p-(4-Hydroxybenzoyl)phenylalanine: A Photoreactive Amino Acid Analog Amenable to Radioiodination for Elucidation of Peptide−Protein Interaction Application to Substance P Receptor†

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ABSTRACT: Benzoylphenylalanine, a photoreactive phenylalanine analog that can be incorporated into a peptide during solid-phase synthesis, is a useful probe for investigating the interactions of bioactive peptides with their receptors. This probe, however, lacks versatility because it is not detectable by Edman sequencing and because it cannot be labeled with radioiodine, requiring radiolabeling of the peptide ligand at a site distal to the photoreactive amino acid. The separation of the radioisotope and photoaffinity labels along the primary sequence limits identification of the photoinsertion site to a peptide fragment rather than a specific amino acid of the receptor protein. We have now synthesized *p*-(4-hydroxybenzoyl)phenylalanine by a synthetic route involving reaction of 4-(chloromethyl)benzoic anhydride with phenol in polyphosphoric acid to give the 4-(chloromethyl)benzoyl ester of 4-(chloromethyl)-4′-hydroxybenzophenone followed by reaction of the benzophenone derivative with ethyl acetamidocyanoacetate and subsequent hydrolysis of the product to give *p*-(4-hydroxybenzoyl)phenylalanine. The novel photolabile amino acid was incorporated into substance P (replacing Phe^8 or Lys^3) to give 11-mer peptides that bind with high (nM) affinity and specificity to the substance P receptor. Radioiodination of the substance P analogs resulted in the incorporation of ^{125}I at the photoreactive amino acid residue, yielding probes of high (∼2000 Ci/mmol) specific activity. Subsequent photolysis of the radiolabeled peptides in the presence of substance P receptor caused covalent attachment of the peptide to the receptor with high photoinsertion yield (≈30%); photolabeling was abolished in the presence of excess unlabeled SP. *p*-(4-Hydroxybenzoyl)phenylalanine retains *p*-benzoylphenylalanine's high insertion yield and low reactivity with water, but in contrast allows placement of radioiodine and the photoactive moieties within the same residue, providing the ability to identify the specific site(s) of interaction, and identification of the residue by Edman sequencing. This novel amino acid may be useful in the elucidation of the interaction of a variety of peptides with their receptors.

Photoaffinity labeling has been an important method for the direct elucidation of intermolecular interactions in biological systems (Bayley, 1983) since its introduction by Westheimer and co-workers (Singh et al., 1962) more than three decades ago. A variety of photophores have been employed, most relying on photoconversion of diazo compounds, azides, or diazirines to nitrenes or carbenes (Bayley, 1983). These reactive intermediates have the disadvantage of reacting rapidly with water, leading to very low photoinsertion yields in most cases. The introduction of benzophenones as photoactivatable reagents by Galardy et al. (1973) led to recognition of a significant advantage of this photophore over the nitrene and carbene precursors: the excited state of the benzophenones, a triplet biradical, is essentially inert toward water. Other advantages of this photophore over previous ones include stability to ambient light, activation at longer wavelengths (thus minimizing photolytic damage to proteins), and a much wider range of chemical stability. The photochemistry of benzophenones has been reviewed (Dorman & Prestwich, 1994). Introduction of the amino acid 4-benzoylphenylalanine (BPA[1]) by Kauer et al. (1986) allowed direct incorporation of a benzophenone photophore at a defined position into peptides by solid-phase synthesis, bringing a significant advance in

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[1] Abbreviations: BHSP, Bolton-Hunter substance P; BPA, 4-benzoylphenylalanine; BSA, bovine serum albumin; CIMS, chemical ionization mass spectrometry; CP-96,345, (2*S*,3*S*)-*cis*-2-(diphenylmethyl)-*N*-[(2-methoxyphenyl)methyl]-1-azabicyclo[2.2.2]octan-3-amine; DMEM, Dulbecco's Modified Eagle's Medium; dmptu, dimethylphenylthiourea; dptu, diphenylthiourea; ESIMS, electrospray ionization mass spectrometry; FBS, fetal bovine serum; Fmoc, fluoren-9-ylmethoxycarbonyl; Fmoc-OSu, fluoren-9-ylmethoxycarbonyl *N*-hydroxysuccinimide ester; HBPA, *p*-(4-hydroxybenzoyl)phenylalanine; MALDMS, matrix-assisted laser desorption mass spectrometry; NK-1R, neurokinin-1 receptor (also known as substance P receptor); NMR, nuclear magnetic resonance; PAGE, polyacrylamide gel electrophoresis; PTH, phenylthiohydantoin; R_f, relative mobility; RIA, radioimmunoassay; RP-HPLC, reverse phase high performance liquid chromatography; SP, substance P; SPR, substance P receptor; TFA, trifluoroacetic acid; TLC, thin layer chromatography; UV, ultraviolet.

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the application of photoaffinity labeling to the study of peptide−protein interactions (Adams et al., 1995; Behar et al., 1996; Blanton et al., 1994; Bosse et al., 1993; Boyd et al., 1991a,b, 1994, 1996; Gao et al., 1995; Garcia et al., 1994; Gergel et al., 1994; Kage et al., 1993; Kauer et al., 1986; Li et al., 1995a; Macdonald et al., 1996; McNicoll et al., 1992; Miller & Kaiser, 1988; O'Neil et al., 1989; Servant et al., 1993; Williams & Shoelson, 1993; Zhang et al., 1996).

The large majority of receptors for bioactive peptides transduce signals through guanine nucleotide binding proteins. The question of which region(s) of a G-protein-linked receptor interact with which region(s) of its peptide agonist has been difficult to approach. With neuropeptide ligands such as substance P (MW $\approx$ 1.3 kDa), many contacts between ligand and receptor are involved as compared to much smaller nonpeptide ligands. Thus, useful data from mutagenesis experiments are more difficult to acquire because of the extremely large number of mutants required for thorough analysis, and because mutations causing loss of function could reflect a change in receptor conformation distant from the binding site (Huang et al., 1995). In contrast, photoaffinity labeling offers a uniquely powerful approach by directly identifying regions of the receptor in close contact with an identified amino acid of the agonist.

Photolabeling of substance P receptor (SPR, also known as NK-1R, a member of the G-protein-coupled receptor family involved in pain modulation and inflammation (Pernow, 1983; Otsuka & Yashika, 1993)) by a substance P (SP) analog is desirable for several reasons. SP, a member of the tachykinin peptide family, is a high affinity ligand (0.5−1 nM) with a single binding site on SPR. SPR has been cloned from several species (human, mouse, rat, and guinea pig) and shows a high degree of sequence homology between them (Gerard et al., 1993), but expression levels remain low and purification is difficult. Mutagenesis studies of SPR have suggested that both extracellular and transmembrane domains are important for agonist binding (e.g., Cascieri et al., 1994; Fong et al., 1992a,b, 1993, 1994a,b; Gether et al., 1993a−c, 1994; Huang et al., 1994a,b; Jensen et al., 1994; Sachais et al., 1993; Yokota et al., 1992; Zoffman et al., 1993).

Benzoylphenylalanine sparked a revolution in photoaffinity labeling (Dorman & Prestwich, 1994), but has the distinct disadvantage that the high specific activity radiolabel must be located distal to the photoactive residue in the primary structure. Furthermore, neither the BPA amino acid nor its phenylthiohydantoin (PTH) analog is detectable using standard amino acid analysis or Edman sequencing protocols (Kauer et al., 1986), making identification of the photoinsertion site difficult. In the present study, these difficulties have been overcome by synthesis of a modified BPA, *p*-(4-hydroxybenzoyl)phenylalanine (HBPA). Incorporation of the hydroxyl group in the *para* position of BPA results in a photoreactive amino acid which is detectable by protein sequencing and amenable to radiolabeling with iodine. Low levels of receptor expression and ease of postsynthetic incorporation make ^{125}I the radioisotope of choice in studies of peptide receptors. Photolabeling with a peptide incorporating HBPA into various positions will allow the specific identification of the photoinsertion sites by radiosequencing, making purification-independent elucidation of peptide receptor binding sites now practical. In this paper we describe the synthesis and characterization of *p*-(4-hydroxybenzoyl)phenylalanine, the incorporation of HBPA into the peptide substance P, radioiodination of the HBPA analogs of SP, and the photolabeling of substance P receptor using the peptide analogs.

