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Photoactivatable, biologically-relevant phenols with sensitivity toward 2-photon excitation†

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Spatio-temporal release of biologically relevant small molecules provides exquisite control over the activation of receptors and signaling pathways. This can be accomplished *via* a photochemical reaction that releases the desired small molecule in response to irradiation with light. A series of biologically-relevant signaling molecules (serotonin, octopamine, capsaicin, *N*-vanillyl-nonanoylamide, estradiol, and tyrosine) that contain a phenol moiety were conjugated to the 8-bromo-7-hydroxyquinolinyl (BHQ) or 8-cyano-7-hydroxyquinolinyl (CyHQ) photoremovable protecting groups (PPGs). The CyHQ caged compounds proved sensitive toward 1PE and 2PE processes with quantum efficiencies of 0.2–0.4 upon irradiation at 365 nm and two-photon action cross sections of 0.15–0.31 GM when irradiated at 740 nm. All but one BHQ caged compound, BHQ-estradiol, were found to be sensitive to photolysis through 1PE and 2PE with quantum efficiencies of 0.30–0.40 and two photon cross sections of 0.40–0.60 GM. Instead of releasing estradiol, BHQ-estradiol underwent debromination.

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Introduction

Biological effectors containing a phenol functional group are ubiquitous in nature. Phenols are found in neurotransmitters, synthetic neural agonists and antagonists, steroids, and amino acids, among other classes of compounds (Fig. 1). The physiological function of these phenols is quite broad. For example, serotonin (5-HT) plays a role in the modulation of mood, appetite, and sleep, and there is a growing body of evidence that it influences left-right patterning in the embryonic development of vertebrates.[1–3] Octopamine is primarily an insect neurotransmitter involved in reinforcing reward encoding and certain behaviors.[4] Capsaicin, the active ingredient in hot peppers, is a TRPV1 ion channel agonist, and heterologously expressed TRPV1 channels have been activated by *in vivo* application of capsaicin to effect behavioral changes.[5] *N*-Vanillyl-nonanoylaminde (VNA) is an equipotent TRPV1 agonist.[6] Steroids are involved in myriad signaling pathways[7] and have been recently implicated in neurogenesis.[8] Amino acids, tyrosine in particular, are involved in the regulation of MAPK and MEK/ERK through phosphorylation and dephosphorylation.[9] Developing ways to artificially activate phenols in model biological systems would enable the conduct of experiments aimed at understanding the role that they play in physiology at the molecular level.

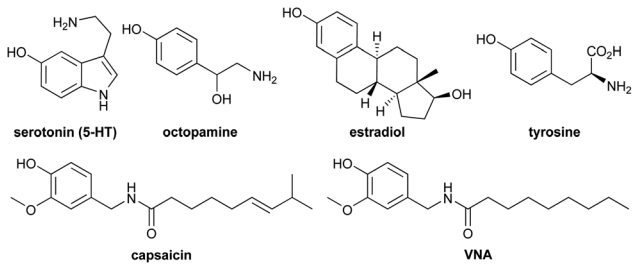

Fig. 1 Some biologically active phenols.

One method of activating biological effectors is through the use of a photoremovable protecting group (PPG). This strategy typically employs a covalent linkage between the PPG and a biologically relevant functional group on the desired biological effector; it has been used successfully to "cage" carboxylates, phosphates, alcohols, aldehydes and ketones, diols, thiols, amines, and phenols.[10] A major limitation faced when using a PPG on a phenol is that most PPGs require a carbonate linkage between the PPG and the phenol to effect release. A common way around this design problem has been to instead protect a different functional group, typically a primary or secondary amine. In either case, this limitation necessitates a decarboxylation step from either the phenol (~4 ms)[11] or the amine (~0.4 s)[12,13] to return the biological effector to full activity. Direct release of phenols enables the study of biological pro-

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† Electronic supplementary information (ESI) available: Synthetic procedures for the preparations of **1b**, **2–7**, **10b**, and **11–16**; ^{1}H NMR and ^{13}C NMR spectra; experimental procedures for determining ε, Q_u, and δ_u; and individual photolysis reaction progress curves. See DOI: 10.1039/c5pp00334b

cesses faster than the rate(s) of decarboxylation; an example of this is the *ortho*-carboxynitrobenzyl (*o*-CNB) PPG, which has demonstrated the ability to directly release phenols with a time constant of 16 μs, albeit with a low quantum efficiency (0.03).[14]

