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Competing pathways in the photo-Favorskii rearrangement and release of esters: Studies on fluorinated *p*-hydroxyphenacyl GABA and glutamate phototriggers

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Abstract

Three new trifluoromethylated *p*-hydroxyphenacyl (PHP) caged γ -aminobutyric acid (GABA) and glutamate (Glu) derivatives have been examined for their efficacy as photoremovable protecting groups in aqueous solution. By replacing hydrogen with fluorine, e.g., a *m*-trifluoromethyl or a *m*-trifluoromethoxy vs. *m*-methoxy substituents on the PHP chromophore, modest increases in the quantum yields for release of the amino acids GABA and glutamate were realized as well as improved lipophilicity. The PHP triplet undergoes a photo-Favorskii rearrangement with concomitant release of the amino acid substrate. Deprotonation competes with the rearrangement from the triplet excited state and yields the PHP conjugate base that, upon reprotonation, regenerate the starting ketoester, a chemically unproductive or “energy wasting” process. Employing picosecond pump–probe spectroscopy, GABA derivatives **2** – **5** are characterized by short triplet lifetimes, a manifestation of their rapid release of GABA. The bioavailability of released GABA at the GABA_A receptor improved when the release took place from *m*-OCF₃ (**2**) but decreased for *m*-CF₃ (**3**) when compared with the parent PHP derivative. These studies demonstrate that pK_a and lipophilicity exert significant but sometimes opposing influences on the photochemistry and biological activity of PHP phototriggers.

Introduction

Photoremovable protecting groups or “phototriggers” have found many applications in chemistry and biology and have become a subject of wide interest.^{1,2} They diminish or block the normal reactivity of an active compound, which can be restored upon exposure to light. When employed in this capacity, light is regarded as a ‘traceless reagent’ that can be administered along specific spatial, temporal, and concentration coordinates.^{1,3} These features are exploited, for example, through the release of the carboxyl group of the amino acids glutamate, GABA, or glycine in order to investigate neuronal processes in cell signaling, kinetic and mechanistic studies of the central nervous system.⁴

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Supporting Information **Available**: Synthetic procedures, ¹H, ¹³C, and ¹⁹F NMR spectra and HRMS data for **2**, **3** and **6** - **12** are available free of charge via the Internet at <http://pubs.acs.org>.

Since our discovery that the *p*-hydroxyphenacyl (pHP) chromophore will serve as a photoremovable protecting group,⁵ several biologically relevant substrates such as phosphates (ATP⁵ and GTP⁶), thiols (Protein Kinase A⁷ and glutathione⁸), carboxylates (γ -aminobutyric acid (GABA)⁹ and glutamate (Glu))¹⁰ and including the C-terminus of the oligopeptide, bradykinin^{11,12} and examples of functional group protection in synthesis² have been reported. This has prompted us to investigate the mechanistic features and substituent effects on the reaction. Interestingly, the reaction results in a significant and useful¹³ blue shift due to the rearrangement of the chromophore to *p*-hydroxyphenylacetic acid (**6**) as a primary product (eq. 1). The reaction mechanism has been studied in detail, predominantly by Phillips and co-workers¹⁴ and by us.¹⁵ We have shown that the reaction of pHP diethyl phosphate proceeds via a very short-lived triplet state T₁ (³ τ = 60 ps in wholly aqueous solution) and that the substrate is released concomitantly with the decay of T₁. Questions remain regarding the nature and significance of competing pathways and the effect of substitution on the chromophore on the release of substrates. In particular, the quantum yields for substrate release are often substantially less than unity, suggesting that chemically non-productive pathways may contribute to the normal photophysical decay of the excited chromophore. Only a very weak fluorescence is observed with this chromophore.^{14d}

(1)

In previous studies, it was demonstrated that the introduction of electron donating groups such as OCH₃ (**4** and **5**) shifts the π, π^* absorption of the chromophore to longer wavelengths ($\lambda_{\text{max}} > 300$ nm), whereas electron withdrawing *meta*-substituents (CO₂Me or CONH₂) have little influence on the λ_{max} .^{1,9} None of these substituents significantly affected the lifetime of the triplet state in contrast to their effects on the quantum yields.^{1,16} Electron withdrawing groups improve the quantum yields for the photo-Favorskii rearrangement whereas electron donors are lower vis-à-vis the parent pHP derivatives.

