New Phototriggers 9: *p*-Hydroxyphenacyl as a C-Terminal Photoremovable Protecting Group for Oligopeptides

Richard S. Givens,*,† Jörg F. W. Weber,† Peter G. Conrad, II,† György Orosz,‡ Sarah L. Donahue,§ and Stanley A. Thayer§

Contribution from the Department of Chemistry, University of Kansas, Lawrence, Kansas 66045, Eötvös Loránd University, Budapest 112, H-1518, Hungary, and Department of Pharmacology, University of Minnesota Medical School, Minneapolis, Minnesota 55455

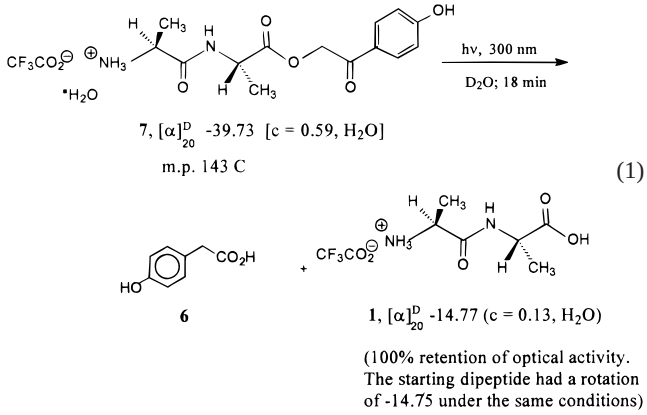
Received March 29, 1999. Revised Manuscript Received January 21, 2000

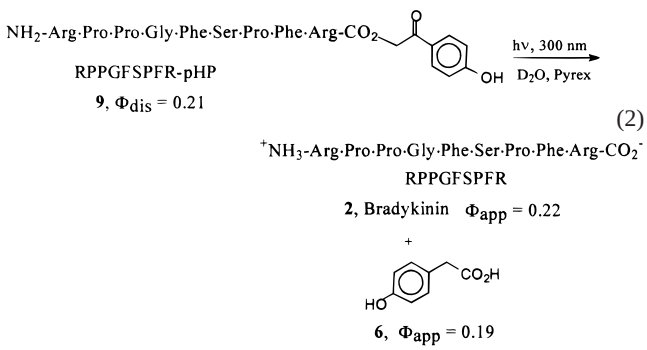
Abstract: In our search for a more versatile protecting group that would exhibit fast release rates for peptides, we have designed and developed the *p*-hydroxyphenacyl (pHP) group as a new photoremovable protecting group. We report the application of this protecting group for the dipeptide Ala-Ala (**1**) and for the nonapeptide bradykinin (**2**), two representative peptides that demonstrate C-terminus "caging" and photorelease. The synthesis of these *p*-hydroxyphenacyl esters was accomplished in good yields by DBU-catalyzed displacement of bromide from *p*-hydroxyphenacyl bromide. As in the case of caged γ-amino acids **11** (pHP glu) and **12** (pHP GABA) and caged nucleotide **17** (pHP ATP) reported earlier,[1,2] irradiations of the *p*-hydroxyphenacyl esters of **1** and **2** actuate the release of the peptides with rate constants that are consistently greater than 10^8 s^{-1} and appearance efficiencies (Φ_{app}) that range from 0.1 to 0.3. Release of the substrate is accompanied by a deep-seated rearrangement of the protecting group into the near-UV silent *p*-hydroxyphenylacetic acid (**6**). Quenching studies of pHP Ala-Ala (**7**) with either sodium 2-naphthalenesulfonate or potassium sorbate gave good Stern−Volmer kinetics yielding a rate constant for release of 1.82×10^8 s^{-1}. Quenching of the phosphorescence emission from pHP Ala-Ala (**7**, E_T = 70.1 kcal/mol) and pHP GABA (**12**, E_T = 68.9 kcal/mol) were also observed. The biological efficacy of bradykinin released from pHP bradykinin (**9**) was examined on single rat sensory neurons grown in tissue culture. A single 337 nm flash (<1 ns) released sufficient bradykinin from the *p*-hydroxyphenacyl protected nonapeptide to activate cell-surface bradykinin receptors as indicated by a rapid increase in the intracellular calcium concentration. A selective antagonist of type 2 bradykinin receptors blocked the biological response. From these results, it is apparent that flash photolysis of *p*-hydroxyphenacyl protected peptides provides a powerful tool for the rapid and localized activation of biological receptors.

Introduction

We report here the efficacy of a photorelease strategy for two peptides, the dipeptide Ala-Ala (**1**) and the nonapeptide bradykinin (**2**), employing *p*-hydroxyphenacyl (pHP) as a photoremovable protecting group for C-terminus carboxylate groups (eqs 1 and 2). Studies with the triplet quenchers sodium 2-naphthalenesulfonate or potassium sorbate gave good linear Stern−Volmer quenching from which a triplet lifetime of 5.5 ns was derived. Phosphorescence studies indicated a triplet energy for the *p*-hydroxyphenacyl chromophore of 69−71 kcal/mol. To demonstrate the physiological efficacy of the pHP phototrigger, we photolyzed *p*-hydroxyphenacyl bradykinin (**9**) in dorsal root ganglion (DRG) preparations producing an in vitro increase of intracellular calcium, that is,$[Ca^{2+}]_i$.

Earlier, we had reported[1,2] that *p*-hydroxyphenacyl serves as an efficient phototrigger for the release of the nucleotide ATP (**5**) from **17** and of two γ-amino acids, the γ-carboxyl group of glutamic acid (**3**) and γ-aminobutyric acid (GABA, **4**) from **11** and **12**, respectively (eqs 3 and 4). However, the initial attempts



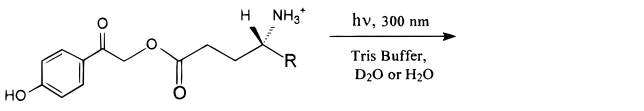


* Corresponding author. E-mail: rgivens@ukans.edu.
† University of Kansas.
‡ Eötvös Loránd University.
§ University of Minnesota Medical School.

(1) (a) Givens, R. S.; Park, C.-H. *Tetrahedron Lett.* **1996**, *37*, 6259−6262. (b) Park, C.-H.; Givens, R. S. *J. Am. Chem. Soc.* **1997**, *119*, 2453−2463.

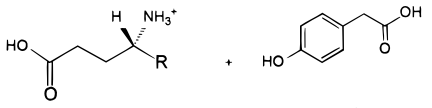
(2) Givens, R. S.; Jung, A.; Park, C.-H.; Weber, J.; Bartlett, W. *J. Am. Chem. Soc.* **1997**, *119*, 8369−8370.

10.1021/ja991014b CCC: $19.00
Published on Web 03/10/2000



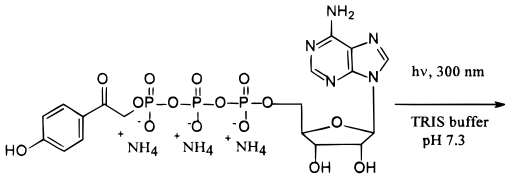
11, R = CO_2^- (*p*-hydroxyphenacyl Glu)
12, R = H (*p*-hydroxyphenacyl GABA)

(3)



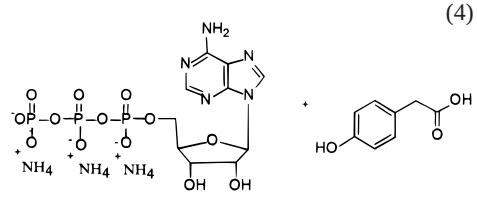
3, R = CO_2^- (Glu)
4, R = H (GABA)

6



17, pHP-ATP $\Phi_{dis} = 0.37$ $k_r = 6.9 \times 10^8\ s^{-1}$

(4)



5, ATP $\Phi_{ATP} = 0.30$ **6**, $\Phi_{pa} = 0.31$

to extend this strategy to α-amino acids failed because of the hydrolytic instability in aqueous or buffered media[2] of the corresponding *p*-hydroxyphenacyl esters. Our studies on the more stable γ-amino acids, phosphates, and nucleotide derivatives did establish, however, that the photochemical deprotection in aqueous media was accompanied by a concomitant rearrangement of the protecting group to *p*-hydroxyphenylacetic acid (**6**). The rearrangement was proposed to occur via the *p*-hydroxyphenacyl triplet excited state, suggesting a very rapid release rate estimated to be at least $10^7-10^8\ s^{-1}$ as derived from Stern−Volmer quenching results with sodium 2-naphthalenesulfonate. A recent report[3] challenged our mechanism for the rearrangement, posing instead that the rearrangement occurs directly from the excited singlet state by an initial transfer of the phenolic proton to the ketone carbonyl followed by expulsion of the ester with concomitant reorganization of the *p*-quinone methide (either as its ground state or excited singlet state?) to the spirodienedione. Our results are not in accord with this mechanism.

To gain a better understanding of the mechanism and to explore the application of the *p*-hydroxyphenacyl photoprotection of peptides and oligopeptides, we selected the dipeptide Ala-Ala (**1**) as a model, chosen for the following reasons: First, we sought to overcome the hydrolytic instability of *p*-hydroxyphenacyl esters of α-amino acids, which we reasoned was due to the enhanced electrophilicity of the ester carbonyl caused by the adjacent protonated α-amino group. The peptide functionality would not be extensively protonated at physiological pH and thus would lack this driving force for hydrolysis. Second, the stability of the chiral center of the amino acid to the protection−deprotection sequence could be evaluated with Ala-Ala. Finally, the ease of synthesis, product identification, and the ready analysis of the progress of "uncaging" by a variety of techniques such as NMR and RP-HPLC would be relatively straightforward with this model.

Similarly, bradykinin (**2**) was selected because it represents a more challenging target in our program to demonstrate the efficacy and versatility of *p*-hydroxyphenacyl for a protection−deprotection sequence. This nonapeptide is representative of many less accessible, yet biologically significant oligopeptides. Bradykinin, a naturally occurring, potent modulator of pain, inflammation, and vasodilator responses,[4,5] is a neuroactive peptide produced after local tissue injury by proteolytic processing of plasma kininogen.[6] Bradykinin is one of the most active pain-producing chemical mediators liberated during tissue damage. It activates cell surface B2 receptors located on sensory nerve endings, producing a direct excitation of nociceptors as well as a secondary release of other chemical mediators that sensitize nociceptors and produce vasodilation.[7,8] Thus, the photorelease of bradykinin from the protected oligopeptide **9** will enable precise temporal and spatial activation of bradykinin receptors, which in turn would facilitate studies on the transduction of pain.