The kinetics of photolysis are important in design considerations from two different standpoints. The first is in regard to spatial resolution, and the second is in regard to the kinetics of the biological process under investigation. Spatial resolution in 3-dimensions is primarily achieved through two-photon excitation (2PE): the nearly simultaneous absorbance of two photons of light to reach an excited state restricts the volume of excitation to the focal point of the laser, which can provide spatial resolution down to one femtoliter.[10] The exquisite spatial resolution provided by 2PE can only be achieved when the kinetics of photolysis are faster than the rate of diffusion ($\sim 3.3 \times 10^{-6}$ cm^2 s^{-1}),[15] otherwise diffusional spread results in a release volume larger than the excitation volume. When photolysis kinetics are slower than the rate of diffusion, the sensitivity of the PPG toward 2PE must raise accordingly, and a reported threshold of 0.10 GM[16] for biological application may be insufficient for certain PPGs and a revised threshold of >3 GM[17] may be warranted. The photorelease of the effector must be faster than the physiological process under study. This is akin to watching a video of sprinters in a 50 m dash at a low *vs.* a high frame rate. If the frame rate is 0.1 frames per s, one would see the runners lined up for the start and the next image would show the racers post finish. Using a frame rate of 10 frames per s would capture nearly all of the action in between the start and finish. Likewise, to investigate a biological process, the photolysis reaction needs to be faster than the action of the biological effector on the system.

The kinetics of biological processes involving the phenols shown in Fig. 1 have been studied and occur on a range of timescales. For example, the estrogen receptor activation rate constant is $\sim 1.7 \times 10^6$ M^{-1} s^{-1},[18] tyrosine phosphorylation by the tyrosine kinase Lck occurs with an apparent Michaelis rate constant k_{cat} of 2.8×10^{-3} s^{-1},[19] and octopamine binds to its receptor with an on-rate constant of $\sim 3.2 \times 10^5$ M^{-1} s^{-1}.[20] Capsaicin induced channel opening of the TRPV1 channel has three phases with the fastest occurring in 0.46 ms.[21] There are two major types of 5-HT receptors: the 5-HT$_3$ receptor, a ligand gated ion channel with kinetics of channel opening equal to 4×10^2 s^{-1},[22] and six G-protein coupled receptor subtypes (5-HT$_{1,2,4-7}$), which display binding on timescales in the 100–500 ms range.[23]

Photoactivatable versions of each of the biologically-relevant phenols shown in Fig. 1 have been previously reported (Fig. 2). The neurotransmitter 5-HT has been protected at the phenol by the α-carboxynitrobenzyl (CNB)[14] and the nitrophenethyl (NPE)[24] PPGs and at the amine with CNB,[14] through a carbamate linkage with NPE (Tocris cat. #3991), and as a complex with the ruthenium bipyridyl PPG.[25] More recently, we reported the synthesis and photochemistry of an 8-bromo-7-hydroxyquinoline (BHQ)-protected 5-HT (**1a** Fig. 3).[3] Octopamine has not been protected on its phenol. Rather, a carbamate linkage between the amine and several 7-aminocoumarinyl derivatives[26] and a pyrenylmethyl PPG[27] have been reported. Several strategies for protecting capsaicin and VNA have been developed. It has been protected at the phenol using CNB and 7-bromo-8-hydroxycoumarinyl derivatives,[28] whereas VNA has been protected at the phenol with NB,[29] DMNB,[29,30] NVOC,[11,29] and MeOPA[29,30] or the amide nitrogen with NB or DMNB.[11] There are two previously reported photoactivatable forms of estradiol, one protected by DMNB at the phenol and one protected by NPEC at the C-17 hydroxyl group.[31] Tyrosine has been protected at the phenol with CNV[32] and the amine with PMOC.[27]