EXPERIMENTAL PROCEDURES

Synthesis

*4-(Chloromethyl)benzoyl Chloride (**II**).* A mixture of 4-(chloromethyl)benzoic acid (**I**; 50 g, 0.29 mol) and oxalyl chloride (100 g, 0.79 mol) in anhydrous benzene (100 mL) was stirred at 25 °C and then refluxed for 2 h. Unreacted oxalyl chloride and benzene were removed by distillation. Traces of the remaining reagents were removed under reduced pressure to give 4-(chloromethyl)benzoyl chloride (**II**) as a colorless liquid. The acid chloride was used in the next step without further purification.

*4-(Chloromethyl)benzoic Anhydride (**III**).* A solution of 4-(chloromethyl)benzoyl chloride (**II**; 14.2 g, 0.075 mol) in anhydrous pyridine (36 mL, 0.45 mol) was warmed gently over a steam bath for 10 min. The reaction mixture was poured over a slurry of ice (75 g) and concentrated HCl (37.5 mL). The solid residue was filtered under suction on a Buchner funnel and washed successively with three 6 mL portions of methanol followed by three 6 mL portions of benzene. The residue was dried under high vacuum to give 4-(chloromethyl)benzoic anhydride (**III**; 10.6 g, 88% yield).

*4-(Chloromethyl)-4′-hydroxybenzophenone 4-(Chloromethyl)benzoyl Ester (**IV**).* A mixture of the anhydride (**III**, 32.3 g, 0.1 mol) and phenol (3.76 g, 0.04 mol) in polyphosphoric acid (100 g) was stirred at 150 °C for 2 h. The mixture was cooled to 80 °C and poured over stirred crushed ice (500 g). The resulting precipitate was filtered on a Buchner funnel and thoroughly washed with water. The solid was suspended in water (400 mL) and the pH adjusted to 7.0 under vigorous stirring with 10 M NaOH. After stirring for 4 h, the suspension was extracted twice with 300 mL portions of chloroform (NaCl was added to separate the phases). The combined chloroform extracts were taken to dryness on a rotary evaporator. The residue was stirred in benzene (50 mL) and filtered. The solution was evaporated on a rotary evaporator and the residue dried under high vacuum to give 10.4 g of solid. Analytical thin layer chromatography of the product (silica gel, benzene) showed two major spots with R_f 0.14 and 0.41 which were identified as 4-(chloromethyl)-4′-hydroxybenzophenone 4-(chloromethyl)benzoyl ester (**IV**) and phenyl *p*-(chloromethyl)-benzoate, respectively, by NMR.

The crude solid was suspended in benzene:hexane 1:1 v/v (100 mL), heated, and decanted after cooling. The supernatant was applied to a column of silica gel G (70 g) equilibrated with benzene:hexane 1:1 to separate the benzophenone derivative from the phenyl benzoate contaminant. The column was eluted with the same solvent (250 mL), followed by benzene:hexane 3:1 (350 mL) and finally with benzene. Fractions (50 mL) were collected. Fractions containing the component with R_f 0.14 (silica gel plate developed with benzene) were pooled and dried on a rotary evaporator. The residue (2.4 g; 15% yield based on starting phenol) was recrystallized from absolute ethanol to give 2.2 g of colorless needles (mp 137−138 °C).

Assignments for the NMR spectrum ($CDCl_3$) were made by inspection. The spectrum was consistent with the structure for the product 4-(chloromethyl)-4′-hydroxy-

benzophenone 4-(chloromethyl)benzoyl ester (**IV**) having chemical shifts δ (ppm downfield from internal tetramethylsilane) of 4.65 ($ClCH_2$), 7.54 (H-3 of both the hydroxybenzophenone and the benzoyl ester rings), 8.22 (H-2 of the benzoyl ester ring), and 7.36 (H-2), 7.91 (H-2′), and 7.82 (H-3′) of the hydroxybenzophenone ring. Elemental analysis of **IV** (66.4% C, 4.0% H, 16.7% Cl) was consistent with the proposed structure $C_{22}H_{16}O_3Cl_2$.

N-Acetyl-α-cyano-p-(4-hydroxybenzoyl)-D,L-phenylalanine Ethyl Ester (V). A stirred mixture of 4-(chloromethyl)-4′-hydroxybenzophenone 4-(chloromethyl)benzoyl ester (**IV**, 798 mg, 2 mmol), ethyl acetamidocyanoacetate (701 mg, 4.12 mmol), potassium carbonate (382 mg, 2.76 mmol), and potassium iodide (8.3 mg, 0.05 mmol) in acetone was refluxed in the dark under argon for 20 h. After cooling, the solid precipitate was filtered and washed with acetone. TLC (chloroform:ethanol 94:6) showed a major spot with R_f 0.24 and several minor spots. The crude product was purified by preparative TLC to give 360 mg (47% yield) of a glassy solid.

The NMR spectrum ($CDCl_3$) for the product was consistent with the structure for *N*-acetyl-α-cyano-*p*-(4-hydroxybenzoyl)-D,L-phenylalanine ethyl ester (**V**). [The phenolic ester resulting from the S_N2 reaction was probably saponified during the overnight alkaline reflux.] Chemical shifts δ (ppm): 7.64, 7.04 (H-3 of the phenylalanine and hydroxybenzoyl rings, respectively), 7.89 (H-2, H-2′), 4.32 (CH_2), 2.14 ($NHCOCH_3$), 3.49 (OCH_2CH_3), and 1.32 (OCH_2CH_3). Elemental analysis of **V** (63.3% C, 5.1% H, 7.2% N) was consistent with the proposed stucture $C_{21}H_{20}NO_5$.

p-(4-Hydroxybenzoyl)-D,L-phenylalanine (HBPA) (VI). The crude reaction product from the above reaction (TLC-purification resulted in loss of material, apparently because the crude material also contained the phenolic ester of *N*-acetyl-α-cyano-*p*-(4-hydroxybenzoyl)-D,L-phenylalanine, **V**) was suspended in 8 M HCl (5 mL) and refluxed under argon in the dark for 20 h. After drying on a rotary evaporator, the residue was suspended in water (10 mL) and the pH adjusted to 7.0 with 1 M NaOH. After vigorous stirring for 1 h, the precipitate was filtered and washed with water. The dried precipitate was resuspended in ethanol (15 mL) and heated to boiling. After cooling, the precipitate was filtered, washed with ethanol, and dried under high vacuum to give 427 mg (75% yield based on the 4-(chloromethyl)-4′-hydroxybenzophenone 4-(chloromethyl)benzoyl ester, **IV**) of *p*-(4-hydroxybenzoyl)phenylalanine (**VI**). TLC (silica gel, butanol:acetic acid:water 4:1:5) showed one spot with R_f 0.5.

The chemical shifts δ (ppm) determined by NMR spectroscopy ($CDCl_3$) for the product were 7.49, 7.72 (H-2, H-3 of the phenylalanine ring), 7.76, 6.91 (H-2, H-3 of the hydroxybenzoyl ring), 3.80 ($C_\alpha H$), and 3.10, 3.40 ($C_\beta H_2$). These results are consistent with the structure of the final product *p*-(4-hydroxybenzoyl)phenylalanine (**VI**). Elemental analysis (65.8% C, 5.4% H, 4.8% N) and mass spectrometry (ESIMS; $(M + 1)^+$ of 286.1) were consistent with the proposed structure $C_{16}H_{15}NO_4$.

N-Fmoc-p-(4-hydroxybenzoyl)-D,L-phenylalanine. *p*-(4-Hydroxybenzoyl)phenylalanine (1.4 mmol) was dissolved in 5 mL of 10% sodium bicarbonate and treated with Fmoc-OSu (1.4 mmol) in acetone (5 mL) for 18 h. Thin layer chromatography (silica gel, dichloromethane:methanol:acetic acid 93:5:2; $R_f = 0.3$) showed the reaction to be ≥95% complete. Acetone was removed by evaporation. The clear yellow aqueous solution was diluted to a final volume of 25 mL and extracted with ether (2 × 20 mL). Ethyl acetate was added, and the mixture was acidified to pH ∼ 2. The organic phase was evaporated, and the residue was coevaporated with dichloromethane (2 × 50 mL). The solid obtained was crystallized from dichloromethane (30 mL). The yield was 92%.

