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Bioorganic & Medicinal Chemistry 14 (2006) 1287–1293
Bioorganic & Medicinal Chemistry

Design, synthesis, and evaluation of novel galloyl pyrrolidine derivatives as potential anti-tumor agents

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Received 5 August 2005; revised 13 September 2005; accepted 14 September 2005
Available online 4 October 2005

Abstract—A series of novel galloyl pyrrolidine derivatives were synthesized as potential anti-tumor agents. Their inhibiting activities on gelatinase (MMP-2 and -9) were tested with succinylated gelatin as the substrate. Structure–activity analyses demonstrate that introduction of longer and more flexible side chains at the C_4 position of the pyrrolidine ring brings higher activity against gelatinase. Free phenol hydroxyl group is more favorable than the methylated one, which confirms the important role of the phenol hydroxyl group when inhibitors interact with gelatinase. In particular, (2*S*,4*S*)-4-(3-(3,4-dimethoxyphenyl)acrylamido)-*N*-hydroxy-1-(3,4,5- trimethoxybenzoyl)pyrrolidine-2-carboxamide (**18**) stood out as the most attractive compound (IC_{50} = 0.9 nM). The anti-metastasis model of mice bearing H_{22} tumor cells was used to evaluate their anti-tumor activities in vivo. The assay in vivo revealed that most of these inhibitors displayed favorable inhibitory activities (inhibitory rate >35%) and no significant toxic effects were observed. The inhibition for 62.37% of **19** indicates the strategy used to design MMP inhibitors (MMPIs) of galloyl pyrrolidine derivatives as potential anti-tumor agents is promising.

1. Introduction

Satisfactory curative chemotherapeutic agents with novel action mechanisms against metastatic cancer are still urgently needed. By sequencing the human genome, we have identified an enormous number of targets associated with cancers. Based on this historically global work, anti-tumor drugs with high affinity and selectivity are expected to be rationally designed against the corresponding targets. Matrix metalloproteinases (MMPs) have been suggested to be one of the important targets for cancer therapy.[1,2]

MMPs, a family of calcium- and zinc-dependent endopeptidases, have been proved to play crucial roles in some physiological conditions of arthritis, periodontitis, heart failure, endothelial cell configuration, tumor proliferation, invasion, angiogenesis, and metastasis.[3,4] Importantly, they are also found to be present at high levels in malignant tumor cells and associated with an aggressive malignant phenotype and poor prognosis in cancer patients.[5–8] Therefore, moderately manipulating the over-expression of MMPs may be useful in the control of cancer at various stages.[9]

To date, at least 26 structurally related members have been found in the mammal MMPs gene family, and they manipulate so many physiological processes that blocking all MMPs is usually not favored as a positive therapy. It is desirable to find MMPIs that are highly selective for certain MMPs associating with cancers.[10] MMP-2 and -9 (gelatinase) are among those meeting this requirement.[11,12] Herein, we designed galloyl pyrrolidine derivatives as novel MMPIs with the activity of inhibiting both MMP-2 and -9.

2. Inhibitor design

Inhibitors containing zinc-binding groups (ZBGs) should be designed to inhibit the activity of MMPs. This class of inhibitors can interact with the zinc ions of zinc-dependent metalloenzymes and sequentially inhibit the metastatic spread of tumors and block the processes of tumor neovascularization.[13,14]

There are two hydrophobic domains beside the catalytic activity center of MMPs, called pockets S_1' and S_2', respectively.[15] S_1' is the key domain to characterize the selectivity of various MMPs and appears to be responsible for much of the observed substrate specificity of a given MMP.[16] Herein, we designed MMPIs with galloyl functional group to extend into the S_1' pocket.

Keywords: Galloyl pyrrolidine derivatives; Gelatinase inhibitor; MMPI; Anti-tumor agents.
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doi:10.1016/j.bmc.2005.09.031

4-Hydroxyproline is the major specific amino acid of collagens, which are substrates of MMPs. So, derivatives of 4-hydroxyproline may specifically recognize and interact with the active sites of MMPs in a competitive manner. On the other hand, gallic acid-bearing polyphenols have been known to have broad biological activities, especially anti-tumor and antioxidative properties.[17,18] According to the 'combination principles', if we link hydroxyproline with gallic acid, the resulting galloyl pyrrolidine scaffold should be capable of inhibiting the enzymatic activity of gelatinase.

3. Chemistry

The synthesis of target compounds was carried out efficiently following the procedures presented in Scheme 1. It was accomplished upon acylation of 3,4,5-trimethoxybenzoic acid with excess $SOCl_2$ (reflux, 3 h) and subsequent nucleophilic substitution with the esterified 4-L-hydroxyproline (Py/CH_2Cl_2, 1.1 equiv, Et_3N, −5 °C, 3.5 h) to give the key intermediate **4**. The use of sulfonyl chloride for mesylation to provide the desired product **5** and subsequent S_N2 reaction upon treatment with the azide group (anhydrous DMF, 55 °C, 10 h) caused the conformational reversal of the C_4* chirality to produce exclusive compound **6**. The preparation of the amino group by catalytic hydrogenation of the azide ($-N_3$) (5% Pd-C/$CaCO_3$, rt, 10 h) was carried out to afford **7** with the unaltered conformation of C_4*, and the acylation of **7** with various hydrophobic substituents (halogenated alkylcarbonyl or sulfonyl) (CH_2Cl_2, Et_3N, 2 equiv) to provide the desired compounds **8**–**17**.

