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Synthesis and Use of New Fluorogenic Precipitating Substrates.

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Abstract: New fluorogenic esterase, glycosidase, aryl sulfatase, microsomal dealkylase, guanidinobenzoatase and alkaline phosphatase substrates have been prepared which precipitate at the site of enzyme activity, both *in vitro* and *in vivo*.

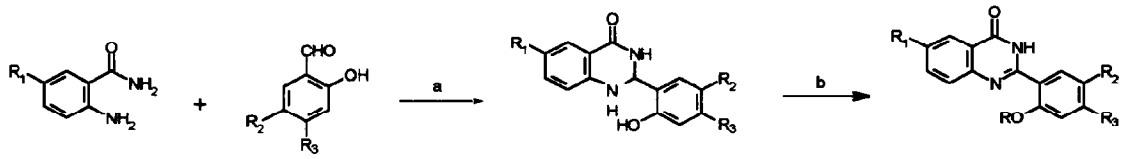
Detection of enzyme activity in biological fluids, chemical samples, individual live cells, or whole organisms is the subject of considerable research effort.[1] Information about metabolism, disease state, the identity of microorganisms, the success of genetic manipulations, or the quantity of drugs or toxins can be gained from evaluating the activity of certain enzymes.[2] In addition, enzyme conjugates often serve as sensitive bioanalytical tools.[3] Our approach to improving the detection of such enzyme activity has been to develop a series of new, stable, water soluble, non-toxic, synthetic enzyme substrates that become highly fluorescent upon enzyme action and precipitate at the site of enzyme activity without translocation.

Recently 2-aryl substituted 4-(3H)-quinazolinones have been synthesized for use in the preparation of fluorescent compositions of inks and enamels.[4] These fluorescent dyes have the properties of exhibiting intense luminescence in the solid state, high extinction coefficients, large Stokes shifts due to intramolecular hydrogen bonding interactions in the excited state, and significant photostability. We have now investigated the derivatization of several 2-hydroxyaryl 4-(3H)-quinazolinones by glycosylation, esterification, and alkylation for the production of new synthetic enzyme substrates.

2-Hydroxyaryl 4-(3H)-quinazolinones were prepared by the heating an anthranilamide **1** and an appropriate aromatic salicylaldehyde **2** in ethanol to reflux (1.0 h) in the presence of catalytic p-toluenesulfonic acid (pTsOH) followed by *in situ* oxidation with a suitable oxidizing agent such as dichlorodicyanoquinone (DDQ), at reflux, until thin layer chromatography showed disappearance of the blue fluorescent dihydro-derivative **3** (Scheme 1). Alternately, equimolar amounts of suitably derivatized isatoic anhydrides with salicylamides were heated at reflux (150°C, 4.0 h) in the presence of catalytic base (KOH or NaOH) in an inert solvent (DMF).[5] Water of reaction was not collected. In either case, after cooling, the insoluble fluorescent dyes **4** precipitated from the reaction mixture in good yield (70-90%) and were isolated by filtration and washing with ethanol. New fluorophores **4a-d** with modified fluorescent emission wavelengths (Table 1) were produced by functional elaboration at the 4'-, 5'-, and/or 6-positions. Fluorogenic enzyme substrates **5a-f** (Table 2) were prepared from fluorophore **4a** as described below.

Glycosylation reactions of these 2-hydroxyaryl-4-(3H)-quinazolinones were carried out using standard Koenigs-Knorr methodology.[6] The final substrates were obtained from the fully protected glycosides (1.0 mmole) by stirring in methanol (100 mL), methylene chloride (30 mL) and aqueous potassium carbonate (250µL of a 1M solution) at room temperature for 1 h followed by deionization using a mixed bed ion-exchange resin (1g IRC-50

and 1 g IRA-93). In this manner, the 2-O-ß-D-Gal[7] (**5a**), -ß-D-GlcU, -ß-D-Glc, -α-D-Man, -α-L-Fuc, -α-D-Neu5-N-Ac, -ß-D-Glc(1-4)ß-D-Glc, -ß-D-Xyl, and -ß-D-GlcNAc derivatives were prepared.



