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Synthesis, evaluation and structural studies of antiproliferative tubulin-targeting azetidin-2-ones

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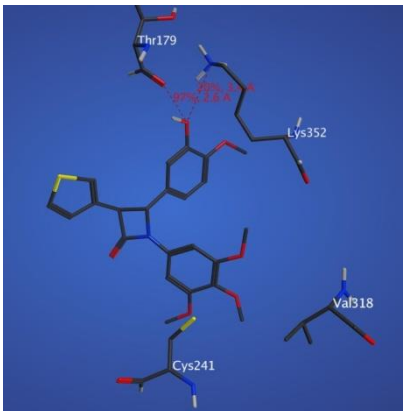
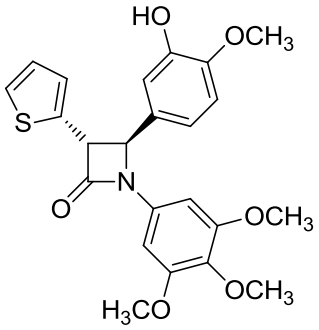
Graphical Abstract:





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Abstract: A series of azetidin-2-ones substituted at positions 2, 3 and 4 of the azetidinone ring scaffold were synthesised and evaluated for antiproliferative, cytotoxic and tubulin binding activity. In these compounds, the *cis* double bond of the vascular targeting agent combretastatin A-4 is replaced with the azetidinone ring in order to enhance the antiproliferative effects displayed by combretastatin A-4 and prevent the *cis*/*trans* isomerization that is associated with inactivation of combretastatin A-4. The series of azetidinones was synthetically accessible via the Staudinger and Reformatsky reactions. Of a diverse range of heterocyclic derivatives, 3-(2-thienyl) analogue **28** and 3-(3-thienyl) analogue **29** displayed the highest potency in human MCF-7 breast cancer cells with IC_{50} values of 7nM and 10nM respectively, comparable to combretastatin A-4. Compounds from this series also exhibited potent activity in MDA-MB-231 breast cancer cells and in the NCI60 cell line panel. No significant toxicity was observed in normal murine breast epithelial cells. The presence of larger, bulkier groups at the 3-position, for example 3-naphthyl derivative **21** and 3-benzothienyl derivative **26**, resulted in relatively lower antiproliferative activity in the micromolar range. Tubulin-binding studies of **28** (IC_{50}=1.37μM) confirmed that the molecular target of this series of compounds is tubulin. These novel 3-(thienyl) β-lactam antiproliferative agents are useful scaffolds for the development of tubulin-targeting drugs.

Key words: Combretastatin A-4 analogues, colchicine, β-lactam, azetidinone, antiproliferative, cytotoxicity, tubulin, structure-activity, Staudinger reaction, Reformatsky reaction.

Abbreviations

BBB	Blood-brain barrier
CA-4	Combretastatin A-4
CA-4P	Combretastatin A-4 phosphate
DAMA-colchicine	*N*-Deacetyl-*N*-(2-mercaptoacetyl)-colchicine
DCM	Dichloromethane
GTP	Guanidine triphosphate
HRMS	High Resolution Molecular Ion Determination
IR	Infra Red
LDA	Lithium diisopropylamide
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NCI	National Cancer Institute
NMR	Nuclear magnetic resonance
SAR	Structure-Activity Relationship
TBAF	Tetrabutylammonium fluoride
TBDMS	*tert*-Butyldimethylchlorosilane
TLC	Thin layer chromatography
THF	Tetrahydrofuran
TMCS	Trimethylchlorosilane
TMS	Tetramethylsilane

1. Introduction

The mitotic phase of cell division relies on assembly of the mitotic spindle. Microtubules are the main constituent of the mitotic spindle and are composed of the α-β heterodimeric protein tubulin.[1] They are highly dynamic structures that alternate between periods of growing and shrinking through the addition or removal of tubulin subunits at the ends of microtubules.[2] Microtubules are a highly-validated target in cancer therapy and a large number of chemically diverse substances bind to tubulin and alter microtubule polymerization and dynamics in diverse ways.[3] These ligands can be broadly divided into two categories: those that inhibit the formation of the mitotic spindle, e.g. colchicine (**1**, Figure 1) and the vinca alkaloids, and those that inhibit the disassembly of the mitotic spindle once it has formed, e.g. paclitaxel and epothilone.[4] Tubulin-binding compounds, such as paclitaxel and vinblastine, are in widespread clinical use for various types of cancer.[3]

The combretastatins are a group of tubulin-binding diaryl stilbenes isolated from the stem wood of the South African tree *Combretum Caffrum*.[5] There is no written evidence of use of the plant for treating cancer amongst the indigenous people of Africa.[6] A number of constituent stilbenes were found to inhibit the growth of colon cancer cells and were strong inhibitors of tubulin polymerisation.[5] Combretastatin A-4 (**2a**, Figure 1) and combretastatin A-1 (**2b**, Figure 1) exhibited potent anticancer activity against a panel of human cancer cell lines from diverse origins, including leukaemic, breast and multi-drug resistant cancers.[4] Stilbenes **2a** and **2b** inhibit the formation of the mitotic spindle by binding to the colchicine-binding site of tubulin and were also shown to exhibit anti-vascular properties in vivo, probably by increasing tumor-vessel permeability.[7, 8] However, **2a** and **2b** display poor water solubility rendering them unsuitable for clinical use and water-soluble prodrugs, including combretastatin A-4-phosphate (**2c**, Figure 1), are in clinical trials, for example evaluation of **2c** for advanced anaplastic thyroid cancer and in combination with chemotherapy for advanced solid tumours.[9-11] **2c** displays excellent water solubility, good stability and cell growth inhibitory activity comparable to that of **2a**.[11]

However, the biological activity of **2a** is lost if isomerization to the inactive *trans* form occurs, for example during storage.[12, 13] Many conformationally restricted analogues of **2a** are known, in which the *cis* double bond is replaced by a heterocycle, thereby locking the two aryl rings in a *cis*-like configuration relative to each other. A diverse range of heterocycles as replacements for the double bond have been reported including benzoxepins,[14] oxadiazolines,[15] imidazoles,[16] combretoxazolones,[17] combretocyclopentenones[13] and thiophenes,[18] and are the subject of many reviews.[4, 12, 19] Previously, β-lactam containing compounds were reported to have anticancer activity[20, 21] and the β-lactam ring scaffold has been investigated as a template for analogues of **2a**.[22-24] We have recently reported a series of antiproliferative, tubulin-binding β-lactam compounds, where **3a**, **3b** and **3c** (Figure 1) emerged as the most potent agents with activity comparable to **2a**.[24] A 3-phenyl ring substituent improved the potency of this series of β-lactam compounds compared to either 3-methyl or 3,3-dimethyl substitution or β-lactams unsubstituted at C-3. β-Lactam **3b** was shown to induce rapid apoptosis in vitro in leukaemic HL-60 cells and also induced apoptosis in *ex vivo* samples from patients with chronic myeloid leukaemia, including those positive for the T315I mutation displaying resistance to imatinib mesylate and dasatinib.[25] Due to the potency of the 3-phenyl substituted compounds **3a** and **3b**, a series of novel analogues with diverse carbocycles and heterocycles to replace the phenyl ring were developed and evaluated for their antiproliferative activity and tubulin effects. These novel compounds reported herein contain carbocyclic, heterocyclic or modified aryl substituents at position 3 of the β-lactam ring while the aryl rings A and B present in **2a** are retained at positions 1 and 4 of the azetidinone scaffold. The rigid β-lactam ring structure facilitates a similar spatial arrangement between the two aryl rings at N-1 and C-4 as is observed for the *cis* configuration of **2a**.

Insert Figure 1

2. Results and Discussion

2.1 Chemistry

The synthetic routes for target β-lactam preparation are illustrated in Schemes 1 – 6. The compounds chosen for initial investigation all contained the 3,4,5-trimethoxyphenyl (mimicking ring A of **2a**) as the β-lactam N-1 substituent, together with the 4-methoxyphenyl ring as the β-lactam C-4 substituent. Two routes for the β-lactam ring-forming reaction were employed. The Staudinger reaction requires appropriately substituted acetic acids or acid chlorides and imines[26] (Scheme 3; Routes I, II and III), while the Reformatsky reaction requires an organozinc species (derived from an α-bromoester) and an imine (Scheme 6).[27] In two cases, the desired acetic acid precursors for β-lactam preparation were not commercially available. To prepare selected 2-thienyl containing derivatives, the appropriately substituted 2-thienyl aldehydes were converted to the corresponding acetic acids through the use of tetraethyl dimethylaminomethylenediphosphonate (**4**, Scheme 1).[28] It was previously reported that the most convenient and efficient method to produce this aminodiphosphonate reagent was the reaction of dimethylchloroformiminium chloride with 2.2 equivalents of triethyl phosphite.[29] The *in-situ* generated iminium ion reacts with triethyl phosphite to generate **4** in high yields of up to 76% (Scheme 1). This reagent reacts with the aldehyde of interest to form an enamine phosphonate, which is hydrolysed with strong acid to produce the substituted acetic acids **5a** and **5b** (Scheme 1).

Substituted acetic acids were required in the Staudinger reaction for the preparation of β-lactams in two procedures. Firstly, the acid chloride could be generated from the acid for use in a traditional Staudinger reaction. Alternatively, direct preparation of β-lactams from substituted acetic acids by the Staudinger route using an acid-activating agent is possible (see below). In the first option, generation of the acid chloride (**6a**, **6b**) from the corresponding substituted acetic acid was achieved by chlorination with thionyl chloride. The chlorination reactions were monitored by IR until absorption was observed between ν 1780 cm^{-1} and ν 1815 cm^{-1}, due to carbonyl stretching in the acid chloride. Acid chlorides **6a** and **6b** were synthesised in high yield and were immediately used in the following β-lactam forming reaction without further purification (Scheme 3).

The preparation of imine precursors **8a** – **8f** is achieved in high yield by condensation of the appropriately substituted aldehydes and anilines (Scheme 2).[30] In the case of 3-hydroxy-4-methoxybenzaldehyde, the hydroxyl group was first protected using the TBDMS silyl ether group and then used for the preparation of Schiff base **8b**.[31]

The preparation of target β-lactams **10** – **27** is illustrated in Scheme 3. β-Lactam synthesis was primarily carried out using the Staudinger reaction, which is a cycloaddition reaction between a ketene and an imine under basic conditions, where the ketene can be generated from an acid chloride.[32] β-Lactams **10** – **18** were prepared by this method (Scheme 3, route I). A modified procedure for preparation of the 3-thienyl compound **19** was employed, as the standard Staudinger reaction conditions were unsuccessful. β-Lactam **19** was obtained in 48% yield using milder conditions with overnight stirring at room temperature (Scheme 3, route II).[33] The stereochemistry of products from the Staudinger reaction depends on numerous factors, including the reaction conditions, the order of addition of the reagents and the substituents present on both the imine and on the acid chloride.[20, 32, 34] The *trans* products were isolated exclusively in all but one case, as evident from the representative ^{1}H NMR spectrum of compound **13** where the H-3 and H-4 were identified at δ 4.47 ppm and δ 4.90 ppm respectively as a pair of coupled doublets, $J_{3,4}$ = 2.5 Hz. The formation of the *trans* isomer is likely due to steric hindrance when two aryl rings present in the β-lactam structure at C-3 and C-4. The sole exception in this series of compounds was the 3-methyl-3-phenyl substituted β-lactam **11**, which was obtained as a mixture of *cis*/*trans* isomers (ratio 1:1.13 *cis*:*trans*) and separated by crystallization from ethanol. The presence of structural isomers was confirmed by X-ray crystal structures of both isomers of **11** (Figure 2). Both enantiomers of **11** can be seen in the crystal structure of the *trans* isomer (Figure 2b). The X-ray crystal structure of 3,3-diphenyl β-lactam **12** is illustrated in Figure 3.

Insert Figures 2 and 3

The β-lactam ring scaffold could also be generated directly from the appropriately substituted acetic acid and imine precursors using an acid-activating agent in a one-step reaction, without generation and isolation of the acid chloride (Scheme 3, route III). Many acid-activating agents are known in literature,

e.g. Mukaiyama's reagent, *p*-toluene-sulfonyl chloride and various phosphorous derived reagents.[32] Triphosgene has been reported for the synthesis of β-lactams and was employed as an acid-activating agent in synthesis of compounds **20** - **27**.[35, 36]

The phenolic products **28** and **29** were obtained on treatment of the silyl ethers **14** and **27** respectively with tetrabutylammonium fluoride at 0 ˚C (Scheme 4).[31] Separation of the silylated β-lactams **14** and **27** from the silylated imine in the final reaction mixture was difficult and hence removal of the silyl protecting group was carried out before subsequent purification. Reduction of the nitro group in compound **19** to the corresponding amine **30** was achieved by treatment with zinc dust and glacial acetic acid (Scheme 5).[37]

Preliminary biochemical assessments of β-lactams **10** – **30** in MCF-7 human breast cancer cells revealed potent antiproliferative activity for thiophene containing compounds **13**, **24**, **28**, **29** and **30** (Table 2). On the basis of these results, further sulfur-containing β-lactam analogues were prepared. 3-Unsubstituted β-lactam **9** was used as a precursor for a variety of substitution reactions at C-3. Compound **9** was obtained by Reformatsky reaction of imine **8a** with ethylbromoacetate and zinc using microwave technology and TMCS as the zinc-activating agent as we have previously reported.[24, 27] Deprotonation of **9** with LDA at -78 °C followed by reaction with aldehydes[32, 38] was successful for the preparation of secondary alcoholic derivatives **31** – **34** (Scheme 6). Further treatment of compounds **32** and **33** by oxidation with pyridinium chlorochromate[39] yielded ketone analogues **35** and **36** (Scheme 7). Transformation of alcoholic derivatives **32** and **34** by dehydration with tosyl chloride in pyridine[38] delivered corresponding vinylogous analogues **37** and **38** (Scheme 8). Although formation of *E*/*Z* isomers at the 3-position double bond is possible for **37** and **38**, only one isomer was obtained in each case, possibly due to steric hindrance between the thiophene ring and aryl substituents at positions 3 and 4 of the azetidinone ring. The products **37** and **38** were assigned the *Z* configuration, by comparison of the signal for H-α in the ^{1}H NMR spectrum (δ 6.45 ppm and δ 6.38 ppm for compounds **37** and **38** respectively) with reported values for Hα in related *E* and *Z* 3-methylenesubstituted azetidin-2-ones.[21]

All β-lactam compounds **10** – **27** were obtained as enantiomeric mixtures and separation by chiral liquid chromatography was demonstrated for selected compounds **13** and **33**, indicating a 1:1 mixture of the two enantiomers for both compounds (Figure 10, Supplementary Information). We have previously demonstrated stability of 3-phenyl β-lactams over the pH range 4 – 9.[24] Preliminary stability studies of 3-(2-thienyl) β-lactams with aryl, naphthyl and thienyl substituents at C-4 (compounds **13**, **16**, **17** and **18**) were carried out in acidic, neutral and basic pH conditions. The half-lives for these compounds were determined to be greater than 24 hours at pH values of 4, 7.4 and 9, with the compounds being least stable at pH 4 for all four analogues assessed.

