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Novel Antitumor Prodrugs Designed for Activation by Matrix Metalloproteinases-2 and -9

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Abstract: Enzyme prodrug monotherapy takes advantage of the selectivity and specificity of enzymes that are differentially active in the immediate environment of tumor cells. Matrix metalloproteinases-2 and -9 (MMP-2 and -9, respectively) are cell-surface Zn-dependent endoproteases associated with diverse processes throughout tumor formation and progression. These enzymes have demonstrated high ratios of tumor- to nontumor-associated activity and may represent candidates for antitumor prodrug activation. Our MMP targeting strategy was to prepare and evaluate two classes of enzyme prodrugs, peptides and sequence-similar peptidomimetics, and determine which would be substrates for the enzymes and thus suitable for further *in vitro* and *in vivo* evaluation. We selected representatives of three structurally and mechanistically distinct classes of compounds for delivery, doxorubicin, several auristatins (novel synthetic members of the dolastatin class of tubulin polymerization inhibitors), and CBI-TMI (a duocarmycin class minor groove binder). The drugs were acylated on available amines with the broadly recognized MMP substrate P3−P1′ sequence acetyl L-prolyl-L-leucyl-glycyl-L-leucine, or with a peptidomimetic analogue. From a panel of four peptides and four peptidomimetics, two compounds, both peptides, were found to be substrates, with specific activities in the range of 1−20 nmol min^{-1} mg^{-1}. For MMP-9, complete conversion took place in 4−16 h; proteolysis by MMP-2 was considerably slower. Cleavage occurred, as predicted, at the Gly−Leu bond to liberate a leucyl drug, and no other intermediates or cleavage products were observed. Although the MMP-9 proteolysis products were equipotent with the parent leucyl drugs, the prodrugs were not differentially active against MMP-2 or -9-expressing versus nonexpressing cell lines during a 4 h exposure. Our data can be interpreted in light of the current understanding of the structural and mechanistic factors governing MMP-2 and -9 proteolysis.

Keywords: Matrix metalloproteinase; MMP-2; MMP-9; doxorubicin; auristatin; duocarmycin; peptide; peptidomimetic; antitumor; prodrug

Introduction

To increase the effectiveness and decrease the systemic toxicity of cancer chemotherapy, a substantial body of research has surrounded enzyme prodrug monotherapy, a therapeutic modality directed toward the use of tumor-specific enzymes for the release of active drugs from inactive prodrugs. The success of an enzyme prodrug strategy relies on the identification of suitable enzymes differentially active in the immediate environment of tumor cells. The matrix metalloproteinases (MMPs) are a family of approximately two dozen Zn-dependent enzymes, many of which have been demonstrated to have functions essential for tumor formation, invasion, and progression to the metastatic phenotype.[1,2] Proteolysis by MMPs is highly regulated by several mech-

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anisms, including posttranslational modifications, endogenous inhibitors, and cell-surface requirements for activity.[2] The increased MMP activity in tumorogenic, as compared to normal, processes has made these proteases attractive targets for inhibitors such as the clinical candidates Batimastat, Marimastat, and Prinomastat.[2−5] None of the originally promising candidates have demonstrated the anticipated benefits in patients, in part because the designs of the clinical trials did not take into account all of the complex, and in some cases anti- as well as pro-tumorogenic, functions of these enzymes.[1,4,6−8]

The multiple functions of the MMPs, and increases in MMP activity during the initial stages of transformation, are characteristics that make the MMPs problematical targets for inhibition-based therapy but make this class of proteases good candidates for enzyme prodrug monotherapy. MMP-2 (Gelatinase A, 72 kDa Collagenase IV) and MMP-9 (Gelatinase B, 95 kDa Collagenase IV) are highly similar in their architecture and substrate recognition properties, and share many characteristics in their activation and regulation pathways.[1,9] MMP-2 and -9 must be localized on specific cell-surface receptors to be activated, and the activation mechanisms require the coordinate actions of several proteins.[1,7,10−12] These two metalloproteases are associated with both the early and later stages in tumor progression and are particularly recognized for their roles in invasiveness in a wide variety of solid tumors. MMP-2 and -9 substrate sequences have been used in prodrug strategies attempting to release doxorubicin,[13−15] melphalan,[16,17] camptothecin, vinblastine,[18] and a recombinant anthrax toxin[19] as the cytotoxic agents.

The MMP targeting strategy described here is distinguished from the previous work by the preparation of both peptide and sequence-similar peptidomimetic anticancer drug derivatives for evaluation as prodrug candidates. We selected representatives of three structurally and mechanistically distinct classes of compounds for delivery, doxorubicin, several auristatins (novel synthetic members of the dolastatin class of tubulin polymerization inhibitors),[20] and CBI-TMI (a duocarmycin class minor groove binder).[21] As shown in Figure 1, the drugs were acylated on available amines with the broadly recognized MMP substrate sequence acetyl L-prolyl-L-leucyl-glycyl-L-leucine (AcProLeuGlyLeu), or with the peptidomimetic analogue. This moiety occupies the P3−P1′ substrate sites in relation to the scissile Gly−Leu bond, according to the nomenclature of Schechter and Berger.[22] The site of acylation on each drug was intended to provide both convenient attachment of the targeting peptide and, by

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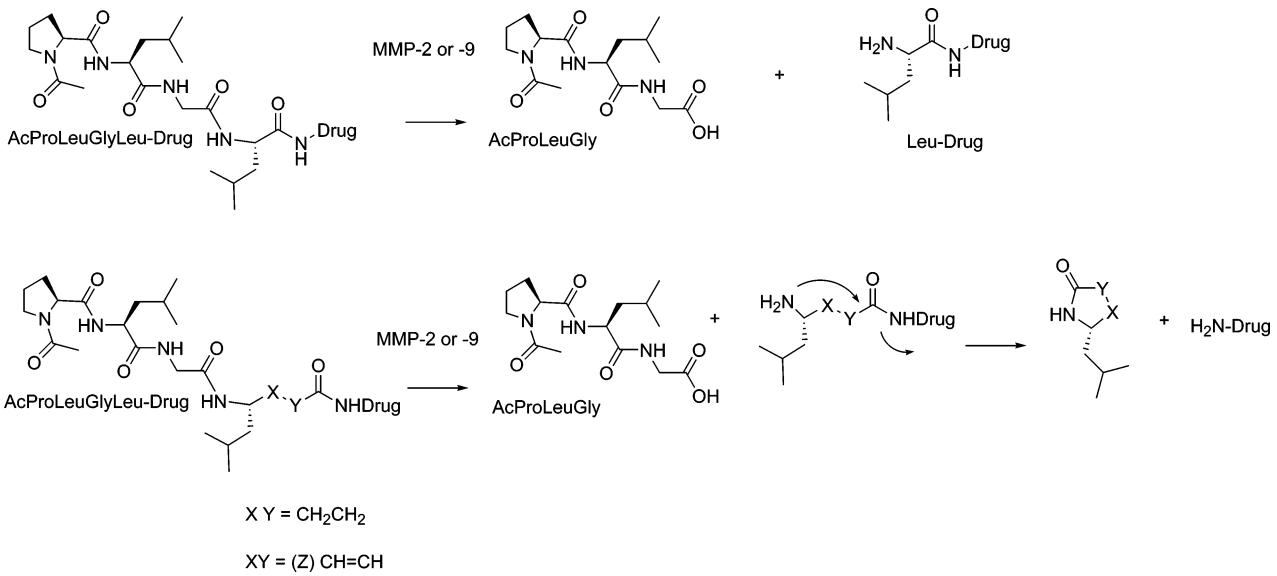

Figure 1. Prodrugs acylated with the peptide AcProLeuGlyLeu would be expected to cleave at the Gly−Leu bond and release a leucyl drug. The peptidomimetic leucyl analogues would be expected to cleave after glycine, allowing the free amino group to attack the amide δ carbonyl and release the free drug.

masking a critical amino group, a significant reduction in the cytotoxicity of the drug.

Prodrugs derivatized with the peptide AcProLeuGlyLeu would be expected to resemble more closely the physiological substrates of the enzymes, but upon proteolysis would retain the residual P1′ leucine on the drug. Doxorubicin is a well-validated example of a drug whose activity is minimally compromised by acylation with leucine: L-leucyldoxorubicin (LeuDox) has been shown to be a potent antitumor agent *in vitro* and *in vivo*.[23,24] This tolerance, however, is by no means a general phenomenon. Compounds of the duocarmycin class are known to be significantly deactivated upon N-acylation,[25] and therefore require a different structural solution to the problem of proteolytic release. For the auristatins, the effect of an N-terminal leucine was not known, and would have to be experimentally determined. On the basis of the predicted effect that a residual leucine might have on the antitumor potency of the released drug, we selected for our initial prodrug screening one peptidyl analogue of doxorubicin, both peptidyl and peptidomimetic analogues of the auristatins, and exclusively a peptidomimetic analogue of the duocarmycin CBI-TMI. For prodrugs in which the leucine would be retained after proteolysis, the corresponding leucyl drug was also prepared. This panel of compounds was synthesized and evaluated *in vitro* as substrates for MMP-2 and MMP-9, with the goal of identifying those compounds that are suitable for further investigation, *in vitro* and *in vivo*, as candidates for enzyme prodrug monotherapy.

Experimental Section

Chemistry. General Section. Unless otherwise indicated, all anhydrous solvents were commercially obtained and stored in Sure-seal bottles under nitrogen. All other reagents and solvents were purchased as the highest grade available and used without further purification. NMR spectra were recorded on a Varian Mercury 400 MHz instrument. Chemical shifts (δ) are reported in parts per million (ppm) referenced to tetramethylsilane at 0.00, and coupling constants (J) are reported in hertz. Low-resolution mass spectral data were acquired on a Micromass ZMD mass spectrometer interfaced with an HP Agilent 1100 high-performance liquid chromatography instrument for LC−MS. Products were eluted on a Phenomonex 2.0 mm × 150 mm, 4 μm, 80 Å MAX RP synergi column using a linear gradient of mobile phase B (CH_3CN) in phase A (0.05% aqueous HCO_2H) at a flow rate of 0.4 mL/min. Unless otherwise specified, the reported retention times (t_R) are those from LC−MS. High-resolution (exact mass) data were obtained at the University of Washington Medicinal Chemistry Mass Spectrometry Center (Seattle, WA) on a Bruker APEXIII 47e [FT(ICR)]-MS instrument. Both analytical and preparative high-performance liquid chromatography (HPLC) were conducted on a Varian model 210 instrument using ProStar software. For analytical HPLC, the stationary phase that was used was a Phenomonex 4.6 mm × 150 mm, 4 μm, 80 Å MAX RP synergi column. Products were eluted on either acidic linear gradients (designated gradient A) of mobile phase B (CH_3-

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CN) in phase A (0.05% aqueous TFA), or neutral linear gradients (designated gradient N) of mobile phase B (CH_3CN) in phase A (5.0 mM $NH_4H_2PO_4$) at a flow rate of 1.0 mL/min. For preparative HPLC, the stationary phase that was used was a Phenomonex 10 mm × 250 mm, 4 μm, 80 Å semipreparative synergi column, using the acidic gradient of phase A at a flow rate of 4.6 mL/min. All peaks were monitored at 256 and 215 nM. Radial chromatography was performed on a Chromatotron instrument (Harrison Research, Palo Alto, CA); preparative thin-layer chromatography was performed on Whatman 20 cm × 20 cm, 500 $\mu\mu$, 60 Å silica gel plates, and all other preparative normal phase purifications were done by standard flash silica gel chromatography using Whatman Science 60 Å 230−400 mesh silica gel as an adsorbent.