1 **2** **3**

4 (a-d) R = H
5 (a-f) R_1, R_2, R_3 = H

Scheme 1 *Reagents:* a. p-TsOH/EtOH; b. DDQ/EtOH

Table 1

Compound	R_1	R_2	R_3	EX (nm)	EM (nm)	yield(%)*	m.p. (°C)
4a	H	H	H	375	490	64	297-298
4b	H	H	OMe	375	550	89	290-292
4c	H	OMe	H	375	450	74	284-286
4d	Cl	Cl	H	375	510	97	>350

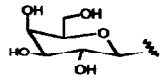
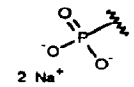
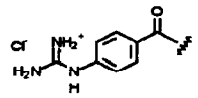
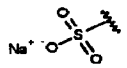
* unoptimized yields

Phosphorylation reactions were carried out essentially using the procedure of Perich and Johns.[8] The di-t-butyl-protected phosphate products were typically obtained in high yield (99% for the 2-(2'-di-t-butylphosphoryloxyphenyl)-4-(3H)-quinazolinone derivative). Removal of the t-butyl protecting groups was carried out using anhydrous 4M HCl/dioxane at -20°C. The resulting free acid was either isolated by filtration at room temperature directly from the reaction mixture, without further purification,[9] or the disodium (**5b**) or diammonium salts could be prepared by neutralization with concentrated aqueous NaOH or NH_4OH solutions to pH 8.5, respectively.

Esterase and lipase substrates (for example: **5c**) were prepared by esterification of the base fluorophore with an appropriate acyl anhydride at reflux for 2 hours using dry pyridine as solvent and catalyst. After solvent removal, the resulting solids were purified by silica column chromatography using chloroform as eluent.[10]

Attempts to directly alkylate the hydroxyl group of the base fluorophores proved unsuccessful. Therefore, instead, microsomal dealkylase substrates (for example: 2-(2'-ethoxyphenyl)-4-(3H)-quinazolinone, **5d**) were prepared by a stepwise procedure. A mixture of anthranilamide and 2-alkoxybenzaldehyde was suspended in methanol and heated to reflux for 3 hours. After cooling, the Schiff's base was isolated by vacuum filtration, suspended in ethanol containing catalytic p-toluenesulfonic acid, and heated to reflux for 1 hour. The resulting dihydroquinazolone was treated as above with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) for 1 hour, and after cooling to room temperature, the precipitated solid was filtered and washed with methanol. The products were recrystallized from methanol to yield colorless solids [11]

Table 2

Compound	R	Yield (%)*
5a		62
5b		76
5c	Ac	68
5d	Et	75
5e		91
5f		88

* unoptimized yields

The guanidinobenzoatase substrate (**5e**) was prepared in a similar manner to that described by Livingston.[12] Under anhydrous conditions a mixture of a 2-(2'-hydroxyphenyl)-4-(3H)-quinazolinone, dicyclohexylcarbodiimide, and p-guanidinobenzoic acid in anhydrous dimethylformamide and dry pyridine were allowed to stir at room temperature for 18 h, the reaction mixture filtered, evaporated to a clear yellow oil and crystallized by trituration with chloroform. The resulting colorless nonfluorescent solid was purified by reversed phase MPLC chromatography to yield the product.[9]

Aryl sulfatase substrates (for example: 2-(2'-sulfonyloxyphenyl)-4-(3H)-quinazolinone, sodium salt, **5f**) were prepared by treatment of a base fluorophore with chlorosulfonic acid and pyridine at 0°C followed by heating at 60°C for 24 hours. Following solvent removal *in vacuo* the residue was dissolved in water, neutralized to pH 7.0 with NaOH and the product purified by gel filtration chromatography on lipophilic Sephadex LH 20, elution using water. Product-containing fractions were combined and lyophilized to a colorless solid.[9]

Our present results indicate that these compounds exhibit moderate to good enzyme kinetic turnover rates and excellent specificity as substrates for their respective enzymes (i.e. *E. coli* ß-galactosidase and ß-glucuronidase, calf intestine alkaline phosphatase, prostatic acid phosphatase, ß-glucosidase (plant), porcine liver esterase, etc.) both *in vitro* and *in vivo*.[13] We are currently examining further uses for these new substrates in enzyme linked assays[14] and for detection of endogenous and cloned enzyme activities both in individual cultured cells and in tissues.[15]

