Synthesis of an Inhibitor-Tethered Resin for Detection of Active Matrix Metalloproteinases Involved in Disease
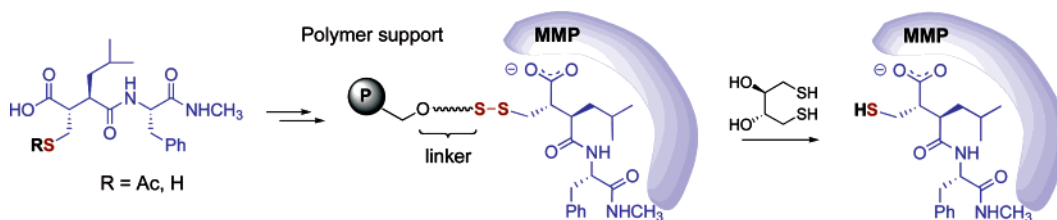
Dusan Hesek,[†] Marta Toth,[†,‡] Viktor Krchňák,[†] Rafael Fridman,[‡] and Shahriar Mobashery*[,†]

Department of Chemistry and Biochemistry and the Walther Cancer Research Center, University of Notre Dame, Notre Dame, Indiana 46556, and Department of Pathology, Wayne State University School of Medicine, Detroit, Michigan 48201

mobashery@nd.edu

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Matrix metalloproteinases (MMPs), of which 26 members are known in humans, are implicated in a number of diseases. Their activity is strictly controlled, but when the biological control over the activity is lost, disease processes set in. In an attempt to delineate what MMP activity has gone awry in what diseases, including metastatic cancers that are of special interest to our laboratories, we conceived and synthesized two chromatographic resins incorporated with a multifunctional broad-spectrum inhibitor for MMPs. The broad-spectrum inhibitor contains three sterogentic centers and was synthesized in 13 steps. Two structural variants of the inhibitors were linked to the polymer support via disulfide moieties. These resins are intended for use in cellular systems to selectively fish out from a complex mixture of all cellular proteins the active MMP forms important for the specific disease for identification.

Introduction

Matrix metalloproteinases (MMPs) are a group of 26 important zinc-dependent endopeptidases involved in a series of physiological processes, including tissue remodeling and development, inflammation, and angiogenesis, among others.[1] These enzymes are highly regulated. They are expressed as inactive zymogenic enzymes that undergo proteolytic processing in becoming active. When activated, their activities are held in check by tissue inhibitors of matrix metalloproteinases (TIMPs), protein inhibitors for MMPs. When these controlled cellular events go awry, pathological processes ensue, which include development of cancer and its metastasis, neurological and cardiovascular ailments, arthritis, and inflammatory diseases. Whereas the involvement of these enzymes in many of these processes are implicated from a series of

biochemical and genetic analyses, it is generally not known what MMPs might play major roles in various diseases. The complicating factor is the necessary posttranslational modifications that need to take place for the active forms of these enzymes to manifest their activities.

Of special interest to our laboratory are the molecular events that lead to cancer metastasis. MMPs are often expressed pericellularly, and they degrade the constituents of the extracellular matrix around the cancerous tissue providing access to the vasculature, a route that these cells use for spread in the body. Whereas recent evidence argues for the critical involvement of MMP-2, -9, and -14 in cancer metastasis,[2,3] the full picture of what cancers express what MMPs is not in hand. It is critical for us to understand which of these enzymes are present in cancer in order to be able to target these enzymes for selective inhibition. In this vein, we hasten to add that broad-

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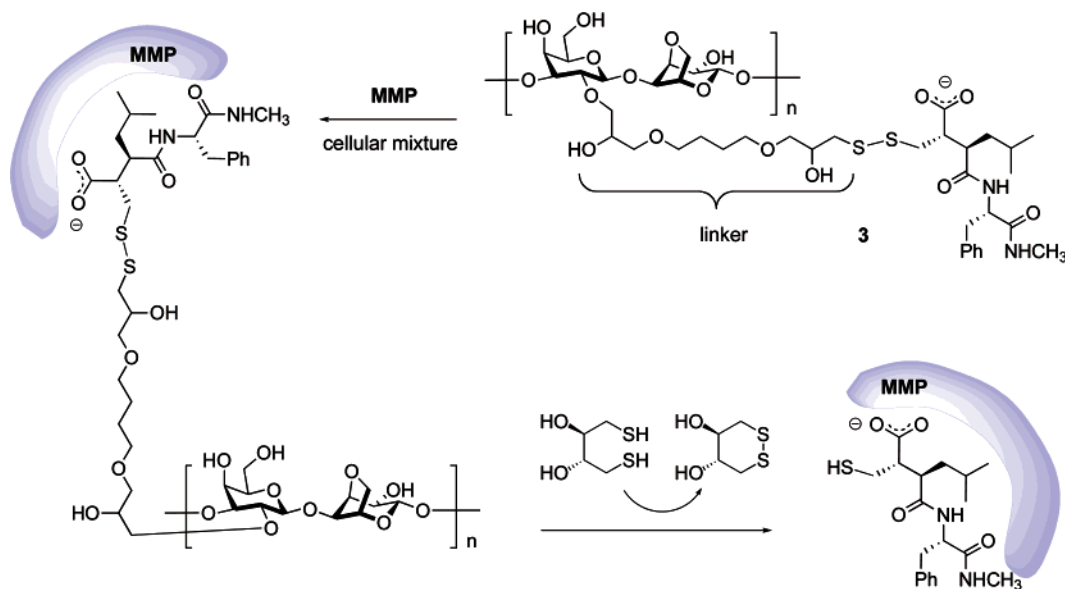
spectrum MMP inhibitors are widely known.[4,5] Unfortunately, their use in clinical trials have been largely ineffectively. A primary problem has been the large breadth of activity that resulted in side effects.[6,7] Hence, selective inhibitors of these enzymes are highly sought.[8,9]

In light of the availability of a number of broad-spectrum inhibitors for MMPs, we conceived of a general strategy in identification of the active forms of MMPs in various tumors. We envisioned that a broad-spectrum inhibitor could be tethered to a solid support. This resin then could be used with extracts from tumors to "fish out" the active form(s) of the MMP(s) involved in the specific cancer from cellular mixture (Scheme 1). The elution from the resin and the subsequent identification of the given MMP (by peptide sequencing or mass spectrometry) would furnish the identity of the protease. This identification would be followed up by biochemical experiments to elucidate the critical roles of the MMP in cancer metastasis (and other diseases).

