Modular Design of Biotinylated Photoaffinity Probes: Synthesis and Utilization of a Biotinylated Pepstatin Photoprobe

Bryant A. Gilbert[†] and Robert R. Rando*

Contribution from the Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, 240 Longwood Avenue, Boston, Massachusetts 02115

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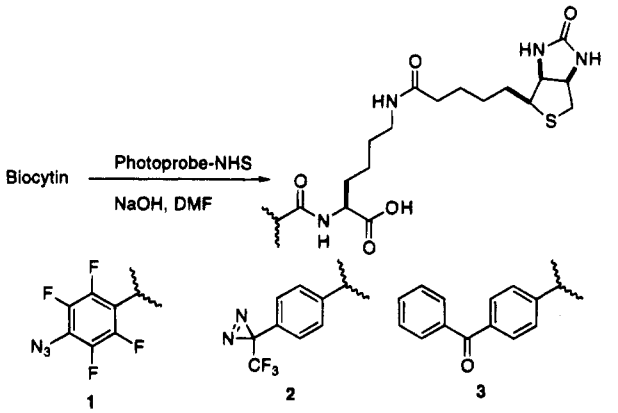
Abstract: A novel modular design is presented for the introduction of biotinylated photoprobes containing either 4-azidotetrafluorobenzamide, 4-(1-azi-2,2,2-trifluoroethyl)benzamide, or 4-benzoylbenzoylamide. The use of biotinylated affinity labels offers several advantages over radiolabeled probes by virtue of their exploitation of the biotin–avidin system of detection and purification. A biotinylated benzoylbenzoyl photoprobe of pepstatin (BBB-pepstatin, **5**) was synthesized in three steps from pepstatin. The photoprobe is a competitive inhibitor of porcine pepsin, with an apparent dissociation constant of 31 pM. Western blotting of BBB-pepstatin-photolabeled porcine pepsin, renin, cathepsin D and human renin, and cathepsin D could be detected with an avidin–horseradish peroxidase label. Routinely, 7 pM of aspartic protease could be photolabeled and detected with this system. The pepstatin photoaffinity probe is also very selective; the probe failed to label cysteine protease (papain), metalloprotease (carboxypepitase A), and serine protease (chymotrypsin and trypsin). To further establish the utility of the biotinylated probe, BBB-pepstatin-photolabeled porcine pepsin was purified by monomeric avidin chromatography. This probe should be useful for the identification of unknown cytosolic and membrane-bound aspartic proteases.

Introduction

Photoaffinity labeling is an important biochemical method for the covalent mapping of ligand receptors and enzyme active sites.[1,2] The main properties desired of a photoactivatable group are chemical stability prior to photoactivation and the photogeneration of highly reactive intermediates.[3] While a large number of photoactivatable reagents have been described in the literature, three families of probes are commonly in use: the aryl azides,[4] the aryl diazirines,[5] and the aryl ketones.[6] The judicious choice of which photolabile group to use will depend on the particular application being considered. This inherent "trial and error" approach to photoaffinity probe design not only is a synthetically intensive task but is prohibitively costly if radiolabeled probes of high specific activity are required. We report here the modular design and synthesis of biotinylated photoprobes containing the three different photoaffinity labeling moieties (Scheme 1). Our design provides the flexibility to introduce the photolabel at either the N or C terminus of an active-site ligand and allows for the nonradioactive detection of photolabeled proteins via biotin–avidin conjugated technologies.[7] Avidin binds biotin with a K_d of 10^{-15} M,[8] allowing for the detection of exceedingly small amounts of probe. Moreover, the biotin label also allows for the purification of the labeled protein using avidin affinity chromatography.[7] This methodology is applied to the synthesis of an aryl ketone photoprobe

Scheme 1



based on pepstatin[9] for the photolabeling, detection, and purification of aspartic proteases. This class of proteases process active-site aspartic residues which act as acid–base catalysts during proteolysis. Aspartic proteases were chosen as initial targets for two reasons: (1) there are no generally useful affinity labeling reagents available for them, and (2) aspartic proteases, which include renin[10] and the HIV protease, are of great pharmacological importance.[11]

Results and Discussion

Pepstatin strongly inhibits several aspartic proteases, including cathepsin D,[12] pepsin,[13] renin,[14] and the HIV protease.[11] The tight binding and high specificity of pepstatin and its analogs