2.2 Biological Results and Discussion

2.2.1 Antiproliferative effects

The series of β-lactam analogues of **2a** were initially evaluated for their antiproliferative activity in human MCF-7 breast cancer cells using the MTT cell viability assay.[40] The previously reported lead compound, **3a**, showed potent activity in this cancer cell line with an IC_{50} of 0.034 μM and further investigation established **3a** and **3b** as potential lead development candidates for the treatment of leukaemia.[24, 25] To establish a more detailed SAR and further examine the effects of 3-substitution on antiproliferative activity, the phenyl ring at the 3-position of **3a** was replaced with a wide variety of carbocyclic and heterocyclic substituents, while retaining the N-1, C-4 substituents of **3a** (Table 1).

The most potent β-lactams in MCF-7 cells were those with 3-(2-thienyl) (**13**) and 3-(3-thienyl) (**24**) substituents with IC_{50} values of 64 nM and 60 nM respectively. Replacement of the sulfur atom of **24** with oxygen (furan analogue **23**) led to a two-fold decrease in activity. Introduction of multiple and/or larger substituents at this position led to substantial decrease in activity compared to substitution with a phenyl ring, for example diphenyl **12** (IC_{50} of 43.17 μM), 1-naphthyl **20** (11.32 μM), 2-naphthyl **21** (2.47 μM) and methyl indole **22** (6.59 μM). The bulky benzothiophene analogue **26** has a much greater IC_{50} value of 0.85 μM than the corresponding thiophene derivative **13** (IC_{50} value of 0.064 μM). This is in line with previous observations that poly-substitution of the C-3 phenyl ring led to decreased activity.[24] A cyclohexane ring at position 3 of the β-lactam (**10**) showed decreased activity of over 100-

fold compared to the 3-phenyl substituted compound **3a**. Cyclohexane-substituted **10** has a higher cLogP value of 4.87 compared to 3.88 for **3a**, indicating a marked increase in hydrophobicity. It is possible that this property contributes to its relative lack of antiproliferative activity. Disubstitution at the 3-position yielded the two compounds with the least antiproliferative activity in MCF-7 cells, 3-methyl-3-phenyl substituted **11** (IC_{50} of 43.45 μM; evaluated as a mixture of *cis*/*trans* isomers) and 3,3-diphenyl substituted **12** (IC_{50} of 43.17 μM). From these results, it can be deduced that substituents larger than a phenyl ring at the 3-position are detrimental to the antiproliferative activity of this series of compounds.

Compounds **28** (IC_{50} = 7 nM) and **29** (IC_{50} = 10 nM) with additional hydroxyl groups at the 3-position of the 4-phenyl ring, analogous to **2a**, displayed increased potency in MCF-7 cells over their respective parent compounds, **13** and **24**, of 9-fold and 10-fold respectively. A dose response graph for **13**, **29** and **2a** is shown in Figure 4. β-Lactam **30**, in which the phenolic moiety of **28** is replaced with an amino substituent, was marginally more potent than **13** but less active than **28** (IC_{50} values of 42 nM, 64 nM and 7 nM respectively). Both **28** and **30** offer the possibility of further modification to form ester or amide prodrugs *via* their phenolic and amino groups.

Insert Figure 4

The effect of introduction of a carbon spacing atom between the thiophene ring and C-3 of the β-lactam ring was investigated (compounds **14**, **32** – **37**). Secondary alcohols **32** and **33** and ketone derivatives **35** and **36** (IC_{50} values = 1.17 μM, 0.99 μM, 0.95 μM and 0.47 μM respectively) were over 16-fold less potent than **13** and **24**. Methylene **14** and alkene **37** have IC_{50} values of 1.64 μM and 4.05 μM respectively, confirming that extension of the distance between the thiophene and β-lactam ring has a detrimental effect on the potency of this series.

Analogues of **2a** with a thiophene ring at the 3-position and naphthyl substituents at the 4-position (**16** and **17**) in place of the 3-hydroxy-4-methoxyphenyl moiety were assessed for antiproliferative activity as the naphthyl moiety has been previously shown to be a good replacement for the B-ring of **2a**.[37] However both 4-(2-naphthyl) β-lactam **16** and 4-(1-naphthyl) β-lactam **17** displayed decreased IC_{50} values of 0.12 μM and 0.62 μM compared to 0.007 μM for **28**. Replacement of the 4-position

substituted phenyl ring with a thiophene ring (**18**) led to decreased activity (IC_{50} value = 0.91 μM) indicating that, while a C-3 thiophene ring is advantageous for activity, such a substitution is not tolerated at C-4.

The most active analogues in the MCF-7 antiproliferative studies (**13**, **28**, **29** and **30**) were subsequently evaluated against human MDA-MB-231 breast cancer cells and exhibited submicromolar IC_{50} values. Of the four compounds tested, 3-(3-thienyl) β-lactam **29** was the most potent (IC_{50} = 49 nM) and showed improved activity compared to the lead compound **3a** (IC_{50} = 78 nM) (Table 3).

2.2.2 Further biochemical assessment: NCI60 cell line screen, cytotoxicity and tubulin polymerisation

Compounds **13, 28** and **30** were chosen for specific analysis and further development (screening in the National Cancer Institute (NCI) 60-cell line panel, determination of cytotoxicity, tubulin binding and molecular modelling) based on the analysis of their drug-like properties from a Tier-1 profiling screen (based on experimentally determined solubility and chemical stability together with together with predictions of permeability, metabolic stability, Pgp substrate status, blood-brain barrier partition, plasma protein binding and human intestinal absorption properties which indicated the suitability of these compounds for further development). These compounds satisfy Lipinski's 'rule of five' for drug-like properties e.g. molecular weights of **13**, **28** and **30** are less than 500, the number of oxygen/nitrogen atoms is less than 10, the number of hydrogen bond donors is less than 5 and the cLogP values are 2.78, 1.88 and 1.95 respectively (<5), implying that they are moderate lipophilic-hydrophobic drugs and are suitable candidates for further investigation.

3-Thienyl β-lactam **13** was screened using the NCI60 panel of cell lines (Table 5, supplementary information)[41] and exhibited IC_{50} values of less than 10 nM in 25 of the 56 cell lines, and IC_{50} values of less than 51 nM in 47 of the cell lines. The mean GI_{50} value for **13** across all cell lines is 27.54 nM [log GI_{50}=(-7.56M)]. The anti-proliferative activity of **13** was particularly potent for all three leukaemic cell lines (<10 nM) and for CNS, melanoma and breast cell lines, indicating a wide-range of potential

therapeutic applications. The mean LC_{50} for **13** across the range of cell lines is >100 μM indicating minimal cytotoxicity. In addition, matrix COMPARE analysis[42, 43] (measuring the correlation between two compounds with respect to their differential antiproliferative activity) demonstrated good correlation between **13**, **3b** and **2a** (*r*=0.76 and 0.61 respectively). However, this algorithm does not distinguish between different tubulin-based mechanisms of action.[44] The COMPARE algorithm was also used to compare the differential antiproliferative activities of **13** to compounds with known mechanisms of action in the NCI Standard Agent Database[4] and showed correlations to vincristine, paclitaxel, maytansine and rhizoxin, all of which affect microtubule polymerization (Table 4).

2.2.3 Evaluation of toxicity in normal murine mammary epithelial cells

Further toxicity measurements were carried out on 3-(2-thienyl) β-lactam **28**, the most potent antiproliferative β-lactam in antiproliferative assessment with MCF-7 cells. Toxicity studies in healthy mouse mammary epithelial cells at two different cell concentrations were carried out (25,000 cells/mL and 50,000 cells/mL harvested from mid- to late- pregnant CD-1 mice and cultured as described previously[45, 46]). These results indicate a favorable toxicity profile for **28** in comparison to **2a**. The IC_{50} value for both compounds was greater than 10 μM indicating minimal toxicity for this compound (Figure 5) (Table 7 and Figure 12, Supplementary Information).

Insert Figure 5

2.2.4 Tubulin polymerization studies

The effects of representative β-lactam CA-4 analogue (compound **28**) which demonstrated potent antiproliferative effects in vitro was assessed on the assembly of purified bovine tubulin. The ability of **2a** to effectively inhibit the assembly of tubulin was assessed as a positive control. Tubulin polymerisation was determined by measuring the increase in absorbance over time at 340 nm. The V_{max} value offers the most sensitive indicator of tubulin/ligand interactions and hence fold-changes in V_{max} values for polymerisation curves of the compound with reference to ethanol control were calculated.

Tubulin polymerization studies on **28** showed a 3.2-fold reduction in the V_{max} at 10 μM compared to a 6-fold reduction for **2a** tested as a control. The IC_{50} value for **28** for the inhibition of V_{max} was calculated to be 1.37 ± 0.85 μM, while an IC_{50} value of 6.25 ± 2.53 μM was obtained for the effect in overall polymer mass (calculated from area under the polymerization curve) (Figure 6). This confirms that the molecular target of these antiproliferative β-lactams is tubulin.

Insert Figure 6

2.3 Structural Studies, Molecular Modeling and Rationalization of Biochemical Activity

Based on the 3D structural similarity between the ligands **1**, **2a** and the β-lactam analogues reported in this study, we propose that the binding site for these compounds is most likely to be the colchicine site, as it has been demonstrated that **2a** and many reported examples of the structurally related conformationally constrained **2a** analogues bind at the colchicine site.[47-49] The colchicine-binding site in tubulin is mainly buried in the β-subunit of tubulin, whilst maintaining some limited interactions with the α-subunit. The H7 and H8 α-helices, the T7 loop and the S8 and S9 β-strands contribute to the binding site and interact with the colchicine-site ligand.[50] Two of the most important residues for colchicine-binding are Val318 and Cys241. Val318 tubulin variants have reduced sensitivity to **1**, and **1** substituted with more reactive groups instead of the methoxys can be crosslinked with Cys241.[51, 52] The Thr179 residue has also been highlighted as being important, though not critical, for binding.[53] Previously reported β-lactams **3a** and **3b** both show interactions with Val318 and Cys241, while **3b** has an additional hydrogen-bonding interaction with Thr179.[24]

The antiproliferative assessment of the β-lactam compounds **10** – **38** (Table 2) established a clear trend, where 2- and 3-thiophene substituents at C-3 proved extremely potent (e.g. compounds **13**, **28**, **29** and **30**). In contrast, bulkier substituents at the 3-position of the azetidinone ring (e.g. 3,3-diphenyl substituted analogue **12**) led to a substantial decrease in activity, even though the required substitution pattern of rings A and B were preserved. To rationalize this observation, molecular structures of compounds **11** and **12**, determined by single-crystal X-Ray crystallography, were examined (Figures 2

and 3). The structures revealed a conformation for the azetidinones **11** and **12** in which the two aromatic rings located at N-1 and C-4 are not coplanar. The observed dihedral angle between Ring A and Ring B in the X-ray crystal structures of these analogues is -61.7 ° for compound **12** (Figure 3). For compound **11**, a dihedral angle of 73.4 ° is observed for the *cis* isomer while values of 62.7 ° and -66.1 ° are calculated for the two enantiomers of the *trans* isomer (Figure 2). These values are very different to the dihedral angle previously observed for **3a** of 46.9 °,[24] and are also higher than the values for **1** (55 °)[51] and **2a** (53 °)[54]. It is possible that this difference in orientation between the two rings is one of the factors leading to the decreased antiproliferative activity observed for **11** and **12**. When these compounds **11** and **12** are docked computationally in the colchicine-binding site of tubulin, the reason for the decreased biochemical activity in vitro becomes apparent. The docked conformations of both **3a** and **12** and of **1** and **12** (Figure 7) reveal that **12** is predicted to be orientated differently to both **3a** and **1** within the binding site. The N-1 trimethoxyphenyl rings of **3a** and **12** adopt similar positions in the binding site but the C-4 4-methoxyphenyl ring lies in a different plane, projecting backwards for **1** and **3a**, but forward for **12**. The 3,3-diphenyl rings of analogue **12** occupy the same part of the binding site as the 4-(4-methoxyphenyl) ring of **3a**, indicating that this part of the colchicine-binding site of tubulin can accommodate a larger volume and explains the relative switch in orientation for **12** compared to **1** and **3a**. This may account for the loss in antiproliferative activity seen with **12** and any other related analogues with a bulky substitution pattern at this position (e.g. compounds **11** **20**, **21** and **22**), as the potential for forming a binding interaction with Thr179 is lost and microtubule dynamics may not be as dramatically affected. However, interactions between the N-1 trimethoxyphenyl ring of **12** and both Val318 and Cys241 are maintained, accounting for the residual antiproliferative activity of this compound.

Insert Figure 7

In contrast to 3,3-diphenyl β-lactam **12**, virtual molecular docking of the most potent β-lactam from this series, 3-(2-thienyl) β-lactam **28,** predicts a docked conformation similar to that previously predicted for **3b** (Figure 8, also figure 11 in supplementary information).[24] The dihedral angle between the N-1

and C-4 phenyl rings of **28**, calculated from the energy minimized structure rather than a crystal structure, is 61.8°. The predicted 2D interactions of the ligand with the protein are shown in figure 9.[55] Residues Cys241 and Val318 interact with the trimethoxyphenyl ring of **28**. Hydrogen bonding of the phenolic group of ring B to Thr179 and Lys352 contributes to the strong tubulin binding of this compound observed in vitro and is suggested to account for the increased antiproliferative activity seen with the phenolic compounds **28** and **29**. Further hydrophobic interactions between the 3-(2-thienyl) ring and the colchicine site residues (Figure 9) reinforce the binding to the protein, for example with Val181, Leu248 and Ala250. These interactions, in contrast to those of β-lactam **12**, may stabilize the binding of **28** and provide a rational basis for the potent antiproliferativea nd tubulin-binding activity displayed by these compounds.