Results

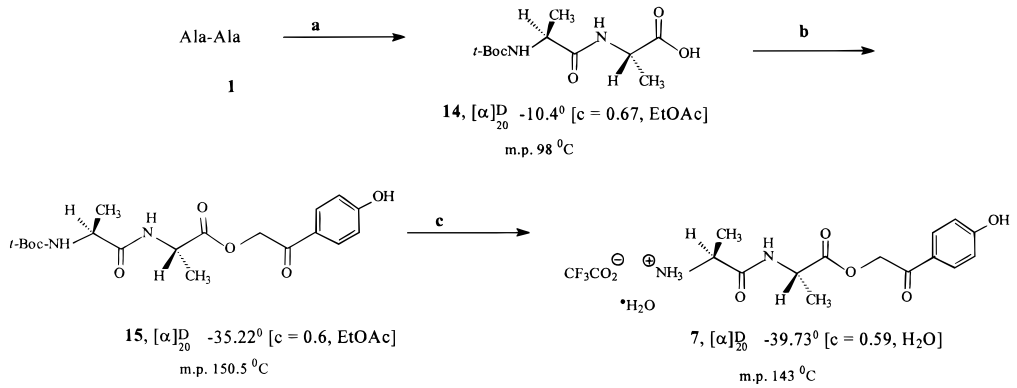
p-Hydroxyphenacyl Ala-Ala (**7**) was synthesized as shown in Scheme 1. Protection of the N terminus by standard Boc chemistry[9] was followed by treatment of the N-protected dipeptide **14** with 2-bromo-4′-hydroxyacetophenone (**8**). Removal of the Boc group with TFA afforded the C-terminal-protected Ala-Ala **7** in an overall unoptimized yield of 46% for the three steps.

Synthesis of the "caged" bradykinin **9** began with the solid-phase synthesis of protected bradykinin **10** employing 2-chlorotrityl resin[10] using an Fmoc strategy. The peptide was released from the resin by hydrolysis at the C-terminus attachment using dichloromethane−methanol−acetic acid (8:1:1), freeing the carboxylic acid **16** while preserving the protecting groups on the remaining functional groups.[11] The C-terminal carboxylate was subsequently converted to the fully protected *p*-hydroxyphenacyl ester **10** by reaction with 2-bromo-4′-hydroxyacetophenone (**8**) and DBU in dry DMF, conditions similar to those employed for Ala-Ala (**1**, see Scheme 1). The fully protected *p*-hydroxyphenacyl bradykinin (**10**) was then treated with a deprotection cocktail of 88% TFA, 7% thioanisole, and 5% H_2O to remove the *N*-Boc-, *tert*-butyl-, and *N*-Pbf-protecting groups, leaving intact the *p*-hydroxyphenacyl ester. The overall yield of **9** from the esterification−deprotection steps followed by purification by RP-HPLC was 60%.

Photolyses of the caged peptides were conducted in either aqueous (H_2O or D_2O) or buffered media. For the model *p*-hydroxyphenacyl Ala-Ala (**7**), the photolysis reaction was followed by RP-HPLC and by ^1H NMR. The ^1H NMR spectrum initially displayed only the starting ester that, after periodic irradiations of the sample, eventually dissolved into a spectrum

(3) Zhang, K.; Corrie, J. E. T.; Munasinghe, V. R. N.; Wan, P. *J. Am. Chem. Soc.* **1999**, *121*, 5625−5632.
(4) Dray, A. *Can. J. Physiol. Pharmacol.* **1997**, *75*, 704−712.
(5) Hornig, B.; Drexler, H. *Drugs* **1997**, *54*, 42−47.
(6) Raidoo, D. M.; Bhoola, K. D. *Pharmacol. Ther.* **1998**, *79* 105−127.
(7) Cesare, P.; McNaughton, P. *Curr. Opin. Neurobiol.* **1997**, *7*, 493−499.
(8) Hall, J. M. *Gen. Pharmacol.* **1997**, *28*, 1−6.
(9) Song, Y.; Niederer, D.; Lane-Bell, P. M.; Lam, L. K. P.; Crawley, S.; Palcic, M. M.; Pickard, M. A.; Pruess, D. L.; Vederas, J. C. *J. Org. Chem.* **1994**, *59*, 5784−5793.
(10) Barlos, K.; Gatos, D.; Stauros, K.; Papaphotiv, G.; Schäfer, W.; Wenquing, Y. *Tetrahedron Lett.* **1989**, *30*, 3947−3950.
(11) Bódi, J.; Süli-Vargha, H.; Ludányi, K.; Vékey, K.; Orosz, G. *Tetrahedron Lett.* **1997**, *38*, 3293−3296.

Scheme 1[a]



[a] (a) $(t\text{-Boc})_2O$, THF 0 °C, 97%; (b) *p*-hydroxyphenacyl bromide (**8**), DBU, 15 °C, 1,4-dioxane, 62%; (c) TFA, 4 h, 0 °C, 78%.

Table 1. Quantum Efficiencies for the Photolysis of *O*-(4-hydroxyphenacyl)-Ala-Ala, Trifuoroacetate Salt (**7**) in D_2O, Determined by NMR and HPLC

entry	method[a]	ϕ_{dis} (**7**)	ϕ_{app} [Ala-Ala (**1**)]	ϕ_{rear} [4-hydroxyphenylacetic acid (**6**)]
1	NMR	0.25	0.24	0.21
2	NMR	0.33	0.31	0.29
3	NMR[b]	0.23	0.21	0.20
4	HPLC[c]	0.26		0.27
	average[d]	0.267 (0.043)	0.253 (0.051)	0.24 (0.044)

[a] The ^{1}H NMR analyses were by integration of the absorptions at 7.2 (**6**), 5.4 (**7**), and 4.2 ppm (**1**). The NMR analyses were performed at 2, 4, 6, 8, 10, and 12 min. A single run of 18 min was included in this study. The slope of the concentrated vs time was used to determine the quantum efficiency.[2] Each entry in the table represents at least four and as many as eight determinations. For the HPLC analysis, aliquots were removed at 2, 4, 6 and 8 min to determine the slope. Each aliquot was run in triplicate (see Experimental for additional details). [b] DMF (2.9 ppm) and acetonitrile (1.9 ppm) used as internal standards. [c] Anthranilic acid was used as an internal standard. [d] Standard deviations are given in parentheses.

of the two photoproducts, Ala-Ala (**1**) and *p*-hydroxyphenylacetic acid (**6**).[12] RP-HPLC also revealed another product, 2,4′-dihydroxyacetophenone (**13**) that was formed in low yield (<10%) even at 100% conversion. The released dipeptide was shown to be identical to and gave the same optical rotation as the Ala-Ala employed in the synthesis ($[\alpha]_D = -14.77°$ vs $[\alpha]_D = -14.75°$ (obtained from Sigma[13])). Quantum efficiencies were measured by both ^{1}H NMR (for Φ_6, Φ_1, and Φ_7) and by RP-HPLC (for Φ_6 and Φ_7, only) and are given in Table 1.

Quenching studies with **7**, employing either sodium 2-naphthalenesulfonate or potassium sorbate, gave essentially identical Stern−Volmer quenching results (Table 2). A triplet lifetime[14] of 5.5 ns was determined based on the diffusion-controlled quenching rate of the disappearance of the pHP ester (**7**) and the appearance of Ala-Ala (**1**), a value consistent with our earlier studies on the γ-amino acid esters of L-glutamate and GABA.[2] Assuming that the dipeptide is released directly from the triplet (i.e., from ^{3}**7**), the rate constant for Ala-Ala formation can be estimated to be $1.82\ (\pm 1) \times 10^8\ s^{-1}$, which is the rate of decay of ^{3}**7**.

Phosphorescence emission spectra were measured for *p*-hydroxyphenacyl Ala-Ala (**7**) and GABA (**12**), and a series of *p*-hydroxyacetophenone derivatives including the parent *p*-hydroxyacetophenone (**17**),[15] α,*p*-dihydroxyacetophenone (**13**), the α,α-dimethyl-α-acetate ester (**18**), the α-phenylacetate ester (**19**), and diethyl *p*-hydroxyphenacyl phosphate (**20**).[16] The triplet energies ranged from 68.9 to 71 kcal/mol for the series whether measured in diethyl ether:isopentane:ethanol (EPA; 5:5:2) or ethylene glycol:water (EG:W; 2:1) glasses (Table 3). Quenching of the phosphorescence emission of **7** and **12** by 0.005−0.07 M 2-naphthalenesulfonate ($E_T = 60.5$ kcal/mol (EG:W; 2:1)) or of **7** by 0.001 M piperylene ($E_T = 59$ kcal/mol ($CHCl_3$)) was observed.[16]

In a separate experiment, the hydrolytic stability of the *p*-hydroxyphenacyl Ala-Ala (**7**) was determined in aqueous buffer (Table 4). The ester showed no measurable hydrolysis (by RP-HPLC) in aqueous media in the absence of buffer or in Ringer's solution I (pH 6.5); however, **7** did slowly hydrolyze over a 24 h period in TRIS buffer (pH 7.3) to give 2,4′-dihydroxyacetophenone (**13**).

The photorelease of bradykinin (**2**) was demonstrated by monitoring the photolysis of a 3 mM solution of *p*-hydroxyphenacyl bradykinin (**9**) in D_2O in a Pyrex vessel at 300 nm by RP-HPLC as shown in Figure 1 and eq 2. Under these conditions, bradykinin release was complete in less than 8 min, yielding bradykinin (**2**) and the rearranged *p*-hydroxyphenylacetic acid (**6**) along with a trace of **13** (<10% of **13** was detected at 98% conversion of **9**). The released oligopeptide was shown to be identical to bradykinin by FAB-MS, exact mass, CD, and comparison of spectral data and RP-HPLC retention volumes when compared with an authentic sample of bradykinin.[17] As shown in Figure 2, the retention of the chirality of the bradykinin obtained from the photolysis reaction was established by a CD comparison with an authentic sample.[17]

Quantum efficiencies were also measured for the photorelease of *p*-hydroxyphenacyl bradykinin (Φ_{dis}, **9**), for the appearance of bradykinin (Φ_{brad}, **2**), *p*-hydroxyphenylacetic acid (Φ_{pa}, **6**) and the hydrolysis product (Φ_{hydr}, **13**) (Table 5).[18]

(12) Givens, R. S.; Weber, J. F. W.; Jung, A. H.; Park, C.-H. Caged Compounds. In *Methods in Enzymology*; Marriott, G., Ed.; Academic Press: New York, 1998; Vol. 291, pp 1−29. See Figure 1.

