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Evaluating Prodrug Strategies for Esterase-Triggered Release of Alcohols

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Prodrugs are effective tools in overcoming drawbacks typically associated with drug formulation and delivery. Those employing esterase-triggered functional groups are frequently utilized to mask polar carboxylic acids and phenols, increasing drug-like properties such as lipophilicity. Herein we detail a comprehensive assessment for strategies that effectively release hydroxy and phenolic moieties in the presence of an esterase. Matrix metalloproteinases (MMPs) serve as our proof-of-concept target. Three distinct ester-responsive protecting groups are incorporated into MMP proinhibitors containing hydroxy moieties. Analytical evaluation of the proinhibitors demonstrates that the use of a benzyl ether group appended to the esterase trigger leads to considerably faster kinetics of conversion and enhanced aqueous stability when compared with more conventional approaches where the trigger is directly attached to the inhibitor. Biological assays confirm that all protecting groups effectively cleave in the presence of esterase to generate the active inhibitor. The superior reaction-based prodrug strategies presented here should serve as a platform for esterase-responsive prodrug design in the future.

Introduction

Prodrugs, or chemically modified versions of bioactive substances, represent a class of pharmaceuticals that are particularly useful in overcoming barriers to drug formulation.[1] These barriers often involve difficulties associated with drug delivery and poor pharmacokinetic properties including, but not limited to, poor solubility, chemical instability, and inadequate oral absorption.[1] A stimulus-responsive promoiety can be appended to a drug to render it inactive until a chemical or enzymatic transformation event occurs, leading to metabolic conversion to the desired active agent. The use of a prodrug strategy often improves the physiochemical and/or pharmacokinetic properties of a drug.[1b] The combined benefits associated with prodrugs mentioned above have made them increasingly popular, with approximately 10% of all drugs approved worldwide classified as prodrugs.[1a,2]

The most common prodrug approach to deliver pharmacologically potent compounds is through esterase bioconversion.[1b] The esterases involved in drug metabolism are mainly localized in the liver; among these are carboxyl- and butyryl-cholinesterase, which can recognize acetate and phenylacetate groups as substrates.[3] Ester-based prodrugs have previously been shown to improve the properties of small-molecule drugs including solubility, stability, and oral bioavailability.[3c] Prodrugs containing ester promoieties are generally easy to synthesize, further adding to the appeal of this approach. Esterase-activated prodrugs effectively mask polar moieties with a nonpolar ester bond, often increasing lipophilicity, and thus membrane permeability.[3c] The vast majority of ester prodrugs mask carboxylic acids, with fewer accounts documenting their use to release hydroxy and phenolic moieties upon hydrolysis. In the latter cases, the hydroxy moiety (hydroxy or phenol) is directly esterified, and esterase bioconversion leads to release of the drug.

Metalloenzyme inhibitors are a class of compounds that can greatly benefit from a prodrug approach. In fact, the most clinically successful metalloenzyme prodrugs involve alkyl and aryl ester-modified carboxylates that target angiotensin converting enzyme (ACE). In the case of enalapril (marketed as Vasotec, Merck), the ethyl ester prodrug is metabolically converted by esterases to the free carboxylic acid that can bind to the catalytic Zn^{II} ion and attenuate enzyme activity.[1b] Other reports of metalloenzyme prodrug development include matrix metalloproteinase (MMP) inhibitors.[4] MMPs are a family of more than 20 zinc(II)-dependent endopeptidases that are capable of degrading all components of the extracellular matrix. MMP expression and activity is a highly regulated process under normal physiological conditions.[5] Overexpression and misregulation of MMPs have implicated these proteases in a number of pathologies, including arthritis and tumor cell metastasis.[6] Broad-spectrum and isoform-selective MMP inhibitors (MMPIs) have previously been developed, but these have seen limited clinical success due, in part, to undesired side effects from off-target inhibition and poor bioavailability.[7] Thus, MMPIs stand to benefit from a prodrug strategy. For this reason, MMPs were chosen as our targets of interest for proof-of-concept studies regarding esterase activation of hydroxy functionalities.

