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Original article

Lead identification of conformationally restricted β-lactam type combretastatin analogues: Synthesis, antiproliferative activity and tubulin targeting effects

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ABSTRACT

The synthesis and study of the structure–activity relationships of a series of rigid analogues of combretastatin A-4 are described which contain the 1,4-diaryl-2-azetidinone (β-lactam) ring system in place of the usual ethylene bridge present in the natural combretastatin stilbene products. The 1,4-diaryl-2-azetidinones are unsubstituted at C-3, or contain methyl substituent(s) at C-3. The most potent compounds **12d** and **12e** display antiproliferative activity at nanomolar concentrations when evaluated against the MCF-7 and MDA-MB-231 human breast carcinoma cell lines. **12d** exerts antimitotic effects through an inhibition of tubulin polymerisation and subsequent G_2/M arrest of the cell cycle in human MDA-MB-231 breast cancer cells, with similar activity to that of CA-4. These novel β-lactam compounds are identified as potentially useful scaffolds for the further development of antitumour agents which target tubulin.

1. Introduction

Microtubules are cytoskeletal structures that are formed by the self-assembly of α and β tubulin heterodimers and are involved in many cellular functions [1]. Their most important role in the formation of the mitotic spindle, which is intimately involved in cell division. Antimitotic agents are one of the major classes of cytotoxic drugs for cancer treatment and microtubules are a significant target for many natural product anticancer agents [2]. The three characterised binding sites of tubulin are the taxane domain, the vinca domain and the colchicine domain and many compounds interact with tubulin at these known sites.

Paclitaxel binds to tubulin at the taxane site and was the first compound found to stabilise tubulin by promoting the assembly of tubulin heterodimers into microtubules [3]. The vinca alkaloids, vinblastine and vincristine bind at the vinca domain resulting in depolymerisation of microtubules and destruction of mitotic spindles at high concentrations [4]. Colchicine (**1**, Fig. 1) also depolymerises microtubules at high concentrations and stabilizes microtubules dynamics at low concentrations; however it binds at a separate site to the vinca alkaloids, the colchicine domain [5,6].

The combretastatins were obtained from the African willow tree *Combretum caffrum*. Pettit et al. [7] showed that combretastatin A-4 (**2**, Fig. 1) (CA-4) potently inhibited microtubule activity by binding to tubulin at the colchicine site, thereby interfering with cell growth and proliferation. CA-4 acts as a vascular targeting agent and induces blood flow reduction and subsequent tumour cell death [4,8–10]. In contrast to colchicine, the anti-vascular effects of CA-4 *in vivo* are apparent well below the maximum tolerated dose, offering a wide therapeutic window. The disodium phosphate salt of CA-4 **3** has been developed with improved solubility [11,12] and is currently in phase II clinical trials for the treatment of thyroid cancer [13–15]. Hydrolysis *in vivo* by endogenous non-specific phosphatases under physiological conditions affords CA-4 [11,12]. The *cis*-double bond in CA-4 is readily converted to the more stable *trans* geometry during storage or use, resulting in a dramatic decrease in antitumour activity [16–18].

Many structural modifications to CA-4 have been reported including variation of the A and B-rings substituents (Fig. 1) [19–21]. Most modifications of the B-ring result in decreased bioactivity; the exception is the removal of the 3′-hydroxy group, which results in a compound with almost the same potency as CA-4

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Abbreviations

CA-4	combretastatin A-4
DEPT	distortionless enhancement by polarization transfer
EGTA	ethylene glycol tetraacetic acid
EI	electron Impact
ER	estrogen receptor
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
GTP	guanidine triphosphate
HMQC	heteronuclear multiple quantum coherence
HRMS	high resolution mass spectrometry
IR	infra red
LRMS	low resolution mass spectra
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NMR	nuclear magnetic resonance
NOE	nuclear overhauser effect
PBS	phosphate buffer saline
SAR	structure–activity relationship
THF	tetrahydrofuran
TMS	tetramethylsilane

in both tubulin polymerisation and colchicine binding assays [18,22]. Substitution of the 3′-OH with an amino group results in a compound with equipotent bioactivity as CA-4 and good water solubility [23]. The 3,4,5-trimethoxy substituted pattern in ring A is optimal for bioactivity of CA-4 as it coincides with the trimethoxyaryl ring of colchicine [20]. A large number of analogues with a modified bridge have also been reported. These include saturation of the olefinic bridge, and replacement with $-NHCH_2-$, $-CH_2NH-$, $-CONH-$, $-OCH_2-$, $-CH_2O-$, $-O-$ and many others.

Isomerisation of the *cis*-double bond in CA-4 has led to intensive investigations of rigid ring modifications [10,19,20,24], including imidazole **4** [25], 1,3-dioxolane **5** [26], furazan (1,2,5-oxadiazole) **6** [27], heteroarylcoumarin **7** [28] and 3-aroyl-6-methoxyindole **8** [29] analogues (Fig. 1). In addition to tumour cell growth inhibition, most of these non-isomerisable compounds have been shown to be capable of binding to and depolymerising tubulin. The anticancer activity of some β-lactam-containing compounds has been reported [30], including the non-isomerisable combretastatin analogues **9** and **10** [31]. We have previously reported antiproliferative activity of compounds containing the β-lactam scaffold which exhibited antiestrogenic effects in MCF-7 cells [32]. It was decided to examine a range of compounds containing the β-lactam ring as potential tubulin targeting agents. These novel compounds are unsubstituted at C-3, or contain methyl substituent(s) at C-3. The β-lactam ring provides a useful scaffold structure with a similar spatial arrangement between the two phenyl rings as observed in the *cis* conformation of CA-4.

2. Results and discussion

2.1. Chemistry

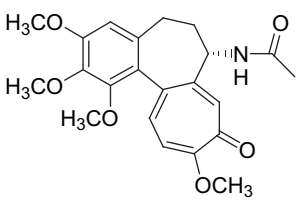
The general procedure for the synthesis of the required β-lactams is illustrated in Scheme 1. The compounds which were initially chosen for synthesis contained the 3,4,5-trimethoxyphenyl (Ring A) and 3-hydroxy-4-methoxyphenyl (Ring B) substituents as the β-lactam N-1 and C-4 substituents, which are present in Combretastatin A-4. The Reformatsky β-lactam synthesis requires the appropriately substituted Schiff bases **11a** and **11c** which were obtained by condensation of the appropriate amine and aldehyde. Schiff bases **11a** and **11c** were then protected by treatment with *tert*-butyldimethylchlorosilane to yield the respective silyl ether products **11b** and **11d**. The racemic β-lactam products **12a** and **12g** were obtained in moderate yields by reaction of imines **11b** and **11d** with ethylbromoacetate in the presence of zinc and trimethylchlorosilane using the conditions similar to those reported by Palomo et al. [33].