Insert Figures 8 and 9

Summary and conclusion

Building on previous work where the β-lactam ring scaffold was utilized to replace the isomerisable double-bond of **2a**, further investigations to determine a comprehensive SAR of antiproliferative β-lactams has led to the new discovery of novel analogues with significant antiproliferative and tubulin-binding activity. A trend for small ring heterocyclic systems at the 3-position ring leading to increased potency was determined, with larger ring systems such as naphthyl, indole and benzothiophene leading to significantly less potent anti-proliferative activities. 3-(2-Thienyl) and 3-(3-thienyl) derivatives **28** and **29** displayed the most potent antiproliferative activity in MCF-7 and MDA-MB-231 breast cancer cell lines with low nanomolar antiproliferative IC_{50} values, and **28** was shown to be minimally toxic to normal murine epithelial cells. The molecular target of β-lactam **28** was confirmed to be tubulin, and this compound displayed an IC_{50} value of 1.37 μM for inhibition of tubulin polymerization. The 2-thienyl and 3-thienyl containing compounds reported herein will be evaluated in further in vitro and in vivo studies to develop their potential vascular targeting and antiangiogenic applications.

3. Experimental Section

3.1 Chemistry: Experimental Methods

All reagents were commercially available and were used without further purification unless otherwise indicated. Tetrahydrofuran (THF) was distilled immediately prior to use from Na/Benzophenone under a slight positive pressure of nitrogen, toluene was dried by distillation from sodium and stored on activated molecular sieves (4 Å) and dichloromethane was dried by distillation from calcium hydride prior to use. IR spectra were recorded as thin films on NaCl plates or as KBr discs on a Perkin-Elmer Paragon 100 FT-IR spectrometer. ^{1}H and ^{13}C NMR spectra were obtained on a Bruker Avance DPX 400 instrument at 20 ^{o}C, 400.13 MHz for ^{1}H spectra, 100.61 MHz for ^{13}C spectra, in $CDCl_3$ (internal standard tetramethylsilane) by Dr. John O'Brien and Dr. Manuel Ruether in the School of Chemistry, Trinity College Dublin. Low resolution mass spectra were run on a Hewlett-Packard 5973 MSD GC–MS system in an electron impact mode, while high resolution accurate mass determinations for all final target compounds were obtained on a Micromass Time of Flight mass spectrometer (TOF) equipped with electrospray ionization (ES) interface operated in the positive ion mode at the High Resolution Mass Spectrometry Laboratory by Dr. Martin Feeney in the School of Chemistry, Trinity College Dublin. Elemental analysis was carried out in the microanalytical laboratory, University College Dublin, Belfield, Dublin 4. Thin layer chromatography was performed using Merck Silica gel 60 TLC aluminium sheets with fluorescent indicator visualizing with UV light at 254 nm. Flash chromatography was carried out using standard silica gel 60 (230-400 mesh) obtained from Merck. All products isolated were homogenous on TLC. Analytical high-performance liquid chromatography (HPLC) to determine the purity of the final compounds was performed using a Waters 2487 Dual Wavelength Absorbance detector, a Waters 1525 binary HPLC pump, a Waters In-Line Degasser AF and a Waters 717plus Autosampler. The column used was a Varian Pursuit XRs C18 reverse phase 150 x 4.6mm chromatography column. Samples were detected using a wavelength of 254 nm. All samples were analyzed using acetonitrile (70%): water (30%) over 10 min and a flow rate of 1 mL/min. Unless otherwise indicated, the purity of the final products was ≥ 95% (see table 6, supplementary information). Chiral liquid chromatography was carried out on selected compounds using a Chiral-AGP^{TM} 150x4.0 mm column supplied by ChromTech Ltd. (now supplied by Chiral Technologies Europe) with a Chiral-

AGP^{TM} guard column and the same Waters hardware as used above for purity testing. Gradient elution was used beginning with 10% of organic phase and finishing with 90% of organic phase over a period of 20 minutes. The organic mobile phase was 2-propanol and the aqueous phase was a sodium phosphate buffer. The sodium phosphate buffer, consisting of 10 mM sodium dihydrogen orthophosphate dihydrate (NaH_2PO_4) in HPLC-grade water, was made up to pH 7.0 using sodium hydroxide. The flow rate was 0.5 mL/min and detection was carried out at 225 nm.

3.1.1 3-(*tert*-Butyldimethylsilanyloxy)-4-methoxybenzaldehyde. To a solution of 3-hydroxy-4-methoxybenzaldehyde (0.02 mol) and dimethyl-*tert*-butylchlorosilane (0.024 mol) in dry CH_2Cl_2 (60 mL) was added 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) (0.032 mol). The resulting mixture was stirred at room temperature under a nitrogen atmosphere until complete on thin layer chromotagraphy. The solution was then diluted with CH_2Cl_2 (80 mL) and washed successively with water (60 mL), 0.1M HCl (60 mL) and saturated aqueous $NaHCO_3$ (60 mL). The organic layer was removed and dried by filtration through anhydrous sodium sulphate, Na_2SO_4. 3-(*tert*-Butyldimethylsilanyloxy)-4-methoxybenzaldehyde was isolated as a brown oil (yield 93.8%)[56]; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.19 (s, 6H, -$SiCH_3$), 1.02 (s, 9H, -CH_3), 3.91 (s, 3H, OCH_3), 6.97 (d, 1H, J=8.52 Hz, ArH), 7.39 (s, 1H, ArH), 7.48 – 7.50 (m, 1H, ArH), 9.83 (s, 1H, -CHO); ^{13}C NMR (100 MHz, $CDCl_3$) δ 18.43 (-$SiCH_3$), 25.65 (-CH_3), 55.58 (OCH_3), 111.19, 120.05, 126.39, 130.17, 145.57, 156.65 (ArC), 191.01 (CHO); HRMS (M^++H): $C_{14}H_{22}O_3S$ requires 266.1338; found: 266.1349

3.1.2 Tetraethyl dimethylaminomethylenediphosphonate (4): To a chilled solution of dimethylformamide (97.9 mmol) in diethyl ether (150 mL) was added dropwise with stirring a solution of oxalyl chloride (97.9 mmol) in diethyl ether (20 mL). Following addition, the mixture was allowed to warm to room temperature and stirred for 1 hour. Triethyl phosphite (215 mmol) was then added dropwise with stirring. After one hour the mixture was concentrated under reduced pressure. The product was obtained as a yellow oil in 75.5% yield.[28]; δ 0.92 (m, 12H, 4xCH_3), 2.19 (s, 6H, 2xCH_3), 2.90 (t, 1H, 4xCH), 3.68 – 3.80 (m, 8H, CH_2); HRMS (M^++Na): $C_{11}H_{27}NNaO_6P_2$ requires 354.1211; found: 354.1218

3.1.3 General method for preparation of enamine phosphonate

To a suspension of NaH (33 mmol) in dry toluene (20 mL) was added dropwise with stirring a solution of **4** (16.7 mmol) in dry toluene (20 mL). After one hour, a solution of the appropriate aldehyde (16.7 mmol) in dry toluene (20 mL) was added. The mixture was stirred at 50 ˚C for one hour and then concentrated. The residue was partitioned between ethyl acetate and water and the aqueous layer was extracted with ethyl acetate three times. The residue was purified by column chromatography (hexane: ethyl acetate gradient) to afford the clean product.

3.1.3.1 (2-Benzo[b]thiophen-2-yl-1-dimethylaminovinyl)phosphonic acid diethyl ester was obtained by reaction of benzo[b]thiophene-2-carbaldehyde with tetraethyl dimethylaminomethylenediphosphonate (**4**). The product was obtained as a dark orange oil (56.7% yield).[28, 29] ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.36 (t, 6H, 2xCH_3), 2.69 (s, 6H, 2xCH_3), 4.12 – 4.22 (m, 4H, 2xCH_2), 7.31 – 7.40 (m, 3H, ArH), 7.73 – 7.78 (m, 2H, ArH) ; HRMS (M^++Na): $C_{16}H_{22}NNaO_3PS$ requires 362.0956; found: 362.0946

3.1.3.2 [1-Dimethylamino-2-(5-methylthiophen-2-yl)vinyl]phosphonic acid diethyl ester was obtained by reaction of 5-methyl-thiophene-2-carbaldehyde with tetraethyl dimethylaminomethylenediphosphonate (**4**). The product was obtained as a dark orange oil (22.5% yield); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.32 (t, 6H, 2xCH_3), 2.45 (s, 3H, CH_3), 2.60 (s, 6H, 2xCH_3), 4.06 – 4.13 (m, 4H, 2xCH_2), 6.63 (s, 1H, CH), 6.98 (d, 1H, J=3.52, ArH), 7.18 (d, 1H, J=12.04, ArH); HRMS (M^++Na): $C_{13}H_{22}NNaO_3PS$ requires 326.0956; found: 326.0972

3.1.4 General procedure for hydrolysis of enamine phosphonates: The appropriate enamine phosphonate was refluxed in 10M HCl (50 mL) for 30 minutes. The mixture was poured onto ice water (200 mL) and extracted with ethyl acetate (twice). The combined organic extracts were dried over anhydrous Na_2SO_4 and concentrated to give the desired product.

3.1.4.1 Benzo[b]thiophen-2-yl-acetic acid (5a) was obtained from (2-benzo[b]thiophen-2-yl-1-dimethylamino-vinyl)phosphonic acid diethyl ester as a light brown powder (61.2% yield); Mp: 130°C (lit. 140 - 142°C[29]); IR (KBr disk) ν_{max}: 1715.55 cm^{-1} (-C=O); ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.99 (s,

2H, CH_2), 7.24 – 7.37 (m, 3H, ArH), 7.74 – 7.76 (m, 1H, ArH), 7.81 – 7.83 (m, 1H, ArH); ^{13}C NMR (100 MHz, $CDCl_3$) δ 35.39 (CH_2), 122.18, 123.36, 124.06, 124.19, 124.39, 135.03, 139.60, 140.03 (ArC), 175.87 (C=O)

3.1.4.2 (5-Methylthiophen-2-yl)acetic acid (5b) was obtained as a brown oil from [1-dimethylamino-2-(5-methylthiophen-2-yl)-vinyl]-phosphonic acid diethyl ester (1% yield) and was used immediately in the subsequent reaction without further purification[29]; IR (KBr disk) ν_{max}: 1705.90 cm^{-1} (-C=O); ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.49 (s, 3H, CH_3), 3.83 (s, 2H, CH_2), 6.65 (d, 1H, J=2.52, ArH), 6.77 (d, 1H, J=2.52, ArH); ^{13}C NMR (100 MHz, $CDCl_3$) δ 15.29 (CH_3), 35.25 (CH_2), 124.99, 127.19, 131.63, 139.95, 177.04 (C=O)

3.1.5 General method for chlorination of acetic acid derivatives: The appropriate acetic acid (10 mmol) was brought to reflux with thionyl chloride (12 mmol) in chloroform (30 mL). The reaction was monitored by I.R. until absorption appeared between 1780cm^{-1} and 1815cm^{-1}. The solvent was evaporated under reduced pressure.

3.1.5.1 2-Phenylpropionyl chloride (6a)[57] was prepared from phenylpropionic acid in 92.2% yield as a pale yellow oil and was used immediately in the subsequent reaction without further purification; IR (KBr) ν_{max}: 1784.17 cm^{-1} (-C=O, acid chloride).

3.1.5.2 3-Thiophen-2-yl-propionyl chloride (6b)[58] was prepared from 3-thiophen-2-yl-propionic acid (10 mmol) and was used immediately in the subsequent reaction without further purification (pale yellow oil, 90.9% yield); IR (KBr) ν_{max}: 1782.84 cm^{-1} (-C=O, acid chloride)

3.1.6 General method for imine formation

The appropriate amine (10 mmol) was heated at reflux with the appropriate aldehyde (10 mmol) in ethanol (50 mL) for 3 hours. The reaction mixture was reduced *in vacuo* and the resulting solution was left to stand until solid product crystallised. The resulting imine was recrystallised from ethanol.

3.1.6.1 N-(4-Methoxybenzylidene)-3,4,5-trimethoxybenzenamine (8a) was synthesised by reacting 3,4,5-trimethoxybenzenamine with 4-methoxybenzaldehyde. The product was obtained as pale yellow crystals (yield 87%); mp: 120°C[24]; IR (KBr disk) ν_{max}: 1604.66 cm^{-1} (C=N); ^{1}H NMR (400 MHz,

$CDCl_3$) δ 3.86 (s, 3H, OCH_3), 3.87 (s, 3H, OCH_3), 3.90 (s, 6H, 2x OCH_3) 6.47 (s, 2H, ArH) 6.98 (d, 2H, J=9.2 Hz, ArH) 7.84 (d, 2H, J=9.2 Hz, ArH) 8.40 (s, 1H, -CH=N); ^{13}C NMR (100 MHz, $CDCl_3$) δ 55.35 (OCH_3), 56.00 (OCH_3), 60.94 (OCH_3), 98.00, 114.13, 128.97, 130.39, 135.97, 148.22, 153.45, 159.03(ArC), 162.20 (CH=N); Elemental analysis: Found: C, 67.73; H, 6.35; N, 4.63; $C_{17}H_{19}NO_4$ requires C, 67.76; H, 6.36; N, 4.65%.

3.1.6.2 [3-(*tert*-Butyldimethylsilanyloxy)-4-methoxybenzylidene](3,4,5-trimethoxyphenyl)amine (8b) was synthesised by reaction of 3-(*tert*-butyldimethylsilanyloxy)-4-methoxybenzaldehyde with 3,4,5-trimethoxybenzenamine. The product was obtained as a yellow solid. Yield 64%, mp: 105°C[24]; IR (KBr disk) ν_{max} 1619.77 cm^{-1}, 1579.73 cm^{-1} (C=N); ^{1}H NMR (400 MHz, $CDCl_3$) δ δ 0.20 (s, 6H, 2xCH_3), 1.03 (s, 9H, $C(CH_3)_3$), 3.87 – 3.91 (m, 12H, 4xOCH_3), 6.48 (s, 2H, ArH), 6.93 (d, 2H, J=8.04 Hz, ArH), 7.43 – 7.47 (m, 1H, ArH), 8.35 (s, 1H, CH=N); ^{13}C NMR (100 MHz, $CDCl_3$) δ –5.04(CH_3-Si-CH_3), 18.03(CH_3-C-CH_3), 25.27($C(CH_3)_3$), 54.98(OCH_3), 55.63(OCH_3), 97.62, 110.94, 119.71, 123.48, 128.95, 135.53, 144.87, 147.94, 153.05, 153.59 (ArC), 158.84(C=N); HRMS (M^++H): $C_{23}H_{34}NO_5Si$ requires 432.2206; found: 432.2213

3.1.6.3 (4-Methoxy-3-nitrobenzylidene)(3,4,5-trimethoxyphenyl)amine (8c) was synthesised by reaction of 3,4,5-trimethoxyphenylamine and 4-methoxy-3-nitrobenzaldehyde. The product was obtained as a yellow powder (yield 88%); mp: 162 – 163°C[24]; IR (KBr disk) ν_{max} 1616.90cm^{-1}, 1580.79cm^{-1}(C=N); ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.89 (s, 3H, OCH_3), 3.93 (s, 6H, 2x OCH_3), 4.06 (s, 3H, OCH_3), 6.52 (s, 2H, ArH), 7.21 (d, 1H, J=8.52 Hz, ArH), 8.13 (dd, 1H, J=8.52 Hz, J=2.48 Hz, ArH), 7.39 (d, 1H, J=2.48 Hz, ArH), 8.45 (s, 1H, (C=N); ^{13}C NMR (100 MHz, $CDCl_3$) δ 55.70 (OCH_3), 56.40 (OCH_3), 60.59 (OCH_3), 97.75, 113.20, 125.59, 128.52, 133.29, 146.59, 153.19, 154.41 (ArC), 155.66 (C=N). Elemental analysis: Found: C, 58.91; H, 5.25; N, 7.95; $C_{17}H_{18}N_2O_6$ requires C, 58.96; H, 5.24; N, 8.09%.

