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4-Alkylidene-azetidin-2-ones: Novel Inhibitors of Leukocyte Elastase and Gelatinase

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Abstract—In addition to their antibiotic potency, β-lactams have recently been investigated as inhibitors of serine proteinase such as leukocyte elastase (LE), released by inflammatory cells. We describe the synthesis of a series of 4-alkylidene-β-lactams, and investigate how substitutions on C-3, C-4, and *N*-1 of the β-lactam ring affect the activity of human LE and gelatinases MMP-2 and MMP-9. LE activity was measured using a chromogenic substrate, while gelatin-zymography assay was used to evaluate gelatinase activity. We demonstrate that C-4 unsaturation on the β-lactam ring determines the degree of biological activity, with a selectivity over LE by 3-[1-(*tert*-butyldimethylsilyloxy)-ethyl] derivatives (lowest IC_{50} was 4 μM), and over gelatinase MMP-2 by C-3-unsubstituted 4-[1-ethoxycarbonyl]-ethylidene-β-lactams (lowest IC_{50} was 60 μM). (3*S*)-3-[(1*R*)-1-hydroxyethyl]-4-(1-ethoxycarbonyl)-ethylidene-azetidin-2-one inhibits gelatinase MMP-9. The compounds tested showed no cytotoxicity against NIH-3T3 murine fibroblasts. This is the first example of beta-lactams inhibiting metallo-proteinases instrumental in cancer invasion and angiogenesis. These molecules are good candidates for prototype drugs showing selective antibiotic, anti-inflammatory, and anti-invasion properties.

Introduction

The clinical demand for new, more specific and potent inhibitors of proteases is growing, especially for enzymes—such as human leukocyte elastase (LE) and mammalian gelatinases—whose activities have proven to be instrumental in a number of severe acute and chronic pathologies, including inflammation and cancer invasion.[1,2] While a number of synthetic inhibitors of gelatinases have been abandoned because of their side effects,[3] more recently some vegetable secondary metabolites—commonly present in human diet—showed good inhibitory activity.[4–8] A comparative investigation has highlighted some chemical moieties crucial for the inhibitory potential of these molecules toward either LE or gelatinases;[9] this knowledge may be useful in designing new and more potent side-effect-free inhibitors.

A similar comparative approach should prove useful for other classes of compounds, such as β-lactams. Initially developed and widely employed as antimicrobial drugs, they have been recently used as inhibitors of some serine enzymes produced by viruses, fungi and mammals. In fact, studies have demonstrated the biological activity of β-lactams against human LE, cytomegalovirus protein, prostate specific antigen, thrombin, herpes virus, co-enzyme A independent transacylase, γ-aminobutyric acid (GABA) aminotransferase, and cytosolic phospholipase A_2.[10] As regards monocyclic β-lactam derivatives, some structural requirements have been recognized as crucial for the inhibitory activity,[11] but there has been little investigation to date of the possibility of activating their azetidinone ring through unsaturated systems directly linked to the β-lactam ring.[12–15]

In a continuation of our research on the synthesis of biologically active compounds[16,17] and in particular β-lactams,[18–22] we describe here the synthesis of a series of 4-alkylidene-β-lactams exhibiting activity against LE and gelatinases MMP-2 and MMP-9 at micromolar concentration. We investigated the effect of substitution on C-3, C-4, and *N*-1 positions of the β-lactam ring on inhibitory activity. In particular we studied 3-[1-

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(*tert*-butyldimethylsilyloxy)-ethyl]-, 3-chloro-, 3-bromo- and 3-H-4-alkylidene-azetidin-2-ones obtained from diazoketones and diazoesters. For substitution on the β-lactam nitrogen atom, together with *N*-H derivatives, we synthesized *N*-acyl and *N*-alkyl-azetidin-2-ones. Scheme 1 shows all β-lactams tested. To our knowledge, no β-lactams with anti-gelatinase activity have been previously described.

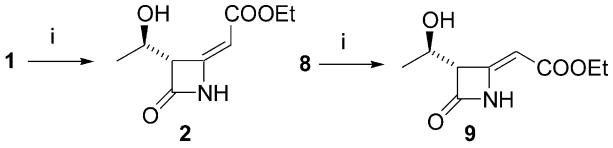
Results and Discussion

The Lewis acid-catalyzed reaction of 4-acetoxy-azetidinones with α-diazo carbonyls represents the key step in the synthesis of 4-alkylidene-azetidinones.[23,24] Thus compounds **1**, **8**, **11**, **12**, **13**, **14**, **15**, and **18** were prepared starting from commercially available (3*R*,4*R*)-4-acetoxy-3-[(1*R*)-1-(*tert*-butyldimethylsilyloxy)-ethyl]-azetidin-2-one and 4-acetoxy-azetidin-2-one, while compounds **16** and **17** were prepared starting from (3*R*,4*R*)-4-acetoxy-3-bromo- and (3*R*,4*R*)-4-acetoxy-3-chloro-azetidin-2-one,[25–30] respectively (Scheme 2).