The ^{1}H NMR spectrum of **12a**, the C-3 proton at δ 2.91(dd, $J = 2.0$ Hz, 15.0 Hz) is in a *trans* orientation to H-4 which appears as a double doublet at δ 4.86 ($J = 2.0$ Hz, 5.4 Hz) [34]. The second C-3 proton is observed at δ 3.49 (dd, $J = 5.4$ Hz, 15.4 Hz) and is *cis* to H-4. The silyl protecting group was removed from both β-lactams **12a** and **12g** using TBAF to afford **12d** (62%) and **12j** (39%) respectively, the β-lactam analogues of CA-4. The related 3-methylazetidinones **12b** and **12h** were similarly obtained by Reformatsky reaction of Schiff bases **11b** and **11d** using ethyl-2-bromopropionate as the α-bromoester (Scheme 1). These azetidinones were obtained as mixtures of *cis*/*trans* isomers (3:2) which were not separated. The 3,3-dimethyl analogues **12c** and **12i** were prepared in a similar manner by reaction of the Schiff bases **11b** and **11d** with ethyl-2-bromoisobutyrate.

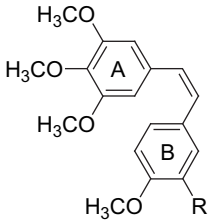
The *tert*-butyldimethylsilyl protecting group is removed from compounds **12b**, **c**, **h**, and **i** using TBAF to yield the phenolic products **12e**, **f**, **k**, and **l** respectively. The diastereomeric composition of compound **12e** was demonstrated by chiral HPLC, (rt 3.39 min and 4.11 min for *trans* isomers and 4.33 min and 4.66 min for *cis* isomers, assigned from integration). The effect of product stereochemistry on the biochemical activity of the β-lactams reported in this study will be considered in the future development of these compounds. (The stereoselective activity of the cholesterol lowering β-lactam drug ezetimibe(1-(4-fluorophenyl)-(3*R*)-[-3-(4-fluorophenyl)-(3*S*)-hydroxypropyl]-4*S*-(4-hydroxyphenyl)-2-azetidinone) has been reported [35,36]).

Because of the observation that the 3,4,5-trimethoxy ring substitution (Ring A) is optimal for bioactivity in all analogues of CA-4 in literature, compounds **12d**–**f** and **12j**–**l** were examined for their antiproliferative effects in both MCF-7 and MDA-MB-231 cell lines with the aim of discovering if the placement of this critical 3,4,5-trimethoxyphenyl ring on the N-1 or C-4 positions resulted in significantly different antiproliferative activities, (see Results and discussion section, Table 1). Results in both cell lines concurred, showing the compounds with the 3,4,5-trimethoxyphenyl ring (Ring A) on the N-1 position being significantly more potent than the compound with the 3,4,5-trimethoxyphenyl substituent (Ring A) on the C-4 position, as previously reported by Sun et al. with other β-lactam compounds [31]. From our initial investigations we established that positioning of the 3,4.5-trimethoxyphenyl ring on the rigid β-lactam ring system for these compounds, was an important consideration and required that any further β-lactams synthesised would contain the 3,4,5-trimethoxyphenyl ring at the N-1 position.

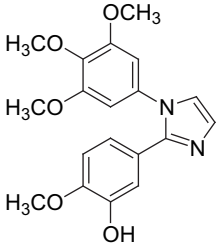
Structural modification of the initially synthesised β-lactam products **12d**–**f** was then investigated with a view to further optimization of the CA-4 analogue β-lactam template for antiproliferative activity. The synthesis of products **12m**–**x** with modification of the C-4 aryl ring substituents is shown in Scheme 2. The 3-unsubstituted β-lactam **12d** contains the 3-hydroxy-4-methoxyphenyl ring at the C-4 position; which is also present in CA-4 (Ring B). A variety of different β-lactams **12m**–**v**, were obtained by using different substitution patterns on the C-4 phenyl ring (Ring B), e.g. 4-F, 4-OCH_3, 2,3,4-trimethoxy, 3,4,5-trimethoxy and 3,4-dimethoxy. The aryl ring at C-4 was also replaced with the alternative 6-methoxy-2-naphthyl and 2-thiophene ring systems (compounds **12w**–**x**). The required imines



1 Colchicine



2 R = OH, Combretastatin A-4
3 R = OPO_3Na_2



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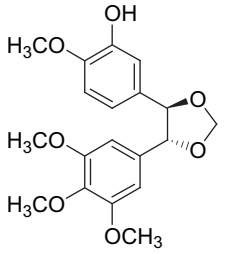

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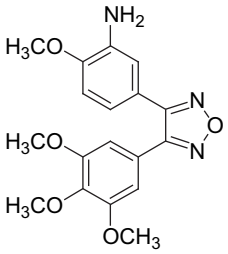

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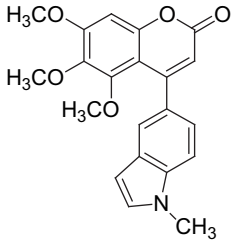

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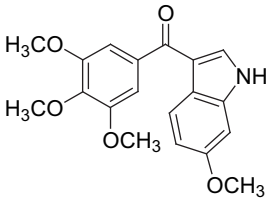

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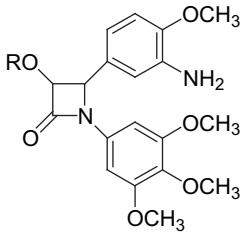


9 R = CH_3
10 R = H

Fig. 1. Colchicine (**1**), combretastatin A-4 (**2**), combretastatin A-4 phosphate (**3**) and related antitubulin compounds **4**–**10**.

11e–**11l** were prepared by condensation of 3,4,5-trimethoxyaniline with the appropriate aldehyde. The β-lactam products **12m**–**o**, **12r**–**t** and **12w**–**x** were obtained in moderate yields by reaction of imines **11e**–**11l** with ethylbromoacetate in the presence of zinc and trimethylchlorosilane. Enantiomeric separation of the racemic product **12o** was achieved by chiral HPLC (rt 7.88 min and 8.92 min).

The 3-methyl and 3,3-dimethyl compounds **12p**, **12q**, **12u** and **12v** were obtained in a similar manner using ethyl-2-bromopropionate and ethyl-2-bromoisobutyrate as the α-bromoesters respectively. The nature of the Schiff base influences the yields obtained in these Reformatsky reactions. While good yields were obtained (e.g. up to 83% for compound **12p**), we have found that after optimization, lower yields of β-lactam products e.g. **12n** (28%) and **12s** (16%) are consistently obtained from the Schiff bases **11f** and **11i** both having *ortho* methoxy substituents, which may be causing some steric hindrance for the imine in forming the β-lactam ring.