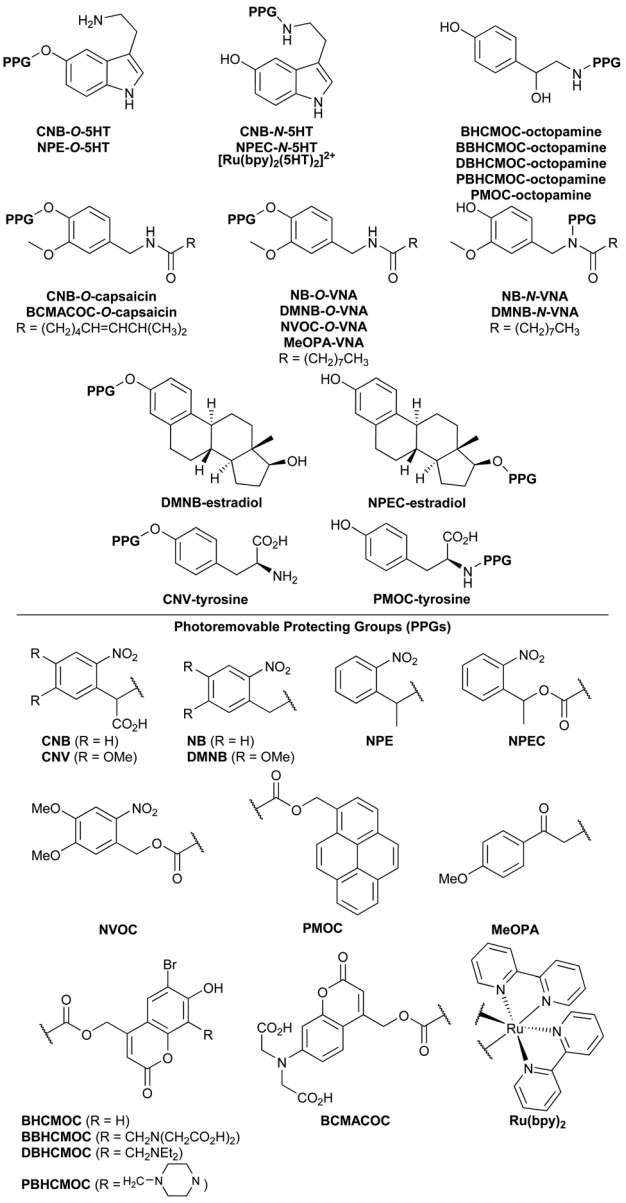


Fig. 2 Photoactivatable, biologically relevant phenols from the literature.

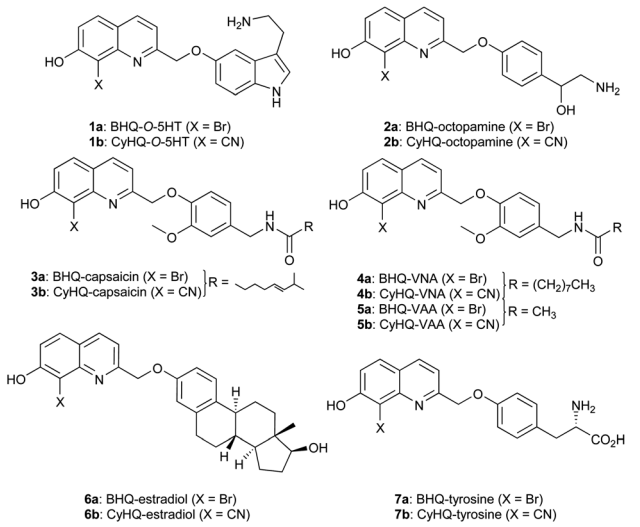


Fig. 3 Photoactivatable, biologically relevant phenols prepared.

0.003–0.05)[11,14,25,27,28,32] or the quantum efficiencies are not reported.[24,29–31] The value of λ_{max} is less than 300 nm for the NB-,[11] CNB-,[14] and NPEC-conjugated[31] effectors, which raises the potential for damaging biological tissues during photoactivation experiments. The coumarinyl-protected compounds have the highest quantum efficiencies at biologically useful wavelengths (λ_{max} > 365 nm),[26,28] but they are linked to the respective biological effectors through carbonate or carbamate linkages, which significantly slows the release kinetics,[12,13] because the resulting carbonate or carbamate must decarboxylate to reveal the active biological effector. Nitrobenzyl PPGs also show slow release kinetics, especially with poor leaving groups.[33] Only the coumarinyl derivatives,[26,28] NV-VNA,[11] and the $[Ru(bpy)_2(5HT)_2]^{2+}$ complex[25] photolyze readily by 2PE.