L-Leucyldoxorubicin (1). To a suspension of L-leucine (15 g, 114.30 mmol) in 200 mL of CH_2Cl_2 was added trimethylsilyl chloride (29 mL, 228.60 mmol). The reaction mixture was refluxed for 1 h, cooled to ambient temperature, and treated sequentially with DIEA (39.8 mL, 228.60 mmol) and *p*-anisylchlorodiphenylmethane (37.08 g, 120.02 mmol). The reaction mixture was stirred at ambient temperature for 16 h, then treated with MeOH (23.3 mL, 571.50 mmol), and stirred at ambient temperature for 1 h. The volatiles were removed *in vacuo*, and the residue was partitioned between pH 5.5 phosphate buffer and EtOAc. The organic phase was dried and concentrated *in vacuo* to give the crude monomethoxytrityl (Mmtr) L-leucine that was used directly in the subsequent reaction: LC−MS (ES) *m*/*z* 404.30 [$(M + H)^+$], 402.38 [$(M - H)^-$]; t_R = 9.45 min. To a suspension of doxorubicin HCl (Meiji Pharmaceuticals, 260 mg, 0.37 mmol) in 4 mL of DMF was added *N*-methylmorpholine (NMM, 0.05 mL, 0.45 mmol). To the resulting solution was added an ice-cooled mixture of the above Mmtr L-leucine (225 mg, 0.56 mmol), NMM (0.05 mL, 0.45 mmol), and isobutyl chloroformate (0.06 mL, 0.45 mmol). The reaction was allowed to proceed at ambient temperature for 16 h in the dark and monitored by HPLC: t_R = 8.05 min (gradient N). Additional isobutyl chloroformate (0.12 mL, 0.90 mmol) in 2 mL of DMF was added, and the reaction was allowed to proceed for an additional 16 h. The crude reaction mixture was applied directly to a 2000 μm radial chromatography plate and the product purified by elution with a gradient of MeOH and CH_2Cl_2 (from 0:100 to 10:90). The volatiles were removed *in vacuo*, and Mmtr L-leucine was deprotected by treatment with a mixture of TFA (0.05 mL) and triethylsilane (0.10 mL) in 2 mL of CH_2Cl_2. After removal of the volatiles *in vacuo*, a portion of the crude free amine was purified by RP-HPLC to give **1** (21.5 mg, 0.03 mmol): HRMS *m*/*z* $[M + H]^+$ calcd for $C_{33}H_{41}N_2O_{12}$ 657.2660, found 657.2641.

Acetyl-L-prolyl-L-leucyl Glycine (2). Fmoc glycyl Wang resin (Advanced Chemtech, Louisville, KY, 5 g, 3 mmol) was allowed to swell in CH_2Cl_2 for 2 h and then treated with two successive cycles of 20% (v/v) piperidine in DMF (30 mL) for 30 min each, followed by three washes of DMF and three of CH_2Cl_2. Fmoc L-leucine was coupled to the resin using of 2.5 equiv of Fmoc L-leucine, 2.5 equiv of *O*-7-azabenzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU), 2.5 equiv of 1-hydroxy-7-azabenzotriazole (HOAt), and 5 equiv of DIEA dissolved in 20 mL of DMF. The ninhydrin test showed the coupling to be complete in 4 h, and the resin was washed with three treatments of DMF, two of MeOH, and three of CH_2Cl_2. The Fmoc group was removed as described above, and Fmoc L-proline was added using the same protocol. Following removal of the N-terminal Fmoc group, the resin was treated with a solution of acetic anhydride and DIEA (30 equiv each) in 30 mL of DMF. The crude acetyltripeptide resin was washed as described above and dried *in vacuo*, and the peptide was cleaved using a TFA/water/triisopropylsilane mixture (95:2.5:2.5, 5 mL/g of dry resin) for 3 h at ambient temperature. After the volatiles were removed *in vacuo*, the crude peptide was triturated with diethyl ether, filtered, and washed with additional ether, to give **2** (424 mg, 43% based on original resin loading) that was sufficiently pure to be used directly in all fragment coupling reactions: ^{1}H NMR (400 MHz, CD_3CN) δ 7.40−7.45 (br, 1H), 6.90−7.11 (br, 1H), 4.30−4.41 (m, 1H), 4.22−4.28 (m, 1H), 3.76−3.87 (m, 2H), 3.48−3.65 (m, 2H), 2.12−2.22 (m, 2H), 2.09 (s, 3H), 1.81−1.90 (m, 4H partially obscured by CH_3CN signals), 1.55−1.70 (m, 2H), 0.88−0.98 (m, 6H); t_R = 6.80 min (gradient A); LC−MS (ES) *m*/*z* 253.10 [$(M - Gly + H)^+$], 328.23 [$(M + H)^+$], 350.20 [$(M + Na)^+$], 326.34 [$(M - H)^-$]; t_R = 5.60 min.

Acetyl L-Prolyl-L-leucyl-glycyl-L-leucyldoxorubicin (3). A solution of **2** (5.0 mg, 15.3 μmol) in 0.5 mL of DMF was treated with DIEA (8 μL, 46 μmol) and DEPC (2.6 μL, 16.8 μmol) for 5 min at ambient temperature. The activated tripeptide acid solution was added to solid **1** (12.0 mg, 18.3 μmol). The resulting solution was stirred at ambient temperature for 16 h and then purified directly by radial chromatography as described for **1** to give **3** (5 mg, 28%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.80 (br, 2H), 7.90−8.10 (br, 2H), 7.07−7.90 (br, 1H), 7.05−7.55 (m, 4H), 5.50−5.60 (m, 1H), 5.25−5.40 (m, 1H), 4.40−4.60 (m, 14H), 4.10 (s, 3H), 3.30−3.90 (m, 5H), 2.00−2.22 (s, 3H over m, 6H), 1.00−1.18 (m, 17H), 0.89−0.90 (m, 6H); t_R = 8.13 min (gradient N); HRMS *m*/*z* $[M + Na]^+$ calcd for $C_{48}H_{63}N_5O_{16}$-Na 988.4168, found 988.4157.

L-Valyl-L-dolaisoleucyl-L-dolaproinyl-L-phenylalanyl Methyl Ester (4). A solution of Boc dolaproinylphenylalanine methyl ester[26] (3.44 g, 7.68 mmol) and Cbz-valyldolaisoleuine *tert*-butyl ester[26] (3.35 g, 7.68 mmol) in 10 mL of CH_2Cl_2 was treated with 10 mL of TFA for 2 h at ambient temperature. The volatiles were removed *in vacuo*, and the residual oily residue was triturated with a 1:1 mixture of toluene and xylenes, dried *in vacuo*, and redissolved in 10 mL of CH_2Cl_2. To this was added sequentially DIEA (3.5

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mL, 30.72 mmol) and DEPC (1.75 mL, 11.52 mmol). The reaction mixture was stirred at ambient temperature for 16 h, and the volatiles were removed *in vacuo*. The residue was dissolved in EtOAc and washed sequentially with 0.1 N HCl, water, saturated $NaHCO_3$, and water, dried, and concentrated *in vacuo* to give a residue that was purified on silica gel (from 80:20 to 100:0 EtOAc/hexanes mixture) to give the Cbz-protected tetrapeptide intermediate (3.5 g, 4.56 mmol). Atmospheric-pressure hydrogenation of this material in 100 mL of EtOH over 10% Pd-C (200 mg) for 16 h, followed by filtration through MeOH-washed Celite and concentration of the filtrate *in vacuo*, gave **4** (2.81, 58%): $t_R = 9.45$ min (gradient N); HRMS *m/z* $[M + H]^+$ calcd for $C_{34}H_{57}N_4O_7$ 633.4227, found 633.4214; HRMS *m/z* $[M + Na]^+$ calcd for $C_{34}H_{56}N_4O_7Na$ 655.4047, found 655.4048.

Acetyl L-Prolyl-L-leucyl-glycyl-L-valyl-L-dolaisoleucyl-L-dolaproinyl-L-phenylalanyl Methyl Ester (5). Tetrapeptide **4** (40 mg, 0.06 mmol) was acylated with AcProLeuGly as described for **3**. The crude product was purified via RP-HPLC to give pure **5** (10 mg, 17%): $t_R = 8.03$ min (gradient N); HRMS *m/z* $[M + Na]^+$ calcd for $C_{49}H_{79}N_7O_{11}Na$ 964.5735, found 964.5727; HRMS *m/z* $[M + K]^+$ calcd for $C_{49}H_{79}N_7O_{11}K$ 980.5475, found 980.5487.

L-Leucyl-L-valyl-L-dolaisoleucyl-L-dolaproinyl-L-phenylalanyl Methyl Ester (6). Tetrapeptide **4** (57 mg, 0.09 mmol) was acylated with Boc L-leucine (24.7 mg, 0.10 mmol) as described for **3**. The crude product was purified via RP-HPLC to give the N^{α}-Boc-protected intermediate pentapeptide, to which was immediately added a solution of 4 N HCl-dioxane (1 mL, 4 mmol). The reaction mixture was stirred for 3 h at ambient temperature, and the volatiles were removed *in vacuo* to give the free amine hydrochloride salt **6** (11 mg, 16%): $t_R = 9.86$ min (gradient N); HRMS *m/z* $[M + H]^+$ calcd for $C_{40}H_{68}N_5O_8$ 746.5068, found 746.5054; HRMS *m/z* $[M + Na]^+$ calcd for $C_{40}H_{67}N_5O_8Na$ 768.4887, found 768.4882.

