Electrophilic aromatic substituted luciferins as bioluminescent probes for glutathione S-transferase assays†

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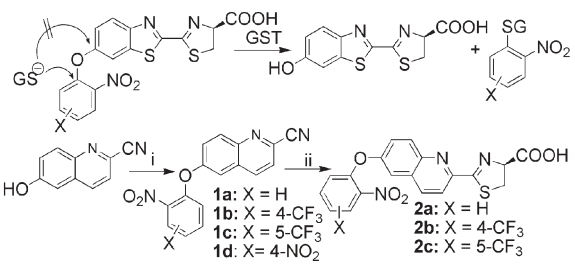
New highly sensitive latent bioluminescent luciferin substrates were designed and synthesized for monitoring mammalian glutathione S-transferase (GST) and *Schistosoma japonicum* enzyme activities.

The glutathione S-transferases (GSTs) represent a major group of detoxification enzymes that catalyze the nucleophilic addition of the tripeptide glutathione (GSH) to many xenobiotics and endogenous electrophiles.[1] The level of expression of GSTs is a crucial factor in determining the susceptibility to cancer chemotherapy. Three classes of GST isozymes, Alpha (A), Mu (M) and Pi (P) are often found over-expressed in drug-resistant tumor or tumor cell lines.[1,2] Thus, there continues to be a need for highly sensitive probes for monitoring mammalian GST isozyme activity in tumor cells to examine anti-cancer drug resistance. Currently, only a few methods for assaying GST activity are available,[3,4] 1-chloro-2,4-dinitrobenzene (CDNB)[3] being the most prevalent method, but its use is often limited by low sensitivity (absorbance), high background (non-enzymatic GSH conjugation) and lack of selectivity for isozymes. The generic advantages of low background and high sensitivity found in luciferase-coupled bioluminescent assays[5] led us to search for bioluminogenic GST substrates as a possible approach to a highly sensitive and selective mammalian GST assay.

We previously demonstrated that chemical modification of the 6-hydroxyl group of luciferin is an effective means to approach bioluminescent assays for enzymes of interest.[5] Relying on the observation that various electrophilic nitrophenyl compounds are detoxified by GSTs[6] with a catalytic reactivity depending on the electrophilicity of the substrate to the GSH anion, we designed a series of electrophilic *o*-nitrophenyl luciferin or quinolinyl luciferin ether derivatives. Relying on the hypothesis that the delocalization of the negative charge on the phenoxide oxygen is correlated to the pK_a values of the phenols,[7] it seemed reasonable to use the pK_a as a simple indicator of the electrophilicity at the phenol carbon site. We therefore envisioned free luciferin would be liberated by GSH attack on the ether carbon site of the *o*-nitrophenyl ring due to the significantly higher pK_a values of the 6-hydroxy group of luciferin and quinolinyl luciferin (8.5 and 9.4)[8] than the pK_a 7.21[9] of *o*-nitrophenol (Scheme 1). We anticipated both the reactivity and isozyme selectivity could be modulated by the effects of substituents in the nitrophenyl ring or by converting the nucleofugic group (ether bond) to a better leaving group.