To combine the advantages of the red shift by *m*-methoxy substitution and the improved quantum yields from electron withdrawing groups, we introduced trifluoromethyl and trifluoromethoxy groups and compared the photochemistry and biological efficacy with the methoxy, and the unsubstituted pHP GABA analogs. Among the factors that are attendant with introducing a trifluoromethyl substituent are the significant increase in polarity due to the pronounced electron withdrawing inductive contribution of F,¹⁷ a relatively modest increase in steric size, and a potential improvement in biocompatibility.¹⁸ Although the trifluoromethyl group has rarely been exploited as a substituent on phototriggers,¹⁹ we reasoned that it would modify the reactivity without greatly disturbing the π -system of the chromophore. We report here the synthesis and photochemistry of three trifluoromethyl modifications, CF₃O-pHP GABA (**2**), CF₃-pHP GABA (**3**), and CF₃-pHP Glu (**8**), to test this hypothesis. We have also probed the differences between **2** and **3** on GABA release at the GABA_A receptor using whole-cell patch clamp monitoring of neurons in cortical slices and compared these derivatives with our previous study employing unsubstituted pHP GABA (**1**) and *meta* CH₃O-pHP GABA (**4**).⁹

Results and Discussion

Synthetic Studies

The synthetic sequence for **3** shown in Scheme 1 is the general route used for all three of the new derivatives. Benzyl protection of commercially available 4-bromo-2-trifluoromethylphenol (**9**) with BnBr/K₂CO₃/CH₃CN, followed by acetylation with a Stille protocol²⁰ using Pd(PPh₃)₄/tributyl(1-ethoxyvinyl)-stannane in toluene, and hydrolysis of the resulting enol ether gave the fluorinated acetophenone **10**. The Stille coupling of protected *p*-bromophenols to stannyl vinyl ethers served as a high yielding, general route for us to synthesize a variety of *p*-hydroxyacetophenones²¹ as opposed to traditional acylation methodology. α -Bromination with dioxane dibromide in dichloromethane (DDB/CH₂Cl₂), followed by esterification with N-Boc protected GABA and deprotection of the phenol and amine groups with Pd/C (10% w/w)/H₂ in EtOAc and 1:1 TFA/CH₂Cl₂, respectively, afforded CF₃-pHP GABA (**3**) in 55% overall yield from **9**. Essentially the same route was followed for the synthesis of OCF₃-pHP GABA (**2**, 57%) and CF₃-pHP Glu (**8**, 38%).

Photochemical Studies

All three derivatives, **2**, **3**, and **8**, quantitatively released the amino acid upon irradiation at $\lambda > 300$ nm in aqueous media under ambient conditions. The Favorskii rearrangement products **6** (R₁ = OCF₃ or CF₃; R₂ = H) along with small amounts of the minor product, *p*-hydroxybenzyl alcohols **7**, were characterized by ¹H, ¹³C, and ¹⁹F NMR, mass spectral analysis, and for certain cases, by comparison with authentic samples. In the case of the formation of *p*-hydroxy-*m*-trifluoromethoxybenzyl alcohol **7** from **2**, for example, the reaction products were also verified by NMR analysis after spiking the photolysis mixture with an independently synthesized sample of **7**. Another potential photohydrolysis byproduct, 2-hydroxy-1-(4-hydroxy-3-trifluoromethoxyphenyl)ethanone (**12**), that is structurally related to products that had been reported by others,²² was shown not to be present by ¹H and ¹³C NMR analysis by addition of an authentic sample to the photolysis mixture.

Quantitative analyses of the major products GABA and the substituted *p*-hydroxyphenylacetic acid, along with unreacted caged GABA, were accomplished using LC/MS/MS equipped with a C18 RP column (mobile phase gradient 1:1 MeOH/H₂O with added NH₄HCO₂ and acetaminophen as the internal standard). This highly sensitive LC/MS/MS method for quantitative analysis coupled with rapid data collection made possible the accurate analyses of the reaction profiles for quantum yields, quenching experiments, and media effects on the photochemistry at low conversions and using less than a few milligrams of caged GABA. The quantum yield data are given in Table 1.

The photorelease quantum yield of the trifluoromethoxy derivative **2** in unbuffered aqueous solution ($\phi = 0.09$) is approximately twice that of the methoxy (**4**) or dimethoxy (**5**) counterparts, whereas that of the trifluoromethyl analog **3** ($\phi = 0.17$) is nearly the same as that of the parent pHP GABA (**1**). Stern–Volmer studies at high concentrations of sorbate quenched these reactions, confirming a short-lived triplet reactive intermediate for pHP esters **2** and **3** (see Experimental) in accord with our earlier results with **1**.^{5,9,23} In HEPES buffer, pH 7.3 (1–9 mM pHP GABA), the quantum yields were slightly lower, which we attribute to pH and salt effects,²⁴ a suggestion supported by dramatic changes in the quantum yields when the pH is varied. In the absence of buffers, the pH of the photolysis solution decreases with increasing conversion due to the production of two carboxylic acids of lower pK_a's than the pHP phenol. Quantum yields are dependent on the pH of the solution (vide infra).

Increasing the pH to 8.2 or 9, values significantly above the pK_a of **2** or **3**, lowered the quantum yields by half or more. The observed strong red shift in the absorption spectra at the higher pH is due to the formation of the conjugate bases of these phenolic chromophores and photorelease from these is decidedly less efficient as was reported earlier.^{15,16}

None of these photoreactions is appreciably quenched by air, a manifestation of the very short lifetimes and high reactivities of the pHP triplet excited state. The inefficiency of O₂ quenching was tested by purging the photolysis solution with Ar (see 2, Table 1).