The choice of inhibitor was made on the basis of the availability of the complex of the inhibitor with MMP. At the beginning of this research in our laboratory, there were 25 three-dimensional structures available for catalytic domains of MMPs bound to various inhibitors. We inspected these with the intention of identifying an inhibitor that would have a moiety exposed to the milieu in its complex with the MMP such that the functionality could be elaborated synthetically for attachment to the resin. A suitable choice appeared to be batimastat (**1**). The thienyl moiety of batimastat in complex with MMP-12 is fully exposed to the solvent.[10] This arrangement is expected to be the same with other MMPs, based on structural similarities

of these enzymes. This appeared as the desirable point of attachment to the resin (Sepharose 6B).

The inhibitor is tethered to a linker, which in turn is attached to the Sepharose resin in structure **2**. We also prepared the corresponding carboxylated derivative (non-hydroxamate) **3**. As will be described, both resins appear to serve the purpose.
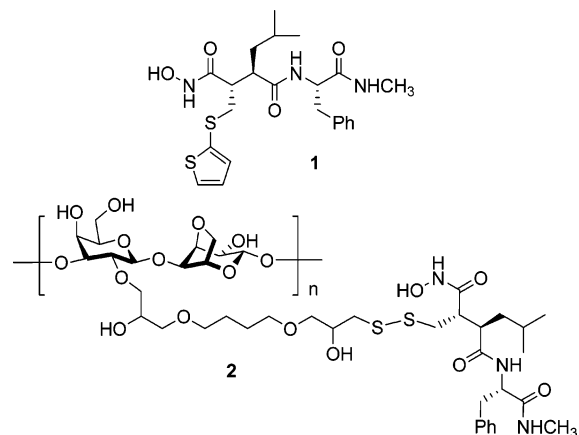
As depicted in Scheme 1, the MMP that is present in the uninhibited active form binds to the inhibitor-tethered resin and all other cellular constituents will be washed away. Subsequently, we envisioned that reduction of the disulfide bond (by any number of reagents, including thiols) would release the MMP along with the inhibitor. The protein will then be detected by mass spectrometry or by N-terminal sequencing. The synthesis of these functionalized resins commence with the commercially available epoxy-activated Sepharose 6B. This resin is of special interest for work with proteins as it shows negligible nonspecific interactions with proteins. A derivatization of the oxirane functionality allows immobilization of ligands via an epoxide ring-opening reaction with a suitable nucleophile present on a ligand molecule, as will be detailed below.

Results and Discussion

The target molecules (**2** and **3**) are complex entities requiring enantiomerically pure intermediates in their preparations through-

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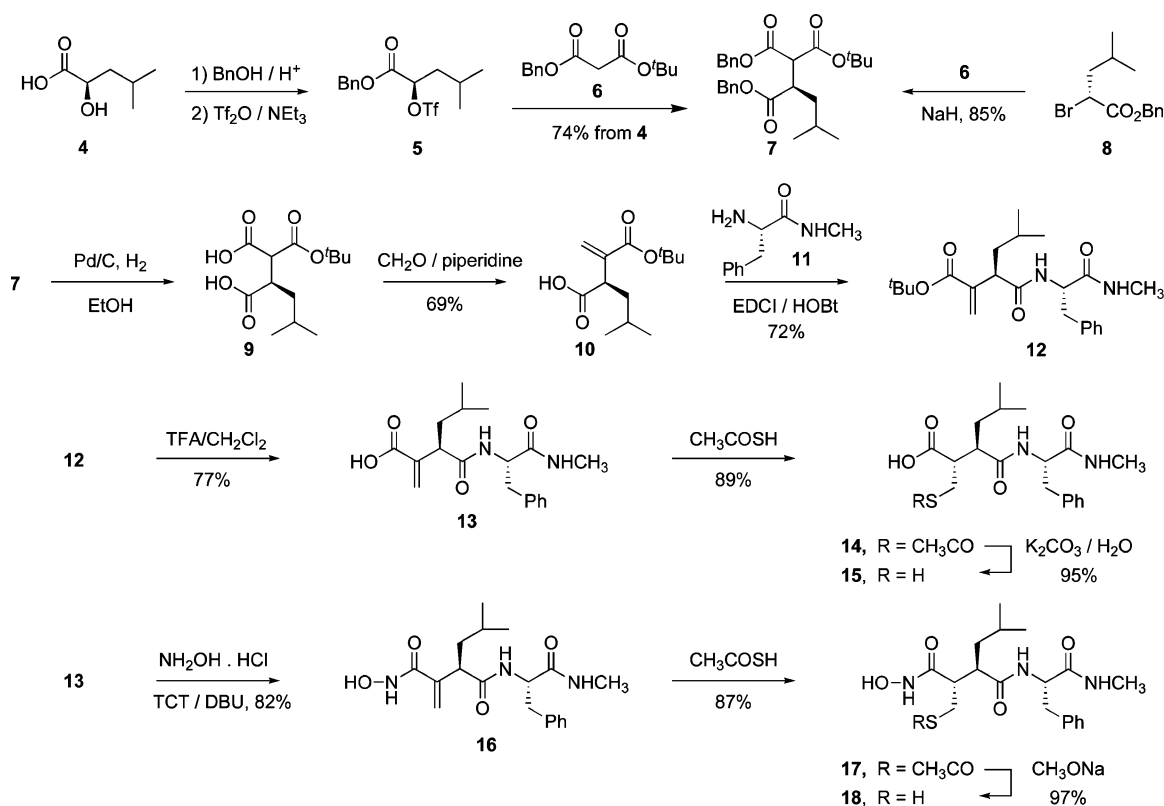(7) Coussens, L. M.; Fingleton, B.; Matrisian, L. M. *Science* **2002**, *295*, 2387−2392.
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SCHEME 2