(13) Obtained from Sigma Chemical Co, St. Louis, MO.

(14) The value of 41 M^{-1} from the Stern−Volmer slope (Table 2) was divided by $7.4 \times 10^9\ M^{-1}\ s^{-1}$, the calculated rate of diffusion in H_2O (Simons, J. P. *Photochemistry and Spectroscopy*; Wiley-Interscience: New York, 1971; pp 212−213) to give the triplet lifetime.

(15) The parent *p*-hydroxyacetophenone has an $E_T = 70.5$ kcal/mol measured by the phosphorescence S → T excitation and 0, 0 emission bands: Kearns, D. R.; Case, W. A. *J. Am. Chem. Soc.* **1966**, *88*, 5087−5097.

(16) The phosphorescence emission spectra of **20** and Na^+ naphthalene-2-sulfonate (**2**) in alcohol water glasses were reported earlier: Givens, R. S.; Athey, P. S.; Matuszewski, B.; Kueper, L. W., III; Xue, J−y.; Fister, T. *J. Am. Chem. Soc.* **1993**, *115*, 6001−6012. The E_T of **2** is reported to be 60 kcal/mol from the S → T absorption spectra (Treinin, A.; Hayon, E. *J. Am. Chem. Soc.* **1976**, *98*, 3884−3891) and that of *(E)*-piperylene is reported to be $E_T = 59$ kcal/mol from its S → T absorption spectrum in $CHCl_3$ (Kellogg, R. E.; Simpson, W. T. *J. Am. Chem. Soc.* **1965**, *87*, 4230−4234).

(17) Obtained from Fluka Chemical Co.

Table 2. Stern−Volmer Quenching Data for the Photolysis of *O*-(4-hydroxyphenacyl)-Ala-Ala (**7**) by Sodium 2-Naphthalenesulfonate and Potassium Sorbate

(A) [Na^+ 2-naphthalenesulfonate] [M]	Φ_{dis} (pHP Ala-Ala, **7**)	Φ_{acid} (**6**)	$\Phi_{Ala-Ala}$ (**1**)
0	0.267 (0.043)[a]	0.24 (0.10)	0.253 (0.051)
0.0197	0.174 (0.044)	0.133 (0.10)	0.151 (0.03)
0.0364	0.13 (0.026)	0.09 (0.003)	0.11 (0.01)
0.0450	0.098 (0.04)	0.076 (0.009)	0.099 (0.005)
slope (K_{SV})[M^{-1}][b]	36 (2.0)	54 (3.7)	32 (1.8)
τ (ns)[c]	4.9 (0.3)	7.3 (0.5)	4.3 (0.2)
	$\tau = 5.5$ ns (av)		
k_r, 10^8 (s^{-1})[d]	2.0 (1.7)	1.4 (0.7)	2.3 (1.0)
	$k_r = 1.82\ (\pm 1.1) \times 10^8\ s^{-1}$ (av)		

(B) [K^+ sorbate] [M]	Φ_{dis} (pHP-Ala-Ala, **7**)	Φ_{acid} (**6**)	$\Phi_{Ala-Ala}$ (**1**)
0.00	0.27 (0.02)[e]	0.24 (0.016)	0.25 (0.01)
0.025	0.12 (0.01)	0.11 (0.007)	0.11 (0.006)
0.050	0.066 (0.016)	0.0663 (0.007)	0.053 (0.005)
0.125	0.038 (0.01)	0.0531(0.005)	0.042 (0.004)
slope (K_{SV})[M^{-1}][b]	49 (4)	36 (8)	38 (11)
τ (ns)[c]	6.6 (0.5)	4.9 (1.1)	5.1 (1.5)
	$\tau = 5.5$ ns (av)		
k_r, 10^8 (s^{-1})[d]	1.5 (0.2)	2.0 (0.9)	2.0 (0.7)
	$k_r = 1.82\ (\pm 0.6) \times 10^8\ s^{-1}$ (ave.)		

[a] Standard deviations are given in parentheses. See Table 1 and experimental for details. [b] The Stern−Volmer slope (K_{SV}) was determined from a plot of the reciprocal of the quantum efficiency vs the 2-naphthalenesulfonate concentration. [c] The lifetime (τ) of the triplet was determined from $\tau = K_{SV}/k_{diff}$, where k_{diff} is 7.4×10^9 M^{-1} s^{-1} (ref 14). [d] The rate constant (k_r) was determined from $k_r = \tau^{-1}$, which assumes that dissociation is the major pathway out of the triplet. [e] Slopes for the concentrations vs time were converted to relative quantum efficiencies using the values from Table 1. Standard deviations are given in parentheses. See Table 1 and experimental for details.

Table 3. Phosphorescence Emission for a Series of *p*-Hydroxyacetophenones in EPA, Water:Ethanol:Methanol and Ethylene Glycol:Water Glasses at 77 K

ketone	solvent[a]	concn (M)	λ_{ex} (nm)	λ_{em} (nm)	$\lambda_{0.0}$ (est)	E_T (kcal/mol)
7	EPA	(0.003)	302	435	408	70.1
12	EG:W	(0.05)	345	445	415	68.9
13	EPA	(0.03)	350	435	407	70.2
17	EPA	(0.02)	324	425	405	70.6 (70.5)[b]
18	EPA	(0.01)	309	429	403	71.0
19	EPA	(0.02)	305	435	415	68.9
20	EPA	(0.02)	320	428	405	70.6
2-NS	WAM	(0.012)	274	510	485	60.5 (60)[c]
7, 2-NS	EG:W	(0.003, 0.005)	302	435[d]	410	- - - - -
7, piperylene	EPA	(0.003, 0.001)	320	440[e]	415	- - - - -
12, 2-NS	EG:W	(0.1−0.05, 0.07)	316	440[f]	415	- - - - - -

[a] EPA = 5:5:2 ether:isopentane:ethanol; EG:W = 2:1 ethylene glycol:water; WAM = 1:2:2 water:ethanol:methanol. [b] See ref 15. [c] See ref 16. [d] The intensity was reduced by 50%. [e] The intensity was reduced by 50%. [f] The intensity was reduced by 75%

Table 4. Half-life of *O*-(*p*-Hydroxyphenacyl)-Ala-Ala (**7**) in H_2O, D_2O, and Buffered Media

medium	pH	τ_2	k_{hydr}
Tris buffer	7.3	214 min	$5.39 \times 10^{-5}\ s^{-1}$
Ringer I[a]	6.5	>24 h	
H_2O	7.0	>24 h	
D_2O	7.0	>24 h	

[a] Ringers Solution I: 600 mg of NaCl, 30 m of KCl, and 20 mg of $CaCl_2$ were dissolved in 100 mL of H_2O, resulting in a pH of 6.5.

To test the efficacy of the release of bradykinin in vitro, we chose to examine bradykinin-evoked excitatory responses in rat dorsal root ganglion neurons grown in primary tissue culture. Excitation of sensory neurons produces an increase in $[Ca^{2+}]_i$.[19] $[Ca^{2+}]_i$ was measured in single neurons using the Ca^{2+}-sensitive dye indo-1 as described in the Experimental Section. Upon binding Ca^{2+}, the fluorescence emission maximum for indo-1 shifts from 405 to 490 nm and thus, $[Ca^{2+}]_i$ is related to the ratio of these two signals in manner that is independent of dye concentration, photobleaching, and optical path length (see Experimental Section for calculations). As shown in Figure 3A, a 60 s application of 100 nM bradykinin evoked a rapid and transient increase in $[Ca^{2+}]_i$ as indicated by the simultaneous decrease in fluorescence detected at 405 nm and increase at 490 nm. Approximately one-third of the DRG neurons are known to express bradykinin receptors, consistent with our finding that 37% ($n = 40$) of the cells responded. The 50% effective concentration (EC_{50}) for bradykinin activation of sensory neurons is 6 nM, and thus, the response to 100 nM bradykinin defines the maximal response for a given cell.[20] The $[Ca^{2+}]_i$ rose from a basal level of 38 ± 11 nM to peak at 282 ± 99 nM ($n = 14$). Cells with robust responses to bradykinin were allowed to recover for 20 min, and then 10 nM *p*-hydroxyphenacyl bradykinin was perfused into the bath. To trigger the photolysis reaction, light from a nitrogen laser was directed through a single optical fiber positioned approximately 50 μm from the soma. A single flash (<1 ns) of UV light (337 nm) evoked a $[Ca^{2+}]_i$ transient in 8 of 9 cells with a mean peak amplitude of 115 ± 45 nM. The absence of detectable bleaching of the indo-1 (absorbance maximum at 350 nm), as indicated by the raw fluorescence intensity values reported in Figure 3A (lower panel), suggests that significant uncaging occurred with flash intensities well tolerated by biological systems. Flashes applied to cells in the absence of pHP bradykinin failed to elicit a detectable change in $[Ca^{2+}]_i$. The selectivity of the flash-induced response was further tested in the experiment shown in Figure 3B. Following an initial control response to bath-applied bradykinin (100 nM), the cell was washed for 20 min, and then, 10 nM pHP bradykinin and the bradykinin receptor antagonist HOE 140[21] were added to the bath. In the presence of HOE 140, flash photolysis of pHP bradykinin failed to increase $[Ca^{2+}]_i$. In this cell the flash produced a slight bleaching

(18) The recent report by Zhang et al.[3] also reports trace of photohydrolysis reactions competing with the rearrangement for a series of simple ester analogues of these *p*-hydroxyphenacyl peptides.

(19) Stucky, C. L.; Thayer, S. A.; Seybold, V. S. *Neuroscience* **1996**, *74*, 1111−1123.

(20) Burgess, G. M.; Mullaney, I.; McNeill, M.; Dunn, P. M.; Rang, H. P. *J. Neurosci.* **1989**, *9*, 3314−3325.

