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Image contrast agents activated by prostate specific antigen (PSA)

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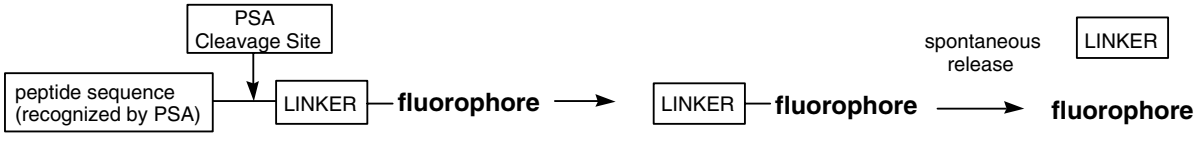
Abstract—A family of image contrast agent conjugates designed to undergo enzymatic activation has been synthesized. The agents underwent activation both with enzymatically active prostate specific antigen and α-chymotrypsin, releasing free fluorophore via cleavage of a three-component system.

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]

Our preferred choice for the inert linker is the *p*-aminobenzyl alcohol pioneered by Katzenellenbogen,[9] having previously employed this method for enzyme mediated cytotoxin release.[10] 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 of appropriate fluorophores (Scheme 2).[11]



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

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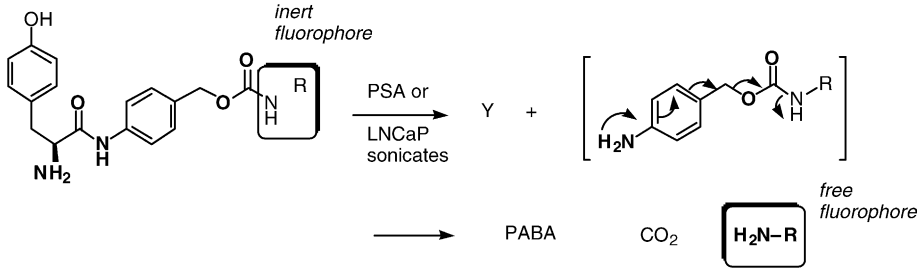
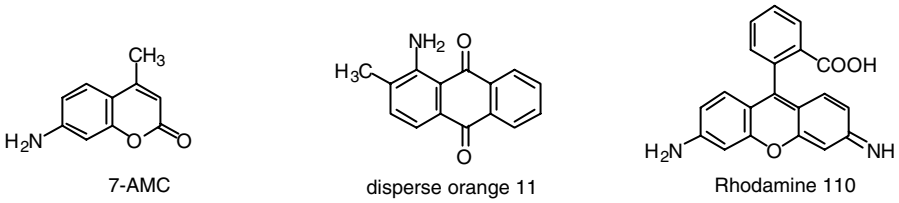


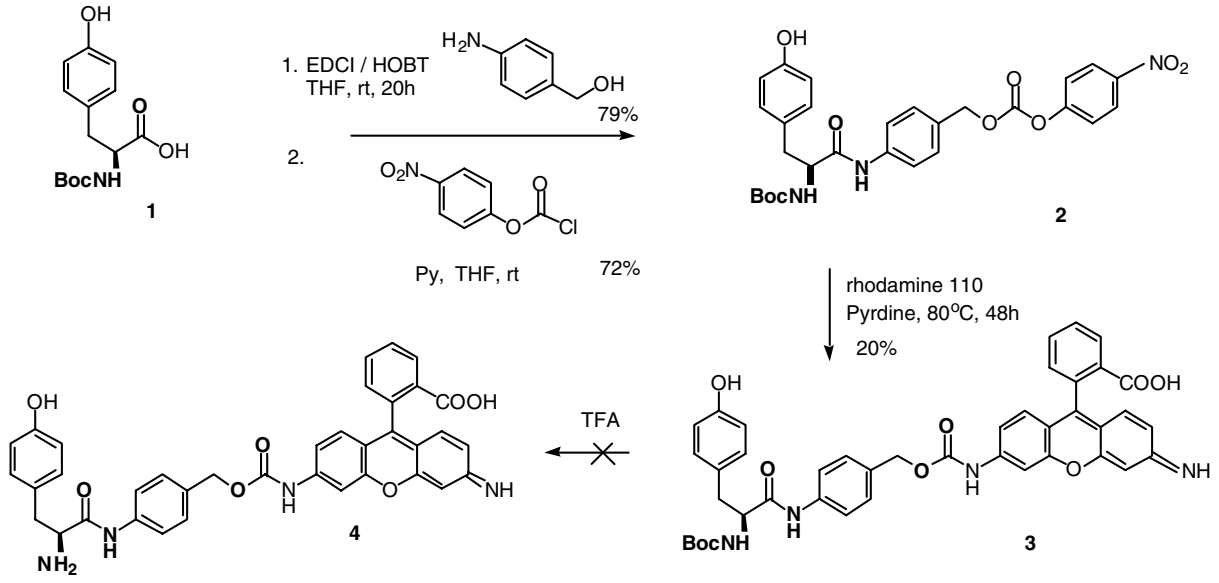
Scheme 2. PSA mediated release of Tyr-linker conjugates.