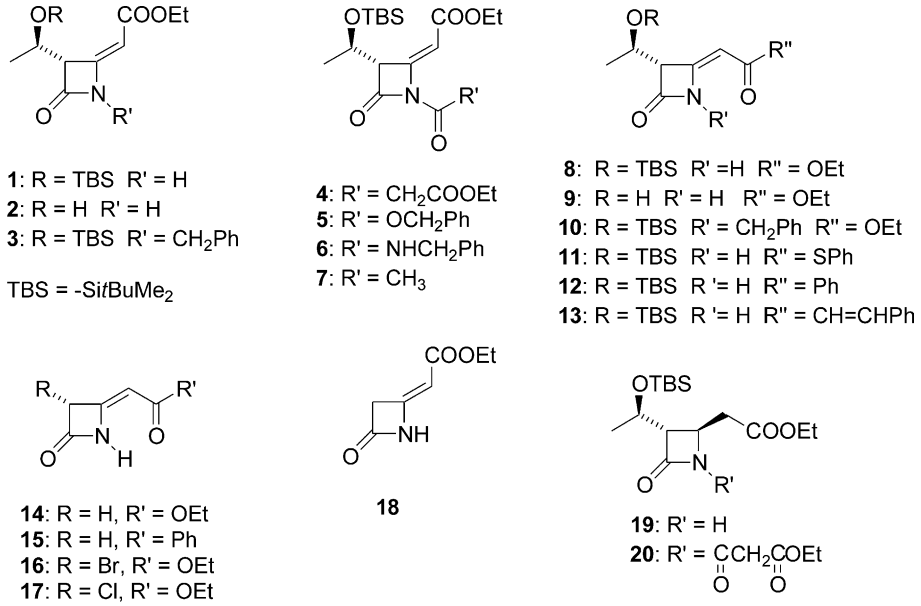
The reaction of 4-acetoxy-azetidinones with α-diazo-carbonyls proceeds smoothly yielding *E* and/or *Z* isomers[31] of the corresponding 4-alkylidene-β-lactams, depending on the diazo-compound and the Lewis acid. Critical to the success of the reaction was a stoichiometric amount of $TiCl_4$ or $AlCl_3$, and an excess of the diazo-compound associated with a requirement for trimethylsilyl protection of the β-lactam nitrogen atom. The diastereomeric products were easily separated by column chromatography. Treatment of **1** and **8** with HCl in acetonitrile produced deprotected derivatives **2** and **9** (Scheme 3).

The *N*-alkylated compounds **3**, **10** were easily obtained by treatment of **1** and **8** with benzyl bromide and solid K_2CO_3 in acetone (Scheme 4).

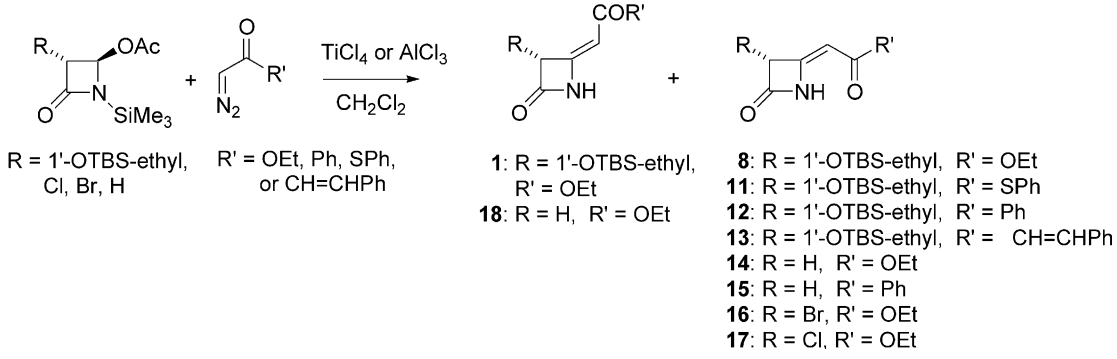
The synthetic pathway to obtain *N*-acylated-4-alkylidene derivatives presented some problems and required



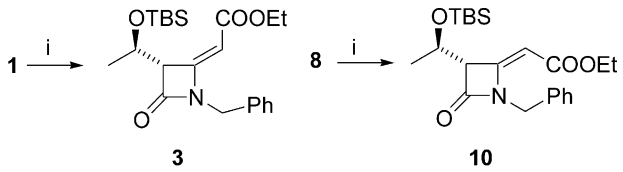
Scheme 3. Reagents: (i) HCl 1 M, CH_3CN.



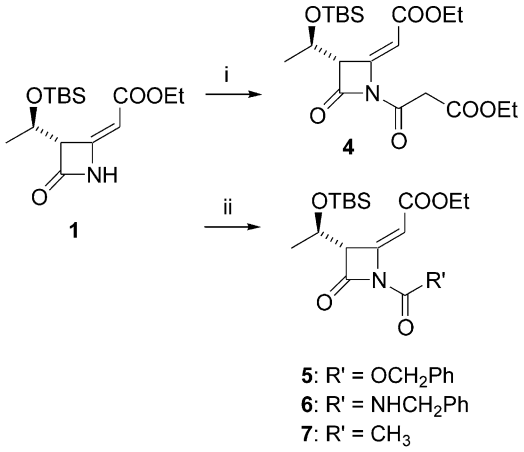
Scheme 1.



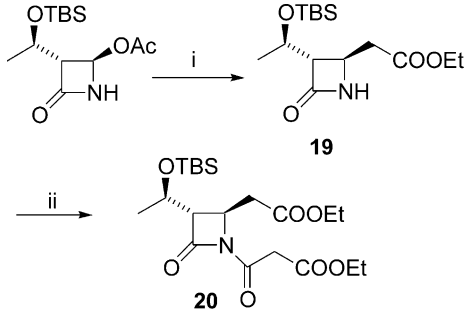
Scheme 2.



Scheme 4. Reagents: (i) BnBr, K_2CO_3, acetone.



Scheme 5. Reagents: (i) ethyl malonyl chloride, benzene reflux; (ii) K_2CO_3, acetone and: CbzCl for **5**, benzyl isocyanate for **6**, and Ac_2O for **7**.