3.1.6.4 3,4,5-Trimethoxy-N-(naphthalen-2-ylmethylene)aniline (8d) was synthesised using 3,4,5-trimethoxyphenylamine and 2-naphthaldehyde as a yellow solid (78% yield); mp:: 132-136 °C; IR (KBr disk) ν_{max}: 1626.22 and 1581.26 cm^{-1} (C=N); ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.91 (s, 3H, OCH_3), 3.95

(s, 6H, 2xOCH_3), 6.29 (s, 2H, ArH), 7.58 (m, 2H, ArH), 7.94 – 7.96 (m, 3H, ArH), 8.84 (m, 2H, ArH), 8.67 (s, 1H, HC=N); Elemental analysis: Found: C, 74.68; H: 6.02; N: 4.31; $C_{20}H_{19}NO_9$ requires C, 74.65, H, 5.98, N, 4.26

3.1.6.5 3,4,5-Trimethoxy-N-(naphthalen-1-ylmethylene)aniline (8e) was synthesised using 3,4,5-trimethoxyphenylamine and 2-naphthaldehyde as a yellow solid (77% yield); mp: 108-116 °C; IR (KBr disk) ν_{max}: 1625.74, 1610.62 and 1583.40 cm^{-1} (C=N); ^{1}H-NMR (400 MHz, $CDCl_3$): δ 3.92 (s, 3H, OCH_3), 3.96 (s, 6H, 2xOCH_3), 6.61 (s, 2H, ArH), 7.59 – 7.67 (m, 3H, ArH), 7.96 (d, 1H, J=8.52 Hz, ArH), 8.02 (d, 1H, J=8 Hz, ArH), 8.12 (m, 1H, ArH), 9.05 (d, 1H, J=8.52 Hz, ArH), 9.15 (s, 1H, HC=N); Elemental analysis: Found: C, 74.67, H, 5.97, N, 4.30; $C_{20}H_{19}NO_9$ requires C, 74.65, H, 5.98, N, 4.26

3.1.6.6 3,4,5-Trimethoxy-N-(thiophen-2-ylmethylene)aniline (8f) was synthesised from 3,4,5-trimethoxyphenylamine and thiophene-2-carbaldehyde as a yellow solid (81% yield); mp: 92-98 °C; IR (KBr disk) ν_{max}: 1617.78 and 1584.53 cm^{-1} (C=N); ^{1}H-NMR (400 MHz, $CDCl_3$): δ 3.88 (s, 3H, OCH_3), 3.92 (s, 6H, 2xOCH_3), 6.52 (s, 2H, ArH), 7.16 – 7.18 (m, 1H, ArH), 7.52 – 7.55 (m, 2H, ArH), 8.61 (s, 1H, HC=N); Elemental analysis: Found: C, 60.62; H, 5.44; N, 5.01; $C_{14}H_{15}NO_3S$ requires C, 60.63; H, 5.45; N=5.05

3.1.7 4-(4-Methoxyphenyl)-1-(3,4,5-trimethoxyphenyl)azetidin-2-one (9): Zinc powder (0.927g, 15 mmol) was activated using trimethylchlorosilane (0.65 mL, 5 mmol) in anhydrous benzene (5 mL), by heating for 15 minutes at 40 °C and subsequently for 2 minutes at 100 °C in a microwave. After cooling, N-(4-methoxybenzylidene)-3,4,5-trimethoxybenzenamine **(8a)** (10 mmol) and ethyl 2-bromoacetate (12 mmol) were added to the reaction vessel and the mixture was placed in the microwave for 30 minutes at 100°C. The reaction mixture was filtered through Celite to remove zinc, then diluted with CH_2Cl_2 (50 mL). This solution was washed with saturated ammonium chloride solution (20 mL) and 25% ammonium hydroxide (20 mL), and then with dilute HCl (40 mL), followed by water (40 mL). 4-(4-Methoxyphenyl)-1-(3,4,5-trimethoxyphenyl)azetidin-2-one (**9**) was obtained as green crystals (yield 43%); mp: 70-71°C; IR (NaCl film) ν_{max}: 1747.5 cm^{-1} (C=O, β-lactam); ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.85 (dd, 1H, J= 2.48 Hz, 12.56 Hz, H-3), 3.48 (dd, 1H, J=5.52 Hz, J=9.56 Hz, H-4), 3.65 (s, 6H,

2xOCH_3), 3.70 (s, 3H, OCH_3), 3.73 (s, 3H, OCH_3), 4.88 (d, 1H, J= 2.76 Hz, H-4), 6.53 (s, 2H, ArH), 6.86 (d, 2H, J=8.56 Hz, ArH), 7.26 (d, 2H, J=8.56 Hz, ArH); ^{13}C NMR (100 MHz, $CDCl_3$): δ 46.36 (C-3), 53.56 (OCH_3), 54.78 (OCH_3), 55.23 (OCH_3), 55.49 (OCH_3), 60.36 (C-4), 93.92, 113.58, 126.83, 129.48, 133.62, 133.68, 152.94, 159.29 (ArC), 164.14 (C=O); HRMS (M^++Na): Found 366.1330; $C_{19}H_{21}NO_5Na$ requires 366.1317.

3.1.8 General method I for β-lactam preparation: The appropriate imine (5 mmol) and triethylamine (15 mmol) were added to dry CH_2Cl_2 (50 mL) and the mixture was brought to reflux at 60°C. Once refluxing, the appropriately substituted acid chloride (7.5 mmol) was injected dropwise through a rubber stopper. This mixture was refluxed for 3 hours. The mixture was washed firstly with distilled water (50 mL) (twice) and then with saturated aqueous sodium bicarbonate solution (50 mL). The organic layer was dried by filtration through anhydrous sodium sulfate. The organic layer containing the product was reduced *in vacuo*. The pure product was isolated by flash column chromatography over silica gel (hexane: ethyl acetate gradient).

3.1.8.1 3-Cyclohexyl-4-(4-methoxyphenyl)-1-(3,4,5-trimethoxyphenyl)azetidin-2-one (10) was obtained from 2-cyclohexylacetyl chloride and N-(4-methoxybenzylidene)-3,4,5-trimethoxybenzenamine **(8a)** as a white powder (15.0% yield); Mp: 144ºC; IR (NaCl film) ν_{max}: 1744.47cm^{-1} (C=O, β-lactam); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.14 – 1.32 (m, 5H, CH_2), 1.68 - 1.78 (m, 3H, CH_2), 1.82 – 1.90 (m, 2H, CH_2), 2.05 – 2.09 (m, 1H, CH), 2.96 (m, 1H, H-3), 3.71 (s, 6H, 2xOCH_3), 3.77 (s, 3H, OCH_3), 3.81 (s, 3H, OCH_3), 4.69 (d, 1H, J=2.52 Hz, H-4), 6.54 (s, 2H, ArH), 6.89 – 6.93 (m, 2H, ArH), 7.28 – 7.31 (m, 2H, ArH); ^{13}C NMR (100 MHz, $CDCl_3$) δ 25.77 (CH_2), 25.92 (CH_2), 26.22 (CH_2), 30.71 (CH_2), 30.92 (CH_2), 38.28 (CH), 55.31 (OCH_3), 55.94 (OCH_3), 58.93 (C-3), 60.93 (OCH_3), 66.11 (C-4), 94.45, 114.51, 127.23, 130.28, 134.05, 134.08, 153.42, 159.57 (ArC), 167.47 (C=O); HRMS (M^++Na): $C_{25}H_{31}NO_5Na$ requires 448.2100; found 448.2101

3.1.8.2 4-(4-Methoxyphenyl)-3-methyl-3-phenyl-1-(3,4,5-trimethoxyphenyl)azetidin-2-one (11) was obtained from 2-phenylpropionyl chloride **(6a)** and N-(4-methoxybenzylidene)-3,4,5-trimethoxybenzenamine **(8a)** as a white powder (29.4% yield); Mp: 183ºC; IR (NaCl film) ν_{max}: 1737.24

cm^{-1} (C=O, β-lactam); ^1H NMR (400 MHz, $CDCl_3$) (*cis* isomer) δ 1.91 (s, 3H, CH_3), 3.72 – 3.73 (m, 9H, 3xOCH_3), 3.79 (s, 3H, OCH_3), 5.00 (s, 1H, H-4), 6.66 (d, 2H, J=7 Hz, ArH), 6.94 (d, 2H, J=8 Hz, ArH), 7.09 – 7.13 (m, 5H, ArH); ^{13}C NMR (100 MHz, $CDCl_3$) (*cis* isomer) δ 24.34 (CH_3), 54.68 (OCH_3), 55.52 (OCH_3), 60.50 (OCH_3), 64.01 (C-3), 68.32 (C-4), 94.53, 113.23, 126.28, 126.81, 127.59, 128.10, 133.60, 133.87, 137.32, 152.96, 158.84 (ArC), 168.80 (C=O); ^1H NMR (400 MHz, $CDCl_3$) (*trans* isomer) δ 1.91 (s, 3H, CH_3), 3.73 (s, 6H, 2xOCH_3), 3.80 (s, 3H, OCH_3), 3.85 (s, 3H, OCH_3), 5.19 (s, 1H, H-4), 6.64 (d, 2H, J=7 Hz, ArH), 6.97 (d, 2H, J=8 Hz, ArH), 7.12 (m, 1H, ArH), 7.28 – 7.34 (m, 3H, ArH), 7.40 – 7.44 (t, 2H, ArH), 7.55 – 7.56 (m, 2H, ArH); ^{13}C NMR (100 MHz, $CDCl_3$) (*trans* isomer) δ 19.20 (CH_3), 54.86 (OCH_3), 55.52 (OCH_3), 55.58 (OCH_3), 60.53 (OCH_3), 62.13 (C-3), 66.56 (C-4), 94.68, 113.23, 113.80, 125.43, 126.08, 126.81, 126.90, 127.59, 127.82, 128.10, 128.47, 133.24, 141.43, 153.01, 159.13 (ArC), 168.77 (C=O); HRMS (M^++H): $C_{26}H_{28}NO_5$ requires 434.1967; found 434.1953

3.1.8.3 4-(4-Methoxyphenyl)-3,3-diphenyl-1-(3,4,5-trimethoxyphenyl)azetidin-2-one (12) was obtained 2,2-diphenylacetyl chloride and N-(4-methoxybenzylidene)-3,4,5-trimethoxybenzenamine (**8a**) as a white crystalline material (70.3% yield); Mp: 167ºC; IR (KBr disk) ν_{max}: 1729.20 cm^{-1} (C=O, β-lactam); ^1H NMR (400 MHz, $CDCl_3$) δ 3.72 – 3.74 (m, 9H, 3xOCH_3), 3.78 (s, 3H, OCH_3), 5.74 (s, 1H, H-4), 6.68 – 6.72 (t, 4H, ArH), 7.06 – 7.09 (m, 5H, ArH), 7.16 – 7.18 (m, 2H, ArH), 7.29 – 7.32 (t, 1H, ArH), 7.39 – 7.43 (m, 2H, ArH), 7.67 (d, 2H, J=7.52 Hz, ArH); ^{13}C NMR (100 MHz, $CDCl_3$) δ 54.74 (OCH_3), 55.53 (OCH_3), 58.06 (OCH_3), 60.50 (OCH_3), 66.94 (C-4), 71.53 (C-3), 94.70, 113.35, 126.33, 126.38, 126.78, 127.00, 127.54, 127.91, 128.35, 128.44, 133.26, 134.02, 136.77, 140.44, 152.94, 159.00 (ArC), 166.70 (C=O); HRMS (M^++Na): $C_{31}H_{29}NO_5Na$ requires 518.1943; found 518.1962

3.1.8.4 4-(4-Methoxyphenyl)-3-thiophen-2-yl-1-(3,4,5-trimethoxyphenyl)azetidin-2-one (13) was obtained from 2-(thiophen-2-yl)acetyl chloride and N-(4-methoxybenzylidene)-3,4,5-trimethoxybenzenamine (**8a**) as a white powder (4.6% yield); Mp: 115ºC; IR (NaCl film) ν_{max}: 1756.78cm^{-1} (C=O, β-lactam); ^1H NMR (400 MHz, $CDCl_3$) δ 3.72 (s, 6H, 2xOCH_3), 3.77 (s, 3H,

OCH_3), 3.82 (s, 3H, OCH_3), 4.47 (d, 1H, J=2.5 Hz, H-3), 4.90 (d, 1H, J=2.5 Hz, H-4), 6.59 (s, 2H, ArH), 6.95 (d, 2H, J=8.56 Hz, ArH), 7.01 – 7.03 (t, 1H, ArH), 7.08 (d, 1H, J=3.48 Hz, ArH), 7.26 (d, 1H, ArH, J=5 Hz), 7.36-7.38 (d, 2H, ArH, J=8.52 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 54.93 (OCH_3), 55.58 (OCH_3), 59.78 (C-3), 60.52 (OCH_3), 64.12 (C-4), 94.46, 113.86, 114.27, 124.43, 124.87, 125.29, 126.82, 126.90, 128.29, 133.19, 134.11, 135.70, 148.98, 153.06, 159.60 (ArC), 163.98 (C=O); HRMS (M^++Na): $C_{23}H_{23}NO_5NaS$ requires 448.1195; found 448.1186

3.1.8.5 4-(3-((*tert*-Butyldimethylsilyl)oxy)-4-methoxyphenyl)-3-(thiophen-2-yl)-1-(3,4,5-trimethoxyphenyl)azetidin-2-one (14) was obtained from 2-(thiophen-2-yl)acetyl chloride and [3-(*tert*-butyldimethylsilanyloxy)-4-methoxybenzylidene](3,4,5-trimethoxyphenyl)amine (**8b**) as a brown oil and was desilylated to form **33** without further purification (crude yield: 4.3%).