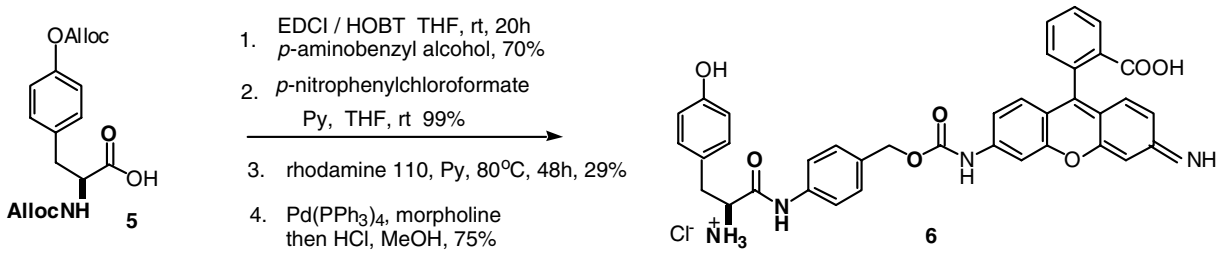
For our initial studies three readily available fluorophore dyes were selected—aminomethyl coumarin (7-AMC), disperse orange 11, and rhodamine 110.

Commencing with commercially available Boc-tyrosine, carbodiimide coupling with *p*-aminobenzyl alcohol, followed by reaction with *p*-nitrophenylchloroformate gave carbonate **2** without incident (Scheme 3). Coupling with free rhodamine gave **3** cleanly, albeit in low yield. However, all attempts to unmask the carbamate group resulted in decomposition of the molecule, rendering **4** unisolable. Remedy was found using the alternate bis-alloc substrate **5**, which under analogous conditions gave the intermediate carbonate, and subsequently underwent rhodamine coupling and unmasking using the Pd route,[12] to allow isolation of the hydrochloride salt **6** in good yield, and which was soluble in assay buffer media (Scheme 4).

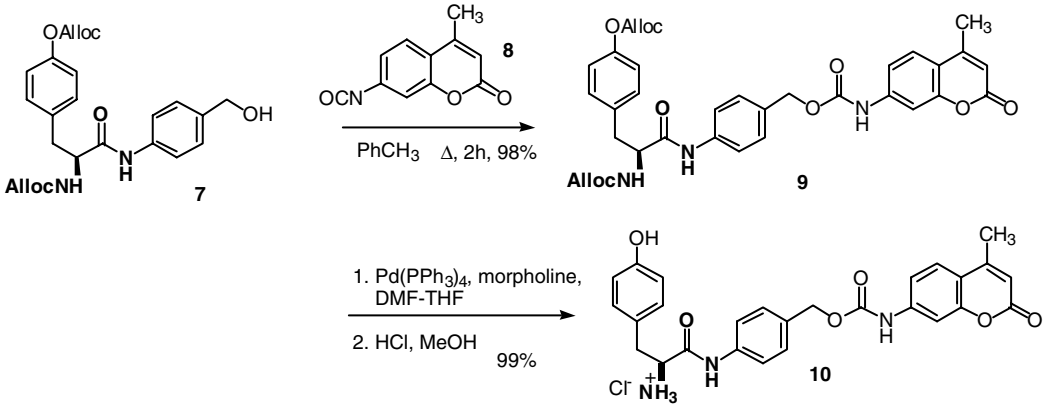
With the alloc route in hand, the coumarin analog **10** was next prepared. This involved coupling of the carbonate used in Scheme 4 (**7**) with isocyanate **8** to give the masked analog **9**, which underwent clean deprotection to give **10** on workup (Scheme 5). Finally, hoping to exploit the benefits of intramolecular hydrogen bonding, the anthraquinone conjugate **14** was assembled. This necessitated selective removal of the quinone carbonyl group of aminomethylanthraquinone to allow formation of the required carbamoyl building block **11** (Scheme 6). Conversion to the *p*-nitrophenyl carbamate was inefficient, giving a complex mixture, which allowed only low recovered yields of **12**. However, reaction with phosgene followed by coupling with **7** gave alloc protected adduct **13** directly, reoxidation taking place during workup. This compares favorably with the corresponding conversion of **12** to **13** and proved reliable on scale-up. Finally, unmasking allowed isolation