Recently, prodrug strategies utilizing glucose and hydrogen peroxide-responsive triggering groups were investigated.[8] In

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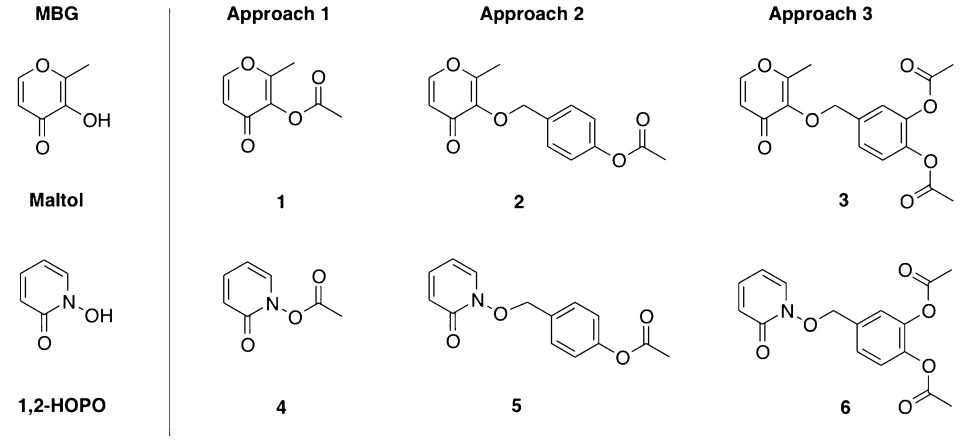
an attempt to expand the understanding and chemical tools available for prodrug design, three esterase-responsive strategies were examined, measuring both the aqueous stability and release kinetics for each. For this study, different promoieties were coupled to distinct metal binding groups (MBGs) that serve as the core scaffold for metalloenzyme inhibitors. The three different approaches for release of the MBG were studied in the presence of an esterase to identify the best system for the development of potential prodrugs.

Results and Discussion

Assessment of ester-responsive triggers

The approaches investigated here consist of the following ester-responsive promoieties, all of which are appended to the hydroxy group of the MBG: 1) direct acetylation, 2) a benzyl ether protecting group containing an acetylated phenol, and 3) a doubly acetylated catechol-based linker (Scheme 1). Approach 1 is comparable to successful strategies widely reported for masking hydroxy groups, where the direct appendage of the ester moiety inactivates the drug. Approaches 2 and 3 represent reaction-based strategies wherein the stimulus event (deacetylation) initiates an elimination reaction that leads to release of the inhibitor. Previous studies indicate that the benzyl ether linkage is superior to the more prevalent carbonate linkage in prodrug design with respect to kinetics of release and stability, thus this linkage was incorporated into our prodrug study here.[9] Approach 2 has been previously reported to release phosphonates in the presence of esterase;[10] however, the utility of this design has not been thoroughly studied.



Scheme 1. Model prodrugs appended with three distinct release strategies.

