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PAPER

Exploring the potential of the β-thiolactones in bioorganic chemistry†

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A series of novel peptide-based β-thiolactones were synthesized and assayed for cytotoxicity against several human cancer cell lines, where they showed greater activity than the corresponding β-lactones and β-lactams. Several of the β-thiolactones prepared showed strong inhibitory activity *in vitro* against human cathepsins B and L.

Introduction

In recent years the β-lactones (2-oxetanones) have come to be recognised as potent inhibitors of a number of biological processes with present applications and considerable future potential as therapeutic agents for several human disease states (Fig. 1).[1–3] For example, tetrahydrolipstatin (Orlistat), an inhibitor of pancreatic and gastric lipases, is a current treatment for obesity[4] that has recently attracted renewed attention by its ability to inhibit the thioesterase domain of fatty acid synthase thereby potentially paving the way for its use as an anti-tumor antibiotic.[5,6] Various other naturally occurring β-lactones,[7–13] their analogs,[14–16] and precursors[17,18] also exhibit strong inhibitory activity of the 20S proteasome, thereby opening a further avenue for their development as anti-cancer and even anti-malarial drugs. β-Lactones are also known to inhibit a variety of cysteine proteases[19,20] and by virtue of this activity have recently demonstrated potential as antibacterial agents.[21,22] Not surprisingly, in view of both their fascinating structure and reactivity and their evident potential as therapeutic agents, the chemistry of the β-lactones has been[2,23–32] and continues to be widely investigated.[33,34,35–41] However, the high reactivity of the β-lactones as both acylating and alkylating agents which provides the basis for their biological activity is not without its problems. Thus, β-lactones not surprisingly have relatively short half lives in aqueous media and under physiological conditions, with that of omuralide, for example, being 13 min at 37 °C and pH 8 in HEPES buffer.[42] Thus prompted, Hogan and Corey synthesized a β-lactam analog of salinosporamide A and omuralide with the hope that the greater longevity in water/serum would compensate for reduced reactivity resulting in an improved pharmacological profile.[43] In our laboratory, we have been interested in the chemistry of the β-thiolactones (2-oxothietanes)[44] and report here on exploratory studies, based on their unique reactivity profile, aimed at their development as β-lactone surrogates.

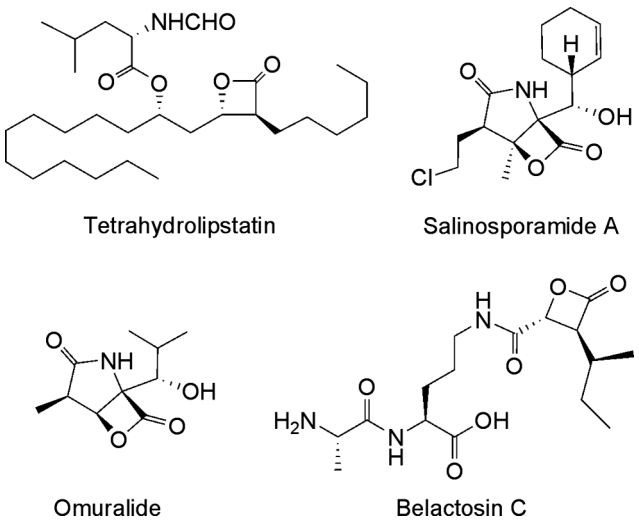

Fig. 1 Salinosporamide A, omuralide, tetrahydrolipstatin and belactosin C.

β-Lactones are planar species both in the crystal and in the gas phase,[24,45,46] whereas β-thiolactones are puckered reflecting the longer C–S bonds and the reduced resonance delocalization of the sulfur lone pairs onto the carbonyl system.[47–50] An X-ray crystal structure of β-thiolactone **1** (Fig. 2), revealing an acute angle of 77.6° for C2–S1–C4, and a puckered conformation of the four-membered ring matching confirms these observations. The IR spectra of β-lactones and β-thiolactones differ more than those of the corresponding esters and thioesters in that the β-lactones typically exhibit carbonyl absorption bands in the region 1810–1840 cm^{-1} while the β-thiolactones absorb at 1740–1750 cm^{-1},

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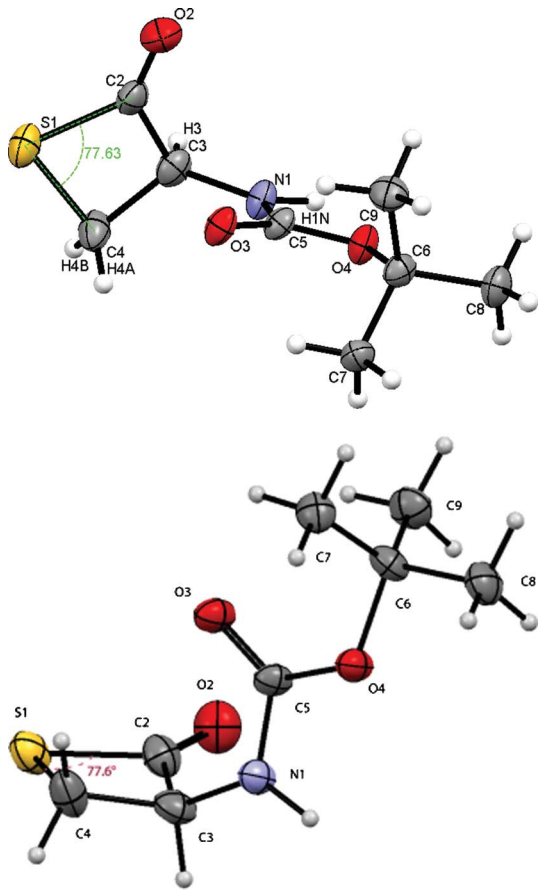


