

Synthesis of arylidene-substituted gelastatin analogues and their screening for MMP-2 inhibitory activity

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Abstract—A series of arylidene-substituted gelastatin analogues were synthesized in a divergent manner. Each analogue was obtained as a mixture of isomers. Calculation methods were devised to deduce the MMP-2 inhibitory activity of each isomer from the activity of an isomeric mixture and its composition. This protocol is suitable for rapidly generating a variety of arylidene-substituted gelastatin analogues and screening them for highly active inhibitors.

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1. Introduction

Matrix metalloproteinases (MMPs), a family of Zn-containing endopeptidases that mediate the breakdown of connective tissue, have attracted a wide interest as therapeutic targets.¹ Of about 20 different members of MMPs known so far, MMP-2 and MMP-9, also known as gelatinase A and B, respectively, are reported to be associated with invasion and metastasis in several cancers.² Inhibitors of these two enzymes are therefore considered to be possible anti-cancer agents.

Our interest in finding MMP inhibitors was drawn to gelastatin A and B (**1**), which had been reported to exhibit, as a 2:1 mixture, MMP inhibitory activities at sub-micromolar level (Scheme 1).³ Initially, we synthesized gelastatin analogues (**2**) in which benzylidene group replaced the conformationally flexible triene unit of the natural products.⁴ The MMP inhibition assay of the synthetic analogues enabled us to establish that (i) the benzylidene ring is an effective bioisostere of the triene chain in this case, and (ii) the bioactive geometry of the lead structure is (*Z*). These observations led us to launch a medicinal chemistry program in order to survey various substitution patterns on the aromatic ring of the benzylidene-substituted gelastatin analogues.

2. Results and discussion

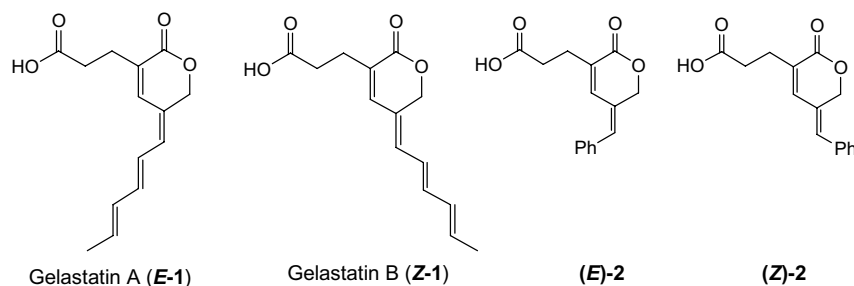
2.1. Synthesis

Our initial synthesis of the benzylidene-substituted gelastatins employed a synthetic scheme in which the benzylidene group was present at the outset and each synthetic route leading to the respective (*E/Z*)-isomeric products was parallel, but set apart from each other at an early stage of the synthesis (Scheme 2). It was, in retrospect, not an efficient synthetic pathway, even for the *then* targets, the benzylidene-substituted gelastatins. However, under the circumstances at that time when the identity of the bioactive geometry had not yet been known with the natural (*E/Z*)-isomeric gelastatins, and there had been, a priori, no guarantee at the outset that the two (*E/Z*)-isomeric benzylidene-substituted gelastatin products (**2**) would be separable at the final stage, the synthetic scheme had seemed a dependable choice, which, as it turned out later, served our purposes adequately.

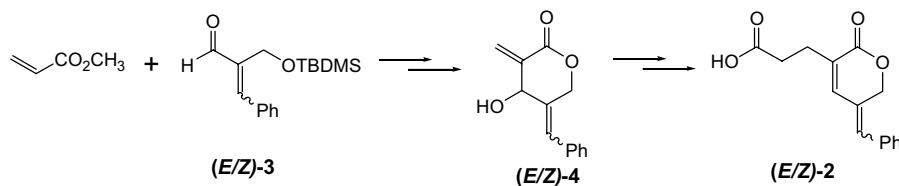
As for our present objective of synthesizing a range of arylidene-substituted gelastatin analogues, our original synthetic scheme seemed rather inefficient. Clearly, we would need a more divergent synthetic strategy whereby variously substituted aryl groups would be introduced on a common intermediate at a later stage of the synthesis. To this end, we devised a synthetic plan in which aryl units would be attached to the *exo*-methylene intermediate via Heck procedure at the last stage (Scheme 3). We were mindful from the outset that the Heck reaction would not be highly stereoselective in this case and the

Keywords: Gelastatin analogues; MMP-2 inhibitor; Screening.

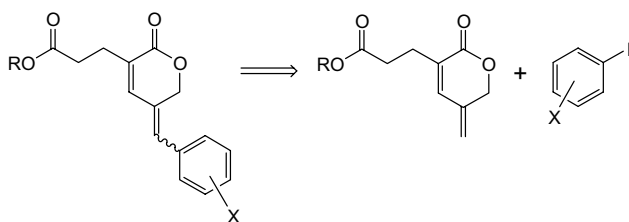
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Scheme 1.



Scheme 2.



Scheme 3.

resulting (*E/Z*)-isomeric products may not be easily separable. But we made a calculated decision that at this phase of the medicinal chemistry project, the ability to diverge from a common intermediate to various targets offered by the present scheme outweighed the potential problems of having to deal with the products of geometrical isomeric mixture. The bioactive geometry had already been established in our earlier work, and the fact that the two geometric isomers, (*E*)- and (*Z*)-benzylidene-substituted gelastatins (**2**), were distinguishable on a silica TLC plate provided a little comfort.⁴

The required *exo*-methylene intermediate (the substrate for the Heck reaction) was prepared via reaction sequence generally identical to that for the benzylidene-substituted gelastatins, that is, Baylis–Hillman reaction followed by Claisen rearrangement. Initial attempt while leaving the *exo*-methylene unit intact revealed that the functional group interfered at the Baylis–Hillman reaction step. A modification was therefore made in which the *exo*-methylene function was masked as a hydroxymethyl group (Scheme 4).