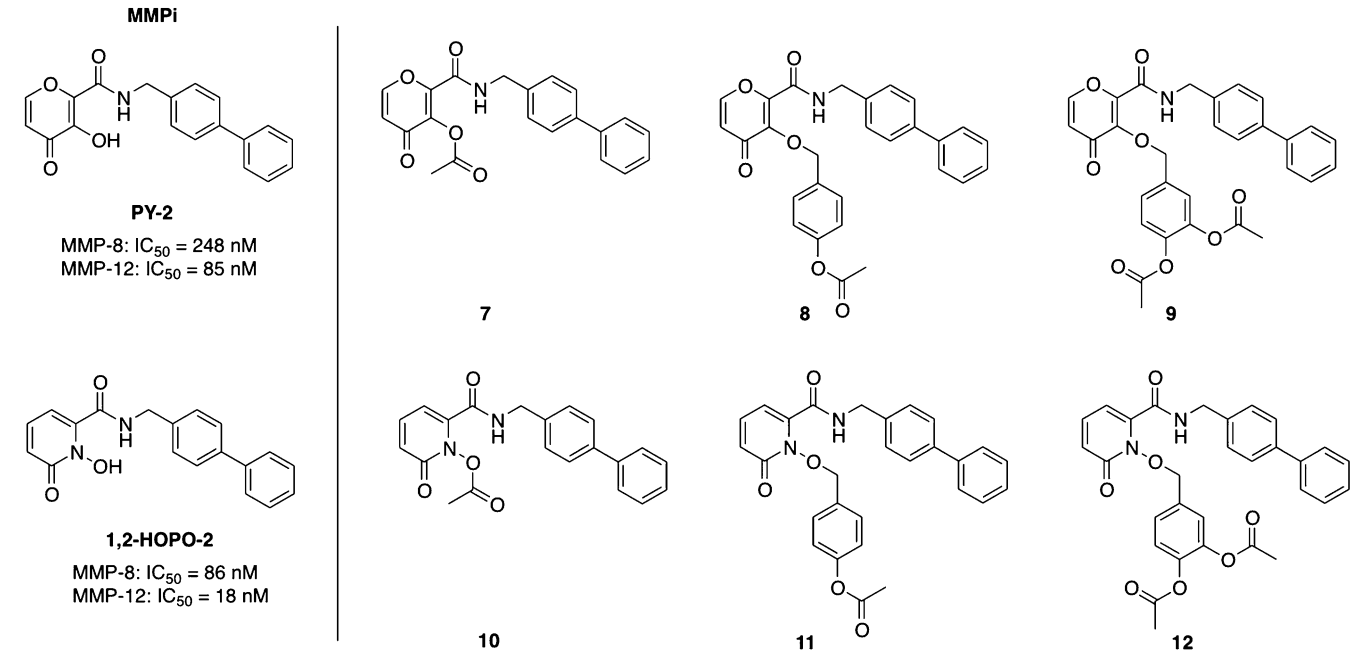
The syntheses of compounds **1**–**6** were straightforward and relatively high yielding. The reactivity of **1**–**6** with an esterase was analyzed via UV–Vis absorption spectroscopy. Upon addition of porcine liver esterase (PLE), the absorbance of the reaction mixture was monitored. The emergence of a new spectrum with a λ_{max} coinciding with that of the parent MBG was observed, indicating complete conversion to the respective MBG, maltol and 1-hydroxy-2-pyridinone (1,2-HOPO) was achieved (Figure S1–S6 in the Supporting Information). This trend was observed for **1**–**6**, demonstrating that all three different protective approaches are effective for esterase-mediated conversion.

It is worth noting that approaches 2 and 3 generate side products that are released upon cleavage. That is, a deacetylation event by esterase at the *para* position leads to a spontaneous cascade reaction releasing a quinone-methide intermediate in both approaches. Quinone-methides are electrophilic Michael acceptors that react rapidly with water to generate 4-hydroxybenzyl alcohol (approach 2) and 3,4-dihydroxybenzyl alcohol (approach 3). This work did not study the effects of these side products; however, 4-hydroxybenzyl alcohol is a known neuroprotective agent,[11] while 3,4-dihydroxybenzyl alcohol is found in virgin olive oil, suggesting an innocuous nature for each.[12]

Applying strategies to full-length inhibitors

The successful conversion of the proMBGs to the parent chelators prompted the exploration to full-length matrix metalloproteinase proinhibitors (proMMPIs). Previous studies in our laboratory led to the discovery of MMP-8/MMP-12-specific inhibitors termed PY-2 and 1,2-HOPO-2 (Scheme 2). The biphenyl backbone of these MMPIs selects against MMPs possessing shallow S1′ pockets, leading to semi-selective inhibition of deep-pocket MMPs with IC_{50} values in the low nanomolar range (Scheme 2).[13] The addition of an esterase-responsive protecting group to the two MMPI was performed in the same manner as compounds **1**–**6**.

Conversion of proMMPIs **7**–**9** was monitored via analytical HPLC, due to unclear spectral overlap between proinhibitors and parent inhibitors observed via UV–Vis spectroscopy. Treating compounds **7**–**9** with PLE produced HPLC traces corresponding to an authentic sample of PY-2 (Figure 1; see also Figures S8 and S9 in the Supporting Information), indicating successful prodrug release. proMMPIs **10**–**12** were similarly converted by PLE as evidenced by UV–Vis absorption spectroscopy, where the emergence of spectral features matching that of the MMPI 1,2-HOPO-2 were clearly observed (Figure 2; see also Figure S7 in the Supporting Information). The final absorbance spectrum shown in Figure 2 contains both 1,2-HOPO-2 and 4-hydroxybenzyl alcohol in a 1:1 ratio (see above), so that the resulting spectrum possesses features of both compounds. This side product was not detected via HPLC monitoring at 260 nm, further demonstrating the value of absorption spec-



Scheme 2. Full-length proMMPI appended with the three protecting strategies.