Hydroxyquinoline derivatives have demonstrated the ability to efficiently release a number of biologically relevant functional groups, including carboxylates, phosphates, diols, and thiols, through 1PE and 2PE.[34–37] The hydroxyquinoline PPG has also been shown to directly release phenols without the use of a carbonate linker,[3] and the timescale, measured for BHQ-OAc, is on the order of nanoseconds,[38] thus making this PPG suitable for use in studying temporally resolved signaling events. By directly releasing phenols, photoactivatable versions of relevant phenol-containing biological effectors with potentially rapid release kinetics are now possible. Indeed, 5-HT was recently caged and subsequently released in *Xenopus* embryos to provide temporal resolution in the examination of left-right patterning defects caused by abnormal distribution of the monoamine in the developing embryo.[3,39]

We describe the synthesis of representative biologically active photactivatable phenols (Fig. 3), their sensitivity toward both 1PE and 2PE with subsequent release of the phenol, and their hydrolytic stability in the dark. The phenols are comprised of 5-HT, octopamine, capsaicin, VNA, estradiol, and tyrosine.

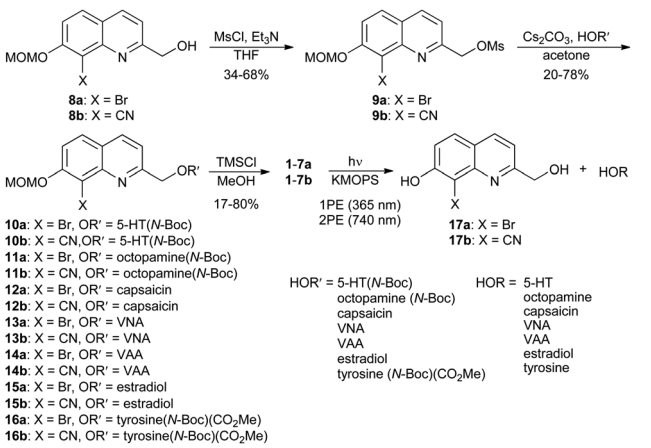
Results and discussion

We protected the biologically relevant phenols using two hydroxyquinoline-based PPGs, BHQ and CyHQ (Scheme 1). Compound **1a** was prepared as previously reported.[3] The other compounds were synthesized from MOM-protected BHQ-OH (**8a**) and CyHQ-OH (**8b**), which were prepared by published protocols.[36,38] The hydroxyl group on **8a** or **8b** was activated with methanesulfonyl chloride to produce mesylate **9a**, which has been previously reported,[3] or **9b**, respectively, in high yield. Displacement of the methanesulfonate with the desired phenol using cesium carbonate in acetone produced each of the protected caged phenols **10–16**. Deprotection conditions depended upon the protecting groups involved, but generally, trimethylsilyl chloride in methanol effected global deprotection, which was followed by HPLC purification to provide the photoactivatable phenols **1–7**.

The caged capsaicinoids **3a** and **4a** were too insoluble in neutral aqueous buffer, so BHQ-VAA (**5a**), which is sufficiently soluble, was used as a surrogate for assessing the photophysical and photochemical properties of the photoactivatable capsaicin and VNA. The CyHQ-protected capsaicinoids were much more water-soluble, but for direct comparison, CyHQ-VAA (**5b**) was photolyzed as a surrogate for **3b** and **4b**.

Both BHQ- (**a** series) and CyHQ-protected (**b** series) phenols had a maximum wavelength of absorption near 370 nm, although comparing the average values of ε (2480 *vs.* 5660 M^{-1} cm^{-1} for BHQ and CyHQ, respectively) shows that the CyHQ series had around twice the molar absorptivity than the BHQ-protected phenols (Table 1).

Irradiation of the **a** and **b** series of caged compounds **1**, **2**, **5**, and **7** plus **6b** with 365 nm light in KMOPS buffer (pH 7.2) released the corresponding phenols in 55–65% yield and with average quantum efficiencies of 0.29 for the BHQ series and 0.36 for the CyHQ series (Fig. 4). The chemical yields reported are similar to those of a variety of other caged compounds.[10] Several factors can reduce the chemical yield of the photolysis:



Scheme 1 Synthesis and photolysis of hydroxyquinoline-protected phenols.