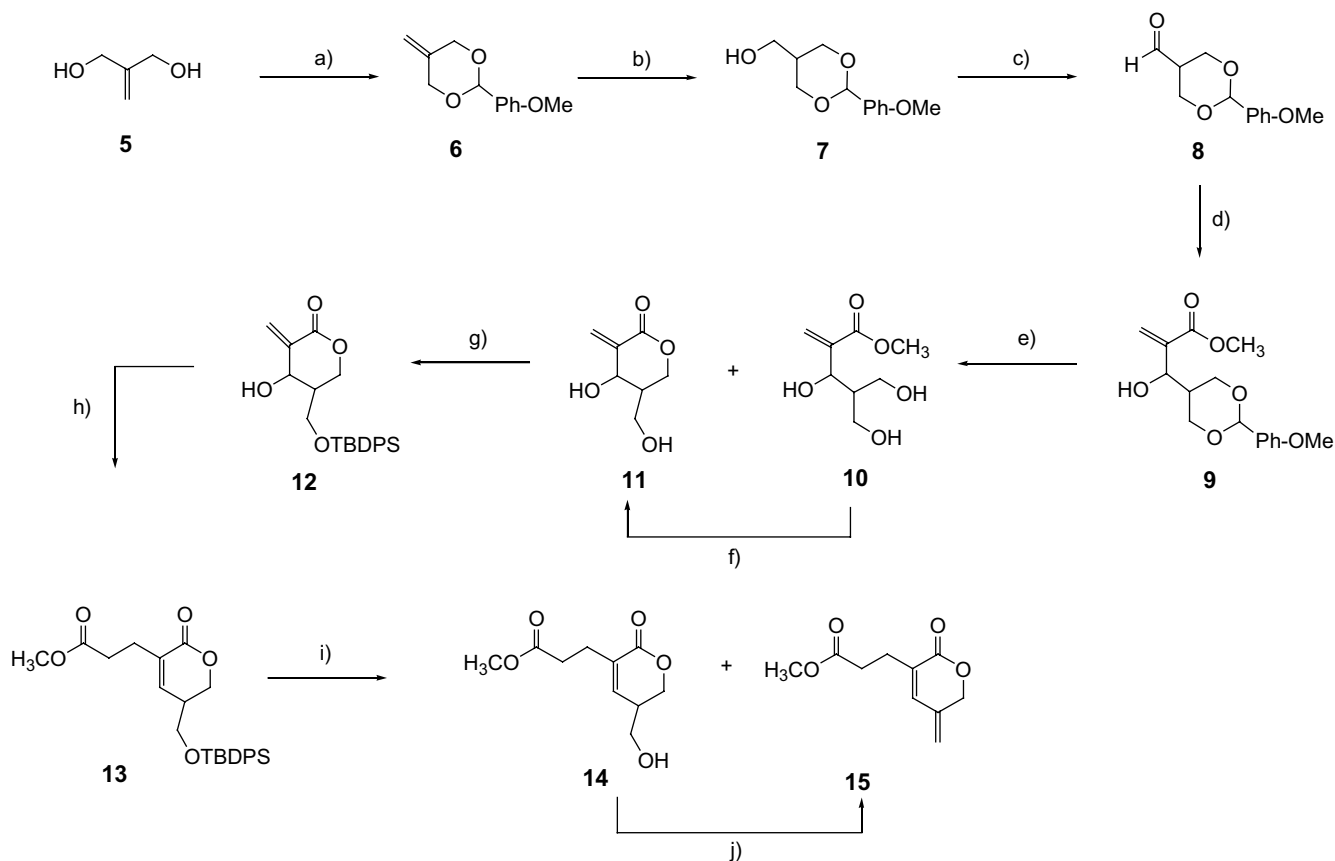
The aldehyde component of the Baylis–Hillman reaction was prepared from 2-methylene-1,3-propanediol (**5**), one of the hydroxymethyl groups of which was the masked *exo*-methylene, while the methylene group itself of this starting material was to become the required aldehyde function. Thus, the diol was protected using anisaldehyde (93%).

The choice of this acid-labile acetal protecting group was necessary to obtain a high yield of the deprotected product at a later step. Hydroboration-oxidative work-up (78%) followed by Swern oxidation (86%) yielded the desired aldehyde (**8**).

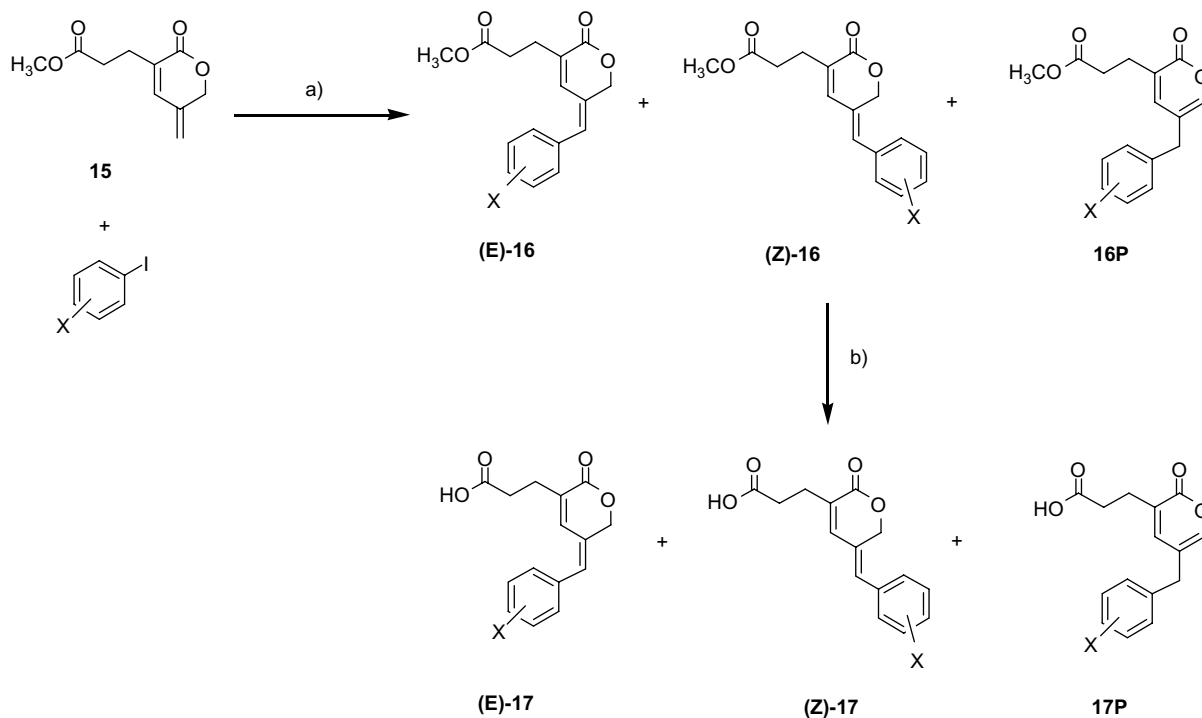
Baylis–Hillman reactions are often handicapped by slow reaction rates.⁵ Efforts to overcome this problem have identified several additives and sets of reaction conditions that reportedly speed up the useful C–C bond-forming process. Our recent contribution in this area has been to use ionic liquids as additive/solvent.⁶ In particular, we reported that [bmim][PF₆] brought about a net effect of more than two-fold rate increase when DABCO-promoted Baylis–Hillman reactions were performed in the presence of La(OTf)₃ and triethanolamine. Under these conditions, the aldehyde (**8**) was coupled to methyl acrylate to give the desired Baylis–Hillman adduct (**9**) in 60% yield.

Mild acidic deprotection of the acetal group yielded a mixture of two compounds, the methyl ester triol (**10**) and the lactone diol (**11**). Upon treating the mixture with TBAF, the former was converted to the latter to result in an overall yield of 72% from the Baylis–Hillman adduct. The primary hydroxyl function in **11** was selectively protected with a bulky silyl group (TBDPS-Cl, 65%). Claisen rearrangement with trimethyl orthoacetate yielded the desired γ,δ -unsaturated ester (**13**, 76%). Treatment with TBAF yielded the desilylated product (**14**, 54%), together with the *exo*-methylene product (**15**, 34%) through a concurrent vinylogous β -elimination. The former product was converted to the latter via two-step sequence of tosylation followed by elimination (84%).

The *exo*-methylene compound (**15**) was then subjected to the Heck protocol (Scheme 5). When the reaction was performed with iodobenzene in the presence of Pd(PPh₃)₄ and triethylamine, a Heck adduct was



Scheme 4. Reagents and conditions: (a) anisaldehyde, TsOH; 93%; (b) (1) 9-borabicyclo[3.3.1]nonane (9-BBN), (2) NaOH, H₂O₂; 78%; (c) (COCl)₂, DMSO, triethylamine; 86%; (d) methyl acrylate, 1,4-diazabicyclo[2.2.2]octane (DABCO), triethanolamine, La(OTf)₃, [bmim][PF₆]; 60%; (e) 0.01 N H₂SO₄; (10:11 = 1:2); (f) Bu₄NF (TBAF); 72% (from 9 to 11); (g) (*t*-Bu)Ph₂SiCl (TBDPS-Cl), DMAP, triethylamine; 65%; (h) MeC(OMe)₃, propionic acid; 76%; (i) Bu₄NF (TBAF); 54% (from 13 to 14); 34% (from 13 to 15); (j) (1) TsCl, (2) 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU); 84%.