Initial attempts at the synthesis of *o*-nitrophenyl luciferin ether compounds *via* a two-step reaction sequence, that is, nucleophilic substitution of 1-chloro-2-nitro-benzene derivatives with 6-hydroxy-2-cyanobenzothiazole, followed by condensation with D-cysteine were unsuccessful due to the unexpected nucleophilic reaction of sulfur in the benzothiazole ring with 1-chloro-2-nitro-benzene. However, a similar method employed with 6-hydroxy-2-cyanoquinoline resulted in the target *o*-nitroaromatic quinolinyl luciferin ether compounds **2a**, **2b** and **2c** (Scheme 1). The activities of mammalian isozymes A1-1, M1-1 and P1-1 with compounds **2a–2c** as substrates and GSH as a co-substrate were examined in a two-step assay format. The resulting luminescence with these compounds in the presence of GSH and GST enzyme above GSH control signal indicated that quinolinyl luciferin was released by GST-catalyzed nucleophilic substitution. The products from GST/GSH reactions were analyzed by LC-MS, and only free luciferin and GSH-nitrophenyl adduct were observed with no *o*-nitrophenol or luciferin–GSH adduct detectable, confirming that the GSH only attacked the ether carbon site of the *o*-nitrophenyl ring. GSH control (non-enzymatic) for compounds **2a–2c** yielded very low net signals with net signal-to-background ratios <0.1. Compounds **2b** and **2c** exhibited reactivity with isozyme A1-1 and M1-1 with signal-to-background ratios of 14 and 11 for A1-1, 46 and 170 for M1-1, respectively, whereas none of the compounds showed appreciable reactivity toward isozyme P1-1. Given that compound **2a** showed lack of reactivity toward any GST isozyme and



Scheme 1 The release of luciferin from *o*-nitroaromatic luciferin ethers by nucleophilic reaction with GSH catalyzed by GST and syntheses of *o*-nitroaromatic quinolinyl luciferin ethers.

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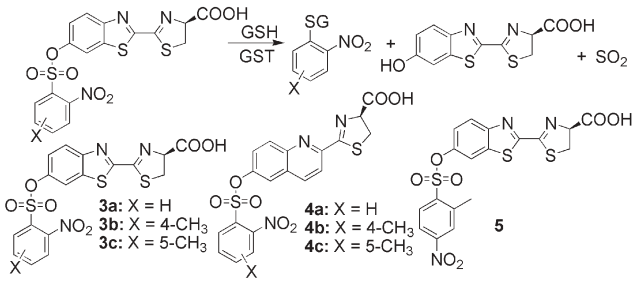
† Electronic supplementary information (ESI) available: Synthetic procedures and characterizations for compounds **2a–c, 3a–3c, 4a–4c** and **5**; protocols for GST enzyme assays and determination of kinetic parameters for GST isozymes with compound **3c**; LC-MS spectra for analyzing the products from the GST reactions. See DOI: 10.1039/b610682j



Scheme 2 The release of luciferin from aromatic sulfonyl luciferin by nucleophilic reaction with GSH catalyzed by GST and structures of compounds **3a–3c, 4a–4c** and **5**.

compounds **2b** and **2c** exhibited higher reactivity, we thought the introduction of a better electron withdrawing group than trifluoromethyl would enhance the reactivity. However, the result that D-cysteine reacted directly with the 2,4-dinitrophenyl ether **4a** during the second step of the condensation reaction implied that inserting stronger electron withdrawing groups on the aromatic ring would increase the signal-to-background from non-enzymatic GSH reactions or other thiols and thus limit the usefulness of these compounds.

We next turned our attention to other nucleofugic groups as an alternative means to activate the nucleophilic reaction. Sulfonate esters have previously been shown to enhance nucleophilic reactivity significantly, presumably because of their increased polarizability.[10] We therefore prepared a series of luciferin sulfonates, anticipating that free luciferin would be released by the loss of sulfur dioxide after nucleophilic attack of GSH on the C–S carbon site of the substituted aromatic ring (Scheme 2). The reactivity of compounds **3a–3c, 4a–4c** and **5** toward three mammalian GSTs was measured by employing the above two-step assay. The luminescent signals produced with compounds **3a–3c, 4a–4c** and **5** were much greater (generally 10–20 fold) than