The NMR spectrum ($CDCl_3$) was consistent with the structure of the final product *N*-Fmoc-*p*-(4-hydroxybenzoyl)phenylalanine. The chemical shifts δ (ppm) for the aromatic protons are almost identical (all within 0.01 ppm) to those for the underivatized amino acid. The chemical shift δ (ppm) for $C_\alpha H$ shifted to 4.40, and for $C_\beta H_2$ shifted only slightly to 3.10 and 3.34. For the Fmoc group, the chemical shifts δ (ppm) were 6.19 (H-1), 7.36 (H-3, H-12), 7.67 (H-4, H-5, H-10, H-11), 7.45 (H-6, H-9), and 4.25 (CH_2OCO).

Mass spectrometry (CIMS) gave an $(M + 1)^+$ of 507.5 as expected for Fmoc-HBPA. The extinction coefficient for Fmoc-HBPA at 300 nm was determined by absorbance spectroscopy to be 18700 cm^{-1} M^{-1} in acetonitrile:dimethyl sulfoxide 3:1.

L-HBPA[8]-substance P. The peptide synthesis of the SP analog (HBPA[8]-SP) ArgProLysProGlnGlnPhe(L-HBPA)-GlyLeuMet-NH_2 was performed using a standard Fmoc solid-phase synthetic strategy (Maggio et al., 1992) by Quality Controlled Biochemicals, Inc. (Hopkinton, MA). The crude synthetic peptide was HPLC purified using a Synchrom C_{18} column (250 × 4.6 mm). The crude racemic peptide D,L-HBPA[8]-SP had two major UV-absorbing (215 nm) peaks of approximately equal intensity (Figure 2), eluting at 51.0 and 54.4% acetonitrile. Peptide diastereomers containing D- or L-HBPA were identified as described for previous work with D,L-isomers of BPA (Li et al., 1995a). The L-HBPA[8]-SP elutes earlier than D-HBPA[8]-SP on RP-HPLC (Li et al., 1995a; Blanton et al., 1994). The first peak was more active than the second with both a SP specific antibody and SPR, as expected for the L-diastereomer (Figures 5, 6). HBPA[3]-SP was prepared in the same manner but with HBPA substituted for lysine (SP position 3).

The isolated L-HBPA[8]-SP was analyzed for purity and correct structure by amino acid analysis, mass spectroscopy, and sequencing. The peptide sequence was confirmed by Edman sequencing and mass spectrometry (MALDMS) gave m/z 1467 [$(M + 1)^+$] as expected. The PTH derivative of HBPA eluted from the sequencer column under standard conditions at 15.3 min (Figure 3). The extinction coefficient for the peptide (L-HBPA[8]-SP) was determined to be 16600 cm^{-1} M^{-1} at 292 nm in acetonitrile:dimethyl sulfoxide (6:1) by absorbance spectroscopy, using amino acid analysis for quantitation of the peptide. Like BPA (Li et al., 1995a), HBPA was not detected by conventional amino acid analysis (ion-exchange using postcolumn ninhydrin detection), but the remaining amino acids gave the expected molar ratios for SP with HBPA substituted for Phe[8] [$Arg^{1.03}$ (1), $Pro^{2.07}$ (2), $Lys^{1.03}$ (1), $Gln^{2.14}$ (2), $Phe^{1.08}$ (1), $Gly^{1.05}$ (1), $Leu^{1.04}$ (1), $Met^{0.87}$ (1)].

Radioiodination of HBPA[8]-SP. The radioligand [^{125}I]-L-HBPA[8]-SP was prepared using general peptide iodination techniques previously described (Too & Maggio, 1991). Typically, 10 nmol of dry peptide was dissolved in 50 μl of 0.5 M borate buffer, pH 8.0, and vortex-mixed with 1 mCi of $Na^{125}I$ (10 μl; Amersham IMS-30). Chloramine-T (10 μg) was added, and the mixture was vortex-mixed for 90 s before the reaction was quenched with 100 μg of $Na_2S_2O_5$.

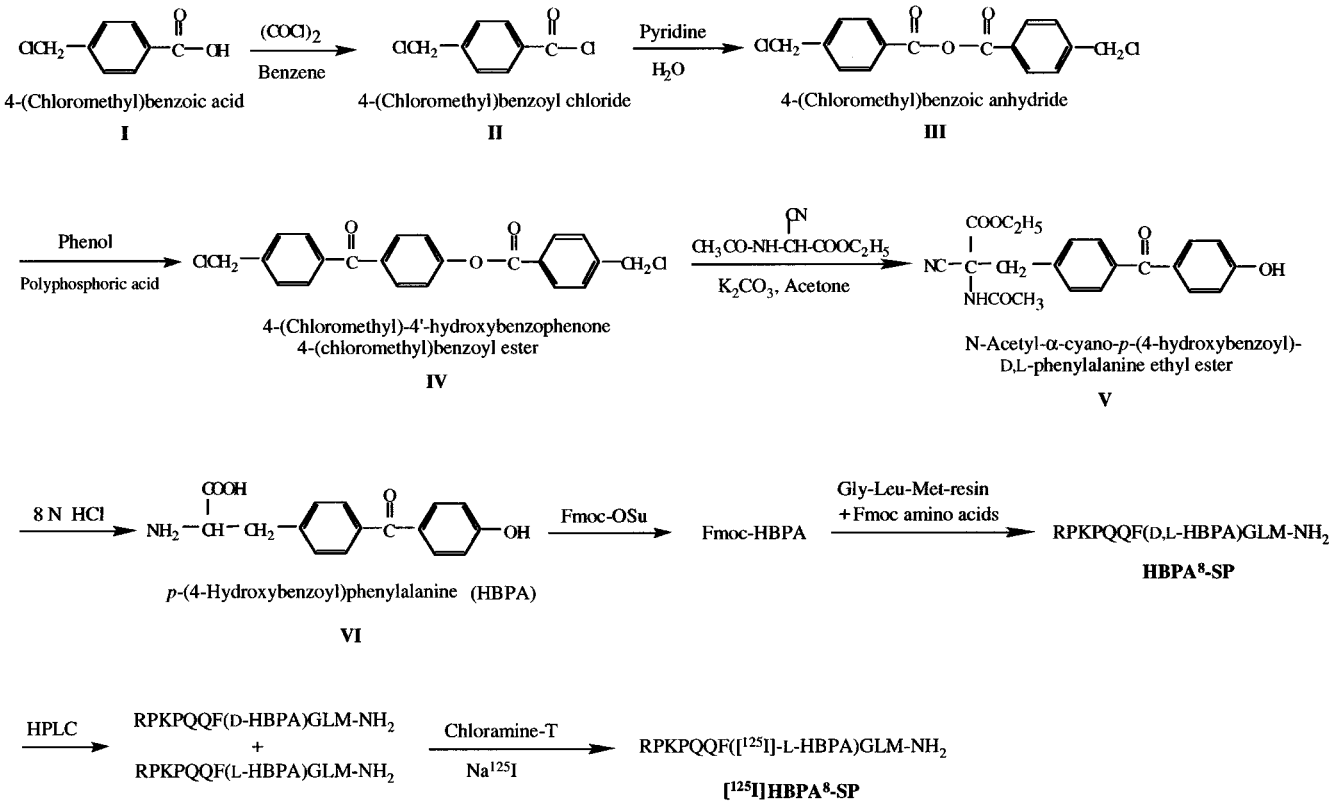

FIGURE 1: Scheme for synthesis of HBPA. 4-(Chloromethyl)benzoyl chloride (**II**) is made by refluxing 4-(chloromethyl)benzoic acid with oxalyl chloride in benzene. Mixing this compound with pyridine with gentle heating using a steam bath followed by ice quench gave 4-(chloromethyl)benzoic anhydride (**III**). 4-(Chloromethyl)benzoic anhydride (**III**) is reacted with phenol in polyphosphoric acid to give the 4-(chloromethyl)benzoyl ester of 4-(chloromethyl)-4′-hydroxybenzophenone (**IV**). Reacting **IV** with ethyl acetamidocyanoacetate and subsequent hydrolysis with concentrated HCl gives racemic *p*-(4-hydroxybenzoyl)phenylalanine (HBPA, **VI**). D,L-HBPA is derivatized using standard Fmoc chemistry for incorporation into the peptide (position 8 of substance P) by solid-phase synthesis. Radioiodination of L-HBPA^8-SP is accomplished using Chloramine-T. See Experimental Procedures for details.