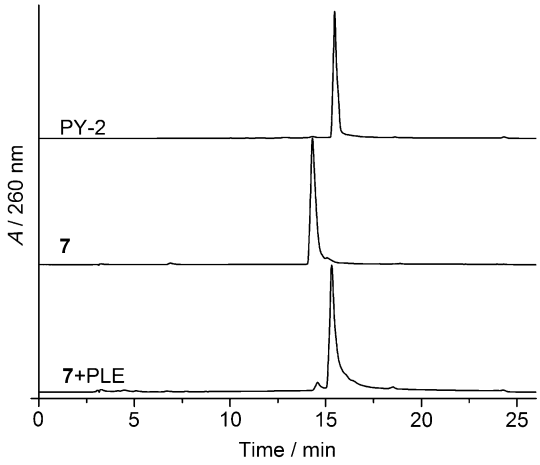


Figure 1. HPLC traces of PY-2 (top), **7** (middle) and **7** after treatment with PLE for 1 h (bottom). The retention time of **7** + PLE matches that of an authentic sample of PY-2 (t_R = 15.4 min), indicative of deprotection.

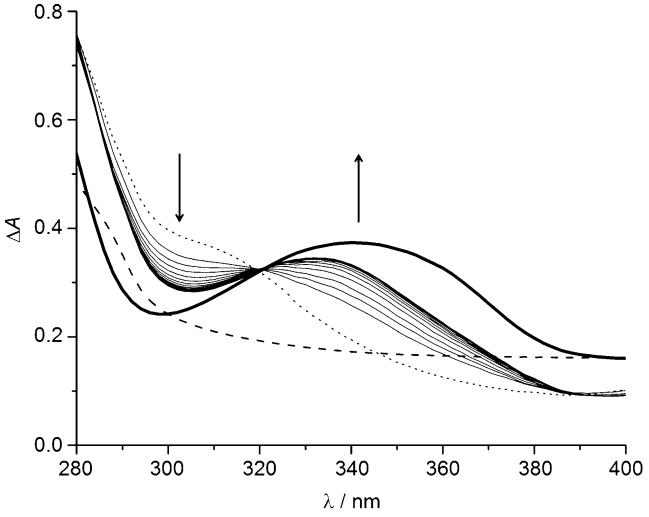
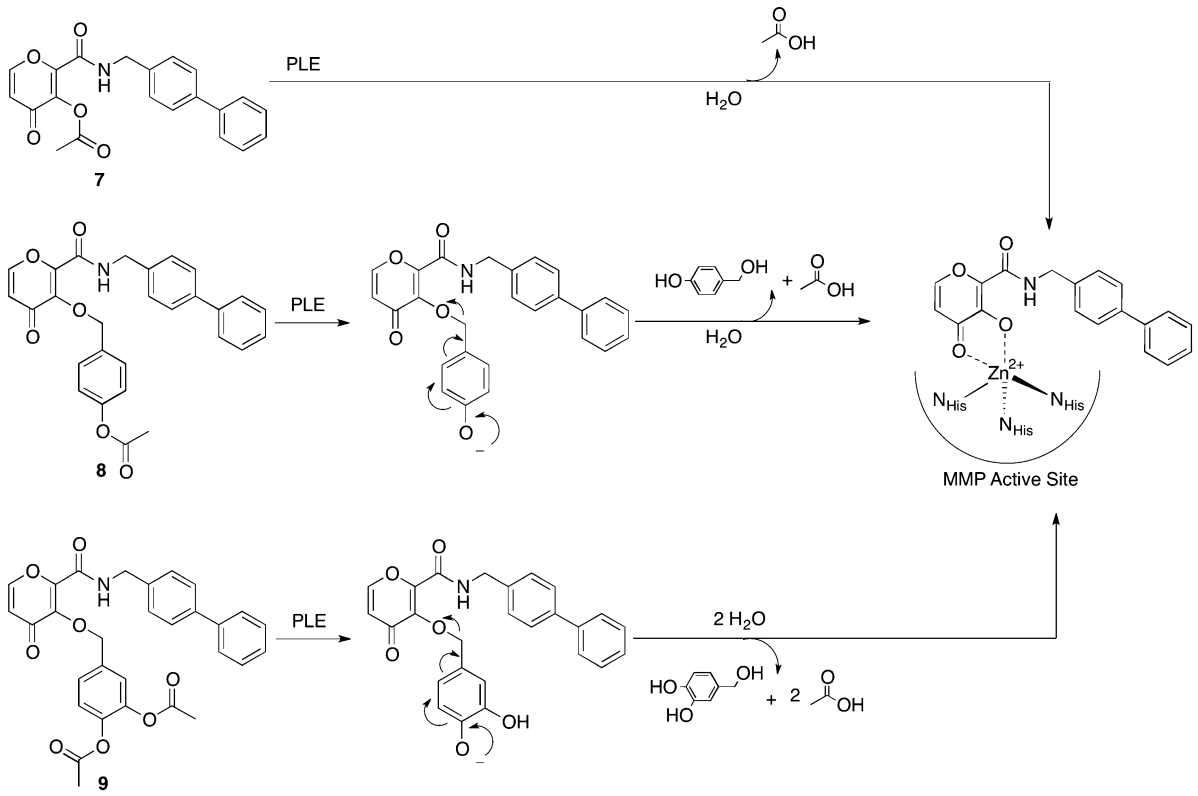


