (5%, 2 ml), $NaHCO_3$ (5%, 1 ml), and then the organic extracts were dried over Na_2SO_4. After condensation in vacuo, the residue was purified by silica gel chromatography (ethyl acetate–hexanes; 2:1 eluent) to give **2**, (216 mg, 78.5%) as a white solid m.p. 142–14 °C (dec); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.45 (d, $J = 8.1$ Hz, 2H), 7.28 (d, $J = 8.1$ Hz, 2H), 7.06 (d, $J = 8.1$ Hz, 2H), 6.68 (d, $J = 8.1$ Hz, 2H), 4.55 (s, 2H), 4.33 (m, 1H), 2.78–3.40 (m, 2H), 1.40 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 176.1, 175.9, 160.6, 160.0, 141.5, 141.2, 141.1, 134.4, 131.9, 131.5, 124.6, 124.5, 119.3, 83.8, 67.8, 61.2, 41.9, 31.8; MS (ESI) *m/z* 387 $(M+H)^+$; HRMS (ESI) calcd for $C_{21}H_{27}N_2O_5$, $(M+H)^+$ *m/z* 387.1920, found 387.1916.

2.2. Boc-L-tyrosyl 4-(4-nitrophenoxycarboxyl)methylanilide (3)

To a solution of alcohol **2** (200 mg, 0.5175 mmol) in fresh distilled anhydrous THF (10 ml) were added 4-nitrophenyl chloroformate (110 mg, 0.5434 mmol) and anhydrous pyridine (44 μl, 0.5434 mmol). The mixture was stirred at rt for 12 h. The resulting precipitate was filtered and the filtrate was evaporated. The residue following evaporation was purified by silica gel chromatography (ethyl acetate–hexanes; 1:1 eluent) to give **3**, (204.3 mg, 71.7%) as a white solid m.p. 175–179 °C; ^{1}H NMR (300 MHz $CDCl_3$) δ 8.31 (d, $J = 8.7$ Hz, 2H), 7.53 (d, $J = 8.7$ Hz, 2H), 7.45 (d, $J = 8.7$ Hz, 2H), 7.39 (d, $J = 8.7$ Hz, 2H), 7.07 (d, $J = 8.7$ Hz, 2H), 6.69 (d, $J = 8.7$ Hz, 2H), 5.24 (s, 2H), 4.35 (m, 1H), 2.8–3.8 (m, 2H), 1.40 (s, 9H); ^{13}C NMR (375 MHz $CDCl_3$) δ 172.0, 164.0, 156.0, 150.4, 140.6, 138.4, 138.2, 135.2, 131.8, 131.8, 130.4, 130.2, 128.8, 127.7, 125.9, 125.1, 120.8, 120.286, 115.6, 115.3, 115.1, 69.0, 57.0, 54.0, 39.0, 27.5; calcd for $C_{28}H_{29}N_3O_9$: C, 60.98; H, 5.30; N, 7.62; found: C, 61.31; H, 5.54; N, 7.26.

2.3. L-Tyrosyl 4-(*N*,*N*-bis(2-hydroxyethyl)carbamoyl)-methylanilide (4)

A solution of diethanolamine (0.276 g, 2.6 mmol) in anhydrous DMF (5.0 ml) was added to a solution of **3** (0.32 g, 0.58 mmol) in anhydrous DMF (5.0 ml). The mixture was stirred at rt for 42 h, and then quenched with water (50 ml). The mixture was extracted with ethyl acetate (3×30 ml) and the combined extracts were washed with brine (1×15 ml), and then dried (Na_2SO_4). The solution was concentrated in vacuo and the residue was purified by flash chromatography (10% methanol in chloroform) to give Boc-L-tyrosyl 4-(*N*,*N*-bis(2-hydroxyethyl)-carbamoyl) methylanilide (0.163 g, 36%) as a hygroscopic oil. The entire anilide (0.314 mmol) was treated with trifluoroacetic acid (6 ml) under argon, stirred for 12 h, and then the TFA was removed by aspiration. The resulting gum was purified by chromatography (1:9 methanol–chloroform) to give **4**, (166.5 mg, 99%) as a colorless foam; ^{1}H NMR (300 MHz, d_6-acetone): δ 7.77–6.83 (m, 10H); 5.35 (s, 1H); 4.65 (s, 2H); 3.96 (m, 4H); 3.75 (m, 1H); 3.55 (m, 1H); 3.40 (m, 7H); 3.01 (s, 1H); 2.85 (s, 1H); calcd for $C_{21}H_{27}N_3O_6$: C, 60.42; H, 6.52; N, 10.06; found: C, 60.77; H, 6.73; N, 9.71.