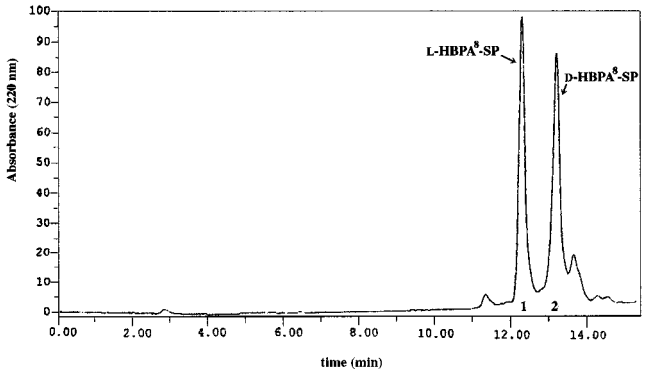

FIGURE 2: HPLC purification of HBPA^8-SP. The crude synthetic peptide ArgProLysProGlnGlnPhe(D,L-HBPA)GlyLeuMet-NH_2 was purified by HPLC on a Synchrom C_{18} (250 × 4.6 mm) column with a flow rate of 1.5 mL/min using a 5−65% acetonitrile gradient. The L-HBPA^8-SP diastereomer elutes at 51.0% acetonitrile and the D-HBPA^8-SP diastereomer elutes at 54.4% acetonitrile. The synthesis produced a racemic mixture consisting of 43.5% L-HBPA^8-SP, 44.7% D-HBPA^8-SP (quantitated by integration of UV peaks), and minor contaminants. Purification by HPLC produced optically pure L-HBPA^8-SP.

To separate the peptide from free ^{125}I, the mixture was diluted and acidified with 0.525 mL of 60 mM trifluoroacetic acid (TFA), with the addition of 25 μl of 2% bovine serum albumin solution to limit nonspecific adsorption. The mixture was then applied to an activated C_{18} Sep-Pak cartridge (Waters), and the iodide and peptide were eluted from the cartridge with a series of 0.5 mL portions of 10 mM TFA in increasing alcohol (ethanol:methanol, 1:1) content. The iodide eluted immediately, whereas the peptide was retained until the alcohol content reached about 60%. The peptide fractions (containing both oxidized and reduced methionine), eluting with 60−90% alcohol, were pooled and reduced in volume under a nitrogen stream. After the addition of 20% (v/v) β-mercaptoethanol, the sample was heated at 90 °C for 2 h to reduce methionine sulfoxide to native methionine. Further purification was achieved by reverse phase HPLC on a Vydac C_{18} column. The eluate was collected in fractions during shallow acetonitrile gradient elution, and the fractions were counted for radioactivity. The reduced (methionine) monoiodinated (HBPA) peptide eluted in a well-resolved peak (at 32% acetonitrile), predictably later than the original unlabeled peptide (at 30% acetonitrile) or the oxidized products, but prior to the reduced diiodinated peptide. The reduced monoiodinated [^{125}I]HBPA^8-SP (specific activity ~2000 Ci/mmol; 1 Ci = 37 GBq) was protected from oxidation with 0.5% (v/v) β-mercaptoethanol added immediately after purification and stored at −20 °C until use. The amount of free ^{125}I in the final tracer was determined by quantitative separation of free ^{125}I and the ^{125}I-peptide using an activated C_{18} Sep-Pak cartridge (Waters) as described above. The complete synthetic scheme is shown in Figure 1. HBPA^3-SP was synthesized, purified, and radiolabeled using the same method as described above.

Biochemical Procedures

Radioimmunoassay for D-HBPA^8-SP and L-HBPA^8-SP. Anti-SP antibody (Too et al., 1989), [^{125}I]BHSP, and D- or L-HBPA^8-SP were incubated in RIA buffer (50 mM sodium phosphate buffer, pH 7.5, with 0.1% BSA and 1 mM sodium

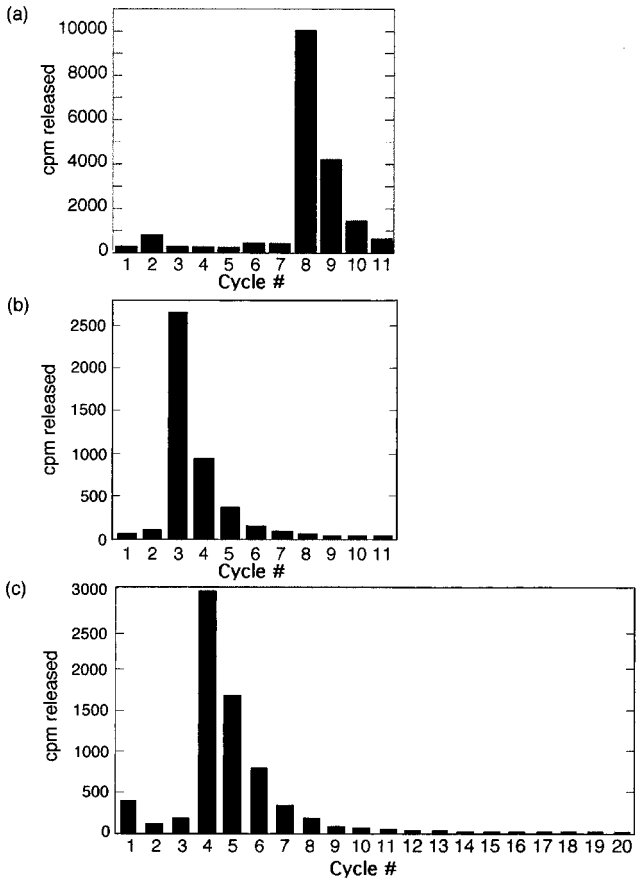

FIGURE 3: Sequence analysis of HBPA-SP peptides. (a) The counts from the peptide [^{125}I]HBPA^{8}-SP are released in cycle no. 8, corresponding to the HBPA amino acid, as expected. A minor lag in elution is observed in the following cycles. (b) The counts from [^{125}I]HBPA^{3}-SP are released in cycle no. 3, corresponding to the HBPA amino acid, as expected; and (c) the counts from the purified peptide fragment containing the BSA•HBPA^{3}-SP adduct are released in cycle 4 defining the photoinsertion site of [^{125}I]HBPA^{3}-SP into BSA. This release demonstrates that a PTH-HBPA•amino acid derivative can be released using standard Edman sequencing conditions.

azide) in a total volume of 0.4 mL. The titer of the antibody was determined by incubating 3.3 pM [^{125}I]BHSP with the anti-SP antibody in RIA buffer overnight at 4 °C. Assays were worked up at 4 °C by adding 0.5 mL of activated charcoal suspension (20 g/L activated charcoal in 50 mM sodium phosphate, pH 7.5, 10% heat-inactivated human serum, 1 mM sodium azide), incubating for 15 min, and centrifuging at 3000*g* for 15 min to separate bound tracer (supernatant) from free tracer (charcoal pellet). The displacement curve for HBPA^{8}-SP was determined at 1/160000 antibody titer with 3.3 pM [^{125}I]BHSP and L-HBPA^{8}-SP or D-HBPA^{8}-SP with incubation at 4 °C overnight. The assay was worked up as described above.

Curve fitting was performed using Kaleidagraph and fit by the following equation:

$$B = T_b(1/(1 + L/IC_{50})) + N_b$$

where B is the percent binding, T_b is the percent total binding, L is the ligand concentration, IC_{50} is the concentration at which 50% of the binding is displaced, and N_b is the percent nonspecific binding.