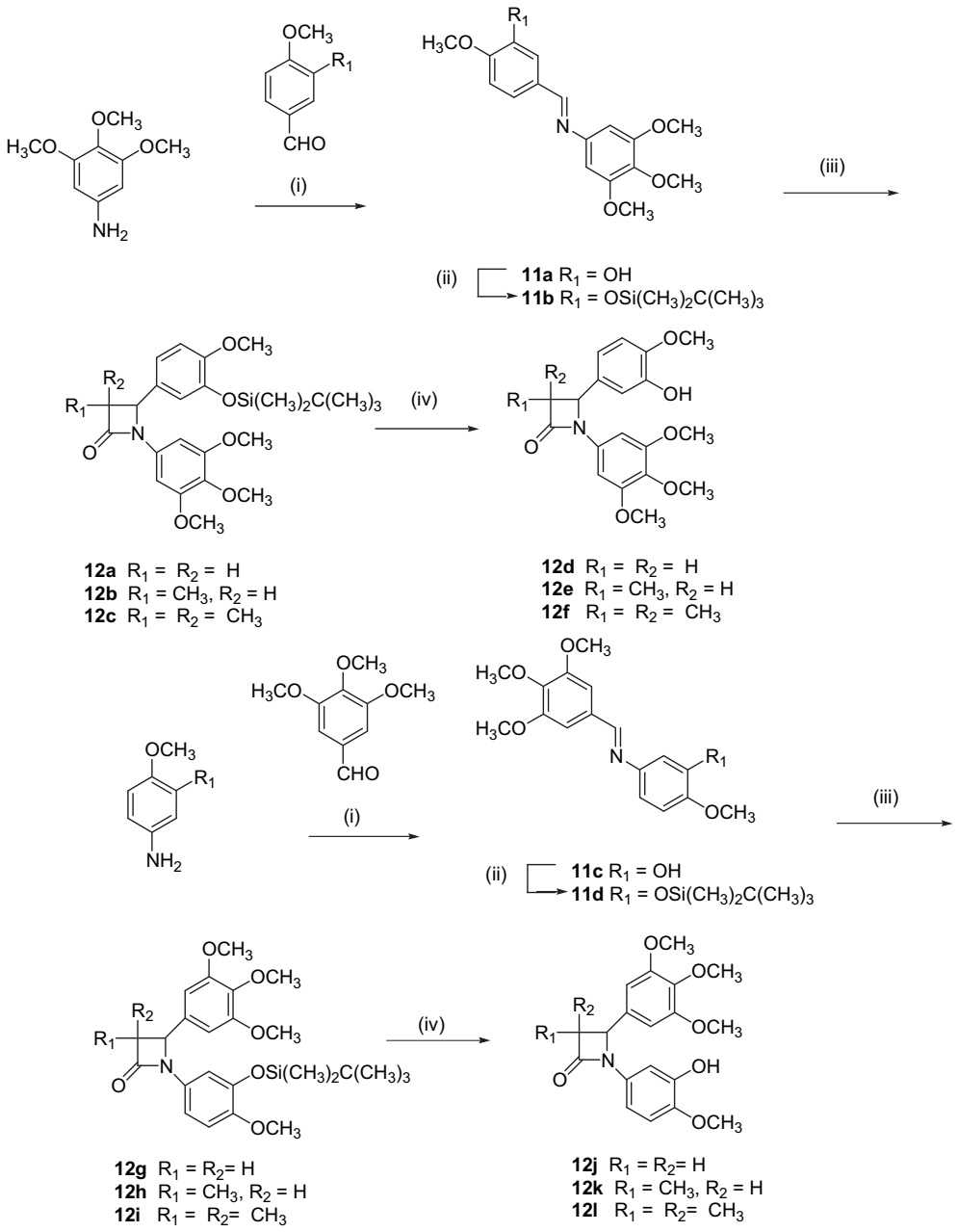
To further investigate the contribution of the β-lactam carbonyl group to the activity of this compound class, the novel thione analogues **13a**–**c** were prepared in moderate yield by reaction of the compounds **12o**–**q** with Lawesson's reagent (2,4-bis-(4-methoxyphenyl)-1,2,3,4-dithiaphosphetane-2,4-disulfide) (Scheme 2). The characteristic C═S absorption band was observed in the region 1590–1595 cm^{-1} for these products.

2.2. Biochemical studies

2.2.1. Antiproliferative activity in MCF-7 and MDA-MB-231 breast cancer cells

The first aim of the biochemical evaluation of the compounds was to determine if the position of the 3,4,5-trimethoxyphenyl ring on the β-lactam scaffold at N-1 or C-4, which is critical in the activity of colchicines and combretastatin type compounds, effected the potency of these β-lactam compounds. Compounds **12d**–**f** and **12j**–**l** were screened for their antiproliferative activity using two breast cancer cell lines, the ER expressing (ER dependent) MCF-7 human breast cancer and the ER lacking (ER independent) MDA-MB-231 human breast cancer cell lines, using the MTT (tetrazolium) based viability assay. The drug concentration required to inhibit the cell growth by 50% (IC_{50}) following incubation of the cells in the culture medium for 72 h was determined and the results are displayed in Table 1. The IC_{50} values obtained for Combretastatin CA-4 in this assay are 31 nM for MCF-7 and 43 nM for MDA-MB-231 and are in good agreement with the reported values for Combretastatin CA-4 using the MTT assay on human MCF-7 and MDA-MB-231 breast cancer cell lines [7,37,38].

From the results in Table 1 it is evident that the 3,4,5-trimethoxyphenyl ring located at the N-1 position of the β-lactam ring is



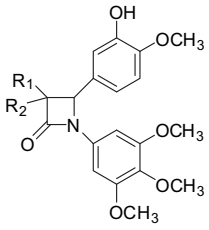
Scheme reagents and conditions: (i) EtOH, reflux, 2.5h.(ii) $(CH_3)_3C(CH_3)_2SiCl$, K_2CO_3, CH_2Cl_2, DBU, 20°C. (iii) $BrCH_2CO_2Et$, $BrCH(CH_3)CO_2Et$ or $Br(CH_3)_2CCO_2Et$, Zn, $(CH_3)_3SiCl$, C_6H_6, reflux.(iv) $(CH_3CH_2CH_2CH_2)_4NF$, THF, 0°C.

Scheme 1. Synthesis of compounds **12a**–**l**.