Scheme 6. Reagents: (i) Zn, THF, $BrCH_2COOEt$, reflux; (ii) ethyl malonyl chloride, benzene reflux.

careful analysis of reagents and reaction conditions. This difficulty is due to higher reactivity of the β-lactam ring in *N*-acylated compounds towards nucleophilic reagents. Treatment of **1** with ethyl malonyl chloride in benzene at reflux gave the corresponding acylated compound **4** (Scheme 5). Any attempt to synthesize other *N*-acylated analogues by this procedure failed. We thus obtained derivatives **5**, **6** and **7** starting from *E*-isomer **1** and benzyl chloroformate, benzyl isocyanate and acetic anhydride, under lightly basic conditions (Scheme 5). Application of this synthetic procedure to the *Z*-isomer **8** yielded only by-products derived from a β-lactam ring expansion.[32]

Even C-4-saturated analogues have been prepared: compound **19** was prepared from (3*R*,4*R*)-4-acetoxy-3-[(1*R*)-1-(*tert*-butyldimethylsilyloxy)-ethyl]-azetidin-2-one using metallic Zn and ethyl 2-bromo-acetate. Its subsequent acylation with ethyl malonyl chloride gave **20** (Scheme 6).[33,34]

All compounds were purified by silica gel chromatography and new derivatives fully characterized (see Experimental).

The inhibition exerted on human HLE and gelatinase MMP-2 and MMP-9 by the various molecules was assayed as described in the Experimental; two examples are given in Figure 1. The majority of the tested compounds showed inhibitory activity and the results are shown in Table 1, which also gives the IC_{50} values.

The type of inhibition and the K_i over HLE was also determined for compound **4**, which showed the lowest IC_{50}: the inhibition exerted is dose-dependent and non-competitive, as determined by double-reciprocal plotting of the results obtained at different β-lactam concentrations; the plots share a common -1/Michaelis constant (K_m) on the abscissa, and the calculated K_i is 10 μM. For compounds **1** and **12** at concentrations ≥ 100 μM, the absorbance of these molecules was interferring with the colorimetric and/or zymographic assays; in addition, the inhibition exerted on HLE did not maintain a constant slope throughout the measurements, and, for this reason, in these cases the IC_{50} is not given.

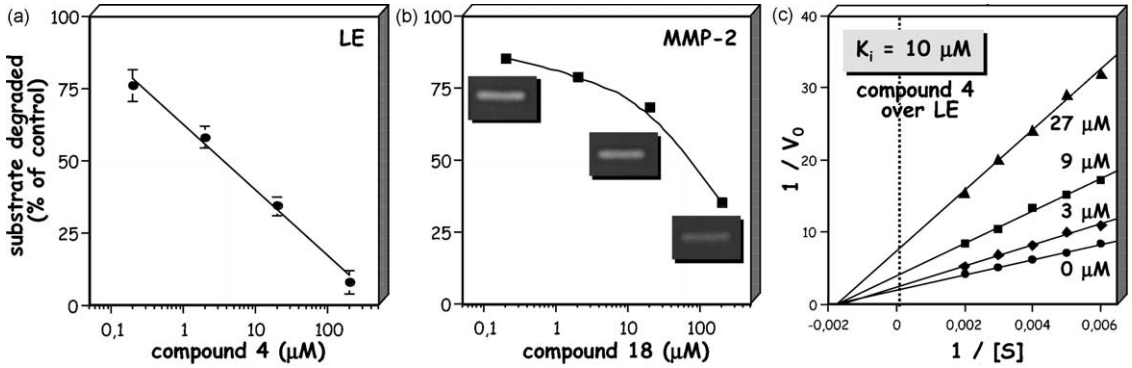
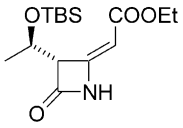
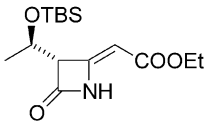
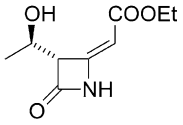
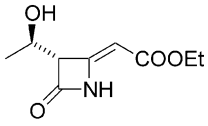
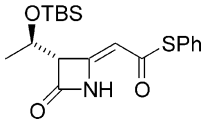
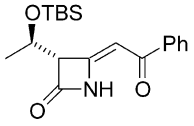
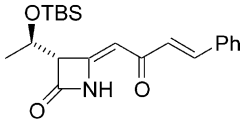
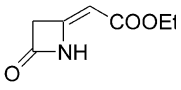
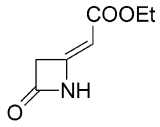
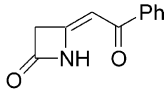
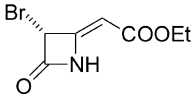
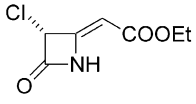


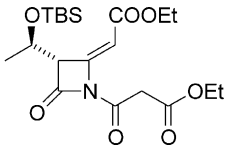
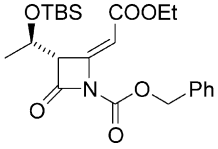
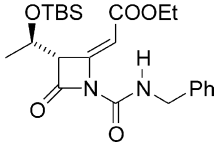
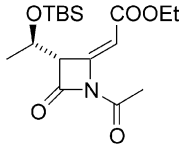
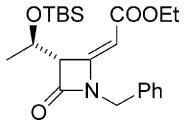
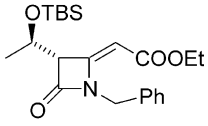
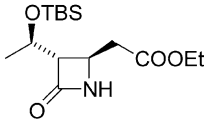
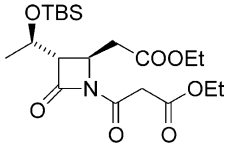
Figure 1. Inhibition of LE and metallo-proteinase-2 (MMP-2) by β-lactams **4** and **18**. In the first case (a) a chromogenic substrate (0.4 mM) was incubated at 37 °C with HLE (5 mU) in the presence of increasing concentration of compound **4** (the most efficacious against LE), and the absorbance monitored at 405 nm (values at 60 min are plotted). Each point represents the mean of three digestions. In the second case (b) MMP-2-containing medium was electrophoresed in gelatin-SDS-PAGE, and the zymography developed overnight in the presence of increasing amount of compound **18** (the most efficacious against MMP-2). The MMP-2 digestion bands (inserts) were quantitated by densitometry. Example of duplicate experiment: double-reciprocal plot (c) demonstrating noncompetitive inhibition of LE (5 mU) by increasing concentration of compound **4** (S = substrate). The points represent the mean values of triplicate samples, with SD < 10%.