Time Resolved Pump-probe Studies—Picosecond transient absorption spectra were obtained for compounds **1–5**. The samples were excited at 266 nm (pulse width 200 fs) and probed by delayed supercontinuum pulses covering a range of 300–650 nm. The main features in the transient spectra of **1** up to 15 ps are the absorptions by the excited singlet and triplet states of the pHP chromophore, which are shown for **1** in Figure 1. Global analysis of the spectral evolution with increasing delay up to 2 ns of the probe pulse provided the singlet and triplet lifetimes, $^1\tau = 2.3$ ps and $^3\tau = 0.34$ ns, respectively.

The transient spectrum of the excited singlet state exhibits an absorption maximum around 315 nm, but it is overlaid by stimulated emission, which is responsible for the negative peak observed at 420 nm (dotted red curve in Figure 1). Because both of these transitions arise from the same state, they exhibit the same kinetic behavior. Singlet–triplet intersystem crossing (ISC) results in a rise of the strong triplet–triplet absorption band of **31** at 400 nm (Figure 1). A similar 400 nm absorption band was observed under similar conditions with *p*-hydroxyacetophenone (pHA) that is quenchable with potassium sorbate and has previously been assigned as due to the triplet–triplet absorption for pHA.²³ Similar triplet–triplet absorption spectra were observed for the other four pHP derivatives, i.e., a maximum at 400 to 420 nm with a shoulder at ~520 nm. Sorbate quenching of the 400 nm bands of the pHP derivatives likewise established them as triplet–triplet absorptions in agreement with our previous studies^{15,23a} and those by the Phillips group^{14,23b,c} derived from time-resolved transient absorption and resonance Raman spectral analyses with pHP phosphate and carboxylate esters.

The pump-probe time scan from 20 ps to 1.96 ns for **2** in water is shown in Figure 2, and the species spectrum attributed to the triplet of **2** is given in Figure 3 (solid green line). Also shown in Figure 3 is the spectrum of a species that persists after the decay of the triplet, $\lambda_{\text{max}} \approx 340$ nm (dotted blue line). A decay rate constant of $k_{\text{dec}} \sim 6.5 \times 10^6 \text{ s}^{-1}$ was determined for the 340-nm transient by nanosecond flash photolysis of **2** in water containing ca. 10 % ACN, similar to the results obtained with pHP phosphates.^{15,23}

In order to identify the 340-nm transient we examined the transient spectra of pHA in aqueous solution. Pump–probe spectra showed the formation of both the 400-nm absorption due to the triplet and a 340-nm transient a lifetime exceeding that accessible by the delay line (2 ns). Nanosecond LFP of pHA showed that neither the lifetime nor the amplitude of the 340-nm transient were affected by the addition of up to 10 mM sorbate, whereas the lifetime of the 400 nm triplet transient was reduced by more than tenfold ($k_q \approx 2 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$). Thus, the 340-nm transient is not a triplet. It showed first order decay kinetics ($^1\tau = \sim 10^{-5} \text{ s}^{-1}$), and, with the addition of 10^{-4} M HCl in a solution containing 10 mM sorbate, the lifetime of the 340-nm transient was quenched ($k_{\text{Hsorb}} = 4.6 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$), suggesting that it is the ground state anion of pHA. Indeed the onset of the absorption by pHA[−] follows the shape of the 340-nm transient down to 350 nm, but it continues to rise to its maximum at 325 nm. The shape of the transient absorption (Figure 3, dotted line) with an apparent maximum at 340 nm is, however, distorted by the onset of absorption by ground state pHA below 350 nm.

On the basis of Förster's cycle,²⁵ triplet excited pHA is predicted to be very strong acids, $pK_a \approx -3.6$.^{23a} Ionization of triplet pHA in aqueous solution occurs on a time scale of 10 ns.^{23c} This explains why quenching of the pHA triplet with up to 10 mM sorbate does not reduce the yield of the ground state anion absorbing at 340 nm. Acceptor-substituted pHP derivatives such as the pHP GABAs are likely to have a somewhat lower pK_a . Thus, we attribute the pathway competing with Favorskii rearrangement to be triplet state deprotonation of the phenol^{15,23,25} forming the triplet conjugate base of pHP GABA. Subsequent IC of the anion triplet and neutralization regenerates the starting pHP ester. The overall process must be viewed as a competing "energy wasting" sequence responsible for the diminished release quantum yields. This and other competing pathways from the triplet lower the release yield in the order $R_2CO_2^- < R_2O_2PO_2^- < RSO_3^-$. Together, these results suggest that better leaving groups influence the partitioning of the triplet more favorably toward the photo-Favorskii pathway.^{14d,15,23a,26}

Thus, the short triplet lifetimes of less than a nanosecond in wholly aqueous solution observed for the pHP esters can be attributed to a facile heterolysis of the leaving group. As reported earlier, water plays a significant role on the rate and quantum yield.²¹ For example, the triplet lifetime of pHP diethyl phosphate with its better leaving group, decreases dramatically as the proportion of water in acetonitrile is increased (Table 3 and Figure 4).^{14,15,23a} Nevertheless, the quantum yields for the disappearance of the pHP phosphate and appearance of the rearranged phenylacetic acid remain significantly below unity. These observations confirm that an additional process is competing with the rearrangement/release process in aqueous environments.