Binding Studies of [^{125}I]HBPA^{8}-SP. P388D$_1$ cells (a nontransfected murine macrophage/monocyte cell line (Dawe & Potter, 1957)) that express a high density of functional SP receptors (Li et al., 1995a; Persico et al., 1988; Li et al., 1995b) but no detectable levels of other tachykinin receptors (Too, H.-P., & Maggio, J. E., unpublished results)) were inoculated (5×10^5 cells/well) on FBS precoated 24-well plates and cultured overnight in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS. Protease inhibitors (0.004% bacitracin, 0.0002% chymostatin, 0.0004% leupeptin) and 0.1% BSA was added to the confluent cells ($\sim 1 \times 10^6$ cells/well in DMEM and 10% FBS) and incubated for 30 min at room temperature (25 °C). The cells were then washed twice (0.5 mL/well) with ice-cold buffer (DMEM + 20 mM HEPES, pH 7.2) and incubated with 0.5 mL of buffer on ice for at least 10 min in the presence of protease inhibitors and 0.1% BSA. The radioactive ligand ([^{125}I]HBPA^{8}-SP or [^{125}I]BHSP), in the presence or absence of unlabeled SP, CP-96,345 (a nonpeptide NK-1R antagonist), or D-HBPA^{8}SP was added to a final concentration of 25 pM ($\sim 8 \times 10^4$ cpm/mL) and incubated for 2 h. Nonspecific binding is defined as binding in the presence of 10 μM SP. After incubation, the cells were washed twice with 0.5 mL/well phosphate-buffered saline (104 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.2), and then solubilized by 0.5 mL of lysis buffer (1% Nonidet P-40, 0.2% SDS, 150 mM NaCl, 50 mM Tris, pH 8.0) for 20 min and transferred for γ counting. Unlabeled SP was stored as 10 mM stock solutions in dimethyl sulfoxide. Curve fitting was performed as described above. Binding studies were also performed with [^{125}I]HBPA^{3}-SP.

Photolabeling of SPR in P388D$_1$ Cells with [^{125}I]HBPA^{8}-SP. Photolysis was performed as previously described (Li et al., 1995a; Blanton et al., 1994) with the following modifications. P388D$_1$ cells (5×10^6 cells/5 mL) were inoculated on FBS-pretreated dishes (60 mm) in DMEM with 10% FBS and grown overnight. The cultured cells ($\sim 1 \times 10^7$) were incubated with protease inhibitors and 0.1% BSA for 30 min at room temperature (25 °C). The cells were then washed twice (5 mL) with ice-cold buffer (DMEM + 20 mM HEPES, pH 6.7) and incubated with 5 mL of buffer on ice for at least 10 min, in the presence of protease inhibitors and 0.1% BSA. The photolabile radioligand, in the presence or absence of unlabeled SP or CP-96,345, was added to a final concentration of 2 nM ($\sim 6 \times 10^6$ cpm/mL) and incubated 2 h at 4 °C. The dishes were then frozen by placement on crushed dry ice. The frozen sample was irradiated under nitrogen for 2 h using a focused HBO 100-W mercury short arc lamp through an optical filter to eliminate light below 310 nm. A second filter removed infrared wavelengths (>900 nm) to minimize sample heating during photolysis (Li et al., 1995a).

After photolysis, the sample was thawed, and any remaining attached cells were scraped from the bottom of the plate. The sample was transferred to microfuge tubes and centrifuged at 16000*g* for 30 min. The pelleted cells were hypotonically lysed by resuspending in 0.3 mL of 5 mM Tris-HCl, pH 8.0, for 15 min at room temperature. Then the samples were homogenized and centrifuged at 500 g for 15 min to remove debris. The resulting supernatants were sedimented at 16000*g* for 30 min and the membrane pellets stored at −20 °C until analysis. These experiments have also been performed with [^{125}I]HBPA^{3}-SP, giving similar results.

To confirm that [^{125}I]HBPA photo-cross-linked to an amino acid of a target protein could be released during Edman sequencing as the PTH derivative, a 10% BSA

solution was photolabeled with [^{125}I]HBPA^3-SP for 15 min on ice and digested with *Staphylococcus aureus* V8 protease, and the HPLC-purified labeled fragment was sequenced by Edman degradation.

Sequence Analysis. Amino-terminal sequence analysis was performed with an Applied Biosystems Model 477A protein sequencer, using gas-phase cycles. Samples of HPLC-purified radioiodinated and non-iodinated HBPA^8-SP were mixed and immobilized on chemically modified, glass fiber filter discs (Beckman Instruments) rather than Polybrene-treated filters to improve sequencing yields of hydrophobic peptides (Pedersen et al., 1992). [^{125}I]HBPA^3-SP and the labeled fragment from V8 enzymatic digests of [^{125}I]HBPA^3-SP photo-cross-linked to BSA were sequenced in the same manner. Initial yield (I_o) and repetitive yield (R) were calculated by nonlinear, least-squares regression of the observed release (M) for each cycle (n) using the equation $M = I_oR^n$.

Miscellaneous. SDS-PAGE was performed on 10% acrylamide gels according to Laemmli (1970). V8 protease digests of proteins were performed as described in Li et al. (1995a). Mass spectroscopy of HBPA and Fmoc-HBPA was performed by Harvard Microchem Facility (Cambridge, MA) using a Finnegan TSQ700 triple quadrupole mass spectrometer. Mass spectroscopy (MALDMS) of the HBPA-SP peptides was performed by Quality Controlled Biochemicals, Inc. (Hopkinton, MA). NMR spectra were recorded using a Varian Unity Spectrometer at 500 MHz in $CDCl_3$ using tetramethylsilane as an internal standard.

RESULTS

Synthesis and Characterization of HBPA. HBPA (**VI**) was successfully synthesized (Figure 1) in three steps from 4-(chloromethyl)benzoic anhydride (**III**). The Friedel−Crafts condensation of 4-(chloromethyl)benzoic anhydride (**III**) and phenol in polyphosphoric acid produced 4-(chloromethyl)-4-hydroxybenzophenone esterified with 4-(chloromethyl)benzoic acid (**IV**). The reaction of the ester intermediate with ethyl acetamidocyanoacetate under alkaline conditions resulted in the expected reaction with the cyano compound accompanied by the hydrolysis of the ester to give the major product **V**. Acid hydrolysis of the cyano intermediate **V** yielded the *p*-(4-hydroxybenzoyl)phenylalanine (**VI**) photoprobe. The amino acid analog was coupled for peptide synthesis using standard Fmoc strategy. Results from both NMR spectroscopy and elemental analysis on the intermediates **IV**, **V** and the final product **VI** were consistent with those expected for the compounds and confirmed using appropriate standards. Mass spectrometry on HBPA (**VI**), Fmoc-HBPA, L-HBPA^8-SP, and L-HBPA^3-SP gave the expected $(M + 1)^+$ peaks (286.1, 507.5, 1467, and 1487, respectively).

The synthesis of HBPA was first attempted by the route previously described for preparation of BPA (chlorination of the methyl group of *p*-methylbenzophenone; Kauer et al., 1986) and by several similar routes. All these routes failed due to the chlorination of the aromatic ring rather than the desired chlorination of the methyl group of the various starting materials.

The amino acid analog (HBPA) was easily incorporated into positions 3 and 8 of the neuropeptide substance P by standard solid-phase synthesis. Both D,L-HBPA^8-SP and D,L-HBPA^3-SP were synthesized; separation of the diastereomers was easily accomplished using RP-HPLC (Figure 2). The identities of the peptides were confirmed by amino acid analysis, sequencing (Figure 3), immunochemistry (Figure 5), and receptor binding (Figure 6). Radioiodination of the L-diastereomer at the HBPA amino acid was accomplished using Chloramine-T and confirmed by sequencing of the radiolabeled peptide. Sequencing of [^{125}I]HBPA^8-SP both before and after photolysis showed the majority of the counts associated with cycle no. 8 with a slight lag in cycles no. 9 and no. 10 (Figure 3a). Edman degradation of [^{125}I]HBPA^3-SP resulted in a major release of counts in cycle no. 3 (Figure 3b), as expected. To show feasibility of the proposed method of analysis, a concentrated solution of BSA (10%) was photolabeled with [^{125}I]HBPA^3-SP. The photolabel inserted into a single site as determined by both SDS-PAGE and RP-HPLC after V8 enzymatic digestion of the sample and subsequently by sequencing. This experiment demonstrated the ability of the HBPA•amino acid adduct to be released in sequencing as a PTH derivative. The PTH derivative of the photoinserted HBPA•BSA adduct is released in cycle no. 4 (Figure 3c), demonstrating the potential utility of this method in mapping binding sites. PTH-HBPA was observed under standard Edman sequencing conditions at 15.3 min (Figure 4), between valine and tryptophan. In contrast, PTH-BPA was not detectable (Li et al., 1995a; Blanton et al., 1994). After radioiodination, the peptides were stable for both binding and photolabeling studies for more than a month. Additionally, the amount of free iodide present after one month of storage at −20 °C was less than 1%.