Acetyl L-Prolyl-L-leucyl-glycyl-L-leucyl-L-valyl-L-dolaisoleucyl-L-dolaproinyl-L-phenylalanyl Methyl Ester (7). Tetrapeptide **4** (309 mg, 0.488 mmol) was acylated with Fmoc L-leucine (181 mg, 0.51 mmol) as described for **3**. The Fmoc intermediate was purified by radial chromatography (from 0:100 to 5:95 MeOH/CH_2Cl_2 mixture) to give 353 mg (0.36 mmol, 74%). The Fmoc group was removed by treatment with 5 mL of a 2:3 diethylamine/CH_2Cl_2 mixture for 2 h at ambient temperature. Volatiles were removed *in vacuo*, and the crude free amine was acylated with Fmoc glycine (118 mg, 0.40 mmol) using the standard coupling conditions. The product was purified by radial chromatography (from 0:100 to 5:95 MeOH/CH_2Cl_2 mixture) to give 296 mg (0.29 mmol, 80%): $t_R = 12.5$ min (gradient N); MS (ES) *m/z* 1025.69 $[(M + H)^+]$, 1047.68 $[(M + Na)^+]$. The Fmoc group was removed using the previously described procedure, and the free amine was acylated with Fmoc L-leucine (153 mg, 0.43 mmol) as described for **3** but with bromotrispyrrolidinophosphonium hexaflurophosphate (PyBroP, 269 mg, 0.58 mmol) in place of DEPC as the coupling agent. The product was purified via radial chromatography (from 0:100 to 5:95 MeOH/CH_2Cl_2 mixture) to give 310 mg (0.27 mmol, 94%), which was deprotected and acylated with Fmoc L-proline (101 mg, 0.30 mmol) using the PyBroP modification of the standard conditions. Purification of the Fmoc prolyl intermediate via radial chromatography (from 0:100 to 5:95 MeOH/CH_2Cl_2 mixture), and removal of the Fmoc group under standard conditions gave the free amine, which was immediately dissolved in CH_2Cl_2 and treated sequentially with DIEA (0.14 mL, 0.82 mmol) and acetic anhydride (1 mL, 0.11 mmol). The reaction was complete in 5 min (by HPLC), and the volatiles were removed *in vacuo*. A portion of the crude product was purified by RP-HPLC to give **7** (28 mg, 0.03 mmol): $t_R = 9.26$ min (gradient N); HRMS *m/z* $[M + H]^+$ calcd for $C_{55}H_{91}N_8O_{12}$ 1055.6756, found 1055.6756; HRMS *m/z* $[M + Na]^+$ calcd for $C_{55}H_{90}N_8O_{12}Na$ 1077.6576, found 1077.6525; HRMS *m/z* $[M + K]^+$ calcd for $C_{55}H_{90}N_8O_{12}K$ 1093.6315, found 1093.6267.

***N,N*-Dimethyl-L-valyl-L-isoleucyl-L-valyl-L-dolaisoleucyl-L-dolaproinyl-L-phenylalanyl-4-(4′-L-Leucylamidophenyl) Amide (9).** To a solution of **8**[27] (42.0 mg, 0.05 mmol) in 3 mL of CH_2Cl_2 were added sequentially Boc L-leucine monohydrate (15.0 mg, 0.06 mmol), HATU (28.0 mg, 0.07 mmol), and pyridine (6.0 μL, 0.07 mmol). The reaction mixture was stirred at ambient temperature for 96 h, diluted with additional CH_2Cl_2, washed successively with water and saturated $NaHCO_3$, dried, and concentrated *in vacuo* to an oil. Purification by radial chromatography (from 0:100 to 10:90 MeOH/CH_2Cl_2 mixture) gave the pure Boc-protected intermediate (51 mg, 97%): $t_R = 13.10$ min (gradient N); LC−MS (ES) *m/z* 1063.74 $[(M + H)^+]$. To a solution of this in 3 mL of DMF was added 4 N HCl in dioxane (3 mL), and the deprotection was allowed to proceed at ambient temperature for 2 h. Removal of the volatiles *in vacuo* gave a residue that was purified by precipitation with successive treatments of diethyl ether and anhydrous HCl in diethyl ether. The precipitate was collected and washed with diethyl ether to give **9** as the dihydrochloride salt (50 mg, 97%): HRMS *m/z* $[M + H]^+$ calcd for $C_{53}H_{87}N_8O_8$ 963.6647, found 963.6627.

***N,N*-Dimethyl-L-valyl-L-isoleucyl-L-valyl-L-dolaisoleucyl-L-dolaproinyl-L-phenylalanyl-4-(4′-acetyl-L-prolyl-L-leucyl-glycyl-L-leucylamidophenyl) Amide (10).** Amine **9** (49 mg, 0.05 mmol) was acylated with **2** as described for **3**. The crude product was purified via RP-HPLC to give pure **10** (34 mg, 56%): $t_R = 10.53$ min (gradient N); HRMS *m/z* $[M + Na]^+$ calcd for $C_{68}H_{109}N_{11}O_{12}Na$ 1294.8155, found 1294.8134.

2-(3-{1-[(4-{[2-(4-*tert*-Butoxycarbonylamino-6-methylhept-2-ynolylamino)-3-methylbutyryl]methylamino}-3-methoxy-5-methylheptanoyl)pyrrolidin-2-yl]-3-methoxy-2-methylpropionylamino}-3-phenyl)propionic Acid Methyl Ester (11). To a −10 °C solution of acid **12**[28] (97 mg, 0.38

(27) Senter, P. D.; Doronina, S. O.; Toki, B. E. Drug Conjugates and their Use for Treating Cancer, an Autoimmune Disease and an Infectious Disease. PCT US 03 24209, 2003.

(28) Reetz, M. T.; Strack, T. J.; Kanand, J.; Goddard, R. Stereospecific Synthesis of Chiral Alkynogous Amino Acids. *Chem. Commun.* **1996**, 733−734.

mmol) and **4** (289 mg, 0.46 mmol) in 1.5 mL of CH_2Cl_2 were added DIEA (0.16 mL, 0.91 mmol) and PyBroP (248 mg 0.53 mmol). The reaction mixture was allowed to equilibrate to ambient temperature overnight, and then the reaction was quenched by addition of saturated aqueous $NaHCO_3$. The reaction mixture was diluted with additional CH_2Cl_2 and washed successively with 0.1 N HCl, water, and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The crude product was purified by radial chromatography on a 4000 μm plate, eluting with a gradient from a 1:99 mixture to a 5:95 mixture of MeOH and CH_2Cl_2, followed by a second chromatography on a 2000 μm plate to give **11** (130 mg, 52%): ^{13}C NMR (400 MHz, $CDCl_3$) δ 174.39, 172.69, 170.29, 154.87, 152.84, 136.81, 129.38, 128.85, 127.10, 86.55, 86.14, 81.87, 80.29, 78.44, 60.59, 59.30, 58.22, 53.91, 53.66, 52.39, 47.79, 44.86, 43.94, 37.74, 37.61, 33.69, 31.61, 28.53, 26.12, 25.13, 24.92, 22.60, 22.27, 19.78, 17.46, 16.14, 14.27, 11.23; LC−MS (ES) *m*/*z* 870.62 [(M + H)$^+$], 868.77 [(M − H)$^-$]; t_R = 8.94 min.

2-(3-{1-[(4-{[2-(4-Acetylprolylleucylglycyamino-6-methylhept-2-ynolylamino)-3-methylbutyryl]methylamino}-3-methoxy-5-methylheptanoyl)pyrrolidin-2-yl]-3-methoxy-2-methylpropionylamino}-3-phenyl)propionic Acid Methyl Ester (13). To a solution of **11** (130 mg, 0.15 mmol) in 1 mL of CH_2Cl_2 was added TFA (1 mL, 13 mmol). The reaction mixture was stirred for 1 h at ambient temperature, and 0.2 mL of MeOH was added before the volatiles were removed *in vacuo*. The crude product was triturated with diethyl ether and dried *in vacuo* overnight: LC−MS (ES) *m*/*z* 770.53 [(M + H)$^+$], 768.66 [(M − H)$^-$]; t_R = 6.19 min. To a solution of the amine TFA salt and **2** (55 mg, 0.17 mmol) in 0.4 mL of CH_2Cl_2 were added DIEA (0.091 mL, 0.53 mmol) and DEPC (0.05 mL, 0.30 mmol). The reaction mixture was stirred at ambient temperature for 2 h, diluted with additional CH_2Cl_2, washed successively with citric acid, $NaHCO_3$, and brine, dried, and concentrated *in vacuo* to a residue that was purified by radial chromatography on a 1000 μm plate using a gradient of CH_2Cl_2 and MeOH from 98:2 to 95:5, to give **13** (68 mg, 42%): ^{13}C NMR (400 MHz, $CDCl_3$) δ 174.43, 173.01, 169.09, 153.10, 136.79, 129.41, 128.93, 128.70, 127.15, 86.87, 81.89, 78.49, 76.39, 60.62, 60.39, 59.32, 58.25, 53.92, 53.57, 52.45, 48.72, 47.83, 43.97, 43.69, 43.41, 39.65, 39.41, 37.65, 26.67, 25.38, 15.17, 24.95, 23.19, 22.70, 21.90, 19.77, 17.66, 16.17, 11.35; LC−MS (ES) *m*/*z* 1079.70 [(M + H)$^+$], 1077.86 [(M − H)$^-$]; t_R = 7.89 min.

2-{3-[1-(4-{[2-(4-*N*-Acetylprolylglycylleucylamino-6-methylheptanoylamino-6-methylhept-2-enoylamino)-3-methylbutyryl]methyamino}-3-methoxyheptanoyl)pyrrolidin-2-yl]-3-methoxy-2-methylpropionylamino}-3-phenylpropionic Acid Methyl Ester (14). To a solution of alkyne **13** (29 mg, 0.027 mmol) in 1 mL of EtOH was added 5% Pd-$BaSO_4$ (9 mg), and the reaction mixture was flushed with hydrogen gas for 1 min. Quinoline (220 μL of a 1% ethanolic solution, 0.019 mmol) was added via syringe, and the reaction mixture was placed under a balloon of hydrogen and stirred for 5 h. The mixture was filtered, the solvent removed *in vacuo*, and the residue purified by radial chromatography on a 1000 μm plate using a gradient of CH_2Cl_2 and MeOH from 98:2 to 92:8, to give **14** (5.7 mg, 20%): ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.40−8.50 (m, 1H), 8.22−8.30 (m, 1H), 8.08−8.20 (m, 1H), 7.90−8.04 (m, 1H), 7.52−7.72 (m, 1H), 7.14−7.58 (m, 5H), 5.84−5.91 (m, 1H), 5.66−5.72 (m, 1H), 5.32−5.42 (m, 1H), 3.90−4.55 (m, 7H), 3.65 (s, 3H), 3.49−3.43 (m, 7H), 3.44 (s, 3H), 3.45 (s, 3H), 3.4 (s, 3H), 2.80−3.40 (m, 10H), 1.95 (s, 3H), 1.20−2.45 (m, 13H), 0.78−1.00 (m, 27H); LC−MS (ES) *m*/*z* 1103.72 [(M + Na)$^+$], 1081.73 [(M + H)$^+$], 1079.82 [(M − H)$^-$]; t_R = 7.84 min; HRMS *m*/*z* [M + Na]$^+$ calcd for $C_{57}H_{92}N_8O_{12}Na$ 1103.6732, found 1103.6721.

2-{3-[1-(4-{[2-(4-*N*-Acetylprolylglycylleucylamino-6-methylheptanoylamino)-3-methylbutyryl]methyamino}-3-methoxyheptanoyl)pyrrolidin-2-yl]-3-methoxy-2-methylpropionylamino}-3-phenylpropionic Acid Methyl Ester (15). To a solution of alkyne **13** (9 mg, 0.008 mmol) in 0.5 mL of EtOH was added Pd-$CaCO_3$ (3 mg), and the reaction mixture was flushed with hydrogen gas for 15 min, then placed under a balloon of hydrogen, and stirred overnight. The catalyst was filtered, the solvent removed *in vacuo*, and the residue purified by RP-HPLC to give **15** (3.5 mg, 37%): LC−MS (ES) *m*/*z* 1083.68 [(M + H)$^+$], 1081.75 [(M − H)$^-$]; t_R = 7.79 min; HRMS *m*/*z*: [M + H]$^+$ calcd for $C_{57}H_{95}N_8O_{12}$ 1083.7069, found 1083.7087; HRMS *m*/*z*: [M + Na]$^+$ calcd for $C_{57}H_{94}N_8O_{12}Na$ 1105.6889, found 1105.6886.