* To whom correspondence should be addressed.
[†] Current address: Department of Chemistry, Portland State University, Portland, OR 97207-0751.
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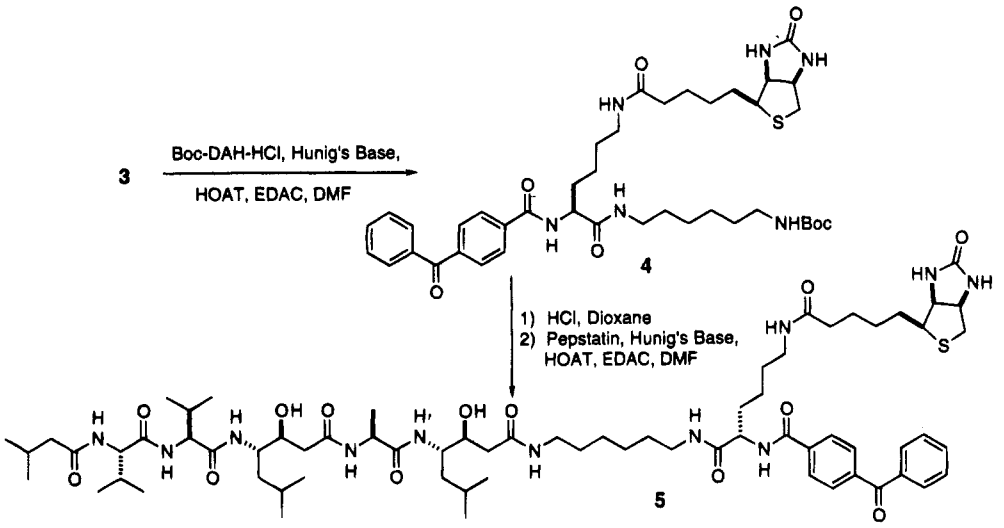
Scheme 2



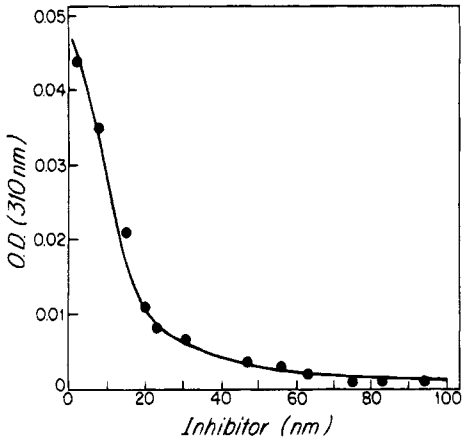


Figure 1. Titration of porcine pepsin activity with BBB-pepstatin (**3**). The assays were started by the addition of substrate (89 μM) to a preincubated mixture of pepsin (23 nM) at various concentrations of photoprobe **3**. Each point represents the absorbance after 5 min and is the mean of three determinations as described in the Experimental Section.

has led to the speculation that the statine (4-amino-3-hydroxy-6-methylheptanoyl) moiety of the inhibitor may serve as a transition state analog of these enzymes.[15] It is anticipated that incorporation of the statine functionality into a photoaffinity label would generate a highly specific tight binding inhibitor for the labeling of aspartic proteases. To establish our modular design of biotinylated photoprobes as useful tools for the biochemist, we have incorporated the 4-benzoylbenzoate **3** moiety into a pepstatin probe. The treatment of commercially available biocytin in aqueous NaOH (1.1 equiv) with the *N*-hydroxysuccinimide ester (1.1 equiv) of 4-azidotetrafluorobenzoate,[16] 4-(1-azi-2,2,2-trifluoroethyl)benzoate,[17] or 4-benzoylbenzoate[18] provided the corresponding biocytin photoprobes **1**, **2**, and **3** in excellent yields (Scheme 1). Incorporation of the biotinylated benzoylbenzoyl photoprobe **3** into the C terminus of pepstatin required the introduction of a diaminohexyl spacer to reverse the polarity of the peptide coupling (Scheme 2). EDAC (1.2 equiv)-mediated condensation of **3** with *N*-Boc-diaminohexane–HCl (1.1 equiv) in the presence of HOAT[19] (1.3 equiv) and Hünig's base (1.2 equiv) provided the *N*-Boc-protected probe **4** in 86% yield. Deprotection of **4** with 4 N HCl–dioxane generated the corresponding HCl salt, which was coupled to pepstatin with EDAC (1.2 equiv), HOAT (1.3 equiv), and Hünig's base (1.2 equiv) to provide the biotinylated benzoylbenzoyl pepstatin photoprobe (**5**, BBB-pepstatin) in 65% yield.

The inhibition curve for the titration of pepsin activity with BBB-pepstatin is shown in Figure 1. The shape of the curve indicates that the probe forms a tight 1:1 complex with the enzyme, similar to the complex formed between pepstatin and pepsin.[17] The pepsin activity was assayed at the absorbance of 310 nm for the hydrolysis of the (*p*-nitro-Phe)–Phe bond in the pepsin substrate H-Phe-Gly-(*p*-nitro-Phe)-Phe-Ala-Phe-OMe developed by Medzihradszky and co-workers.[20] The apparent dissociation constant (K_d) for the complex was calculated using the residual enzymatic activity in the region of stoicheiometric inhibition, i.e. when the concentration of enzyme is equal to the concentration of probe. Using the method developed by Green and Work,[21] the apparent K_d of the BBB-pepstatin–pepsin complex is 31 pM, comparing favorably to the 45 pM K_d of pepstatin–pepsin.[22]