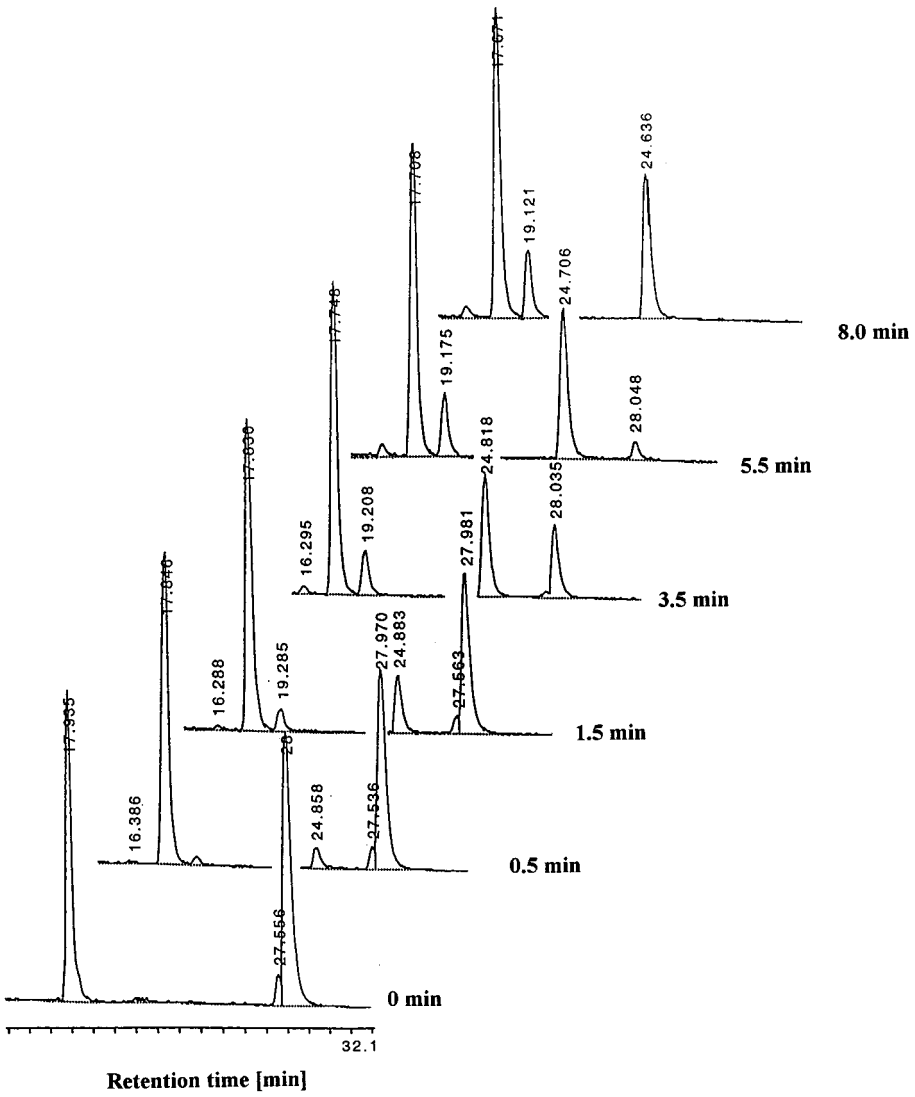


Figure 1. Sequential recordings of the RP-HPLC analysis of the irradiation of *p*-hydroxyphenacyl bradykinin (**9**) in D_2O. Irradiation was conducted in a Pyrex tube with 4 RPR-300 nm lamps, aliquots removed at 0.5, 1.0, 1.5, 3.5, 5.5, and 8.0 min which were analyzed by RP-HPLC on a 1.0 cm Vydac C-18 protein/peptide column using a gradient of 0.1% TFA (5−50% over 45 min) in acetonitrile. Hemimellitic acid (0.85 mg, 2.93 mM) was added as an internal standard. Retention times were 16.3 min for **13**, 17.7 min for hemimellitic acid (i.s.), 19.2 min for **6**, 24.7 min for **2** and 27.9 for **9**. Additional details are given in the Experimental Section.

of the indo-1 as indicated by the decrease in fluorescence intensity detected at both 405 and 490 nm (Figure 3b lower traces).

Discussion

In our earlier reports on the applications of *p*-hydroxyphenacyl as a photoremovable protecting group,[1,2] we noted that simple α-amino acids were not amenable to this approach because the protected esters readily hydrolyzed in aqueous buffer. We further speculated that the pHP ester hydrolysis may have been enhanced by the protonated amine group at the pHs employed (6.5−7.5) in our photochemical studies. To prevent or minimize this factor, we examined two peptides, that is, the dipeptide Ala-Ala and the nonapeptide bradykinin, in which the α-amino group would be less basic and therefore would be far less likely to be protonated.

In fact, the Ala-Ala ester (**7**) proved sufficiently stable to satisfy our requirements for a model application of the *p*-hydroxyphenacyl group as a phototrigger for the C terminus of peptides. As shown in Table 4, the rate of hydrolysis was slow, even in TRIS buffer. This observation and the efficient photorelease reaction allowed us to extend our studies to bradykinin, an oligopeptide that plays a major role in the nociceptive and vascular response to tissue injury.

The syntheses of these and other *p*-hydroxyphenacyl-protected carboxylates are generally accomplished through an S_N2 displacement of bromide from 2-bromo-4′-hydroxyacetophenone catalyzed by DBU. For synthetic oligopeptides, the incorporation of the photoremovable group can be administered on the C terminus of the otherwise fully protected oligopeptide after release of the carboxylate terminus from the solid support, controlled-pore glass beads, or resin employed in the synthesis.[9,10] The resulting *p*-hydroxyphenacyl esters are stable to the standard TFA deprotection cocktails employed to remove the common side chain and amine-protecting groups employed in peptide synthesis as demonstrated in the synthesis of pHP Ala-Ala and bradykinin shown in Schemes1 and 2.

(21) Hock, F. J.; Wirth, G. Henke, S. Breipohl, G.; Konig, W.; Knolle, J.; Schoolkens, B. A. *Br. J. Pharmacol.* **1991**, *102*, 769−773. HOE 140 (D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-D-Tic-Oic-Arg) is a selective agonist for the B2 bradykinin receptors.

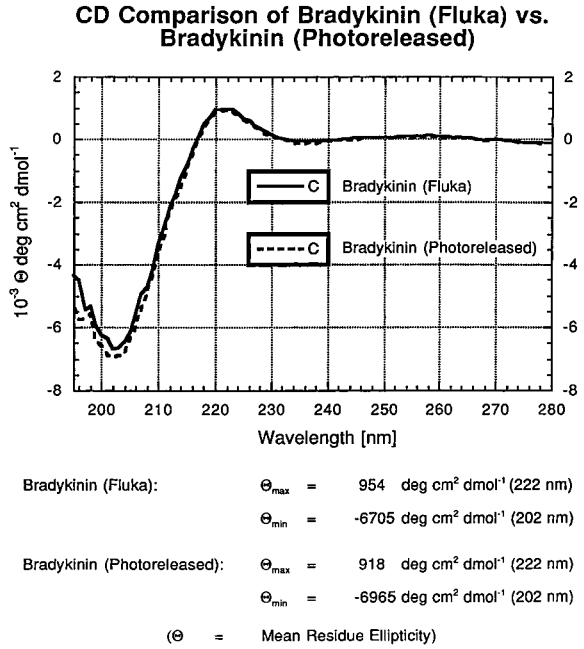


Figure 2. Comparison of the CD spectra of bradykinin (**2**) isolated by HPLC from the photolysis of *p*-hydroxyphenacyl bradykinin (**9**) with authentic bradykinin obtained from Fluka.

Table 5. Quantum Efficiencies[a] for the Photolysis of *p*-Hydroxyphenacyl Bradykinin (**9**) in D_2O

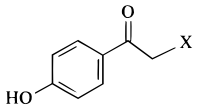
φ_{dis} (pHP-bradykinin, **9**)	φ_{app} (bradykinin, **2**)	φ_{rear} (**6**)	φ_{hydr} (**13**)
0.205 (0.021)	0.219 (0.022)	0.193 (0.013)	0.021 (0.003)

[a] Standard deviations in parentheses. See Experimental Section and footnote *a*, Table 1, for additional details on the HPLC analysis. Hemimellitic acid was used as an internal standard.

The photorelease of the two peptides was examined in aqueous and in buffered media. Both gave release and disappearance efficiencies of slightly greater than 20% along with the appearance of the rearranged *p*-hydroxyphenylacetic acid (**6**) as the only other major photoproduct (eqs 1 and 2; Tables 1 and 5). A minor amount of photohydrolysis was also observed, but this amounted to less than 10% of the released peptide.

Quenching studies with sodium 2-naphthalenesulfonate and potassium sorbate (Table 2) demonstrated the intermediacy of the triplet of pHP Ala-Ala (**^{3}7**) in the photodeprotection process. The rate constant, 1.82×10^8 s^{-1}, derived from the Stern−Volmer slope is essentially the same as the release rate obtained in our earlier studies on *p*-hydroxyphenacyl ATP (**17**)[1].

Phosphorescence emission studies (Table 3) on a series of hydroxyphenacyl esters including pHP Ala-Ala (**7**), pHP GABA (**12**), pHP acetate (**18**), pHP phenylacetate (**19**) and pHP diethyl phosphate (**20**) showed triplet energies of 68.9−70.6 kcal/mol in EPA (5:5:2) and ethylene glycol:H_2O (2:1) glasses at 77 K, a value that is >3 kcal/mol above those of the triplet quenchers employed here (56−60 kcal/mol) under conditions that assured efficient triplet energy transfer to the quencher.