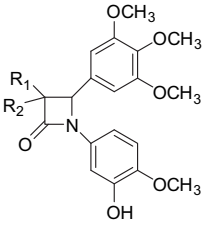
the structural arrangement for optimum activity as previously observed by Sun et al. with other β-lactam compounds [31]. All three compounds **12d**–**f** (in which the 3,4,5-trimethoxyphenyl ring is located at the N-1 position) are much more potent in both cell lines than the corresponding compounds **12k** and **12l** (where the 3,4,5-trimethoxyphenyl ring is located at the C-4 position) and therefore this template was chosen for the further compounds examined. The 3-unsubstituted compound **12j** was the most active of the products with 3,4,5-trimethoxy ring located at the C-4 position (IC_{50}value = 0.130 μM in MCF-7 cells). It is interesting to note that the most active compounds described here (**12d**, **12e**) are considerably more potent as antiproliferative agents than the previously reported 3-hydroxy, 3-methoxy and 3-acetoxy substituted β-lactams [31]. There is little difference in antiproliferative activity between the 3-unsubstituted compound **12d** and 3-methyl compound **12e**, both demonstrating low nanomolar activity in both MCF-7 cell line (IC_{50} = 17 nM and 10 nM respectively) and MDA-MB-231 cell line (IC_{50} = 54 and 47 nM respectively). These values are similar to that obtained for CA-4 (IC_{50} = 3 nM for MCF-7 and IC_{50} = 43 nM for MDA-MB-231 cell line). However, the introduction of the 3,3-dimethyl substitution in compound **12f** results in significant loss of activity with IC_{50} = 0.25 μM for MCF-7 cells and IC_{50} = 0.27 μM for the MDA-MB-231 cell line.

The cytotoxic effect of these β-lactam compounds on proliferating cells was determined in the lactose dehydrogenase (LDH) assay [39]. Cytotoxicity values in the range 2–15% were obtained

Table 1
Antiproliferative effects of β-lactam compounds **12d**–**f**, **12j**–**l** in MCF-7 cells and MDA-MB-231 cells.



12d-f



12j-l

Compound Number	R_1	R_2	Antiproliferative activity[a,b] MCF-7 cells IC_{50} value (μM)	Cytotoxicity % death[c] 10 μM	Antiproliferative activity[a], [b] MDA-MB-231 cells IC_{50} value (μM)	Cytotoxicity % death[c] 10 μM
12d	H	H	0.017 ± 0.002	9	0.054 ± 0.046	16
12e[d]	CH_3	H	0.010 ± 0.0032	15	0.047 ± 0.041	25
12f	CH_3	CH_3	0.25 ± 0.08	12	0.27 ± 0.15	20
12j	H	H	0.130 ± 0.054	2.3	1.67 ± 0.06	6.6
12k[d]	CH_3	H	2.96 ± 0.61	17	13.03 ± 4.36	0
12l	CH_3	CH_3	4.04 ± 2.35	11.5	3.19 ± 2.56	12.1
2 (CA-4)	–	–	0.0031	5.5	0.043	4.3

[a] IC_{50} values are half maximal inhibitory concentrations required to block the growth stimulation of MCF-7 or MDA-MB-231 cells. Values represent the mean ± S.E.M (error values × 10^{-6}) for three experiments performed in triplicate.
[b] The IC_{50} values obtained for combretastatin CA-4 in this assay are 0.0031 μM for MCF-7 and 0.043 μM for MDA-MB-231 and are in good agreement with the reported values for combretastatin CA-4 using the MTT assay on human MCF-7 and MDA-MB-231 breast cancer cell lines, (see Refs. [7,37,38]).
[c] Lactate dehydrogenase assay: Following treatment of the cells, the amount of LDH was determined using LDH assay kit from Promega. Data is presented as % cell lysis at compound concentration of 10 μM, (see Ref. [39]).
[d] 3-Methyl substituted compounds **12e** and **12k** isolated and tested as *cis*/*trans* mixture.

for compounds **12d**–**f** and **j** at a concentration of 10 μM in MCF-7 cells and 6–25% for the same compounds in MDA-MB-231 cells at a concentration of 10 μM. CA-4 was determined to have cytotoxicity value of 5.6% in MCF-7 cells and 4.3% in MDA-MB-231 cells at 10 μM concentration.

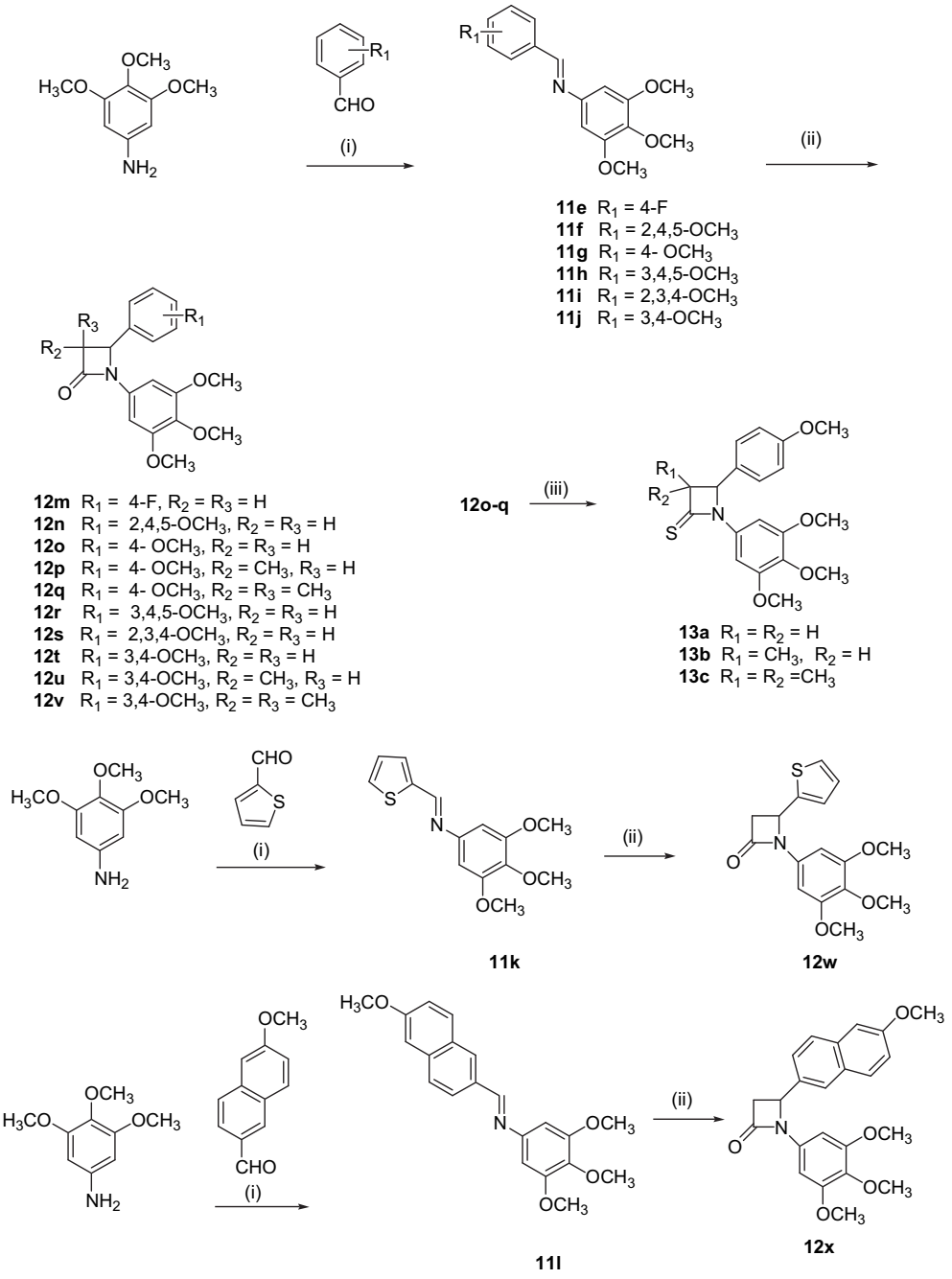
The antiproliferative results in MCF-7 and MDA-MB-231 breast cancer cell lines for the second series of compounds synthesised **12m**–**x**, and **13a**–**c** are displayed in Table 2. All compounds contain the 3,4,5-trimethoxyphenyl substituent located at N-1 with the C-3 position unsubstituted, or mono or dimethyl substituted. The effects on antiproliferative activity of a number of aryl substitution patterns are investigated for the aryl group at C-4, including 2,3,4-trimethoxy, 3,4,5-trimethoxy, 3,4-dimethoxy, 4-methoxy and 4-fluoro are displayed in Table 2. There is no significant loss in activity observed on removal of the C-3 phenolic group from the most potent compounds **12d** and **12e** as shown in the results obtained for **12o** and **12p** with IC_{50} values of 39 nM and 47 nM respectively. These compounds also showed reduced cytotoxicity values in the LDH assay of 1.5% and 5.7% respectively at 10 μM concentration. The related 3,3-dimethyl-2-azetidinones **12q** and **12v** show good antiproliferative effects on MCF-7 cells (IC_{50} = 265 nM and 344 nM respectively). Introduction of the 3,4-dimethoxyphenyl substituent at C-4 results in intermediate antiproliferative activity for compound **12t** (IC_{50} value = 1.64 μM), with moderate activity also obtained for the 3-methyl substituted analogue **12u** (IC_{50} value = 2.96 μM) and also for compound **12x** (containing the 6-methoxy-2-naphthyl substituent at C-4), with IC_{50} value of 1.51 μM. The introduction of the fluoro substituent in **12m** resulted in loss of activity (IC_{50} value = 25.9 μM). The presence of two trimethoxyphenyl ring systems in the β-lactam products e.g. compound **12s** resulted in a moderate reduction in activity (IC_{50} = 0.31 μM). However for the related 1,4-bis(trimethoxyphenyl) substituted compounds **12n** and **12r** a significant reduction in the antiproliferative activity was observed with the IC_{50} values in the high μM range. The replacement of aryl ring at C-4 position with the heterocyclic 2-thiophene ring in **12w** also resulted in much reduced antiproliferative activity with an IC_{50} value of 21.6 μM and notably higher cytotoxicity of 20%. A similar trend was observed for antiproliferative activity of these compounds in the MDA cell line, with compounds **12o** and **12p** again displaying the most potent antiproliferative activity (IC_{50} = 210 and 127 nM respectively) with low cytotoxicity (1.1% and 4.5% respectively, at 10 μM concentration).