3.1.8.6 4-(4-Methoxyphenyl)-3-(thiophen-2-ylmethyl)-1-(3,4,5-trimethoxyphenyl)azetidin-2-one (15) was obtained from 3-thiophen-2-yl-propionyl chloride (**6b**) and N-(4-methoxybenzylidene)-3,4,5-trimethoxybenzenamine (**8a**) and isolated as a yellow oil in 0.6% yield; IR (NaCl film) ν_{max}: 1746.67 cm^{-1} (C=O, β-lactam); ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.30 – 3.36 (m, 1H, CH_2), 3.40 – 3.44 (m, 1H, CH_2), 3.49 – 3.54 (m, 1H, H-3), 3.72 (s, 6H, 2xOCH_3), 3.78 (s, 3H, OCH_3), 3.81 (s, 3H, OCH_3), 4.71 (d, 1H, J=2.5 Hz, H-4), 6.54 (s, 2H, ArH), 6.86 – 6.89 (m, 3H, ArH), 6.94 – 6.97 (m, 1H, ArH), 7.16 – 7.19 (m, 3H, ArH); ^{13}C NMR (100 MHz, $CDCl_3$) δ 28.32 (CH_2), 54.84 (OCH_3), 55.56 (OCH_3), 60.25 (C-3), 60.49 (C-4), 94.33, 113.99, 123.71, 125.32, 126.63, 126.85, 128.89, 133.37, 139.45, 153.01, 159.23 (ArC), 165.95 (C=O); HRMS (M^++Na): $C_{24}H_{25}NO_5NaS$ requires 462.1351; found 462.1333

3.1.8.7 1-(3,4,5-Trimethoxyphenyl)-4-(naphthalen-2-yl)-3-(thiophen-2-yl)azetidin-2-one (16) was obtained from 2-(thiophen-2-yl)acetyl chloride and 3,4,5-trimethoxy-N-(naphthalen-2-ylmethylene)aniline (**8d**) as a brown oil (9.1% yield); IR (KBr disk) ν_{max}: 1754.84 cm^{-1} (C=O); ^{1}H-NMR (400 MHz, $CDCl_3$): δ 3.70 (s, 6H, 2xOCH_3), 3.78 (s, 3H, OCH_3), 4.59 (d, 1H, J=2.0 Hz, H-3), 5.15 (d, 1H, J=2.0 Hz, H-4), 6.66 (s, 2H, ArH), 7.07 – 7.08 (m, 1H, ArH), 7.13 – 7.14 (m, 1H, ArH), 7.29 (s, 1H, ArH), 7.34 – 7.35 (m, 1H, ArH), 7.54 – 7.57 (m, 2H, ArH), 7.88 – 8.03 (m, 4H, ArH); ^{13}C-NMR (400 MHz, $CDCl_3$): δ 55.59 (OCH_3), 59.75 (OCH_3), 60.51 (OCH_3), 64.12 (C-3), 64.64 (C-4), 94.46, 114.27,

122.35, 125.02, 125.07, 125.47, 126.26, 126.41, 126.96, 127.46, 129.17, 132.89, 133.06, 133.25, 133.94, 135.55, 153.13 (ArC), 163.88 (C=O); HRMS (M^++Na): $C_{23}H_{23}NO_5NaS$ requires 468.1245; found 468.1227

3.1.8.8 1-(3,4,5-Trimethoxyphenyl)-4-(naphthalen-1-yl)-3-(thiophen-2-yl)azetidin-2-one (17) was obtained from 2-(thiophen-2-yl)acetyl chloride and 3,4,5-trimethoxy-N-(naphthalen-1-ylmethylene)aniline (**8e**) as a brown oil (15.2% yield); IR (KBr disk) ν_{max}: 1755.27 cm^{-1} (C=O); ^1H NMR (400 MHz, $CDCl_3$): δ 3.71 (s, 6H, 2xOCH_3), 3.83 (s, 3H, OCH_3), 4.54 (d, 1H, J=2.5 Hz), 5.77 (d, 1H, J=2.5 Hz), 6.69 (s, 2H, ArH), 7.07 (m, 1H, ArH), 7.14 (s, 1H, ArH), 7.38-7.40 (m, 1H, ArH), 7.49 – 7.59 (m, 4H, ArH), 7.88 – 7.98 (m, 3H, ArH); ^{13}C NMR (100 MHz, $CDCl_3$): δ 55.70 (OCH_3), 55.77 (OCH_3), 59.51 (OCH_3), 60.55 (C-3), 61.86 (C-4), 94.71, 122.39, 125.12, 125.22, 125.81, 126.29, 126.37, 127.06, 128.50, 128.73, 130.01, 131.89, 133.33, 133.49, 134.30, 135.96, 153.21 (ArC), 164.17 (C=O); HRMS (M^++Na): $C_{26}H_{23}NO_4NaS$ requires 468.1245 found 468.1269

3.1.8.9 1-(3,4,5-Trimethoxyphenyl)-3,4-di(thiophen-2-yl)azetidin-2-one (18) was obtained from 2-(thiophen-2-yl)acetyl chloride and 3,4,5-trimethoxy-N-(thiophen-2-ylmethylene)aniline (**8f**) as a brown oil (6.7% yield); IR (KBr disk) ν_{max}: 1755.61 cm^{-1} (C=O); ^1H NMR (400 MHz, $CDCl_3$): δ 3.78 (s, 6H, 2xOCH_3), 3.81 (s, 3H, OCH_3), 4.68 (d, 1H, J=2 Hz), 5.23 (d, 1H, J=2 Hz), 6.67 (s, 2H, ArH), 7.06- 7.09 (m, 1H, ArH), 7.08 (d, 1H, J=3.52 Hz, ArH), 7.23 -7.24 (m, 1H, ArH), 7.33 (m, 1H, ArH), 7.42 (m, 2H, ArH); ^{13}C NMR (100 MHz, $CDCl_3$): δ 56.07 (OCH_3), 60.98 (C-3), 61.07 (C-4), 94.9, 98.33, 125.58, 125.97, 126.20, 126.37, 127.41, 127.48, 133.44, 135.60, 140.69, 153.57 (ArC), 163.96 (C=O); HRMS (M^++Na): $C_{26}H_{23}NO_4NaS$ requires 424.4889; found 424.0628

3.1.9 General method II for β-lactam preparation: The appropriate imine (10 mmol) and acetyl chloride (10 mmol) were added to CH_2Cl_2 (50 mL) under nitrogen and the mixture was left stirring for 2 hours. Triethylamine (10 mmol) was added dropwise. The mixture was left to stir overnight. The mixture was washed firstly with distilled water (50 mL) (twice) and then with saturated aqueous sodium bicarbonate solution (50 mL). The organic layer was dried by filtration through anhydrous sodium

sulfate. The organic layer containing the product was collected and reduced *in vacuo*. The pure product was isolated by flash column chromatography over silica gel (hexane: ethyl acetate gradient).

3.1.9.1 4-(4-Methoxy-3-nitrophenyl)-3-thiophen-2-yl-1-(3,4,5-trimethoxyphenyl)azetidin-2-one (19) was obtained from 2-(thiophen-2-yl)acetyl chloride and (4-methoxy-3-nitrobenzylidene)(3,4,5-trimethoxyphenyl)amine (**8c**) as a brown powder (48.4% yield); Mp: 123ºC; IR (KBr disk) ν_{max}: 1742.06cm^{-1} (C=O, β-lactam); ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.76 (s, 6H, 2xOCH_3), 3.79 (s, 3H, OCH_3), 4.00 (s, 3H, OCH_3), 4.49 (d, 1H, J=2.5 Hz, H-3), 4.98 (d, 1H, J=2.5 Hz, H-4), 6.57 (s, 2H, ArH), 7.03 – 7.05 (m, 1H, ArH), 7.10 (d, 1H, J=3 Hz, ArH), 7.18 (d, 1H, J=8.52 Hz, ArH), 7.33 (d, 1H, J=5 Hz, ArH), 7.60 – 7.63 (dd, 1H, ArH), 7.94 (s, 1H, ArH); ^{13}C NMR (100 MHz, $CDCl_3$) δ 55.78 (OCH_3), 56.31 (OCH_3), 59.75 (C-3), 60.52 (OCH_3), 62.92 (C-4), 94.48, 114.25, 123.13, 125.28, 125.66, 127.07, 128.82, 130.78, 132.59, 134.56, 134.77, 139.45, 152.80 (ArC), 163.33 (C=O); HRMS (M^++Na): $C_{23}H_{22}N_2O_7NaS$ requires 493.1045; found 493.1047

3.1.10 General method III for β-lactam preparation: The appropriate acetic acid (15 mmol) was refluxed for 30 minutes with triphosgene [bis(trichloromethyl) carbonate] (5 mmol) in dry CH_2Cl_2 (50 mL). A solution of the appropriately substituted imine (10 mmol) in dry CH_2Cl_2 (10 mL) was added dropwise to the refluxing solution. Triethylamine (30 mmol) was added. The reaction mixture was heated at reflux for 5 hours and stirred at room temperature overnight. The mixture was washed firstly with distilled water (twice) (50 mL) and then with saturated aqueous sodium bicarbonate solution (50 mL). The organic layer was dried over anhydrous sodium sulphate. The pure product was isolated by flash column chromatography over silica gel (hexane:ethyl acetate gradient).

3.1.10.1 4-(4-Methoxyphenyl)-3-naphthalen-1-yl-1-(3,4,5-trimethoxyphenyl)azetidin-2-one (20) was obtained from 2-(naphthalen-1-yl)acetic acid and N-(4-methoxybenzylidene)-3,4,5-trimethoxybenzenamine (**8a**) as a pale yellow crystalline powder (6.9% yield); Mp: 164ºC; IR (NaCl film) ν_{max}: 1741.44 cm^{-1} (C=O, β-lactam); ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.72 (s, 6H, 2xOCH_3), 3.77 (s, 3H, OCH_3), 3.87 (s, 3H, OCH_3), 4.78 (d, 1H, J=2.4 Hz, H-3), 4.98 (d, 1H, J=2.4 Hz, H-4), 6.63 (s, 2H, ArH), 7.00 (d, 2H, J=8.52 Hz, ArH), 7.36 – 7.46 (m, 6H, ArH), 7.77 (d, 1H, J= 7.04 Hz, ArH), 7.84 (d,

1H, J=8.52 Hz, ArH), 7.89 (d, 1H, J=8.04 Hz, ArH); ^{13}C NMR (100 MHz, $CDCl_3$) δ 54.94 (OCH_3), 55.54 (OCH_3), 60.51 (OCH_3), 62.00 (C-3), 63.33 (C-4), 94.39, 114.30, 123.29, 123.82, 125.28, 125.58, 125.96, 127.47, 128.00, 128.46, 128.97, 131.16, 131.21, 133.29, 133.44, 134.01, 153.05, 159.65 (ArC), 165.27 (C=O); HRMS (M^++Na): $C_{29}H_{27}NO_5Na$ requires 492.1787; found 492.1774

3.1.10.2 4-(4-Methoxyphenyl)-3-naphthalen-2-yl-1-(3,4,5-trimethoxyphenyl)azetidin-2-one (21) was obtained from 2-(naphthalen-2-yl)acetic acid and N-(4-methoxybenzylidene)-3,4,5-trimethoxybenzenamine (**8a**) as a white solid (2.5% yield); Mp: 150ºC; IR (NaCl film) ν_{max}: 1739.65 cm^{-1} (C=O, β-lactam); ^{1}H NMR (600 MHz, $CDCl_3$) δ 3.73 (s, 6H, 2xOCH_3), 3.78 (s, 3H, OCH_3), 3.84 (s, 3H, OCH_3), 4.45 (d, 1H, J=2.52 Hz, H-3), 4.94 (d, 1H, J=2.48 Hz, H-4), 6.64 (s, 2H, ArH), 6.95 – 6.97 (m, 2H, ArH), 7.36 – 7.42 (m, 3H, ArH), 7.48 – 7.50 (m, 2H, ArH), 7.80 – 7.88 (m, 4H, ArH); ^{13}C NMR (100 MHz, $CDCl_3$) δ 55.41, 56.06 (OCH_3), 61.00 (OCH_3), 63.94 (C-3), 65.27 (C-4), 94.89, 114.75, 125.04, 126.23, 126.52, 127.39, 127.74, 127.88, 128.97, 129.30, 132.14, 132.87, 133.48, 133.76, 134.51, 153.55, 160.02 (ArC), 165.65 (C=O); HRMS M^++Na): $C_{29}H_{27}NO_5Na$ requires 492.1787; found 492.1790

3.1.10.3 4-(4-Methoxyphenyl)-3-(1-methyl-1H-indol-2-yl)-1-(3,4,5-trimethoxyphenyl)azetidin-2-one (22) was obtained from 2-(1-methyl-1H-indol-2-yl)acetic acid and N-(4-methoxybenzylidene)-3,4,5-trimethoxybenzenamine (**8a**) as a yellow solid (11.9% yield); Mp: 77 – 78 ºC; IR (NaCl film) ν_{max}: 1747.66 cm^{-1} (C=O, β-lactam); ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.75 (s, 6H, NCH_3, OCH_3), 3.80 (s, 3H, OCH_3), 3.81 (s, 3H, OCH_3), 3.87 (s, 3H, OCH_3), 4.52 (d, 1H, J=2.0 Hz, H-3), 4.94 (d, 1H, J=2.0 Hz, H-4), 6.66 (s, 2H, ArH), 6.99 (d, 2H, J=8.52 Hz, ArH), 7.17 (m, 2H, ArH), 7.29 (m, 1H, ArH), 7.40 – 7.42 (m, 4H, ArH); ^{13}C NMR (100 MHz, $CDCl_3$) δ 32.34 (NCH_3), 54.92 (OCH_3), 55.56 (OCH_3), 57.49 (C-3), 60.52 (OCH_3), 63.22 (C-4), 94.32, 107.51, 109.67, 114.21, 118.51, 119.16, 121.72, 126.33, 126.59, 126.92, 129.36, 133.61, 133.90, 136.84, 153.07, 159.39 (ArC), 166.09 (C=O); HRMS (M^++H): $C_{28}H_{29}N_2O_5$ requires 473.2076; found 473.2075

3.1.10.4 3-Furan-3-yl-4-(4-methoxyphenyl)-1-(3,4,5-trimethoxyphenyl)azetidin-2-one (23) was obtained from 2-(furan-3-yl)acetic acid and N-(4-methoxybenzylidene)-3,4,5-trimethoxybenzenamine