Table 1 Photophysical and photochemical data for the photoactivatable phenols

Photoactivatable phenol	λ_{max} (nm)	Molar absorptivity (ε)[a]	Quantum efficiency (Q_u)	Sensitivity ($Q_u \times \varepsilon$)	2-Photon uncaging action cross-section (δ_u)[b]
BHQ-*O*-5HT **1a**[3]	368	2000	0.30	600	0.50
CyHQ-*O*-5HT **1b**	372	5700	0.39	2223	0.23
BHQ-octopamine **2a**	368	2700	0.36	972	0.49
CyHQ-octopamine **2b**	372	5400	0.34	1944	0.18
BHQ-VAA **5a**	370	2700	0.18	486	0.61
CyHQ-VAA **5b**	368	6100	0.41	2501	0.24
BHQ-estradiol **6a**	369	2400	—	—	—
CyHQ-estradiol **6b**	373	6200	0.29	1798	0.24
BHQ-tyrosine **7a**	366	2600	0.32	832	0.61
CyHQ-tyrosine **7b**	371	4900	0.38	1862	0.36

[a] M^{-1} cm^{-1}, λ = 370 nm. [b] GM = 10^{-50} cm^4 s per photon.

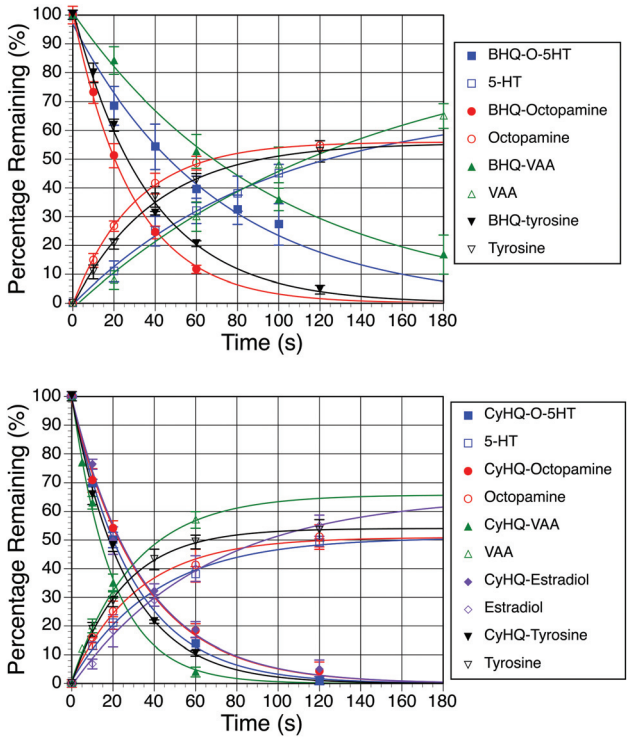


Fig. 4 Time courses for the photolysis of the photoactivatable phenols at 365 nm (1PE) and the rise of the biologically active phenols. Top: BHQ series, Bottom: CyHQ series. The percentage remaining is the average of at least 3 runs, and the error bars represent the standard deviation of the measurement. Lines are least-squares fits of a single exponential decay or a single exponential rise to max.

the caged compounds might undergo undesired photoreactions,[38] the effectors might possess some photosensitivity and react further to form secondary photoproducts,[40] or undesired secondary photoproducts might be generated through the interaction of the spent PPG and the biological effector, or a combination of all of these. The BHQ- and CyHQ-protected phenols have similar quantum efficiencies, but the sensitivity, which is quantified by multiplying the quantum efficiency by the molar absorptivity ($Q_u \times \varepsilon$) was much greater for the CyHQ-protected compounds, owing to their much larger extinction coefficients.

BHQ-protected estradiol **6a** did not photolyze to the expected products of **17a** and estradiol. Instead, debromination of **6a** was the primary reaction pathway, a phenomenon that has been observed previously.[41] To further investigate the debromination reaction, we irradiated at 365 nm a model compound, BHQ-OH (**17a**), which did not undergo photoinduced cleavage of the C–O bond, but lost bromine instead with a quantum efficiency of 0.12 in pH 7.2 KMOPS buffer. The extent of debromination in the BHQ series of photoactivatable phenols can be significant. A factor that contributes to the extent of debromination is the leaving group ability, and effectors that are poor leaving groups (high pK_a) conjugated to PPGs tend to be inert.[10] The calculated pK_a of estradiol was found to be 10.27 ± 0.60 (ACD/Labs version 11.02 accessed through SciFinder). All of the other phenols have calculated pK_a values lower than this value (10.09 ± 0.40 for 5-HT, 9.97 ± 0.26 for octopamine, 10.02 ± 0.31 for VAA, and 9.09 ± 0.10 for tyrosine), which might be the threshold for leaving group ability for BHQ-protected effectors. The ACD/Labs-calculated values were used to provide an estimated pK_a of the phenols under identical conditions and avoid issues arising from the variations in conditions found in the literature reporting pK_a values from experimental titrations.