For the rearrangement pathway, as we had previously proposed, an adiabatic carbon-oxygen bond heterolysis is accompanied by deprotonation of the phenol^{15,21,23} to generate the oxyallyl-phenoxy triplet biradical ³**14** ($\lambda_{max} = 445$ and 420 nm, $\tau \sim 0.6$ ns). This biradical was also observed, but barely detectable in the pump-probe absorption spectra of the present series (see the SI for the biradical triplet-triplet transient absorption spectrum for ³**14**). Its weak signals were overlaid by the strong triplet absorptions, which are longer-lived than for **1** (Table 2). The two competing pathways for pHP GABA are summarized in Scheme 2.^{15,21}

Finally, the effects of pH on the quantum yields prompted further investigations of **2** and **3**. Photolysis of the conjugate base of the trifluoromethyl pHP GABA derivatives demonstrated a decided diminution in the quantum yields at pH's above the pK_a of the phenolic group. The lower photoreactivity of the phenolates may follow a completely different mechanistic pathway, although the photoproducts remain the same.

The replacement of methyl with trifluoromethyl is accompanied by a significant increase in the lipophilicity of the two GABA derivatives for **2** and **3**, nearly doubling the $ClogP$, as determined by the partitioning between 1-octanol and water, compared with the unsubstituted pHP GABA (Table 4). The effects of increased lipophilicity on the photoreactions of **2** and **3** were reflected by the quantum yields in 1-octanol and in mixtures of CH₃CN/H₂O or DMSO/H₂O. In 1-octanol, **2** and **3** had quantum yields for disappearance of 0.14 and 0.10, respectively. In aqueous-organic mixed solvents, the quantum yields increased with increasing water content in accord with earlier observations with mixed acetonitrile:H₂O solvent mixtures,^{14,15,21,23} Typically, for biologically benign solvents like CH₃CN or DMSO, the quantum yield for **2** increased by a factor of 4 upon addition of 10% (v:v) H₂O, i.e., increasing from 0.03 in dry CH₃CN to 0.12 in 10% H₂O:CH₃CN. Above 25% H₂O, the quantum yields remained relatively constant.

Results of CF₃ Substituent Effect on Photorelease of GABA in Biological Studies

The trifluoromethoxy and trifluoromethyl pHP caged GABA's **2** and **3** were tested for their efficacy in whole-cell patch clamp studies in neurons in cortical slices of mice²⁷ and compared with earlier results for the methoxy (**3**) and dimethoxy (**4**) GABA derivatives.⁹ Local photolysis with short UV light pulses (10–50 ms) delivered through a small-diameter optical fiber produced transient whole-cell inward currents. As shown in Figures 5 and 6, increasing the concentrations of the photolyzed derivative generated currents of larger amplitude and longer duration.

To elucidate whether photolysis of these new derivatives was evoking activation of GABA_A receptor, the effect of the specific GABA_A receptor antagonist SR 95531 (Gabazine; Tocris, Ellisville, MI) on the photolysis responses and the reversal potential of **2** and **3** were determined. SR 95531 completely abolished photolysis-induced membrane currents (10 μM, Fig. 5 B and 6 B), indicating that the response is mediated by activation of GABA_A receptor. Current-voltage relationships of responses revealed a reversal potential of -14.2 ± 2.9 mV ($n=3$, Fig. 5 C) for **2**. This value is close to the theoretical value of -20 mV as calculated by the Nernst equation for the chloride concentration between internal (60 mM) and external (133 mM) solution. Taken together, these results demonstrate that CF₃O-pHP GABA and CF₃-pHP GABA photolysis-evoked membrane currents are mediated by specifically activating the GABA_A chloride receptor channel.

To determine whether non-photolyzed CF₃O-pHP GABA could act as an agonist for GABA_A receptors, the effect of CF₃O-pHP GABA on membrane input resistance was measured by monitoring current responses to short 5 mV depolarizations from a holding potential of -70 mV in the presence and the absence of GABA (100 μM) or CF₃O-pHP GABA (100 μM). As expected, GABA (100 μM) reduced the membrane input resistance due to its activation of GABA_A chloride channels. In contrast, CF₃O-pHP GABA had no effect on the membrane input resistance (Fig. 5 D), indicating that only photolyzed CF₃O-pHP GABA, but not CF₃O-pHP GABA itself activates GABA_A chloride receptor channels. A parallel study on CF₃-pHP GABA (**3**) gave the same result (Fig. 6 C).