(**8a**) as brown crystals (4.9% yield); Mp: 127ºC; IR (NaCl film) ν_{max}: 1743.69 cm^{-1} (C=O, β-lactam); ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.75 (s, 6H, 2xOCH_3), 3.80 (s, 3H, OCH_3), 3.85 (s, 3H, OCH_3), 4.34 (d, 1H, J=2.5 Hz, H-3), 5.06 (d, 1H, J=2.5 Hz H-4), 6.35 (d, 1H, J=3.28 Hz, ArH), 6.41 (t, 1H, ArH), 6.62 (s, 2H, ArH), 6.96 (d, 2H, J=4.52 Hz, ArH), 7.36 – 7.38 (m, 2H, ArH), 7.45 (d, 1H, J=0.76 H, ArH); ^{13}C NMR (100 MHz, $CDCl_3$) δ 55.38 (OCH_3), 56.05 (OCH_3), 58.67 (C-3), 60.97 (OCH_3), 61.43 (C-4), 94.93, 108.75, 110.69, 114.68, 127.37, 128.82, 133.75, 134.58, 142.86, 147.50, 153.53, 160.03 (ArC), 163.38 (C=O); HRMS (M^++H): $C_{23}H_{24}NO_6$ requires 410.1604; found 410.1605

3.1.10.5 4-(4-Methoxyphenyl)-3-thiophen-3-yl-1-(3,4,5-trimethoxyphenyl)azetidin-2-one (24) was obtained from 2-(thiophen-3-yl)acetic acid and N-(4-methoxybenzylidene)-3,4,5-trimethoxybenzenamine (**8a**) as a off-white powder (19.2% yield); Mp: 130ºC; IR (KBr disk) ν_{max}: 1750.82 cm^{-1} (C=O, β-lactam); ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.74 (s, 6H, 2xOCH_3), 3.79 (s, 3H, OCH_3), 3.85 (s, 3H, OCH_3), 4.35 (d, 1H, J=2.52 Hz, H-3), 4.87 (d, 1H, J=2.52 Hz, H-4), 6.61 (s, 2H, ArH), 6.96 – 6.98 (m, 2H, ArH), 7.09 – 7.11 (m, 1H, ArH), 7.29 – 7.30 (m, 1H, ArH), 7.36 – 7.40 (m, 3H, ArH); ^{13}C NMR (100 MHz, $CDCl_3$) δ 54.93 (OCH_3), 55.56 (OCH_3), 60.09 (OCH_3), 60.52 (C-3), 62.89 (C-4), 94.34, 114.25, 122.01, 125.85, 126.42, 126.85, 133.33, 134.20, 153.05, 159.52 (ArC), 164.89 (C=O); HRMS (M^++Na): $C_{23}H_{23}NO_5NaS$ requires 448.1195; found 448.1189

3.1.10.6 4-(4-Methoxyphenyl)-3-(5-methylthiophen-2-yl)-1-(3,4,5-trimethoxyphenyl)azetidin-2-one (25) was obtained from 2-(5-methylthiophen-2-yl)acetic acid (**5b**) and N-(4-methoxybenzylidene)-3,4,5-trimethoxybenzenamine (**8a**) as a brown oil (3.1% yield); IR (NaCl film) ν_{max}: 1736.68 cm^{-1} (C=O, β-lactam); ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.50 (s, 3H, CH_3), 3.74 (s, 6H, 2xOCH_3), 3.84 – 3.87 (m, 6H, 2xOCH_3), 4.41 (d, 1H, J=2.5 Hz, H-3), 4.89 (d, 1H, J=2.5 Hz, H-4), 6.60 (s, 2H, ArH), 6.67 – 6.71 (m, 3H, ArH), 6.86 (d, 1H, ArH, J=3.52 Hz), 6.96 (d, 2H, ArH, J=8.76 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 15.36 (CH_3), 55.38 (OCH_3), 56.04 (OCH_3), 60.51 (OCH_3), 60.97 (C-3), 61.00 (OCH_3), 64.62 (C-4), 94.93, 114.70, 125.30, 125.73, 127.26, 128.89, 133.73, 140.03, 153.45, 153.52, 160.02 (ArC), 164.71 (C=O); HRMS (M^++H): $C_{24}H_{26}NO_5S$ requires 440.1532; found 440.1535

3.1.10.7 3-Benzo[b]thiophen-2-yl-4-(4-methoxyphenyl)-1-(3,4,5-trimethoxyphenyl)azetidin-2-one (26) was obtained from 2-(benzo[b]thiophen-2-yl)acetic acid (**5a**) and N-(4-methoxybenzylidene)-3,4,5-trimethoxybenzenamine (**8a**) as a white solid (5.6% yield); Mp: 118ºC; IR (NaCl film) ν_{max}: 1747.58 cm^{-1} (C=O, β-lactam); ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.75 (s, 6H, 2xOCH_3), 3.81 (s, 3H, OCH_3), 3.86 (s, 3H, OCH_3), 4.56 (d, 1H, J=2.0 Hz, H-3), 5.02 (d, 1H, J=2.0 Hz, H-4), 6.63 (s, 2H, ArH), 6.99 (d, 2H, J=8.52 Hz, ArH), 7.35 – 7.41 (m, 5H, ArH), 7.76 (d, 1H, J=7.04 Hz, ArH), 7.83 (d, 1H, J=7.52, ArH); ^{13}C NMR (100 MHz, $CDCl_3$) δ 54.95 (OCH_3), 55.60 (OCH_3), 60.35 (OCH_3), 60.53 (C-3), 63.60 (C-4), 94.48, 114.33, 121.82, 121.99, 123.16, 124.10, 124.17, 126.84, 128.16, 133.13, 134.20, 136.49, 139.11, 153.10, 159.68 (ArC), 163.36 (C=O); HRMS (M^++H): $C_{27}H_{26}NO_5S$ requires 476.1532; found 476.1537

3.1.10.8 4-(3-((tert-Butyldimethylsilyl)oxy)-4-methoxyphenyl)-3-(thiophen-3-yl)-1-(3,4,5-trimethoxyphenyl)azetidin-2-one (27) was obtained from 2-(thiophen-3-yl)acetic acid and [3-(*tert*-butyldimethylsilanyloxy)-4-methoxybenzylidene](3,4,5-trimethoxyphenyl)amine (**8b**) as a brown oil and was desilylated to form **29** without further purification (crude yield: 79.5%).

3.1.11 General method IV for preparation of β-lactams 28 – 29: To a solution of the appropriately protected phenol (10 mmol) in THF (50 mL) was added 1.5 equivalents of 1M tetrabutylammonium fluoride. The solution was stirred in an ice-bath for 15 minutes. The reaction mixture was diluted with ethyl acetate (100 mL) and quenched with 10% HCl (100 mL). The layers were separated and the aqueous layer was extracted with ethyl acetate (2 x 50 mL). The organic layer was then washed with water (100 mL) and brine (100 mL) and was dried with sodium sulphate. The pure product was isolated by flash column chromatography over silica gel (hexane: ethyl acetate gradient).

3.1.11.1 4-(3-Hydroxy-4-methoxyphenyl)-3-thiophen-2-yl-1-(3,4,5-trimethoxyphenyl)azetidin-2-one (28) was obtained from 4-(3-((*tert*-butyldimethylsilyl)oxy)-4-methoxyphenyl)-3-(thiophen-2-yl)-1-(3,4,5-trimethoxyphenyl)azetidin-2-one (**14**) as brown crystals (1.3% overall yield); Mp: 113-114ºC; IR (KBr disk) ν_{max}: 1721.07cm^{-1} (C=O, β-lactam); ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.76 (s, 6H, 2xOCH_3), 3.80 (s, 3H, OCH_3), 3.94 (s, 3H, OCH_3), 4.48 (d, 1H, J=2.5 Hz, H-3), 4.87 (d, 1H, J=2.5 Hz, H-4), 5.75 (s, 1H, OH), 6.62 (s, 2H, ArH), 6.89 – 6.95 (m, 2H, ArH), 7.01 – 7.03 (m, 3H, ArH), 7.31 – 7.32 (m,

1H, ArH); ^{13}C NMR (100 MHz, $CDCl_3$) δ 55.58 (OCH_3), 55.61 (OCH_3), 59.70 (OCH_3), 60.51 (C-3), 64.07 (C-4), 94.50, 110.60, 111.46, 117.36, 124.86, 125.28, 126.87, 129.54, 133.18, 134.15, 135.68, 145.93, 146.53, 149.32, 153.06 (ArC), 163.90 (C=O); HRMS (M^++Na): $C_{23}H_{23}NO_6SNa$ requires 464.1144; found 464.1124

3.1.11.2 4-(3-Hydroxy-4-methoxyphenyl)-3-thiophen-3-yl-1-(3,4,5-trimethoxyphenyl)azetidin-2-one (29) was obtained from 4-(3-((*tert*-butyldimethylsilyl)oxy)-4-methoxyphenyl)-3-(thiophen-3-yl)-1-(3,4,5-trimethoxyphenyl)azetidin-2-one (**27**) as a pale pink solid (18.6% yield); Mp: 151 – 152ºC; IR (KBr disk) ν_{max}: 1739.65 cm^{-1} (C=O, β-lactam), 3187.91 cm^{-1} (-OH); ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.76 (s, 6H, 2xOCH_3), 3.80 (s, 3H, OCH_3), 3.93 (s, 3H, OCH_3), 4.34 (d, 1H, J=2.2, H-3), 4.82 (d, 1H, J=2.2 Hz, H-4), 5.77 (s, 1H, OH), 6.63 (s, 2H, ArH), 6.89 – 6.96 (m, 2H, ArH), 7.02 (m, 1H, ArH), 7.09 – 7.11 (m, 1H, ArH), 7.29 – 7.31 (m, 1H, ArH), 7.39 – 7.41 (m, 1H, ArH); ^{13}C NMR (100 MHz, $CDCl_3$) δ 56.05 (OCH_3), 56.09 (OCH_3), 60.48 (C-3), 60.98 (OCH_3), 63.32 (C-4), 94.87, 111.06, 111.98, 117.81, 122.44, 126.33, 126.85, 130.43, 133.78, 134.65, 146.39, 146.93, 153.53 (ArC), 165.28 (C=O); HRMS (M^++Na): $C_{23}H_{23}NO_6SNa$ requires 464.1144; found 464.1153

3.1.12 4-(3-Amino-4-methoxyphenyl)-3-thiophen-2-yl-1-(3,4,5-trimethoxyphenyl)azetidin-2-one (30): To 4-(4-methoxy-3-nitrophenyl)-3-thiophen-2-yl-1-(3,4,5-trimethoxyphenyl)azetidin-2-one (**19**) (10 mmol) in glacial AcOH (5 mL) was added metallic zinc dust (10 equiv.). The mixture was stirred for 6 days at room temperature under nitrogen until TLC indicted formation of product. The residue was filtered through Celite and was extracted with dichloromethane. The amino compound was isolated using a hexane and ethyl acetate gradient column. and was obtained as a brown residue (48.5% yield); IR (NaCl film) ν_{max}: 1749.94 cm^{-1} (C=O, β-lactam); ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.76 (s, 6H, 2xOCH_3), 3.80 (s, 3H, OCH_3), 3.89 (s, 3H, OCH_3), 4.49 (d, 1H, J=2 Hz, H-3), 4.83 (d, 1H, J=2.52 Hz, H-4), 6.64 (s, 2H, ArH), 6.78 – 6.81 (m, 3H, ArH), 7.03 – 7.05 (m, 1H, ArH), 7.08 – 7.09 (m, 1H, ArH), 7.29 – 7.31 (m, 1H, ArH) ; ^{13}C NMR (100 MHz, $CDCl_3$) δ 55.59 (OCH_3), 56.07 (OCH_3), 60.13 (C-3), 60.98 (OCH_3), 64.84 (C-4), 94.93, 110.53, 111.56, 116.43, 125.25, 125.70, 127.31, 129.34, 133.81,

134.50, 136.36, 136.96, 147.76, 153.50 (ArC), 164.60 (C=O); HRMS (M^++H): $C_{23}H_{25}N_2O_5S$ requires 441.1484; found 441.1471

3.1.13 General procedure for synthesis of β-lactams 31 – 34. A solution of 4-(4-methoxyphenyl)-1-(3,4,5-trimethoxyphenyl)azetidin-2-one **9** (2.5 mmol) in dry THF (20 mL) was stirred at -78 ˚C under a nitrogen atmosphere. A 2M lithium diisopropylamide (5 mmol) solution was added quickly and the mixture was stirred for 5 minutes at -78 ˚C. A solution of the appropriate aldehyde (3.75 mmol) in dry tetrahydrofuran (5 mL) was added slowly to the reaction mixture. The reaction was stirred at -78 ˚C for 30 minutes after which the reaction mixture was allowed to heat up to room temperature. It was poured into a saturated sodium chloride solution (50 mL). This solution was extracted with ethyl acetate, the organic layer was separated and was dried over anhydrous sodium sulphate. The pure product was isolated by flash column chromatography over silica gel (hexane: ethyl acetate gradient).