Scheme 5. Reagents and conditions: (a) Pd(OAc)₂, PPh₃, AgOAc; (b) LiOH/H₂O, MeOH/H₂O.

obtained. Structural elucidation suggested that the dehydropalladation step had taken place away from the benzene ring to produce none of the desired (*E/Z*)-benzylidene-substituted gelastatin methyl esters (*E/Z*-**16a**) but the α -pyrone type product (**16Pa**). Replacement of the base by AgOAc directed some of the dehydropalladation away from the oxygen function to yield a 1:1:3 mixture of (*E*)-**16a**, (*Z*)-**16a**, and **16Pa** (72%).⁷ Further optimizations led to the Heck reaction conditions of Pd(OAc)₂/PPh₃/AgOAc in acetonitrile at 40–50 °C, under which a 1:1:2.5 mixture of the three isomeric products was formed (84%).

A series of aryl iodides were subjected to the optimized Heck conditions to produce the corresponding adducts (**16**), each as a mixture of the aforementioned three isomers. The results are summarized in Table 1.⁸ These aryl groups were chosen roughly based on the Topliss scheme to represent a range of hydrophobicity and electronic properties of the substituents.⁹ The isomeric mixture of each Heck adduct was treated with LiOH to hydrolyze the methyl ester function selectively. The carboxylic acid products (**17**) were obtained, again, each as a mixture of three isomers [(*E*)-**17**, (*Z*)-**17**, and the α -pyrone type (**17P**)], but in a different ratio from the methyl ester starting material (Table 1). It appears that some of the isomers underwent selective decompositions under the hydrolytic conditions.

While the isomeric products were in most cases distinguishable from one another on a silica TLC plate, and generally more so at the final carboxylic acid stage (**17**) than at the methyl ester intermediates (**16**), a complete separation of the isomers was not easily achieved, and as had been feared beforehand, repeated cycles of chromatography would be needed even for a partial separation of these isomers.

2.2. MMP inhibitory activity

The MMP inhibition assay was performed, as previously described, using a protocol based on the cleavage of the fluorogenic peptide MCA-Pro-Leu-Gly-Dpa-Ala-Arg-NH₂.¹⁰ As our initial results with the benzylidene-substituted gelastatins had already shown that the synthetic analogues exhibited ca. 10-fold more potent inhibitory activities with MMP-2 than with MMP-9, the assays

were done only against purified MMP-2 in the present study.

As mentioned above, separation of the isomeric products [(*E*)-, (*Z*)-, and the α -pyrone type] turned out to be very challenging. While some of the (*E*)-isomers, the highest running of the three isomers, could be obtained in pure form through silica column chromatography, the more bioactive (*Z*)-isomers were particularly difficult to purify. Faced with the prospect of protracted purification step, we sought to explore whether the mixture of the isomers might be employed in the MMP inhibitory assay and meaningful conclusions could still be deduced with respect to the bioactivity of *each isomer*.

In the course of the MMP inhibitory assay with our synthetic materials, every sample, pure or isomeric mixture, exhibited a perfect sigmoidal dependence of % inhibition on the log *C* (concentration), 10–90% inhibition falling within the range of log IC₅₀ ± 1.¹¹ This strongly suggests that the synthetic analogues are all reversible competitive inhibitors and their inhibitory activities are related to their binding energies alone. A test case to check the validity of this assumption was provided by the samples of (unsubstituted) benzylidene-gelastatins (**17a**), for which we had in our possession each of the all three isomers [(*E*)-**17a**, (*Z*)-**17a** (same as (*E/Z*)-**2**, respectively) and **17Pa**] in pure form as well as a mixture of these isomers.

Each of the three isomeric benzylidene-gelastatins, (*E*)-**17a**, (*Z*)-**17a**, and **17Pa**, was assayed against MMP-2 and observed to exhibit an inhibitory activity of IC₅₀ 21.5, 2.0, and 163 μ M, respectively. At the same time, a 2:3:3 mixture of the three isomers was assayed to exhibit IC₅₀ 9.54 μ M. From the activity of the individual isomer, it can be calculated that the 2:3:3 mixture of these three isomers (all competitive inhibitors) would exhibit IC₅₀ 18.7 μ M.¹¹ The calculated MMP-2 inhibitory activity of this mixture was practically comparable to the actually observed value, mere 0.4 kcal/mol (or ca. 6%) off in terms of the binding energy. Therefore, we felt confident enough with our assumption to proceed with other derivatives.

The synthetic arylidene-gelastatin analogues, all reversible competitive MMP-2 inhibitors, would more likely than not exhibit a uniform pattern among the three

Table 1. The Heck reactions and the subsequent hydrolysis

Entry	Substituent	Product	Compound 16		Compound 17	
			<i>E:Z:P</i>	Yield (%)	<i>E:Z:P</i>	Yield (%)
1	H	a	1:1:2.5	84	4:3:3	70
2	4-Cl	b	3:2:8	79	3:3:1	61
3	4-OMe	c	2:2:5	82	4:3:2	66
4	4-CH ₃	d	2:2:5	81	3:3:2	71
5	3,4-Cl ₂	e	3:2:5	76	3:2:2	56
6	4-CF ₃	f	4:3:1	34	5:1:1	88
7	4-NO ₂	g	2:2:5	44	3:1:3	69
8	4-OH	h	3:2:6	11	5:1:4	51
9	2,4-Cl ₂	i	4:2:5	66	6:5:8	74
10	Benzo ^a	j	1:1:2	78	2:1:1	54

^a Derived from the Heck adduct with 1-iodonaphthalene.

isomers [(*E*)-, (*Z*)-, and the α -pyrone type], the same pattern as has been observed for the benzylidene–gelastatin case (**17a**). From the observed collective activity of an isomeric mixture and its composition, and interpolating from the inhibitory activities (i.e., the binding energy differences) observed with (*E*)-**17a**, (*Z*)-**17a**, and **17Pa**, we would then be able to calculate MMP inhibitory activity of each individual isomer. While this second assumption (of a uniform pattern among the three isomers) had been admittedly on a questionable scientific ground at the outset, it was pretty much substantiated by subsequent observations (vide infra).

Thus, each series of the synthetic arylidene-substituted gelastatin analogues was assayed against MMP-2 as a mixture of the three isomers. The composition was analyzed by ^1H NMR at the same time. From these and the hypothesized inhibitory activity differences among the three isomers, MMP inhibitory activity of each isomer was calculated.¹¹ The results are summarized in Table 2. Meanwhile, silica column chromatography of each mixture provided relatively easily some of the (*E*)-isomers, the highest running of the three isomers, in pure form. These pure samples were also assayed against MMP-2 and the results are included in the Table. The calculated activity values of the (*E*)-isomers are all in remarkably good agreement with those actually observed with the pure samples. This agreement offers a firm support for our hypotheses. The calculated activity values of the (*Z*)-isomers are therefore considered to be reliable as well. Accordingly, 4-methoxy- and 4-methyl-substituted arylidene–gelastatin analogues are judged to be among the most active synthetic compounds identified in this study, each (*Z*)-isomer [(*Z*)-**17c** and (*Z*)-**17d**, respectively] is estimated to exhibit ca. IC_{50} 0.1 μM against MMP-2.