Table 1. LE, MMP-2 and MMP-9 inhibition of 4-alkylidene-β-lactams

Compd		LE		MMP-2		MMP-9	
		Inhibition % at 100 μM	IC_{50} (μM)	Inhibition % at 200 μM	IC_{50} (μM)	Inhibition % at 200 μM	IC_{50} (μM)
1		66	Nd	53	150	28	—
8		58	9	42	—	25	—
2		30	—	56	85	52	150
9		22	—	41	—	32	—
11		86	20	51	150	37	—
12		68	Nd	0	—	0	—
13		100	20	0	—	0	—
14		2	—	37	160	51	—
18		28	—	65	60	20	—
15		0	—	0	—	0	—
16		0	—	0	—	0	—
17		32	—	0	—	0	—

(*continued on next page*)

Table 1 (*continued*)

Compd		LE		MMP-2		MMP-9	
		Inhibition % at 100 μM	IC_{50} (μM)	Inhibition % at 200 μM	IC_{50} (μM)	Inhibition % at 200 μM	IC_{50} (μM)
4		82	4	37	—	37	—
5		89	30	0	—	0	—
6		89	6	0	—	0	—
7		44	25	0	—	0	—
3		0	—	0	—	0	—
10		23	—	0	—	0	—
19		0	—	0	—	0	—
20		20	—	0	—	0	—

Nd, not determined, due to non-constant slope of the inhibition exerted.

Cytotoxicity tests were performed only for two β-lactams showing noteworthy inhibition of LE, compounds **4** and **8**. The viability of NIH-3T3 murine fibroblasts cultured in presence of β-lactams was not significantly impaired by the presence of the tested molecules. At 50 μM (concentration 5- and 100-fold the IC_{50} of epigallocatechin-3-gallate (EGCG) against gelatinase MMP-2 and LE, respectively),[4–6] the ratio of dead cells after 24 h was not increased compared with the control, while the cell proliferation (as number of viable cells) was lowered by 19 and 8%, respectively, in triplicate experiments (SD $<10\%$).

Regarding inhibition activity, we first investigated 4-alkylidene-β-lactams **1** and **8** with the hydroxy group protected as *tert*-butyldimethylsilyl derivative (TBS). Both stereoisomers inhibit LE, and in particular **8** showed a good IC_{50} value (9 μM), about 22-fold less potent than epigallocatechin-3-gallate (EGCG, $IC_{50} = 0.4$ μM),[6] but as potent as other β-lactam inhibitors of LE, such as the azetidin-2-one L-680,833 ($IC_{50} = 9$ μM),[35] selected as reference compounds. The corresponding OH derivatives **2** and **9** exhibited a potency lower than the OTBS precursors, indicating that a lipophylic character is preferred for LE inhibition. As regards gelatinases, β-lactams **1** and **2** gave satisfactory values of IC_{50} over MMP-2 (IC_{50} 150 and 85 μM, respectively), and **2** over MMP-9, with IC_{50} 150 μM. These results are particularly interesting since, to our knowledge, this is the first example of β-lactams active as inhibitors of gelatinases.

The absence of a substituent on the C-3 position of the β-lactam ring, as in compounds **14** and **18**, causes a severe loss of activity over LE, but they exhibited an interesting selectivity over MMP-2 (IC_{50} 160 μM for **14,** and 60 μM for **18**). As regards the influence of the C-4 double bond geometry on the activity over MMP-2, *E* isomers (**1**, **2** and **18**) were found to be more potent than *Z* isomers (**8**, **9** and **14**). In a tentative explanation of this result, it should be recalled that we revealed the presence in *Z*-alkylidene-β-lactams (but not in the *E* isomers) of an intramolecular hydrogen bond between the NH and the ethoxycarbonyl group on the C-4 side chain.[24] This could cause the reduction of activity in *Z* isomers, suggesting the possibility of a specific binding interaction through the β-lactam NH group. This interaction should be more efficient in the *E* isomers given the absence of the intramolecular H-bond.

The *S*-phenyl-carbonyl derivative **11** showed good activity over LE and MMP-2, whereas the benzoyl (**12**) and the cinnamoyl analogue (**13**) maintained the LE activity, but they were found to be inactive over MMPs. Indeed, the presence of an ester or a thioester on the C-4 side chain is crucial. This requirement occurs even for C-3 unsubstituted β-lactams, where substitution of the ethoxycarbonyl side chain (compound **14**) with a benzoyl one (compound **15**) resulted in a complete loss of activity.

N-acyl and *N*-alkyl-β-lactams were then examined. *N*-acyl derivatives showed an improvement of LE inhibition activity; in particular compounds **4** and **6** showed the best values of IC_{50} in the series (4 and 6 μM, 10-fold that of EGCG and better than L-680,833). The *N*-acetyl (**7**) and the *N*-carbobenzyloxy (**5**) derivatives were less potent and *N*-alkyl-β-lactams **3** and **10** were inactive over LE. Neither *N*-acyl nor *N*-alkyl-4-alkylidene-β-lactams are active against gelatinases, thus reinforcing the hypothesis for a specific interaction of the β-lactam NH group with gelatinases, as discussed above. In order to attribute the inhibitory activity confidently to the presence of a C=C double bond on the C-4 position of the β-lactam ring, we tested the saturated analogues on C-4, compound **19**, and the corresponding *N*-acyl derivative **20**: the former was totally inactive over LE and MMPs, while the latter showed a weak activity on LE alone.