The photoaffinity labeling of porcine and human aspartic proteases with BBB-pepstatin is summarized in Figures 2 and 3. Figure 2 shows the SDS-PAGE Coomassie Blue stained gel (A) and its Western blot (B) of photolabeled porcine pepsin (lanes 1–3), renin (lanes 4–6), and cathepsin D (lanes 7–9). The probe was preincubated with the enzymes for 15 min before photolysis. All photolysis reactions were conducted in Pyrex test tubes. The samples were irradiated for 30 min at 0 °C with a uranium-filtered 450 W Hanovia high-pressure mercury lamp. The samples were concentrated before electrophoresis on 12.5% (w/v) polyacrylamide gel (run under nonreducing denaturing conditions). The protein was transferred from the SDS-PAGE gel to nitrocellulose using a Trans-Blot Semi-Dry Transfer Cell system from Bio-Rad. The blotted biotinylated proteins were detected by the exposure of photographic film to the chemiluminescence of luminol oxidation by avidin-conjugated horseradish peroxidase.[23] The probe is able to label and detect these aspartic proteases without the use of radioactivity (lanes 2, 5, and 8). To demonstrate that the photoprobe was labeling the

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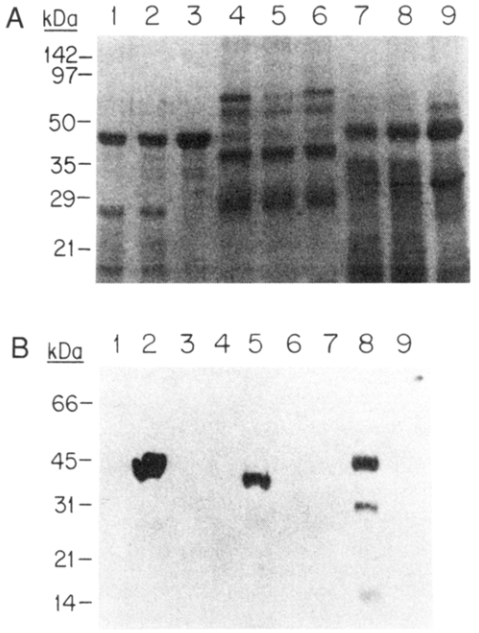

Figure 2. Photochemical labeling of porcine aspartic proteases pepsin (35 kDa), renin (36 kDa), and cathepsin D (43 kDa) with BBB-pepstatin (**3**). The proteases were photolabeled with the biotinylated photoprobe (300 nM) before electrophoresis on 12.5% (w/v) polyacrylamide gel (run under nonreducing denaturing conditions). After electrophoretic transfer, the biotinylated proteins were revealed with avidin−horseradish peroxidase as described in the Experimental Section. (A) The Coomassie Blue stained SDS-PAGE gel of the photolabeled proteases: lane 1, prephotolysis of photoprobe + pepsin; lane 2, photolysis of photoprobe + pepsin; lane 3, pepstatin + pepsin, then probe + photolysis. Lanes 4−6 and lanes 7−9 represent renin and cathepsin D samples that were treated in the same way as the pepsin samples applied to lanes 1−3. The multiple bands observed for renin and cathepsin D are due to either impurities in the commercial preparations and/or autoproteolysis of the samples. (B) The Western-blot analysis of the photolabeled proteases. Lanes 2, 5, and 8 represent BBB-pepstatin photolabeled pepsin, renin, and cathepsin D as detected by avidin-conjugated horseradish peroxidase. The weakly detected lower molecular mass band in lane 8 probably represents a proteolytic generated fragment of cathepsin D's active site.

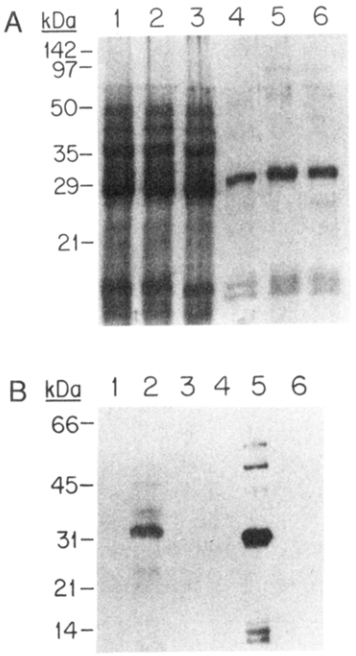

Figure 3. Photochemical labeling of human aspartic proteases renin (44 kDa) and cathepsin D (45 kDa) with BBB-pepstatin (**3**). The proteases were photolabeled and detected as described in the Experimental Section. (A) The Coomassie Blue stained SDS-PAGE gel of the photolabeled proteases: lane 1, prephotolysis of photoprobe + renin; lane 2, photolysis of photoprobe + renin; lane 3, pepstatin + renin, then probe + photolysis. Lanes 4−6 represent cathepsin D samples that were treated in the same manor as for renin. The multiple bands observed for renin and cathepsin D are due to impurities in the commercial preparations. (B) The Western-blot analysis of the photolabeled proteases. Lanes 2 and 5 represent BBB-pepstatin photolabeled renin and cathepsin D as detected by avidin-conjugated horseradish peroxidase. The weakly detected higher and lower molecular mass bands in lane 5 probably represent fragments of cathepsin D's active site.