Considering that the inhibitors designed should have good ZBG to interact with zinc ions, we prepared several compounds with hydroximic acid functional group by treating with NH_2OK (CH_3OH, rt, 24 h) upon ester exchange reaction. Besides, toward the goal of comparing the inhibitory activity of free phenol hydroxyl group against gelatinase, compound **16** with free polyphenols was also prepared to contrast with **15**.

4. Molecular modeling studies

R/*S* configuration of C_4* was optimized with the Powell Energetic Gradient method built in the Sketch/Build Edit model (SYBYL6.91,[19] Linux 7.3). The predicted conformation of **5** was comparable with the known MMPI (marimastat), suggesting affinity to the same spatial regions (S_1' and S_2'). As shown in Figure 1, both the methyl sulfonyl ester group of **5** and the methyl amide group of marimastat can adjust their flexibility to extend into the S_1' pocket with their preponderant conformations.

To further understand the interactions of hydrophobic R_1 group with the S_1' pocket, the preferred pharmacophore docking studies were out via the FlexX flexible-Dock program. An X-ray crystallographic structure of MMP-2 complexed with a hydroximic acid inhibitor **SC-74020** (**I_{52}**)[20,21] was used as a template, the ligands docked with the cavity of S_1' pocket by superposing **16** and **I_{52}** molecules. We compared the binding modes of docking R_1 group or gallic acid portion into the S_1' pocket (Fig. 2). Binding energies of the two interaction modes were calculated. The lower binding energy of R_1 group indicated that it is reasonable to insert the R_1 group into the S_1' pocket. Moreover, the flexible mobility of R_1 could undergo torsional motion to allow their individual low-energy conformation and well-oriented interaction with the catalytic domain of MMP, and the bulk of R_1 was also required to be moderate to accommodate the S_1' pocket.

Although the computed information partially supported our assumption, the exact binding model of the galloyl pyrrolidine derivatives with MMP should be obtained from further X-ray crystal studies.

5. Structure–activity relationship studies of the galloyl pyrrolidine series

5.1. Effect of the length of the side chains linked to the pyrrolidine ring at C_4

As demonstrated in Table 1, to some extent, the longer the R_1 the more strongly the compounds inhibit the enzyme. The side chain of **16** is much longer than that of **6**, so **16** (IC_{50} = 2.1) can insert into the S_1' pocket and interact with the enzyme preferably.

5.2. Effect of H-bond interactions of the R_1 groups with the enzyme

The R_1 group of **4** (hydroxyl) is too small to occupy the S_1' pocket entirely, thus it cannot form steady H-bond interactions with the active domain of enzyme. Therefore, compound **4** cannot chelate with zinc commendably (IC_{50} > 1000 nM).

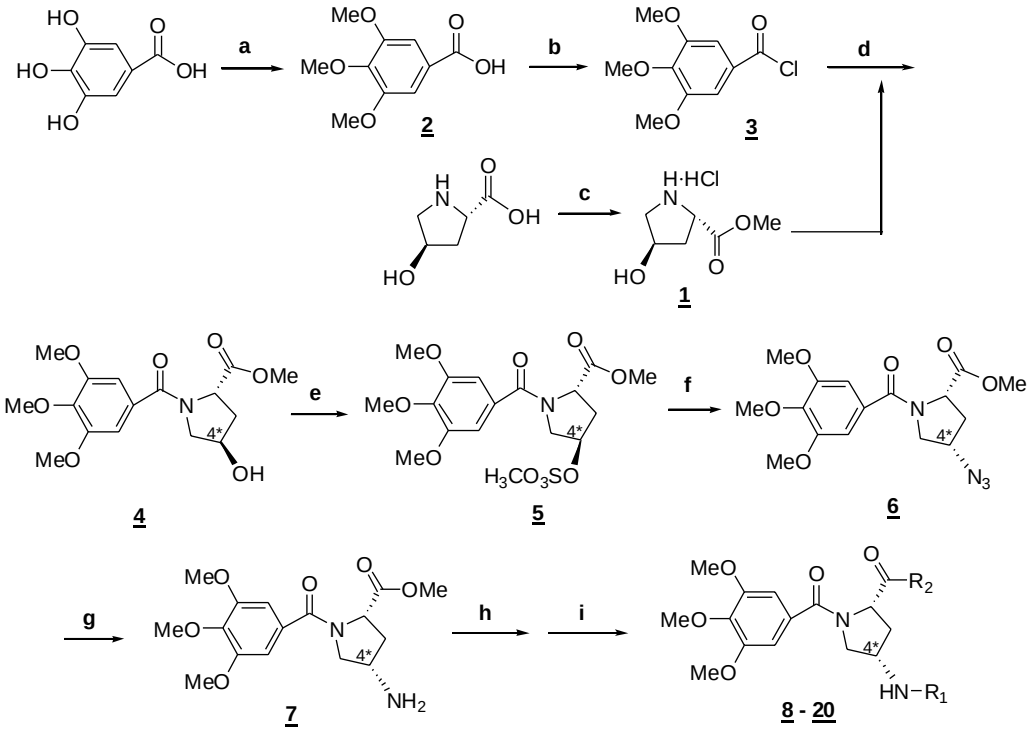
The activity of **8** is better than that of **10**, which implies that the sulfonyl amide was better than that of acryl. This is also because the sulfonyl group is easier to form hydrogen bonds with the active sites of the enzyme. So, it is favorable to introduce an appropriate side chain of R_1 that could establish few H-bond interactions with the active binding sites of the enzyme.