Conclusions

In this report, we have described the synthesis of 4-alkylidene-β-lactams and their evaluation as inhibitors of LE and gelatinases MMP-2 and MMP-9. We demonstrated that the presence of the C-4 unsaturation on the β-lactam ring was crucial for the biological activity. Investigation on C-3, C-4 and *N*-1 substitution disclosed a selectivity over LE by 3-[1-(*tert*-butyldimethylsilyloxy)-ethyl]-4-[1-(ethoxycarbonyl)-ethylidene]-azetidin-2-ones (compounds **1**, **4**, **5**, **6**, **7**, and **8**), and over MMP-2 by C-3-unsubstituted 4-[(1-ethoxycarbonyl)-ethylidene]-azetidin-2-ones (compounds **14** and 1**8**). Only compound **2** effectively inhibits MMP-9. *N*-Acyl-β-lactams were more effective than *N*-H analogues over LE. The best results were obtained with compound **18** (IC_{50} 60 μM over MMP-2) and compound **4** (IC_{50} 4 μM, and K_i 10 μM over LE), in the latter being typical of non-competitive inhibition.

This is the first example of β-lactams inhibiting metalloproteinases instrumental in cancer invasion and angiogenesis.

These results may represent a first step in designing new β-lactams combining in the same molecule enhanced and possibly differentiated antibiotic and anti-inflammatory properties, to be used in the treatment of various pathologies.

Routine tests for determining the antibiotic potential of all tested molecules are in progress, and subsequent tests will be performed on molecules of selection in order to exclude cytotoxicity, as already reported for compounds **4** and **8**.

Experimental

General

Commercial reagents were used as received without additional purification. Compounds **1**, **2**, **8**, **9**, **12**, **13**, **14**, **15**, **16**, **17** and **18** have been prepared according to refs 23 and 24. *S*-phenyl diazothioacetate was prepared according to ref 36. Anhydrous solvents were obtained commercially and used without further drying. ^1H and ^{13}C NMR values were recorded on a Varian Mercury 400, Inova 300 or Gemini 200 instrument using a 5-mm probe. All chemical shifts have been quoted relative to deuterated solvent signals, δ in ppm, *J* in Hz. FT-IR: Nicolet 205 measured as films or Nujol mull between NaCl plates and reported in cm^{-1}. TLC: Merck 60 F_{254}. Column chromatography: Merck silica gel 200–300 mesh. GC–MS: HP 5890 II-HP 5971, column: HP 5 (30 m, 0.25 mm, 0.25 μm) temperature programme: 50 °C (2 min), 10°C/min, 250 °C. Elemental analyses were performed at the Istituto Geologia Marina C.N.R.

Bologna, Italy and were within 0.4% of the calculated values. The $[\alpha]_D^{25}$ were determined with a Perkin-Elmer 343 polarimeter. Melting points were determined using a Büchi apparatus and are uncorrected.

Supporting information

^{1}H NMR and ^{13}C NMR for all new compounds (**3**, **4**, **5**, **6**, **7**, **10**, **11**, **19**, **20**) are available.

Ethyl {(2*E*),(3*S*)-1-benzyl-3-[(1*R*)-1-(*tert*-butyldimethylsilanyloxy)-ethyl]-4-oxo-azetidin-2-ylidene}-acetate (3). Compound **1** (0.31 g, 1 mmol) and benzyl bromide (0.12 mL, 1 mmol) were dissolved in anhydrous acetone (10 mL); K_2CO_3 (0.14 g, 1 mmol) was added and the reaction mixture was stirred for 2 h. Then K_2CO_3 was filtered, the solvent was removed and the crude oily residue was immediately purified by flash chromatography (ethyl acetate–cyclohexane 5:95). Product **3** (0.323 g, 80%) was isolated as a pale yellow oil; ^{1}H NMR ($CDCl_3$, 200 MHz): δ 0.11 (s, 3H), 0.12 (s, 3H), 0.92 (s, 9H), 1.17 (d, J = 6.4 Hz, 3H), 1.26 (t, J = 7.2 Hz, 3H), 4.13 (q, J = 7.2 Hz, 2H), 4.18 (m, 1H), 4.45 (d, J = 16 Hz, 1H), 4.60 (d, J = 16 Hz, 1H), 4.65 (dq, J = 2.2 and J = 6.4 Hz, 1H), 5.10 (d, J = 1.4 Hz, 1H), 7.22–7.4 (m, 5H). ^{13}C NMR ($CDCl_3$, 50.29 MHz): δ −4.8, −4.6, 14.4, 18.0, 19.8, 25.8, 44.2, 59.8, 65.0, 65.2, 90.5, 127.7, 128.0, 128.8, 134.1, 155.5, 165.7, 167.5. $[\alpha]_D^{25}$ = +76 (*c* 1.5, $CHCl_3$). IR (film): 2925, 1806, 1706, 1653, 1262 cm^{-1}. GC–MS: R_t = 23.4 min; *m*/*e* 388 (M^+−15, 1), 346 (44), 185 (9), 143 (7), 115 (4), 91 (100), 73 (24), 59 (5). Anal. ($C_{22}H_{33}NO_4Si$) C, H, N.

Ethyl {(2*E*),(3*S*)-3-[(1*R*)-1-(*tert*-butyldimethylsilanyloxy)-ethyl]-2-ethoxy carbonylmethylene-4-oxo-azetidin-1-yl}-3-oxo-propionate (4). Compound **1** (0.13 g, 0.415 mmol) and ethyl malonyl chloride (0.09 mL, 0.715 mmol) were dissolved in anhydrous benzene (3 mL). The reaction mixture was refluxed for 3 h, then the solvent was removed and the crude oily residue was immediately purified by flash chromatography (ethyl acetate–cyclohexane 10:90). Product **4** (0.035 g, 20% yield) was isolated as a pale yellow oil; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.11 (s, 3H), 0.12 (s, 3H), 0.90 (s, 9H), 1.22 (d, J = 6.4 Hz, 3H), 1.26–1.32 (m, 6H), 3.80 (s, 2H), 4.21 (m, 4H), 4.28 (dd, J = 4.5 Hz, J = 1.2 Hz, 1H), 4.72 (m, 1H), 6.55 (d, J = 1.2 Hz, 1H). ^{13}C NMR ($CDCl_3$, 75.45 MHz): δ −5.0, −4.7, 14.0, 14.2, 17.9, 19.7, 25.6, 43.5, 60.4, 61.9, 65.2, 65.2, 100.9, 147.9, 162.1, 165.3, 165.7, 165.9. $[\alpha]_D^{25}$ = + 61 (*c* 1.06, $CHCl_3$). IR (film): 2932, 1836, 1724, 1665 cm^{-1}. Anal. ($C_{20}H_{33}NO_7Si$) C, H, N.