Fluorenemethoxycarbonylphenylenediamine (16). A suspension of phenylenediamine dihydrochloride (506 mg, 2.8 mmol) in CH_2Cl_2 was cooled in an ice bath, and DIEA (1.46 mL, 8.4 mmol) and fluorenemethoxycarbonyl chloride (723 mg, 2.8 mmol) were added sequentially. The cooling bath was removed, and the reaction mixture was stirred at ambient temperature for 2 h. The precipitated product was collected by filtration, washed by suspending in CH_2Cl_2 and hexane, and filtered again to give **16** (562 mg, 61%): t_R = 9.11 min (gradient A); ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.6 (br, 1H), 9.2 (br, 1H), 7.89 (d, J = 7.2 Hz, 2H), 7.72 (s, 2H), 7.42 (d, J = 3.6 Hz, 2H), 7.34 (d, J = 6.8 Hz, 2H), 7.15 (s, 2H), 6.75 (s, 1H), 6.51 (s, 2H), 4.26−4.34 (m, 3H); LC−MS (ES) *m*/*z* 331 [(M + H)$^+$], 372 [(M + CH_3CN + H)$^+$]; t_R = 6.97 min.

4,4-*tert*-Butoxycarbonylamino-6-methylhept-2-ynoyl-(fluorenemethoxycarbonylamino)benzene (17). To a −10 °C solution of **12** (354 mg, 1.39 mmol) in 1 mL of THF were added, sequentially, NMM (306 μL, 2.78 mmol) and isobutyl chloroformate (144 μL, 1.11 mmol). The reaction mixture was stirred for 30 min, and then additional THF (0.5 mL) followed by **16** (229.2 mg, 0.7 mmol) suspended in 4 mL of THF was added. The mixture was allowed to equilibrate to ambient temperature overnight, the reaction quenched with saturated $NaHCO_3$, and the reaction mixture diluted with CH_2Cl_2. The organic phase was washed successively with $NaHCO_3$, 1 mM citric acid, and brine and dried, and the solvent was removed *in vacuo* to give a crude solid residue. Recrystallization from ethyl acetate gave **17** (145 mg); a second crop (86 mg) was obtained by addition

of hexane to the mother liquors, for a combined yield of 59%: ^{1}H NMR [400 MHz, $(CD_3)_2CO$] δ 9.60 (s, 1H), 8.85 (br, 1H), 7.75 (d, J = 7.3 Hz, 2H), 7.60 (d, J = 6.7 Hz, 2H), 7.46 (d, J = 8.8 Hz, 2H), 7.38 (br, 1H), 7.27−7.31 (m, 3H), 7.19−7.23 (m, 3H), 4.44 (dd, J = 8.2 Hz, 1H), 4.36 (d, J = 7.0 Hz, 2H), 4.17 (t, J = 6.9 Hz, 1H), 1.80 (m, 1H), 1.62 (m, 2H), 0.94 (2d, J = 2.2 Hz, 6H); LC−MS (ES) m/z 590.27 [$(M + Na)^+$], 568.29 [$(M + H)^+$], 468.26 [$(M - Boc + H)^+$], 566.33 [$(M - H)^-$]; t_R = 9.25 min.

4,4-[(Acetylprolylleucylglycyl)amino-6-methylhept-2-ynoyl(fluorenemethoxycarbonylamino)]benzene (18). Neat **17** (140 mg, 0.25 mmol) was treated with TFA (2.2 mL, 24 mmol) at 0 °C for 5 min to remove the Boc protecting group. Excess TFA was removed *in vacuo* and the amine salt precipitated by trituration with diethyl ether: LC−MS (ES) m/z 468.22 [$(M + H)^+$]; t_R = 6.40 min. After being dried *in vacuo* overnight, the crude amine was stirred with tripeptide acid **2** (90 mg, 0.28 mmol) in 1 mL of CH_2Cl_2, and sequentially, DIEA (152 μL, 0.88 mmol) and DEPC (83 μL, 0.5 mmol) were added. The reaction mixture was stirred at ambient temperature for 3 h and then diluted with $CHCl_3$. The organic phase was washed successively with water, 1 mM citric acid $NaHCO_3$, and brine, dried, and concentrated *in vacuo*. The product was purified by radial chromatography on a 1000 μm plate using a gradient of CH_2Cl_2 and MeOH from 98:2 to 95:5, to give **18** (102 mg, 53%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.4 (s, 1H), 7.76 (d, J = 7.3 Hz, 2H), 7.66 (br, 1H), 7.60 (br d, 2H), 7.28−7.51 (m, 10H), 7.0 (br, 1H), 4.82 (dd, J = 4 Hz, 1H), 4.41−4.49 (m, 3H), 4.22−4.26 (m, 2H), 4.07 (dd, J = 6.4 Hz, 1H), 3.80 (dd, J = 5.6 Hz, 1H), 3.54−3.60 (m, 1H), 3.40−3.48 (m, 1H), 2.03 (s, 3H) over 1.60−2.20 (m, 10H), 0.90 (two d, J = 6.0 Hz); LC−MS (ES) m/z 777.36 [$(M + H)^+$]; t_R = 8.26 min.

4,4-[(Acetylprolylleucylglycyl)amino-6-methylhept-2-enoyl(fluorenemethoxycarbonylamino)]benzene (19). A solution of **18** (102 mg, 0.13 mmol) in 5 mL of EtOH was partially hydrogenated to the (*Z*)-olefin using Pd-$BaSO_4$ (35 mg) and quinoline (11 μL, 0.91 mmol) by the procedure described for **14**. The product was isolated as a white solid (77 mg, 76%) and was used without further purification: ^{1}H NMR (400 MHz, CD_3CN) δ 10.10 (s, 1H), 7.28−7.90 (m, 16H), 5.90 (d, J = 10.0 Hz, 1H), 5.60 (d, J = 10.0 Hz, 1H), 5.02−5.00 (m, 1H), 3.51−4.49 (m, 10H), 2.10 (s, 3H), over 1.60−2.20 (m, 10H), 0.90 (two d, J = 6.0 Hz, 6H); LC−MS (ES) m/z 779.32 [$(M + H)^+$], 777.34 [$(M - H)^-$], 555.31 [$(M - Fmoc - H)^-$]; t_R = 8.17 min.

4,4-[(Acetylprolylleucylglycyl)amino-6-methylhept-2-enoic Acid (4-{2-[3-(1-{4-[(2,2-Dimethylamine-3-methylbutyrylamin-3-methylbutyrylamino)-3-methoxy-5-methylheptanoyl]pyrrolin-2-yl}-3-methoxy-2-methylpropionylamino)-3-phenylpropionylamino]})phenylamide (21). Protected phenylenediamine **19** (77 mg, 0.10 mmol) was treated with 20% piperidine in DMF (3 mL, 30 mmol) for 5 min, and the reaction mixture was then poured into water (100 mL) and extracted with $CHCl_3$ (3 × 15 mL). The organic phase was washed with water, dried, and concentrated *in vacuo*. The product was purified by radial chromatography on a 1000 μm plate using a gradient of CH_2Cl_2 and MeOH from 97:3 to 95:5, to give to give the free amine (40 mg, 72%) as a pale yellow solid. A portion of the amine (33 mg, 0.06 mmol) was acylated with **20**[29] (56 mg, 0.07 mmol), DEPC (32 μL, 0.13 mmol), and DIEA (36 μL, 0.21 mmol) using the procedure described for **3**. Purification by radial chromatography (from 95:5 to 99:10 CH_2Cl_2/MeOH mixture), followed by preparative TLC (90:10 CH_2Cl_2/MeOH mixture), gave **21** (20 mg, 22%). An analytical sample was obtained from a portion by final purification on RP-HPLC: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.9 (br, 1H), 7.20−7.80 (m, 9H), 5.95 (d, J = 10.0 Hz, 1H), 5.45 (t, J = 10.0 Hz, 1H), 3.45 and 3.31 (two s, 3H), over 3.03−4.90 (17H), 0.89−2.60 (m, 84H); t_R = 9.25 min (gradient A); LC−MS (ES) m/z 1284.72 [$(M + H)^+$]; t_R = 6.34 min; HRMS m/z [$M + H]^+$ calcd for $C_{69}H_{110}N_{11}O_{12}$ 1284.8335, found 1284.8387; HRMS m/z [$M + Na]^+$ calcd for $C_{69}H_{109}N_{11}O_{12}Na$ 1306.8155, found 1306.8171.

4-*tert*-Butoxycarbonylamino-6-methylhept-2-ynoic Acid [1-Chloromethyl-3-(5,6,7-trimethoxy-1*H*-indole-2-carbonyl)-2,3-dihydro-1*H*-benzo[*e*]indol-5-yl]amide (22). A solution of **12** (46 mg, 0.18 mmol) and NMM (40 μL, 0.36 mmol) in 0.3 mL of THF was cooled to −10 °C. Isobutyl chloroformate (19 μL, 0.16 mmol) was added, and the reaction mixture was stirred at −10 °C for 30 min. A solution of CBI-TMI[21] in 1 mL of THF was added, and the reaction mixture was allowed to equilibrate to ambient temperature over the course of 3 h. TLC showed unconverted CBI-TMI, and additional **12** (46 mg, 0.18 mmol) was activated as described above and added. The reaction mixture was stirred overnight, the reaction quenched by addition of saturated $NaHCO_3$, and the mixture extracted with $CHCl_3$. The organic phase was washed successively with 1 M $KHSO_4$, saturated $NaHCO_3$, and brine, dried, and concentrated *in vacuo*. Purification by radial chromatography (1000 μm plate, 75:25 hexanes/EtOAc mixture) gave **22** (21 mg, 33%): ^{1}H NMR (400 MHz, CD_3CN) δ 9.83 (br, 1H), 8.91 (br, 1H), 8.67 (s, 1H), 7.15−8.15 (m, 2H), 7.40−7.65 (m, 2H), 7.06 (s, 1H), 6.95 (s, 1H), 5.77 (br, 1H), 4.66−4.80 (m, 3H), 3.86 (s, 9H), 3.75−3.82 (m, 1H), 4.20−4.40 (m, 2H), 1.80−1.90 (m, 1H), 1.65−1.70 (m, 2H), 1.45 (s, 9H), 0.98 (d, J = 6 Hz, 6H); LC−MS (ES) m/z 603 [$(M - Boc + H)^+$], 703 [$(M + H)^+$], 725 [$(M + Na)^+$], 701 [$(M - H)^-$]; t_R = 9.23 min.