2.4. (Bis)alloc tyrosyl 4-(hydroxymethyl)anilide (8)

To a solution of bis-alloc tyrosine (2.26 g, 6.476 mmol) in anhydrous THF (50 ml) were added 4-aminobenzylalcohol (800 mg, 6.476 mmol), EDCI (1.5 g, 7.77 mmol), and then HOBT (874 mg, 6.476 mmol). The mixture was stirred at rt for 12 h, and then condensed in vacuo. The residue was dissolved in ethyl acetate (30 ml) and the solution was washed with HCl (5%, 2 ml), $NaHCO_3$ (5%, 1 ml), and then the organic extracts were dried over Na_2SO_4. After condensation in vacuo, the residue was purified by silica gel chromatography (ethyl acetate–hexanes; 2:1 eluent) to give the title compound (2.09 g, 71%) as a pale solid m.p. 177–119 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.45 (d, J = 8.7 Hz, 2H), 7.27–7.32 (m, 4H), 7.08 (d, J = 8.7 Hz, 2H), 5.80–6.10 (m, 2H), 5.13–5.42 (m, 4H), 4.70 (dd, 1H, J = 5.7, 1.5 Hz), 4.55 (s, 2H), 4.49 (m, 1H), 2.90–3.20 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.0, 157.0, 154.0, 150.0, 138.0, 137.0, 135.0, 133.0, 132.0, 130.3, 127.4, 121.0, 120.0, 118.0, 117.0, 69.0, 65.0, 63.0, 57.0, 38.0; MS (ESI) m/z 455 $(M+H)^+$; HRMS (ESI) calcd for $C_{24}H_{27}N_2O_7$ $(M+H)^+$ m/z 455.1818, found 455.1827.

2.5. Bis (alloc)-L-tyrosyl 4-(4-nitrophenoxycarboxyl)-methylanilide (6)

To a solution of **8** (100 mg, 0.22 mmol) THF (5 ml) were added 4-nitrophenyl chloroformate (57 mg, 0.28 mmol) and anhydrous pyridine (23 μl, 0.28 mmol). The mixture was stirred at rt for 12 h. then the formed precipitate was filtered, and the filtrate was recovered and evaporated. This residue was purified by silica gel chromatography (ethyl acetate–hexanes; 1:2 eluent) to give **6** (143.3 mg, 98.8%) as a white solid m.p. 87–90 °C (dec); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.25 (d, J = 9.0 Hz, 2H), 7.30–7.44 (m, 6H), 7.23 (d, J = 9.0 Hz, 2H), 7.10 (d, J = 8.7 Hz, 2 H), 5.78–6.09 (m, 2H), 5.65 (d, J = 5.7 Hz, 1H), 5.10–5.50 (m, 5H), 4.72 (d, J = 5.7 Hz, 2H), 4.53–4.60 (m, 3H), 3.14 (s, 1H), 3.11 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.2, 156.1, 155.8, 155.1, 153.8, 152.7, 150.5, 145.8, 137.9, 134.3, 132.4, 131.2, 130.7, 130.6, 129.9, 126.9, 126.4, 125.5, 122.5, 122.0, 121.7, 120.7, 119.8, 118.5, 116.1, 70.9, 69.5, 66.5, 55.5, 37.5; MS (ESI) m/z 620 $(M+H)^+$; HRMS (ESI) calcd for $C_{31}H_{30}N_3O_{11}$ $(M+H)^+$ m/z 620.1880, found 620.1892.