5.3. Effect of the flexibility of the side chain linked to the pyrrolidine ring at C_4

Compounds **11** and **19** showed higher activities, which might be owing to the flexibility of the hexanyl group, which is flexible enough to match the 3-D structure of the enzyme.

5.4. Effect of the ZBG

Comparing **15** and **16**, it is believed that the free phenol hydroxyl group is favored. An explanation was that the phenol hydroxyl group as ZBG was easier to bind zinc ions. As per our initial designing, compounds bearing hydroxamate groups (**18**–**20**) gave the most satisfactory



8	R_1=SO_2CH_3	R_2=OCH_3	**9**	R_1=$SO_2C_6H_4CH_3$-p	R_2=OCH_3
10	R_1=$COCH_2CH_3$	R_2=OCH_3	**11**	R_1=$CO(CH_2)_4CH_3$	R_2=OCH_3
12	R_1=COC_5H_4N-3'	R_2=OCH_3	**13**	R_1=$CO(CH_2)_2COOH$	R_2=OCH_3
14	R_1=$COCH_2C_6H_5COOH$	R_2=OCH_3	**15**	R_1=$COCH{=}CHC_6H_3(OMe)_2$-3',4'	R_2=OCH_3
16	R_1=$COCH{=}CHC_6H_3(OH)_2$-3',4'	R_2=OCH_3	**17**	R_1=$COC_6H_2(OH)_3$-3',4',5'	R_2=OCH_3
18	R_1=$COCH{=}CHC_6H_3(OMe)_2$-3',4'	R_2=NHOH	**19**	R_1=$NHCO(CH_2)_4CH_3$	R_2=NHOH
20	R_1=$SO_2C_6H_4CH_3$-p	R_2=NHOH			

Scheme 1. The synthesis of galloyl pyrrolidine derivatives. Reagents and conditions: (a) $(CH_3)_2SO_4$, NaOH; (b) $SOCl_2$, benzene; (c) MeOH, HCl (g); (d) dry Py, Et_3N; (e) MsCl, Et_3N, CH_2Cl_2; (f) NaN_3, DMF; (g) 5% Pd-C/H_2, EtOH, rt; (h) Et_3N, CH_2Cl_2, R-Cl (R = alkylcarbonyl or sulfonyl group); (i) NH_2OK, MeOH.

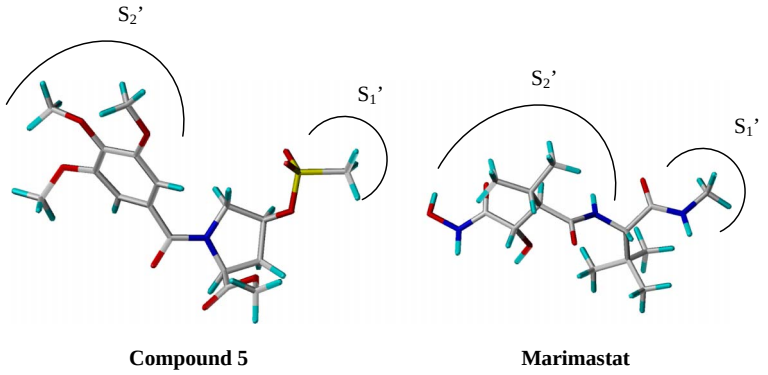


Figure 1. Comparison of preponderant conformations between compound **5** and marimastat.

inhibitory activities in vitro with IC_{50} values of 0.9–6.7 nmol.

Free phenol hydroxyl substituents (**16**) gave more potent activity than compounds bearing methoxy substituents (**15**), with a 30-fold improvement in gelatinase inhibition. But it seemed that more phenol hydroxyl substituents did not result in further enhancement in potency (**15** vs **17**).

6. Conclusions

It can be concluded that the galloyl pyrrolidine derivatives designed are a new class of gelatinase (MMP-2, -9) inhibitors endowed with significant anti-tumor activity. Several compounds are interesting as they display favorable inhibitory potency (inhibitory rate >35%) and no obvious toxic effects were observed.

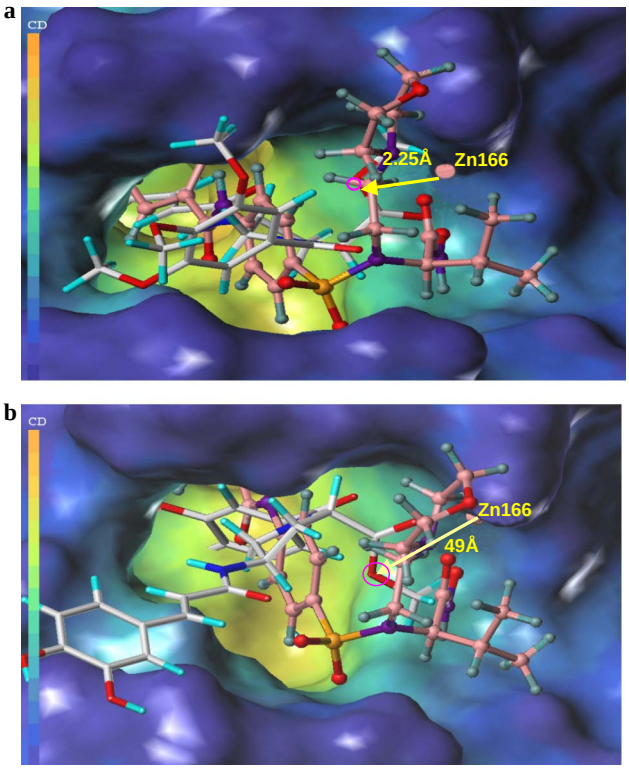


Figure 2. Predicted binding mode by superposing **16** (color of red-cyan) and $\mathbf{I_{52}}$ (color of red-orange) when bound to the cavity of the S_1' pocket of MMP-2 in the cavity depth (CD) on surface map. (a) R_1 group of **16** inserts into the S_1' pocket; (b) it is unreasonable to insert the gallic acid portion into the S_1' pocket.