out. Intermediate **7** was initially synthesized by a slight modification of the previously described route from the patent literature[11,12] using benzyl *tert*-butyl malonate (**6**). D-α-Hydroxyisocaproic acid (**4**) was converted to the corresponding triflate ester **5**, which was then used to prepare **7**, as shown in Scheme 2. Although the alkylation step produced **7** in moderate yield (72−77%), the reaction was accompanied by 30−35% epimerization of the stereogenic center. A different entry to the key building block (*R*)-1,1-dibenzyl 2-*tert*-butyl 4-methylpentane-1,1,2-tricarboxylate from *tert*-butyl-(2*R*)-bromo-4-methylpentanoate, itself prepared from D-leucine, has been described.[13,14] Unfortunately, this route also gave 20−25% epimerization, but the yield of the reaction was consistently higher (88%). These difficulties prompted us to explore an alternative strategy in the synthesis utilizing benzyl (2*R*)-bromo-4-methylpentanoate (**8**), which was converted into **7** in 85% yield, similar to a method reported previously for a related reaction.[13]

Hydrogenolysis of **7** produced **9**[15] in enantiomerically pure form for the first time. Compound **9** underwent decarboxylation under basic condition to give the intermediate (2*R*)-2-(*tert*-butyloxycarbonyl)methyl-4-methylpentanoic acid that was converted in situ to the corresponding acrylic derivative **10** by treatment with 37% aqueous formaldehyde in the presence of piperidine.[16,17] This reaction was optimized for multigram scale.

Conversion of the acrylate **10** to **12** was accomplished by coupling of **11** by the water-soluble carbodiimide EDCI in the presence of hydroxybenzotriazole (HOBt). The *tert*-butyl group of **12** was removed by treatment with acid under standard conditions (TFA/CH₂Cl₂) to give **13**.[18] The reaction of **13** to **14** was carried out with thiolacetic acid in a Michael-type reaction adapting a newly developed method of the addition of thionucleophiles to α,β-unsaturated systems.[19,20] It was envisioned that the thioester **14** derived from α,β-unsaturated acid **13** could be an appropriate intermediate to regioselectively introduce a hydoxylamido functionality at the terminal carboxyl group. Therefore, the direct conversion of carboxylic acid **14**[21] to the corresponding hydroxamic acid derivative **17** was performed with either excess of hydroxylamine or *O*-silyl hydroxylamine using an EDCI-mediated coupling protocol in a presence of HOBt.[22] Although the reactions proceeded in moderate yield, the presence of base readily initiates a saponification of the labile thiocarbonyl functionality to prematurely unmask the sulfhydryl group giving thiol **18**, which in turn

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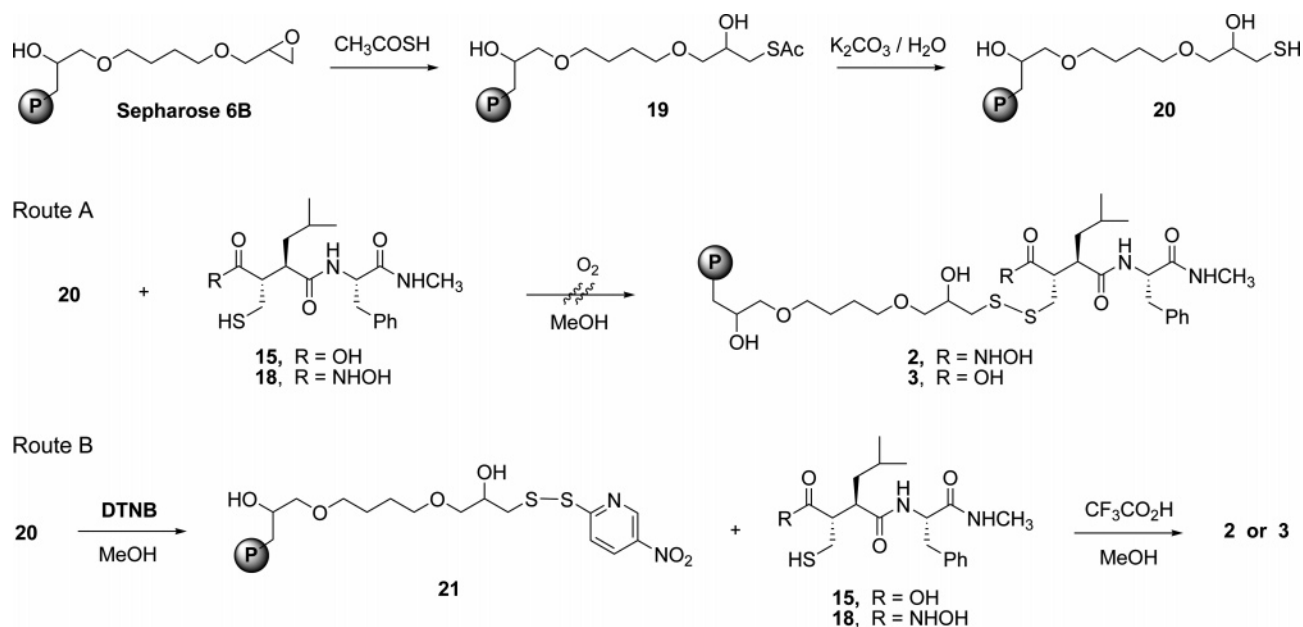(12) Campion, C.; Davidson, A. H.; Dickens, J. P.; Crimmin, M. J. In *PCT Int. Appl.*; WO 9005719 (British Bio-Technology Ltd., UK, 1990).
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SCHEME 3