enzyme via the short-lived photogenerated triplet biradical, the probe was first irradiated in solution and then the enzyme was added in the dark (lanes 1, 4, and 6). To prove that the probe was labeling the enzyme by specific binding at its active site and not by a nonspecific interaction, the enzyme was first preincubated with pepstatin (a specific active-site inhibitor of the enzyme) before introduction of the photoprobe and photolysis (lanes 3, 6, and 9). The labeling reaction is very specific for the pepstatin binding site (lanes 3 6, and 9) and does not involve a long-lived photogenerated reactive intermediate (lanes 1, 4, and 6). Similar results were also obtained for the BBB-pepstatin photolabeling of human renin and cathepsin D (Figure 3). Human cathepsin D is a lysosomal enzyme that is isolated from the spleen as a heterogenous mixture of single-chain (45 kDa) and double-chain (31 and 14 kDa) isozymes.[24] The Western-blot analysis of the photolabeled protease (Figure 3B, lane 5), clearly indicates the presence of the single-chain (45 kDa) protein in the commercial preparation. Moreover, the 31 kDa fragment was predominantly labeled over the 14 kDa band by the photoprobe, suggesting that the former fragment might contain the enzyme's active site. Human renin[25] has also been shown to exist as mixture of single-chain (44 kDa) and double-chain forms analogous to cathepsin D. Fusek and co-workers[25] have postulated that, through immunoblotting with the anti-human renin antibody, the double-chain form contains a 22 and 18 kDa fragment. Our Western-blot analysis of the photolabeled protease (Figure 3B, lane 2), also labeled a band at 44 kDa, but in contrast to the immunoblotting, we find a 31 kDa fragment labeled by the probe.

The photolabeling is very specific for only the targeted proteases. The Coomassie Blue stained SDS-PAGE gels in both Figures 2 and 3 indicate multiple bands of proteins in the commercial proteases preparations which were not labeled by the photoprobe. To further establish the selectivity of the probe, a series of representative proteases (papain (cysteine), carboxypepitase A (metallo), chymotrypsin, and trypsin (serine)) were subjected to photolabeling (Figure 4). The Western lot of the photolabeled proteases (B) indicates that only pepsin, lane 1 (photolysis of photoprobe + pepsin), was labeled under the experimental conditions. Therefore under the conditions of the experiments the photoprobe is completely specific for pepsin.

The sensitivity for the detection of photolabeled enzyme with the avidin−horseradish peroxidase label was explored in Figure 5. Pepsin at various concentrations (70−0.7 pM) was photolabeled with the biotinylated photoprobe (300 nM) before SDS-PAGE electrophoresis. The Western-blot analysis of the photolabeled pepsin SDS-PAGE gel (B) clearly indicates (lanes 1 and 3) that the avidin−horseradish peroxidase label could detect BBB-pepstatin photolabeled pepsin at less than 7 pM enzyme.

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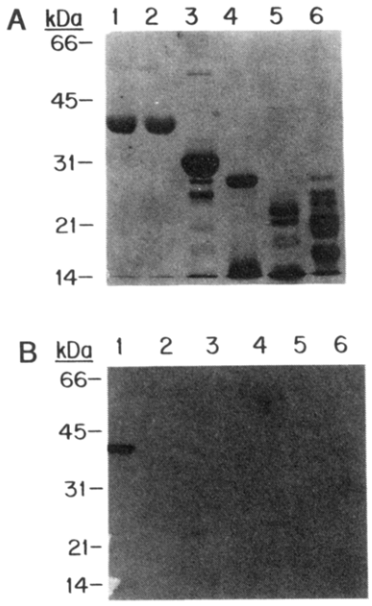

Figure 4. Selectivity of BBB-pepstatin (**3**) photochemical labeling. A series of representive proteases were photolabeled with the biotinylated photoprobe (300 nM) before electrophoresis on 12.5% (w/v) polyacrylamide gel (run under nonreducing denaturing conditions). After electrophoretic transfer, the biotinylated proteins were revealed with avidin–horseradish peroxidase as described in the Experimental Section. (A) The Coomassie Blue stained SDS-PAGE gel of the photolabeled proteases: lane 1, photolysis of photoprobe + pepsin; lane 2, pepstatin (10 μM) + pepsin, then probe + photolysis. Lanes 3–6 represent photolysis of carboxypeptidase A (29 kDa), chymotrypsin (28 kDa), trypsin (24 kDa), and papain (21 kDa) that were treated in the same way as the pepsin sample applied to lane 1. The multiple bands observed for the proteases are due to either impurities in the commercial preparations and/or autoproteolysis of the samples. (B) The Western-blot analysis of the photolabeled proteases. Lane 1 represents BBB-pepstatin-photolabeled pepsin.