13, X =OH
17, X = H
18, X = $OCOCH_3$; α, α-dimethyl
19, X = $OCOCH_2Ph$
20, X = $OPO(OCH_2CH_3)_2$

These and earlier results of Falvey,[22] Savéant,[23a] and Wayner[23b,c] suggest a mechanism involving release of the carboxylate group from the phenacyl triplet. Electrochemical

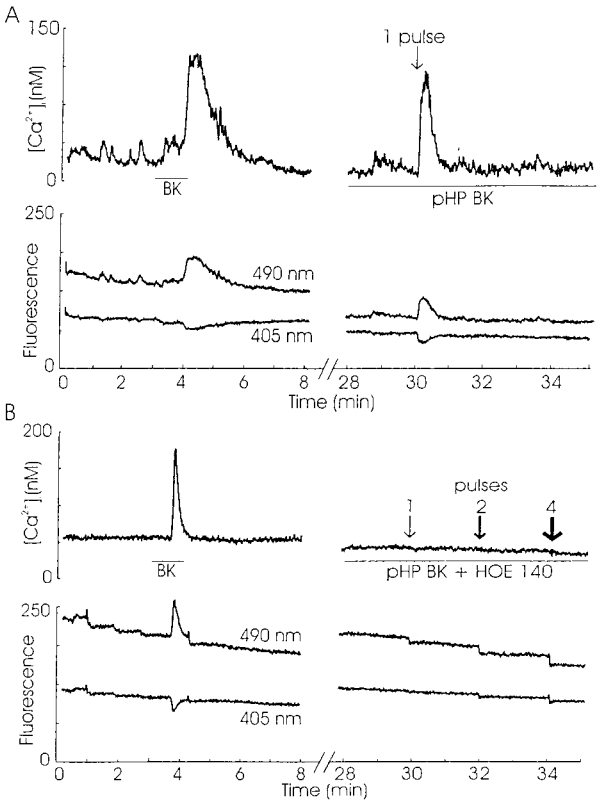


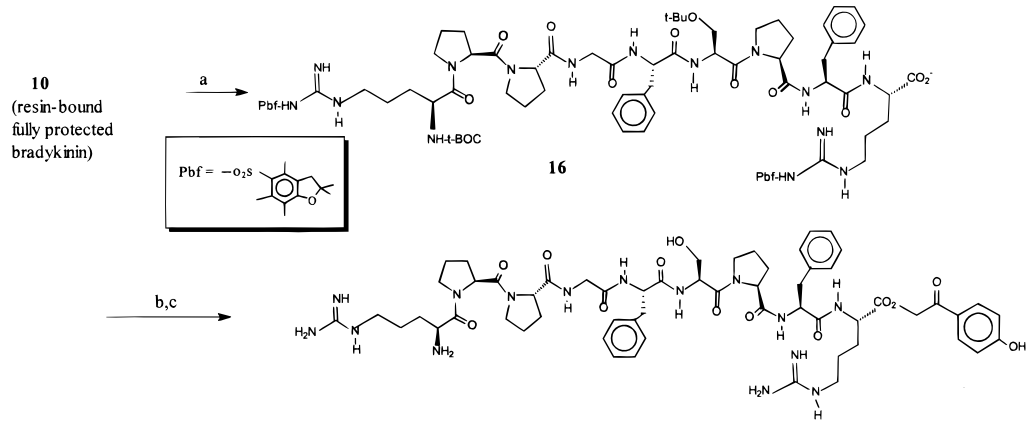
Figure 3. Photolysis of *p*-hydroxyphenacyl bradykinin (9) evoked a neuronal response. Bradykinin-induced increases in $[Ca^{2+}]_i$ were measured in single rat DRG neurons with indo-1-based photometry as described in the Experimental Section. Indo-1 fluorescence at 490 nm (unbound) and 405 nm (calcium-bound) is plotted in the lower panels. Drugs were applied by superfusion at the times indicated by the horizontal bars. Cells were not stimulated during the 20 min gaps in the recordings. (A) Bradykinin (100 nM, 60 s) produced an increase in $[Ca^{2+}]_i$ that rapidly desensitized. Following a 20 min recovery period, p-hydroxyphenacyl bradykinin was applied and at the time indicated by the arrow, a single pulse of UV light (1 ns, 337 nm) from a nitrogen laser was delivered to the cell by a single optical fiber positioned approximately 50 μm from the cell soma. The flash evoked a second $[Ca^{2+}]_i$ response. A representative recording from 8 experiments is shown. (B) Bradykinin (100 nM, 60 s) produced an increase in $[Ca^{2+}]_i$ that rapidly desensitized. Following a 20 min recovery period, *p*-hydroxyphenacyl bradykinin in combination with 300 nM HOE 140,[20] a B2 type bradykinin receptor antagonist, were applied and at the times indicated by the arrows, 1, 2, or 4 pulses of UV light (1 ns, 337 nm) were delivered to the cell. The flashes failed to elicit a second $[Ca^{2+}]_i$ response in the presence of the receptor antagonist. A representative recording from four experiments is shown.

studies coupled with laser flash photolyses by Andrieux, Savéant et al.,[23a] and Wayner[23b,c] of several substituted phenacyl derivatives led Andrieux and Savéant to suggest that the bond fragmentation is assisted by an electron transfer from the π^* orbital to the leaving group's antibonding σ^* orbital followed by bond cleavage and release of a carboxylate ion. We observe no decarboxylation in our studies with C-terminal-protected peptides nor with our previously reported deprotection reactions with pHP glutamate and pHP GABA.[2,12] The absence of decarboxylation products in these studies militates against

(22) Banerjee, A.; Falvey, D. E. *J. Am. Chem. Soc.* **1998**, *120*, 2965−2966.

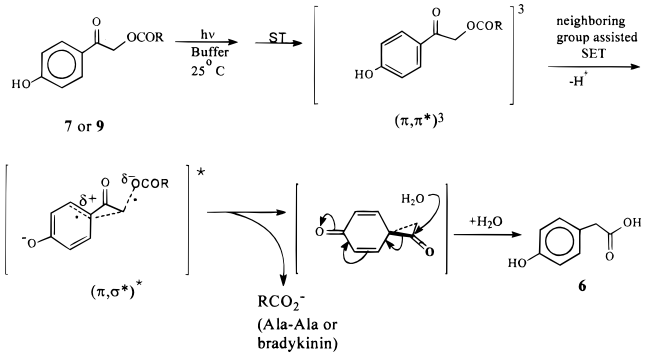
(23) (a) Andrieux, C. P.; Savéant, J.-M.; Tallec, A.; Tradivel, R.; Tardy, C. *J. Am. Chem. Soc.* **1996**, *118*, 9788−9789. (b) Andersen, M. L.; Long, W.; Wayner, D. D. M.; *J. Am. Chem. Soc.* **1997**, *119*, 6590−6595. (c) Mathivanan, N.; Johnston, L. J.; Wayner, D. D. *J. Chem. Phys.* **1995**, *99*, 8190−8195.

Scheme 2[a]



[a] (a) CH_2Cl_2, CH_3OH, CH_3CO_2H, (8:1:1), 2 h (quantitative); (b) *p*-hydroxyphenacyl bromide (**8**), DBU, DMF, rt, 24 h (72%); (c) 88% TFA, 7% thioanisole, 5% H2O, 2.5 h (84%).

Scheme 3



formation of a free carboxyl radical intermediate, possibly formed from direct homolysis of the CH_2-O bond. These results are also consistent with the studies reported earlier by us[1,2] and recently by Falvey[22] but contrast with an earlier result of Sheehan and Umezawa,[24] where partial decarboxylation was observed for some *p*-methoxyphenacyl esters, especially for the phthaloyl protected α-amino group on glycine. We have suggested the mechanism paralleling that of Andrieux and Savéant shown in Scheme 3 which involves participation of the CH_2-O σ^* orbital followed by carboxylate disengagement.[12]

A recent mechanistic proposal by Zhang, Corrie, Munasinghe, and Wan[3] suggested that a proton transfer occurs from the phenolic hydroxy group to the carbonyl from the initial singlet state of the pHP ester and that it is this intermediate *p*-quinone methide that releases the carboxylate through a cyclic transfer of the proton to the ester carbonyl with concomitant participation of the phenoxy ligand to produce the diene−dione (Scheme 4). The electronic state of the *p*-quinone methide (excited or ground state??) was left undefined.[3]

In fact, our mechanism has not addressed the state of protonation of the phenolic group, which would be expected to be altered in the excited-state singlet or triplet. A proton transfer to solvent may precede or occur in concert with the migration of the aryl moiety. We have determined, however, that the reaction does proceed from a quenchable triplet as shown now with two different quenchers. Our observed quenching of the reaction and of the phosphorescence emission by sodium 2-naphthalenesulfonate and by potassium sorbate (potassium 2,4-hexadienoate), the latter quencher has an even lower triplet energy and does not absorb above 280 nm. The small competitive absorption by 2-naphthalenesulfonate, which we estimate is 6% at equimolar concentrations of quencher and pHP Ala-Ala and only 16% at the highest concentration of quencher employed in this study, does not account for the >60% quenching we observed. The quenching of both the disappearance of **7** and the appearance of the two products by triplet−triplet energy transfer clearly demonstrates that the triplet of **7** is the intermediate excited state leading to release of the dipeptide and rearrangement of the phenacyl nucleus.

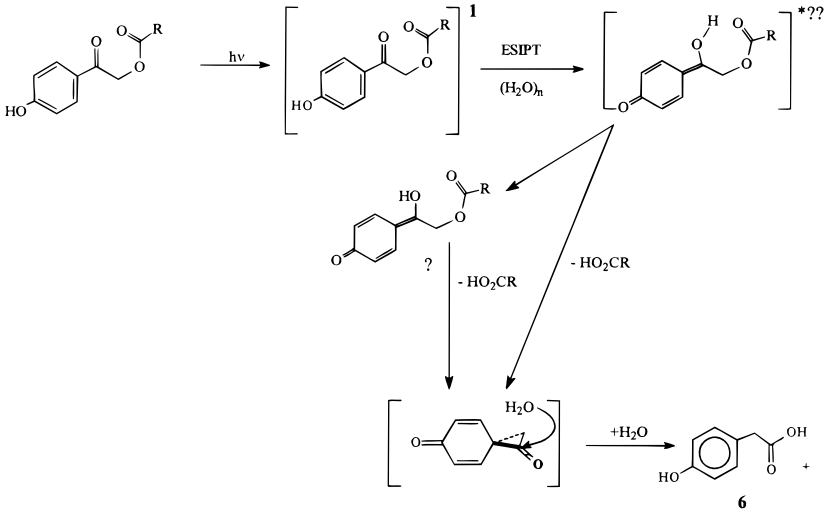
The biological utility of pHP bradykinin was demonstrated in an experiment in which a single nanosecond pulse at 337 nm released sufficient bradykinin to excite sensory neurons. It was not possible to precisely calculate the amount of energy delivered to the vicinity of the cell in this system, although uncaging occurred with laser pulses that did not appear to produce any observable damage to the cell. Furthermore, our optical method for $[Ca^{2+}]_i$ measurement relied on the fluorescent indicator indo-1, a dye that absorbs at 337 nm, but was not significantly bleached at pulses that efficiently uncaged pHP bradykinin. Clearly, pHP bradykinin is sufficiently stable that it can be introduced into biological systems without degradation and yet retains adequate photoreactivity to uncage at UV energies that spare the cells from toxicity. The actual efficiency of release can only be approximated using $[Ca^{2+}]_i$ as the bioassay because the amplitude of bradykinin-induced response is dependent on factors in addition to concentration. The concentration response relationship for bradykinin-induced responses in these cells is quite steep because of a threshold that results from amplification of signaling through the second messenger cascade. Furthermore, the response depends not only on bradykinin concentration but also the rate of its application because the receptor rapidly desensitizes. We can estimate that at least 30% of the 10 nM pHP bradykinin present was released in biologically active form because the threshold to elicit a response by application of bradykinin to the bath is 3 nM.