Figure 2. Absorption spectra of **11** in the presence of porcine liver esterase (PLE) monitored every 30 s for 8 min. The dotted line represents initial absorbance spectrum. The dashed line represents an authentic spectrum of 4-hydroxybenzyl alcohol, and the bold solid line depicts the absorption of an authentic sample of 1,2-HOPO-2. The arrows indicate spectral change over time.

troscopy for these studies. Nevertheless, both methods successfully show the responsiveness of the proMMPIs to esterase with release of the parent inhibitors observed in every case. A summary of the deprotection mechanisms for each esterase-activated prodrug approach for PY-2 is shown in Scheme 3.

Hydrolytic stability studies of full-length proMMPIs

ProMMPIs were evaluated for aqueous stability under simulated physiological conditions (50 mM HEPES, pH 7.4). An initial HPLC trace was obtained immediately after preparation in aqueous buffer, and a second trace was collected after 24 h incubation at 37 °C. After 24 h, approximately 35% of **7** was hydrolyzed to PY-2, while **10** underwent rapid, complete hydrolysis to 1,2-HOPO-2 (data not shown). However, compounds **8**, **9**, **11**, and **12** were all >90% stable to hydrolysis under these simulated physiological conditions for 24 h. These measurements clearly demonstrate the superior hydrolytic stability of the benzyl ether linkage (approaches 2 and 3) over direct acetylation (approach 1) for these inhibitors.



Scheme 3. Deprotection mechanisms of proMMPI **7**–**9** by esterase to generate PY-2, a potent inhibitor of MMP-8 and MMP-12.

Release kinetics

To determine the sensitivity of these compounds to esterase in a quantitative fashion, pseudo-first-order kinetic measurements were performed using UV–Vis absorption spectroscopy (Table 1). Pyrone-based proMBGs **1**–**3** and the full-length proMMPIs **7**–**9** were evaluated to compare the three prodrug approaches. Compounds **2** and **3** displayed similar rate of conversion with k_{obs} values of $245 \pm 8\ s^{-1}$ and $280 \pm 97\ s^{-1}$, respectively. Surprisingly, these rates were more than 25 times faster than that observed for directly acetylated proMBG **1** ($k_{obs} = 9 \pm 0.4\ s^{-1}$). A similar trend was observed for the proMMPIs, where directly acetylated compound **7** displayed slower kinetics than the proMMPIs containing the acetylated trigger appended via either benzyl ether linker (**8** and **9**). Liberation of **8** and **9** was approximately 4 times and 8 times faster than that of **7**, respectively. These values are consistent with previous reports showing that the rate of deprotection is enhanced with the presence of electron-donating substituents on the aromatic ring.[14] Overall, these findings highlight that the kinetic rates of release can be greatly attenuated by using different promoieties.