resulted in the formation of undesired byproducts,[23] includes the corresponding symmetric disulfide. Alternative approaches to **17** by the use of different coupling methods (e.g., DCC, POCl$_3$, or pivaloyl-mixed anhydride) starting from **14**, led to a number of unsuccessful outcomes. Consequently, the hydroxamate was introduced first to give compound **16**, which was then readily converted to derivative **17**. A new method for activation of the carboxylic function using chloro-triazine derivatives as a coupling reagent in the presence of base has been developed. Under this condition, compound **13** was transformed to **16** using 2-chloro-4,6-dimethoxy-1,3,5-triazine (CDMT) or 2,4,6-trichloro[1,3,5]triazine (TCT) as a triazine-based coupling reagent.[24,25] Following addition of thioacetic acid[22] to **16**, the reaction proceeded with high stereocontrol to provide thioester **17**.

A new method for immobilization of the hydroxamate (**2**) and carboxylate (**3**) MMP ligands was developed. Under this protocol the ligands are attached to the resin by a heterodisulfide tether in resins **2** and **3**.

First, we evaluated the direct formation of the disulfide from two thiol-containing synthons, as shown in Scheme 3. Briefly, we allowed the epoxy-activated Sepharose to react with thioacetic acid to open the epoxide ring and to form the Sepharose-supported thioester **19**, which was then cleaved under strictly oxygen-free conditions with base to give the desired thiol intermediate **20** (route A, Scheme 3). Resin **20** was then treated with ligands **15** or **18** containing the free thiol, which was generated in situ from the corresponding esters **14** or **17** by sonication for 5 min under an atmosphere of argon and transferred via cannula to **20**. The swollen mixed resin was placed in a flask, and then oxygen was used to purge the residual argon from the reaction mixture. After overnight incubation under an oxygen atmosphere, the resin was thoroughly washed

with methanol and then with water. However, the quality of the derivatized resin prepared by this oxidative method varied from attempt to attempt and was not very reproducible. This method suffered from side reactions, namely dimerization of the hydroxamate ligand and cross-linking of the sulfhydryl-derivatized Sepharose.

To prevent the formation of the undesired symmetric disulfides, we developed an alternative, but more reliable, procedure in which one of the thiol counterparts was activated for unambiguous reaction with the second thiol component. The nonoxidative method is based on the two consecutive steps of disulfide exchange reactions and typically involves an introduction of 2-nitrophenyl, 2-pyridinesulfenyl, or 3-nitro-2-pyridine-sulfenyl residues, respectively.[26] To selectively form the disulfide bridge between the ligand counterparts and the derivatized Sepharose, and thus eliminate the formation of symmetrical disulfides, we activated the sulfhydryl group of resin **20** for the subsequent reaction with the free thiol-containing ligands. With regard to the intermolecular disulfide bridge formation, we selected the two-step procedure entailing the use of a 2,2′-dithiobis(5-nitropyridine) protocol, since this versatile reagent had been used before for the formation of unsymmetrical disulfide bonds in peptides.[27] Under this protocol, the initial synthetic step of thiol-containing resin **20** was identical with the previous procedure. The derivatized support was then treated with carboxylate ligand **18** containing the free thiol group in the presence of acid (route B, Scheme 3). A catalytic amount of TFA promotes, by protonation of the pyridine nitrogen, the selective nucleophilic cleavage by the ligand sulfur **18** to give the target derivative **3**. After overnight incubation, the resin was thoroughly washed with methanol, and then with water to remove the last traces of reagents and the excess of the ligand. The product resin can be stored at 5 °C until needed for use in the biochemical experiments.

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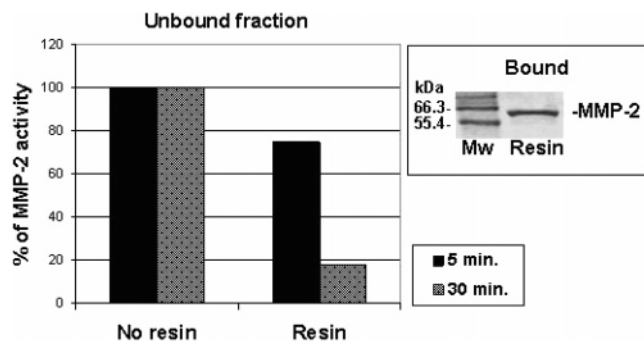
FIGURE 1. MMP-2 binding to inhibitor-tethered resin. Active human recombinant MMP-2 was incubated without or with inhibitor-tethered resins **2** or **3**. To monitor MMP binding to the resin the unbound fractions were tested for MMP-2 activity and the bound fraction was eluted from resin, resolved by SDS−PAGE, and stained by Coomassie blue. The recovered enzyme ("bound") exhibited the requisite MMP-2 activity expected of the amount bound to the resin.