4-(Acetylprolylleucylglycyl)amino-6-methylhept-2-ynoic Acid [1-Chloromethyl-3-(5,6,7-trimethoxy-1*H*-indole-2-carbonyl)-2,3-dihydro-1*H*-benzo[*e*]indol-5-yl]amide (23). Neat **22** (21 mg, 0. 03 mmol) was treated with TFA (0.3 mL, 3.9 mmol) at 0 °C for 5 min. The TFA was removed *in vacuo*, and the residue was triturated with diethyl ether to a white solid that was dried *in vacuo* overnight. The crude amine TFA salt was stirred with **2** (10 mg, 0.031 mmol) and DIEA (17 μL, 0.10 mmol) in 0.2 mL of CH_2Cl_2. To this was added DEPC (10 μL, 0.57 mmol), and the reaction

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mixture was stirred for 1 h and then diluted with $CHCl_3$. The organic phase was washed successively with water, saturated citric acid, $NaHCO_3$, and brine, dried, and concentrated *in vacuo*. Purification by radial chromatography (1000 μm plate, from 97:3 to 95:5 CH_2Cl_2/MeOH mixture) gave **23** (13 mg, 50%): ^{1}H NMR (400 MHz, CD_3CN) δ 9.85 (s, 1H), 9.64 (d, J = 8.4 Hz, 1H), 8.75 (s, 1H), 8.02 (d, J = 8.0 Hz, 1H), 7.93 (d, J = 7.6 Hz, 1H), 7.70 (s, 1H), 7.57−7.60 (m, 1H), 7.48−7.50 (m, 1H), 7.20−7.35 (m, 2H), 7.09 (s, 1H), 6.98 (s, 1H), 4.85−4.95 (m, 1H), 4.60−4.80 (m, 1H), 4.25−4.35 (m, 2H), 4.15−4.20 (m, 1H), 3.86 (s, 9H), over 3.83−3.95 (m, 2H), 3.50−3.70 (m, 2H), 2.18 (s, 3H), 1.60−2.00 (m, 10H), 0.96−1.00 (dd, J = 6.4 Hz, 6H), 0.89 (d, J = 4.8 Hz, 3H), 0.81 (d, J = 6.4 Hz, 3H); LC−MS (ES) *m/z* 913 [(M + H)$^+$], 935 [(M + Na)$^+$], 660 [(M − AcProLeu + H)$^+$], 911 [(M − H)$^-$], 875 [(M − HCl − H)$^-$]; t_R = 8.07 min.

4-(Acetylprolylleucylglycyl)amino-6-methylhept-2-(*Z*)-enoic Acid [1-Chloromethyl-3-(5,6,7-trimethoxy-1*H*-indole-2-carbonyl)-2,3-dihydro-1*H*-benzo[*e*]indol-5-yl]amide (24). Alkyne **23** (10 mg, 0.01 mmol) was hydrogenated under the Lindlar conditions described for **14** to give **24** (2.6 mg, 29%): ^{1}H NMR (400 MHz, CD_3CN) δ 8.75 (s, 1H), 8.00−8.02 (m, 1H), 7.90−7.99 (m, 1H), 6.95−7.80 (m, 9H), 6.00−6.20 (m, 1H), 5.80−5.95 (m, 1H), 5.20−5.40 (m, 1H), 4.80−4.90 (m, 2H), 3.89 (s), over 3.50−4.50 (m, total of 18H), 2.18 (s, 3H), 1.60−2.00 (m, 10H), 0.80−1.00 (m, 12H); t_R = 11.25 min (gradient A); LC−MS (ES) *m/z* 914.48 [(M + H)$^+$], 912.60 [(M − H)$^-$]; t_R = 8.04 min; HRMS *m/z* [M + Na]$^+$ calcd for $C_{48}H_{60}N_7O_9NaCl$ 936.4039, found 936.4033; HRMS *m/z* [M + K]$^+$ calcd for $C_{48}H_{60}N_7O_9ClK$ 932.5778, found 932.3788; HRMS *m/z* [M − H + 2Na]$^+$ calcd for $C_{48}H_{59}N_7O_9Na_2Cl$ 958.3858, found 958.3873.

***In Vitro* MMP-2 and -9 Substrate Assay.** MMP-2 and -9 proteolysis was assayed by a modification of a published procedure.[30] The purified enzymes (Calbiochem, San Diego, CA) were activated for each assay by 1.25 mM *p*-aminophenylmercuric acetate (APMA, from Calbiochem, dissolved in 0.1 M NaOH prior to use) in 50 mM HEPES buffer (pH 7.4) for 3 h at 37 °C.[30] The activated enzyme solution was added to a reaction mixture consisting of 100 mM HEPES (pH 7.4), 150 mM NaCl, 0.1% Brij 35, 20 μM $ZnSO_4$, and 10 mM $CaCl_2$. The assay mixture was added to a DMSO solution of substrate. The initial substrate concentrations ranged from 50 to 100 μM as determined by the solubility of the test compound in the assay medium. The enzyme to substrate molar ratios were approximately 1:400 for all assays. The enzyme reaction was allowed to proceed at 37 °C, and aliquots were removed for analysis at time points between 0.5 and 16 h and reactions quenched with 50 mM EDTA. The aliquots were analyzed by RP-HPLC on a Waters alliance 2695 instrument equipped with a Waters 2996 diode array detector. The stationary phase that was used was a Phenomonex 4.6 mm × 150 mm, 4 μm, 80 Å MAX RP synergi column. Products were eluted using a linear gradient from 20 to 95% mobile phase B (CH_3CN) in phase A (0.05% aqueous TFA) over the course of 10 min at a flow rate of 1.0 mL/min. Peaks were monitored at a wavelength determined from the full ultraviolet spectrum of each compound; 290 nm was used for compound **3**, and 266 nm was used for compound **10**. Enzymatic cleavage of dinitrophenyl L-prolyl-L-leucyl-glycyl-L-leucyl-L-tryptophanyl-L-alanyl-D-argininamide (Dnp PLGLWA[D]Arg-NH_2, Bachem, King of Prussia, PA) was included as a positive control for enzyme activity in all assays.[31] The specific activities against the commercial substrate in these experiments were 2070 nmol min^{-1} mg^{-1} for MMP-9 and 1000 nmol min^{-1} mg^{-1} for MMP-2. Ilomastat (GM6001, Chemicon International, Temecula, CA) was used at a final assay concentration of 50 μM for the inhibition experiments.[32]

***In Vitro* Analysis of Drug and Prodrug Activity.** HT 1080 fibrosarcoma cells were obtained from ATCC (Manasas, VA) and cultured in DMEM with 10% FBS and 2 mM L-glutamine (all from Invitrogen). This cell line has previously been shown to express MMP-2 and MMP-9,[19] and this was confirmed in our laboratory by gelatin zymography following the published method[19] (data not shown). Twenty-four hours prior to being exposed, cells were plated at a density of 5000 cells/well in 0.1 mL of complete medium in 96-well microplates. Prior to addition of drugs, the culture medium was aspirated, and cultures were washed twice with serum-free medium. Serum-free medium (100 μL) was added to cultures, followed by drugs diluted in serum-free medium. Cultures were then incubated for 4 h at 37 °C. At the completion of this exposure, cultures were washed three times with serum-containing medium, and the final volume was adjusted to 0.2 mL/well. Cultures were grown for 96 h. Inhibition of growth was assessed with resazurin (Sigma, St. Louis, MO) conversion, and fluorescence was measured on a Fusion HT plate reader (Perkin-Elmer, Meridian, CT).

Results

Chemistry. Doxorubicin Derivatives. Doxorubicin was converted to L-leucyldoxorubicin (LeuDox) by a modification of a published procedure.[33] The P3−P1 peptide AcProLeuGly (**2**) was prepared by conventional solid-phase peptide chemistry and appended to LeuDox (**1**) using DEPC and DIEA as shown in Scheme 1.

Auristatins. MMP-2 and -9 accept a variety of hydrophobic aliphatic residues in the P1′ position.[34] The auristatins,

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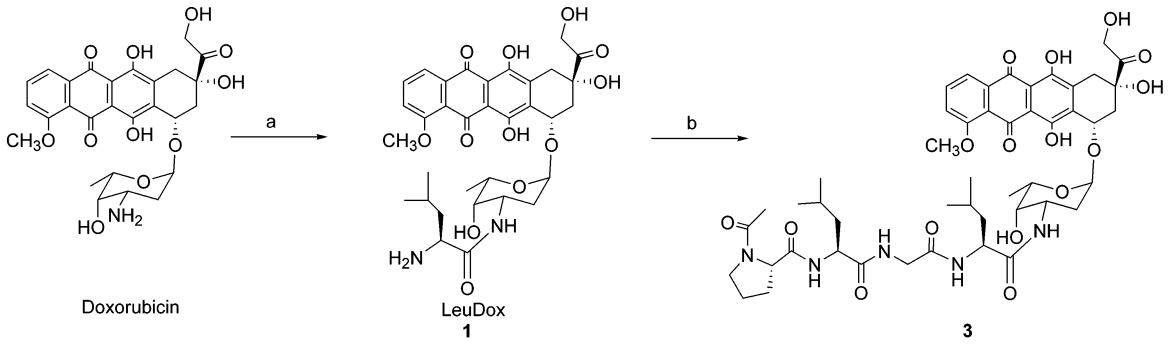
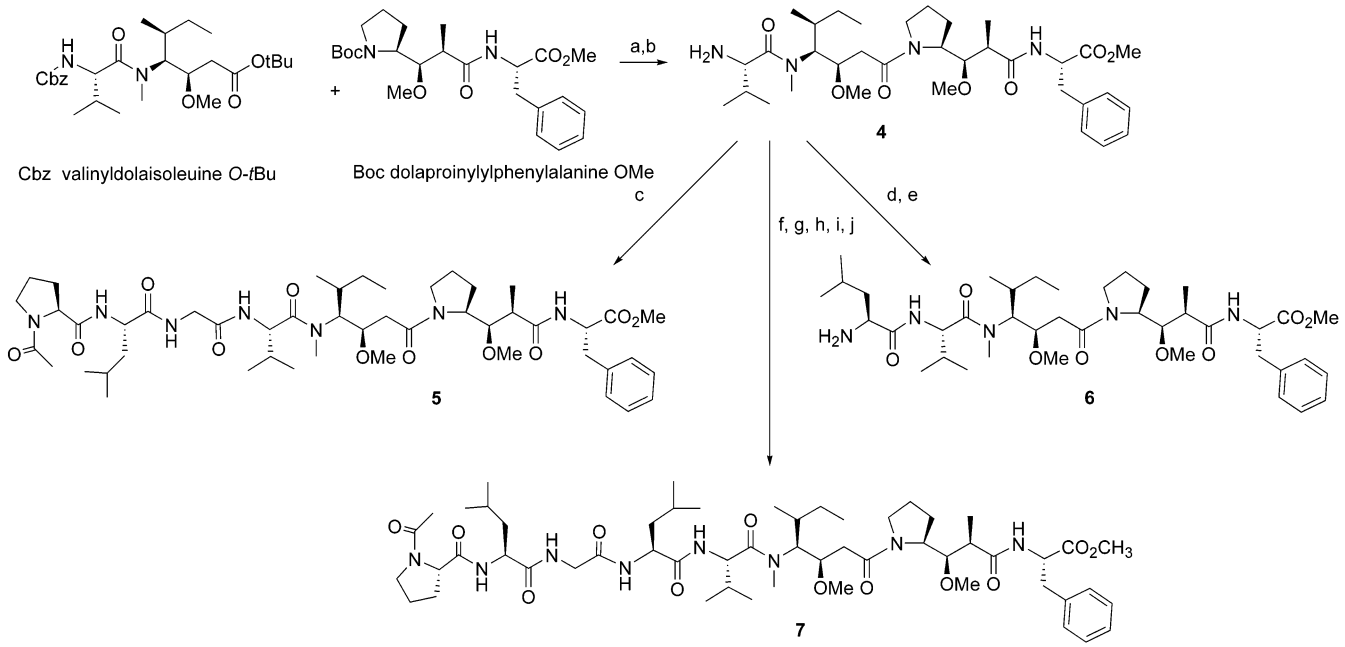
Scheme 1 [a]



[a] (a) (i) DEPC, DIEA, DMF, Mmtr-L-leucine, DMF; (ii) 90:10 CH_2Cl_2/TFA, cat. Et_3SiH; (b) **2**, DEPC, DIEA, DMF.