In order to compare the relative sensitivity of photolyzed **2** and **3** with the other caged GABA's, peak amplitudes of membrane currents elicited by photolysis of **2** and **3** at increasing concentrations were plotted as concentration-response curves, which were fitted by Hill's equation. The best fit curves showed EC₅₀ values of 49.2 μM, 119.8, and 93.4 μM for **2**, **3**, and **4**, respectively (Figure 7 and Table 4), demonstrating that photorelease from CF₃O-pHP GABA is more effective in eliciting GABA responses than either CF₃-pHP GABA or CH₃O-pHP GABA.

Conclusions

We have shown that the competing 'energy wasting' triplet pathway is the deprotonation of the phenol group in the photolysis of pHP GABA derivatives. In aqueous phase photolyses at low to moderate pH, it is the triplet state that undergoes the photo-Favorskii rearrangement concomitantly releasing the substrate. The efficiency of the triplet excited state rearrangement pathway depends on several factors, the most important of which is the nucleofugality of the caged substrate and the pK_a of the chromophore. The carboxylate leaving group, e.g. GABA release, is less effective than phosphates,^{5,14-16,21-23} for example, as expressed by the higher quantum yields for phosphate release. In fact, carboxylates are among the least efficient substrates ($\phi_d = 0.04 - 0.20$) we have reported. Additional factors also influence the partitioning of the pathways, including the water content of the solvent, pH, pK_a of the pHP, and media effects.

Evidence that the conjugate bases are much less efficient chromophores for photorelease was reinforced with both trifluoromethyl derivatives through studies at pH 9.0, well above the pK_a 's of the *p*-hydroxyphenacyl derivatives, and at 350 nm where only the conjugate base absorbs. Thus, substituents on the chromophore can attenuate the effective pH range and wavelength region available for photorelease by their influence on the pK_a of the *p*-hydroxy group. In the present case, electron withdrawing substituents on the chromophore sufficiently lower the pK_a to a point where the conjugate base is the only tautomer present at pH 9. Consequently, the quantum yields are depressed to <0.02 due to the poor reactivity of this tautomer. In a similar manner, photoinduced deprotonation to the conjugate base is an unproductive pathway.

The fluoro substitution did improve the lipophilicity of the chromophore, a feature that may have future implications regarding transport of caged compounds across membranes in biological studies. A test on the photochemical efficacy in more lipophilic environments such as octanol, acetonitrile, and DMSO showed that photorelease occurs, but the quantum yields improved with added H_2O as a co-solvent as was the case for acetonitrile- H_2O mixtures.^{14c} As a further test, the efficacy of fluorinated pHP GABA derivatives were tested for controlled release of GABA as an antagonist at the $GABA_A$ receptor in whole-cell, patch clamp studies with neurons in cortical slices. Photorelease of GABA from CF_3O -pHP **2** was $>50\%$ more effective as an agonist than its non-fluoro analog. This was not true for the CF_3 derivative, which was less effective by ca.30% relative to the unsubstituted pHP GABA in spite of its greater quantum yield.

Further studies on the complex effects that substituents have on the photochemical and photophysical properties of the pHP chromophore, including ionic strength and leaving group effects on the partitioning of the two dominant triplet pathways are needed to more fully understand the efficacy of the pHP phototrigger reaction and are currently being pursued by our groups.

Experimental Section

Quantitative photolysis conditions for determination of quantum yields and Stern–Volmer quenching constants (K_{SV}) were as follows: The lamp light output (in mEinstein/min) was established using the potassium ferrioxalate method.²⁸ Milligram quantities of caged compounds and caffeine or acetamidophenol were weighed out on a Fisher brand Microbalance and dissolved in 4 mL of 18 MΩ ultrapure water, salt solutions of various concentrations, buffers with or without adjusted ionic strengths, or purified organic solvents were then added to a 10 mm × 75 mm quartz tube and vortexed, resulting in a homogenous solution of the caged compound and internal standard. The same tubes and mixing procedures were employed for actinometry (*vide infra*). Concentrations of the caged compounds ranged from 1–9 mM. At these concentrations, the absorbance was greater than 4 through the excitation range of the 300 nm lamps, assuring the complete absorption by the sample at low conversion over the irradiation wavelength range employed. These tubes were then placed in a Rayonet MGR 100 carousel within a Rayonet Photochemical Reactor equipped with two 3000 Å, 15 W lamps. 100 μL samples were removed at 30 s intervals up to 5 min using a 250 μL micro syringe after vortexing the reaction tube and the sample diluted to 1 mL with water using 1 mL volumetric flasks. The samples were thoroughly mixed before LC/MSMS analysis. Each run gave linear time dependent-conversions up to $<20\%$ conversion of the pHP ester.