We tested the ability of resins **2** and **3** to bind purified human recombinant active MMP-2. The enzyme and inhibitor-tethered resin were incubated, followed by analyses of both the unbound and bound fractions. We measured the relative activity of MMP-2 in the unbound fractions by following peptide substrate hydrolysis with fluorescence spectrophotometer (Figure 1). The MMP-2 activity decreased in the unbound fraction by incubation time as the active enzyme bound to the inhibitor-tethered resin. Most of the active MMP-2 bound to the resin within 30 min of incubation time.

Experimental Section

Compounds **7** and **8** were prepared by published literature methods.[11,13] The optical purities of synthesized derivatives were determined by high-performance liquid chromatography (HPLC) analysis using (*S,S*)-Whelk-O 1 column (250 × 4.6 mm, L × i.d.) and monitored with a photodiode array spectrometer equipped with the Millennium software. An isocratic condition of mixed solvents system (hexane/CH_2Cl_2/EtOH/AcOH = 70:20:10:0.1, v/v) was used for 15 min, and the mobile-phase flow rate of the HPLC pump was 0.8 mL/min. A 6-μL of sample solution was subject to HPLC for analysis, and the retention data at room temperature were as follows: **7** (t_R = 4.3 min), **8** (t_R = 4.0 min), **10** (t_R = 4.2 min), **11** (t_R = 4.9 min), **12** (t_R = 5.0 min), **13** (t_R = 5.7 min), **14** (t_R = 5.8 min), **16** (t_R = 6.1 min), **17** (t_R = 6.6 min).

(2*RS*)-2-(*tert*-Butoxycarbonyl)-(3*R*)-3-isobutylsuccinic Acid (9). A slurry of 10% Pd−C (0.8 g) in water (1 mL) was added to a solution of (1*RS*)-*tert*-butyl (2*R*)-1,2-dibenzyl-4-methylpentane-1,1,2-tricarboxylate[11] (**7**, 4.5 g, 10 mmol) in ethanol (60 mL), under an atmosphere of nitrogen. The reaction vessel was aspirated to purge it of any other dissolved gases, and then hydrogen was bubbled through the vigorously stirred mixture for 5 h at 45 °C. The catalyst was removed by filtration through Celite, and the filtrate was evaporated under reduced pressure to leave a crude product **8** as a mixture of isomers. Recrystallization of the crude material from ether/hexane (1:8) gave the desired isomer (2*RS*)-2-(*tert*-butoxycarbonyl) (3*R*)-3-isobutylsuccinic acid (**9**, 2 g, 73%; >99% ee): ^{1}H NMR (compound **9**, 600 MHz, 10% MeOD-d_4 in CDCl$_3$) δ 0.79 (d, *J* = 6.8 Hz, 3H), 0.83 (d, *J* = 6.3 Hz, 3H), 1.18 (ddd, *J* = 13.6, 9.7, 3.9 Hz, 1H), 1.33 (s, 10H), 1.46−1.51 (m, 1H), 1.55−1.59 (m, 1H), 2.95 (td, *J* = 10.2, 4.3 Hz, 1H), 3.43 (d, *J* = 10.2 Hz, 1H); ^{13}C NMR (151 MHz, 10% MeOD-d_4 in CDCl$_3$) δ 20.9, 23.1, 25.5, 27.3, 39.3, 42.6, 55.6, 82.3, 167.6, 170.3, 176.3; HRMS (FAB) for $C_{13}H_{22}O_6$ [M + H]$^+$ calcd 275.1495, found 275.1479.

(2*R*)-2-[1-(*tert*-Butoxycarbonyl)vinyl]-4-methylpentanoic Acid (10). (2*RS*)-2-(*tert*-Butoxycarbonyl)-(3*R*)-3-isobutylsuccinic acid (**9**, 2.74 g, 10 mmol) was treated with piperidine (1.1 mL, 11 mmol) and a solution of formaldehyde (38%, 5 mL) in EtOH (25 mL). The mixture was stirred at room temperature for 10 h. The solvent was removed in vacuo to give an oil, which was dissolved in ethyl acetate (50 mL) and washed with 0.1 N HCl and water, followed by concentration in vacuo to yield an oil (2.02 g). The crude material was purified by flash chromatography (hexane/EtOAc = 3:2) to afford 1.66 g (69% yield) as a single desired enantiomer of **10**: ^{1}H NMR (compound **10**, 300 MHz, CDCl$_3$) δ 0.94 (dd, *J* = 9.5, 6.3 Hz, 7H), 1.51 (s, 9H), 1.53−1.68 (m, 2H), 1.79 (dd, *J* = 7.5, 6.1 Hz, 1H), 3.57 (t, *J* = 7.3 Hz, 1H), 5.69 (s, 1H) 6.31 (s, 1H); ^{13}C NMR (75 MHz, CDCl$_3$) δ 22.2, 22.4, 25.7, 27.9, 39.7, 45.0, 81.5, 126.1, 139.5, 165.4, 179.6; LC/MS *m/z* 241 (M − H)$^-$; HRMS (FAB) for $C_{13}H_{22}O_4$ [M + H]$^+$ calcd 243.1596, found 243.1606.