3.1.13.1 3-(Furan-3-yl-hydroxymethyl)-4-(4-methoxyphenyl)-1-(3,4,5-trimethoxyphenyl)azetidin-2-one (31) was obtained as a yellow oil by reaction of furan-3-carbaldehyde and 4-(4-methoxyphenyl)-1-(3,4,5-trimethoxyphenyl)azetidin-2-one (**9**) in 50.2% yield; IR (NaCl film) ν_{max}: 1740.12 cm^{-1} (C=O, β-lactam), 3453.40 cm^{-1} (-OH); ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.44 – 3.49 (m, 1H, H-3), 3.77 (s, 6H, 2xOCH_3), 3.81 (s, 3H, OCH_3), 3.82 (s, 3H, OCH_3), 4.84 (d, 0.6H, J=2.52 Hz, H-3), 5.08 (d, 0.4H, J=2 Hz, H-4), 5.14 (t, 0.6H), 6.55 (m, 3H, ArH), 6.86 – 6.91 (m, 2H, ArH), 7.18 – 7.25 (m, 2H, ArH), 7.39 – 7.41 (m, 1.3H, ArH); ^{13}C NMR (100 MHz, $CDCl_3$) δ 53.00 (OCH_3), 54.83 (C-4), 54.88 (OCH_3), 55.51 (OCH_3), 55.59 (OCH_3), 55.83 (OCH_3), 57.32 (C-3), 59.98 (OCH_3), 60.48 (OCH_3), 63.31, 64.52, 64.79, 65.14 (CH), 94.26, 94.32, 108.16, 108.81, 114.02, 114.12, 125.25, 125.83, 126.97, 127.00, 128.48, 128.81, 133.15, 133.97, 139.00, 139.81, 143.19, 143.23, 153.00, 159.17, 159.39 (ArC), 164.79 (C=O), 164.81 (C=O); HRMS (M^++Na): $C_{24}H_{25}NO_7Na$ requires 462.1529; found 462.1509

3.1.13.2 3-(Hydroxythiophen-2-yl-methyl)-4-(4-methoxyphenyl)-1-(3,4,5-trimethoxyphenyl)azetidin-2-one (32) was obtained as a light yellow powder from thiophene-2-carbaldehyde and 4-(4-methoxyphenyl)-1-(3,4,5-trimethoxyphenyl)azetidin-2-one (**9**) in 12.4% yield by

the above method; Mp: 96ºC; IR (KBr disk) ν_{max}: 1745.90 cm^{-1} (C=O, β-lactam), 3436.73 cm^{-1} (-OH); ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.53 – 3.56 (m, 1H, H-3), 3.71 (s, 6H, 2xOCH_3), 3.77 – 3.81 (m, 6H, 2xOCH_3), 4.83 (d, 0.6H, J=2.5 Hz, H-4), 5.20 (d, 0.4Hz, J=2.5 Hz, CH), 5.41 (d, 0.6H, J=6.52 Hz, CH), 5.61 (d, 0.4H, J=3.92 Hz, CH), 6.55 (d, 2H, J=6 Hz, ArH), 6.83 – 6.89 (m, 2H, ArH), 6.92 – 6.96 (m, 1H, ArH), 7.00 – 7.02 (m, 0.6H, ArH), 7.14 – 7.17 (m, 2.5H, ArH), 7.26 – 7.29 (m, 1H, ArH); ^{13}C NMR (100 MHz, $CDCl_3$) δ 54.81 (OCH_3), 54.86 (OCH_3), 55.51 (C-4), 55.54, 55.59, 55.63, 57.54 (C-3), 60.49 (OCH_3), 65.50, 65.94, 66.04, 68.02 (CH), 94.31, 94.39, 94.63, 113.58, 113.91, 114.04, 123.55, 124.55, 124.91, 125.34, 126.42, 126.44, 126.85, 127.05, 128.26, 128.80, 133.09, 133.12, 133.92, 134.01, 143.48, 144.59, 152.96, 153.00, 153.05, 159.11, 159.33 (ArC), 164.64 (C=O); HRMS (M^++Na): $C_{24}H_{25}NO_6NaS$ requires 478.1300; found 478.1292

3.1.13.3 3-(Hydroxythiophen-3-yl-methyl)-4-(4-methoxyphenyl)-1-(3,4,5-trimethoxyphenyl)azetidin-2-one (33) was obtained from thiophene-3-carbaldehyde and 4-(4-methoxyphenyl)-1-(3,4,5-trimethoxy-phenyl)azetidin-2-one (**9**) as a yellow powder (43.6% yield) by the above method; Mp: 69ºC; IR (NaCl film) ν_{max}: 1745.08 cm^{-1} (C=O, β-lactam), 3438.10 cm^{-1} (-OH); ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.85 (broad s, 0.4H), 3.01 (broad s, 0.4H), 3.48 – 3.52 (m, 1H, H-3), 3.71 (s, 6H, 2xOCH_3), 3.77 (m, 6H, 2xOCH_3), 4.82 (d, 0.6H, J=2 Hz, H-4), 5.08 (d, 0.6H, J=2 Hz, H-4), 5.25 (d, 0.5H, J=6 Hz), 5.45 (s, 0.5H), 6.55 (s, 2H, ArH), 6.81 – 7.02 (m, 2H, ArH), 7.02 (m, 1H, ArH), 7.15 – 7.17 (m, 2H, ArH), 7.28 – 7.41 (m, 2H, ArH); ^{13}C NMR (100 MHz, $CDCl_3$) δ 54.80 (OCH_3), 54.87 (OCH_3), 55.56 (C-4), 55.59, 57.44 (C-3), 59.22, 60.49, 65.12, 65.64, 66.34, 66.45, 67.96 (CH), 76.81, 94.28, 94.34, 94.60, 113.59, 113.91, 114.07, 120.70, 122.41, 125.00, 125.97, 126.14, 126.19, 126.72, 126.84, 128.48, 128.89, 133.20, 133.91, 133.96, 141.61, 142.25, 152.99, 153.05, 159.01, 159.32 (ArC), 164.97 (C=O), 165.53 (C=O); HRMS (M^++Na): $C_{24}H_{25}NO_6NaS$ requires 478.1300; found 478.1282

3.1.13.4 3-[Hydroxy-(5-methylthiophen-2-yl)-methyl]-4-(4-methoxyphenyl)-1-(3,4,5-trimethoxyphenyl)azetidin-2-one (34) was obtained by reaction of 5-methylthiophene-2-carbaldehyde and 4-(4-methoxyphenyl)-1-(3,4,5-trimethoxyphenyl)azetidin-2-one (**9**) as a white powder (16.5% yield) by the above method; Mp: 186ºC; IR (KBr disk) ν_{max}: 1736.68 cm^{-1} (C=O, β-lactam), 3500 cm^{-1} (-OH);

^{1}H NMR (400 MHz, $CDCl_3$) δ 2.48 (s, 3H, CH_3), 2.79 (s, 1H, OH), 3.53 – 3.55 (dd, 1H, H-3), 3.73 (s, 6H, 2xOCH_3), 3.78 (s, 3H, OCH_3), 3.82 (s, 3H, OCH_3), 4.83 (d, 0.8H, J=2.52 Hz, H-4), 5.19 (d, 0.2H, J=2 Hz, CH), 5.31 (d, 0.8 Hz, J=6.56 Hz, CH), 5.50 (s, 0.2H), 6.56 (s, 2H, ArH), 6.65 (m, 1H, ArH), 6.87 – 6.95 (m, 3H, ArH), 7.17 – 7.20 (m, 1H, ArH), 7.29 (s, 1H, ArH); ^{13}C NMR (100 MHz, $CDCl_3$) δ 14.95 (CH_3), 54.86 (OCH_3), 55.54 (OCH_3), 57.61 (C-3), 60.50 (OCH_3), 65.34 (C-4), 68.21 (CH), 94.30, 113.94, 114.04, 124.41, 125.02, 126.90, 128.38, 133.20, 140.24, 140.82, 153.01 (ArC), 163.54 (C=O); HRMS (M^++Na): $C_{25}H_{27}NO_6NaS$ requires 492.1457; found 492.1473

3.1.14 General procedure for oxidation of alcohols 32 and 33: Pyridinium chlorochromate (10 mmol) was suspended in anhydrous dichloromethane (15 mL). The appropriate alcohol (**32**, **33**) (15 mmol, 1.5 equiv.) was dissolved in anhydrous dichloromethane (20 mL) and was added to the pyridinium chlorochromate suspension. The solution became briefly homogenous before depositing the black insoluble reduced reagent and was stirred for a further 2 hours. The reaction mixture was then diluted with 5 volumes of anhydrous ether. The solvent was decanted and the black residue was further washed with ether until the entire oxidised product was removed. The solvent was removed *in vacuo* and the product was isolated by flash column chromatography over silica gel using a hexane: ethyl acetate gradient elution.

3.1.14.1 4-(4-Methoxyphenyl)-3-(thiophene-2-carbonyl)-1-(3,4,5-trimethoxyphenyl)azetidin-2-one (35) was prepared from 3-(hydroxythiophen-2-yl-methyl)-4-(4-methoxyphenyl)-1-(3,4,5-trimethoxyphenyl)azetidin-2-one (**32**) as a yellow powder (27.3% yield); Mp: 123 ˚C; IR (KBr disk) ν_{max}: 1751.87 cm^{-1} (β-lactam -C=O), 1655.95 cm^{-1} (C=O); ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.73 (s, 6H, 2xOCH_3), 3.78 (s, 3H, OCH_3), 3.83 (s, 3H, OCH_3), 4.71 (d, 1H, J=2.52 Hz, H-3), 5.65 (d, 1H, J=2.52 Hz, H-4), 6.57 (s, 2H, ArH), 6.96 (d, 2H, J=8.56, ArH), 7.21 – 7.23 (t, 1H, ArH), 7.41 (d, 2H, J=8.52 Hz, ArH), 7.76 (d, 1H, J=4.52 Hz, ArH), 8.01 (d, 1H, J=4 Hz, ArH); ^{13}C NMR (100 MHz, $CDCl_3$) δ 54.92 (OCH_3), 55.41 (OCH_3), 55.54 (C-4), 60.51 (OCH_3), 68.20 (C-3), 94.39, 114.24, 127.20, 127.87, 128.19, 132.94, 134.21, 134.58, 135.02, 142.35, 153.03 (ArC), 159.51 (C_2=O), 159.61 (C_2=O), 182.86 (C=O); HRMS (M^++Na): $C_{24}H_{23}NO_6NaS$ requires 476.1144; found 476.1141

3.1.14.2 4-(4-Methoxyphenyl)-3-(thiophene-3-carbonyl)-1-(3,4,5-trimethoxyphenyl)azetidin-2-one (36) was prepared from 3-(hydroxythiophen-3-yl-methyl)-4-(4-methoxyphenyl)-1-(3,4,5-trimethoxyphenyl)azetidin-2-one (**33**) as a white solid (18.6% yield); Mp: 143 – 144 ˚C; IR (KBr disk) ν_{max}: 1735.14 cm^{-1} (β-lactam -C=O), 1673.80 cm^{-1} (C=O); ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.74 (s, 6H, 2xOCH_3), 3.79 (s, 3H, OCH_3), 3.84 (s, 3H, OCH_3), 4.68 (d, 1H, J=2.44 Hz, H-3), 5.67 (d, 1H, J=2.44 Hz, H-4), 6.58 (s, 2H, ArH), 6.96 (d, 2H, J=8.32, ArH), 7.37 – 7.43 (m, 3H, ArH), 7.70 (d, 1H, J=1 Hz, ArH), 8.43 – 8.44 (m, 1H, ArH); ^{13}C NMR (100 MHz, $CDCl_3$) δ 55.38 (OCH_3), 55.69 (OCH_3), 56.00 (C-4), 60.98 (OCH_3), 69.45 (C-3), 94.85, 114.70, 126.64, 127.12, 127.64, 128.45, 133.45, 134.69, 135.34, 140.91, 153.51 (ArC), 160.05 (-C_2=O), 160.25 (-C_2=O), 184.60 (-C=O); HRMS (M^++Na): $C_{24}H_{23}NO_6NaS$ requires 476.1144; found 476.1124

3.1.15 General procedure for dehydration of alcohols 32 and 34: A solution of appropriate alcohol (**32**, **34**) (10 mmol) and tosyl chloride (20 mmol) in dry pyridine (50 mL) was heated at reflux for 5 hours under a nitrogen atmosphere. After cooling, ice/water (50 mL) was added and the mixture was extracted twice with chloroform (50 mL). The combined organic extracts were washed twice with dilute hydrochloric acid (50 mL) and once with water (50 mL), dried with anhydrous sodium sulfate and solvent evaporated *in vacuo*. The pure product was isolated by flash column chromatography over silica gel (hexane: ethyl acetate gradient).

3.1.15.1 (*Z*)-4-(4-Methoxyphenyl)-3-thiophen-2-ylmethylene-1-(3,4,5-trimethoxyphenyl)azetidin-2-one (37) was prepared from 3-(hydroxythiophen-2-yl-methyl)-4-(4-methoxyphenyl)-1-(3,4,5-trimethoxyphenyl)azetidin-2-one (**32**) in 30.0% yield as a yellow oil; IR (KBr disk) ν_{max}: 1721.18 cm^{-1} (-C=O); ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.77 (s, 6H, 2xOCH_3), 3.80 (s, 3H, OCH_3), 3.84 (s, 3H, OCH_3), 5.37 (s, 1H, H-4), 6.45 (s, 1H, CH), 6.68 (s, 2H, ArH), 6.95 (d, 2H, J=9.04 Hz, ArH), 7.08 – 7.10 (m, 1H, ArH), 7.39 – 7.42 (m, 2H, ArH), 7.45 (d, 1H, J=4.52 Hz, ArH), 7.71 (d, 1H, J=3.52 Hz, ArH); ^{13}C NMR (100 MHz, $CDCl_3$) δ 54.90 (OCH_3), 55.54 (OCH_3), 60.53 (OCH_3), 62.01 (C-4), 93.96, 114.09, 120.91, 127.51, 127.93, 128.23, 128.96, 131.16, 133.70, 136.99, 137.60, 153.06 (ArC), 159.67 (C=O), 159.82 (C=O); HRMS (M^++Na): $C_{24}H_{23}NO_5NaS$ requires 460.1195; found 460.1189

3.1.15.2 (*Z*)-4-(4-Methoxyphenyl)-3-(5-methylthiophen-2-ylmethylene)-1-(3,4,5-trimethoxyphenyl)azetidin-2-one (38) was prepared from 3-[hydroxy-(5-methylthiophen-2-yl)-methyl]-4-(4-methoxyphenyl)-1-(3,4,5-trimethoxyphenyl)azetidin-2-one (**34**) as a brown oil (9.0% yield); IR (KBr disk) ν_{max}: 1725.19 cm^{-1} (-C=O); ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.54 (s, 3H, CH_3), 3.77 – 3.84 (m, 12H, 4xOCH_3), 5.35 (s, 1H, H-4), 6.38 (s, 1H, CH), 6.68 – 6.74 (m, 2H, ArH), 6.94 – 6.96 (m, 2H, ArH), 7.35 – 7.41 (m, 3H, ArH); ^{13}C NMR (100 MHz, $CDCl_3$) δ 15.69 (CH_3), 55.35 (OCH_3), 56.05 (OCH_3), 56.17 (OCH_3), 58.67 (OCH_3), 62.43 (C-4), 94.33, 94.93, 114.34, 114.52, 114.67, 118.58 , 121.85 (-CH-), 126.19, 127.37, 128.39, 128.89, 132.28, 134.29, 135.59, 136.49, 145.21, 153.51 (ArC), 160.08 (C=O), 160.51 (C=O); HRMS (M^++H): $C_{25}H_{26}NO_5S$ requires 452.1532; found 452.1527

3.2 Biochemistry: Experimental methods

3.2.1 MTT assay procedure

All assays were performed in triplicate for the determination of mean values reported. Compounds were assayed as the free bases isolated from reaction. The human breast tumour cell line MCF-7 was cultured in Eagles minimum essential medium in a 95%O_2/5% CO_2 atmosphere with 10% fetal bovine serum, 2mM L-glutamine and 100 µg/mL penicillin/streptomycin. The medium was supplemented with 1% non-essential amino acids. MDA-MB-231 cells were maintained in Dulbecco's Modified Eagle's medium (DMEM), supplemented with 10% (v/v) Fetal bovine serum, 2mM L-glutamine and 100 µg/mL penicillin/streptomycin (complete medium). Cells were trypsinised and seeded at a density of 2.5 x 10^4 cells/mL in a 96-well plate and incubated at 37ºC, 95%O_2/5% CO_2 atmosphere for 24 h. After this time they were treated with 2 µL volumes of test compound which had been pre-prepared as stock solutions in ethanol to furnish the concentration range of study, 1 nM–100 µM, and re-incubated for a further 72 h. Control wells contained the equivalent volume of the vehicle ethanol (1% v/v). The culture medium was then removed and the cells washed with 100 µL phosphate buffered saline (PBS) and 50 µL MTT added, to reach a final concentration of 1 mg/mL MTT added. Cells were incubated for 2 h in darkness at 37ºC. At this point solubilization was begun through the addition of 200 µL DMSO and the cells maintained at room temperature in darkness for 20 min to ensure thorough colour diffusion before

reading the absorbance. The absorbance value of control cells (no added compound) was set to 100 % cell viability and from this graphs of absorbance versus cell density per well were prepared to assess cell viability using GraphPad Prism software[59].