The same procedures were used for the actinometry using the same volumes in the sample tubes. Complete light absorption by the ferrioxalate solutions was accomplished following

to the procedures of Hatchard and Parker.²⁹ A linear response for light output was also obtained in each series.

Quantitative analysis was achieved by LC/UV or HPLC/MS/MS. The LC/MS/MS instrument was equipped with a triple quadrupole, electro spray ionization mass spectrometer, outfitted with an autosampler. UV-Vis detection consisted of a dual wavelength detector set at 220 and 240 nm. The reservoirs used were as follows: A) 99% water, 1% methanol, 10 mM ammonium formate and 0.06% formic acid. B) 99% methanol, 1% water, 10 mM ammonium formate, and 0.06% formic acid. The column was a reverse-phase (C18), 4 μ m mesh, and 50 mm. Injections of 100 μ L were made with an automated sampler for each run for a total of 3 injections per vial. A mobile phase gradient was utilized to optimize compound separation. The flow rate was set at 300 μ L/min. Data analysis was performed using MassLynx software. Smoothing functions were used for peak analysis of the chromatographic peaks. Calibration curves to obtain R values from linear least-squares regression were determined at concentrations of the reactants and products in photolyses by systematic increases of pHP-caged GABA, free GABA, and *p*-hydroxyphenylacetic acid concentrations to determine correlations with internal standards caffeine or 4-acetamidophenol. The quantum efficiencies were then calculated from the ratio of the reactant or product concentrations to the photons absorbed using the actinometer values obtained as indicated above. A minimum of three independent, quantitative iterations were conducted for each compound to underpin the veracity of reported quantum efficiencies.

The Stern–Volmer quenching method²⁹ was employed to determine the triplet lifetimes of pHP derivatives. Potassium sorbate served as the quenching agent. Solutions of pHP-GABA, 0.001–0.01 M, were diluted with sorbate solutions of increasing concentration (0–0.1 M), and photolyzed under the aforementioned conditions to ascertain the change in quantum efficiencies of GABA release. Stern–Volmer constants, K_{SV} , were determined from the slope of ϕ_0/ϕ vs. $[Q]$. To determine the triplet lifetime, τ^3 , the rate of quenching, k_q , was assumed to be the rate of bimolecular diffusion, $k_{diff} = 7.2 \times 10^9 \text{ s}^{-1}$ (water). The K_{SV} values in H₂O were for **2**, 20 M⁻¹ and for **3**, 33 M⁻¹.

Femtosecond transient absorption spectra were measured with the pump-supercontinuum probe (PSCP) technique.³⁰ The sample was excited with a frequency-quadrupled pulse from a Ti/Sa laser system (775 nm, pulse energy 0.8 mJ, full width at half maximum (*fwhm*) 150 fs, operating frequency 426 Hz) described previously.³¹ The output at 540 nm was frequency doubled to 266 nm and after compression provided pump pulses with an energy of 1 μ J and <100 fs pulse width. A probe beam continuum was produced by focusing the 775 nm 1 mm in front of a CaF₂ of 2 mm path length. The second harmonic (400 nm) of the fundamental generated a supercontinuum probe in the range 270–690 nm. The pump and probe were focused in a 0.2 mm spot on the sample flowing in an optical cell of 0.4 mm thickness. The probe signal was spectrally dispersed and registered with a photodiode array (512 pixels). The pump-probe cross-correlation was well below 100 fs over the whole spectrum. The experimental transient spectra $\Delta A(\lambda, t)$ were corrected for the chirp of the supercontinuum and for the solvent contribution.

Biological Activity. Experimental Determinations: Experimental procedures were in accordance with US National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh. Mice (aged postnatal days 10–11) were anesthetized with isoflurane and decapitated. The brain was quickly removed from the mouse skull and immersed into ice-cold artificial cerebrospinal fluid (ACSF) which contained 1 mM Kynurenic acid and was composed of (in mM): 124 NaCl, 26 NaHCO₃, 10 Glucose, 5 KCl, 1.25 KH₂PO₄, 1.3 MgSO₄ and 2 CaCl₂ (pH 7.4 when aerated with 95% O₂/5% CO₂). The brain was blocked and coronal slices (300 μ m

thick) were obtained from the cortex using a vibrating microtome (Leica, VT 1200). Slices were incubated in an interface-type chamber in 95% O₂/5% CO₂ atmosphere for 1 hour at room temperature (20-25 °C) before commencing electrophysiological recordings.