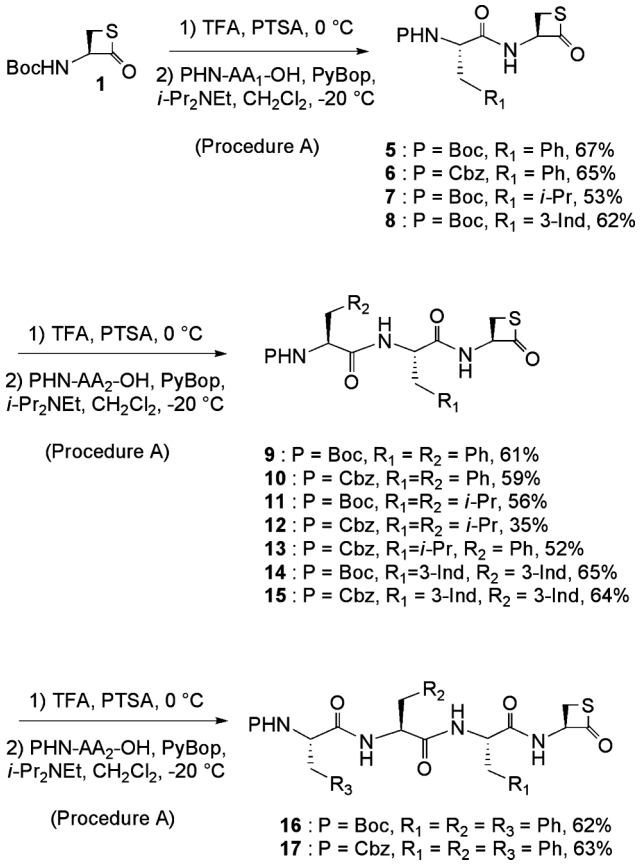
Fig. 2 Two views of the X-ray crystal structure of β-thiolactone **1**.

indicative of reduced strain in the latter. The different structural and spectroscopic characteristics of the β-lactones and β-thiolactones are reflected in their differing reactivities with the former undergoing S_N2-type ring opening at the β-position with a broad spectrum of soft nucleophiles[20,24,30,31] including amines, while the latter are more prone to attack at the carbonyl carbon.[44,51] These differences in reactivity, and the generally lower tendency of the thiolactones toward hydrolysis in our hands, suggested that β-thiolactone analogs of β-lactones would have generally greater persistence in aqueous media than the β-lactones but would retain much of the acylating capabilities of the latter toward biological targets perhaps providing a useful compromise in stability and reactivity intermediate between the β-lactones and the β-lactams. To our knowledge a β-thiolactone analog of omuralide is presently the only member of the β-thiolactone family claimed to have displayed any kind of biological activity.[52]

Synthesis

In this initial survey of the potential of the β-thiolactones in bioorganic chemistry, which draws inspiration from the work of Overkleeft[53–55] and others[56] on peptide-based proteasome and enzyme inhibitors and from the belactosins,[10] we targeted a series of simple mono-, di- and tripeptidyl systems carrying β-thiolactones as well as their lactam and lactone analogs for synthesis.

The synthesis of the target compounds started from known heterocyclic structures: β-thiolactones (**1** and **2**),[44,53,57] β-lactone (**3**)[57,58] and β-lactam (**4**).[59,60] To this end the Boc group of β-thiolactone (**1**) was cleaved in neat TFA at 0 °C in the presence of PTSA and the resulting amine used directly in a sequence of peptide coupling reactions (procedure A) with suitable *N*-protected amino acids and PyBop® as reagent in the presence of *i*-Pr_2NEt at –20 °C (Scheme 1, procedure A). Further elongation of the so-obtained peptides was then accomplished by iterative *N*-Boc deprotection and coupling reactions in good yields giving a series of tri- and tetrapeptide-based β-thiolactones (Scheme 1).



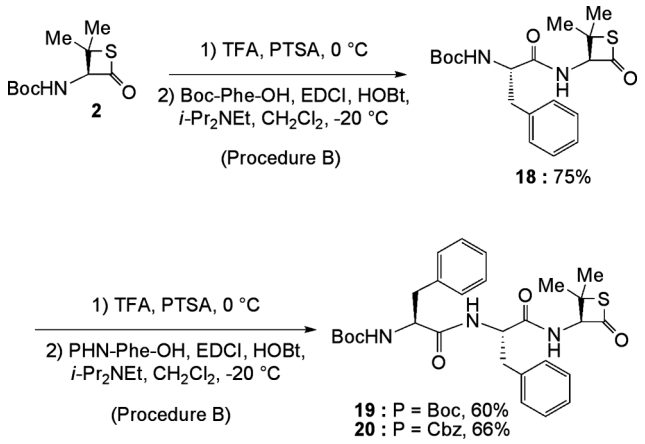
Scheme 1 Synthesis of peptidyl β-thiolactones **5–17**.

An analogous reaction sequence was applied to the penicillanic acid derived β-thiolactone (**2**) except that a carbodiimide-based coupling was the method of choice for the formation of the peptide bonds (Scheme 2, procedure B).

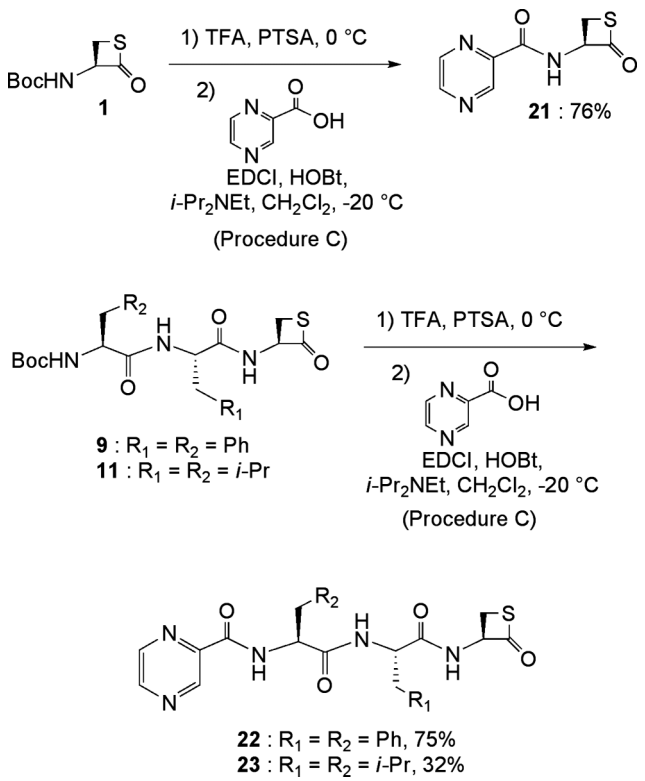
Variations on these protocols were then applied to prepare a series of related peptide-based β-thiolactones (**21–25**) bearing the polar pyrazine-2-carboxamide at the N-terminal position. These systems were accessed either from pyrazine-2-carboxylic acid at –20 °C (Scheme 3) with EDCI/HOBt (procedure C), or from pyrazine-2-carbonyl-L-phenylalanine[53] at –30 °C with PyBop® (Scheme 4, procedure A).