2.6. Bis(alloc) L-tyrosyl rhodamine

Carbonate **6** (69 mg, 0.112 mmol) and rhodamine 110 (40 mg 0.112 mmol) were dissolved in anhydrous pyridine (10 ml) and the solution was stirred at 80 °C for 2 days. The solution was evaporated to dryness and the residue was purified by silica gel chromatography (ethyl acetate–hexanes; 2:1 eluent) to give the title compound (26 mg, 28.6%) as a yellow oil together with recovered rhodamine (16 mg); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.34 (s, 1 H), 7.98 (d, J = 7.2 Hz, 2 H), 7.50–7.70 (m, 5H), 7.00-7.40 (m, 8H), 6.68(d, J = 8.4 Hz, 1H), 6.64 (d, J = 8.7 Hz, 1H), 6.49 (d, J = 8.4 Hz, 1H), 6.43 (s, 1H), 6.20 (d, J = 8.1 Hz, 1H), 5.70–6.12 (m, 2H), 5.12–5.50 (m, 4H), 5.06 (s, 2H, CH_2), 4.72 (dd, J = 5.7, 0.9 Hz, 2H), 4.50–4.70 (m, 3H), 3.11 (s, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.5, 170.4, 156.8, 155.8, 154.0, 153.8, 152.8, 152.2, 150.8, 150.2, 149.2, 140.5, 137.5, 135.5, 134.5, 132.8, 132.2, 131.3, 130.6, 129.9, 129.2, 128.9, 127.8, 127.0, 125.2, 124.2, 121.5, 120.5, 119.8, 118.2, 115.8, 114.5, 113.8, 111.9, 108.2 106.5, 101.8, 69.2, 66.8, 66.2, 56.5, 38.2; MS (ESI) m/z 811 $(M+H)^+$, HRMS (ESI) calcd for $C_{45}H_{39}N_4O_{11}$ $(M+H)^+$ m/z 811.2615, found 811.2606.

2.7. L-Tyrosyl rhodamine (7)

A catalytic amount of $Pd(PPh_3)_4$ (~1 mg) and morpholine (0.5 ml) was added to a degassed solution of bis(alloc)-L-tyrosyl rhodamine (14.7 mg, 0.0182 mmol) in anhydrous THF (5 ml). The solution was stirred under argon for 48 h, and then the resulting red precipitate was collected by centrifugation, and washed with ethyl acetate (2 × 5 ml). The solid was then exposed to a solution of hydrochloric acid (0.5 M, 2 ml) in ethyl acetate (5 ml) followed by methanol (1 ml). The resulting (yellow) solution was condensed in vacuo solution and the residue was purified by silica gel chromatography (ethyl acetate–methanol; 10:1 eluent) to give **6**, (8.7 mg, 74.7%) as a yellow solid m.p. 213–216 °C; ^{1}H NMR (300 MHz, CD_3OD) δ 8.38 (d, J = 5.1 Hz, 1H), 8.23 (s, 1H), 7.80–7.92 (m, 1H), 7.50-7.70 (m, 4H), 7.30–7.48 (m, 2H), 7.20–7.26 (m, 2H), 7.15 (d, J = 5.1 Hz, 1H), 6.90–7.10 (m, 2H), 6.75 (d, J = 5.7 Hz, 1H), 6.66 (t, J = 5.1 Hz, 1H), 5.22 (s, 2H), 4.23 (m, 1H), 2.84–3.28 (m, 2H); ^{13}C NMR (75 MHz, CD_3OD) δ 176.2, 171.5, 161.0, 142.0, 141.8, 137.0, 136.7, 136.5, 135.9, 135.8, 135.6, 135.5, 135.4, 134.9, 134.5, 134.3, 134.2, 132.9, 132.8, 131.4, 128.8, 124.9, 121.4, 121.1, 120.9, 119.5, 108.2, 70.8, 61.1, 40.7; MS (ESI) m/z 643 $(M+H-2HCl)^+$, HRMS (ESI) calcd for $C_{37}H_{31}N_4O_7$ $(M+H-2HCl)^+$ m/z 643.2193, found 643.2215.

2.8. (Bis)alloc tyrosyl 4-(hydroxymethyl)anilide coumarin conjugate (10)

A solution of (bis)alloc tyrosyl 4-(hydroxymethyl)anilide (**8**, 0.100 g, 0.22 mmol) and coumarin isocyanate (**9**, 66 mg, 0.33 mmol) in toluene (10 ml) was refluxed for 2 h. The solution was condensed in vacuo and the residue was crystallized from CH_2Cl_2–MeOH:Et_2O to give **10** (138 mg, 98%) as a white solid m.p. 139–142 °C; ^{1}H NMR ^{1}H NMR (CD_3OD, 500 MHz) δ 7.45 (d, 2H, J = 10 Hz), 7.41–7.36 (m, 3H), 7.28-7.24 (m, 3H), 7.18–7.14 (m, 3H), 7.02–6.98 (m, 3H), 6.09 (d, 1H, J = 1.5 Hz), 5.94-5.86 (m, 1H), 5.82–5.74 (m, 1H), 5.36–5.31 (m, 1H), 5.26–5.23 (m, 1H), 5.18 (d, 1H, J = 17.5 Hz), 5.12–5.07 (m, 3H), 4.65–4.63 (m, 2H), 4.45–4.44 (m, 2H), 4.39 (t, 1H, J = 7 Hz), 3.53 (br s, 3H), 3.04 (dd, 1H, J = 13.7, 7 Hz), 2.96 (dd, 1H, J = 13.7, 7 Hz); MS (ESI) m/z 656 $(M+H)^+$.