Sufficient hydrolytic stability of the PPG-effector conjugate is required, so that there is no problem with leakage of the activated effector prior to photoactivation. Experiments where the caged compound is subjected to aqueous environments for hours or days require robust resistance to hydrolysis in the dark. Such experiments, might include those that use CyHQ-estradiol for neurogenesis or gene regulation studies or BHQ/CyHQ-tyrosine when controlling kinase cascades *via* phosphorylation of tyrosine residues. All of the compounds were examined for their ability to resist hydrolysis under simulated physiological conditions. Solutions of the relevant compound (100 μM) in KMOPS pH 7.2 were prepared, stored in the dark at room temperature, analyzed periodically over a period of 1 week, and in all cases showed minimal (<3%) hydrolysis over the measurement time frame.

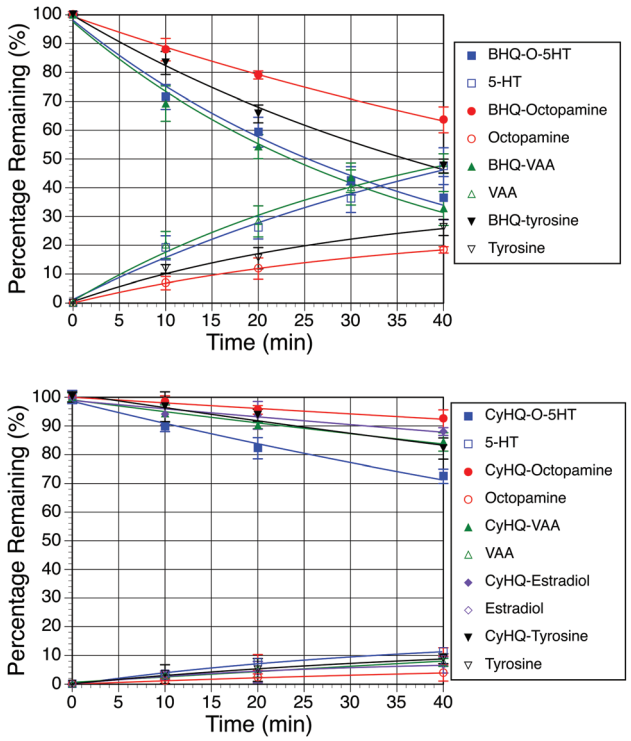


Fig. 5 Reaction progress curves for the photolysis of the photoactivatable phenols at 740 nm (2PE). Top: BHQ series, Bottom: CyHQ series. The percentage remaining is the average of at least 3 runs, and the error bars represent the standard deviation of the measurement. Lines are least-squares fits of a single exponential decay or a single exponential rise to max.

To assess the sensitivity of the photoactivatable phenols to 2PE, **1**, **2**, **5**, **6b**, and **7** were photolyzed at 740 nm using a femtosecond-pulsed, mode-locked Ti:sapphire laser. HPLC or uHPLC was used to monitor the reaction progress and from the decay curves (Fig. 5) and the 2PE-generated fluorescence output of fluorescein as an external standard, the 2-photon uncaging action cross-sections (δ_u) were calculated as previously described (Table 1).[3,16] The BHQ derivatives were more sensitive to 2PE than the CyHQ derivatives, having average values of δ_u of 0.55 GM compared to 0.25 GM for the CyHQ derivatives. The cross-sections reported are similar to that of previously reported quinoline- and coumarin-based PPGs and significantly higher than most nitrobenzyl and nitroindoline derivatives.[10,42–44] BHQ and CyHQ are sufficiently sensitive to be applied to the photoactivation of biologically relevant phenols *in vivo* through 2PE.