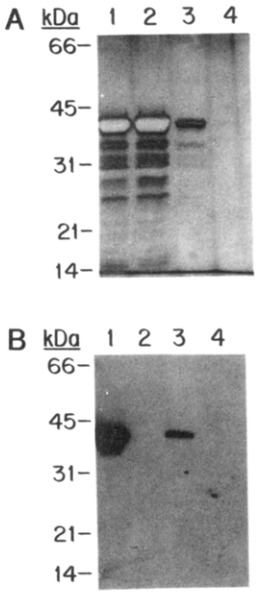

Figure 5. Sensitivity of BBB-pepstatin (**3**) photochemical labeling of porcine pepsin (35 kDa). Pepsin at various concentrations (70–0.7 pM) was photolabeled with the biotinylated photoprobe (300 nM) before electrophoresis on 12.5% (w/v) polyacrylamide gel (run under nonreducing, denaturing conditions). After electrophoretic transfer, the biotinylated proteins were revealed with avidin–horseradish peroxidase as described in the Experimental Section. (A) The Daiichi Silver Stain-II SDS-PAGE gel of the photolabeled protease: lane 1, photolysis of photoprobe + pepsin (70 nM); lane 2, pepstatin (10 μM) + pepsin (70 nM), then probe + photolysis. Lanes 3 and 4, represent photolabeling of pepsin at 7 and 0.7 pM, respectively, in the presence of BBB-pepstatin (**3**). (B) The Western-blot analysis of the photolabeled pepsin SDS-PAGE gel. Lanes 1 and 3 represent BBB-pepstatin-photolabeled pepsin at 70 and 7 pM concentrations of enzyme as detected by avidin-conjugated horseradish peroxidase.

One potential application of the biotinylated pepstatin photoprobe would be for the isolation of new aspartic proteases using the strong binding interaction of biotin and avidin.[8] The SDS-PAGE Coomassie Blue stained gel (A) and its Western blot (B) of the monomeric avidin purification of BBB-pepstatin-labeled porcine pepsin is shown in Figure 6. Porcine pepsin was photolabeled as described above (lane 1). The biotinylated enzyme was then immobilized on monomeric avidin coupled to agarose.[7] The column was washed with 4 vol of PBS buffer (lanes 2–5). The biotinylated enzyme was then eluted from the column with PBS buffer containing 2 mM free biotin (lanes 6–9), to provide the pure biotinylated photolabeled enzyme in fraction 7 (lane 7).

Summary

A novel modular design is introduced for the synthesis of biotinylated photoprobes containing either 4-azidotetrafluorobenzamide, 4-(1-azi-2,2,2-trifluoroethyl)benzamide, or 4-benzoylbenzoylamide. A benzoylbenzoyl biotinylated photoprobe of pepstatin was prepared. The probe could routinely label and detect small quantities (~7 pM) of either porcine or human aspartic protease. The use of the biotinylated affinity labels offers several advantages over radiolabeled probes by virtue of their exploitation of the biotin–avidin systems for detection and purification. The possibility thus exists that this probe could be useful in the identification of unknown aspartic proteases, as well as for mapping active-site regions of known aspartic proteases.

Experimental Section

Materials and Methods. Porcine pepsin, renin, and cathepsin D were purchased from Sigma Chemical. Human Cathepsin D and renin was obtained from Calbiochem. Immobilized monomeric avidin–agarose was obtained from Pierce Chemical. Avidin-conjugated horseradish peroxidase and prestained and biotinylated molecular-weight markers were from Bio-Rad. Daiichi Silver Stain-II was purchased from Integrated Separation Systems. Nitrocellulose (0.45 μm pore size) was obtained from Schleicher and Schuell. The luminol ECL Western blotting detection reagents were purchased from Amersham Life Sciences. Pepstatin was procured from Fluka Chemical and H-Phe-Gly-His-(*p*-nitro-Phe)-Phe-Ala-Phe-OMe was acquired from Bachem Bioscience. Other reagents, unless otherwise stated, were purchased from Aldrich Chemical.