In particular, compound **18** shows the best inhibitory activity in the anti-metastasis assay in vivo, and thus can be regarded as a lead in further development of anticancer galloyl pyrrolidine derivatives.

7. Materials and methods

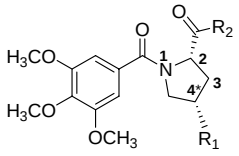
7.1. General

Reaction courses and product mixtures were routinely monitored by thin-layer chromatography (TLC) on silica gel (precoated GF_{254} plates) and visualized with iodine. Flash chromatography was performed using 200–300 mesh silica gel and the solvent system was indicated in the procedure. All solvents were of reagent grade and, when necessary, were purified and dried by standard methods. Melting points were determined with a capillary apparatus with no correction. Infrared spectra were recorded in the range of 4000–600 cm^{-1} using a Nicolet Nexus 470FT spectrometer, and KBr disks were used as indicated. ^1H NMR spectra were recorded on a Bruker AM-300 spectrometer, with chemical shifts (δ) given in ppm upfield from Me_4Si as internal standard, and the spectra were recorded in $CDCl_3$ solvents. Electrospray ionization mass spectrometry (ESI-MS) was performed on an API 4000 spectrometer.

7.2. Chemistry

7.2.1. (2*S*,4*R*)-Methyl 4-hydroxy-2-pyrrolidinecarboxylate hydrochloride (1). The synthesis of compound **1** was accomplished according to the published procedure.[22]

Table 1. Structure–activity relationship of the galloyl pyrrolidine derivatives **4**–**20**



Compound	R_1	R_2	IC_{50} (nmol)	log *P*	4*	MR
4	OH	OMe	>1000	4.14e−002	*S*	8.3548
5	OSO_2CH_3	OMe	541.4 ± 22.6	−0.4158	*S*	9.6911
6	N_3	OMe	162.3 ± 11.5	[a]	*S*	9.1304
7	NH_2	OMe	nd[a]	nd[a]	*S*	8. 9571
8	$NHSO_2CH_3$	OMe	262.5 ± 17.6	0.5908	*S*	9.9067
9	$NHSO_2C_6H_4CH_3$-*p*	OMe	113.1 ± 13.4	2.332	*S*	12.4179
10	$NHCOCH_2CH_3$	OMe	451.7 ± 23.5	0.2467	*S*	9.9975
11	$NHCO(CH_2)_4CH_3$	OMe	15.7 ± 1.5	1.4986	*S*	11.3889
12	$NHCOC_5H_4N$-3′	OMe	71.9 ± 12.3	0.1534	*S*	11.3700
13	$NHCO(CH_2)_2CO_2H$	OMe	76.1 ± 9.8	−0.7391	*R*	10.6501
14	$NHCOCH_2C_6H_5CO_2H$	OMe	103.4 ± 13.4	1.4348	*R*	12.0449
15	NHMc	OMe	62.0 ± 5.5	1.579	*R*	14.0211
16	NHCa	OMe	2.1 ± 0.4	1.0528	*R*	13.0935
17	NHGa	OMe	49.3 ± 5.3	0.322	*R*	12.0404
18	NHMc	NHOH	0.9 ± 0.2	0.6875	*R*	11.9498
19	$NHCO(CH_2)_4CH_3$	NHOH	7.7 ± 0.4	0.7513	*R*	11.2938
20	$NHSO_2C_6H_4CH_3$-*p*	NHOH	6.7 ± 0.17	1.4258	*R*	12.3267

Mc, $COCH{=}CHC_6H_3(OCH_3)_2$-3′,4′; Ca, $COCH{=}CHC_6H_3(OH)_2$-3′,4′; Ga, $COC_6H_2(OH)_3$-3′,4′,5′; 4* is the chiral center; MR was determined by the Chem3D Ultra 7.0 program.

[a] nd, not determined.

7.2.2. 3,4,5-Trimethoxybenzoic acid (2). To a stirred solution of gallic acid (5 g, 29.4 mmol) in 50 mL of 4 N NaOH was added $(CH_3)_2SO_4$ (8.9 g, 71 mmol) dropwise, keeping the inside temperature under 20 °C. The solution was allowed to stir at 30–40 °C for 20 min, another gallic acid (5 g) and 50 mL of 4 N NaOH were successively added. The resulting mixture was slowly heated to 90 °C and maintained at this temperature for 1 h. After refluxing for another 2 h, the reaction mixture was allowed to cool to rt. The solution was acidified with 2 N HCl to pH 2, filtered, and the solid was washed with water. The crude product was recrystallized with 50% EtOH to give compound **2** (40.8 g, 65%) as a white needle crystal: mp 168–171 °C. ^{1}H NMR δ 3.97 (s, 9H), 7.23 (s, 2H), 12.11 (s, 1H).