The replacement of the β-lactam carbonyl group with the thione in compounds **13a**–**c** resulted in a reduction in the observed antiproliferative activity; the most active thione compound **13c** was found to be IC_{50} = 0.35 μM for MCF-7 cells and IC_{50} = 1.33 μM for MDA-MB-231 breast cancer cells. This could be related to the difference in lipophilicity (e.g. cLogP for **12q** = 3.36 compared with cLogP for **13c** = 2.86 [40]).

The influence of the rigid β-lactam ring scaffold structure on the activity of the above compounds was investigated. The antiproliferative effect of the two Schiff bases **11a** and **11c** (related in structure to the most potent β-lactam **12d** and the related **12j**) was determined in MCF-7 breast cancer cells. Neither of the Schiff bases show any significant antiproliferative activity at concentrations up to 50 μM, which is in sharp contrast to the corresponding β-lactams **12d** and **12j**. As already observed by Cushman et al. the lack of activity of these Schiff bases probably derives from their *trans* geometry [41].

2.2.2. Evaluation of G_2/M arrest in MDA-MB-231 cells exposed to compound *12d*

The extent of G_2/M arrest and apoptosis (sub-G_1 peak) induced by compound **12d** in MDA-MB-231 cells was statistically quantified by flow cytometric analysis. The fluorescent dye, propidium iodide (PI) intercalates with the DNA and hence, the amount of fluorescence measured per cell is proportional to the DNA content. Cells were harvested after 24, 48 or 72 h and analysed for DNA content by flow cytometry. Table 3 shows the percentage of cells in each phase of the cell cycle over the three different time scales.



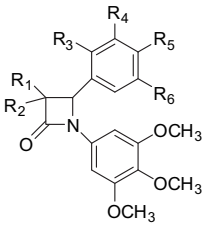
Scheme 2. Synthesis of compounds **12m–x**.

The results obtained for compound **12d** show a large increase of cells in the G_2/M phase at concentrations 100 nM, 1 μM and 10 μM after 24 h of exposure (highlighted in Table 3). This increase is accompanied by a corresponding reduction in the G_1 phase. At a concentration of 10 nM, there appears to be little effect on the cells giving results similar to the vehicle value. After 48 h a significant increase in G_2/M peak (highlighted) is again seen at 100 nM and above but not to the same degree as seen after 24 h as highlighted in Table 3. This is due to the parallel increase in sub-G_1 phase indicating induction of apoptosis. After 72 h a decrease in G_2/M and in G_1 phase is accompanied by a large increase in sub-G_1 peak. These results indicate that this compound's mechanism of action may indeed be by targeting the microtubules. The ability of these β-lactam compounds to bind to and depolymerise tubulin was then investigated.

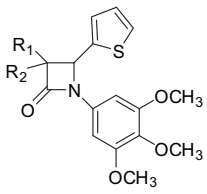
2.2.3. Tubulin polymerisation study

The effects of representative β-lactam combretastatin A-4 analogues on the assembly of purified bovine tubulin were evaluated. The assay was optimised using a polymerisation enhancer (paclitaxel) and polymerisation suppressor (nocodazole) (data not shown). Compound **12d** which demonstrated potent anti-proliferative effects (low nanomolar) *in vitro* and compound **12h**

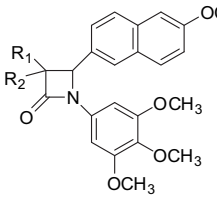
Table 2
Antiproliferative effects of β-lactam compounds **12m**–**x**, **13a**–**c** in MCF-7 cells and MDA-MB-231 cells.