The *N*-Hydroxysuccinimidyl esters of 4-azidotetrafluorobenzoate,[16] 4-(1-azi-2,2,2-trifluoroethylbenzoate,[17] and 4-benzoylbenzoate[18] were prepared as reported. The K_d of BBB-pepstatin for porcine pepsin was calculated by the method of Green and Work[21] using the substrate H-Phe-Gly-His-(*p*-nitro-Phe)-Phe-Ala-Phe-OMe developed by Medzihradszky[20] and co-workers for pepsin. Pepsin activity was assayed at the absorbance of 310 nm for the hydrolysis of the (*p*-nitro-Phe)–Phe bond in the heptapeptide substrate at 25 °C in 0.04 M formate buffer (pH 4.0).[22] Gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) was carried out without disulfide bond reduction as described by Laemmli[26] with 12.5% (w/v) polyacrylamide gels using the Mighty Small Apparatus (Hoefer Scientific). Prestained and biotinylated molecular-weight markers were included in each gel. Protein bands were visualized by staining with Coomassie brilliant blue. Western blotting was carried out by the method of Towbin and co-workers.[27] The transfer of protein from SDS-PAGE gel to nitrocellulose

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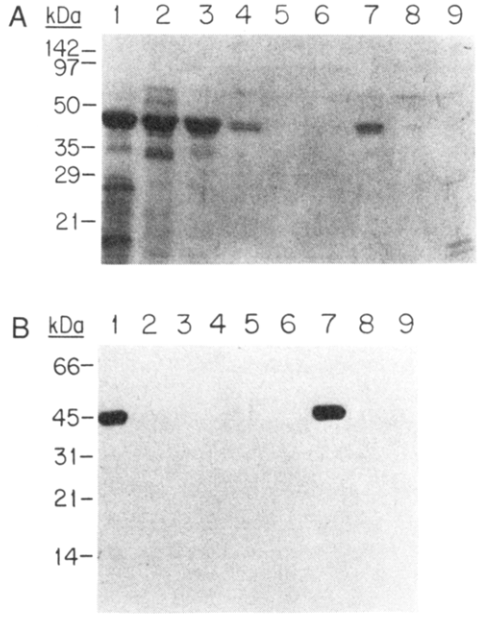

Figure 6. Monomeric avidin purification of BBB-pepstatin photolabeled porcine, pepsin (35 kDa). (A) The Coomassie Blue stained SDS-PAGE gel of the photolabeled protease avidin column washing: lane 1, photolysis of photoprobe (300 nM) + pepsin (140 pM); lanes 2−5 column volume washing with PBS buffer; lanes 6−9, fraction washing with PBS buffer + 2 mM biotin. (B) The Western-blot analysis of the photolabeled protease avidin column washing. After electrophoretic transfer, the biotinylated proteins were revealed with avidin−horseradish peroxidase as described in the Experimental Section. Lane 7 represents BBB-pepstatin-photolabeled pepsin purified by monomeric avidin chromatography.

was performed using a Trans-Blot Semi-Dry Transfer Cell from Bio-Rad in 25 mM glycine/192 mM Tris/20% methanol at a constant 15 V for 37 min. The Western-blotted biotinylated proteins were detected by exposure of photographic film to the chemiluminescence of luminol oxidation by avidin-conjugated horseradish peroxidase.[24]

Proton nuclear magnetic resonance (^{1}H NMR) spectroscopy was obtained with a Varian VRX 500S spectrometer operating at a proton frequency of 499.843 MHz. Dimethyl sulfoxide (DMSO-d_6) was used as the ^{1}H NMR solvent. The residual proton absorption of the deuterated solvent was used as the internal standard. All ^{1}H NMR chemical shifts are reported as δ values in parts per million (ppm), and the coupling constants (J) are given in hertz. The splitting pattern abbreviations are as follows: s, singlet; d, doublet; t, triplet; q, quartet; br, broad; m, multiplet; dd, doublet of doublets. For the spectrophotometric measurements, a Perkin-Elmer Lambda 3B UV/vis spectrophotometer was employed. FAB (fast atom bombardment) mass spectra were performed in a JEOL mass spectrometer JMS-SX102.

General Procedure for the Synthesis of Biocytinylated Photoprobes. The appropriate *N*-hydroxysuccinimide photoprobe ester (1.2 mmol) in DMF (2.0 mL) was added slowly to a solution of biocytin (1.0 mmol) and sodium hydroxide (1.1 mmol) in water (1.0 mL). Additional DMF was added if a precipitate appeared. The solution was stirred at room temperature overnight. Water (10 mL) was added, and the solution extracted with ether (3 × 20 mL) and hexanes (20 mL). The solution was acidified with 10% citric acid, and the precipitate was collected by filtration. The precipitate was washed with ether and dried overnight under vacuum to give the desired product.

N^{α}-(4-Azidotetrafluorobenzoyl)-L-biocytin (1). *N*-Hydroxysuccinimidyl 4-azidotetrafluorobenzoate[16] was treated as described above to provide **1** as a white crystalline material in 85% yield: TLC R_f = 0.3 (5% MeOH, 0.1% AcOH in DCM); ^{1}H NMR (DMSO-d_6) δ 9.17 (1H, d, J = 7.6 Hz), 7.74 (1H, t, J = 5.9 Hz), 6.39 (1H, s), 6.33 (1H, s), 4.33−4.26 (2H, m), 4.11−4.08 (1H, m), 3.09−3.05 (1H, m), 3.03−2.98 (2H, m), 2.79 (1H, dd, J = 12.5, 4.9 Hz), 2.55 (1H, d, J = 11.5 Hz), 2.01 (2H, t, J = 6.9 Hz), 1.80−1.74 (1H, m), 1.66−1.56 (2H, m), 1.48−1.24 (9H, m); FABHRMS (glycerol) *m/e* (M + H)$^+$, 590.1809; calculated molecular weight for $C_{23}H_{27}F_4N_7O_5S$ requires 589.