7.2.3. 3,4,5-Trimethoxybenzoic chloride (3). To a stirred solution of **2** (21.2 g, 100 mmol) in benzene (320 mL) was added dropwise $SOCl_2$ (40 mL, 548 mmol). The reaction mixture was refluxed for 3 h, and the solvent was evaporated to give 3,4,5-trimethoxybenzoic chloride as a pale yellow oil.

7.2.4. (2*S*,4*R*)-Methyl-4-hydroxy-1-(3,4,5-trimethoxybenzoyl)pyrrolidine-2-carboxylate (4). A mixture of compound **1** (1.85 g, 11 mmol) and Et_3N (3 mL, 22 mmol) in pyridine (20 mL) was stirred at rt for 20 min and filtered. The filtrate was cooled to −5 °C, and then was added dropwise a solution of **3** (2.30 g, 10 mmol) in CH_2Cl_2 (10 mL). After the mixture was stirred at −5 °C for 3.5 h, the white precipitate was removed and concentrated to dryness. The residue was purified by flash chromatography (petroleum ether/ethyl acetate 4:1 to 1:4) to give **4** (2.7 g, 79.6%) as a pale yellow solid: mp 139.2–140.4 °C. ^{1}H NMR δ 2.03–2.16 (m, 2H), 3.55–3.60 (m, 1H), 3.77 (s, 3H), 3.81–3.91 (m, 2H), 3.85 (m, 9H), 3.97 (s, 1H), 4.50 (m, 1H), 4.81 (t, J = 7.8 Hz, 1H), 6.79 (s, 2H). ESI-MS: *m*/*z* (rel intensity) 338.5.

7.2.5. (2*S*,4*R*)-Methyl-4-(methoxysulfonyloxy)-1-(3,4,5-trimethoxybenzoyl)pyrrolidine-2-carboxylate (5). Compound **4** (3.39 g, 10 mmol) was dissolved in CH_2Cl_2 (5 mL) at 0 °C under nitrogen atmosphere. Et_3N (4.5 mL, 32 mmol) and methanesulfonyl chloride (1.26 g, 11 mmol) were successively added. The mixture was stirred at rt for another 4 h. The solution was partitioned between ethyl acetate (50 mL) and water (20 mL). The organic phase was washed with saturated $NaHCO_3$ solution (15 mL), water (10 mL), and brine (10 mL), and dried over anhydrous $MgSO_4$. The solvent was removed in vacuo to give the crude product as a pale yellow oil, which was purified by flash chromatography (petroleum ether/ethyl acetate, 4:1 to 1:2) to afford **8** (3.5 g, 83.9%) as a white crystal: mp 156.7–158.9 °C. ^{1}H NMR δ 2.33 (m, 1H), 2.02–2.04 (m, 1H), 3.03 (s, 3H), 3.81 (s, 3H), 3.86–3.87 (m, 9H), 3.91–4.11 (m, 2H), 4.83 (d, J = 6.6 Hz, 1H), 5.29 (m, 1H), 6.78 (s, 2H). ESI-MS: *m*/*z* (rel intensity) 415.8.

7.2.6. (2*S*,4*S*)-Methyl-4-azido-1-(3,4,5-trimethoxybenzoyl)pyrrolidine-2-carboxylate (6). Compound **5** (4.17 g, 10 mmol) was dissolved in anhydrous DMF (15 mL) and ground NaN_3 (695 mg, 10.7 mmol) was added in one portion. Then the reaction mixture was heated at 55 °C under nitrogen atmosphere for 10 h. After the reaction mixture was cooled to 23 °C, it was partitioned between ethyl acetate (50 mL) and water (20 mL). The organic portion was washed with water (10 mL) and brine (10 mL), dried over anhydrous $MgSO_4$, filtered, and concentrated. The residue oil was purified by flash chromatography (hexane/ethyl acetate 5:1 to 3:1) to afford compound **6** (2.3 g, 63.2%) as a pale yellow oil. IR (KBr): ν 2937 (CH_3), 2103.45 (N_3), 1727 (C=O), 1644 and 1586, 1513 (C=C). ^{1}H NMR δ 1.92–1.95 (m, 1H), 2.25–2.26 (m, 2H), 3.47–3.50 (m, 2H), 3.88 (s, 9H), 4.22 (s, 1H), 4.501 (m, 1H), 4.62 (t, J = 2.8 Hz, 1H), 7.24 (s, 2H). ESI-MS: *m*/*z* (rel intensity) 363.1.

7.2.7. (2*S*,4*S*)-Methyl-4-amino-1-(3,4,5-trimethoxybenzoyl)pyrrolidine-2-carboxylate (7). Compound **6** (11 g, 30 mmol) in EtOH (120 mL) was hydrogenated over 5% palladium on calcium carbonate (3 g, 30 mmol) at rt under an atmospheric pressure of hydrogen for 10 h. The reaction mixture was filtered through a Celite pad, and the filtrate was concentrated in vacuo to give the crude product as a brown oil, which was purified by flash chromatography (chloroform/ethanol 4:1 to 1:4), to afford **7** (2.32 g, 60.8%) as a sheet crystal: mp 179–180 °C. IR (KBr): ν 3345.94, 3257.87 (NH_2), 2936 (CH_3), 1726, 1641 (C=O) and 1590, 1514 (C=C). ^{1}H NMR δ 2.04–2.06 (m, 1H), 2.56 (m, 1H), 3.23 (s, 3H), 3.86 (s, 9H), 4.12 (m, 1H), 4.57 (d, J = 7.0 Hz, 2H), 4.80–4.90 (b, 2H), 6.91 (s, 2H). ESI-MS: *m*/*z* (rel intensity) 339.4.