Conclusions

In summary, pHP bradykinin is a useful reagent for studying the kinetics and localization of bradykinin-mediated signaling processes. Both the efficiency of the release and the lack of side reactions suggest that the *p*-hydroxyphenacyl group is ideal for biochemical kinetic studies at the molecular and physiologi-

(24) Sheehan, J. C.; Umezawa, K. *J. Org. Chem.* **1973**, *38*, 3771−3774.

Scheme 4



cal level. In fact, the chemical properties of *p*-hydroxyphenacyl bradykinin (**9**) indicate that such compounds are well suited for activating biological processes by flash photolytic release of the substrate.

The results reported here and earlier show that the *p*-hydroxyphenacyl group can be confidently employed as a C-terminal photoremovable protecting group for oligopeptides and provides a general approach to developing reagents for studying the interactions of oligopeptides with biological systems.

Finally, the photochemical reaction proceeds through a short-lived, quenchable triplet ($^3\tau \approx 5.5$ ns) with a rate constant of 1.8×10^8 s^{-1}, sufficiently rapid that studies of most fast biological processes can be initiated with this phototrigger.

Experimental Section

General Procedures. All NMR spectra are reported in ppm (δ) from tetramethylsilane (^{1}H and ^{13}C). Melting points are uncorrected. TEA was dried by refluxing over KOH and distilled under Ar, collecting the middle fraction and storing in the dark. Pyridine and DMF were dried over phosphorus pentoxide and distilled under reduced pressure, collecting the middle fraction, which was stored over NaOH or molecular sieves, respectively. Benzene was distilled from sodium/benzophenone ketyl prior to use. Technical grade diethyl ether, MeOH, CH_2Cl_2, and THF were distilled from CaH_2.

Synthesis of 2-Bromo-4′-hydroxyacetophenone (8). 2-Bromo-4′-hydroxyacetophenone (**8**) was prepared by the modification of the method of Durden and Juorio[25] which we reported earlier.[12]

Synthesis of *p*-Hydroxyphenacyl-Ala-Ala (7). Boc-Ala-Ala (14). Boc-Ala-Ala (**14**) was prepared by the method of Song et al.[9] Ala-Ala (**1**, 300 mg, 1.87 mmol) was converted to Boc-Ala-Ala (**14,** 470 mg, 97% yield): mp 98 °C; ^{1}H NMR (300 MHz, acetone-d_6) $\delta = 7.40$ (s, 1H), 6.07 (s, 1H), 4.40 (m, 1H), 4.11 (m, 1H), 1.37 (s, 9H), 1.32 (d, 3H, $J = 6.5$ Hz), 1.27 (d, 3H, $J = 6.5$ Hz).

***N*-Boc-*O*-(4-hydroxyphenacyl)-Ala-Ala (15).** To a solution of 2-bromo-4′-hydroxyacetophenone (**8**) (328 mg, 1.5 mmol) dissolved in 3 mL of 1,4-dioxane and cooled to 15 °C was added 397 mg (1.5 mmol) of Boc-Ala-Ala (**14**) dissolved in 17 mL of 1,4-dioxane. This was followed by dropwise addition (10 min) of 1,8-diazabicyclo[5.4.0]-undec-7-ene (DBU, 269 mg, 1.8 mmol). The reaction mixture was allowed to warm to room temperature, and the mixture was stirred overnight. Thin-layer chromatography indicated that the reaction was complete. The solvent was removed in vacuo, and the crude product was purified by silica gel column chromatography (EtOAc:hexane = 1:1). After collection of appropriate fractions for the product, the solvent was concentrated to ~10 mL to give white crystals of *N*-Boc-*O*-(4-hydroxyphenacyl)-Ala-Ala (**15**) (374 mg, 62%): mp 150.5 °C; $[\alpha]_D = -35.2°$ ($c = 0.6$, ethyl acetate); IR (KBr) 3420, 3371, 3333, 3260, 2977, 3937, 1733, 1714, 1686, 1608, 1587, 1518, 1459, 1450, 1429, 1367,1331, 1280, 1250, 1212, 1165, 1094, 1070, 1022, 969, 842, 828, 802, 565 cm^{-1}; ^{1}H NMR (300 MHz, acetone-d_6) $\delta = 9.39$ (s, 1H), 7.86 (d, 2H, $J = 8.8$ Hz), 7.50 (m, 1H), 6.91 (d, 2H, $J = 8.8$ Hz), 6.04 (m, 1H), AB ($\delta_A = 5.46$, $\delta_B = 5.29$, $|J_{AB}| = 16.3$ Hz, CH_2), 4.56 (m, 1H), 4.14 (m, 1H), 1.44 (d, 3H, $J = 7.3$ Hz), 1.35 (s, 9H), 1.26 (d, 3H, 7.1 Hz); ^{13}C NMR (100 MHz, acetone-d_6) $\delta = 190.9$, 173.3, 173.0, 163.4, 156.2, 130.6, 126.8, 115.8, 78.7, 66.5, 50.1, 48.2, 28.0, 18.3, 17.7; FAB-MS *m*/*z* (relative intensity) 395 (M + 1, 14), 339 (18), 295 (100), 224 (58), 143 (43), 121 (30). Exact mass Calcd for $C_{19}H_{27}O_7N_2$ (M + H): 395.1818. Found: 395.1801.

***O*-(4-Hydroxyphenacyl)-Ala-Ala, Trifluoroacetate Salt·H$_2$O (7).** A solution of *N*-Boc-*O*-(4-hydroxyphenacyl)-Ala-Ala (**15**) (389 mg, 1.0 mmol) in 15 mL of trifluoroacetic acid (TFA) was cooled to 0 °C and reacted for 4 h with stirring. The resulting solution was concentrated by a rotary evaporator and residual solvent removal with a high vacuum pump. The crude product was extracted with the solution of H_2O/EtOAc. The aqueous layer was collected, and the water was removed by lyophilization to give a white solid of *O*-(4-hydroxyphenacyl)-Ala-Ala, trifluoroacetate salt·H_2O (**7**) (326 mg, 77.6%): mp 143 °C; $[\alpha]_D = -39.7°$ ($c = 0.59$, H_2O); IR (KBr) 3429, 3357, 2996, 2954, 1738, 1685, 1673, 1608, 1586, 1543, 1520, 1432, 1375, 1280, 1252, 1210, 1186, 1171, 1141, 1060, 970, 846, 828, 806, 728 cm^{-1}; UV−vis (H_2O) λ_{max} (ϵ) 282 nm (13 323); ^{1}H NMR (300 MHz, D_2O) $\delta = 7.82$ (d, 2H, $J = 8.4$ Hz), 6.9 (d, 2H, $J = 8.4$ Hz), 5.43 (s, 2H), 4.57 (m, 1H), 4.04 (m, 1H), 1.47 (two superimposed d = s, 6H; $J = 6$ Hz); ^{19}F NMR (300 MHz, D_2O) $\delta = 111.3$ (s, 3F); ^{13}C NMR (100 MHz, D_2O) $\delta = 194.4$, 174.0, 171.0, 162.3, 131.3, 126.1, 116.1, 67.5, 49.2, 49.0, 16.8, 16.3; FAB-MS (free amine) *m*/*z* (relative intensity) 295 (M + 1, 35), 277 (12), 185 (100), 161 (8). Exact mass Calcd for $C_{14}H_{19}N_2O_5$ (free amine, M + H): 295.1294. Found: 295.1308. Anal. Calcd for $C_{16}H_{21}N_2O_8F_3$: C, 45.07; H, 4.96; N, 6.57. Found: C, 45.09; H, 4.89; N, 6.80.

Synthesis of *p*-Hydroxyphenacyl Bradykinin (9). *N*(α-Boc)-*N*-(*ω*-Pbf)-L-arginyl-L-prolyl-L-prolyl-glycyl-L-phenylalanyl-(*O*-*tert*-butyl)-L-seryl-L-prolyl-L-phenylalanyl-*Nω*-Pbf-L-arginine (16). The partially protected bradykinin (**16**) was synthesized by a solid-phase Fmoc strategy.[10,11] The side chain functional groups on bradykinin were introduced as the protected amino acids, i.e., arginine was protected as its 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) derivative and serine as its tertiary butyl ether. The coupling of the protected amino acids was accomplished using standard Fmoc protocol on 2-chlorotrityl resin (nominal capacity: 1.2 mmol/g). The loading of the resin was 0.6 mmol/g initial capacity as determined by the Fmoc assay. The couplings were carried out by using two equiv of the protected amino acid, 1.9 equiv of *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU) in 1-methyl-2-pyrrolidinone (NMP) or

(25) Durden, D. A.; Juorio, A. V.; Davis, B. A. *Anal. Chem.* **1980**, *52*, 1815−1820.

dimethylformamide (DMF) in the presence of *N*,*N*-diisopropylethylamine (DIEA). When the Kaiser test was positive (usually after 30 min), the resin was washed and coupled again until no free amino groups were detected. The Fmoc group was then cleaved with 30% piperidine in DMF. This process was repeated for each amino acid. The N-terminal arginine was incorporated as the Fmoc-protected derivative, and the Fmoc group was cleaved as described above. The N-terminal amino group was acylated with Boc-pyrocarbonate in the presence of DIEA. Finally, the fully protected oligopeptide was cleaved from the resin by treatment with a mixture of CH_2Cl_2:MeOH:CH_3CO_2H = 8:1:1 which gave complete conversion to **16** within 2 h.

The protected bradykinin carboxylic acid (**16**) was purified by precipitation from $CHCl_3$ with MeOH, and the purity was established by RP-HPLC and TLC (RF in $CHCl_3$:CH_3OH:CH_3CO_2H = 8:1:1) = 0.6; (RF in ethyl acetate:pyridine:CH_3CO_2H:H_2O = 60:20:6:11) = 0.6 on air-dried Mack Kieselgel 60 F_{254} glass plates. The purity of the protected bradykinin carboxylic acid was greater than 90% based on RP-HPLC analysis. The amino acid composition was confirmed by a FAB-MS spectrum.