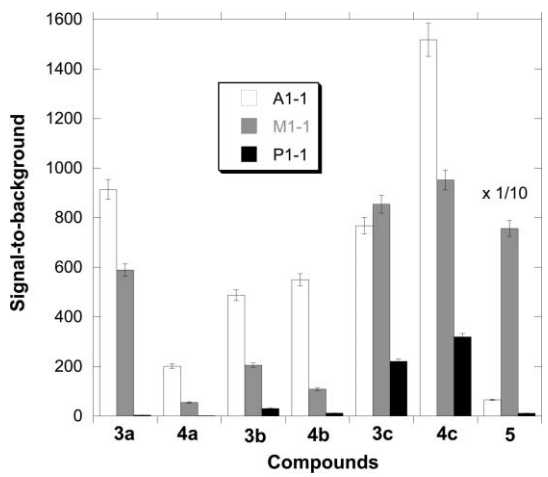


Fig. 1 The relative activities of three mammalian GST isozymes with compounds **3a–3c, 4a–4c** and **5**. Each GST isozyme (~1 μg) was incubated with 100 μM compound and 2.0 mM reduced GSH in 125 mM Hepes, pH 7.5. After incubation for 30 min at room temperature, 100 μl of a proprietary luciferin detection reagent was added and the luminescent signal was measured after 20 min.

compounds **2b** and **2c** (Fig. 1). The incubation of the substrates with GSH in the absence of GST enzyme yielded net signal-to-background ratios that were less than 0.5, and in most cases, the GSH control signal could be neglected due to the large net signals. As before, only free luciferin and a single nitrophenyl–GSH adduct were detected, and no *o*-nitrophenol and luciferin–GSH adduct were observed by HPLC and LC-MS, confirming the mechanism described in Scheme 2. Paired substrates bearing an identical aromatic ring with the two different frameworks (luciferin and quinolinyl luciferin) exhibited similar patterns of selectivity toward GST isozymes, indicating that isozyme selectivities were relatively insensitive to the central core of the luciferin. However, the isozyme selectivity was more dependent on the substituents on the aromatic ring, even the introduction of a small and mild electron donating methyl group changed the selectivity. Specifically, compounds **3a** and **4a** lacking a methyl group exhibited similar selectivity towards GST A1-1 and M1-1 while compounds **3b** and **4b** with a methyl group positioned *para* to the sulfonate showed enhanced preference for isozyme A1-1. Compound **5**, with switched substituents compared to compound **3b**, was more selective toward GST M1-1 whereas much more reactivity with GST P1-1 was observed for compounds **3c** and **4c** with a methyl substituent at the position *meta* to the ether site. Compounds **3c** and **4c** also appeared to be good substrates for all three major human GST isozymes with K_m values for each isozyme, 4.8 ± 0.8, 27.8 ± 6.8 and 91.8 ± 18.2 μM for A1-1, M1-1 and P1-1, respectively, for compound **3c** in the presence of 2.0 mM GSH.

Additionally, it is worthwhile to mention that all of the sulfonate substrates were extremely reactive with GST from *Schistosoma japonicum* which is widely used for producing fusion protein constructs (GST fusion).[11] Specifically, compound **3b** exhibited the greatest signal-to-background ratio, ~11 000, with substantially lower K_m values (39.3 ± 3.1 μM) for GST from *S. japonicum*. The high turnover number of this isozyme for the substrate suggested that compound **3b** would be an excellent substrate for measuring recombinant GST fusion protein production. This would make the evaluation of recombinant protein production much more rapid than currently used methods such as SDS-PAGE analyses or immunochemical methods.

In summary, we designed a series of electrophilic substituted nitrophenyl luciferin ether and sulfonate compounds for monitoring mammalian GST and *S. japonicum* enzyme activities. GST activities were not only influenced by electron-directing group(s), but were highly depending on the leaving ability of the nucleofugic group. The luciferin sulfonates exhibited excellent reactivities toward one or more GST isozymes and the isozyme selectivity is more sensitive to the electrophilic ring rather than the luciferin scaffold. A variety of other possibilities for molecular modifications on such sulfonate substrates should facilitate exploring the complexity of GST enzyme structures, conformations and catalytic mechanisms. The highly sensitive *S. japonicum* could provide a method for rapidly and effectively detecting fusion protein production and allow establishing a coupled, specific GSH assay at cellular levels.

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