Table 1. Pseudo-first-order rates of conversion in the presence of porcine liver esterase (PLE).[a]

Compd	k_{obs} [s^{-1}]	Compd	k_{obs} [s^{-1}]
1	9.0 ± 0.4	**7**	160 ± 12
2	245 ± 8	**8**	742 ± 90
3	280 ± 97	**9**	1249 ± 60

[a] Rate constant (k_{obs}) values were obtained by averaging three independent trials; data represent the mean ± SD.

MMP inhibition studies

To determine the efficacy of these prodrug approaches, the ability of proMMPIs **7**–**12** to inhibit MMP-8 and MMP-12 in the absence and presence of esterase was performed. Compound **10** was excluded in these studies as it was found to be unstable in aqueous buffer upon preparation. MMP activity assays utilizing a cleavable fluorescent resonance energy transfer (FRET) substrate were employed (Figure 3).[15] Before treatment with PLE, compounds **7**, **9**, and **11** showed essentially no inhibition against MMP-8 and MMP-12, while compounds **8** and **12** showed minimal inhibition (< 10%) against these two isoforms. Upon addition of PLE, the percent inhibition increased to 40–50% inhibition for all compounds, indicative of activation to PY-2 and 1,2-HOPO-2. These biochemical assays demonstrate that esterase-responsive prodrugs are an effective class of proMMPIs.

Conclusions

We have demonstrated three different approaches to liberate phenol or hydroxy moieties upon conversion by esterase, using MMP prodrugs as our proof-of-concept system. The

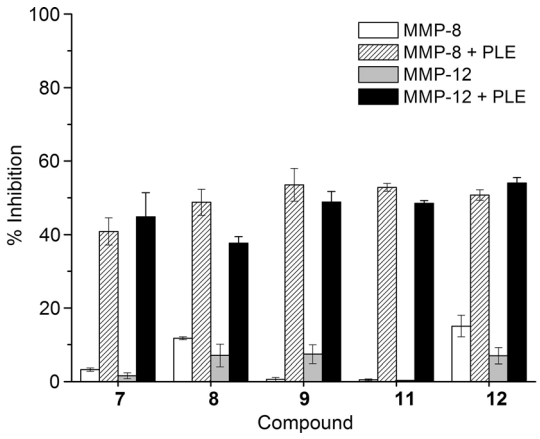


Figure 3. Inhibition assay results for compounds **7**–**9**, **11** and **12** against MMP-8 and MMP-12 and in the absence or presence of porcine liver esterase (PLE).

benzyl ether linkage (approaches 2 and 3) is superior to the conventional direct linkage of the acetate protecting group (approach 1) with respect to kinetics and aqueous stability. Testing of these compounds in a biochemical assay shows no inhibition by the proinhibitors against either MMP-8 or MMP-12. Upon treatment with esterase, the promoieties effectively cleave to generate the active MMPI, which inhibits the targets as expected. We hope that the superior reaction-based strategies presented here will serve as a platform for esterase-responsive prodrug design.

Experimental Section

Synthesis and characterization: The detailed synthesis and characterization of compounds **1**–**12** are provided in the Supporting Information. All chemicals were purchased from commercial suppliers (Sigma–Aldrich, Acros Organics, TCI America) and were used without further purification. Chromatography was performed using a CombiFlash *Rf* 200 automated system from TeledyneISCO (Lincoln, USA). NMR spectra were recorded on a Varian FT 400 NMR instrument. Mass spectrometry (MS) was performed at the Molecular Mass Spectrometry Facility (MMSF) in the Department of Chemistry & Biochemistry at the University of California, San Diego.

UV–vis spectroscopy: Absorption spectra of compounds **1**–**6**, **11**, and **12** were collected on a PerkinElmer Lambda 25 UV–vis spectrophotometer. A 0.05 mM solution of test compound (1.0 mL) in HEPES buffer (50 mM, pH 7.5) was treated with porcine liver esterase (PLE) (3.57 U), and the absorption was monitored over time at room temperature (Figure S1–S7 in the Supporting Information).