In view of the widespread use in medicinal chemistry of morpholinoacetamides, and particularly in the clinical candidate for multiple myeloma Carfilzomib which functions by irreversible proteasome inhibition,[61–63] the derivative **26** was obtained by deprotection of **9** under acidic conditions and acylation of the resulting amine with 2-morpholinoacetyl chloride (Scheme 5).

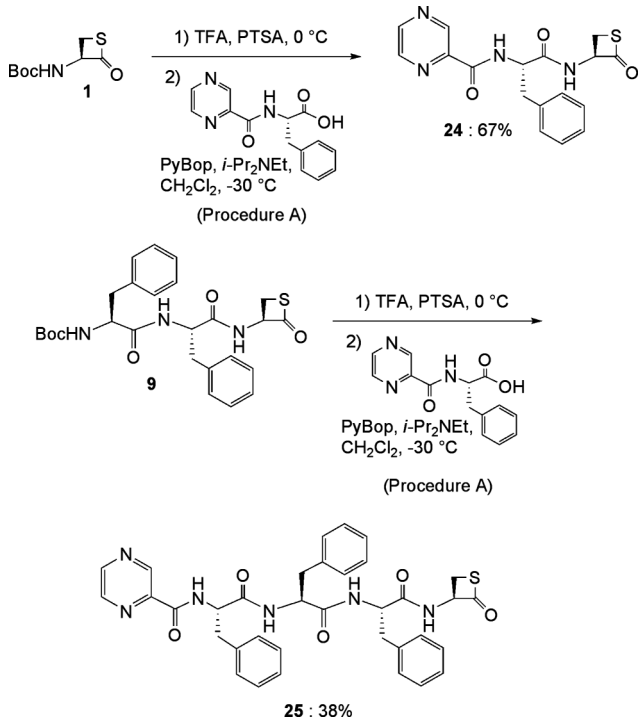
In this manner the preparation of a library of 22 compounds bearing a β-thiolactone subunit at the *C*-terminal position was



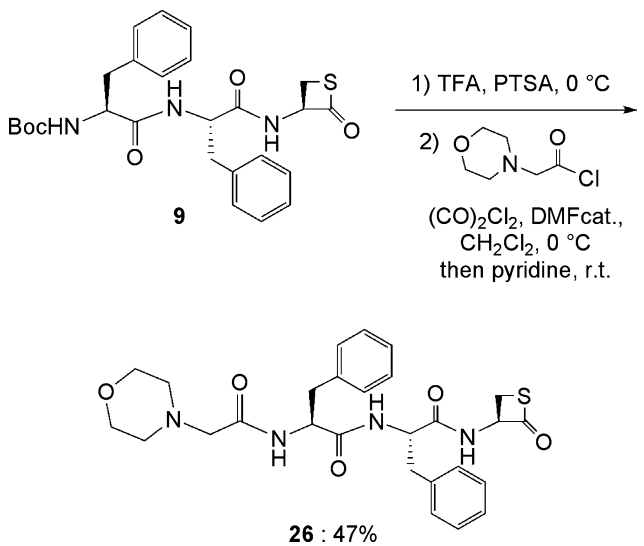
Scheme 2 Synthesis of peptidyl β-thiolactones **18**–**20**.



Scheme 3 Preparation of pyrazine-2-carboxamido terminated peptidyl β-thiolactones **21**–**23**.



Scheme 4 Preparation of pyrazine-2-carboxamido terminated peptidyl β-thiolactones **24** and **25**.

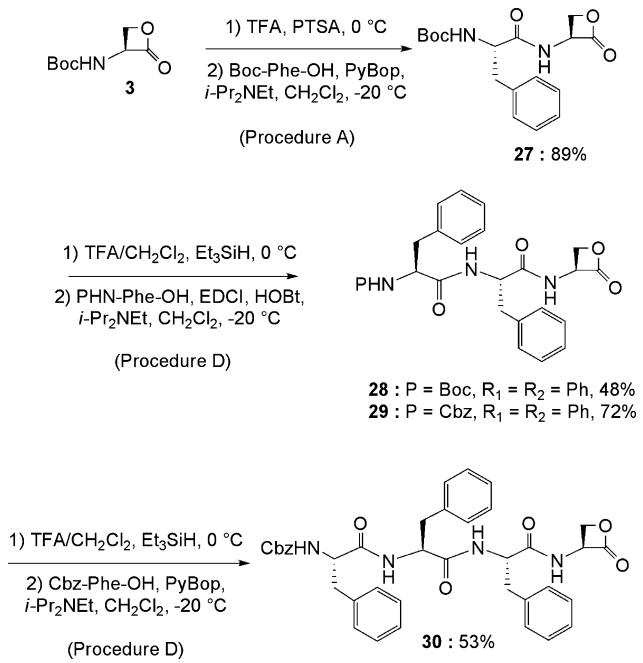


Scheme 5 Synthesis of the 2-morpholinoacetyl terminated β-thiolactones **26**.