3.2.2 Cytotoxicity assay using murine mammary epithelial cells

Mammary glands from 14-18 day pregnant CD-1 mice were used as source and primary mammary epithelial cell cultures were prepared from these. Mammary epithelial cells were isolated as described by us previously.[24] The isolated mammary epithelial cells were seeded at two concentrations. After 24 hours, they were treated with 2 µL volumes of test compound which had been pre-prepared as stock solutions in ethanol to furnish the concentration range of study, 1 nM–100 µM, and re-incubated for a further 72 h. Control wells contained the equivalent volume of the vehicle ethanol (1% v/v). The cytotoxicity was assessed using alamar blue dye as reported previously.[60]

3.2.3 Tubulin polymerization: Tubulin polymerisation was carried out using a kit supplied by Cytoskeleton. It is based on the principal that light is scattered by microtubules to an extent that is proportional to the concentration of the microtubule polymer. Compounds that interact with tubulin will alter the polymerisation of tubulin, and this can be detected using a spectrophotometer. The absorbance at 340nm at 37°C is monitored. The experimental procedure of the assay was performed as described in version 8.2 of the tubulin polymerisation assay kit manual[61].

3.3 Stability studies for compounds 13, 16, 17 and 18: Analytical high-performance liquid chromatography (HPLC) stability studies were performed using a Symmetry® column (C_{18}, 5 µm, 4.6×150 mm), a Waters 2487 Dual Wavelength Absorbance detector, a Waters 1525 binary HPLC pump and a Waters 717plus Autosampler. Samples were detected at wavelength of 254 nm. All samples were analysed using acetonitrile (80%): water (20%) as the mobile phase over 10 min and a flow rate of 1 mL/min. Stock solutions are prepared by dissolving 5mg of compound in 10 mL of mobile phase. Phosphate buffers at the desired pH values (4, 7.4, and 9) were prepared in accordance with the British Pharmacopoeia monograph 2010. 30 µL of stock solution was diluted with 1 mL of appropriate buffer, shaken and injected immediately. Samples were withdrawn and analysed at time intervals of t=0 min, 5

min, 30 min, 60 min, 90 min, 120 min and 21 hours. Retention times were **13**: 2.70 mins; **16**: 3.75 mins; **17**: 3.74 mins; **18**: 3.75 mins.

3.4 Computational Procedures: For ligand preparation, all compounds were built using ACD/Chemsketch v10 to generate SMILES. A single conformer from each string was generated using Corina v3.4 and ensuring Omega v2.2.1 was subsequently employed to generate a maximum of 50 conformations of each compound. For the receptor preparation, the PDB entries 1SA0 were downloaded from the Protein Data Bank (PDB). All waters were retained in both isoforms. Addition and optimisation of hydrogen positions for these waters was carried out using MOE 2007.09 ensuring all other atom positions remained fixed. Using the reported X-ray structure of tubulin co-crystallised with a colchicine derivative, DAMA-colchicine (PDB entry – 1SA0)[51], possible binding orientations of the β-lactam ligands were probed with the docking program FREDv2.2.3 (Openeye Scientific Software)[62]. Docking was carried out using FREDv2.2.3 in conjunction with the PLP scoring function. 3D ligand conformations were enumerated using CORINAv3.4 (Molecular Networks GMBH)[63] followed by generation of multiple conformations using OMEGAv2.2.1 (Openeye Scientific Software)[64]. Each conformation was subsequently docked and scored with PLP as outlined previously[14]. The top binding poses were refined using the LigX procedure (MOE - Chemical Computing Group)[65] together with Postdock analysis (SVL script; MOE) of the docked ligand poses.

3.5 X-ray crystallography: The X-ray crystallography data for crystals was collected on a Rigaku Saturn 724 CCD Diffractometer. A suitable crystal was selected and mounted on a glass fiber tip and placed on the goniometer head in a 123K N2 gas stream. The data set was collected using Crystalclear-SM 1.4.0 software and 1680 diffraction images, of 0.5° per image, were recorded. Data integration, reduction and correction for absorption and polarization effects were all performed using Crystalclear-SM 1.4.0 software. Space group determination, structure solution and refinement were obtained using Crystalstructure ver. 3.8 and Bruker Shelxtl Ver. 6.14 software.[66]

Crystal Data for **11 (*cis*)**: $C_{104}H_{108}N_4O_{20}$, MW 1733.94 (4 molecules). Monoclinic, Space group P-1; *a* = 12.364(4), *b*= 13.084(4), *c* = 14.956(4)A˚, α = 82.867(11)º , β = 72.242(7)º, γ = 84.107(12) º ; *U* = 2280.9) A˚; *Z* = 1; *D*c = 1.262Mg m-3; *m* = 0.087 mm-1; Range for data collection = 1.12–25.00; Reflections collected 35367, Unique Reflections 8025 [*R*int= 0.0486]; Data/restraints/parameters 8025/0/587; Goodness-of-fit on F2 1215; *R* indices (all data) = *R*1 = 0.0728, w*R*2 = 0.1442; Final *R* indices [$I > 2s(I)$] = *R*1 = 0.0642, w*R*2 = 0.1393. CCDC deposition no. **778106**.

Crystal Data for **11 (*trans*)**: $C_{104}H_{108}N_4O_{20}$, MW 1733.94 (4 molecules). Monoclinic, Space group P21/c; *a* = 11.538(3), *b*= 12.295(3), *c* = 18.953(4)A˚, α = γ = 90º, β = 123.08(12) º ; *U* = 2252.8(9) A˚; *Z* = 1; *D*c = 1.278 Mg m-3; *m* = 0.088 mm-1; Range for data collection = 1.12–25.00; Reflections collected 17982, Unique Reflections 3955 [*R*int= 0.0441]; Data/restraints/parameters 3955/0/295; Goodness-of-fit on F2 1244; *R* indices (all data) = *R*1 = 0.0674, w*R*2 = 0.1195; Final *R* indices [$I > 2s(I)$] = *R*1 = 0.0616, w*R*2 = 0.1195. CCDC deposition no. **778108**.

Crystal Data for **12**: $C_{124}H_{116}N_4O_{20}$, Formula MW 1982.21 (4 molecules); Monoclinic, Space group P21/c; *a* = 10.349(3), *b*= 9.828(3), *c* = 26.547(8)A˚, α = γ = 90º, β = 110.277(10) º ; *U* = 2532.8(13) A˚; *Z* = 1; *D*c = 1.300 Mg m-3; *m* = 0.088 mm-1; Range for data collection = 1.12–25.00; Reflections collected 30080, Unique Reflections 4457 [*R*int= 0.0652]; Data/restraints/parameters 4457/0/338; Goodness-of-fit on F2 1222; *R* indices (all data) = *R*1 = 0.0789, w*R*2 = 0.1389; Final *R* indices [$I > 2s(I)$] = *R*1 = 0.0835, w*R*2 = 0.1410.CCDC deposition no. **778107**.

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Supplementary Information Available: NCI60 cell-line data for β-lactam **13**, HPLC purity data for target azetidinone compounds, chiral separation chromatograms for β-lactams **13** and **28**, additional

molecular modeling for β-lactam **28** and cytotoxicity data for **2a** and **28** in murine epithelial cells at 50,000 cells/mL.

Figure, Scheme and Table captions

Figure 1. Structures of small molecule tubulin-binding agents

Figure 2a. Ortep representation of the X-ray crystal structure of β-lactam **11** (*cis* isomer) drawn with 50% thermal ellipsoids

Figure 2b. Ortep representation of the X-ray crystal structure of β-lactam **11** (*trans* isomer; 2 enantiomers shown with relative stereochemistry) drawn with 50% thermal ellipsoids

Figure 3. Ortep representation of the X-ray crystal structure of β-lactam **12** drawn with 50% thermal ellipsoids

Figure 4: Antiproliferative effect of **2a** and 3-(3-thienyl)-β-lactams **13** and **29** in MCF-7 human breast cancer cells.

MCF-7 cells were seeded at a density of 2.5 x 10^4 cells per well in 96-well plates and left for 24 hours to allow the cells to adhere to the surface of the wells. A range of concentrations (0.01 nM-50 μM) of the compound were added in triplicate and the cells left for 72 hours. Control wells contained the equivalent volume of the vehicle ethanol:DMSO (70%:30%) (1% v/v). An MTT assay was performed to determine the level of anti-proliferation. The values represent the mean ± S.E.M (error values) for three experiments performed in triplicate.

Figure 5. Cell viability in healthy murine epithelial cells

Mouse mammary epithelial cells were harvested from mid- to late- pregnant CD-1 mice and cultured. The isolated mammary epithelial cells were seeded at two concentrations. After 24 hours, they were treated with 2 μL volumes of test compound which had been pre-prepared as stock solutions in ethanol to furnish the concentration range of study, 1 nM–100 μM, and re-incubated for a further 72 h. Control wells contained the equivalent volume of the vehicle ethanol (1% v/v). The cytotoxicity was assessed using alamar blue dye.

Figure 6. Inhibition of tubulin polymerisation for β-lactam **28**

Effects of compound **28** on in vitro tubulin polymerisation. Purified bovine tubulin and GTP were mixed in a 96-well plate. The reaction was started by warming the solution from 4 ºC to 37ºC. Ethanol (1%v/v) was used as a vehicle control. The effect on tubulin assembly was monitored in a Spectramax 340PC spectrophotometer at 340nm at 30 second intervals for 60 minutes at 37 ºC. The graph shows one representative experiment. Each experiment was performed in triplicate.

Figure 7. Comparison of the docked conformations of β-lactams **3a**, **12** and DAMA-colchicine.

12 is shown in green; **3a** (left) and DAMA-colchicine (right) are colored by atom with oxygen red, nitrogen blue, carbon grey and sulfur yellow. Protein residues are not shown for clarity.

Figure 8. Docked pose of β–lactam **28** in the colchicine-binding site of tubulin

Docked pose of β–lactam **28** in the colchicine-binding site of tubulin (PDB entry 1SA0). Significant binding residues Thr 179, Lys 241 and Val 318 are indicated. Hydrogens are not shown for clarity. Coloured by atom: Grey (carbon); red (oxygen); blue (nitrogen); yellow (sulfur). Residue numbers are those used by Ravelli et al[51].

Figure 9. 2D representation of binding interactions of β-lactam **28** in the colchicine-binding site of tubulin

2-D rendering of ligand–protein interactions using LigX module of MOE used to create docked structures of **28** in the colchicine-binding site of tubulin[55]. Residue numbers are those used by Ravelli et al[51].

Scheme 1. Synthesis of substituted acetic acids **5a**, **5b**[a]

[a]Reagents and conditions: (a) Diethyl ether, 0°C, 1 hour; (b) 20°C; (c) NaH, toluene, 50°C, 1 hour; (d) 10M HCl, 50°C, 30 mins

Scheme 2. Synthesis of imines **8a** – **8f**[a]

[a]Reagents and conditions: EtOH, reflux, 3 h

Scheme 3. Synthesis of β-lactams **10** - **27**[a]

[a]Reagents and conditions: (a) $SOCl_2$, $CHCl_3$, reflux, 3 h; (b) (Route I) triethylamine, CH_2Cl_2, reflux, 3 h; (c) (Route II) triethylamine, CH_2Cl_2, 18 h; (d) (Route III) triphosgene, triethylamine, anhydrous CH_2Cl_2, reflux, 5 h, 18 h; TBMDS = *tert*-butyldimethylchlorosilyl

Scheme 4. Synthesis of phenolic azetidinones **28**, **29**[a]

[a]Reagents and conditions: (a) TBAF, THF, 0 ˚C, 15 min; TBMDS = *tert*-butyldimethylchlorosilyl

Scheme 5. Synthesis of amino-substituted azetidinone **30**[a]

[a]Reagents and conditions: (a) Zn, CH_3CO_2H, 7 days

Scheme 6. Synthesis of azetidinones **9**, **31**-**33**[a]

[a]Reagents and conditions: (a) Zn, TMCS, benzene, microwave; (b) LDA, dry THF, -78°C; (c) Pyridinium chlorochromate, CH_2Cl_2, 2 h; (d) Tosyl chloride, pyridine, reflux, 5 h

Table 1. Azetidin-2-one combretastatin A-4 analogues[a]

[a]Routes for synthesis were: compounds **10** – **18**: route I; compound **19**: route II; compounds **20** – **27**: route III; Route I: triethylamine, CH_2Cl_2, reflux, 3 h; Route II: triethylamine, CH_2Cl_2, 18 h; Route III: triphosgene, triethylamine, anhydrous CH_2Cl_2, reflux, 5 h, 18 h; [b]TBMDS = *tert*-butyldimethylchlorosilyl

Table 2. Antiproliferative activities of β-lactams in human MCF-7 breast cancer cells

[a]IC_{50} values are half maximal inhibitory concentrations required to block the growth stimulation of MCF-7 cells. Values represent the mean ± S.E.M (error values x 10^{-6}) for at least three experiments performed in triplicate.

[b]The IC_{50} value obtained for **2a** in this assay is 0.0052 μM for MCF-7 which is in good agreement with the reported values for **2a** using the MTT assay on human MCF-7 breast cancer cell line[18, 47, 48, 67]

Table 3. Antiproliferative activities of β-lactams in human MDA-MB-231 breast cancer cells

[a]IC_{50} values are half maximal inhibitory concentrations required to block the growth stimulation of MDA-MB-231 cells. Values represent the mean ± S.E.M (error values x 10^{-6}) for at least three experiments performed in triplicate.

[b]The IC_{50} value obtained for **2a** in this assay is 0.043 μM for MDA-MB-231 which is in good agreement with the reported values for **2a** using the MTT assay on the human MDA-MB-231 breast cancer cell line[68, 69]

Table 4: Standard COMPARE Analysis of β-lactam **13**[a]

[a] The target set was the standard agent database and the target set endpoints were selected to be equal to the seed end points. Standard COMPARE analysis was performed. Correlation values are Pearson correlation coefficients. Vincristine sulfate and rhizoxin appear at different concentrations as they have been tested by the NCI at multiple concentration ranges

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