Scheme 2 [a]



[a] (a) TFA, CH_2Cl_2; (b) DEPC, DIEA, CH_2Cl_2; (c) **2**, DEPC, DIEA, CH_2Cl_2; (d) Boc L-Leu, DEPC, DIEA, CH_2Cl_2; (e) 4 N HCl/dioxane; (f) Fmoc L-Leu, DEPC, DIEA, CH_2Cl_2; (g) (i) $NHEt_2$, CH_2Cl_2; (ii) Fmoc Gly, DEPC, DIEA, CH_2Cl_2; (h) (i) $NHEt_2$, CH_2Cl_2; (ii) Fmoc L-Leu, PyBrop, DIEA, CH_2Cl_2; (i) (i) $NHEt_2$, CH_2Cl_2; (ii) Fmoc L-Pro, PyBrop, DIEA, CH_2Cl_2; (j) (i) $NHEt_2$, CH_2Cl_2; (ii) Ac_2O, DIEA, CH_2Cl_2.

comprised of modified pentapeptides incorporating two internal statine-like residues, have valine derivatives (*N*-methyl or *N*,*N*-dimethyl) at the N-terminus.[20] The branched hydrophobic side chain of the N-terminal valine should, in principle, make this an acceptable P1′ residue for MMP-2 and/or MMP-9. The remainder of the auristatin structure would then occupy the active site cleft and extend into either an exosite or a solvent-exposed pocket. Proteolysis at the Gly−Leu bond would release a primary amine N-terminal valine auristatin analogue. An auristatin having leucine at the N-terminus might be an even better substrate, in which case the Gly−Leu cleavage would release the corresponding leucyl auristatin. To evaluate the effect of the leucine extension, we prepared a truncated auristatin derivative, ValDilDapPheOMe (**4**), the N-terminal leucine analogue, LeuValDilDapPheOMe (**6**), and the corresponding AcProLeuGly prodrugs, **5** and **7**, respectively. As shown in Scheme 2, the assembly of these peptidyl prodrugs followed slightly different routes; **7** was prepared by sequential addition of each residue, whereas **5** was prepared by convergent fragment coupling.

The S1′ subsites in MMP-2 and -9 contain long hydrophobic tunnels,[35,36] and the S2′ subsites are partly solvent exposed.[37,38] Taking advantage of the ability of the catalytic cleft to accommodate an aromatic ring, we prepared derivatives of an auristatin analogue (**8**) that has phenylenediamine at the C-terminus.[27] The terminal amino group enabled us to attach the P1′ leucine in **9**, and the AcProLeuGlyLeu

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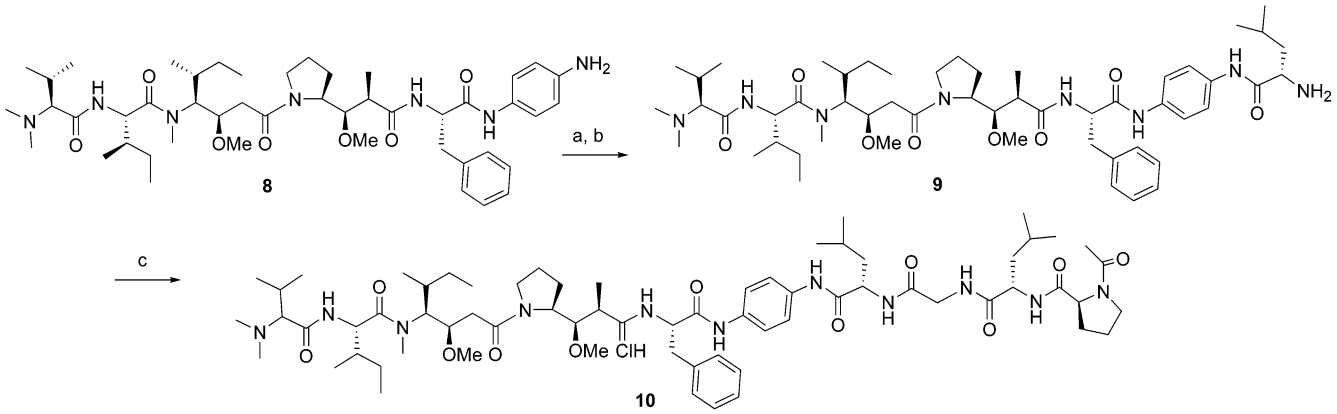
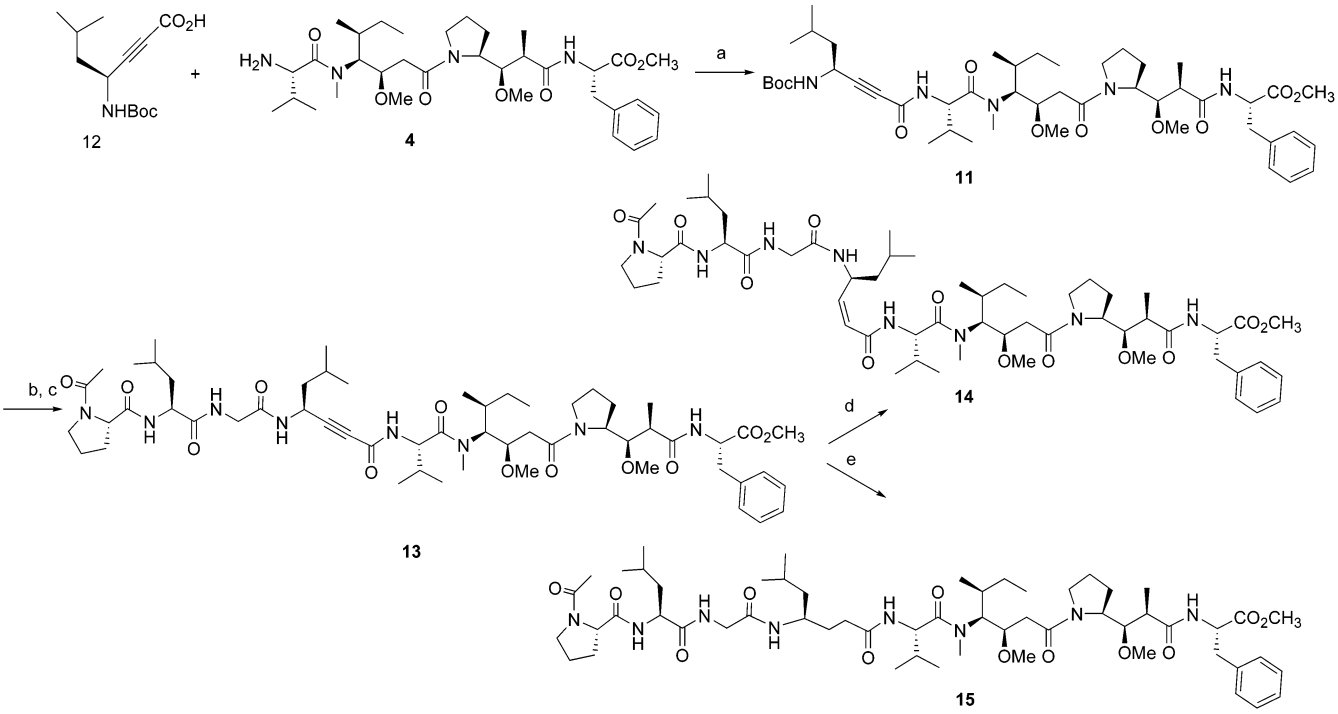
Scheme 3 [a]



[a] (a) HATU, pyridine, Boc-L-leucine; (b) 4 N HCl/dioxane; (c) **2**, DEPC, DIEA, CH_2Cl_2.

Scheme 4 [a]



[a] (a) PyBrop, DIEA, CH_2Cl_2; (b) TFA, CH_2Cl_2; (c) **2**, DEPC, DIEA, CH_2Cl_2; (d) Pd-$BaSO_4$, quinoline, H_2, EtOH; (e) Pd-$CaCO_3$, H_2, EtOH.

sequence in **10**, in an orientation in which the P3−P1′ sequence remains N-to-C, but the directionality of the auristatin peptide backbone reverses thereafter. Compound **10** was prepared by fragment coupling of the leucyl auristatin **9** with **2** (Scheme 3).

To release free drugs with no residual leucine, we prepared leucine mimetics that would undergo lactamization, with concomitant release of drug, upon proteolytic generation of a terminal amine group. To maintain a high degree of structural homology to the peptides, we retained the AcPro-LeuGly sequence in the unprimed sites, and preserved the isobutyl side chain to maintain appropriate hydrophobic contacts in the S1′ pocket. Additionally, the steric effects of the isobutyl group, as well as the conformational restriction imposed by the (*Z*)-olefin in the *Z* α,*β*-unsaturated analogues, were intended to promote cyclization.[39]