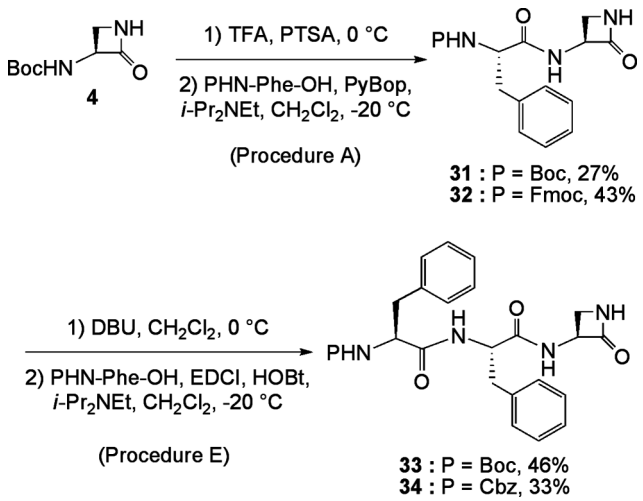
achieved. This library (**5**–**26**) contained various modifications in the amino acid side chains (phenylalanine, tryptophan or leucine) at the N-terminus (Boc, Cbz, pyrazin-2-yl or 2-morpholinoacetyl) and in the nature of the β-thiolactone amino acid precursor (L-cysteine or L-penicillamine). For purposes of comparison a number of analogous peptidyl β-lactones were prepared by a similar approach. Thus, removal of the *N*-Boc group from **3** was accomplished using a TFA/Et_3SiH mixture at 0 °C (Scheme 6, Procedure D). Peptide coupling was then achieved with EDCI/HOBt to give peptides **27**–**30** in good yields (Scheme 6).

Similarly, a number of analogous β-lactams were required. To this end deprotection of **4** with TFA followed by PyBop-mediated peptide bond formation (procedure A) gave rise to the formation of **31** and **32** in 27 and 43% yield, respectively. Interestingly, and in contrast to the β-thiolactone and β-lactone series, subsequent application of acidic deprotection conditions to **31**, followed by peptide coupling, was not effective for the synthesis of the homologous compounds **33** and **34**. However, treatment of the *N*-Fmoc protected dipeptide **32** with DBU (procedure E) followed by standard peptide coupling was successful and gave rise to **33** and **34** in 46% and 33% yield, respectively (Scheme 7).

A close analog of the β-lactone-containing 20S proteasome inhibitor Belactosin C,[8,10] was prepared (Scheme 8) from a known mono ester of (3*R*)-3-hydroxysuccinic acid[64] by esterification with

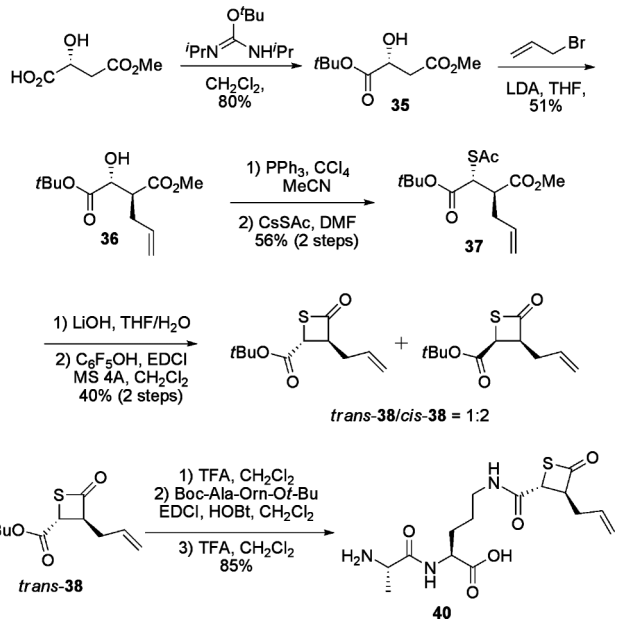


Scheme 6 Preparation of β-lactones **27–30**.



Scheme 7 Synthesis of β-lactams **31–34**.

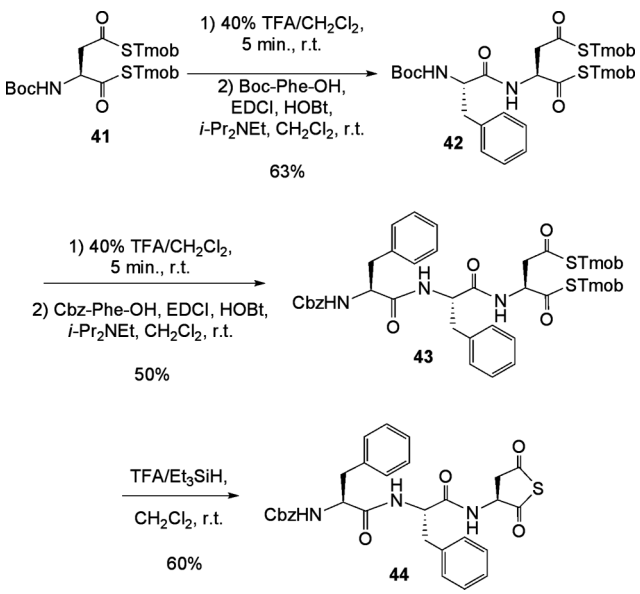
O-*tert*-butyl *N*,*N*-diisopropylisourea[65] to give the corresponding *tert*-butyl ester **35** in 80% yield (Scheme 8). Introduction of an allyl-group by *C*-alkylation of the enolate generated with LDA at –78 °C from **35** then gave the adduct **36** as a single diastereoisomer in 51% yield.[66] Substitution of the alcohol function by a C–S bond with overall retention of configuration was achieved by a double inversion protocol involving an initial chlorination step with the PPh_3/CCl_4 combination,[67] followed by displacement with CsSAc in DMF to give **37** in 56% yield over two steps.[67,68] Saponification of the methyl ester with LiOH and subsequent cyclization *via* the intermediacy of a pentafluorophenyl ester obtained with EDCI and C_6F_5OH resulted in the formation of the corresponding β-thiolactone **38** in 40% yield over two steps as a separable mixture of stereoisomers with a *trans*/*cis* ratio of 1 : 2. The loss of stereochemical integrity in the formation of this β-thiolactone reflects the ease of epimerization of α-mercapto esters and almost



Scheme 8 Synthesis of a β-thiolactone analogue of belactosin C.