3. Conclusion

The synthetic scheme presented in this paper allows a library of arylidene-substituted gelastatin analogues to be generated in a divergent manner. While each analogue is produced as a mixture of isomers, which are not easily separable, we have shown that the bioactivity of each

isomer could be deduced from the activity of a mixture and its composition through a series of calculations. Therefore, this protocol is suitable for rapidly generating a variety of arylidene-substituted gelastatin analogues and screening them for highly active inhibitors. Once the structures of interest have been identified, their (*Z*)-isomers may be prepared following the reaction scheme presented in our earlier work.⁴

4. Experimental

4.1. Conversion of 2-methylene-1,3-propanediol to 2-(4-methoxyphenyl)-5-methylene-[1,3]dioxane (**6**)

2-Methylene-1,3-propanediol (10 g, 113.5 mmol) was dissolved in toluene (100 mL). Anisaldehyde (28 mL, 227 mmol) and TsOH (a catalytic amount) were added. The mixture was heated for 3 h at reflux under N_2 with a Dean–Stark trap to remove water. The mixture was cooled, washed with 10% NaHCO_3 , then with brine. Following drying (Na_2SO_4) and concentration, the crude product was purified on a silica column (hexane–EtOAc 4:1, then 2:1) to afford **6** (21 g, 93%).

Mp 50–51 °C. IR (CH_2Cl_2): 3074, 2972, 2842, 1618, 1518, 1453, 1310, 1247. ^1H NMR (CDCl_3): δ 3.80 (s, 3H), 4.51 (s, 4H), 4.99 (s, 2H), 5.57 (s, 1H), 6.89 (d, J = 8.6 Hz, 2H), 7.42 (d, J = 8.7 Hz, 2H). MS: 207 ($[\text{M}+\text{H}]^+$).

4.2. Hydroboration–oxidation of **6** to give [2-(4-methoxyphenyl)-[1,3]dioxan-5-yl]-methanol (**7**)

Compound **6** (19 g, 91.6 mmol) was dissolved in THF (60 mL). 9-BBN (550 mL of 0.5 M solution in THF) was added dropwise and the mixture was stirred at rt overnight. The mixture was cooled in ice bath. Methanol (200 mL) was added slowly for 2 h, then the mixture was concentrated. The residue was dissolved in THF (150 mL). NaOH (3 M, 200 mL) followed by 30% H_2O_2 (200 mL) was added dropwise. The mixture was stirred at rt for 3 h. It was extracted with EtOAc and the organic phase was washed with brine. Following

Table 2. MMP-2 inhibition activities, observed with the mixture of isomers **17**, and calculated for each individual isomer

Entry	Substituent	Product	Composition (<i>E</i> : <i>Z</i> : <i>P</i>)	Collective IC_{50} (μM)	IC_{50} against MMP-2 (μM)			
					<i>E</i> - 17		<i>Z</i> - 17	17P
					Calcd	Obsd	Calcd	Calcd
1	H	17a	2:3:3	9.54	—	21.5	— ^a	— ^b
2	4-Cl	17b	5:5:6	2.21	2.1	1.4	0.2	16.3
3	4-OMe	17c	5:6:6	0.88	1.0	0.9	0.1	7.5
4	4-CH ₃	17d	3:2:8	2.98	1.2	1.2	0.1	9.4
5	3,4-Cl ₂	17e	1:6:16	6.18	2.8	2.8	0.3	21.1
6	4-CF ₃	17f	4:1:1	20.93	22.9	33.1	2.2	174.5
7	4-NO ₂	17g	1:1:3	64.97	30.7	19.8	2.9	232.4
8	4-OH	17h	4:5:24	164.11	53.6	53.0	5.1	405.6
9	2,4-Cl ₂	17i	8:1:2	125.17	106.8	207.9	10.1	809.9
10	Benzo ^c	17j	2:1:3	50.32	27.0	18.7	2.5	203.1

^a Observed IC_{50} value 2.0 μM .

^b Observed IC_{50} value 163 μM .

^c Derived from the Heck adduct with 1-iodonaphthalene.

drying (Na_2SO_4) and concentration, the crude product was purified on a silica column (hexane–EtOAc 2:1, then 1:3) to afford the diastereomeric mixture **7a** and **7b** (**7a:b** = 1:2.7, 16.1 g, 78%).

Compound **7a**: ^1H NMR (CDCl_3): δ 2.33–2.38 (m, 1H), 3.53 (br, 1H), 3.65–3.74 (m, 2H), 3.69 (s, 3H), 4.02–4.19 (m, 4H), 5.47 (s, 1H), 6.87–6.90 (d, 2H), 7.37–7.43 (d, 2H). MS: 225 ($[\text{M}+\text{H}]^+$).

Compound **7b**: ^1H NMR (CDCl_3): δ 2.33–2.38 (m, 1H), 3.47 (br, 1H), 3.49–3.53 (m, 2H), 3.80 (s, 3H), 4.05–4.31 (m, 4H), 5.37 (s, 1H), 6.87–6.90 (d, 2H), 7.37–7.43 (d, 2H). MS: 225 ($[\text{M}+\text{H}]^+$).

4.3. Oxidation of **7** to give 2-(4-methoxyphenyl)-[1,3]dioxane-5-carbaldehyde (**8**)

Anhydrous dichloromethane (30 mL) was placed in a dry three-neck flask under N_2 . Oxalyl chloride (6.4 mL, 76.2 mmol) was added and the solution was cooled to -80°C . DMSO (16 mL, 228.6 mmol) was added dropwise and the mixture was stirred for 20 min. A solution of **7** (11.4 g, 50.8 mmol, diastereomeric mixture) in dry dichloromethane (70 mL) was added and the mixture was stirred for 30 min, maintaining -80°C . Triethylamine (35 mL, 254 mmol) was then added, and the mixture was warmed to rt, where it was stirred for 12 h. The mixture was washed with water, then with brine. Following drying (Na_2SO_4) and concentration, the crude product was purified on a silica column (hexane–EtOAc 1:2) to afford the diastereomeric mixture **8a** and **8b** (**8a:b** = 1:0.3, 8.94 g, 86%).

Compound **8a**: ^1H NMR (CDCl_3): δ 3.79 (s, 3H), 4.21 (dd, J = 0.9 Hz, 12.2 Hz, 2H), 4.71 (d, J = 12.1 Hz, 2H), 5.50 (s, 1H), 6.84–6.92 (d, 2H), 7.33–7.41 (d, 2H), 10.04 (s, 1H). MS: 223 ($[\text{M}+\text{H}]^+$).

Compound **8b**: ^1H NMR (CDCl_3): δ 3.24–3.17 (m, 1H), 3.79 (s, 3H), 3.96 (dd, J = 12.1, 11.5 Hz, 2H), 4.51 (dd, J = 4.6, 11.5 Hz, 2H), 5.38 (s, 1H), 6.84–6.91 (d, 2H), 7.33–7.42 (d, 2H), 9.67 (s, 1H). MS: 223 ($[\text{M}+\text{H}]^+$).