For electrophysiological recordings, slices were transferred to a chamber mounted to a fixed-stage microscope (Olympus BX50) where they were superfused with ACSF solutions at a rate of 2-3 ml/min using a gravity-driven perfusion system. Whole-cell patch clamp recordings were made in voltage-clamp mode using an Axoclamp-1D amplifier (Molecular Devices, CA) with a Digidata-1440A A/D converter under the control of pCLAMP10 (Molecular Devices). The recording pipettes (resistance of 2-3 MΩ) were constructed from borosilicate glass capillaries (A-M systems, WA) using a P-97 puller (Sutter Instrument, CA). Recording pipettes were filled with pipette solution containing (in mM): 54 D-gluconic acid, 54 CsOH, 56 CsCl, 1 MgCl₂, 1 CaCl₂, 10 Hepes, 11 EGTA, 0.3 Na-GTP, 2 Mg-ATP and 5 QX-314 (pH 7.2, 280 mOsm/L). Recordings were taken at a holding potential of -70 mV, unless otherwise specified. Caged compounds and pharmaceutical drugs were dissolved in ACSF immediately before application.

The area around the recorded neuron was illuminated with UV light using an optical fiber-based system consisting of a fuse-silica fiber (inner diameter 20 μm, Polymicro Technologies Inc.) coupled to a 100W mercury arc lamp.³² The duration of the light pulses was regulated by an electronic shutter (Vincent Associates, NY) located between and the optical fiber. The location of the light spot on the slice was monitored with a CCD camera. Data are presented as mean ± SEM. Results are shown in Figures 4, 5, and 6.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

The species spectra of the singlet (absorption and stimulated emission; red, dotted) and of the triplet (absorption; green, solid) of pHP GABA (**1**) in water determined by global analysis of the spectra taken with delays of 0.6–15 ps using a monoexponential rate law for fitting.

Figure 2.

Pump-probe spectra of **2** in water, reconstructed after factor analysis in the time range from 20 ps to 1.96 ns using the species spectra shown in Fig. 3.

Figure 3.

The species spectra attributed to the triplet (solid, green) and the conjugate base (anion) of pHP (see text) formed from the triplet (dotted, blue) of CF₃O-pHP GABA (**2**) in water determined by global analysis of the spectra using a biexponential fit. These spectra are equal to those taken at delays of 4 ps and 1.96 ns, respectively.

Figure 4. Effect of H_2O on the rate of disappearance of the 390 - 400 nm band. (blue squares are from Hellrung, Wirz, green circles are from Phillips et al., ref. 14 and the red triangles are this work.)

Figure 5.

Dose response of CF₃O-pHP GABA (2) photolysis. (A) Sample traces of whole-cell currents elicited by photolysis of CF₃O-pHP GABA with 50 ms UV light pulses (horizontal bar; UV). (B) The specific GABA_A receptor antagonist Gabazine (10 μM) completely blocked currents evoked by photolysis of CF₃O-pHP GABA (100 μM, flash duration 10 ms). Washout of Gabazine partly restored the initial response. (C) Current-voltage curve: Peak amplitudes of responses are plotted versus holding membrane potential (−70 mV to +20 mV, n=3 neurons). Inset shows sample traces at the various holding potential (100 μM CF₃O-pHP GABA, flash duration 50 ms). (D) CF₃O-pHP GABA has no effect on holding currents or membrane input resistance. Perfusion with ACSF containing 100 μM CF₃O-pHP GABA for 2 min (horizontal bar) did not change membrane input resistance (1), whereas bath application of 100 μM GABA for 2 min decreased the membrane input resistance due to activation of GABA_A-activated chloride channels

Figure 6.

Dose-response of CF₃-pHP GABA (3) photolysis. (A) Examples of membrane current responses of a neuron upon photolysis of CF₃-pHP GABA at concentrations of 50, 100, and 500 μM. (B) Responses elicited by photolysis of CF₃-pHP GABA (200 μM) were blocked by the specific GABA_A receptor antagonist Gabazine (10 μM). Upon 15 min washout of Gabazine, the response partially recovered. These results indicate that photochemically released GABA from CF₃-pHP GABA activated GABA_A receptors. (C) CF₃-pHP GABA (200 μM, application during black bar) itself has no effect on holding currents or membrane input resistance indicating that CF₃-pHP GABA itself did not activate GABA_A receptors. In contrast, in the same neuron, 200 μM GABA elicited a strong inward current and decreased input resistance as indicated by an increase in the amplitude of injected current necessary to produce a 5 mV membrane potential depolarization (example traces to the right).

Figure 7.

Comparison of GABA_A receptor activation by rapid photolysis of pHP GABA. Dose-response curves for CF₃O-pHP GABA **2** (blue, n=7 neurons), CF₃-pHP GABA **3** (red, n=6 neurons), and CH₃O-pHP GABA **4** (black, n=6 neurons) with **4** population data of peak currents normalized to the maximum peak response. Data were fitted by Hill's equation to yield the EC₅₀. EC₅₀ and Hill's coefficient values were: CF₃O-pHP GABA, 49.2 μM, 1.8, n=7 neurons; CH₃O-pHP GABA, 93.4 μM, 1.9, n=6 and CF₃-pHP GABA 119.8 μM, 2.73 n=6.