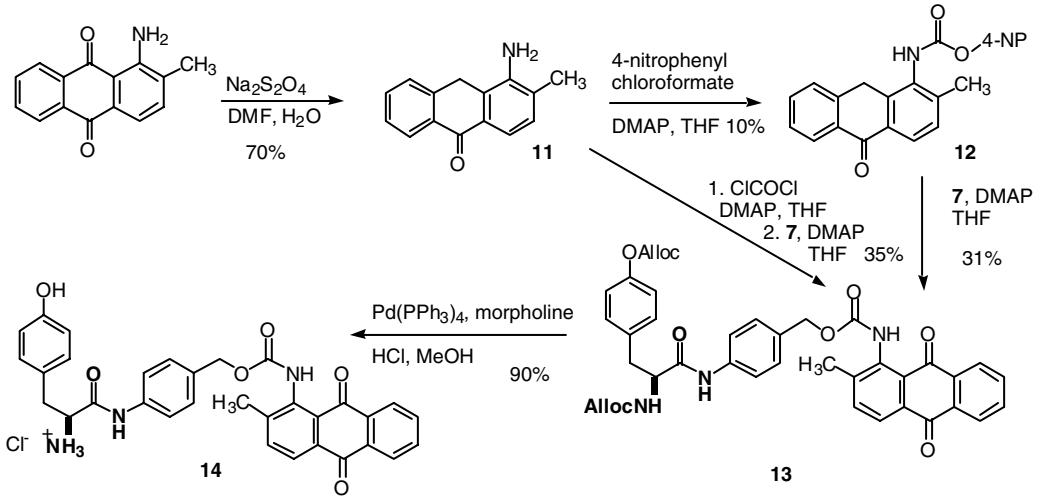
Scheme 3. Initial route to tyrosyl rhodamine conjugate via *p*-aminobenzyl carbamate linker.



Scheme 4. Y-alloc route to tyrosyl rhodamine conjugate.



Scheme 5. Preparation of tyrosyl aminomethylcoumarin conjugate.



Scheme 6. Preparation of tyrosyl aminomethylanthraquinone conjugate.

of the hydrochloride salt of anthraquinone substrate **14** 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 initially probed using fresh, enzymatically active PSA and chymotrypsin, using UV detection to quantitate (and fluorescence in the case of **10**). 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, the most selective of these (HSSKLQ), which will now become the target of future synthetic studies and kinetic analysis.[8] Additionally, linker architecture has been shown to have a marked impact on substrate half-life in three-component systems,[9,12] suggesting that specificity and stability might ultimately be tailored according to desired application. Though the

Table 1. Enzyme mediated release of chromophores[a]

Entry	Substrate	UV λ_{max} conjugate	UV λ_{max} free	Chymotrypsin[b]	PSA[b]
1	**6**	300	497	9	4
2	**10**	328[c]	352[d]	24	10
3	**14**	285	486	18	6

[a] In duplicate, PSA (Cortex Biochem), 20 μL, 0.5 mg/mL, pH 7.4 0.01 M phosphate buffer 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. *p*-Aminobenzyl alcohol was quantified against authentic standards by HPLC (C18 μBondpak, 1 mL/min, 100% *i*PrOH, $t_R = 8.2$ min). Specific activity was determined on the basis of mM released fluorophore per unit time per unit mass of enzyme.
[b] mM/h/mg fluorophore released.
[c] Fluorescence emission λ_{max} 397.
[d] Fluorescence emission λ_{max} 435.

changes in the λ_{max} range between free and bound contrast agents prepared are pronounced, differences in fluorescent characteristics will be of more importance for imaging purposes where differences in quantum yield might be exploited.[13]

Moreover, for application with in vitro and 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,[14] in that the conjugated amino function has a profound influence on its quantum yield.[15] The coupling chemistries described herein for amino substituted fluorophores offer flexibility towards this goal, providing the potential for in situ CCD based near-IR imaging of systemic agents that are locally activated under in vivo conditions.[14]

In summary, a three-component system comprised of enzyme substrate, inert linker and fluorophore has been designed and activation by chymotrypsin and PSA demonstrated. The results support the synthesis and in vitro evaluation of more complex and selective substrates, which will be reported in due course.

Acknowledgements

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