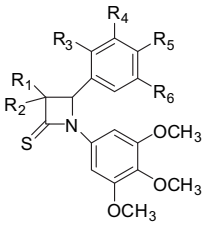
12m-v



12w



12x



13a-c

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Compound Number	R_1	R_2	R_3	R_4	R_5	R_6	Antiproliferative activity MCF-7 cells IC_{50} value (μM)[a], [b]	Cytotoxicity % death 10 μM[c]	Antiproliferative activity MDA-MB-231 cells IC_{50} value (μM)[a], [b]	Cytotoxicity % death 10 μM[c]
12m	H	H	H	H	F	H	25.95 ± 11.95	0	25.95 ± 15.1	6.3
12n	H	H	OCH_3	H	OCH_3	OCH_3	63.74 ± 19.8	2.2	38.5 ± 6.43	0
12o	H	H	H	H	OCH_3	H	0.039 ± 0.013	1.5	0.21 ± 0.17	1.1
12p[d]	H	CH_3	H	H	OCH_3	H	0.047 ± 0.024	5.7	0.127 ± 0.0096	4.5
12q	CH_3	CH_3	H	H	OCH_3	H	0.265 ± 0.010	12	1.15 ± 0.16	16
12r	H	H	H	OCH_3	OCH_3	OCH_3	167.9 ± 121.1	1.9	94.39 ± 18.55	0
12s	H	H	OCH_3	OCH_3	OCH_3	H	0.31 ± 0.036	8.0	2.83 ± 1.98	9.0
12t	H	H	H	OCH_3	OCH_3	H	1.64 ± 0.02	5.0	0.97 ± 0.008	15
12u[d]	H	CH_3	H	OCH_3	OCH_3	H	2.96 ± 0.61	7.5	8.84 ± 0.50	6.1
12v	CH_3	CH_3	H	OCH_3	OCH_3	H	0.344 ± 0.116	14	1.46 ± 0.83	12.0
12w	H	H	–	–	–	–	21.6 ± 14.5	20	34.47 ± 1.50	15
12x	H	H	–	–	–	–	1.51 ± 0.56	13.5	13.18 ± 1.61	18
13a	H	H	H	H	OCH_3	H	1.12 ± 0.10	16	4.50 ± 2.08	3.0
13b[d]	CH_3	H	H	H	OCH_3	H	0.89 ± 0.21	3.2	9.26 ± 2.07	5.1
13c	CH_3	CH_3	H	H	OCH_3	H	0.35 ± 0.012	5.5	1.33 ± 0.11	8.0
2 (CA-4)	–	–	–	–	–	–	0.0031	5.6	0.043	4.3

[a] IC_{50} values are half maximal inhibitory concentrations required to block the growth stimulation of MCF-7 or MDA-MB-231 cells. Values represent the mean ± S.E.M (error values × 10^{-6}) for three experiments performed in triplicate.

[b] The IC_{50} values obtained for combretastatin CA-4 in this assay are 0.0031 μM for MCF-7 and 0.043 μM for MDA-MB-231 and are in good agreement with the reported values for combretastatin CA-4 using the MTT assay on human MCF-7 and MDA-MB-231 breast cancer cell lines (see Refs. [7,37,38]).

[c] Lactate dehydrogenase assay: Following treatment of the cells, the amount of LDH was determined using LDH assay kit from Promega. Data is presented as % cell lysis at compound concentration of 10 μM (see Ref. [39]).

[d] 3-Methyl substituted compounds **12p**, **12u** and **13b** isolated and tested as *cis*/*trans* mixture.

which demonstrated poor antiproliferative effects (high micromolar) were assessed. The ability of combretastatin A-4 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 ν_{max} value offers the most sensitive indicator of tubulin/ligand interactions, hence ν_{max} values were calculated for each test compound. Fold changes in ν_{max} values for polymerisation curves of each test compound with reference to ethanol control were calculated and detailed in Table 4. As anticipated the active β-lactam combretastatin A-4 analogue **12d** inhibited the polymerisation of tubulin whilst the relatively inactive counterpart **12h** did not significantly affect the rate of tubulin polymerisation (Table 4). In more detail, the active β-lactam **12d** when evaluated at 10 μM concentration, reduced the

Table 3
Evaluation of G_2/M arrest in MDA-MB-231 cells exposed to compound **12d**.[a]

Concentration	Time (h)	Sub-G_1 (%)	G_1 (%)	S (%)	G_2/M (%)	Polyploid (%)
Control	24	5.71 ± 3.47	51.20 ± 2.37	10.46 ± 4.19	20.18 ± 3.18	4.53 ± 1.66
10 nM	24	3.72 ± 1.97	48.33 ± 4.18	12.55 ± 2.54	25.61 ± 0.98	2.84 ± 0.59
100 nM	24	4.88 ± 0.85	18.95 ± 6.60	6.05 ± 0.38	59.43 ± 3.13	4.87 ± 4.39
1 μM	24	7.08 ± 4.20	19.22 ± 6.19	6.01 ± 2.90	63.16 ± 4.86	4.31 ± 3.17
10 μM	24	3.18 ± 0.25	29.30 ± 5.95	3.72 ± 0.71	55.36 ± 1.49	3.85 ± 3.83
Control	48	9.80 ± 1.55	50.34 ± 11.09	10.50 ± 0.77	18.81 ± 1.04	5.62 ± 3.06
10 nM	48	11.57 ± 3.06	45.25 ± 7.37	13.36 ± 2.58	23.71 ± 7.81	6.10 ± 0.62
100 nM	48	37.50 ± 0.71	10.76 ± 0.34	8.90 ± 0.85	36.42 ± 3.42	4.61 ± 0.86
10 μM	48	51.27 ± 6.31	14.44 ± 2.28	4.47 ± 0.06	27.25 ± 4.45	2.28 ± 0.06
Control	72	12.92 ± 2.61	44.13 ± 2.31	9.10 ± 2.75	21.51 ± 4.86	5.48 ± 3.26
10 nM	72	15.25 ± 2.03	48.24 ± 2.79	9.08 ± 2.32	17.68 ± 4.58	3.55 ± 1.57
100 nM	72	63.10 ± 20.06	13.90 ± 3.62	5.34 ± 4.32	12.62 ± 9.22	2.99 ± 1.89
1 μM	72	50.53 ± 14.78	14.29 ± 2.20	7.76 ± 0.58	21.24 ± 12.05	5.97 ± 4.71
10 μM	72	46.49 ± 17.49	17.14 ± 3.31	9.66 ± 1.84	21.81 ± 12.40	3.38 ± 2.84

[a] Cell cycle analysis of MDA-MB-231 cells treated with vehicle control (1% (v/v) ethanol), or 10 nM, 100 nM, 1 μM, and 10 μM(final concentrations) of compound **12d** at 24 and 72 h, and at 10 nM, 100 nM and 10 μM(final concentrations) of compound **12d** at 48 h. % MDA-MB-231 cells in each cell cycle phase are shown after exposure to compound **12d**. Cells were analysed with the FACScan flow cytometry. Cells in the sub-G1 peak are indicative of apoptotic cells. Results show a typical experiment which has been repeated three times. Values represent the mean ± standard deviation for three experiments.