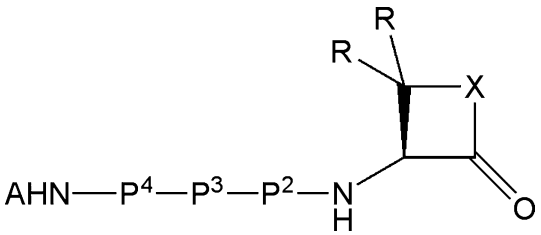
likely intervenes at the level of the saponification step.[68] Cleavage of the *tert*-butyl ester from *trans*-**38** was followed by peptide coupling with Boc-Ala-Orn-*O*-*t*Bu to give the dipeptide **39**, from which removal of both the *t*-butyl ester and the *N*-Boc moiety was achieved with TFA to give the β-thiolactone analogue **40** of Belactosin C in 85% yield over three steps (Scheme 8).

Finally, on the basis of earlier work from our laboratory on the chemistry of cyclic monothioanhydrides,[69,70] it was also of interest to investigate such a system. Thus, starting from the known bis(thioester) Boc-Asp(STmob)-STmob **41** (Tmob = 2,4,6-trimethoxybenzyl),[71] preparation of the tripeptide Cbz-Phe-Phe-Asp(STmob)-STmob **43** was achieved in 32% yield over 4 steps using classical peptide chain elongation methods (Scheme 9). Treatment of **43** with a TFA/Et_3SiH mixture at room



Scheme 9 Synthesis of a peptidyl monothioaspartic anhydride.

Table 1 Antiproliferative activity of some β-thiolactone derivatives of KB cancer cell line *in vitro*. All amino acids have the L-configuration



Entry	Cmpd	A	P^4	P^3	P^2	X	*R*	10 μM[a]	1 μM[a]
1	**1**	Boc-	—	—	—	S	H	87	0
2	**5**	Boc	—	—	Phe	S	H	100	0
3	**6**	Cbz	—	—	Phe	S	H	100	43
4	**7**	Boc	—	—	Leu	S	H	82	0
5	**8**	Boc	—	—	Trp	S	H	100	14
6	**9**	Boc	—	Phe	Phe	S	H	100	84
7	**10**	Cbz	—	Phe	Phe	S	H	100	92
8	**11**	Boc	—	Leu	Leu	S	H	100	0
9	**12**	Cbz	—	Leu	Leu	S	H	99	4
10	**13**	Cbz	—	Leu	Phe	S	H	97	92
11	**14**	Boc	—	Trp	Trp	S	H	100	0
12	**15**	Cbz	—	Trp	Trp	S	H	77	4
13	**16**	Boc	Phe	Phe	Phe	S	H	82	75
14	**17**	Cbz	Phe	Phe	Phe	S	H	94	85
15	**2**	Boc	—	—	Phe	S	Me	29	18
16	**18**	Boc	—	—	Phe	S	Me	24	0
17	**19**	Boc	—	Phe	Phe	S	Me	22	2
18	**20**	Cbz	—	Phe	Phe	S	Me	28	12
19	**21**	Pyrazin-2-yl	—	—	—	S	H	12	0
20	**22**	Pyrazin-2-yl	—	Phe	Phe	S	H	100	4
21	**23**	Pyrazin-2-yl	—	Leu	Leu	S	H	0	0
22	**24**	Pyrazin-2-yl	—	—	Phe	S	H	100	4
23	**25**	Pyrazin-2-yl	Phe	Phe	Phe	S	H	100	16
24	**26**	2-Morpholinoacetyl	—	Phe	Phe	S	H	15	0
25	**3**	Boc	—	—	—	O	H	33	15
26	**27**	Boc	—	—	Phe	O	H	3	0
27	**28**	Boc	—	Phe	Phe	O	H	43	3
28	**29**	Cbz	—	Phe	Phe	O	H	68	0
29	**30**	Cbz	Phe	Phe	Phe	O	H	0	0
30	**4**	Boc	—	—	—	NH	H	8	0
31	**31**	Boc	—	—	Phe	NH	H	0	0
32	**33**	Boc	—	Phe	Phe	NH	H	0	0
33	**34**	Cbz	—	Phe	Phe	NH	H	0	0

[a] Average of the triplicate at concentration 10 or 1 μM.

temperature then afforded the monothioanhydride **44** in 60% isolated yield.[71]

Biology

Twelve mono-, di- and tripeptidyl β-thiolactones (**1**, **5–15**) were first assessed for inhibition of KB cell growth *in vitro* at concentrations of 10 and 1 μM (Table 1, entries 1–12) from which a difference of activity was observed between the different series carrying the phenylalanyl, tryptophanyl side chains. Thus, those β-thiolactones coupled to either Phe or Phe–Phe (Table 1, entries 3, 6, and 7) were active at a concentration of 10 μM and retained most of that activity at 1 μM. On the other hand, β-thiolactones coupled to either leucine (Table 1, entries 4, 8–10) or tryptophan (Table 1, entries 5, 11 and 12), while active at 10 μM were devoid of activity at the higher dilution. In view of these results, we investigated the tetrapeptides **16** and **17**, each carrying three phenylalanine units. Compounds **16** and **17** with the N-terminal Boc and Cbz groups strongly inhibit KB cell proliferation at a concentration of 1 μM (Table 1, entries 13 and 14).

The β,β-dimethyl-substited β-thiolactones (Table 1, entries 15–18) were significantly less potent inhibitors of KB cell growth than their unsubstituted analogs (Table 1, entries 1, 2, 6 and 7). This loss of activity on substitution at the β-position of the thiolactone could simply be indicative of a steric clash with the undetermined target enzyme or protein or may suggest a mechanism of inactivation involving alkylation rather than acylation (Fig. 3).

The influence of polar functionality at the N-terminus was also screened in the form of the pyrazin-2-yl (**21–25**) or morpholinoacetyl groups (**26**) (Table 1, entries 19–24). In the pyrazin-2-carboxamido series better activity again was seen with compounds based on phenylalanine at 10 μM (Table 1, entries 20, 22 and 23)

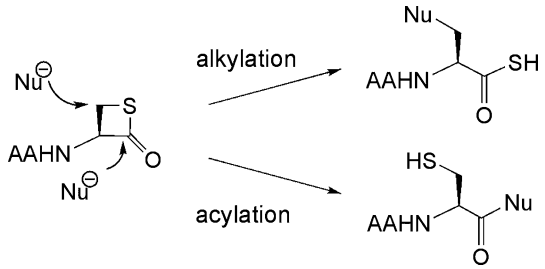


Fig. 3 Two modes of nucleophile capture by β-thiolactones.

than on leucine (Table 1, entry 21) but no such compounds were active at the lower concentration of 1 μM. The morpholinoacetyl derivative **26** (Table 1, entry 24) was inactive at all concentrations tested.