4.4. Baylis–Hillman reaction of **8** to give 2-{hydroxy-[2-(4-methoxyphenyl)-[1,3]dioxan-5-yl]-methyl}-acrylic acid methyl ester (**9**)

Compound **8** (8.5 g, 38.5 mmol, diastereomeric mixture) was placed in a flask. Methyl acrylate (7 mL, 77 mmol), DABCO (4.3 g, 38.5 mmol), $\text{La}(\text{OTf})_3$ (1.1 g, 1.9 mmol), and triethanolamine (2.5 mL, 19.3 mmol) were added successively. A minimum amount of ionic liquid $[\text{bmim}][\text{PF}_6]$ (0.1 mL) was added to keep the mixture homogeneous. An equivalent of methyl acrylate (3.5 mL) was added at every 24 h interval for 5 days. The mixture was diluted with EtOAc and washed with water, then with brine. Following drying (Na_2SO_4) and concentration, the crude product was purified on a silica column (hexane–EtOAc 2:1, then 1:1, then 1:3) to afford the desired product as a mixture of diastereomers **9a** and **9b** (**9a:b** = 1:4, 8.6 g, 60%).

Compound **9a**: ^1H NMR (CDCl_3): δ 2.42–2.57 (m, 1H), 3.28 (d, J = 8.3 Hz, 1H), 3.78 (s, 3H), 3.80 (s, 3H), 3.64–4.50 (m, 5H), 5.50 (s, 1H), 6.0 (s, 1H), 6.39 (s, 1H), 6.86–6.91 (d, 2H), 7.37–7.41 (d, 2H). MS: 309 ($[\text{M}+\text{H}]^+$).

Compound **9b**: ^1H NMR (CDCl_3): δ 2.42–2.57 (m, 1H), 2.71 (d, J = 8.0 Hz, 1H), 3.80 (s, 3H), 3.81 (s, 3H), 3.64–4.50 (m, 5H), 5.35 (s, 1H), 5.80 (s, 1H), 6.31 (s, 1H), 6.86–6.91 (d, 2H), 7.37–7.41 (d, 2H). MS: 309 ($[\text{M}+\text{H}]^+$).

4.5. Deprotection/lactonization of **9** to give 4-hydroxy-5-hydroxymethyl-3-methylene-tetrahydro-pyran-2-one (**11**)

Compound **9** (3.5 g, 11.0 mmol, diastereomeric mixture) was dissolved in 0.01 N H_2SO_4 solution. The mixture was stirred at rt, overnight, then was extracted with EtOAc. Following drying (Na_2SO_4) and concentration, the crude product was purified on a silica column (EtOAc) to afford **10** and **11** (**10:11** = 1:2). This mixture of **10** and **11** was dissolved in THF (50 mL). Bu_4NF (8 mL of 1.0 M solution in THF) was added, and the mixture was stirred at rt for 2 h, then diluted with EtOAc and washed with 10% citric acid and with brine. Following drying (Na_2SO_4) and concentration, the crude product was purified on a silica column (EtOAc) to afford the lactone **11a** and **11b** as a mixture of diastereomers (**11a:b** = 1.5:1, 1.25 g, 72%).

Compound **11a**: ^1H NMR (CDCl_3): δ 2.18–2.35 (m, 1H), 2.97 (br, 1H), 3.75–3.91 (m, 2H), 4.11–4.19 (m, 1H), 4.46 (dd, J = 4.3, 11.6 Hz, 1H), 4.53–4.57 (m, 1H), 6.09 (s, 1H), 6.63 (s, 1H). MS: 159 ($[\text{M}+\text{H}]^+$).

Compound **11b**: ^1H NMR (CDCl_3): δ 2.33–2.38 (m, 1H), 3.04 (br, 1H), 3.75–3.91 (m, 2H), 4.34 (dd, J = 4.4, 11.3 Hz, 1H), 4.59–4.67 (m, 1H), 4.79 (s, 1H), 5.94 (s, 1H), 6.57 (s, 1H). MS: 159 ($[\text{M}+\text{H}]^+$).

4.6. Silylation of **11** to give 5-(*tert*-butyldiphenylsilyl)-oxymethyl)-4-hydroxy-3-methylene-tetrahydro-pyran-2-one (**12**)

DMAP (43 mg, 0.35 mmol) and triethylamine (1.33 mL, 9.57 mmol) were dissolved in anhydrous dichloromethane (15 mL) under N_2 . A solution of **11** (1.38 g, 8.7 mmol, mixture of diastereomers) in dry dichloromethane (25 mL) was added and TBDPS-Cl (2.5 mL, 9.57 mmol) was added dropwise. The mixture was stirred at rt for 4 days, then washed with satd NH_4Cl and with brine. Following drying (Na_2SO_4) and concentration, the crude product was purified on a silica column (hexane–EtOAc 2:1, then 1:2) to afford the diastereomeric mixture **12a** and **12b** (**12a:b** = 1:2, 2.17 g, 65%).

Compound **12a**: ^1H NMR (CDCl_3): δ 1.07 (s, 9H), 2.12–2.18 (m, 1H), 2.47 (d, J = 4.6 Hz, 1H), 3.77–3.86 (m, 2H), 4.11–4.15 (m, 1H), 4.38 (dd, J = 4.3, 10.0 Hz, 1H), 4.48–4.56 (m, 1H), 6.04 (s, 1H), 6.61 (s, 1H), 7.38–7.49 (m, 6H), 7.62–7.66 (m, 4H). MS: 396 ($[\text{M}-\text{H}]^+$).

Compound **12b**: ^1H NMR (CDCl_3): δ 1.07 (s, 9H), 2.33–2.40 (m, 1H), 3.06 (d, J = 3.6 Hz, 1H), 3.77–3.86 (m,

2H), 4.25 (dd, $J = 4.3$, 11.2 Hz, 1H), 4.48–4.56 (m, 1H), 4.74 (s, 1H), 5.94 (s, 1H), 6.60 (s, 1H), 7.38–7.49 (m, 6H), 7.62–7.66 (m, 4H). MS: 396 ($[M-H]^+$).

4.7. Claisen rearrangement of **12** to give 3-[5-(*tert*-butyldiphenylsilyloxymethyl)-2-oxo-5,6-dihydro-2H-pyran-3-yl]-propionic acid methyl ester (**13**)

The lactone **12** (2.17 g, 5.48 mmol, diastereomeric mixture) was dissolved in trimethyl orthoacetate (10 mL) and propionic acid (0.082 mL, 1.1 mmol) was added. The mixture was heated to reflux for 2 days. The mixture was diluted with EtOAc and washed with 10% NaHCO₃, then with brine. Following drying (Na₂SO₄) and concentration, the crude product was purified on a silica column (hexane–EtOAc 3:1) to afford the desired product **13** (1.88 g, 76%).

IR (CH₂Cl₂): 3433, 2957, 2859, 2090, 1720, 1647, 1470, 1429, 1397, 1111. ¹H NMR (CDCl₃): δ 0.99 (s, 9H), 2.41–2.53 (m, 4H), 2.61–2.65 (m, 1H), 3.56 (s, 3H), 3.57–3.60 (m, 2H), 4.32 (d, $J = 4.8$ Hz, 2H), 6.43 (d, $J = 4.2$ Hz, 1H), 7.30–7.37 (m, 6H), 7.54–7.57 (m, 4H). MS: 453 ($[M+H]^+$).

4.8. Deprotection of **13** to give 3-(5-hydroxymethyl-2-oxo-5,6-dihydro-2H-pyran-3-yl)-propionic acid methyl ester (**14**) and 3-(5-methylene-2-oxo-5,6-dihydro-2H-pyran-3-yl)-propionic acid methyl ester (**15**)

TBAF (2 mL of 1.0 M solution in THF) was diluted in THF (6 mL) and molecular sieves were placed for 6 h. to remove water. To this TBAF solution (4 mL) was added compound **13** (157 mg, 0.35 mmol). After 2 h, the mixture was diluted with EtOAc and washed with 10% NaHCO₃, satd NH₄Cl, then with brine. Following drying (Na₂SO₄) and concentration, the crude product was purified on a silica column (hexane–EtOAc 2:1) to afford the desired product **14** (40 mg, 54%) along with **15** (23 mg, 34%).