2.9. L-Tyrosyl 4-(hydroxymethyl)anilide coumarin conjugate (11)

A solution of **10** (50 mg, 76.5 mmol) in DMF–THF (1:1, 4 ml) was treated with morpholine (1 ml) and $Pd(Ph_3)_4$ (10 mg). The mixture was stirred at rt for

1 h and then concentrated in vacuo. The residue was purified by silica gel chromatography (CH_2Cl_2–MeOH–Et_3N; 93:5:2 eluent) to give the title compound (0.041 g, 99%) as a white solid m.p. 183–185 °C;. ^1H NMR (CD_3OD, 500 MHz) δ 7.57 (s, 1H), 7.58–7.50 (m, 3H), 7.39–7.35 (m, 3H), 7.03 (d, $J = 8.0$ Hz, 2H), 6.72 (d, $J = 8.0$ Hz, 2H), 6.15 (br s, 1H), 5.14 (s, 2H), 4.69 (br s, 5H, exch.), 3.60 (dd, $J = 5.5$, 8.0 Hz, 1H), 3.02 (dd, $J = 5.5$, 14.0 Hz, 1H), 2.75 (dd, $J = 5.5$, 14.0 Hz, 1H), 2.42 (s, 3H); ^{13}C NMR (CD_3OD, 75 MHz) δ 170.0, 156.1, 153.9, 153.5, 153.4, 149.6, 142.4, 137.2, 134.2, 132.0, 131.8, 130.6, 130.0, 128.5, 124.9, 120.7, 120.0, 118.9, 117.2, 114.7, 114.5, 111.7, 105.3, 68.8, 56.3, 37.6, 17.9; HRMS calcd for $C_{27}H_{26}N_3O_6$(MH+) *m/z* 488.1822, found 488.1825.

2.10. De-oxy disperse orange 13

Sodium dithionite (14 g, 80.41 mmol) was added to a suspension of the disperse orange 11 (**12**, 2 g, 8.439 mmol) in DMF (100 ml), and water (100 ml) and the mixture was heated to 90 °C over 30 min. The mixture was stirred for 48 h and then cooled to rt The resulting yellow precipitate was filtered and then purified by silica gel chromatography (hexanes–ethyl acetate; 1:1 eluent) to give **13** (1.08 g, 58%) as a yellow solid m.p. 103–105 °C; ^1H NMR (300 MHz, $CDCl_3$) δ 8.30 (dd, 1H, $J = 8.4$Hz, 0.9Hz), 7.78 (d, 1H, $J = 7.5$Hz), 7.54–7.59 (m, 1H), 7.45 (d, 2H, $J = 6.9$Hz), 7.16 (d, 1H, $J = 8.1$Hz), 3.87 (s, 2H), 3.76 (s, 2H), 2.22 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 184.7, 141.8, 139.8, 132.7, 132.0, 130.7, 129.2, 126.0, 127.6, 127.2, 126.8, 124.4, 117.8, 28.4, 18.3; MS (ESI) *m/z* 224.2 $(M+H)^+$; HRMS (ESI) calcd for $C_{15}H_{16}NO$ $(M+H)^+$ *m/z* 224.1075, found 224.1075.