7.2.8. (2*S*,4*S*)-Methyl-4-(methylsulfonamido)-1-(3,4,5-trimethoxybenzoyl)pyrrolidine-2-carboxylate (8). Compound **7** (338 mg, 1 mmol) was dissolved in anhydrous CH_2Cl_2 (2 mL) and Et_3N (0.5 mL) under an atmospheric pressure of nitrogen. A solution of methanesulfonyl chloride (236 mg, 2 mmol) in 2 mL CH_2Cl_2 was added dropwise at 0 °C. The resulting solution was stirred at rt for another 3 h. The reaction mixture was partitioned between CH_2Cl_2 and water, the organic portion was washed successively with 1% HCl, 5% Na_2CO_3, and water, dried over anhydrous $MgSO_4$, filtered, and concentrated in vacuum to give the crude product as a yellow solid, which was purified by flash chromatography (petroleum ether/EtOAc 4:1 to 1:4) to give compound **8** (301 mg, 72.4%) as white crystal: mp 52.5–54.5 °C. ^{1}H NMR δ 1.26–1.36 (m, 1H), 2.09–2.12 (m, 1H), 2.96 (s, 3H), 3.86 (s, 3H), 3.89 (s, 9H), 3.92–3.96 (m, 2H), 4.11–4.17 (m, 1H), 4.64 (s, 1H), 6.76 (s, 2H). ESI-MS: *m*/*z* (rel intensity) 415.0.

7.2.9. (2*S*,4*S*)-Methyl-4-(4-methylphenylsulfonamido)-1-(3,4,5-trimethoxybenzoyl)pyrrolidine-2-carboxylate (9). Yield 69.3%, mp 68.8–70.3 °C. ^{1}H NMR δ 1.82–1.85 (m, 2H), 2.39 (s, 3H), 3.52 (d, J = 10.5 Hz, 2H), 3.80 (s, 3H), 3.85–3.86 (m, 9H), 3.92–4.00 (m, 1H), 4.49 (1H), 6.74 (s, 2H), 7.23 (d, J = 7.5 Hz, 2H), 7.68 (d, J = 7.5 Hz, 2H). ESI-MS: *m*/*z* (rel intensity) 491.6.

7.2.10. (2*S*,4*S*)-Methyl-4-propionamido-1-(3,4,5-trimethoxybenzoyl)pyrrolidine-2-carboxylate (10). Yield 75.7%, mp 142.5–143.9 °C. ^{1}H NMR δ 1.08 (t, J = 7.5 Hz, 3H), 2.13 (q, J = 7.5 Hz, 2H), 2.51–2.56 (m, 2H), 3.56 (t, J = 6.9 Hz, 1H), 3.83 (s, 9H), 3.88–

3.91 (m, 1H), 4.62 (t, J = 8.7 Hz, 2H), 6.77 (s, 2H), 6.87 (d, J = 6.9 Hz, 1H). ESI-MS: *m/z* (rel intensity) 393.5.

7.2.11. (2*S*,4*S*)-Methyl-4-hexanamido-1-(3,4,5-trimethoxybenzoyl)pyrrolidine-2-carboxylate (11). Yield 62.3%, mp 72.5–74.6 °C. ^{1}H NMR δ 0.85 (t, J = 7.2 Hz, 3H), 1.22–1.32 (m, 4H), 1.52–1.62 (m, 2H), 1.93–2.04 (m, 2H), 2.12 (t, J = 4.8 Hz, 2H), 3.59 (t, J = 8.1 Hz, 2H), 3.85 (s, 9H), 4.60–4.69 (m, 2H), 6.74 (s, 2H), 6.86 (d, J = 6.0 Hz, 1H). ESI-MS: *m/z* (rel intensity) 435.5.

7.2.12. (2*S*,4*S*)-Methyl-4-(nicotinamido)-1-(3,4,5-trimethoxybenzoyl)pyrrolidine-2-carboxylate (12). Yield 72.9%, mp 55.0–57.1 °C. ^{1}H NMR δ 2.03–2.30 (m, 2H), 3.79 (s, 3H), 3.83–3.84 (m, 9H), 3.93–4.08 (m, 1H), 4.66 (d, J = 7.8 Hz, 1H), 4.97 (s, 1H), 6.74 (s, 2H), 7.40 (dd, J = 4.5, 7.8 Hz, 1H), 8.03 (d, J = 7.8 Hz, 1H), 8.24 (d, J = 6.0 Hz, 1H), 8.72 (d, J = 4.5 Hz, 1H), 9.06 (s, 1H). ESI-MS: *m/z* (rel intensity) 442.5.