Compound **14**: IR (CH₂Cl₂): 3433, 2090, 1646, 1442, 1410, 1275, 1209, 1137. ¹H NMR (CDCl₃): δ 2.43–2.64 (m, 6H), 3.60 (s, 3H), 3.62–3.68 (m, 2H), 4.32–4.35 (m, 2H), 6.58 (d, $J = 4.4$ Hz, 1H). MS: 215 ($[M+H]^+$).

Compound **15**: Mp 68–70 °C. IR (CH₂Cl₂): 3444, 2957, 2089, 1730, 1649, 1440, 1266, 1178. ¹H NMR (CDCl₃): δ 2.49–2.65 (m, 4H), 3.61 (s, 3H), 4.88 (s, 2H), 5.20 (d, $J = 6.6$ Hz, 2H), 6.92 (s, 1H). MS: 197 ($[M+H]^+$).

4.9. Tosylation/elimination of **14** to give 3-(5-methylene-2-oxo-5,6-dihydro-2H-pyran-3-yl)-propionic acid methyl ester (**15**)

Compound **14** (413 mg, 1.9 mmol) was dissolved in dichloromethane (10 mL). Tosyl chloride (0.7 g, 3.8 mmol) and pyridine (0.2 mL, 3.8 mmol) were added, and the mixture was stirred at rt for 3 days. Following concentration, the crude product was purified on a silica column (hexane–EtOAc 1:1) to afford the desired tosylate. This product (578 mg, 1.5 mmol) was dissolved in dichloromethane (10 mL) and DBU (0.27 mL,

1.8 mmol) was added. The mixture was stirred at rt for 24 h, and then diluted with EtOAc and washed with water, then with brine. Following drying (Na₂SO₄) and concentration, the crude product was purified on a silica column (hexane–EtOAc 1:1) to afford the desired product **15** (290 mg, 84%).

4.10. Heck reaction of **15** to give 3-(5-benzylidene-2-oxo-5,6-dihydro-2H-pyran-3-yl)-propionic acid methyl ester (*E/Z*-**16a**) and 3-(5-benzyl-2-oxo-2H-pyran-3-yl)-propionic acid methyl ester (**16Pa**)

Compound **15** (113 mg, 0.58 mmol) was dissolved in acetonitrile (5 mL). Iodobenzene (0.13 mL, 1.16 mmol), Pd(OAc)₂ (6.5 mg, 0.03 mmol), PPh₃ (15 mg, 0.058 mmol), and AgOAc (97 mg, 0.58 mmol) were added successively. The mixture was heated to 40–50 °C for 24 h. under N₂. The mixture was diluted with EtOAc and washed with water, then with brine. Following drying (Na₂SO₄) and concentration, the crude product was purified on a silica column (hexane–EtOAc 2:1) to afford **16a** as a mixture of three isomers (1:1:2.5, 131 mg, 84%).

Compound (*E*)-**16a**: ¹H NMR (CDCl₃): δ 2.51–2.58 (m, 2H), 2.62–2.67 (m, 2H), 3.60 (s, 3H), 4.89 (s, 2H), 6.55 (s, 1H), 7.01–7.33 (m, 6H).

Compound (*Z*)-**16a**: ¹H NMR (CDCl₃): δ 2.51–2.58 (m, 2H), 2.62–2.67 (m, 2H), 3.63 (s, 3H), 5.29 (s, 2H), 6.55 (s, 1H), 6.94 (s, 1H), 7.01–7.33 (m, 5H).

Compound **16Pa**: ¹H NMR (CDCl₃): δ 2.51–2.58 (m, 2H), 2.62–2.67 (m, 2H), 3.50 (s, 2H), 3.53 (s, 3H), 6.92 (s, 1H), 7.01–7.33 (m, 6H).

Compound (*E*)-**16b**: ¹H NMR (CDCl₃): δ 7.33–7.01 (m, 5H), 6.55 (s, 1H), 4.89 (s, 2H), 3.60 (s, 3H), 2.67–2.62 (m, 2H), 2.58–2.51 (m, 2H).

Compound (*Z*)-**16b**: ¹H NMR (CDCl₃): δ 7.33–7.01 (m, 4H), 6.92 (s, 1H), 6.55 (s, 1H), 5.25 (s, 2H), 3.62 (s, 3H), 2.67–2.62 (m, 2H), 2.58–2.51 (m, 2H).

Compound **16Pb**: ¹H NMR (CDCl₃): δ 7.33–7.01 (m, 5H), 6.92 (s, 1H), 3.53 (s, 3H), 3.50 (s, 2H), 2.67–2.62 (m, 2H), 2.58–2.51 (m, 2H).

Compound (*E*)-**16c**: ¹H NMR (CDCl₃): δ 7.34–6.85 (m, 5H), 6.62 (s, 1H), 4.95 (s, 2H), 3.85 (s, 3H), 3.68 (s, 3H), 2.72–2.59 (m, 4H).

Compound (*Z*)-**16c**: ¹H NMR (CDCl₃): δ 7.34–6.85 (m, 5H), 6.62 (s, 1H), 5.37 (s, 2H), 3.85 (s, 3H), 3.69 (s, 3H), 2.72–2.59 (m, 4H).

Compound **16Pc**: ¹H NMR (CDCl₃): δ 7.34–6.85 (m, 6H), 3.80 (s, 3H), 3.60 (s, 3H), 3.53 (s, 2H), 2.72–2.59 (m, 4H).

Compound (*E*)-**16d**: ¹H NMR (CDCl₃): δ 7.34–6.96 (m, 5H), 6.66 (s, 1H), 4.96 (s, 2H), 3.68 (s, 3H), 2.72–2.66 (m, 2H), 2.64–2.58 (m, 2H), 2.39 (s, 3H).

Compound (Z)-**16d**: ^1H NMR (CDCl_3): δ 7.34–6.96 (m, 5H), 6.66 (s, 1H), 5.37 (s, 2H), 3.69 (s, 3H), 2.72–2.66 (m, 2H), 2.64–2.58 (m, 2H), 2.39 (s, 3H).

Compound **16Pd**: ^1H NMR (CDCl_3): δ 7.34–6.96 (m, 6H), 3.60 (s, 3H), 3.55 (s, 2H), 2.72–2.66 (m, 2H), 2.64–2.58 (m, 2H), 2.34 (s, 3H).

Compound (E)-**16e**: ^1H NMR (CDCl_3): δ 7.50–6.98 (m, 4H), 6.56 (s, 1H), 4.97 (s, 2H), 3.68 (s, 3H), 2.75–2.60 (m, 4H).

Compound (Z)-**16e**: ^1H NMR (CDCl_3): δ 7.50–6.98 (m, 4H), 6.56 (s, 1H), 5.30 (s, 2H), 3.69 (s, 3H), 2.75–2.60 (m, 4H).

Compound **16Pe**: ^1H NMR (CDCl_3): δ 7.35–6.92 (m, 5H), 3.54 (s, 3H), 3.49 (s, 2H), 2.69–2.63 (m, 2H), 2.58–2.53 (m, 2H).

Compound (E)-**16f**: ^1H NMR (CDCl_3): δ 7.69–7.26 (m, 5H), 6.70 (s, 1H), 5.00 (s, 2H), 3.67 (s, 3H), 2.75–2.67 (m, 2H), 2.65–2.59 (m, 2H).