2.11. Disperse orange-4 bis-alloc-L-tyrosyl carbamate (14)

A solution of phosgene (20%) in toluene (1.8 ml, 3.60 mmol) and DMAP (482 mg, 3.60 mmol) were added to a degassed solution of **13** (400 mg, 1.79 mmol) in THF (20 ml). The solution was stirred at rt for 24 h and the excess phosgene was removed with an argon sweep. **8** (750 mg, 1.79 mmol) was then added and the solution was stirred at rt for 24 h. The mixture was filtered and the filtrate was condensed in vacuo. This residue was purified via silica gel chromatography (hexanes–ethyl acetate; 1:1) to give **14** (145 mg, 22%) as a yellow oil; ^1H NMR (300 MHz, $CDCl_3$) δ 10.0 (s, 1H), 8.20–8.30 (m, 2H), 8.10 (d, 1H, $J = 7.5$ Hz), 7.70–7.80 (m, 4H), 7.63 (d, 1H, $J = 7.5$ Hz), 7.30-7.50 (m, 4H), 7.24 (d, 1H, $J = 7.2$ Hz), 7.11 (d, 2H, $J = 8.1$z), 5.80–6.20 (m, 2H), 5.00–5.50 (m, 6H), 4.72 (d, 2H, $J = 5.7$Hz), 4.40–4.60 (m, 3H), 3.10–3.20 (m, 2H), 2.41 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 186.9, 182.7, 169.3, 154.2, 153.7, 150.7, 150.7, 150.5, 142.5, 138.6, 134.5, 134.4, 133.0, 132.7, 132.5, 132.4, 131.3, 130.6, 129.3, 127.6, 127.1, 126.3, 124.8, 124.4, 121.7, 120.4, 119.8, 118.5, 69.4, 67.3, 66.5, 57.2, 38.0, 20.2; MS (MALDI) *m/z* $(M+Na)^+$ 740.84. Also isolated was recovered **8** (639 mg) [yield 83% based on **8**].

2.12. L-Tyrosyl-disperse orange carbamate (15)

A catalytic amount of $Pd(PPh_3)_4$ and of morpholine (50 μl) was added to a degassed solution of **14** (38 mg, 0.053 mmol) in anhydrous THF (5 ml). The solution was stirred under argon for 48 h and the resulting red precipitate was recovered by centrifugation and washed with ethyl acetate (10 ml). The solid was dissolved in methanol (1 ml), the solution was condensed in vacuo, and then the residue was purified via silica gel chromatography (ethyl acetate–methanol; 10:1 eluent) to give **15** (26.1 mg, 90%) as a red solid m.p. 221–224 °C; ^1H NMR (300 MHz, CD_3OD) δ 8.19–8.30 (m, 2H), 8.10–8.15 (m, 1H), 7.82–7.86 (m, 2H), 7.64 (d, 1H, $J = 8.1$Hz), 7.54 (d, 2H, $J = 8.4$Hz), 7.41 (d, 2H, $J = 8.1$Hz), 7.10–7.18 (m, 2H), 6.86–6.92 (m, 1H), 6.76(d, 2H, $J = 8.4$Hz), 5.18 (s, 2H), 4.20 (m, 1H), 3.0–3.4 (m, 2H), 2.40 (s, 3H); MS (MALDI) *m/z* $(M+Na)^+$ 572.86; calcd for $C_{32}H_{27}N_3O_6$: C, 69.93; H, 4.95; N, 7.65; found: C, 70.31; H, 5.06; N, 7.33.

2.13. HSSKLY-tyrosyl-coumarin carbamate conjugate (17)

Pentapeptide NH_2-HSSKL-CO_2H (10 mg, 17.6 μmol), Boc anhydride (8.86 mg, 40 μmol), and Et_3N (16 μl, 106 μmol) were stirred at 40 °C for 12 h in DMF (2 ml). The solution was concentrated in vacuo, the residue was redissolved in DMF–THF (1:1, 10 ml), and then **11** (9 mg, 20.2 μmol), BOP (10 mg, 22 μmol), and Hunigs base (8 μl, 44 μmol). The mixture was stirred at rt for 12 h. The solution was concentrated in vacuo and the residue was dissolved in MeOH (2 ml) and the solution passed through a silica gel plug (6% MeOH, 94% CH_2Cl_2 eluent). Removal of eluents and concentration in vacuo gave the Boc-protected derivative of **17** (21.8 mg, 87%) as a colorless gum; ^1H NMR ($CDCl_3$, 300 MHz) δ 8.41 (m, 1H), 7.62 (s, 1H), 7.65–7.59 (m, 3H), 7.55 (s, 1H), 7.44–7.31 (m, 3H), 7.11 (m, 2H), 6.85 (m, 2H), 6.25 (s, 1H), 5.19 (s, 2H), 5.10 (br s, 13H), 4.65–4.58 (m, 2H), 4.51–4.49 (m, 2H), 4.38 (m, 1H), 4.11–3.96 (m, 2H), 3.97–3.84 (m, 2H), 3.66 (m, 1H), 3.57–3.41 (m, 2H), 3.13 (m, 1H), 2.99 (m, 2H), 2.82 (m, 1H), 2.41 (s, 3H), 2.01–1.87 (m, 1H), 1.83–1.64 (m, 6H), 1.57-1.46 (m, 2H), 1.35 (s, 18H), 0.99–0.94 (m, 6H); (ESI MS 1239 = M^+). A portion of this product (14 mg, 11.1 μmol) was treated with a solution of TFA (25% in CH_2Cl_2, 3 ml), stirred at rt for 3 h, and then condensed in vacuo. The residue was dissolved in DMF (1 ml) and the solution was passed through a short plug of silica gel. The eluents were condensed in vacuo and the residue was washed with CH_2Cl_2 (3×1 ml) then hexanes (3×1 ml) to give **17** (11.4 mg, 99%) as a pale white solid m.p. 168–173 °C (dec); ^1H NMR (CD_3OD, 500 MHz) δ 8.36 (m, 1 H), 7.58 (s, 1H), 7.59–7.51 (m, 3H), 7.49 (s, 1H), 7.41–7.36 (m, 3H), 7.05 (d, $J = 8.0$ Hz, 2H), 6.71 (d, $J = 8.0$ Hz, 2H), 6.17 (br s, 1H), 5.13 (s, 2H), 5.05 (br s, 15 exch. H), 4.61–4.55 (m, 2H), 4.47–4.41 (m, 2H), 4.30 (dd, 1H, $J = 5$, 7 Hz), 4.03–3.91 (m, 2H), 3.92–3.81 (m, 2H), 3.63 (dd, $J = 5.5$, 8.0 Hz, 1H), 3.51–3.36 (m, 2H), 3.05 (dd, $J = 5.5$, 14.0 Hz, 1H), 2.95 (t, 2H, $J = 8$ Hz), 2.77 (dd, $J = 5.5$, 14.0 Hz, 1H, CH), 2.40 (s, 3H, CH_3),