(2*S*)-2-Amino-*N*-methyl-3-phenylpropanamide (11). L-Phenylalanine ethyl ester hydrochloride (4.5 g, 20 mmol) was suspended in THF (150 mL) together with 1.0 equiv (2.8 mL, 20 mmol) of triethylamine. After 15 min, the reaction mixture was filtered, and to the filtrate was added 2.0 M methylamine solution in tetrahydrofuran (60 mL). After 1 h, the reaction mixture was concentrated under reduced pressure, followed by precipitation from hexane to give crude **11** as a free base in 94% yield. The substance **11** was then taken up into CH_2Cl_2 (50 mL) and filtered, and the CH_2Cl_2 solution of enantiomerically pure compound **11** was directly introduced into the reaction step for the preparation of derivative **12** without further purification: ^{1}H NMR (compound **11**, 500 MHz, CDCl$_3$) δ 1.43 (s, 2H) 2.68 (dd, *J* = 13.6, 9.4 Hz, 1H) 2.81 (d, *J* = 5.0 Hz, 3H) 3.28 (dd, *J* = 13.7, 4.1 Hz, 1H) 3.61 (dd, *J* = 9.4, 4.0 Hz, 1H) 7.24 (ddd, *J* = 16.3, 7.8, 6.6 Hz, 3H) 7.28−7.33 (m, 2H); ^{13}C NMR (126 MHz, CDCl$_3$) δ 25.7, 40.9, 56.4, 126.7, 128.6, 129.2, 137.9, 174.6; LC/MS *m/z* 179 [M + H]$^+$; HRMS (FAB) for $C_{10}H_{14}N_2O$ [M + H]$^+$ calcd 179.1184, found 179.1200.

(*R*)-*tert*-Butyl-3-[(*S*)-1-(methylcarbamoyl)-2-phenylethylcarbamoyl]-5-methyl-2-methylenehexanoate (12). A mixture of **10** (2.4 g, 10 mmol), freshly prepared *(2S)*-2-amino-*N*-methyl-3-phenylpropanamide (**11**) (1.9 g, 11 mmol), HOBt (1.49 g, 11 mmol), and DMF (70 mL) was allowed to react with EDC (2.11 g, 11 mmol) at 15 °C. The mixture was stirred at room temperature overnight. The reaction mixture was diluted with EtOAc (200 mL), washed with brine, and then evaporated in vacuo to leave a pale yellow oil. The residue was purified by flash column chromatography (2:1 hexane/EtAc) to give **12** as a white solid (2.9 g, 72%): ^{1}H NMR (compound **12**, 300 MHz, CDCl$_3$) δ 0.80−0.93 (m, 6H), 1.41−1.53 (m, 11H), 1.68 (dd, *J* = 7.4, 6.2 Hz, 1H), 2.71−2.79 (m, 3H), 3.05 (d, *J* = 7.0 Hz, 2H), 3.41 (t, *J* = 7.4 Hz, 1H), 4.57−4.66 (m, 1H), 5.66 (s, 1H), 6.13−6.27 (m, 2H), 6.47 (d, *J* = 8.0 Hz, 1H), 7.15−7.30 (m, 5H); ^{13}C NMR (75 MHz, CDCl$_3$) δ 22.3, 22.5, 25.6, 26.0, 27.9, 37.8, 39.5, 45.6, 54.4, 76.5, 77.0, 77.4, 81.6, 126.1, 126.7, 128.5, 129.1, 136.7, 140.0, 166.1, 171.2, 172.5; LC/MS *m/z* 403 [M + H]$^+$; HRMS (FAB) for $C_{23}H_{34}N_2O_4$ [M + H]$^+$ calcd 403.2597, found 403.2570.

(3*R*)-3-[1-(Methylcarbamoyl)-(2*S*)-2-phenylethylcarbamoyl]-5-methyl-2-methylenehexanoic Acid (13). Trifluoroacetic acid (10 mL) was added to a solution of compound **12** (9.4 g, 23 mol) in CH_2Cl_2 (100 mL) at ice−water temperature and the solution was stirred for 10 h at room temperature. The solvent was removed in vacuo to give oil, which was diluted with anisole, and the solution was evaporated to dryness. The reaction mixture was concentrated under reduced pressure, followed by precipitating from Et$_2$O to give a first crop of **13**. The filtrate containing the remainder of **13** was concentrated and then further purified by flash column chromatography (8:2 CHCl$_3$/MeOH) to give the second crop of product **13** with an overall yield of 77%: ^{1}H NMR (compound **13**, 500 MHz, CDCl$_3$) δ 0.86 (dd, *J* = 9.9, 6.3 Hz, 6H), 1.42−1.51 (m, 2H), 1.62−1.71 (m, 1H), 2.73 (d, *J* = 4.6 Hz, 3H), 2.91−3.00 (m, 2H), 3.97 (t, *J* = 7.2 Hz, 1H), 4.99−5.06 (m, 1H), 5.80 (s, 1H),

6.42 (s, 1H), 7.07−7.10 (m, 2H), 7.12−7.19 (m, 3H), 7.62 (s, 1H), 8.56 (d, $J = 8.8$ Hz, 1H), 11.5 (bs, 1H); ^{13}C NMR (126 MHz, CDCl$_3$): δ 22.7, 22.8, 26.0, 26.6, 39.6, 41.9, 43.4, 54.7, 126.9, 127.4, 128.6, 129.5, 136.5, 139.2, 169.9, 173.2, 173.7; LC/MS *m/z* 345 (M − H)$^-$; HRMS (FAB) for C$_{19}$H$_{26}$N$_2$O$_4$ [M + H]$^+$ calcd 347.1971, found 347.1990.