Compound (Z)-**16f**: ^1H NMR (CDCl_3): δ 7.69–7.26 (m, 4H), 7.02 (s, 1H), 6.71 (s, 1H), 5.33 (s, 2H), 3.70 (s, 3H), 2.75–2.67 (m, 2H), 2.65–2.59 (m, 2H).

Compound **16Pf**: ^1H NMR (CDCl_3): δ 7.69–7.26 (m, 5H), 7.02 (s, 1H), 3.59 (s, 5H), 2.75–2.67 (m, 2H), 2.65–2.59 (m, 2H).

Compound (E)-**16g**: ^1H NMR (CDCl_3): δ 7.42–7.16 (m, 5H), 6.69 (s, 1H), 4.98 (s, 2H), 3.67 (s, 3H), 2.75–2.70 (m, 2H), 2.66–2.61 (m, 2H).

Compound (Z)-**16g**: ^1H NMR (CDCl_3): δ 7.42–7.16 (m, 4H), 7.02 (s, 1H), 6.69 (s, 1H), 5.36 (s, 2H), 3.70 (s, 3H), 2.75–2.70 (m, 2H), 2.66–2.61 (m, 2H).

Compound **16Pg**: ^1H NMR (CDCl_3): δ 7.42–7.16 (m, 5H), 7.01 (s, 1H), 3.59 (s, 5H), 2.75–2.70 (m, 2H), 2.66–2.61 (m, 2H).

Compound (E)-**16h**: ^1H NMR (CDCl_3): δ 7.35–7.08 (m, 5H), 6.97 (s, 1H), 4.91 (s, 2H), 3.60 (s, 3H), 2.68–2.57 (m, 2H), 2.54–2.43 (m, 2H).

Compound (Z)-**16h**: ^1H NMR (CDCl_3): δ 7.35–7.08 (m, 5H), 6.97 (s, 1H), 5.29 (s, 2H), 3.63 (s, 3H), 2.68–2.57 (m, 2H), 2.54–2.43 (m, 2H).

Compound **16Ph**: ^1H NMR (CDCl_3): δ 7.35–7.08 (m, 6H), 3.52 (s, 5H), 2.68–2.57 (m, 2H), 2.54–2.43 (m, 2H).

Compound (E)-**16i**: ^1H NMR (CDCl_3): δ 7.48–7.01 (m, 4H), 6.72 (s, 1H), 5.03 (s, 2H), 3.67 (s, 3H), 2.77–2.59 (m, 4H).

Compound (Z)-**16i**: ^1H NMR (CDCl_3): δ 7.48–7.01 (m, 4H), 6.78 (s, 1H), 5.15 (s, 2H), 3.70 (s, 3H), 2.77–2.59 (m, 4H).

Compound **16Pi**: ^1H NMR (CDCl_3): δ 7.48–7.01 (m, 5H), 3.68 (s, 3H), 3.62–3.59 (m, 2H), 2.77–2.59 (m, 4H).

Compound (E)-**16j**: ^1H NMR (CDCl_3): δ 7.91–7.10 (m, 9H), 5.14 (s, 2H), 3.62 (s, 3H), 2.75–2.55 (m, 4H).

Compound (Z)-**16j**: ^1H NMR (CDCl_3): δ 7.91–7.10 (m, 9H), 5.19 (s, 2H), 3.72 (s, 3H), 2.75–2.55 (m, 4H).

Compound **16Pj**: ^1H NMR (CDCl_3): δ 7.91–7.10 (m, 9H), 4.06 (s, 2H), 3.56 (s, 3H), 2.75–2.55 (m, 4H).

4.11. Hydrolysis of **16a** to give 3-(5-benzylidene-2-oxo-5,6-dihydro-2H-pyran-3-yl)-propionic acid (E/Z-**17a**) and 3-(5-benzyl-2-oxo-2H-pyran-3-yl)-propionic acid (**17Pa**)

The methyl ester **16a** (153 mg, 0.56 mmol, 1:1:2.5 mixture of three isomers) was dissolved in MeOH–H₂O (3:1 v/v, 4 mL) and LiOH monohydrate (5 mg, 0.12 mmol) was added. The mixture was stirred at rt for 24 h and the reaction was quenched by adding 1.0 N HCl. The mixture was diluted with EtOAc and washed with brine. Following drying (Na_2SO_4) and concentration, the crude product was purified on a silica column (hexane–EtOAc 2:1 then EtOAc) to afford **17a** as a mixture of three isomers (4:3:3, 101 mg, 70%).

Compound (E)-**17a**: ^1H NMR (CDCl_3): δ 2.63 (s, 4H), 4.90 (s, 2H), 6.61 (s, 1H), 7.14–7.43 (m, 6H), 7.68–9.38 (br, 1H).

Compound (Z)-**17a**: ^1H NMR (CDCl_3): δ 2.63 (s, 4H), 5.35 (s, 2H), 6.61 (s, 1H), 7.01 (s, 1H), 7.14–7.43 (m, 5H), 7.68–9.38 (br, 1H).

Compound **17Pa**: ^1H NMR (CDCl_3): δ 2.57–2.67 (m, 4H), 3.53 (s, 2H), 7.05 (s, 1H), 7.14–7.43 (m, 6H), 7.45–8.9 (br, 1H).

Compound (E)-**17b**: yellow solid; mp 118–119 °C; ^1H NMR (CDCl_3): δ 7.39–7.17 (m, 5H), 6.62 (s, 1H), 4.97 (s, 2H), 2.71 (s, 4H); FT-IR (CH_2Cl_2 , cm^{-1}) 3445(br), 2361(s), 2088(m), 1647(m), 1489(s), 1405(m), 1198(m); MS *m/e* 293 (M+H).

Compound (Z)-**17b**: ^1H NMR (CDCl_3): δ 7.40–7.01 (m, 5H), 6.63 (s, 1H), 5.32 (s, 2H), 2.71–2.69 (s, 4H).

Compound **17Pb**: ^1H NMR (CDCl_3): δ 7.40–7.01 (m, 6H), 3.57 (s, 2H), 2.71–2.69 (m, 4H).

Compound (E)-**17c**: yellow oil; ^1H NMR (CDCl_3): δ 7.35 (s, 1H), 7.21 (d, 2H, $J = 8.6$ Hz), 6.93 (d, 2H, $J = 8.7$ Hz), 6.61 (s, 1H), 4.93 (s, 2H), 3.84 (s, 3H), 2.71 (s, 4H); FT-IR (CH_2Cl_2 , cm^{-1}) 3445(br), 2959(m), 2925(s), 2853(m), 2063(m), 1699(m), 1511(s), 1409(m), 1256(s), 1178(s), 1030(s); MS *m/e* 288 (M).

Compound (Z)-**17c**: ^1H NMR (CDCl_3): δ 7.34–6.85 (m, 5H), 6.62 (s, 1H), 5.37 (s, 2H), 3.85 (s, 3H), 3.69 (s, 3H), 2.72–2.59 (m, 4H).

Compound **17Pc**: ^1H NMR (CDCl_3): δ 7.34–6.85 (m, 6H), 3.80 (s, 3H), 3.60 (s, 3H), 3.53 (s, 2H), 2.72–2.59 (m, 4H).

Compound (E)-**17d**: yellow oil; ^1H NMR (CDCl_3): δ 7.27 (s, 1H), 7.16–6.96 (m, 4H), 6.57 (s, 1H), 4.87 (s, 2H), 2.63–2.60 (m, 4H), 2.31 (s, 3H); FT-IR (CH_2Cl_2 , cm^{-1}) 3446(br), 2361(s), 2078(m), 1708(m), 1511(s), 1409(m), 1185(m), 1114(s), 1033(m); MS *m/e* 272 (M).