***N*(α-Boc)-*N*(*ω*-Pbf)-L-arginyl-L-prolyl-L-prolyl-glycyl-L-phenylalanyl-(*O*−*tert*-butyl)-L-seryl-L-prolyl-L-phenylalanyl-*N*(*ω*-Pbf)-L-arginine 4-hydroxyphenacyl ester. (10).** To a solution of 1,8-diazabicyclo[4.3.0]-undec-7-ene (DBU, 23.1 mg, 0.15 mmol) dissolved in 2 mL of dry DMF and cooled to 0 °C with ice was added 100 mg (0.058 mmol) of *N*(α-Boc)-*N*(*ω*-Pbf)-L-arginyl-L-prolyl-L-prolyl-glycyl-L-phenylalanyl-(*O*−*tert*-butyl)-L-seryl-L-prolyl-L-phenylalanyl-*N*(*ω*-Pbf)-L-arginine (**16**) under Ar. 2-Bromo-4′-hydroxyacetophenone (**8**, 37.5 mg; 0.17 mmol) was then added, and the mixture was cooled to 0 °C. The reaction mixture was allowed to reach room temperature and was stirred overnight. TLC indicated complete reaction. The solvent was removed in vacuo, and the crude product was first purified by silica gel column chromatography ($CHCl_3$/MeOH/AcOH = 90:8:2) to give a light oil which was further purified by HPLC on a 2.2 cm Vydac -C18 peptide/protein column using a gradient of 0.1% TFA in acetonitrile (50 to 100% over 90 min) at a flow rate of 8 mL/min (product retention time = 35.6 min). The solvent was concentrated to give a white crystalline solid of *N*(α-Boc)-*N*(*ω*-Pbf)-L-arginyl-L-prolyl-L-prolyl-glycyl-L-phenylalanyl-(*O*−*tert*-butyl)-L-seryl-L-prolyl-L-phenylalanyl-*N*(*ω*-Pbf)-L-arginine *p*-hydroxyphenacyl ester (**10**, 78 mg, 72%): ^{1}H NMR (400 MHz, $CDCl_3$) (only signals of the 4-hydroxyphenacyl- and the two phenyl groups are assigned) δ 7.84 (m, 2H), 7.27 (m, 10H), 6.93 (d, 2H, J = 8.6 Hz), 5.33 (m, 2H); FAB-MS *m*/*z* (relative intensity) 1856.1 (M + 1, 100). Exact mass Calcd for $C_{93}H_{128}N_{15}O_{21}S_2$ (M + H): 1854.8850. Found: 1854.8810.

L-Arginyl-L-prolyl-L-prolyl-glycyl-L-phenylalanyl-L-seryl-L-prolyl-L-phenylalanyl-L-arginine 4-Hydroxyphenacyl Ester (Bradykinin 4-Hydroxyphenacyl Ester) (9). To a 25 mL conical glass flask were added 54 mg (0.0291 mmol) of the dry, protected bradykinin 4-hydroxyphenylacetic ester **16**, followed by 2 mL of the deprotection cocktail of 88% TFA, 7% thioanisole, 5% H_2O. The solution was stirred with a glass rod every 15 min for 2.5 h to effect the deprotection. The resulting mixture was concentrated at 30 °C on a rotary evaporator to yield a light yellow syrup which was dissolved in 400 μL of TFA and the resulting solution was taken up into a Pasteur pipet and then squirted forcibly into 12 mL of methyl *tert*-butyl ether (−75 °C) in a 12 mL centrifuge tube. A fine white precipitate formed immediately. After 5 min of centrifugation, the ether was decanted, and the crude product was dispersed thoroughly with a glass rod in 10 mL of fresh ether and centrifuged once again. This process was repeated six times. The crude product was dried overnight to give 35.5 mg as a white pellet. The product was further purified by HPLC on a 2.2 cm Vydac C-18 peptide/protein column using a gradient of 0.1% TFA in acetonitrile (5 to 50% over 45 min) at a flow rate of 8 mL/min (product retention time = 33.6 min). The solvent was concentrated on a rotary evaporator to remove the CH_3CN and the remaining material was lyophilized to give white crystals of bradykinin *p*-hydroxyphenacyl ester (**9**, 29.1 mg, 84%): FAB-MS *m*/*z* (relative intensity) 1194.6 (M + H, 43). Exact mass Calcd for $C_{58}H_{80}N_{15}O_{13}$ (M + H): 1194.6060. Found: 1194.6016. UV−vis (H_2O) λ_{max} (ϵ) 191 nm (117 490), 282 (11 853).

Hydrolytic Stability of Ala-Ala *p*-Hydroxyphenacyl Ester (7). Into a test tube, containing 2 mL of Tris buffer solution (50 mM, pH 7.3) was introduced 2.5 mg (0.006 mmol) of Ala-Ala *p*-hydroxyphenacyl ester and trifluoroacetate salt·H_2O (**7**) containing 3 mg (0.02 mmol) of *p*-hydroxyphenylacetic acid (**6**) as an internal standard. Aliquots (2 μL) were taken after 10, 30, 60, and 80 min and analyzed by HPLC. The buffer system for analytical HPLC was 90% ammonium acetate buffer (50 mM, pH 4.5) and 10% acetonitrile at a flow rate of 1 mL/min through a Bondesil C-18 3 μm 4.6 mm × 5 cm column. The products and disappearance of the ester were monitored at 280 nm. The data were fit to a linear least-squares regression line using a Microcal Origin software program to obtain the half-life and the rate constant for the first-order hydrolysis in tris buffer. The reactions were too slow to monitor in Ringer's solution I, D_2O or in H_2O to obtain rate constants under these conditions. The results are given in Table 4.

General Procedure for Photolysis. All water was distilled and passed through a Nanopure deionizing system. Ammonium acetate (for Ala-Ala) and H_2O:TFA:CH_3CN buffers (bradykinin) were employed for analytical HPLC. The HPLC system employed a UV−vis detector set at 254 or 280 nm, and the separations were carried out on a reverse phase column. All analytical HPLC analyses employed either a solvent gradient or an isocratic elution with a flow rate of 1.0 mL/min. Photolysis was performed on a merry-go-round apparatus equipped with 4 × 300 nm lamps. The light output for the determination of quantum efficiencies was measured by using the potassium ferrioxalate method.[26] All determinations of quantitative measurements were performed at least in triplicate. The data were submitted to linear least-squares regression analysis using a Microcal Origin software program. Standard deviations are given in parentheses, and r values were 0.97512 to 0.99999.

Photolysis of *O*-(4-Hydroxyphenacyl)-Ala-Ala, Trifluoroacetate Salt (7). (A) Quantum Efficiencies by NMR. Into three NMR tubes containing 2 mL of D_2O were introduced 17.6 mg of *O*-(4-hydroxyphenacyl)-Ala-Ala and trifluoroacetate salt (**7**) (0.0394 mmol) containing 0.00296 mmol Ala-Ala as an impurity as determined by ^{1}H NMR. Dimethylformamide (DMF) (8 μl, 0.1034 mmol) was added as an internal standard. The solutions were deaerated for 10 min with argon and irradiated at 300 nm for 8 min with 4 RPR-3000 lamps. ^{1}H NMR spectra (400 MHz) were measured at 2 min intervals during irradiation. The rearrangement product, 4-hydroxyphenylacetic acid (**6**), the free dipeptide Ala-Ala (**1**), and the disappearance of *O*-(4-hydroxyphenacyl)-Ala-Ala (**7**) were identified by the characteristic 400 MHz-^{1}H NMR-signals. Quantitative measurement of the products and starting ester were performed with an internal standard by RP-HPLC (see below) and by integration of characteristic ^{1}H NMR signals for each compound. A very small amount of a photohydrolysis product, 2,4′-dihydroxyacetophenone (**13**), was also formed. Linear least squares regression analyses (vide supra) of the integrated ^{1}H NMR absorptions at 4.2 (**1**), 7.2 ppm (**6**), and 5.4 (**7**), respectively, were carried out for the appearance of Ala-Ala (**1**), *p*-hydroxyphenylacetic acid (**6**) and the disappearance of starting ester **7** (relative to the internal standards DMF and acetonitrile) as a function of time to determine the quantum efficiencies. The results are shown in Table 1.

(B) Test for Chirality Stability. Into an NMR tube containing 1 mL of D_2O was introduced 10 mg (0.0235 mmol) of *O*-(4-hydroxyphenacyl)-Ala-Ala, trifluorocetate salt (**7**), which was synthesized as described above from optically active Ala-Ala ($[\alpha]_D = -35.9°$ (c = 2, 6 N HCl) Ala-Ala (Aldrich)). The solution was deaerated for 10 min with argon and irradiated at 300 nm for 18 min with 16 RPR-300 lamps. Spectra (300 MHz ^{1}H NMR) were measured after 0, 2, 4, 6, 8, 12, and 18 min of irradiation. The conversion of the phototrigger **7** to the rearrangement product, 4-hydroxyphenylacetic acid (**6**) and the free dipeptide Ala-Ala, trifluoroacetate salt (**1**) was complete (see Figure 1). The rotation of the released Ala-Ala was measured directly from the photolysis solution. The measured rotation of Ala-Ala as its trifluoroacetate salt was $[\alpha]_D = -14.75°$ (c = 0.13, H_2O). At 100% conversion as shown in Figure 1, the 0.0235 mmol of Ala-Ala produced from the photolysis of 0.0235 mmol of **7** gave a specific rotation of $[\alpha]_D = -14.77°$ (c = 0.13, D_2O) for Ala-Ala as the trifluoroacetate salt, equivalent to the value obtained from the precursor Ala-Ala.

(26) Hatchard, C. G.; Parker, C. A. *Proc. R. Soc. London* **1956**, *A-235*, 518−522.