(2S,3R)-3-[1-(Methylcarbamoyl)-(2S)-2-phenylethylcarbam-oyl]-2-(2-oxopropyl)hexanoic Acid (14). Compound **13** (0.69 g, 2 mmol) in THF (5 mL) was treated with thioacetic acid (210 μL, 3 mmol) and two drops of a 1:1 solution of triethylamine in THF. The mixture was stirred at room temperature overnight under an atmosphere of nitrogen, followed by the addition of hexane (25 mL) to yield a white solid **14** (0.75 g, 89%): ^{1}H NMR (compound **14**, 500 MHz, DMSO-*d$_6$*) δ 0.69 (d, $J = 6.4$ Hz, 3H), 0.75 (d, $J = 6.4$ Hz, 3H), 0.81−0.87 (m, 1H), 1.21−1.27 (m, 1H), 1.40−1.46 (m, 1H), 2.21 (s, 3H), 2.25−2.31 (m, 2H), 2.38−2.43 (m, 1H), 2.45−2.49 (m, 1H), 2.52 (d, $J = 4.5$ Hz, 3H), 2.74 (dd, $J = 13.5$, 10.2 Hz, 1H), 2.90 (dd, $J = 13.6$, 4.8 Hz, 1H), 4.48−4.53 (m, 1H), 7.06 (t, $J = 7.2$ Hz, 1H), 7.14 (t, $J = 7.4$ Hz, 2H), 7.18−7.22 (m, 2H), 7.82 (d, $J = 4.6$ Hz, 1H), 8.31 (d, $J = 8.5$ Hz, 1H), 12.2 (s, 1H); ^{13}C NMR (126 MHz, DMSO-*d$_6$*) δ 22.2, 24.5, 25.8, 26.2, 29.1, 31.0, 38.2, 47.0, 49.2, 54.7, 126.8, 128.6, 129.7, 138.5, 172.0, 172.4, 174.6, 194.5; LC/MS *m/z* 423 [M + H]$^+$; HRMS (FAB) for C$_{21}$H$_{30}$N$_2$O$_5$S [M + H]$^+$ calcd 423.1954, found 423.1938.

(2R)-N^1-[1-(Methylcarbamoyl)-(2S)-2-phenylethyl]-N^4-hydroxy-2-isobutyl-3-methylenesuccinamide (16). To an ice-cold solution of **13** (3.4 g, 10 mmol) and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU, 1.6 g, 10 mmol) in dry DMF (40 mL) was added 2,4,6-trichloro[1,3,5]triazine (TCT, 0.76 g, 4.2 mmol), and the mixture was stirred under N$_2$ for 1 h. The resulting mixture was added by syringe to a flask containing a suspension of hydroxylamine hydrochloride (3.0 g, 40 mmol) and Et$_3$N (5.6 mL, 40 mmol) in DMF. The mixture was then stirred for 2 h at room temperature. Removal of solvent in vacuo gave an oil that was copiously washed with water to give a solid material, which was then extracted into CH$_2$Cl$_2$. The extract was dried over MgSO$_4$ and concentrated on a rotary evaporator to give **16** (2.9 g, 82%): ^{1}H NMR (compound **16**, 600 MHz, 10% CD$_3$OD in CDCl$_3$) δ 0.65 (d, $J = 5.3$ Hz, 3H), 0.66−0.73 (m, 3H), 1.24 (d, $J = 2.9$ Hz, 2H), 1.37 (m, 1H), 2.52−2.59 (m, 4H), 2.76 (s, 1H), 2.96 (d, $J = 7.8$ Hz, 1H), 3.15−3.23 (m, 1H), 4.38 (s, 1H), 5.18 (s, 1H), 5.48 (s, 1H), 6.98−7.05 (m, 3H), 7.09 (t, $J = 6.8$ Hz, 2H), 7.17 (d, $J = 6.8$ Hz, 1H); ^{13}C NMR (75 MHz, MeOD-*d$_4$*) δ 22.8, 22.9, 26.4, 27.0, 39.0, 41.5, 46.5, 56.2, 126.8, 127.7, 129.4, 130.3, 138.4, 141.3, 170.4, 174.0, 175.2; LC/MS *m/z* 362 [M + H]$^+$; HRMS (FAB) for C$_{19}$H$_{27}$N$_3$O$_4$ [M + H]$^+$ calcd 362.2080, found 362.2101.

S-(2S,3R)-3-[1-(Methylcarbamoyl)-(2S)-2-phenylethylcarbam-oyl]-2-(hydroxycarbamoyl)-5-methylhexyl ethanethioate (17). The derivative **16** (0.72 g, 2 mmol) in THF (5 mL) was treated with thioacetic acid (420 μL, 6 mmol) and two drops of a 1:1 solution of triethylamine in THF. The mixture was stirred at room temperature for 1 day under an atmosphere of nitrogen, followed by removal of solvents to yield a white solid **17** (0.76 g, 87%), which was purified by HPLC. Reversed-phase semipreparative HPLC purification was performed using DeltaPak C-18 column (300 mm × 19 mm) and monitored with a Waters 2996 photodiode array detector: ^{1}H NMR (compound **17**, 500 MHz, 3% CD$_3$OD in CDCl$_3$) δ 0.74−0.82 (m, 6H), 1.03 (ddd, $J = 13.3$, 9.9, 3.6 Hz, 1H), 1.34 (td, $J = 6.5$, 3.1 Hz, 1H), 1.59−1.68 (m, 1H), 2.23−2.28 (s, 3H), 2.70−2.79 (m, 6H), 2.98−3.07 (m, 1H), 3.09−3.18 (m, 1H), 4.84−4.90 (m, 1H), 7.01 (d, $J = 4.8$ Hz, 1H), 7.15 (dt, $J = 8.6$, 4.3 Hz, 1H), 7.19−7.25 (m, 4H), 7.71 (d, $J = 8.6$ Hz, 1H); ^{13}C NMR (126 MHz, 3% MeOD-*d$_4$* in CDCl$_3$) δ 21.2, 23.3, 25.6, 25.9, 28.2, 30.3, 31.2, 39.5, 46.5, 47.3, 54.3, 126.6, 128.3, 129.0, 136.8, 171.6, 172.4, 173.3, 194.5; LC/MS *m/z* 438 [M + H]$^+$; HRMS (FAB) for C$_{21}$H$_{31}$N$_3$O$_5$S [M + H]$^+$ calcd 438.2063, found 438.2083.