N^{α}-(4-(1-Azi-2,2,2-trifluoroethyl)benzoyl)-L-biocytin (2). *N*-Hydroxysuccinimidyl 4-(1-azi-2,2,2-trifluoroethyl)benzoate[17] was treated as described above to provide **2** as a white crystalline material in 91% yield: TLC R_f = 0.2 (5% MeOH, 0.1% AcOH in DCM); ^{1}H NMR (DMSO-d_6) δ 8.70 (1H, d, J = 7.9 Hz), 7.97 (2H, d, J = 6.5 Hz), 7.73 (1H, t, J = 5.5 Hz), 7.40 (2H, d, J = 8.0 Hz), 6.39 (1H, s), 6.33 (1H, s), 4.31−4.24 (2H, m), 4.10−4.07 (1H, m), 3.09−3.04 (1H, m), 3.01−2.95 (2H, m), 2.79 (1H, dd, J = 12.0, 6.0 Hz), 2.54 (1H, d, J = 12.5 Hz), 2.00 (2H, t, J = 6.9 Hz), 1.81−1.72 (2H, m), 1.59−1.53 (1H, m), 1.48−1.21 (9H, m).

N^{α}-(4-Benzoylbenzoyl)-L-biocytin (3). *N*-Hydroxysuccinimidyl 4-benzoylbenzoate[18] was treated as described above to provide **3** as a white crystalline material in 82% yield: TLC R_f = 0.3 (5% MeOH, 0.1% AcOH in DCM); ^{1}H NMR (DMSO-d_6) δ 8.77 (1H, d, J = 7.9 Hz), 8.03 (2H, d, J = 7.5 Hz), 7.80 (2H, t, J = 6.5 Hz), 7.74 (3H, d, J = 7.9 Hz), 7.69 (1H, t, J = 6.5 Hz), 7.56 (2H, t, J = 7.5 Hz), 6.38 (1H, s), 6.33 (1H, s), 4.37−4.33 (1H, m), 4.28−4.25 (1H, m), 4.10−4.07 (1H, m), 3.08−2.97 (3H, m), 2.78 (1H, dd, J = 12.5, 4.9 Hz), 2.54 (1H, d, J = 11.5 Hz), 2.01 (2H, t, J = 6.9 Hz), 1.86−1.72 (2H, m), 1.61−1.52 (1H, m), 1.48−1.23 (9H, m); FABHRMS (glycerol) *m/e* (M + H)$^+$, 581.2434; calculated molecular weight for $C_{30}H_{36}N_4O_6S$ requires 580.

Synthesis of BBB-Pepstatin Photoprobe. 1-(*N*-(N^{α}-(4-Benzoylbenzoyl)-L-biocytinoyl)amino)-6-(*N'*-boc-amino)hexane (4). To a solution of carboxyl acid **3** (390 mg, 0.58 mmol), 1-(Boc-amino)-6-aminohexane hydrochloride (192 mg, 0.76 mmol), diisopropylethylamine (106 mg, 0.82 mmol), and HOAT (120 mg, 0.88 mmol) in DMF (10 mL) was added EDAC (134 mg, 0.70 mmol) in one portion. The mixture was stirred at 0 °C for 2 h, then at room temperature overnight. The reaction was quenched with 3.5% aqueous HCl (50 mL), and the solution was extracted with *n*-butanol (3 × 25 mL). The combined organic layers were washed with brine (2 × 20 mL), dried over $MgSO_4$, filtered, and concentrated to give a white solid. The material was purified by silica gel chromatography (5% MeOH in DCM) to afford amide **4** as a white crystalline solid in 86% yield: TLC R_f = 0.8 (5% MeOH in DCM); ^{1}H NMR (DMSO-d_6) δ 8.56 (1H, d, J = 7.5 Hz), 8.03 (2H, d, J = 7.9 Hz), 7.92 (1H, t, J = 5.5 Hz), 7.78 (2H, d, J = 8.9 Hz), 7.73 (3H, d, J = 7.5 Hz), 7.69 (1H, t, J = 7.9 Hz), 7.56 (2H, t, J = 7.9 Hz), 6.73 (1H, t, J = 5.5 Hz), 6.38 (1H, s), 6.32 (1H, s), 4.39−4.32 (1H, m), 4.26 (1H, t, J = 5.5 Hz), 4.08 (1H, t, J = 5.5 Hz), 3.09−2.96 (4H, m), 2.89−2.84 (2H, m), 2.80−2.74 (1H, m), 2.53 (1H, d, J = 12 Hz), 2.00 (2H, t, J = 6.9 Hz), 1.74 (2H, brs), 1.58−1.52 (1H, m), 1.48−1.19 (26H, m); FABHRMS (glycerol) *m/e* (M + Na^+)$^+$, 801.3985; calculated molecular weight for $C_{41}H_{58}N_6O_7S$ requires 778.