Benzyl (2*E*),(3*S*)-3-[(1*R*)-1-(*tert*-butyldimethylsilanyloxy)-ethyl]-2-ethoxy carbonylmethylene-4-oxo-azetidine-1-carboxylate (5). Compound **1** (0.31 g, 1 mmol) and benzyl chloroformate (0.15 mL, 1 mmol), were dissolved in anhydrous acetone (10 mL) and then K_2CO_3 (0.14 g, 1 mmol) was added with a similar procedure as that described for **3.** Product **5** (0.309 g, 69% yield) was isolated as a pale yellow oil; ^{1}H NMR ($CDCl_3$, 200 MHz): δ 0.09 (s, 3H), 0.10 (s, 3H), 0.87 (s, 9H), 1.23 (d, J = 6.6 Hz, 3H), 1.30 (t, J = 7 Hz, 3H), 4.19 (q, J = 7 Hz, 2H), 4.20 (dd, J = 4.4 Hz, J = 1.8 Hz, 1H), 4.66 (dq, J = 4.4 Hz, J = 6.6 Hz, 1H), 5.33 (s, 2H), 6.28 (d, J = 1.8 Hz, 1H), 7.35 (m, 5H). ^{13}C NMR ($CDCl_3$, 75.45 MHz): δ −5.1, −4.6, 14.3, 17.9, 20.2, 25.6, 60.3, 65.1, 65.4, 68.6, 99.1, 128.3, 128.7, 134.4, 147.3, 148.7, 163.6, 165.9. $[\alpha]_D^{25}$ = +65 (*c* 0.4, $CHCl_3$). IR (film): 2926, 1845, 1752, 1713, 1666 cm^{-1}. Anal. ($C_{23}H_{33}NO_6Si$) C, H, N.

Ethyl {(2*E*),(3*S*)-1-benzylcarbamoyl-3-[(1*R*)-1-(*tert*-butyldimethylsilanyl oxy)-ethyl]-4-oxo-azetidin-2-ylidene}-acetate (6). Compound **1** (0.31 g, 1 mmol) and benzyl isocyanate (0.14 mL, 1 mmol) were dissolved in anhydrous acetone (10 mL) and then K_2CO_3 (0.14 g, 1 mmol) was added, with a similar procedure as that described for **3**. Product **6** (0.268 g, 60% yield) was isolated as a pale yellow oil; ^{1}H NMR ($CDCl_3$, 200 MHz): δ 0.11 (s, 3H), 0.12 (s, 3H), 0.90 (s, 9H), 1.23 (d, J = 6.4 Hz, 3H), 1.30 (t, J = 7.0 Hz, 3H), 4.19 (q, J = 7.0 Hz, 2H), 4.24 (dd, J = 4.4 Hz, J = 1.6 Hz, 1H), 4.44 (dd, J = 5.8 Hz, J = 15.8 Hz, 1H), 4.52 (dd, J = 5.8 Hz, J = 15.8 Hz, 1H), 4.70 (dq, J = 4.4 Hz and J = 6.4 Hz), 1H), 6.44 (d, J = 1.6 Hz, 1H), 6.92 (t, J = 5.8 Hz, 1H), 7.35 (m, 5H). ^{13}C NMR ($CDCl_3$, 50.29 MHz): δ −5.0, −4.6, 14.3, 17.9, 19.9, 25.6, 43.7, 60.2, 64.8, 65.1, 98.6, 127.6, 127.7, 128.7, 137.0, 148.5,[37] 165.9, 167.7. $[\alpha]_D^{25}$ = +69 (*c* 1.5, $CHCl_3$). IR (film): 3377, 2933, 1819, 1739, 1666, 1534 cm^{-1}. GC–MS: R_t = 17.7 min; *m*/*e* 256 (M^+−148, 63), 210 (100), 184 (19), 168 (6), 143 (43), 115 (6), 99 (16), 75 (50), 59 (8). Anal. ($C_{23}H_{34}N_2O_5Si$) C, H, N.

Ethyl {(2*E*),(3*S*)-1-acetyl-3-[(1*R*)-1-(*tert*-butyldimethylsilanyloxy)-ethyl]-4-oxo-azetidin-2-ylidene}-acetate (7). Compound **1** (0.31 g, 1 mmol) and acetic anhydride (0.10 mL, 1 mmol) were dissolved in anhydrous acetone (10 mL). Then K_2CO_3 (0.14 g, 1 mmol) was added with a similar procedure as that described for **3**. Product **7** (0.284 g, 80% yield) was isolated as a pale yellow oil; ^{1}H NMR ($CDCl_3$, 200 MHz): δ −0.07 (s, 3H), 0.03 (s, 3H), 0.80 (s, 9H), 1.30 (t, J = 6.8 Hz, 3H), 1.40 (d, J = 6.4 Hz, 3H), 2.42 (s, 3H), 4.05 (dd, J = 1.4 Hz, J = 1.6 Hz, 1H), 4.20 (q, J = 6.8 Hz, 2H), 4.73 (dq, J = 6.4 Hz, J = 1.6 Hz, 1H), 6.52 (d, J = 1.4 Hz, 1H).^{13}C NMR ($CDCl_3$, 50.29 MHz): δ −5.5, −4.4, 14.4, 17.7, 22.0, 24.1, 25.5, 60.3, 65.0, 65.6, 99.6, 150.3, 165.7, 166.5, 166.7. $[\alpha]_D^{25}$ = −189 (*c* 1.2, $CHCl_3$). IR (film): 2959, 1832, 1726, 1660, cm^{-1}. GC–MS: R_t = 18.2 min; *m*/*e* 340 (M^+−15, 1), 298 (96), 256 (100), 228 (16), 184 (21), 103 (20), 75 (53), 59 (7). Anal. ($C_{17}H_{29}NO_5Si$) C, H, N.