Preparation of the Inhibitor-Tethered Resins 2 and 3. Two different synthetic approaches were attempted, as follows:

Route A (Scheme 3). Commercial epoxy-activated Sepharose 6B resin (560 mg) was washed thoroughly with distilled water (500 mL) before use. The swollen resin was placed in a flask and purged with argon. A solution of CH$_3$COSH (0.5 mL) in 50% MeOH (5 mL) was added to the resin under argon. The resulting suspension was swirled using a gyratory water-bath shaker under argon at 25 °C for 1 day. The thioacetyl-modified resin **19** was filtered and washed with methanol (200 mL) and then with water (200 mL). To generate the intermediate **18**, a solution of compound **17** (180 mg, 0.4 mmol) and K$_2$CO$_3$ (56 mg, 0.4 mmol) in 50% MeOH (1 mL) was sonicated under argon for 5 min and then was transferred via cannula to the swollen resin **19** followed by the addition of the potassium carbonate solution pH 10.8 (15 mL). The suspension was vigorously swirled for 15 min. Subsequently, oxygen was bubbled into the reaction vessel to purge the residual inert gas from the reaction mixture, and after overnight oxidative treatment the modified Sepharose resin **2** was filtered, washed with methanol (5 × 40 mL), followed by 100 mM sodium acetate, 0.5 M NaCl, pH 4.0 (50 mL each, 5×). Finally, the resin was washed with 0.5 M NaCl (100 mL) containing 1% sodium azide and was stored at 5 °C in the same solution.

Route B (Scheme 3). Commercial epoxy-activated Sepharose 6B resin (560 mg) resin was washed thoroughly with distilled water (500 mL) before use. The swollen resin was placed in a flask and purged with argon. A solution of CH$_3$COSH (0.5 mL) in 50% MeOH (5 mL) was added to the resin under argon. The resulting suspension was swirled in a gyratory water-bath shaker under argon at 25 °C for 1 day. The resulting resin **19** was filtered and washed with methanol (200 mL) and then with water (200 mL), followed by the addition of the potassium carbonate solution pH 10.8 (5 mL) to the resin under argon. The resulting suspension was swirled under argon at 25 °C for 1 h and washed with methanol (200 mL) and then with CH$_2$Cl$_2$/MeOH (1:1, 200 mL). To generate the Sepharose-supported disulfide **21**, a solution of 2,2′-dithiobis(5-nitropyridine) (DTNB; 310 mg, 1 mmol) in CH$_2$Cl$_2$ (1 mL) was transferred via cannula to the swollen Sepharose 6B resin **20**, and the mixture was swirled in a gyratory water-bath shaker under argon at 25 °C for 1 day. The resin was filtered, washed with CH$_2$Cl$_2$/MeOH (1:1, 200 mL). To generate the intermediate **15**, a solution of compound **14** (174 mg, 0.4 mmol) and K$_2$CO$_3$ (56 mg, 0.4 mmol) in 50% methanol (1 mL) was sonicated for 10 min, and the resultant mixture was transferred via cannula to the modified resin **21** followed by the addition of TFA (40 μL, 0.5 mmol). The suspension was swirled under argon at 25 °C for 1 day. The inhibitor-tethered resin **3** was filtered and washed with methanol (5 × 40 mL), followed by 100 mM sodium acetate, 0.5 M NaCl, pH 4.0 (50 mL each, 5 times). Finally, the resin was washed with 0.5 M NaCl (100 mL) containing 1% sodium azide and was stored at 5 °C in the same solution.

Binding of MMP-2 to the Inhibitor-Tethered Resin. Recombinant pro-MMP-2 was activated by incubation with 1 mM *p*-aminophenylmercuric acetate (APMA), followed by dialysis against CB buffer (50 mM Tris/HCl pH 7.5, 150 mM NaCl, 5 mM CaCl$_2$ and 0.02% Brij 35) to remove APMA as previously described.[28] Active MMP-2 (100 nM in CB buffer) was incubated at room temperature without or with 10 μL of the inhibitor-tethered resin (0.5 mL final volume). At various times (5, 30, and 60 min), the samples were centrifuged at 15000*g* for 1 min. Aliquots of the supernatants (unbound fractions) were collected, and enzyme activity was measured using the fluorescence-quenching peptide substrate (MOCAc-Pro-Leu-Gly-Leu-A$_2$pr (Dnp)-Ala-Arg-NH$_2$). Substrate hydrolysis was monitored for 15 min by fluorescence spectrophotometer at excitation and emission wavelengths of 328 and 393 nm, respectively, and the percentages of remaining activity were compared. The total of MMP-2 activity (100%) was attributed

(28) Fridman, R.; Toth, M.; Pena, D.; Mobashery, S. *Cancer Res.* **1995**, *55*, 2548−2555.

to an aliquot of MMP-2 incubated under the same conditions in the absence of any resins. At the end of incubation the resins were washed with the CB buffer, the bound MMP-2 was eluted with 50 mM Tris/HCl, 150 mM NaCl, pH 7.5, supplemented with 2.5% β-mercaptoethanol (or (2R,3R)-1,4-dimercaptobutane-2,3-diol), and the samples were subjected to SDS−PAGE. The gel was stained with Coomassie Brilliant Blue for visualization of the proteins.

Supporting Information Available: NMR spectra for the synthetic molecules. This material is available free of charge via the Internet at http://pubs.acs.org.

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