Conclusion

The sensitivity of the hydroxyquinoline PPGs BHQ and CyHQ toward 2PE, combined with sub-microsecond release kinetics provided an opportunity to create a variety of photoactivatable phenolic biological effectors (5-HT, octopamine, capsaicin, VNA, estradiol, and tyrosine). The CyHQ-protected phenols were more sensitive to 1PE owing to their larger molar absorptivities, whereas the BHQ-derivatives were more sensitive to 2PE. Instead of releasing estradiol, BHQ-estradiol was efficiently debrominated upon exposure to 365 nm light. This photochemical reaction pathway dominates when the attached effector is a poor leaving group. Alternative reaction pathways were not observed with the CyHQ-protected phenols. The synthesis of the XHQ-phenol conjugates is modular and can be applied to any biologically relevant phenol, not just the ones described here. The photoactivatable phenols reported will enable the examination of their associated biological processes at the cellular and molecular level. BHQ-*O*-5HT (**1a**) has already been used to study the role of 5-HT in left/right asymmetry in the developing vertebrate embryo.[3,39] Biological applications of the other photoactivatable phenols will be reported in due course.

Experimental

Synthesis

General procedures. MOM-protected BHQ-OH (**8a**)[38] and CyHQ-OH (**8b**)[36] were prepared as previously described, as were **1a**, **9a**, and **10a**.[3] A representative experimental procedure for preparing a photoactivatable phenol is given below. Experimental procedures for preparing each caged phenol can be found in the ESI.† uHPLC and HPLC were carried out on Agilent 1290 and 1260 systems, respectively, with Zorbax Eclipse C-18 reverse phase columns.

(8-Cyano-7-(methoxymethoxy)quinolin-2-yl)methyl methanesulfonate (9b). Alcohol **8b** (0.31 g, 1.2 mmol) was stirred in THF (5 mL). Methanesulfonyl chloride (0.189 ml, 2.44 mmol) and triethylamine (0.51 ml, 3.7 mmol) were added. The reaction progress was monitored by uHPLC, and upon completion, celite was added to the reaction mixture, which was subsequently dried *in vacuo*, loaded onto a column and purified *via* flash chromatography using EtOAc/hexane (1 : 2) to provide **9b** as a white powder (0.132 g, 0.408 mmol, 34%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.23 (d, J = 8.4 Hz, 1H), 8.02 (d, J = 9.2 Hz, 1H), 7.58 (dd, J = 12.6, 8.8 Hz, 2H), 5.55 (s, 2H), 5.47 (s, 2H), 3.59 (s, 3H), 3.29 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 162.5, 157.0, 148.2, 137.5, 133.7, 122.9, 119.1, 116.3, 114.5, 99.5, 95.1, 71.6, 56.9, 38.5; HRMS-ESI (m/z) $[M + H]^+$ calcd for $C_{14}H_{14}N_2O_5S$ 323.06962; found, 323.06966.

***tert*-Butyl(2-(4-((8-cyano-7-(methoxymethoxy)quinolin-2-yl)methoxy)phenyl)-2-hydroxyethyl)carbamate (11b).** *tert*-Butyl (2-hydroxy-2-(4-hydroxyphenyl)ethyl)carbamate (0.043 g, 0.17 mmol) and **9b** (0.050 g, 0.16 mmol) were stirred in acetone (1 mL). Cs_2CO_3 (0.10 g, 0.33 mmol) was added, and the reaction progress was monitored by uHPLC. Upon completion, brine was added and the resulting mixture was extracted with EtOAc. The extract was dried over sodium sulfate, concentrated *in vacuo* and purified *via* flash chromatography eluting with EtOAc/hexane (1 : 2), which yielded **11b** as a yellow residue upon drying (0.025 g, 0.052 mmol, 31%):

^{1}H NMR (600 MHz, CD_3OD) δ 9.05 (d, J = 8.3 Hz, 1H), 8.26 (d, J = 9.1 Hz, 1H), 7.90 (d, J = 8.3 Hz, 1H), 7.64 (d, J = 8.9 Hz, 1H), 7.27 (d, J = 7.9 Hz, 2H), 6.82 (d, J = 7.9 Hz, 2H), 5.27 (s, 2H), 4.82 (dd, J = 11.9, 3.7 Hz, 1H), 3.35 (s, 3H), 3.59–3.20 (m, 2H), 1.53 (s, 9H); ^{13}C NMR (151 MHz, CD_3OD) δ 167.6, 162.0, 157.3, 147.0, 145.5, 140.8, 135.5, 131.6, 126.9, 122.6, 120.0, 117.6, 115.0, 111.9, 97.1, 88.9, 85.0, 69.3, 60.7, 53.9, 46.0, 26.2; HRMS-ESI (m/z) $[M + H]^+$ calcd for $C_{26}H_{29}N_3O_6$ 480.21291; found, 480.21438.