Binding Studies with [^{125}I]HBPA^8-SP. The binding of HBPA^8-SP was first studied using a C-terminal directed antibody to SP (Too et al., 1989) to confirm the identities of the D- and L-diastereomers. A displacement curve for [^{125}I]BHSP by HBPA^8-SP was determined by radioimmunoassay using this SP antibody (Figure 5). The IC_{50} for HBPA^8-SP HPLC peak 1 was 52 ± 8 pM and for HPLC peak 2 was 390 ± 40 pM, as compared to that for SP at 71 ± 6 pM. Thus, peak 1 was confirmed to be the L-HBPA^8-SP diastereomer and peak 2 the D-HBPA^8-SP diastereomer, which is consistent with the HPLC results. Marfey's reagent (Marfey, 1984) has also been used to determine the absolute identity of the diastereomers (Blanton et al., 1994).

Binding studies of the peptide analog with SPR in P388D$_1$ cells confirmed that the photoprobe was a high affinity ligand for SPR as well as the identity of the D- and L-diastereomers. [^{125}I]-L-HBPA^8-SP bound to SPR was displaced by SP and displayed a typical high affinity binding curve for SPR in P388D$_1$ cells (Figure 6). The IC_{50} for SP displacement of [^{125}I]-L-HBPA^8-SP was determined by nonlinear least squares analysis to be 0.11 ± 0.01 nM from four independent experiments, whereas the IC_{50} for D-HBPA^8-SP displacement of [^{125}I]BHSP was 1.50 nm ± 0.34 nM; the IC_{50} for SP displacement of [^{125}I]BHSP was found to be 0.28 ± 0.07 nM. Further binding studies (not shown) with these ligands and others are also consistent with the conclusion that L-HBPA^8-SP and [^{125}I]HBPA^8-SP, like L-BPA^8-SP (Li et al., 1995a), bind with high affinity and specificity to SPR. Results with HBPA substituted in position 3 of the SP peptide also demonstrates high affinity for SPR (not shown).

Photoaffinity Labeling of SP Receptor. Prior to synthesis of HBPA, to address concerns about the possible loss of radioiodine from the new photophore during photolysis, [^{125}I]-3-iodo-4-hydroxybenzophenone was prepared from 4-hydroxybenzophenone by standard oxidative radioiodina-

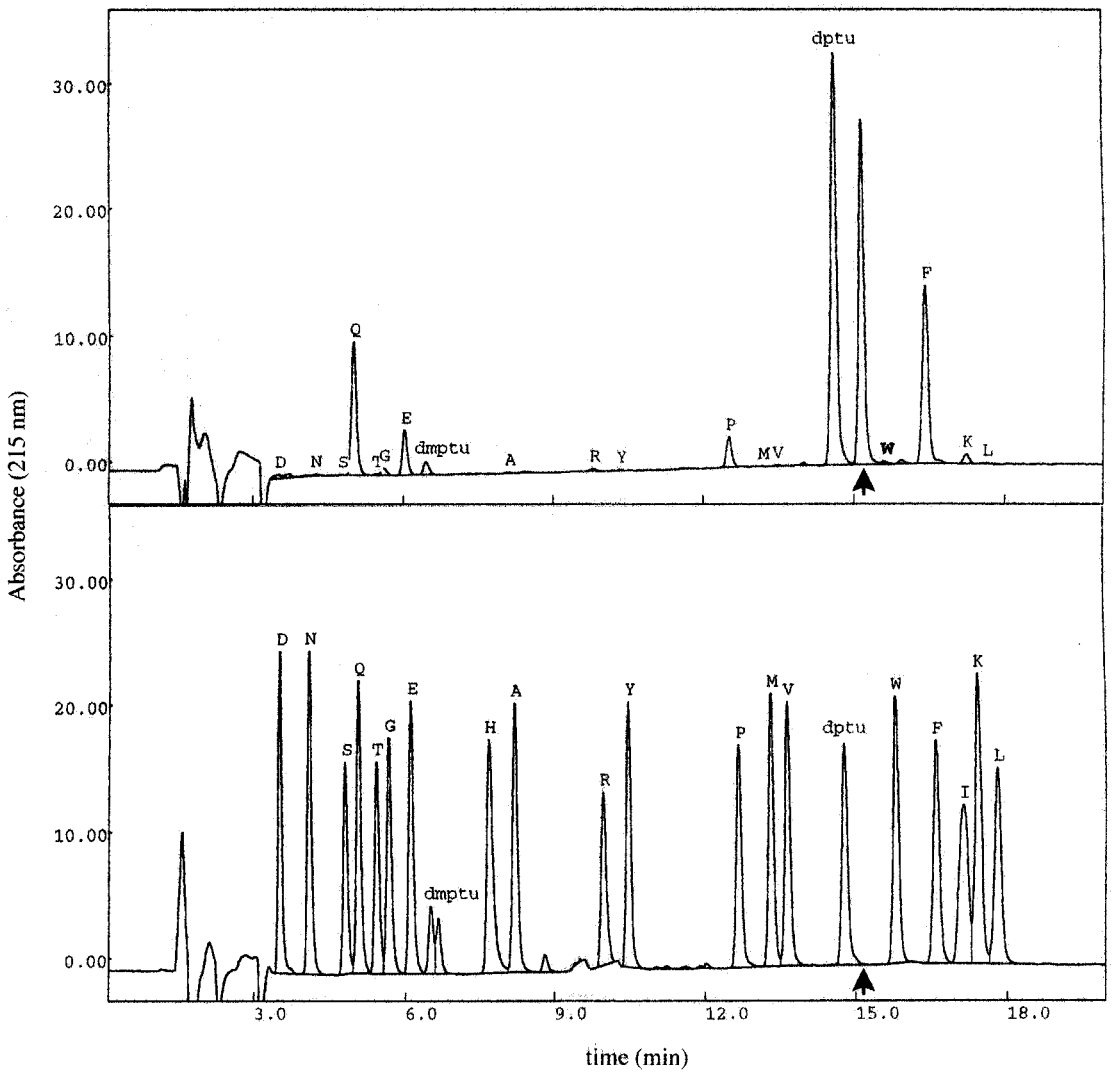

FIGURE 4: Elution pattern from sequencer for PTH•HBPA. The PTH derivative of HBPA elutes at 15.3 min in a standard Edman degradation sequencing run as shown by the arrow in this cycle (no. 8; top panel) of the sequencing of HBPA[8]-SP. This position does not overlap with any other amino acids as seen in the PTH standards (bottom panel), allowing easy detection of the analog. In contrast, PTH-BPA is not detectable. PTH-amino acids are indicated using standard one-letter abbreviations; dptu and dmptu are side products of Edman sequencing.

tion. The molecule was then subjected to photolysis under conditions identical to those used for our receptor photolabeling with [^{125}I]BPA-SP (Li et al., 1995a), but using twice the photolysis time. After photolysis, greater than 98% of the radioiodine remained incorporated in the starting material. Using these same conditions, which gave efficient cross-linking using BPA[8]-SP, only modest photolabeling of SPR by [^{125}I]HBPA[8]-SP was observed. Thus, it was desirable to modify the photolysis conditions to increase the efficiency of photolabeling SPR in P388D$_1$ cells with [^{125}I]HBPA[8]-SP. Increasing the time of photolysis increased the incorporation of [^{125}I]HBPA[8]-SP only negligibly (from $<$1% at 15 min to 2.8% incorporation at 2 h). Freezing the sample prior to and during photolysis resulted in a significant increase in incorporation (to 2.9% for 15 min and 30% for 2 h photolyses). A major broad band ($\sim$98% of total counts) at $\approx$50$-$75 kDa was apparent on SDS-PAGE after photoinsertion of [^{125}I]HBPA[8]-SP in P388D$_1$ cells, with a minor band of the unincorporated [^{125}I]HBPA[8]-SP at the gel front (Figure 7). The broad band is a result of the heterogeneous glycosylation of SP receptor (Li et al., 1995a; Vigna et al., 1994). The photolabeling was completely inhibited by the presence of excess unlabeled SP or a specific nonpeptide SPR antagonist CP-96,345 in parallel experiments (Figure 7), showing specificity. No incorporation of the peptide was observed upon incubation of cells with [^{125}I]HBPA[8]-SP in the dark. Experiments performed with [^{125}I]HBPA[3]-SP gave similar results but with lower levels of incorporation.