Compound (Z)-**17d**: ^1H NMR (CDCl_3): δ 7.35–7.00 (m, 5H), 6.66 (s, 1H), 5.37 (s, 2H), 2.71–2.65 (m, 4H), 2.38 (s, 3H).

Compound **17Pd**: ^1H NMR (CDCl_3): δ 7.35–7.00 (m, 6H), 3.60 (s, 2H), 2.71–2.65 (m, 4H), 2.33 (s, 3H).

Compound (E)-**17e**: yellow solid; mp 130–133 °C; ^1H NMR (CDCl_3): δ 9.90–7.64 (br s, 1H), 7.42–7.01 (m, 4H), 6.49 (s, 1H), 4.90 (s, 2H), 2.64 (s, 4H); FT-IR (CH_2Cl_2 , cm^{-1}) 3431(br), 2362(s), 2080(m), 1711(m), 1469(s), 1407(m), 1201(m), 1113(s), 1031(s), 817(m); MS *m/e* 326 (M–H).

Compound (Z)-**17e**: ^1H NMR (CDCl_3): δ 7.47–6.99 (m, 4H), 6.71 (s, 1H), 5.37 (s, 2H), 2.71–2.59 (m, 4H).

Compound **17Pe**: ^1H NMR (CDCl_3): δ 7.47–6.99 (m, 5H), 3.61 (s, 2H), 2.71–2.59 (m, 4H).

Compound (E)-**17f**: white oil; ^1H NMR (CDCl_3): δ 7.59 (d, 2H, $J = 8.2$ Hz), 7.30 (d, 2H, $J = 8.1$ Hz), 7.20 (s, 1H), 6.62 (s, 1H), 4.93 (s, 2H), 2.64 (s, 4H); FT-IR (CH_2Cl_2 , cm^{-1}) 3446(br), 2087(m), 1647(m), 1410(m), 1325(s), 1260(m), 1166(m), 1113(m); MS *m/e* 326 (M).

Compound (Z)-**17f**: ^1H NMR (CDCl_3): δ 7.61–6.95 (m, 5H), 6.62 (s, 1H), 5.28 (s, 2H), 2.64 (s, 4H).

Compound **17Pf**: ^1H NMR (CDCl_3): δ 7.61–6.95 (m, 6H), 3.53 (s, 2H), 2.64 (s, 4H).

Compound (E)-**17g**: yellow oil; ^1H NMR (CDCl_3): δ 7.41–7.26 (m, 4H), 7.25 (s, 1H), 6.68 (s, 1H), 4.97 (s, 2H), 2.70 (s, 4H); FT-IR (CH_2Cl_2 , cm^{-1}) 3445(br), 2923(m), 2853(s), 2082(m), 1712(m), 1449(m), 1406(s), 1255(m), 1199(m), 1112(s), 1034(s), 751(s).

Compound (Z)-**17g**: ^1H NMR (CDCl_3): δ 7.26–6.86 (m, 5H), 6.68 (s, 1H), 5.12 (s, 2H), 2.70 (s, 4H).

Compound **17Pg**: ^1H NMR (CDCl_3): δ 7.26–6.86 (m, 6H), 3.45 (s, 2H), 2.70 (s, 4H).

Compound (E)-**17h**: yellow oil; ^1H NMR (CDCl_3): δ 7.45–7.27 (m, 5H), 6.61 (s, 1H), 4.91 (s, 2H), 2.65–2.61 (m, 4H); FT-IR (CH_2Cl_2 , cm^{-1}) 3433(br), 2360(m), 2089(m), 1646(m), 1467(s), 1260(s), 1198(m), 1105(s).

Compound (Z)-**17h**: ^1H NMR (CDCl_3): δ 7.47–6.97 (m, 5H), 6.61 (s, 1H), 5.28 (s, 2H), 2.65–2.61 (m, 4H).

Compound **17Ph**: ^1H NMR (CDCl_3): δ 7.47–6.97 (m, 6H), 3.53 (s, 2H), 2.65–2.61 (m, 4H).

Compound (E)-**17i**: white solid; mp 135–137 °C; ^1H NMR (CDCl_3): δ 7.46–7.15 (m, 3H), 7.12 (s, 1H), 6.72 (s, 1H), 5.03 (s, 2H), 2.70 (s, 4H); FT-IR (CH_2Cl_2 , cm^{-1}) 3433(br), 2360(m), 2333(m), 2089(m), 1646(m), 1469(m), 1406(s), 1260(m), 1198(m), 1105(s); MS *m/e* 326 (M–H).

Compound (Z)-**17i**: ^1H NMR (CDCl_3): δ 7.48–7.06 (m, 4H), 6.73 (s, 1H), 5.16 (s, 2H), 2.70 (s, 4H).

Compound **17Pi**: ^1H NMR (CDCl_3): δ 7.48–7.06 (m, 5H), 3.68 (s, 2H), 2.70 (s, 4H).

Compound (E)-**17j**: yellow oil; ^1H NMR (CDCl_3): δ 7.91–7.85 (m, 3H), 7.55–7.29 (m, 4H), 7.23 (s, 1H), 7.16 (s, 1H), 5.13 (s, 2H), 2.71–2.60 (m, 4H); FT-IR (CH_2Cl_2 , cm^{-1}) 3427(br), 2395(s), 2349(s), 1765(s), 1727(m), 1583(m), 1550(s), 1530(s), 1444(s); MS *m/e* 308 (M).

Compound (Z)-**17j**: ^1H NMR (CDCl_3): δ 7.91–7.87 (m, 3H), 7.57–7.23 (m, 5H), 7.16 (s, 1H), 5.19 (s, 2H), 2.77–2.63 (m, 4H).

Compound **17Pj**: ^1H NMR (CDCl_3): δ 7.91–7.23 (m, 9H), 4.06 (s, 2H), 2.77–2.63 (m, 4H).

4.12. MMP-2 inhibition assay

The assay was performed by measuring the fluorescence intensity of 7-methoxycoumarin-4-acetyl-Pro-Leu-Gly, which was produced through the cleavage of a fluorescent synthetic peptide substrate (7-methoxycoumarin-4-acetyl-Pro-Leu-Gly-Leu- β -(2,4-dinitrophenylamino)Ala-Ala-Arg-NH₂ [Sigma]) by MMP-2 from human fibrosarcoma cells (*Boehringer mannheim*).

The enzymatic reactions were performed by placing in 96-well plate the following: test compounds; TNBC buffer solution (25 mM Tris–HCl, pH 7.5, 0.1 M NaCl, 0.01% Brij-35, and 5 mM CaCl_2); MMP-2 (activated with 1 mM of aminophenylmercuric acetate for 30 min at 37 °C just before the enzymatic reaction, final concentration in well: 4.17 nM); and the fluorescent synthetic peptide substrate (final concentration in well: 9.15 μM). The reactions were run for 30 min at 37 °C, and the fluorescence intensity was measured at excitation 328 nm and emission 393 nm by spectrofluorometer. The procedure was repeated three times at a given inhibitor concentration. The inhibition rate (% inhibition) was calculated from the following equation:

$$\% \text{ Inhibition} = [(D - C) - (B - A)] / (D - C) \times 100$$

wherein, *A* represents fluorescence intensity before the reaction with an inhibitor; *B* represents fluorescence

intensity after the reaction with an inhibitor; *C* represents fluorescence intensity before the reaction without an inhibitor; and, *D* represents fluorescence intensity after the reaction without an inhibitor.

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