7.2.13. 3-((3*S*,5*S*)-5-(Methoxycarbonyl)-1-(3,4,5-trimethoxybenzoyl)pyrrolidin-3-ylamino)propanoic acid (13). Yield 70.0%, mp 72.5–75.5 °C. ^{1}H NMR δ 1.97–2.04 (m, 2H), 2.45 (t, J = 6.3 Hz, 2H), 2.65 (t, J = 6.3 Hz, 2H), 3.83 (s, 3H), 3.84–3.86 (m, 9H), 3.91–3.97 (m, 2H), 4.66 (m, 2H), 6.76 (s, 2H), 7.13 (s, 1H). ESI-MS: *m/z* (rel intensity) 437.5.

7.2.14. 4-(2-((3*S*,5*S*)-5-(Methoxycarbonyl)-1-(3,4,5-trimethoxybenzoyl)pyrrolidin-3-ylamino)-2-oxoethyl)benzoic acid (14). Yield 62.3%, mp 73.0–74.5 °C. ^{1}H NMR δ 2.50 (m, 2H), 3.48 (s, 1H), 4.59 (m, 2H), 5.27 (s, 1H), 6.71 (s, 1H), 7.22–7.31 (m, 5H). ESI-MS: *m/z* (rel intensity) 455.6.

7.2.15. (2*S*,4*S*)-Methyl-4-(3-(3,4-dimethoxyphenyl)acrylamido)-1-(3,4,5-trimethoxybenzoyl)pyrrolidine-2-carboxylate (15). Yield 68.9%, mp 176.5–177.0 °C. ^{1}H NMR δ 2.56–2.66 (m, 2H), 3.61–3.74 (m, 2H), 3.84–3.94 (m, 14H), 6.22 (d, J = 15.3 Hz, 1H), 6.76 (s, 1H), 6.85 (d, J = 8.4 Hz, 1H), 7.02 (d, J = 1.8 Hz, 1H), 7.08 (dd, J = 1.8, 8.4 Hz, 1H), 7.13 (d, J = 8.4 Hz), 7.54 (d, J = 15.3 Hz, 1H). ESI-MS: *m/z* (rel intensity) 527.8.

(2*S*,4*S*)-Methyl-4-(3-(3,4-dihydroxyphenyl)acrylamido)-1-(3,4,5-trimethoxybenzoyl)pyrrolidine-2-carboxylate (16). The same procedure, as described for the synthesis of **8**, was followed by using (*E*)-3-[3,4-di(acetyloxy)phenyl]-2-propanoic chloride instead of methanesulfonyl chloride. The crude product was purified by flash chromatography (petroleum ether/EtOAc/acetic acid 30:10:1 to 10:30:1) to give the target compound **16** (58.5%) as a white crystal, which appeared blue in $FeCl_3$ and Fe $(SCN)_3$. mp 137.7–141.3 °C. ^{1}H NMR δ 1.58 (s, 3H), 2.05–2.55 (m, 2H), 2.89 (s, 1H), 2.96 (s, 1H), 3.45–3.52 (m, 1H), 3.81 (s, 3H), 3.88 (s, 6H), 3.92–3.95 (m, 1H), 4.09–4.16 (m, 1H), 6.23 (d, J = 15.3 Hz, 1H), 6.69 (s, 1H), 6.84 (d, J = 8.1 Hz, 1H), 6.92 (d, J = 8.1 Hz, 1H), 7.46 (d, J = 15.3 Hz, 1H), 8.02 (s, 1H). ESI-MS: *m/z* (rel intensity) 499.5.

7.2.16. (2*S*,4*S*)-Methyl-4-(3,4,5-trihydroxybenzamido)-1-(3,4,5-trimethoxybenzoyl)pyrrolidine-2-carboxylate (17). Yield 47.1%, mp 115.6–117.8 °C. ^{1}H NMR δ 2.02–2.06 (m, 1H), 2.55–2.60 (m, 1H), 3.29 (s, 3H), 3.44–3.53 (m, 2H), 3.70 (s, 3H), 3.80 (s, 6H), 3.85–3.87 (m, 2H), 6.76 (s, 1H), 6.80 (s, 1H). ESI-MS: *m/z* (rel intensity) 489.6.

7.2.17. (2*S*,4*S*)-4-(3-(3,4-Dimethoxyphenyl)acrylamido)-*N*-hydroxy-1-(3,4,5-trimethoxybenzoyl)pyrrolidine-2-carboxamide (18). Compound **15** (858 mg, 2 mmol) was dissolved in anhydrous MeOH (7 mL) and a solution of NH_2OK in MeOH (1.5 mL) was added. The resulting solution was stirred at rt for 24 h, then 1.5 g silica gel was added and evaporated to give a pale yellow powder, which was purified by flash chromatography (CH_2Cl_2/MeOH 50:1 to 1:50) to afford the target compound **21** (537 mg, 50.8%) as a pale yellow crystal, which appeared red in $FeCl_3$. ^{1}H NMR δ 1.82–1.90 (m, 2H), 3.30 (s, 1H), 3.69–3.80 (15H), 4.26 (s, 1H), 4.38 (s, 1H), 6.43 (d, J = 15.6 Hz, 2H), 6.82 (s, 2H), 6.96 (d, J = 8.4 Hz, 1H), 7.12 (d, J = 8.4 Hz, 1H), 7.13 (s, 1H), 7.33 (d, J = 15.6 Hz, 2H), ESI-MS: *m/z* (rel intensity) 528.3.