Ethyl {(2*Z*),(3*S*)-1-benzyl-3-[(1*R*)-1-(*tert*-butyldimethylsilanyloxy)-ethyl]-4-oxo-azetidin-2-ylidene}-acetate (10). From compound **8** (0.31 g, 1 mmol) and benzyl bromide (0.12 mL, 1 mmol), a similar procedure as that described for **3**, gave product **10** (0.31 g, 77% yield) as a pale yellow oil; ^{1}H NMR ($CDCl_3$, 200 MHz): δ 0.03 (s, 3H), 0.07 (s, 3H), 0.87 (s, 9H), 1.21 (t, J = 7.0 Hz, 3H), 1.32 (d, J = 6.2 Hz, 3H), 3.59 (d, J = 5.8 Hz, 1H), 4.10 (q, J = 7.0 Hz, 2H), 4.10–4.22 (m, 1H), 5.04 (d, J = 15.8 Hz, 1H), 5.14 (d, J = 15.8 Hz, 1H), 5.20 (s, 1H), 7.20–7.40 (m, 5H). ^{13}C NMR ($CDCl_3$, 75.45 MHz): δ −4.8, −4.3, 14.3, 17.9, 22.3, 25.7, 46.6, 59.8, 64.1, 65.8, 92.2, 127.2, 127.7, 128.4, 136.8, 152.4, 165.0, 168.4. $[\alpha]_D^{25}$ = +21 (*c* 2.57, $CHCl_3$). IR (film): 2926, 1806, 1713, 1646 cm^{-1}.

GC–MS: $R_t = 23.4$ min *m/e* 346 ($M^+ - 57$, 47), 185 (10), 170 (3), 143 (7), 103 (6), 91 (100), 65 (6). Anal. ($C_{22}H_{33}NO_4Si$) C,H,N.

S-Phenyl-{(2Z), (3S)-3-[(1R)-1-(*tert*-butyldimethylsilanyloxy)-ethyl]-4-oxo-azetidin-2-ylidene}-thioacetate (11). To a solution of [3*R*(1′*R*,4*R*)4-acetoxy3-[1-(*t*butyldimethylsilyloxy) ethyl]-azetidin-2-one (0.959 g, 3.343 mmol) in CH_2Cl_2 (33 mL), Et_3N (0.64 mL, 4.346 mmol) and Me_3SiCl (0.465 mL, 3.677 mmol) were added at room temperature. At complete conversion of the starting material (GC monitoring) the solution was brought to 0 °C and 3.34 mL (3.34 mmol) of a 1 M solution of $TiCl_4$ in CH_2Cl_2 were added. After 1 min, a solution of 2 equiv of *S*-phenyl diazothioacetate[35] in 6 mL of CH_2Cl_2 was slowly added dropwise (over 30 min) at 0 °C. The reaction mixture was stirred at room temperature and the conversion monitored by TLC. After 4 h the reaction was quenched in ice cold water and extracted with CH_2Cl_2 (3 × 40 mL), dried on Na_2SO_4 and concentrated. The residue was purified by flash chromatography (cyclohexane–ethylacetate 95:5) to give product **11** (0.62 g, 50% yield) as a pale yellow solid; mp 129–131 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.11 (s, 3H), 0.12 (s, 3H), 0.92 (s, 9H), 1.36 (d, $J = 6.3$ Hz, 3H), 3.73 (d, $J = 5.4$ Hz, 1H), 4.27 (quintet, $J = 6.3$ Hz, 1H), 5.73 (s, 1H), 7.45–7.53 (m, 5H), 8.62 (bs, 1H). ^{13}C NMR ($CDCl_3$, 75.45 MHz): δ −5.0, −4.2, 17.9, 22.4, 25.7, 65.1, 65.4, 97.2, 127.7, 129.2, 129.5, 134.8, 151.4, 166.4, 187.5. $[\alpha]_D^{25} = -24$ (*c* 1, $CHCl_3$). IR (film): 3185, 1819, 1786, 1679, 1633 cm^{-1}. Anal. ($C_{19}H_{27}NO_3Si$) C,H,N.