2-((4-(2-Amino-1-hydroxyethyl)phenoxy)methyl)-7-hydroxyquinoline-8-carbonitrile (2b). Compound **11b** (0.025 g, 0.052 mmol) was dissolved in methanol (1 mL). TMSCl (0.115 mL, 0.918 mmol) was added. The resulting solution was stirred in the dark and monitored by uHPLC. Upon completion, the reaction was concentrated *in vacuo* and purified by HPLC using a 10 minute gradient from 5% CH_3CN/95% H_2O (0.1% TFA) to 100% CH_3CN. Fractions containing only one peak were combined and concentrated *in vacuo* to provide **2b** as a pale yellow solid (0.014 g, 0.042 mmol, 80% yield): ^{1}H NMR (600 MHz, CD_3OD) δ 8.27 (d, J = 8.4 Hz, 1H), 8.01 (d, J = 9.1 Hz, 1H), 7.58 (d, J = 8.3 Hz, 1H), 7.26 (d, J = 8.5 Hz, 2H), 6.82 (d, J = 8.4 Hz, 2H), 5.03 (s, 2H), 4.81 (d, J = 6.5 Hz, 1H), 4.81 (d, J = 12.7 Hz, 1H), 3.12–3.02 (m, 2H); ^{13}CNMR (600 MHz, CD_3OD) δ 163.6, 157.3, 148.4, 137.0, 133.7, 131.6, 126.8, 121.3, 117.7, 116.8, 115.1, 115.0, 94.0, 69.3, 64.7, 47.1, 45.9; HRMS-ESI (m/z) $[M + H]^+$ calcd for $C_{19}H_{17}N_3O_3$ 336.13427; found, 336.13471.

Photochemistry

Determination of the quantum efficiency for one-photon photolysis (Q_u). Briefly, solutions (100 μM) of the relevant substrate in KMOPS pH 7.2 were irradiated in quartz cuvettes (21-Q-10, Starna, Atascadero, CA) with a 365 ± 10 nm LED (Cairn OptoLED Lite). Aliquots (25 μL) were removed at various time points and analyzed by uHPLC (MeCN/H_2O 0.1% TFA: 6 min gradient 5% CH_3CN to 100% CH_3CN, 2 min gradient 100% CH_3CN to 5% CH_3CN), using an external standard to determine concentrations. The reaction progress was plotted and the data fit to a single exponential decay function and Q_u values were calculated according to previously reported protocols.[34] See ESI† for detailed experimental procedure.

Determination of the two-photon uncaging action cross-section (δ_u). Solutions (100 μM) of the substrates in KMOPS were prepared and stored in the dark. Aliquots (25 μL) of this solution were placed in a microcuvette (10 × 1 × 1 mm illuminated dimensions, 25 μL effective filling volume) and irradiated with a fs-pulsed and mode-locked Ti:Sapphire laser (Chameleon Ultra II, Coherent or a Mai Tai HP DeepSee, Spectra-Physics) with 740 nm light at an average power of 220–300 mW measured after passing through the cuvette. Three samples of each substrate were irradiated for various time periods and analyzed by HPLC or uHPLC as described for one-photon experiments. The reaction progress was plotted and the data fit to a single exponential decay function and δ_u values were calculated according to previously reported methods using fluorescein as an external standard.[34] See ESI† for detailed experimental procedure.

Hydrolysis in the dark

Substrates (100 μM) were dissolved in KMOPS and stored in the dark at room temperature. Aliquots (20 μL or 5 μL) were removed and analyzed periodically by HPLC or uHPLC, respectively, as described for one-photon photolysis. None of the compounds synthesized displayed a measurable rate of dark hydrolysis over an approximately 100 h time period.

Acknowledgements

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