The Corey−Fuchs route[40] (Schemes 4 and 5) was the

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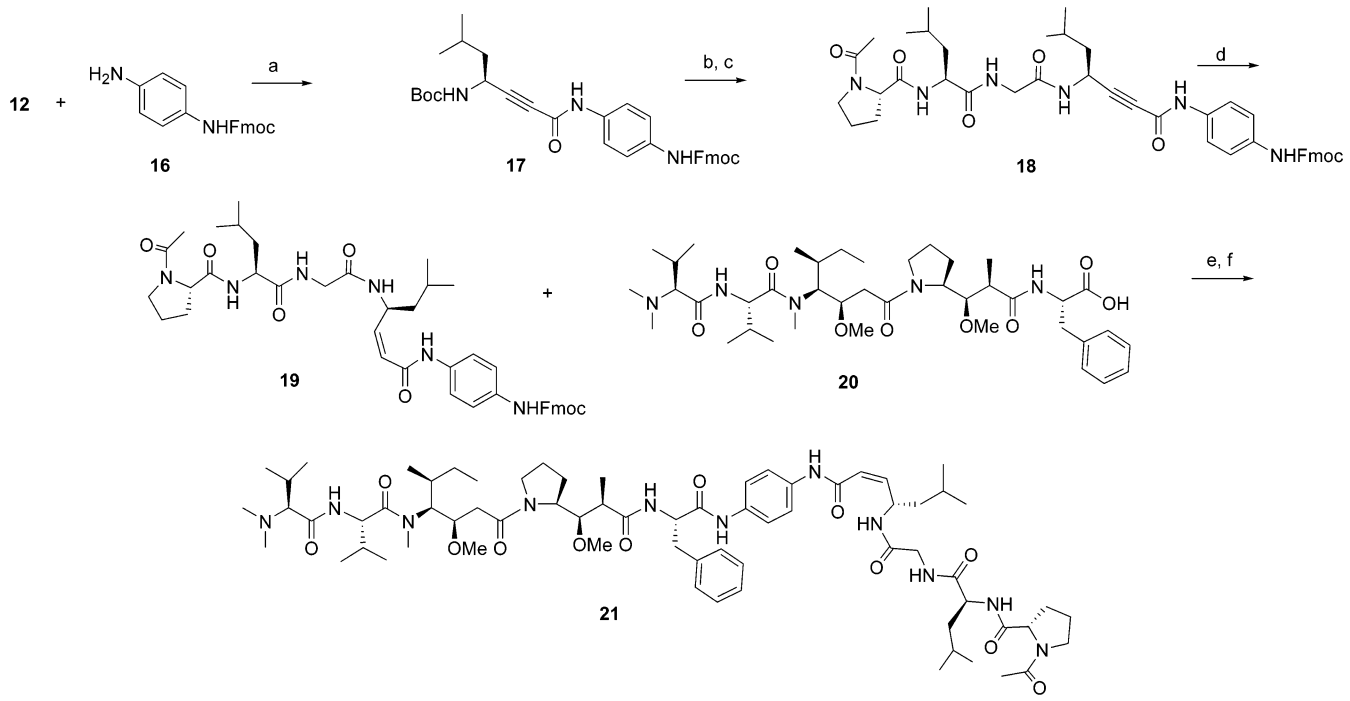
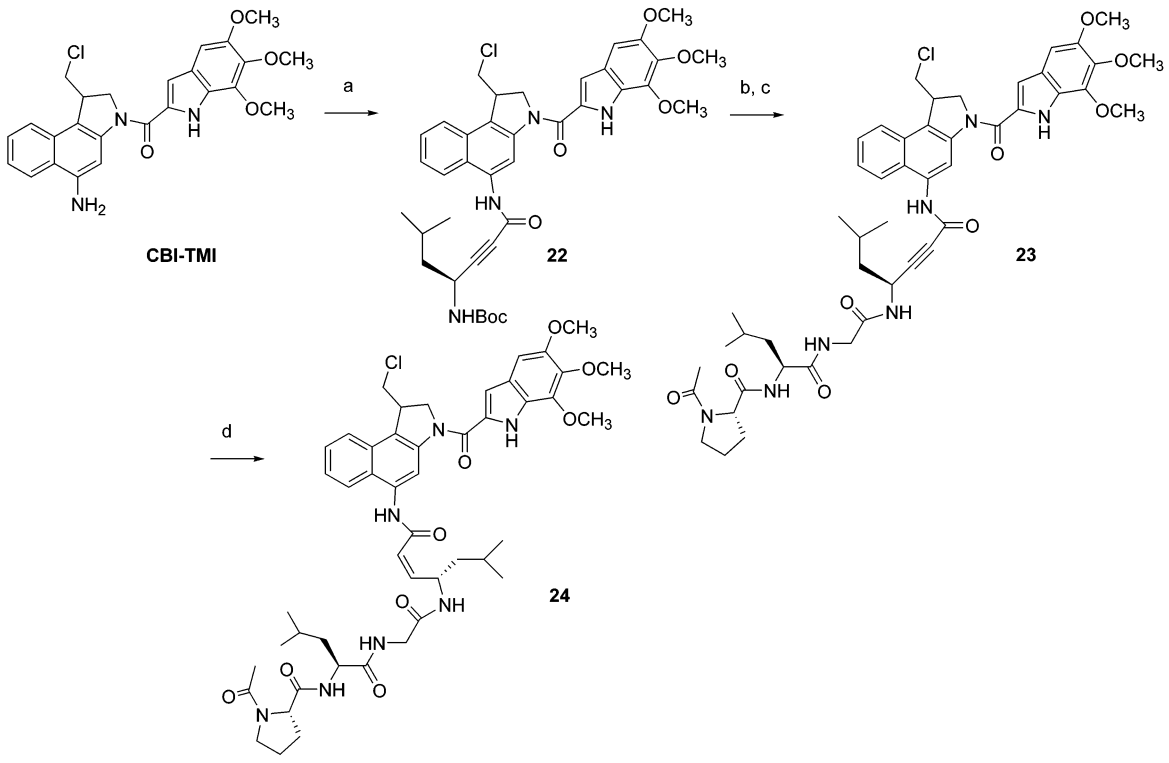
Scheme 5 [a]



[a] (a) *i*BuOCOCl, NMM, THF; (b) TFA, CH_2Cl_2; (c) **2**, DEPC, DIEA, CH_2Cl_2; (d) Pd-$BaSO_4$, quinoline, H_2, EtOH; (e) 20% piperidine/DMF; (f) **20**, DEPC, DIEA, CH_2Cl_2.

Scheme 6 [a]



[a] (a) **12**, *i*BuOCOCl, NMM, THF; (b) TFA, 0 °C; (c) **2**, DEPC, DIEA, CH_2Cl_2; (d) Pd-$BaSO_4$, quinoline, H_2, EtOH.

method of choice for the synthesis of the (*Z*)-olefins as well as the saturated peptidomimetic analogue. Propiolic acid **12**[28] was prepared from Boc L-leucinal and reacted with **4** (Scheme 4). After amine deprotection, the AcProLeuGly moiety was added, and the alkyne was reduced under Lindlar conditions[41] to give **14**. The hydrogenation proved to be very sensitive to the palladium species that was used, and to the presence and timing of addition of the auxiliary poison. Use

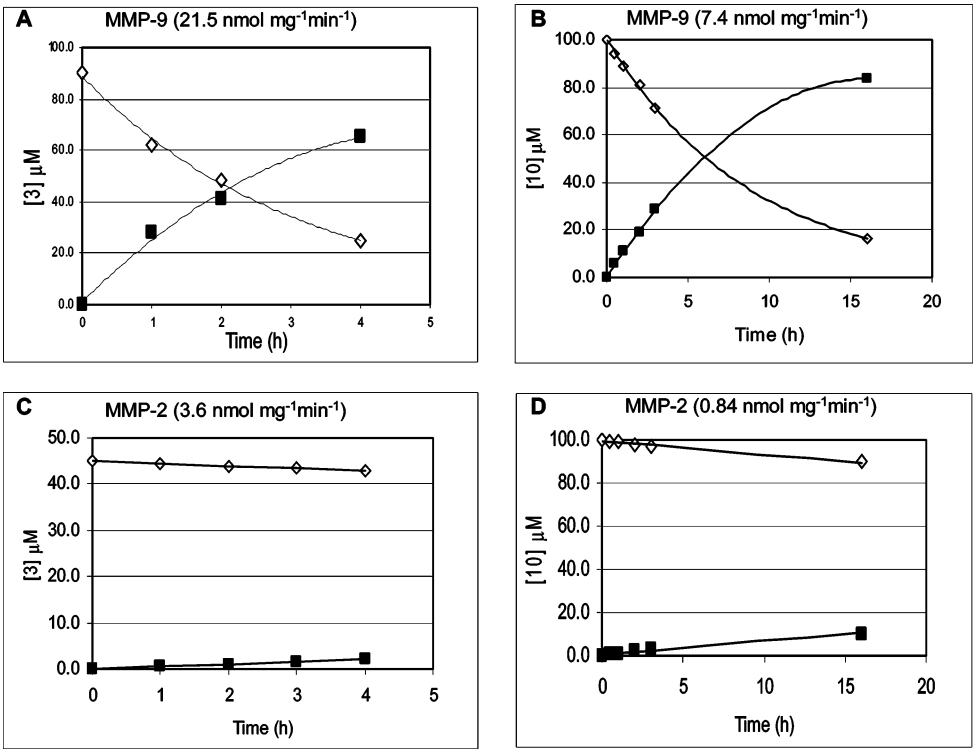


Figure 2. (A) Decrease in the level of prodrug **3** (◇) and concomitant increase in the level of LeuDox **1** (■) and (B) decrease in the level of prodrug **10** (◇) and concomitant increase in the level of leucyl drug **9** (■), treated with MMP-9. Panels C and D show the same conversions mediated by MMP-2 for **3** and **10**, respectively. The specific activities are shown for each experiment. Details of the assay are given in the Experimental Section.

of Pd-$CaCO_3$ in place of Pd-$BaSO_4$, and omitting the quinoline, gave the saturated amide **15**. The partial hydrogenation conditions, once established, could be applied to a variety of substrates. We were able to use a similar route (Scheme 5), introducing the hydrogenation reaction at an earlier stage, to obtain **21** from monoprotected phenylenediamine **16**.

AminoCBI Derivative 24. The *Z* α,*β*-unsaturated analogue **24** was prepared as a peptidomimetic substrate representing the duocarmycin class. As shown in Scheme 6, the *N*-Boc isobutylpropiolic acid **12** was coupled to CBI-TMI[21] via a mixed anhydride. Brief treatment with TFA removed the Boc group, allowing the AcProLeuGly moiety to be added. Partial hydrogenation under the previously established conditions gave **24**.

MMP-2 and -9 Proteolysis *in Vitro*. All peptides and peptidomimetics were evaluated as substrates for MMP-9, and selected compounds were additionally evaluated as substrates for MMP-2 (Figure 2). Two of the peptides, **3** and **10**, were substrates for both MMP-2 and -9, whereas two other peptides (**5** and **7**) and none of the peptidomimetics (**14**, **15**, **21**, and **24**) were cleaved. Specific activities for both **3** and **10** were higher for MMP-9 than MMP-2, which was consistent with specific activities using the known substrate dinitrophenylProLeuGlyLeuTrpAla[D]ArgNH_2.[31]

We characterized the proteolysis products of **10** and **3** by HPLC, by mass spectrometry, and by the cytotoxicity of the MMP-9 digests against HT1080 human fibrosarcoma cells. The enzymes converted the prodrugs to the corresponding leucyl drugs by the predicted cleavage of the Gly−Leu bond, with neither intermediate nor further cleavages observed (Figure 3). The conversions of **10** to **9** and **3** to **1** were abolished with 50 μM Ilosmastat, a broad-spectrum MMP inhibitor,[32] indicating that the cleavage was MMP-mediated. Nonsubstrates, or substrates in the presence of inhibitor, were stable in pH 7.4 buffer for 18 h, the duration of the assay.

Cytotoxicity of MMP-9-Treated Prodrugs. Prodrugs **3** and **10** were incubated with MMP-9 overnight to effect complete hydrolysis. We then determined the cytotoxicity against HT1080 human fibrosarcoma cells of leucyl drugs **1** and **9**, the corresponding untreated peptidyl prodrugs **3** and **10**, and the MMP-9 proteolysis products. As shown in Figure 4, derivatization with AcProLeuGly reduced the cytotoxicity nearly 8-fold for leucyl auristatin **9** and 30-fold for LeuDox **1**. MMP-9 proteolysis completely restored the potencies of the original leucyl drugs.