For comparison purposes five β-lactones (**3**, **27–30**) and four β-lactams (**4**, **31**, **33**, **34**) were also screened for their ability to inhibit KB cell growth. As shown in Table 1 (entries 25–33), the β-lactones displayed only modest inhibition at 10 μM, while the β-lactams were devoid of activity at the same concentration despite the presence of one or more phenylalaninyl residues in all but the simplest members of these two classes of compound. The five-membered ring monothioanhydride **38** proved to be ineffective toward the inhibition of the KB cell line.

Overall, this preliminary *in vitro* screen for the inhibition of KB cell growth showed that tripeptidyl (**9–10**) and tetrapeptidyl β-thiolactones (**16–17**) displaying multiple phenylalanyl residues and a Cbz or Boc group at the N-terminus were the most active compounds. This screen also clearly demarks the increased inhibitory activity of the β-thiolactones as compared to the β-lactones and β-lactams when carried by the identical peptide chains.

Following this initial screening the IC_{50} values (Table 2, entries 1–4) of the most active compounds (**9–10** and **16–17**) were determined for four human cancer cell lines (KB nasopharynx human carcinoma, MDA231 human breast adenocarcinoma, HCT116 colorectal carcinoma and PC3 prostate carcinoma). The tetrapeptides **16** and **17** with their three phenylalanyl residues and the N-terminal Boc or Cbz group at the N-terminus were the most active compounds against this range of cell lines with an IC_{50} in the range of 69 to 200 nM for the HCT116 cell line. Compound **17** was also screened for activity against leukemic HL60 cell lines, where it displayed a comparable IC_{50} value of 200 nM. For all cell lines assayed activities were time dependent, typically requiring 48 to 72 h to achieve full potency.

Table 2 IC_{50} values of compounds **9**, **10**, **16**, **17**, and **40** determined on KB, MDA231, HCT116 and PC3 cancer cell lines *in vitro*

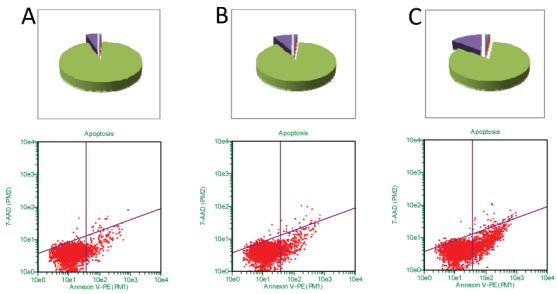
		IC_{50} (μM)[a]			
Entry	Compound	KB	MDA231	HCT116	PC3
1	**9**	0.51	0.98	0.44	1.7
2	**10**	0.54	0.90	0.59	2.8
3	**16**	0.18	0.14	0.20	0.81
4	**17**	0.078	0.072	0.069	0.53
5	**40**	>100	>100	>100	>100

[a] Average of the duplicate.

The belactosin C analog **40** showed a low IC_{50} of >100 μM for cytotoxicity of the same cell lines (Table 2, entry 5) in accordance with the reported values of 51 μM and 200 μM for belactosins A and C on HeLa S3 cells, respectively.[8]

In an attempt to elucidate the activity of compound **17**, necrosis was assayed through the release of LDH[72] from HL60 cells. However, after 24 and 48 h of exposure to **17** at concentrations ranging 0.2 to 5 μM, no significant LDH activity was detectable in the culture medium, indicating that HL60 cells do not enter the necrotic pathway.

Early and late apoptosis was assayed with annexin V-PE and 7-AAD by FACS analysis after 24 and 48 h exposure of HL60 cells to **17**.[73,74] No effect was observed after 24 h and only concentrations of **17** higher than 1 μM elicited a modest increase of the percentage of cells in early apoptosis at 48 h (Fig. 4). This was confirmed by a direct measurement of activation of caspases 3 and 7 activation, which showed no activation after 24 and 48 h. DNA fragmentation, a major event occurring during apoptosis,[75] was monitored by a FACS TUNEL analysis using an anti-BrdU antibody[76] of cells treated with increasing concentrations of **17** but a very minor increase of the fragmented DNA level was noticed.

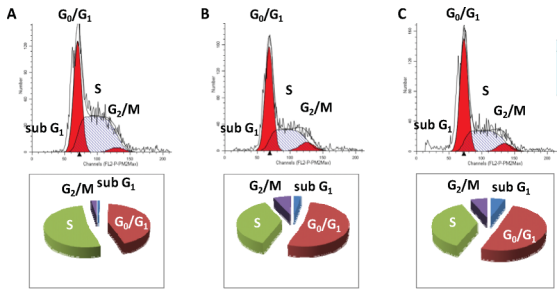


HL60 cells were incubated for 48 h with compound **17** or DMSO, before the addition of 7-AAD and annexin V-PE. Intact (green), early apoptotic (purple), late apoptotic (blue) and dead cells (red) were estimated as the percentage of total events monitored by FACS analysis. HL60 cells treated with vehicle only (A), 1 μM **17** (B) or 5 μM **17** (C).

Fig. 4 FACS analysis of apoptosis in HL60 cells treated with compound **17**.

In cancer cell lines, mitochondrial membranes are hyperpolarized and prevent the traffic of proapoptotic proteins from the mitochondria to the cytoplasm where they can activate the apoptotic cascade.[77] Treatment with compounds known to activate apoptosis is frequently associated with a decrease in the mitochondrial *trans* membrane potential ($\Delta\Psi m$) and can be estimated by FACS with dyes like JC-1 or JC-10, which accumulate as orange aggregates in intact mitochondria but which show green fluorescence in cells with a lower mitochondrial membrane potential.[78] When HL60 cells were treated with compound **17** for 24 and 48 h and the fluorescence of the cell populations was compared to that of untreated cells (treated with DMSO only) no modification of the green/orange fluorescence ratio was observed, indicating that the mitochondrial membrane potential remained unchanged and that efflux of proapoptotic protein was not enhanced after treatment with increasing concentrations of **17**. Overall, it appears that **17** does not derive its activity from the apoptosis pathway.