References and Notes

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7. Analytical data for the 2-O-ß-D-galactoside: ^{1}H NMR (300 MHz, DMSO-d_6) δ: 8.52 (d, 1H); 8.28 (m, 2H); 8.25 (d, 1H); 7.74 (t, 1H); 7.45 (m, 1H); 7.0 (m, 2H); 6.22 (d, 1H,H-1ß, $J_{1,2}$=6.8 Hz); 5.25 (m, 1H); 4.55 (m, 2H); 3.80 (m, 3H); 3.55 (m, 4H); 3.22 (m, 1H). IR (KBr): 1751, 767, 1052, 1080, 1091, 1491, 1580, 1583, 3382, 3390, 3396, 3402, 3410, 3417, 3419 cm^{-1}; m.p.(d) = 160-165°C. TLC: (SiO_2, 1:1 ethyl acetate:hexane) product R_f = 0, starting material at R_f = 0.55, decomposition product at R_f = 0.84.
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9. Analytical data for the free acid 2-(5'-chloro-2'-phosphoryloxyphenyl)-6-chloro-4-(3H)-quinazolinone: TLC (SiO_2, 7:1:1 ethyl acetate:methanol:water) R_f = 0.17. ^{1}H NMR (300 MHz, DMSO-d_6) δ:7.39 (d, 1H); 7.57 (d, 1H); 7.70-7.90 (m, 3H); 8.07 (s,1H); ^{31}P NMR (121.5 MHz, DMSO-d_6) δ:1.2 (s) (ref. H_3PO_4 = 0). Found: C, 43.44; H, 2.38%. Calcd. for $C_{14}H_9O_5N_2Cl_2P$: C, 43.44; H, 2.34%. Analytical data for 2-(2'-sulfonyloxyphenyl)-4-(3H)-quinazolinone and 2-(2'-p-guanidinobenzoyloxy)-4-(3H)-quinazolone were similar and consistent with the proposed structures.
10. Analytical data for 2-(2'-acetoxyphenyl)-4-(3H)-quinazolinone: TLC: (SiO_2, chloroform) R_f = 0.14. ^{1}H NMR (300 MHz, $CDCl_3$) δ:8.31 (d, 1H); 8.07 (d, 1H); 7.81 (m, 2H); 7.62-7.50 (m, 2H); 7.44 (d, 1H); 7.27 (dd, 1H); 2.32 (s,3H,-OAc). Yield: 68%.
11. Analytical data for 2-(2'-ethoxyphenyl)-4-(3H)-quinazolinone: ^{1}H NMR (300 MHz , $CDCl_3$) δ: 8.54 (d, 1H); 8.30 (d, 1H); 7.77 (m, 2H); 7.51-7.43 (m, 2H); 7.15 (t, 1H); 7.05 (d, 1H); 4.30 (q, 2H); 1.60 (t, 3H); Molar Ex. Coeff. = 14.2K (MeOH); TLC (SiO_2, 1:1 ethyl acetate:hexane) R_f = 0.40.
12. Livingston, D.C.; Brocklehurst, J.R.; Cannon, J.F.; Leytus, S.P.; Wehrly, J.A.; Peltz, S.W.; Peltz, G.A.; Mangel, W.F. *Biochemistry* **1981**, *20*, 4298.
13. Kinetic assays for the substrates: The specific activity (k_2, in units of micromole per minute per milligram protein) and Michaelis constant (K_M, in units of millimole) for hydrolysis of the substrates by the enzyme are as follows: Quinazolinone Phosphates: (2-phenyl: k_2 = 188.8, K_M = 5.0), (2-(5'chlorophenyl)-6-chloro: k_2 = 618.6, K_M = 75.0), (2-(3',5'-dimethoxyphenyl): k_2 = 215.6, K_M = 10.8). The specific activities for the glycosidase and esterase substrates were measured with respect to the rates for turnover of the corresponding p-nitrophenyl derivatives, and were in the range of 0.1 - 0.5. All of the specific enzymatic reactions are made using the following buffers: For β-galactosidase: 0.1 M phosphate, pH 7.0 containing 0.11 M 2-mercaptoethanol and 1 mM $MgCl_2$. For alkaline phosphatase: 0.1 M TRIS pH 10.3 containing 50 mM NaCl, 10 mM $MgCl_2$ and 0.1 mM $ZnCl_2$. For acid phosphatase: 0.1 M acetate pH 5.0. For sulfatase: 0.1 M acetate pH 5.0.
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15. Preliminary results indicate the phosphatase substrates are sensitive markers for *in vivo* detection of endogenous phosphatase activities in mammalian lung, kidney, intestine, neural, and retinal tissues; ß-Galactosidase substrates are sensitive probes for measuring activity in CRE BAG 2 (*lac Z* positive) cells, in culture.

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