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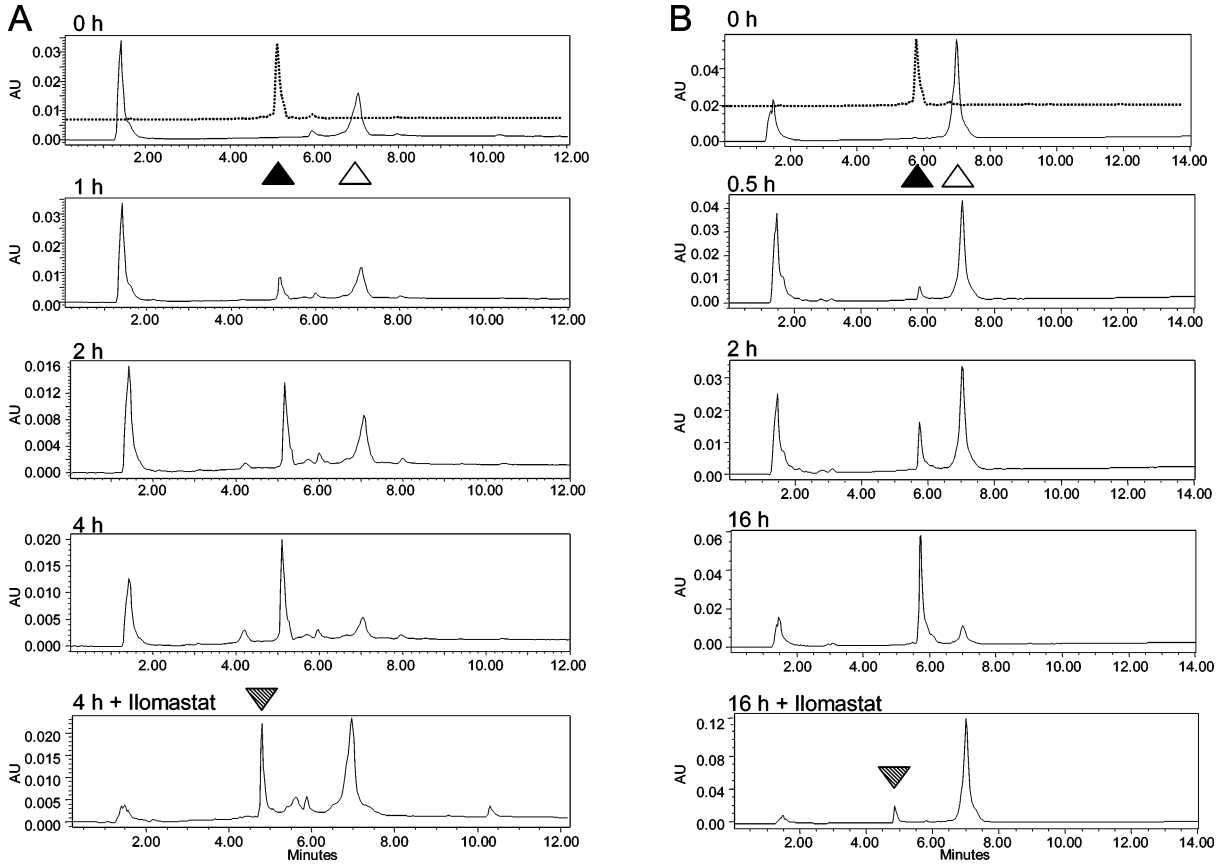

Figure 3. (A) HPLC tracings showing the disappearance of prodrug **3** (△) and the appearance of LeuDox **1** {LC−MS *m*/*z* 657 [(M + H)$^+$]; t_R = 5.66 min} mediated by MMP-9. (B) HPLC tracings for the MMP-9 conversion of prodrug **10** (△) to leucyl drug **9** {LC−MS *m*/*z* 964 [(M + H)$^+$], *m*/2*z* 483 [(M/2 + 2H)$^+$]; t_R = 5.67 min}. The bottom tracings show the respective experiments for **3** and **10** in the presence of 50 μM Ilomastat (gray triangles). The HPLC tracings of authentic **1** and **9** (black triangles) are shown in dotted lines inset into the top tracings of panels A and B, respectively. Details of the assay are given in the Experimental Section.

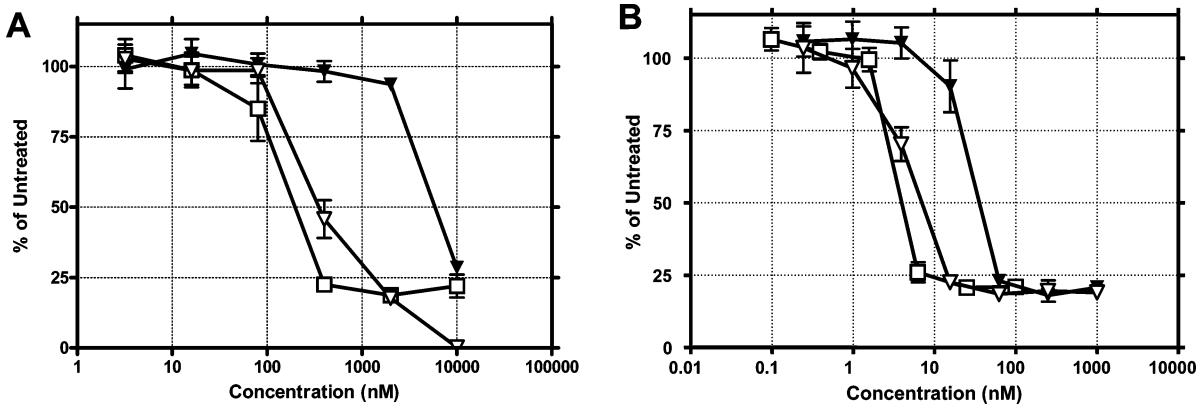

Figure 4. (A) *In vitro* cytotoxicity of prodrug **3** (▼), LeuDox **1** (□), and the MMP-9 proteolysis product of **3** (▽) against HT-1080 cells. (B) The same experiment using prodrug **10** (▼), leucyl drug **9** (□), and the MMP-9 proteolysis product of **10** (▽). Details of the assay are given in the Experimental Section.

Discussion

A panel of four peptides and four peptidomimetic compounds were prepared as potential substrates for MMP-2 and -9. Of these, two compounds, both peptides, were found to be substrates, and had a substantially higher rate of cleavage when cleaved by MMP-9 as opposed to MMP-2. Both MMP-2 and -9 appear to be highly sensitive to the structure and orientation of the P1′ residue. Replacing the carbonyl of substrate **10** with a (*Z*)-olefin, exemplified by compound **21**, abrogated all hydrolysis of the Gly−Leu−mimetic amide bond. Protease sensitivity to structure extends beyond P1′, however, since **7** was also not a substrate despite the peptide-

like structure of the entire compound. Interestingly, the doxorubicin derivative **3** was the best substrate of all the compounds that were evaluated.

Prodrugs **3** and **10** were significantly deactivated by their appended AcProLeuGly moieties and were hydrolyzed at the predicted Gly−Leu bond to restore the fully active leucyl drugs **1** and **9**, respectively. Proteolysis was nearly complete within 4−16 h and showed no evidence of intermediates or side products. While these are encouraging results, the levels of proteolysis were obtained with enzyme concentrations and exposure times that are likely not to be physiologically relevant. In a preliminary *in vitro* cytotoxicity experiment, we were unable to detect any differences in the sensitivities of HT-1080 (MMP-2 and -9-expressing) cells[19] and MB-MDA-453 (not expressing MMP-2 or -9, determined by gelatin zymography) cells to **10**; the IC_{50} values were 30.8 ± 5.22 and 31.7 ± 1.67 nM, respectively. A similar discrepancy between the *in vitro* enzymatic and cytotoxicity results was also observed by Copeland and co-workers for MMP-targeted doxorubicin prodrugs.[13] Several factors might contribute to this observation, including the likelihood that the prodrugs, even without proteolysis of the peptide or peptidomimetic sequence, may penetrate cell membranes and exert weak to modest cytotoxic effects. Some of our analogues that were not substrates had IC_{50} values in the range of 0.1−1 μM (data not shown). For robust enzyme activation, however, it is clear that improved substrate kinetics for the prodrugs are needed to achieve the activities required for therapeutic efficacy.

To guide in the design of more kinetically favorable MMP-targeted prodrugs, we can interpret the results from our first panel of compounds with respect to the functional domains and catalytic mechanisms of MMP-2 and -9. Exosites that lie outside of the catalytic cleft are known to engage dynamically with physiological MMP substrates that require reorientation or unwinding for cleavage. These enzyme exosites may also block the access of potential substrates to the active site.[42] The auristatin analogues **5**, **7**, **10**, **14**, **15**, and **21**, which extend more than 16 Å from the Gly−Leu or −Leu−mimetic bond, are likely to have contacted a participating exosite, and the resulting enzyme−substrate complexes were then incorrect (no cleavage) or suboptimal (inefficient cleavage) for hydrolysis.

For CBI-TMI prodrug **24**, exosite involvement is less likely to account for our results. Here one must look at the active site itself, which in MMP-2 and -9 is long but narrow, ca. 5 Å wide.[42] The planar aromatic duocarmycin structure permits little flexibility in such a narrow cleft, so the correct binding orientation for hydrolysis must be established in the prodrug molecule. MMPs, and metalloproteases in general, use critical contacts on the primed as well as unprimed sides of the scissile bond for substrate specificity and catalysis.[2,43,44] The crystal structure of a reverse hydroxamate inhibitor in the MMP-9 active site shows strong hydrogen bonds between the amide carbonyl of the P1′ occupant and Leu188, and between the NH group of the same amide bond and Pro421.[35] Similar hydrogen bonds were reported for an MMP-2 hydroxamate inhibitor complex.[36] In the peptidomimetic **24**, this amide bond is replaced by the ethylene moiety, and these interactions are lost. In contrast, the peptidyl doxorubicin analogue **3** also has a rigid aromatic ring wider than 5 Å, but retains the P1′−P2′ amide bond, and is a moderately good substrate.

Our preliminary results suggest that MMP-2 and -9 may be exploited to activate antitumor prodrugs, but these prodrugs must be structurally optimized in their binding to the enzymes and must be delivered to areas of high enzyme activity. Both the catalytic cleft and exosite domains must be taken into account to achieve catalytic efficiency. Developing the optimized prodrugs will be the goal of future work.

Abbreviations Used

MMP, matrix metalloproteinase; AcProLeuGly, acetyl L-prolyl-L-leucylglycine; AcProLeuGlyLeu, acetyl L-prolyl-L-leucyl-glycyl-L-leucine; CBI-TMI, 5-amino-3-(3,4,5-trimethoxyindol-2-ylcarbonyl)-1-(chloromethyl)-1,2-dihydro-3*H*-benzyl[*e*]indole; Dnp PLGLWA[D]Arg-NH_2, dinitrophenyl L-prolyl-L-leucyl-glycyl-L-leucyl-L-tryptophanyl-L-alanyl-D-argininamide; DEPC, diethyl cyanophosphonate; HATU, *O*-7-azabenzotriazol-1-yl-*N*,*N*,*N*′,*N*′-bromotrispyrrolidinophosphonium hexaflurophosphate; NMM, *N*-methylmorpholine.

Acknowledgment. We thank Svetlana Doronina, Brian Toki, and Tim Bovee for their contributions to the auristatin chemistry, Darin Gustin for many helpful discussions with regard to the Corey−Fuchs chemistry, and Stephen Alley for a critical reading of the manuscript. We acknowledge the support of this work by NIH Grant 1 R43 CA97840-01.

MP0340183

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