Lastly, we investigated the impact of treatment with compound **17** on cell cycle. KB and HL60 cells were treated during 24 and 48 h with increasing doses of **17** and the cell cycle was examined by FACS with PI as marker (Fig. 5). In both cell lines, the partition of cells remained fairly stable, suggesting that **17** did not significantly affect the cell cycle and did not promote any blockage in the cell division.



HL60 cells were incubated for 48 h with compound **17** or DMSO, before the addition of PI. Results estimate the percentage of cells monitored by FACS analysis. HL60 cells treated with vehicle only (A), 1 μM **17** (B) or 5 μM **17** (C).

Fig. 5 Cell cycle analysis of HL60 cells treated with compound **17**.

The cytotoxic compounds **9**, **10** and **17** were also evaluated for their activity against the chymotrypsin and trypsin-like activity of the 20S proteasome, but all showed only marginal potency of inhibition. Likewise, compound **40** was found to be only a very poor inhibitor of the chymotrypsin and trypsin-like activity of the HL60 cell line 20S proteasome *in vitro* in comparison to the known inhibitor MG-132.[79]

The beneficial effect of multiple phenylalanyl residues and a consideration of the anticipated reactivity of the β-thiolactone moiety focused our attention on the cysteine proteases as potential targets and in particular on the cathepsins B and L. These enzymes are involved in several disease states,[79–81] and are currently important targets of drug discovery programs.[82–85] Thus, several reports have disclosed the importance of the phenylalanyl residue in the P2 position of cathepsin inhibitors as well as of Boc or Cbz groups at the S2 subsite for specificity toward papain-like cysteine proteases.[56,86–89] Cysteine proteases are also known for their affinity towards electrophilic groups such as β-lactones[20,90–93] and β-lactams[94–96] through covalent interaction with their highly nucleophilic active active site thiol. Thus, focusing on structures having phenylalanyl and tryptophanyl residues, we assessed the inhibitory activities of 29 compounds (**1–6**, **8–10**, and **14–22**, **24–31**, **33**, **34**, and **40**) against cathepsins B and L according to a known procedure by using Cbz-Phe-Arg-AMC as specific substrate and E64 as control (Fig. 6).[97]

From this cathepsin inhibition assay, several features emerged (Table 3). No monopeptides, irrespective of structure, showed any activity against either cathepsin. Most of the cysteine-derived di, tri, and tetrapeptidyl β-thiolactones (**5**, **6**, **8–10**, **14–16**, **22**, **25**) inhibited both cathepsins B and L to some varying extent (Table 3, entries 7–9, 14, 15, 19, 20, 22, 26, and 28). None of the β-lactams (**31**, **33–34**) tested inhibited either enzyme (Table 3, entries 13, 18 and 25). For the β-lactones (**27–30**), only cathepsin L was inhibited at the 1 μM concentration of the assay (Table 3, entries 12, 24, and

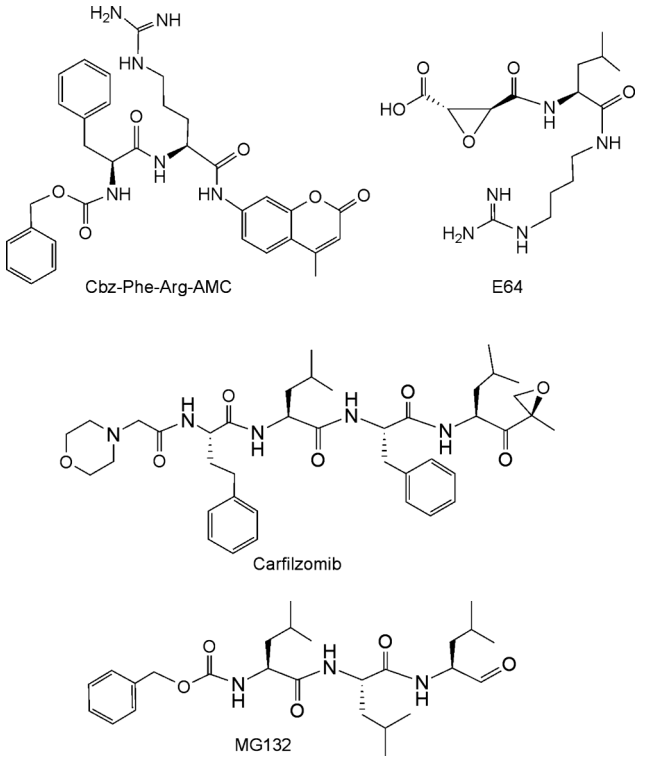


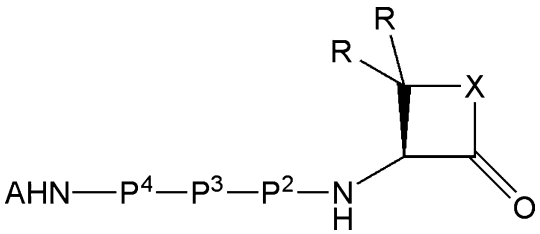
Fig. 6 Structures of Cbz-Phe-Arg-AMC, E64, Carfilzomib, and MG-132.

29), with the exception of tripeptide **28** which also partially inhibits cathepsin B (Table 3, entry 17). The inhibitory activity of the four β-lactones against cathepsin L increased with the length of the peptide chain with the, tetrapeptide **30** showing the best activity (Table 3, entry 29). The β-lactones **29** and **30** displayed a similar cathepsin L inhibition profile to the β-thiolactones **10**, **15–17**, **22**, **25**.