DISCUSSION

We describe in this paper the first reported synthesis of a benzophenone-based photoactivatable amino acid (HBPA) that can be incorporated into peptides by solid-phase synthesis and subsequently radiolabeled to quantitative specific activity with ^{125}I. The ability to radioiodinate the photoreactive residue of a peptide ligand provides a distinct advantage over previous photolabeling methods by allowing the identification of the exact amino acid at the photoinsertion site on the receptor as opposed to the mere identification of a fragment or region of the receptor. The strategy of putting ^{125}I within a photoreactive moiety was introduced by Ruoho and co-workers (Lownder et al., 1988; Morris et al., 1991) for iodoazidophenyl derivatives. HBPA adds to this concept the significant advantages of a photolabile amino acid which can be placed anywhere in a peptide ligand during synthesis and the significant advantage of benzophenone photochemistry, providing an excellent tool for mapping binding sites. The specific interactions between each residue of the peptide ligand and the binding-site amino acids of the receptor can thus be identified by replacing each amino acid of the

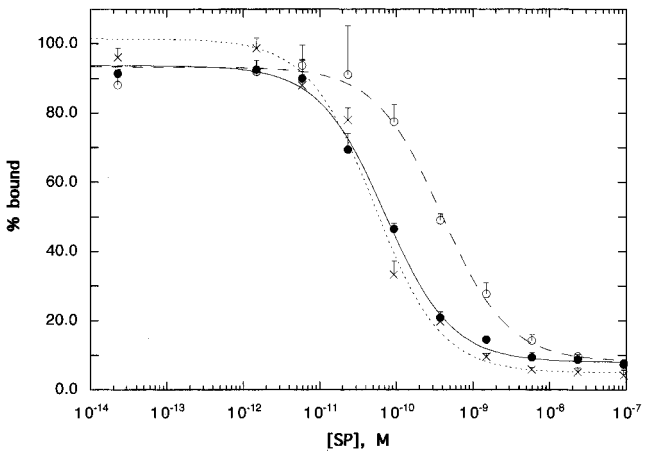

FIGURE 5: Binding of HBPA[8]-SP to anti-substance P antibody. [^{125}I]BHSP was displaced from substance P antibody by the D- and L-diastereomers of HBPA[8]-SP. (●) L-HBPA[8]-SP binds to the antibody with a higher affinity than (○) D-HBPA[8]-SP. The IC_{50} for L-HBPA[8]-SP is 52 ± 8 pM which is very similar to that determined for SP (71 ± 6 pM; (×)). The IC_{50} measured for the D-isomer is significantly larger (∼8×) at 390 ± 40 pM. Anti-substance P antibody was used at a dilution of 1/160000; [^{125}I]BHSP = 3.3 pM. IC_{50} is expressed as the mean ± standard deviation of ≥3 experiments.

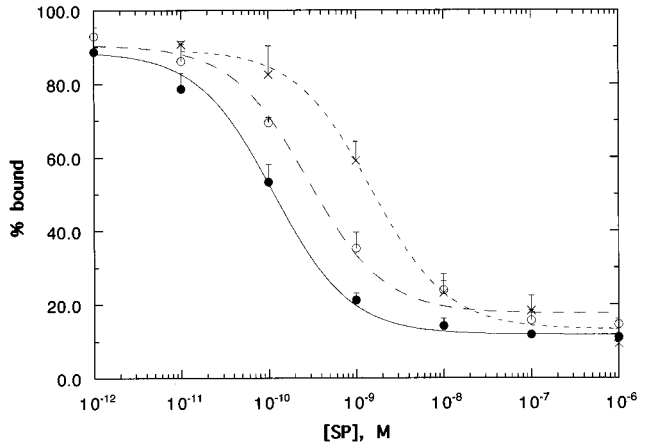

FIGURE 6: Binding of [^{125}I]HBPA[8]-SP to SPR. After pre-incubation on ice with protease inhibitors and BSA, the cells (∼1 × 10^6 cells/sample) were incubated for 2 h on ice with tracer (25 pM [^{125}I]-L-HBPA[8]-SP (●) or [^{125}I]BHSP) (○)) and displaced by SP, or with 25 pM [^{125}I]BHSP and displaced by D-HBPA[8]-SP (×). Percentage of tracer bound was determined by solubilization and counting after washing away excess unbound tracer. IC_{50} for displacement by SP of [^{125}I]-L-HBPA[8]-SP is 0.11 ± 0.01 nM and of [^{125}I]BHSP is 0.28 ± 0.07 nM, and for displacement by D-HBPA[8]-SP of [^{125}I]BHSP is 1.50 ± 0.34 nM. The data shown is the average of four experiments containing triplicate samples in each. IC_{50} is expressed as the mean ± standard deviation of ≥4 experiments.

peptide. We refer to this method as the *p*hoto*a*ffinity *r*esidue *s*canning and *e*lucidation (PARSE technique). Additionally, unlike BPA, HBPA is easily identified using standard peptide sequencing methodology (Figure 4), providing the options of detection by standard sequencing technology or radioisotope tracking of the mapping process. This resolution of HBPA (but not BPA) in the sequencing cycle is most likely due to the differences in polarity between HBPA and BPA. If the additional hydroxyl group on HBPA affects the elution pattern of HBPA in a manner similar to that of tyrosine (10.4 min) and phenylalanine (16.5 min), then the BPA would fail to elute under standard conditions (Figure 4). The retention of the ^{125}I through sequencing and release (Figure 3c) until the photoinsertion site is identified is extremely advantageous, especially in systems where receptor quantity is very limited. The adduct of the photolabel and the photoinserted amino acid after cross-linking and enzymatic digestion is observed by Edman degradation as detected by count release of the PTH derivative (Figure 3c).

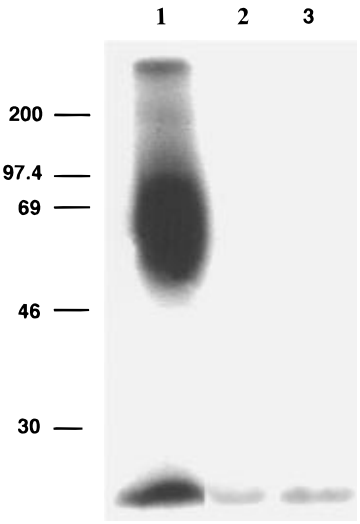

FIGURE 7: Photolabeling of SPR with [^{125}I]HBPA[8]-SP. SPR in P388D_1 cells was labeled by irradiation with UV light for 2 h on dry ice under a nitrogen stream of samples preincubated on ice with 2 nM [^{125}I]HBPA[8]-SP for 2 h in the absence (lane 1) or presence of 10 μM SP (lane 2) or 10 μM CP-96,345 (lane 3). A major broad band of ≈50−75 kDa representing glycosylated SPR is labeled. A minor band representing unincorporated [^{125}I]HBPA[8]-SP runs at the dye front. Labeling is abolished in the presence of cold SP (lane 2) or the specific nonpeptide SPR antagonist CP-96,345 (lane 3). Molecular weight standards: myosin (200 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (69 kDa), ovalbumin (46 kDa), and carbonic anhydrase (30 kDa).