Scheme 1.
Synthetic Strategy for Trifluoromethyl Substituted pHP Amino Acids.

Scheme 2.
Competing Photo-Favorskii and Deprotonation of pHP Esters.

Table 1

UV spectral data, pK_a and disappearance quantum yields^a for pHP GABA derivatives 1–5 and CF₃-pHP Glu (8).

pHP	λ_{max} (log ϵ)	pK_a^b	$\phi_{dis}^{H_2O^c}$	$\phi_{dis}^{pH 7.3^d}$	$\phi_{dis}^{pH 8.2^e}$	λ_{max} (log ϵ) pH 9.0	$\phi_{dis}^{pH 9.0^f}$
1 ^g	282 (4.16), 325 sh	8.0	0.20	0.20	0.09	326 (4.06)	0.0041
2	274 (4.20), 331 (3.54)	6.7	0.09 ^h	0.06	0.02	332 (4.18)	0.0058
3	328 (4.11) ⁱ	5.5	0.17	0.12	0.08	328 (4.14)	0.0081
4	279 (3.97), 307 (3.90), 341 sh	7.85	0.06	NA	0.02	348 (4.05)	NA
5	303 (3.90), 355 (3.55)	7.78	0.03 ^g	NA	NA	366 (4.30)	NA
8	328 (4.11) ^h	5.5	0.18	NA	0.11	328 (4.11)	0.0067

^aQuantum yields for the appearance of GABA were within $\pm 10\%$ of the disappearance quantum yields for pHP GABA, ϕ_{dis} .

^b pK_a 's were determined titrimetrically (see Supporting Information).

^cDeionized water; pH = 7.0.

^d0.01 M HEPES, adjusted to pH = 7.3.

^e0.01 M HEPES adjusted to pH = 8.2.

^f0.01 M TRIS adjusted to pH 9.0, 0.1 M LiClO₄, irradiation λ = 350 nm.

^gSee ref. 1a, Ch. 1.3 and 2.

^hAn identical value was obtained with oxygen-purged solutions (Ar, 30 min).

ⁱThe pK_a (5.5) is below the pH for this pHP GABA derivative.

Table 2

Singlet ($^1\tau$) and Triplet ($^3\tau$) Lifetimes and Rate Constants Obtained from Pump–probe and Stern–Volmer Measurements.

pHP GABA	$^1\tau/\text{ps}$	$^3\tau/\text{ns}$	ϕ_{dis}^a	$k_{\text{pF}}/10^8 \text{ s}^{-1}^b$
H ₂ O				
1	2.3	0.34	0.20	6.00
2		0.46	0.09	1.98
3		0.39	0.17	4.42
4		0.77	0.06	0.78
10% CH ₃ CN/H ₂ O ^c				
2		0.71		
3	3.3	0.39		
4	4.6			
5	3.7	0.36		

^aSee Table 1.

^bCalculated as $k_{\text{I}} = \phi_{\text{I}}/{}^3\tau$, where ϕ_{dis} is the quantum yield for the pHP GABA disappearance and k_{pF} is the rate constant for the photo-Favorskii process (see Scheme 2).

^cData obtained by pump–probe spectroscopy. Ca.10% CH₃CN was added to ensure complete solubility of the substituted pHP GABA.

Table 3Effect of H₂O on the Rate of Disappearance of the 390 – 400 nm Band.

$^3k_{\text{dis}}/\text{S}^{-1}$	mol % H ₂ O ^a	References ^b
1.6×10^{10}	100.00	this work
9.95×10^9	95.14	this work
3.45×10^9	89.77	Phillips ¹⁴
3.30×10^9	74.53	this work
1.89×10^9	66.11	Phillips ¹⁴
1.25×10^9	55.64	Phillips ¹⁴
7.81×10^8	49.38	Phillips ¹⁴
4.17×10^8	34.05	Phillips ¹⁴
1.11×10^8	24.54	Phillips ¹⁴
3.84×10^7	15.03	Hellrung
1.15×10^7	10.00	Hellrung
6.56×10^6	7.51	Hellrung
2.72×10^6	5.04	Hellrung
2.62×10^5	0.00	Hellrung

^aCH₃CN was the cosolvent.^bData are combined from this work and from previous studies (B. Hellrung, J. Wirz unpublished, and Phillips and coworkers (ref. 14) as indicated).

Table 4*ClogP* and EC₅₀ values for Fluoro-pHP GABA **2** and **3**.

pHP	ϕ_{dis} (1-octanol)	<i>ClogP</i>	EC ₅₀ (μM) ^a
1	0.12	1.19	
2	0.14	1.93, -1.59 ^b	49.2
3	0.10	2.02, -1.90 ^a	119.8
4	0.042 ^c	-3.38	93.4

^aEffective concentration for 50% activity. See Figure 7.^bThis value is for the conjugate base of **2** which predominates at pH 7.3.^cSolvent was 1-pentanol.