7.2.18. (2*S*,4*S*)-4-Hexanamido-*N*-hydroxy-1-(3,4,5-trimethoxybenzoyl)pyrrolidine-2-carboxamide (19). Yield 53.6%, mp 183.3–185.3 °C. ^{1}H NMR δ 0.82 (t, J = 3.9 Hz, 3H), 1.21–1.27 (m, 4H), 1.41–1.50 (m, 2H), 1.71–1.78 (m, 1H), 2.02 (t, J = 4.5 Hz, 2H), 2.41–2.46 (m, 1H), 2.49–2.50 (m, 2H), 3.69 (s, 3H), 3.79 (s, 6H), 4.12 (s, 1H), 4.31–4.33 (m, 1H), 6.79 (s, 1H), 8.05 (d, J = 5.4 Hz, 1H). ESI-MS: *m/z* (rel intensity) 436.4.

7.2.19. (2*S*,4*S*)-*N*-Hydroxy-4-(4-methylphenylsulfonamido)-1-(3,4,5-trimethoxybenzoyl)pyrrolidine-2-carboxamide (20). Yield 55.3%, mp 101.5–104.5 °C. ^{1}H NMR δ 2.05–2.27 (m, 2H), 3.61 (m, 1H), 3.78 (s, 3H), 3.81 (s, 6H), 3.88–3.93 (m, 2H), 4.31 (t, J = 5.8 Hz, 1H), 6.55 (d, J = 15.3 Hz, 1H), 6.97 (d, J = 7.8 Hz, 1H), 7.19 (d, J = 7.8 Hz, 1H), 7.30 (s, 1H), 7.35 (d, J = 15.3 Hz, 1H), 7.50 (d, J = 8.1 Hz, 2H), 7.80 (d, J = 8.1 Hz, 2H). ESI-MS: *m/z* (rel intensity) 488.4.

7.3. In vitro gelatinase inhibitory assay

The galloyl pyrrolidine derivatives were submitted for the assessment of the inhibition against gelatinase (MMP-2, -9) in vitro in a 96-well microtiter plate using L-leucyl-*p*-nitroaniline (L-Leu-*p*NA) as a substrate, which was synthesized according to the method of Vijaykumar et al.[23] Inhibitions were evaluated with IC_{50} values (Table 1). The gelatinase (sigma) was a mixture of gelatinase A (MMP-2) and gelatinase B (MMP-9), which are both closely correlated with the tumor. So the mixture was directly used in the in vitro test without separation (see Table 2).

The gelatinase, substrate, and inhibitors were dissolved in sodium borate buffer (pH 8.5, 50 mM) and incubated for 30 min at 37 °C. Then 0.03% 2,4,6-trinitrobenzene sulfonic acid (TNBS, 50 μL) was added and incubated at 37 °C for another 20 min. Absorbance at 450 nm was measured.

Table 2. Anti-tumor activity assay of galloyl pyrrolidine derivatives

Compound	Mice (*n*)	Body weight (g)	Lung weight (g)	Metastasized nodes on lung surface (*n*)	Inhibitory rate (%)
Control	10	23.50 ± 3.42	0.168 ± 0.021	46.5 ± 2.12	
8 (100 mg/kg/day)	9 (10)[a]	18.08 ± 3.27	0.165 ± 0.013	27.1 ± 9.34	41.72
12 (100 mg/kg/day)	9 (10)[a]	21.36 ± 2.42	0.155 ± 0.051	17.9 ± 0.96	61.51
13 (100 mg/kg/day)	10	21.83 ± 1.79	0.154 ± 0.012	25.3 ± 8.32	45.66
14 (100 mg/kg/day)	10	23.17 ± 1.16	0.150 ± 0.042	19.5 ± 0.96	58.06
15 (100 mg/kg/day)	10	22.58 ± 4.25	0.164 ± 0.022	28.0 ± 1.17	39.78
16 (100 mg/kg/day)	10	17.54 ± 3.12	0.168 ± 0.080	28.7 ± 9.63	38.28
18 (50 mg/kg/day)	10	22.90 ± 3.74	0.174 ± 0.052	19.8 ± 5.43	57.42
19 (50 mg/kg/day)	9 (10)[a]	20.00 ± 2.62	0.157 ± 0.089	17.5 ± 5.68	62.37

[a] The animal number in parentheses is the original number, log *P* was determined by the Chem 3D Ultra 7.0 program.

7.4. Anti-tumor activities in vivo

A further assay in vivo was performed in the anti-metastasis model of mice bearing H_{22} carcinoma cells to evaluate anti-tumor activities of the compounds.

Suspension of inhibitors was prepared by homogenizing the tested compounds in excipient (0.5% sodium carboxymethyl cellulose solution, 0.9% benzyl alcohol, and 0.4% Tween 80 in saline). A control solution of the same excipient without inhibitors was also prepared.

Mice bearing H_{22} tumor ascites were injected via the caudal vein and randomly divided into 11 groups. The animals of the control group were treated with the same volume of excipient, while the other groups were given the inhibitors by oral administration, at a dose of 50 (hydroxamates) or 100 mg/kg/day (carboxylates), 6 days/week for 2 weeks. The mice were then weighed and sacrificed for autopsy immediately. The lungs with tumor nodes were removed, weighed, and then placed in bouin stationary solution (saturated 2,4,6-trinitrophenol solution/formaldehyde/glacial acetic acid = 15:5:1). One day later, the metastasized nodes on the surface of lungs were counted.

Acknowledgments

We thank the financial support from Natural Science Foundation of China (Grant No. 20272033) and the Doctoral Foundation of Ministry of Education of the People's Republic of China (Grant No. 20020422024).

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