1.98–1.85 (m, 1H), 1.80–1.62 (m, 6H), 1.55–1.48 (m, 2H), 0.98 (d, 3H, $J = 6$ Hz), 0.92 (d, 3H, $J = 6.5$ Hz); ^{13}C NMR (CD_3OD, 75 MHz) δ 174.5, 172.8, 172.0, 171.0, 170.0, 167.8, 162.2, 161.8, 156.1, 153.9, 153.5, 153.4, 149.6, 142.4, 137.3, 135.0, 134.2, 132.0, 131.8, 130.6, 130.0, 128.5, 126.7, 124.9, 120.7, 120.0, 118.9, 118.9, 117.2, 115.0, 114.7, 114.5, 111.7, 105.3, 68.8, 61.5, 56.3, 56.1, 55.8, 53.2, 52.0, 51.0, 40.3, 39.3, 37.6, 31.1, 26.7, 26.5, 24.8, 22.3, 22.1, 20.6, 17.9; ESI-MS 1040.14 (M^+); $C_{51}H_{65}N_{11}O_{13}$ req. C, 58.89; H, 6.30; N, 14.81; found: C, 59.21; H, 6.55; N, 14.59.

2.14. General procedure for enzymatic assays

In duplicate, PSA (Cortex Biochem), 20 μl, 0.5 mg/ml, pH 7.4, 0.01 M phosphate-buffered saline (PBS) or α-chymotrypsin (Sigma, TLCK-treated, 20 μl, 0.5 mg/ml, pH 7.4, 0.01 M PBS), 160 μl PBS, and 20 μl substrate (1.0 mg/ml; 1:1, EtOH–H_2O) were incubated for 24 h at 37 °C. Duplicate control reactions containing PBS (180 μl) and substrate (20 μl) solutions were incubated under identical conditions. The two cuvettes were placed in a Varian (Cary WinUV) spectrophotometer for 24 h and kinetics were recorded. To determine the release of free fluorophore and linker, substrates and controls were also incubated at 37 °C for 24–120 h and then reactions were terminated by addition of cold trichloroacetic acid (10% v/v). Enzyme was removed by centrifugation (700*g*, 30 min, 4 °C) using Ultrafree-MC tubes (Millipore), and peak areas for control and reaction mixtures were determined by HPLC. Conditions: C-18 reverse-phase column (15 cm); mobile phase A 99% H_2O + 1% TEA; mobile phase B 99% methanol + 1% TEA; linear gradient 10–80%; flow rate 1 ml/min; monitoring wavelength = 253 nm. Specific activity (mmol/h/mg) was determined as: [concn] released substrate (mmol/ml) × total vol. of the assay (ml)/time of the reaction (h) × [concn] enzyme mg/ml × vol enzyme (ml).

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