Ethyl {(3S,2R)-3-[(1R)-1-(*tert*-butyldimethylsilanyloxy)-ethyl]-4-oxo-azetidin-2-yl}-acetate (19). In a double-necked round-bottom flask metallic Zn was warmed under vacuum and, after cooling, [3*R*(1′*R*,4*R*)4-acetoxy3-[1-(*t*butyldimethylsilyloxy) ethyl]-azetidin-2-one (0.861 g, 3 mmol) and THF (30 mL) were added. The solution was heated to reflux and 2-bromoethyl acetate (0.5 mL, 4.5 mmol) was added dropwise. The reaction mixture was stirred for 2.5 h and then quenched in a buffer solution (pH 7.5), the zinc salt was filtered on Celite and the solution was extracted with AcOEt (2 × 20 mL), dried on Na_2SO_4 and concentrated. The residue was purified by flash chromatography (cyclohexane-ethylacetate 7:3) to give product **19** (0.210 g, 23%) as a white crystalline solid; mp = 56–59 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.06 (s, 6H), 0.86 (s, 9H), 1.20 (d, $J = 6.3$ Hz, 3H), 1.26 (t, $J = 7.2$ Hz, 3H), 2.55 (dd, $J = 9.6$ Hz, $J = 16.2$ Hz, 1H), 2.72 (dd, $J = 3.9$ Hz, $J = 16.2$ Hz, 1H), 2.80 (dd, $J = 2.1$ Hz, $J = 5.1$ Hz, 1H), 3.95 (ddd, $J = 2.1$ Hz, $J = 3.9$ Hz, $J = 9.6$ Hz, 1H), 4.15 (q, $J = 7.2$ Hz, 2H), 4.18 (dq, $J = 5.1$ Hz, $J = 6.3$ Hz, 1H), 6.23 (bs, 1H). ^{13}C NMR ($CDCl_3$, 75.45 MHz): δ −5.3, −4.5, 13.9, 17.7, 22.2, 25.5, 39.5, 46.6, 60.6, 63.8, 65.2, 168.0, 170.9. $[\alpha]_D^{25} = +12$ (*c* 1.43, $CHCl_3$). IR (film): 3262, 2930,1763,1736 cm^{-1}. GC–MS: $R_t = 21.2$ min *m/e* 300 ($M^+ - 15$, 2), 258 (100), 214 (25), 170 (23), 115 (25), 75 (70). Anal. ($C_{15}H_{29}NO_4Si$) C, H, N.

Ethyl {(3S,2R)-3-[(1R)-1-(*tert*-butyldimethylsilanyloxy)-ethyl]-2-ethoxycarbonylmethyl-4-oxo-azetidin-1-yl}-3-oxo-propionate (20). Obtained from **19** (0.1 g, 0.32 mmol) and ethyl malonyl chloride (0.07 mL, 0.54 mmol), with a procedure similar to that described for **4**, product **20** (0.051 g, 37% yield) was isolated as a colorless oil; ^{1}H NMR ($CDCl_3$, 200 MHz): δ 0.05 (s, 3H), 0.08 (s, 3H), 0.85 (s, 9H), 1.21–1.32 (m, 9H), 2.76 (dd, $J = 15.4$ Hz, $J = 8.0$ Hz, 1H), 3.10–3.20 (m, 2H), 3.61 (d, J 4 Hz, 1H), 4.49 (m, 1H). ^{13}C NMR ($CDCl_3$, 75.45 MHz): δ −5.2, −4.3, 14.1, 14.0, 17.8, 22.0, 25.6, 36.4, 43.3, 49.3, 60.9, 61.5, 62.9, 65.0, 163.3, 166.0, 166.2, 169.8. $[\alpha]_D^{25} = -63$ (*c* 1.46, $CHCl_3$). IR (film): 2933, 1799, 1746, 1720 cm^{-1}. Anal. ($C_{20}H_{35}NO_7Si$) C, H, N.

Materials for biological assays

Elastase from human leukocytes and elastase substrate *N*-methoxysuccinyl-ala-ala-pro-val *p*-nitroanilide were purchased from Sigma.

Cytotoxicity test

NIH-3T3 murine fibroblasts were used for cytotoxicity tests. Cells (3 × 10^6) were seeded onto 75 cm^2 cell culture flasks and incubated at 37 °C in Dulbecco Modified Eagle's Medium supplemented with 10% fetal calf serum, with or without β-lactams 50 μM (> 10-fold the IC_{50} of compound **4**, the most active against LE). After 24 h, adherent cells were harvested with trypsin-EDTA (0.05–0.2%, Sigma), and added to those previously pelleted from suspension; following Trypan Blue (Sigma) vital staining, alive and dead cells were counted. In order to discriminate between possible cytotoxic and cytostatic effects, the values were subtracted from those from control experiments.

Substrate degradation by HLE

LE was solubilized (250 mU/mL) in Hepes buffer (0.1 M Hepes, 0.5 m NaCl, 10% DMSO, pH 7.8). All the compounds and elastase substrate were freshly prepared 20× in DMSO. Dilutions of the compounds were premixed with the enzyme in micrometers wells, and maintained 15 min at 4 °C. Then, 5 μL of the substrate (8 mM concentrated) were added to 100 μL final volume, and the mixture was incubated at 37 °C. At 20-min intervals, the intensity of the color developed by the digested substrate was measured at 405 nm using a Titertek Mutiskan (Flow Laboratories), and the control background subtracted (in triplicate experiments). The reactions developed linearly for as long as 120 min; data from 60 min were used for determining all IC_{50}s. Double-reciprocal plot of the results allowed the type and K_i of inhibition exerted over LE by compound **4** to be deduced.

Zymographic analysis

Aliquots of gelatinase-containing medium conditioned by human neuroblastoma cells (for MMP-2) or HT-1080 human fibrosarcoma cells (for MMP-9) were assayed as described.[5] Without heating the samples, zymography was performed by electrophoresing 15 μL

of medium in 0.1% gelatin-containing 8% polyacrylamide, in presence of SDS. After electrophoresis, the gels were washed twice for 15 min with 2.5% Triton X-100, incubated overnight at 37 °C in Tris buffer (50 mM Tris–HCl, 200 mM NaCl, 10 mM $CaCl_2$, pH 7.4). For gelatinase inhibition assays, compounds were freshly solubilized in DMSO and diluted in Tris buffer used for developing the zymogram. The gel slab was then cut into slices corresponding to the lanes and then put in different tanks containing the stated concentrations of inhibitors.

The gels then were stained for 30 min with 30% methanol/10% acetic acid containing 0.5% Coomassie Brilliant Blue R-250, and destained in the same solution without dye. Clear bands on the blue background represent areas of gelatinolysis. Digestion bands were quantitated using an image analyzer system with Gel-Doc 2000 and Quantity One software (Bio-Rad), and the densitometric values were expressed as percentage of control bands on the same gel.

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37. This signal is the sum of two carbons that have the same chemical shift. This evidence derives from an integrated ^{13}C spectrum recorded with a delay of 60 s and by gHMBC analyses.