Table 4
Inhibition of tubulin polymerisation in response to β-lactam combretastastin analogues.

Compound	Concentration	Fold inhibition of tubulin polymerisation[a] (ν_{max} ± SEM)
Ethanol (Control)	[1% v/v]	1
CA-4	10 μM	6.0 ± 1.4
12d	10 μM	10.2 ± 2.3
12n	10 μM	1.2 ± 0.2

[a] Effects of **12d** and **12n** 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 C to 37 C. CA-4 (10 μM) was used as a reference, while ethanol (1%v/v) was used as a vehicle control. The effect on tubulin assembly was monitored in a Spectramax 340PC spectrophotometer at 340 nm at 30 s intervals for 60 min at 37 C. Fold inhibition of tubulin polymerisation was calculated using the ν_{max} value for each reaction. The results represent the mean ± standard error of the mean for three separate experiments.

ν_{max} value for the rate of tubulin polymerisation from 6- to 10-fold. This value is comparable if not superior to the rate of inhibition of tubulin assembly (6-fold) observed with combretastatin A-4. These results suggest that the molecular target of the active β-lactam combretastatin A-4 analogues is indeed tubulin.

2.3. Molecular modeling studies

To investigate potential binding modes of these β-lactam compounds, a docking study was carried out using the most potent compound in the series, the 3-unsubstituted β-lactam **12d**. Using the reported X-ray structure of tubulin co-crystallised with the colchicine derivative, N-deacetyl-N-(2-mercaptoacetyl)colchicine (DAMA-colchicine, PDB entry 1SA0) [42], possible binding orientations for **12d** were probed with the docking program FREDv2.2.3 (Openeye Scientific Software) [43]. 3-D conformations were enumerated using CORINAv3.4 (Molecular Networks GMBH) [44] for **12d** and combretastatin A-4 followed by multiple conformations using OMEGAv2.2.1 (Openeye Scientific Software) [45]. Each conformation was subsequently docked and scored with Chemgauss3 as outlined previously by our laboratory [46] The top binding poses were refined using the LigX procedure (MOE – Chemical Computing Group) [47]. Postdock analysis (SVL script; MOE) of the docked poses for **12d** revealed that approximately 10% of the population appeared to dock in a similar orientation to colchicine as illustrated in Fig. 2.

For comparison, the docked pose of CA-4 overlayed with N-deacetyl-N-(2-mercaptoacetyl)-colchicine (DAMA-colchicine) in the tubulin binding site (PDB entry 1SA0) is illustrated in Fig. 3. The strong hydrogen-bonding to Thr179 observed for the 3-hydroxy substituent of **12d** is also observed with combretastatin A-4 as depicted in Fig. 3. (The X-ray crystal structure of the disodium phosphate salt of combretastatin A-4 (**3**) suggests that the conformation of this stilbene is not planar. The crystal structure reveals that the planes of the two phenyl rings are inclined to each other, suggesting a low-energy conformation that may be the one involved in binding at the tubulin receptor site [48]). Importantly, an additional H-bond is seen to occur with residue Lys352 of the active site for **12d**, colchicine and CA-4. Superimposition of **12d**, CA-4 and colchicine as they docked within the active site of 1SA0, illustrates the similar nature of positioning of the trimethoxy substituents (ring A) and also 3-hydroxy-4-methoxy substituents of the B-ring. These binding parallels may rationalise the potency observed for **12d** in its tubulin effects which is seen to be close to that reported for CA-4.

3. Conclusion

We have synthesised a series of β-lactam compounds which show potent antiproliferative activity in breast cancer cells. The 3-unsubstituted compound **12d** and 3-methyl substituted compound **12e** (each with 3-hydroxy-4-methoxyaryl substitution pattern at C-4) were identified as the most potent compounds of the series having low nanomolar activity in both MCF-7 and MDA-MB-231 breast cancer cells. The most effective compound **12d** was shown to arrest cells in the G_2/M phase of the cell cycle. **12d** was also shown to inhibit the polymerisation of tubulin with improved efficacy when compared with combretastatin CA-4.

However, as these compounds were evaluated in this study as racemates, the effect of product stereochemistry on the biochemical activity will be relevant in the future development of these compounds. The β-lactam ring provides a scaffold structure with a similar spatial arrangement between the two phenyl rings as observed in *cis* conformation of combretastatin CA-4. These

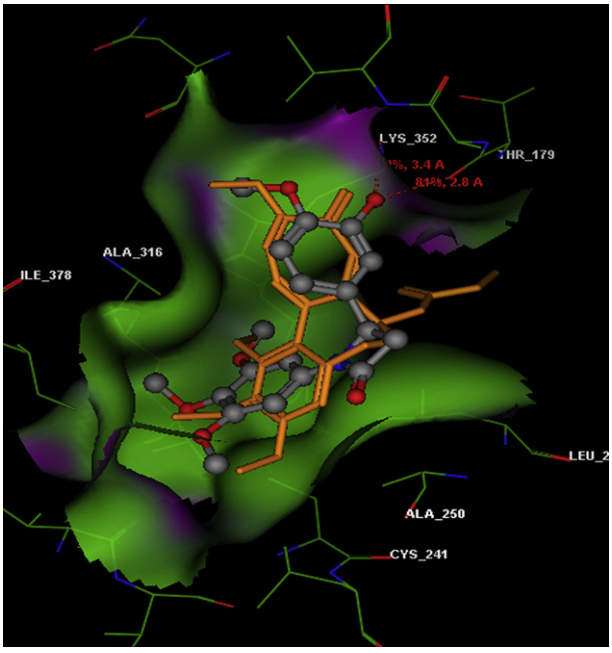

Fig. 2. Docked pose of β-lactam **12d** overlayed with colchicine in the tubulin binding site (PDB entry 1SA0). Hydrogen-bonds were created with an svl script through MOE.

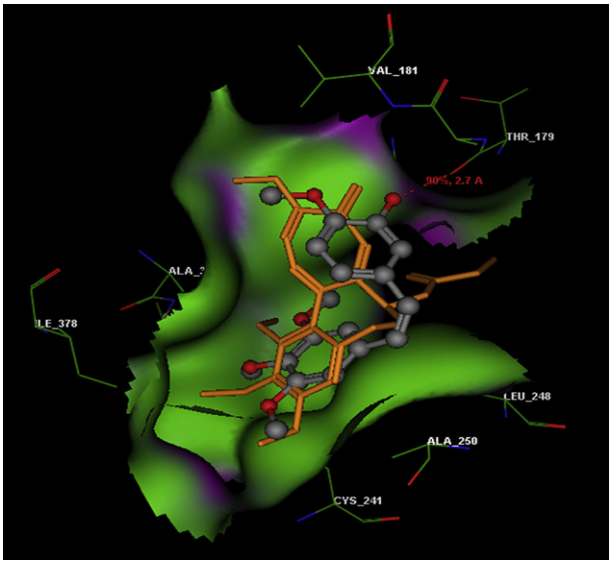

Fig. 3. Docked pose of CA-4 overlayed with colchicine in the tubulin binding site (PDB entry 1SA0).

conformationally restricted β-lactam structures, which unlike the *cis*-stilbene CA-4 are not easily isomerised, are promising lead compounds in the development of new anticancer agents. Further studies will determine the possible antiangiogenic effects of these compounds.