Among the active β-thiolactones, the tripeptidyl systems with a Cbz or pyrazin-2-yl group at the N-terminus (**10**, **15**, **22**, **29**) or the tetrapeptidyl series (**16**, **17**, **25**, **30**) were generally the most potent inhibitors. The tetrapeptide **16** with its β-thiolactone and Boc group displayed the best activity against cathepsin L with 68.2% of inhibition at the 1 μM of the test (Table 3, entry 26), while the most potent inhibitor of cathepsin B at the same concentration (Table 3, entry 19) was the tripeptide **10** retaining the two phenylalanyl residues and a Cbz group at the N-terminus. Interestingly, the penicillanic acid-derived β-thiolactones (**18–20**), with their *gem*-dimethyl groups, showed activity towards cathepsin L (Table 3, entries 10, 16 and 21), with **20** having a similar level of activity to the most potent compounds, but were inactive toward cathepsin B. As with the inhibition of KB cell growth this may simply indicate a steric clash in the case of cathepsin B. Alternatively, it can be construed as signalling a change of mechanism between the two cathepsins by the β-thiolactones. Thus, potentially cathepsin B is inhibited by an alkylation process by the cysteine-derived β-thiolactones, while cathepsin L is captured by an acylation mechanism (Fig. 2) by both the cysteine- and penicillanic acid-derived series.

Modification of the N-terminal position by a 2-morpholinoacetyl group as in the case of **26** was accompanied by a loss of cathepsin inhibitory activity (Table 3, entry 23).

Table 3 Inhibitory activity of compounds **1–6**, **8–10**, **14–22**, **24–31**, **33**, **34**, and **40** toward human recombinant cathepsins B and L



Entry	Cmpd	A	P^4	P^3	P^2	X	*R*	Cathepsin B% inhibition[a]	Cathepsin L% inhibition[a]
1	**E64**	—	—	—	—	—	—	97.3	70.2
2	**1**	Boc	—	—	—	S	H	—	—
3	**2**	Boc	—	—	Phe	S	Me	—	—
4	**21**	—	—	—	—	S	H	—	—
5	**3**	Boc	—	—	—	O	H	—	—
6	**4**	Boc	—	—	—	NH	H	—	—
7	**5**	Boc	—	—	Phe	S	H	52.0[b]	28.1[b]
8	**6**	Cbz	—	—	Phe	S	H	50.1[b]	45.9[b]
9	**8**	Boc	—	—	Trp	S	H	53.6[b]	21.4[b]
10	**18**	Boc	—	—	Phe	S	Me	—	32.6[b]
11	**24**	Pyrazin-2-yl	—	—	Phe	S	H	16.6[b]	—
12	**27**	Boc	—	—	Phe	O	H	—	29.4
13	**31**	Boc	—	—	Phe	NH	H	—	—
14	**9**	Boc	—	Phe	Phe	S	H	42.0[b]	48.9[b]
15	**14**	Boc	—	Trp	Trp	S	H	58.7[b]	49.3[b]
16	**19**	Boc	—	Phe	Phe	S	Me	_	48.9
17	**28**	Boc	—	Phe	Phe	O	H	38.3	35.7
18	**33**	Boc	—	Phe	Phe	NH	H	—	—
19	**10**	Cbz	—	Phe	Phe	S	H	42.2	40.2
20	**15**	Cbz	—	Trp	Trp	S	H	25.5	40.9
21	**20**	Cbz	—	Phe	Phe	S	Me	—	54.4
22	**22**	Pyrazin-2-yl	—	Phe	Phe	S	H	19.0	53.7
23	**26**	2-Morpholinoacetyl	—	Phe	Phe	S	H	—	—
24	**29**	Cbz	—	Phe	Phe	O	H	—	40.4
25	**34**	Cbz	—	Phe	Phe	NH	H	—	—
26	**16**	Boc	Phe	Phe	Phe	S	H	33.9	68.2
27	**17**	Cbz	Phe	Phe	Phe	S	H	—	49.7
28	**25**	Pyrazin-2-yl	Phe	Phe	Phe	S	H	23.8	41.7
29	**30**	Cbz	Phe	Phe	Phe	O	H	—	55.7
30	**40**	NH_2	—	Ala	Orn	S	H	23.9	—

[a] % Inhibition cathepsin at 1 μM (unless otherwise specified) with Cbz-Phe-Arg-AMC as specific substrate. [b] % Inhibition cathepsin at 3.16 μM

The β-thiolactone analogue **40** of belactosin C showed only moderate inhibitory activity toward cathepsin B no doubt due to the inappropriate nature of its backbone for recognition by these enzymes (Table 3, entry 30). Overall, the β-thiolactones showed better activity against both the cathepsins B and L than the comparable β-lactones, which, with one exception, were indifferent to cathepsin B. Analogous β-lactams were essentially inactive against both cathepsisn screened.

Encouraged by these results, we turned to the glioblastoma cell line U87 that, compared to other human cancer cell lines, possesses high cathepsin B and L activities when assayed *in vitro* with specific substrates (cellular cathepsine cellular with RR-AMC as substrate: U87, $3.065 \pm 0.325 \times 10^9$ Fluorescence units/30 min/50,000 cells; KB, 0.519 ± 0.053). When the cytotoxic activity of compound **17** was evaluated in both U87 and KB cell lines, much higher cytotoxicity was observed in KB cells than in U87 cells which were only poorly affected by this compound. This suggests that, in spite of their inhibitory activity towards cathepsin B and L, cathepsin inactivation is unlikely to be implicated in the cell death process provoked by exposure to compound **17**.

Conclusions

Peptide-based β-thiolactones are a group of compounds that are readily prepared and which show interesting time dependent cytotoxicity against a range of human cancer cell lines. Several peptide-based β-thiolactones also showed good inhibitory activity *in vitro* against human cathepsins B and L, members of the cysteine protease family, that was again distinct from the activity of the analogous β-lactones and β-lactams. A variety of tests were performed that appear to exclude necrosis, apoptosis, cell cycle arrest, proteasome inhibition, and even cathepsin inhibition as the root cause of the cytoxicity of the assayed β-thiolactones. Although determination of the precise cytoxic event will require further work, it is clear that the β-thiolactones represent a novel and exciting class of compounds worthy of further investigation in bioorganic and medicinal chemistry.

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