(C) Quantum Efficiencies by HPLC. Into a Pyrex tube containing 5 mL of D_2O were placed 17.6 mg of *O*-(4-hydroxyphenacyl) Ala-Ala, trifluoroacetate salt (**7**) (0.0394 mmol) which contained a trace (0.00296 mmol) of Ala-Ala (**1**) as an impurity and 5.7 mg (0.04256 mmol) anthranilic acid as the internal standard. The solution was deaerated for 10 min with Ar and irradiated at 300 nm. Three 5 μL aliquots at a time were taken at 2 min intervals (for a total of 8 min) and injected on HPLC on a Bondesil C-18 3 μm × 4.6 mm × 5 cm column. The buffer system for analytical HPLC was 90% ammonium acetate buffer (50 mM, pH 4.5) and 10% acetonitrile and the effluent was monitored at 280 nm. All analytical HPLC analyses employed an isocratic flow rate of 1 mL/min for 10 min. The rearrangement product 4-hydroxyphenylacetic acid (**6**) was identified by injection on HPLC with authentic sample. In addition a small amount (<10%) of the hydrolysis product 2,4′-dihydroxyacetophenone (**13**) was also detected. The quantum efficiencies for the disappearance of *O*-(4-hydroxyphenacyl)-Ala-Ala, trifluoroacetate salt (**7**) and the appearance of the 4-hydroxyphenylacetic acid (**6**) were obtained by linear squares regression analyses. The data were submitted to linear least-squares regression analysis using a Microcal Origin software program and the results are shown in Table 1.

(D) Quenching Studies of Ala-Ala *p*-Hydroxyphenacyl Ester, Trifluoroacetate Salt·H_2O (7) with Sodium 2-Naphthalene Sulfonate. Into four NMR tubes containing 2 mL of D_2O were introduced 17.6 mg of Ala-Ala *p*-hydroxyphenacyl ester and trifluoroacetate salt (**7**) (0.0394 mmol) containing 0.00296 mmol of Ala-Ala (**1**) as an impurity. Dimethylformamide (DMF) (8 μL, 0.1034 mmol) was added as an internal standard. To three of these tubes was added sodium 2-naphthalene sulfonate, 9.1 mg (39 μmol), 16.8 mg (73 μmol), or 20.9 mg (91 μmol), respectively. The tubes were deaerated for 10 min with Ar at room temperature and irradiated for a total of 8 min with 4 RPR-3000 Å lamps. ^{1}H NMR spectra (400 MHz) were determined at 2 min intervals during irradiation. The appearance of the rearrangement product, *p*-hydroxyphenylacetic acid (**6**), the free dipeptide Ala-Ala (**1**) and the disappearance of *p*-hydroxyphenacyl Ala-Ala (**7**) were identified and quantitatively determined by integration of characteristic peaks in their ^{1}H NMR spectra. The data were submitted to linear least-squares regression analysis using a Microcal Origin software program, and the results are given in Table 2.

The competitive absorption of sodium 2-naphthalenesulfonate in this quenching study was determined to be relatively minor in the region between 300 to ~320 nm where it overlaps with the absorption of **7**. The emission range of the 300 nm lamps extends to about 335−340 nm. Pyrex test tubes were used, thus filtering out the incident radiation below 295 nm, making the dynamic range essentially ~295−335 nm. In this region, the *p*-hydroxyphenacyl ester has a ϵ that ranges from 7700 at 300 nm to 4000 at 310 nm and then tails to 0 at ~340 nm. The 2-NS absorption spectrum consists of three small absorption λ_{max}'s between 300 and 320 nm, none with ϵ greater than 550. Thus, from the spectral data, the calculated competitive absorption by 2-NS at the highest concentration used was less than 16% that of **7**.

(E) Quenching Studies of Ala-Ala *p*-Hydroxyphenacyl Ester with Potassium Sorbate. Into each of four NMR tubes containing 2 mL of D_2O were introduced Ala-Ala *p*-hydroxyphenacyl ester (15 mg, 0.05 mmol), DMF (5 μL, 0.065 μmol), and increasing amounts of potassium sorbate (0 mg, 0 μmol; 7.6 mg, 50 μmol; 15.3 mg, 100 μmol, 38.0 mg, 250 μmol, respectively). The NMR tubes were placed inside a Pyrex test tube in the merry-go-round apparatus and were irradiated with four RPR-3000 Å lamps. The samples were analyzed at 2 min intervals during the irradiation by 400 MHz ^{1}H NMR using DMF as the internal standard. The disappearance of *p*-hydroxyphenacyl Ala-Ala (**7**) and the appearance of *p*-hydroxyphenylacetic acid (**6**) and Ala-Ala (**1**) were determined (see **A**, vide supra). Stern−Volmer slopes were determined as indicated above and the results are given in Table 2.

Photolysis of Bradykinin 4-Hydroxyphenacyl Ester (9): Quantum Efficiency Determinations. Into a Pyrex tube containing 5 mL of D_2O was dissolved 3.5 mg (0.00293 mmol) bradykinin 4-hydroxyphenacyl ester (**9**) and hemimellitic acid (0.85 mg, 0.00405 mmol) as the internal standard. The solution was deaerated for 10 min with Ar and irradiated at 300 nm for 8 min. Aliquots (100 μL) were taken at 0.5, 1.0, 2.0, and 2.5 min and analyzed by HPLC on a 1.0 cm Vydac C-18 peptide/protein column using a gradient of 0.1% TFA (5 to 50% over 45 min) in acetonitrile at a flow rate of 2 mL/min. The rearrangement product, 4-hydroxyphenylacetic acid (**6**) was identified by co-injection on HPLC with authentic sample. In addition a small amount of the photohydrolysis product 2,4′-dihydroxyacetophenone (**13**) was also observed. The photoreleased bradykinin (**2**) was identified by co-injection with an authentic sample, by FAB-MS and by exact mass of the collected fraction (retention time = 24.8 min): FAB-MS *m/z* (relative intensity) 1060.5 (M, 100). Exact mass calcd for $C_{50}H_{74}N_{15}O_{11}$ (M + H): 1060.5692. Found: 1060.5720. CD spectroscopy (H_2O) (Figure 2): Θ_{max} = 918 deg cm^2 dmol^{-1} (222 nm), Θ_{min} = −6965 deg cm^2 dmol^{-1} (202 nm), for bradykinin (Fluka): Θ_{max} = 954 deg cm^2 dmol^{-1} (222 nm), Θ_{min} = −6705 deg cm^2 dmol^{-1} (202 nm).

At 100% conversion, the product yields of 6 and **13** were 0.00263 and 0.00035 mmol, respectively, in good agreement with the total conversion of **9** to bradykinin and the two byproducts. This also corroborates the high degree of purity of pHP bradykinin (**9**). The quantum efficiencies for the disappearance of bradykinin 4-hydroxyphenacyl ester (**9**) and for the appearance of bradykinin (**2**), 4-hydroxyphenylacetic acid (**6**), and 2,4′-dihydroxyacetophenone (**13**) were obtained by linear least-squares regression analyses using Microcal Origin software as discussed above. These results are shown in Table 4.

Intracellular Ca^{+2} Determinations in Rat Dorsal Root Ganglion: General Procedures for Flash Photolysis of *p*-Hydroxyphenacyl Bradykinin (9). Rat dorsal root ganglion (DRG) neurons were grown in culture as previously described.[19] The intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured in single cells with the Ca^{2+}-sensitive dye indo-1 using previously published procedures and instrumentation.[19] Briefly, cells were loaded with dye by incubation with indo-1 acetoxymethyl ester, which is membrane permanent. De-esterification by cytosolic esterases traps the Ca^{2+}-sensitive, free acid form of indo-1 within the cell. The cells were then placed in a recording chamber on the stage of an epifluorescence microscope and excited with UV light (350 ± 5 nm). The emitted fluorescence was detected at 405 ± 20 and 490 ± 20 nm. The ratio (R) of the 405:490 signals was converted to $[Ca^{2+}]_i$ by the equation $[Ca^{2+}]_i = K\beta(R_{max} - R)/(R - R_{min})$. The constants K, β, R_{max}, and R_{min} were derived as previously described.[19]

Flash photolysis was accomplished with light from a pulsed nitrogen laser (Photon Technologies Inc., GL-2300). Ultraviolet light (337 nm) was focused (plano convex f = 10 cm) onto the end of a single optical fiber (polyimide, core diameter = 50 mm; Ceram Optec Inc.), the terminating end of which was placed approximately 50 μm from the cell soma with a micromanipulator. A single pulse of approximately 1 ns in duration (approximately 2.3 mJ) was sufficient for photolysis.

Phosphorescence Emission Studies. The phosphorescence measurements were performed in either ethylene glycol:H_2O (EG:W; 2:1) or ether:isopentane:ethanol (EPA; 5:5:2) glasses in a rotating cam double monochromator phosphoroscope illuminated with a 200 W xenon−mercury lamp and detected with an IP28 photomultiplier. The solvents were HPLC or spectral grade. The samples were placed in a 0.1 mm quartz tube and cooled to 77 K.

In a typical study, the phosphorescence spectrum of *p*-hydroxyphenacyl γ-aminobutyrate (18−36 mg, 0.05−0.10 mmol) was dissolved in 1.0 mL of EPA or EG:W and cooled to 77 K to form a clear glass. The phosphorescence emission and excitation spectra were measured for each sample. A blank of each solvent was checked for impurities.

In the quenching experiments, a 0.05−0.10 M solution of **7** and 17 mg (0.04 M) of Na^+ 2-naphthalenesulfonate[16] or piperylene[27] in EW was cooled to 77 K and the phosphorescence emission and excitation measured. Table 3 shows the results for **7, 12**, **13**, *p*-hydroxyacetophenone (**17**),[15] α,α-dimethyl-*p*-hydroxyphenacyl acetate (**18**), *p*-hydroxyphenacyl phenylacetate (**19**), diethyl *p*-hydroxyphenacyl phosphate (**20**), and mixtures of Na^+ naphthalene-2-sulfonate with **7** and **12** and mixtures of **7** with piperylene.

(27) The triplet energy of (*E*)-piperylene is reported to be E_T = 60 kcal/mol from the S → T 0, 0 band: Kellogg, R. E.; Simpson, W. T. *J. Am. Chem. Soc.* **1965**, *87*, 4230−4234.

Acknowledgment. We thank the NSF (NSF/O5R-9255223; R.S.G. and IBN9723796; S.A.T.), NIH (DA07340, DA092393, DA11806; S.A.T.) and the Minnesota Medical Foundation (S.A.T.) for partial support of this research. G.O. thanks the Council for International Exchange of Scholars for a Fulbright Fellowship and J.F.W.W. thanks the University of Kansas College of Liberal Arts and Sciences for partial support. We thank Professor Michael Doughty, University of Kansas, for suggesting the bradykinin problem and D. Williamson for helpful advice.

JA991014B