Benzoylphenylalanine-containing analogs of SP efficiently label the NK-1 receptor (Li et al., 1995a; Boyd et al., 1991a,b, 1994, 1996; Kage et al., 1993) and were successful in identifying the regions of insertion of SP into SP receptor (Li et al., 1995a; Boyd et al., 1996; Girault et al., 1996). Agonist-binding domains of the receptor, directly identified by photoaffinity labeling (Li et al., 1995a), did not match those identified as necessary for binding by mutagenesis (Huang et al., 1994a), demonstrating the importance of the photolabeling technique by providing novel information that was complementary to that provided from mutagenesis. A BPA analog of SP has also been employed to identify the region of the nicotinic acetylcholine receptor which interacts with SP (Blanton et al., 1994).

In the first use of BPA for peptide photoaffinity labeling (Kauer et al., 1986), an optically pure peptide ligand was produced by chemical acetylation and enzymatic deacetylation prior to peptide synthesis to resolve L-BPA from D-BPA. Our subsequent work (Li et al., 1995a; Blanton et al., 1994) showed that this step is unnecessary in the case of substance P, as the racemic BPA can be used directly in peptide synthesis and the resulting diastereomeric peptides resolved by RP-HPLC during peptide purification. This result and work by others (Williams & Shoelson, 1993; Shoelson et al., 1993; Miller & Kaiser, 1988) incorporating BPA into other peptides suggests that the resolution of racemic BPA prior to peptide synthesis is generally unnecessary, allowing the more efficient approach of omitting the yield-reducing and somewhat cumbersome steps required to resolve the amino acid. With HBPA, as with BPA (Li et al., 1995a; Blanton et al., 1994; Williams & Shoelson, 1993;

Shoelson et al., 1993; Miller & Kaiser, 1988), resolution of the amino acid prior to solid-phase peptide synthesis of the photolabile SP peptide analog is not required, and resolution of the optically pure peptides is instead carried out by RP-HPLC after its synthesis (Figure 2).

The photoreactive analog of SP was easily radiolabeled to the high specific activity (2000 Ci/mmol) required for studies of peptide receptors by oxidative iodination of the HBPA residue at position 8 (or 3) with $Na^{125}I$ using established methods. Concern that the bulkiness of the HBPA side chain, before or after iodination, might result in a ligand of lower affinity proved unfounded, as the affinity of L-HBPA^8-SP and [^{125}I]-L-HBPA^8-SP for the murine SP receptor was similar to that of native SP (Figure 6). Additionally, the affinity of L-HBPA^8-SP and [^{125}I]-L-HBPA^8-SP for anti-SP antiserum was similar to that of native SP. Both the antibody (Too et al., 1989) and SPR (Maggio, 1994) recognize the C-terminal 5 amino acids of SP (−PhePheGlyLeuMet−NH_2). Both antiserum (Figure 5) and receptor (Figure 6) demonstrated the expected significant preference of L- over D-stereochemistry. Displacement studies (Figures 6 and 7) with unlabeled SP and a nonpeptide antagonist of NK-1/SP receptor demonstrated that L-HBPA^8-SP, like L-BPA^8-SP (Li et al., 1995a), is a specific as well as a high affinity ligand for the receptor. Although the bulkiness of HBPA is well tolerated at several positions in SP, tolerance of substitutions in the general case is both position and ligand/receptor specific. There are positions in some peptides which may not accept the bulk of this amino acid derivative, presenting a limitation to the photoscanning method described above (or any such method).

Concern that [^{125}I]-L-HBPA^8-SP might lose radioiodine during receptor photolabeling and subsequent workup or react to photolysis very differently than L-BPA^8-SP also proved unfounded, as covalent incorporation of ^{125}I into SP receptor proceeded with high efficiency (Figure 7). Quantitation of the ^{125}I and sequencing of the radiolabeled HBPA-SP peptides before and after photolysis showed little loss of ^{125}I. Further support for the photochemical stability of the radioiodine in the hydroxybenzoyl ring was shown by Koch et al. (1994).

Leyva and co-workers (1991) have studied the photochemistry of several photolabeling reagents in the frozen state. Potential advantages of using the frozen state for photolabeling have included slowing of the rapid diffusion of the ligand out of the binding site (Leyva et al., 1991), slowing the rapid loss of iodine observed at warmer temperatures (Leyva et al., 1991; Watt et al., 1989), and selective enhancement of triplet or singlet states giving greater incorporation efficiency (Leyva et al., 1986, 1991). The greater efficiency of incorporation of radioiodinated BPA^3-SP or BPA^8-SP into SPR (Li et al., 1995a) and [^{125}I]HBPA^3-SP into BSA compared to incorporation of [^{125}I]HBPA^3-SP or [^{125}I]HBPA^8-SP into SPR provides some insight into the effects of the frozen state on this system. Both [^{125}I]HBPA^3-SP and [^{125}I]HBPA^8-SP are specific high affinity ligands for SPR, are stable during photolysis to loss of radioiodine, and have a slow off-rate from SPR (not shown). The advantage of using the frozen state with the SPR system could thus result from a steric interaction between the HBPA-SP ligand and SPR which is not observed with BPA-SP and SPR or with HBPA-SP and BSA.

The potential utility for HBPA is underscored by the wide range of peptide receptors that have been the target of photoaffinity labeling with BPA (Adams et al., 1995; Behar et al., 1996; Blanton et al., 1994; Bosse et al., 1993; Boyd et al., 1991a,b, 1994, 1996; Gao et al., 1995; Garcia et al., 1994; Gergel et al., 1994; Girault et al., 1996; Hampe et al., 1996; Kage et al., 1993; Kauer et al., 1986; Keutmann & Rubin, 1993; Li et al., 1995a; Macdonald et al., 1996; McNicoll et al., 1992; Miller & Kaiser, 1988; Nakamoto et al., 1995; O'Neil et al., 1989; Servant et al., 1993; Shoelson et al., 1993; Yamada et al., 1995; Zhang et al., 1996). This novel amino acid may allow the exact site(s) of interaction(s) between peptide and protein receptors of low abundance to be identified in many systems. Its high insertion yield (≈30% in the present example) is especially useful for systems like the neuropeptide substance P which have very low levels of receptor available for study. Although Stull and co-workers (Gao et al., 1995) have identified specific sites of interaction between a [^{3}H]-acetyl-BPA peptide substrate and myosin light chain kinase, this was only possible because BPA was the N-terminal amino acid of the substrate and the target protein was soluble and abundantly available from an overexpressed system. In contrast, HBPA may allow identification of insertion sites on rare proteins with the photolabile amino acid located at any position within the photoligand. Other systems which have significantly lower affinities (i.e., the interaction of SP with an allosteric site on the nicotinic acetylcholine receptor, Blanton et al., 1994) will also find this analog useful in mapping the specific binding site by retaining the advantageous properties associated with BPA (high photoinsertion efficiency, inertness to water) but adding the ability to incorporate a high specific activity label into the photoreactive residue.

The synthesis of HBPA opens a new era in studying peptide receptor interactions by allowing the concept of photoaffinity scanning to be more fully realized. It is the first amino acid that may allow the identification of specific interactions between a receptor of low abundance and a specific residue of its peptide ligand because the photoreactive residue can be radioiodinated while retaining the advantages of benzophenone photochemistry. By synthesis of a series of peptides for cross-linking, it will facilitate construction of a detailed interaction map of peptide receptors and their ligands. The peptides used here, HBPA-containing analogs of SP, bind with approximately the same potency and pharmacological specificity as the native agonist. The ligands are radioiodinatable after solid-phase synthesis and cross-link with high efficiency, and the specific photoinsertion site in the primary sequence of the receptor may be identifiable because the radiolabel does not have to be placed at a site distal to the photoreactive moiety. The novel amino acid, HBPA, can be synthesized in bulk, incorporated into peptides in any position using standard Fmoc chemistry, radioiodinated, photo-cross-linked, and released as a PTH derivative. These properties significantly enhance the ability to detect specific insertion sites of peptide ligands into receptors of low abundance.

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