1-(*N*-(N^{α}-(4-Benzoylbenzoyl)-L-biocytinoyl)amino)-6-(*N'*-pepstatinoylamino)hexane (5). To a solution of urethane **4** (52 mg, 0.066 mmol) in 1,4-dioxane (1.0 mL) was added 4 M HCl in 1,4-dioxane (5.0 mL) at 0 °C. The mixture was stirred at 0 °C for an additional 10 min, than at room temperature for 1 h. The solution was concentrated and the white solid dried under vacuum overnight. The residue was dissolved in DMF (2.0 mL); then HOAT (9.0 mg, 0.066 mmol), diisopropylethylamine (8.5 mg, 0.066 mmol), and pepstatin (30 mg, 0.044 mmol) were sequentially added. The solution was cooled to 0 °C, then EDAC (13 mg, 0.066 mmol) was added. The mixture was stirred at 0 °C for 2 h, then at room temperature overnight. The reaction was quenched with 3.5% aqueous HCl (50 mL), and the solution was extracted with *n*-butanol (3 × 25 mL). The combined organic layers were washed with brine (2 × 20 mL), dried over $MgSO_4$, filtered, and concentrated to give a white solid. The material was purified by silica gel chromatography (10% MeOH in DCM) to afford amide **5** as a white crystalline solid in 65% yield: TLC R_f = 0.5 (5% MeOH in DCM); ^{1}H NMR (DMSO-d_6) δ 8.56 (1H, d, J = 7.5 Hz), 8.03 (2H, d, J = 7.9 Hz), 7.94−7.89 (3H, m), 7.81−7.65 (7H, m), 7.56 (3H, t, J = 7.5 Hz), 7.45 (1H, d, J = 8.9 Hz), 7.31 (1H, d, J = 9.5 Hz), 6.39 (1H, s), 6.33 (1H, s), 4.89−4.80 (1H, m), 4.39−4.32 (1H, m), 4.28−4.04 (5H, m), 3.80 (3H, brs), 3.08−2.91 (3H, m), 2.87 (1H, brs), 2.53 (1H, d, J = 12 Hz), 2.10 (2H, brs), 2.00 (4H, m), 1.95−1.89 (2H, m), 1.70 (2H, brs), 1.50−1.16 (33H, m), 0.85−0.75 (33H, m); FABHRMS (glycerol) *m/e* (M + H)$^+$ 1346.8162; calculated molecular weight for $C_{70}H_{111}N_{11}O_{13}S$ requires 1345.

Photoaffinity Labeling of Aspartic Proteases with BBB-Pepstatin. The following protocol is representative of that used for the photola-

beling of aspartic proteases with BBB-pepstatin. Into a Pyrex test tube (13 × 100 nm) were sequentially added BBB-pepstatin (1 μL, 0.10 mM in DMSO), 289 μL of 0.04 M formate buffer (pH = 4.0), and porcine pepsin (10 μL, 1 mg/mL in 1 mM aqueous HCL). The test tube was gently shaken and allowed to stand at room temperature for 15 min. The sample was then placed 3 in. from a uranium-filtered 450 W Hanovia high-pressure mercury-vapor lamp in an ice-cooled water bath. The sample was irradiated for 30 min, during which the temperature of the sample solution did not exceed 5 °C. The solution was transferred into an Amicon Centricon-10 microconcentrator, and the excess photprobe was removed by two cycles of dilution and concentration by centrifugation at 6500 rpm for 30 min. The concentrated residue was prepared for SDS-PAGE chromatography by dissolving an equal volume of sample into 2 × SDS loading buffer and then heating the mixture at 95 °C for 5 min.

Monomeric Avidin Purification of BBB-Pepstatin-Photolabeled Porcine Pepsin. Porcine pepsin was photolabeled with BBB-pepstatin as described above. The solution was concentrated by one cycle of dilution centrifugation in an Amicon Centricon-10 microconcentrator at 6500 rpm for 30 min. The residue was diluted into PBS buffer (1.0 mL, 0.1 M sodium phosphate, 0.15 M sodium chloride, pH = 7.2) and applied to a monomeric avidin−agarose column (Pierce). The solution was preincubated on the column for 15 min. The column was washed with PBS buffer (4 × 1 mL, 0.1 M sodium phosphate, 0.15 M sodium chloride, pH 7.2) and then with PBS buffer containing 2 mM biotin (4 × 1 mL) to elute the biotinylated photolabeled protein. Each washing column volume (1 mL) was separately collected and concentrated by the previously described Centricon-10 microconcentrator protocol for preparation in SDS-PAGE chromatography.

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