Calculation of kinetic rate constant: Pseudo-first-order rate constants (k_{obs}) were calculated by monitoring absorption over time in the presence of PLE. A 50 μM solution of test compound (1.0 mL) in HEPES buffer (50 mM, pH 7.5) was treated with PLE (0.178 U), and the absorption was monitored over 10–20 min at room temperature; at least 100 spectra were recorded for each sample. The change in absorption was monitored at 274 nm for the maltol series (**1**–**3**) and at 338 nm for the PY-2 series (**7**–**9**), we term A_{max}. The rate constant (k_{obs}) was determined by monitoring the appearance of the absorption peak by plotting the linear slope of $\ln[(A_{max}-A)/(A_{max})]$.

HPLC analysis: Analytical HPLC was performed on a HP Series 1050 system equipped with a Vydac C18 reverse-phase column (218TP, 250×4.6 mm, 5 μm). Separation was achieved with a flow rate of 1 mL min^{-1} and the following mobile phase: 5% MeOH + 0.1% formic acid in H_2O (A) and 0.1% formic acid in MeOH (B). Starting with 95% A and 5% B, an isocratic gradient was run for 15 min to a final solvent mixture of 5% A and 95% B, which was held for 5 min before ramping back down to 95% A and 5% B over 2 min, and holding for an additional 4 min. Compounds were prepared in HEPES buffer (50 mM, pH 7.5) at a concentration of 1 mM. Retention times of compounds PY-2, and 1,2-HOPO-2 were determined under identical HPLC conditions prior to evaluation of esterase cleavage of the protected compounds.

To determine the efficiency of esterase cleavage for the matrix metalloproteinase proinhibitors (proMMPI), a 1 mM solution of test compound (1 mL) in HEPES buffer (50 mM, pH 7.5) was prepared and treated with PLE (50 U). The sample was incubated at 25 °C for 1 h prior to analysis (Figures S8 and S9 in the Supporting Information).

To evaluate the hydrolytic stability of the proMMPI, a 1 mM solution of test compound (1.0 mL) in HEPES buffer (50 mM, pH 7.4) was prepared and a HPLC trace was obtained immediately. The sample was then incubated in the buffer solution for 24 h at 37 °C before a second HPLC trace was obtained. The stability of each sample was determined based on the area under the curve (Figures S11–S14 in the Supporting Information).

MMP inhibition assays: Inhibition values of compounds **7**–**9**, and **11**–**12** were determined using a previously described commercially available fluorescent-based assay kit.[15] MMP activity was measured in 96-well plates using a Bio-Tek Flx800 fluorescent plate reader. Each test compound was dissolved in DMSO to a concentration of 1 mM and diluted in HEPES buffer (50 mM, pH 7.5) to a concentration of 50 μM. Each sample was then treated with PLE such that 50 U of protein was present. This mixture was incubated for 1 h at room temperature. The esterase was removed via micro-centrifugation (25 °C, 5 min, 13 000 rpm) using 10 kDa molecular weight cut-off filters. The filtered esterase-treated compounds were then added to appropriate wells at their respective IC_{50} values. Each well contained 20 μL of MMP-8 or MMP-12 (1.82 U mL^{-1} or 0.35 U mL^{-1}, respectively), 60 μL MMP assay buffer (50 mM HEPES, 10 mM $CaCl_2$, 0.10% Brij-35, pH 7.5), and the esterase-treated MMPI (10 μL). After a 30 min incubation at 37 °C, a reaction was initiated with the addition of 10 μL (40 μM) of the fluorescent substrate (Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2) where Mca = (7-methoxycoumarin-4-yl)-acetyl and Dpa = *N*-3-(2,4-dinitrophenyl)-L-α-β-diaminopropionyl)),and the kinetic activity was monitored every 40 s for 30 min with excitation and emission wavelengths at 335 nm and 405 nm, respectively. Enzymatic activity and thus inhibition was calculated with respect to the control experiment (no inhibitor present). Measurements were performed in duplicate in two independent experiments.

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