4. Experimental

4.1. Chemistry

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, and toluene was dried by distillation from sodium and stored on activated molecular sieves (4 A). 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 °C, 400.13 MHz for ^{1}H spectra, 100.61 MHz for ^{13}C spectra, in either $CDCl_3$, CD_3COCD_3 or CD_3OD (internal standard tetramethylsilane). 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 Department of Chemistry, Trinity College Dublin. 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. Chiral liquid chromatography was carried out on selected compounds using a Chiral-AGP™ 150 × 4.0 mm column supplied by ChromTech Ltd. (now supplied by Chiral Technologies Europe) with a Chiral – AGP™ guard column. The HPLC system consisted of the following components: a Waters 1525 binary HPLC pump, a Waters 2487 Dual Wavelength Absorbance Detector, a Waters In-Line Degasser AF and a Waters 717 plus Autosampler. Gradient elution was used beginning with 10% of organic phase and finishing with 90% of organic phase over a period of 20 min. 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. Compounds **11c** [48], **11g** [31] and **11h** [49] were prepared as previously reported.

4.1.1. 2-Methoxy-5-[(3,4,5-trimethoxyphenylamino)methyl]phenol (***11a***)

A solution of 3-hydroxy-4-methoxybenzaldehyde (10 mmol, 1.36 g) and 3,4,5-trimethoxyaniline (10 mmol, 1.37 g) in ethanol (50 mL) was heated to reflux for 3 h. The reaction mixture was reduced to 25 mL under vacuum, and the solution transferred to a beaker. The mixture was left to stand for 3 h and the Schiff base product crystallised out of the solution. The crude product was then re-crystallised from ethanol and filtered to yield the purified product. Yield 84%, pale yellow crystals, m.p. 176–178° C. IR ν_{max} (KBr) cm^{-1}: 1602.6 cm^{-1} (C=N), 3069.2 cm^{-1} (OH). ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.70 (s, 3H, O–CH_3), 3.81 (s, 3H, O–CH_3), 3.85 (s, 6H, 2× OCH_3), 6.57 (s, 2H, Ar-H), 7.03 (d, 1H, J = 8.2 Hz, Ar-H), 7.29 (dd, 1H, J = 2.0, 8.2 Hz, Ar-H), 7.42 (d, 1H, J = 2.0 Hz, Ar-H), 8.48 (s, 1H, CH=N), 9.33 (s, 1H, OH). ^{13}C NMR (100 MHz, DMSO): δ 55.83 (O–CH_3), 56.09 (O–CH_3), 60.09 (O–CH_3), 98.50, 111.57, 113.57, 122.26, 129.27, 135.50, 146.78, 147.59, 150.79, 153.21 (Aromatic C), 159.42 (CH=N). HRMS: Found: 318.1345; $C_{17}H_{20}NO_5$ requires 318.1341(M^+ + H).

4.1.2. (4-Fluorobenzylidene)-(3,4,5-trimethoxyphenyl)amine (***11e***)

Preparation was as above from 4-fluorobenzaldehyde (10 mmol, 1.24 g) and 3,4,5-trimethoxyaniline (10 mmol, 1.37 g). Yield 72%, pale yellow crystals, m.p. 87–90 °C. IR ν_{max} (KBr) cm^{-1}: 1626.1 cm^{-1} (C=N). ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.87 (s, 3H, O–CH_3), 3.90 (s, 6H, 3× O–CH_3), 6.48 (s, 2H, Ar-H), 7.14–7.29 (m, 2H, Ar-H), 7.88 (m, 2H, Ar-H), 8.44 (s, 1H, –CH=N). ^{13}C NMR (100 MHz, $CDCl_3$): δ 55.98 (O–CH_3), 60.88 (O–CH_3), 98.00, 106.60, 115.73, 115.98, 130.56, 130.65, 131.51, 136.30, 147.58, 153.46, (Aromatic C), 158.04 (CH=N). Elemental Analysis: Found: C, 66.28; H, 5.54; N, 4.73; F, 16.87. $C_{16}H_{16}FNO_3$ requires C, 66.43; H, 5.57; N, 4.84; F, 16.59%.

4.1.3. (2,4,5-Trimethoxybenzylidene)-(3,4,5-trimethoxyphenyl) amine (***11f***)

Preparation as described above from 2,4,5-trimethoxybenzaldehyde (10 mmol, 1.48 g) and 3,4,5-trimethoxyaniline (10 mmol, 1.37 g). Yield 78%, yellow crystals, m.p. 123 °C. IR ν_{max} (KBr) cm^{-1}: 1619.5 cm^{-1} (C=N). ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.84 (s, 3H, O–CH_3), 3.89 (s, 6H, 2× O–CH_3), 3.90(s, 3H, O–CH_3), 3.94 (s, 3H, O–CH_3), 3.95 (s, 3H, O–CH_3), 6.50 (s, 2H, Ar-H), 6.53 (s, 1H, Ar-H), 7.26 (s, 1H, Ar-H), 8.80 (s, 1H, –CH=N). ^{13}C NMR (100 MHz, $CDCl_3$): δ 55.42 (O–CH_3), 55.81(O–CH_3), 56.06(O–CH_3), 57.93 (O–CH_3), 60.62 (O–CH_3), 95.71, 97.87, 108.47, 122.48, 116.78, 125.43, 143.06, 143.39, 153.08, 154.52, (Aromatic C), 155.35 (CH=N). Elemental Analysis: Found: C, 62.68; H, 6.31; N, 3.05. $C_{19}H_{23}NO_6$, requires C, 63.16; H, 6.37; N, 3.88%.

4.1.4. (2,3,4-Trimethoxybenzylidene)-(3,4,5-trimethoxyphenyl) amine (***11i***)

Preparation as described above from 2,3,4-trimethoxybenzaldehyde (10 mmol, 1.48 g) and 3,4,5-trimethoxyaniline (10 mmol, 1.37 g). Yield 74%, yellow crystals, m.p. 178 °C. IR ν_{max} (KBr) cm^{-1}: 1619.6 cm^{-1} (C=N). ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.87 (s, 3H, O–CH_3), 3.91 (s, 9H, 3× O–CH_3), 3.93 (s, 3H, O–CH_3), 3.98 (s, 3H, O–CH_3), 6.48 (s, 2H, Ar-H), 6.80 (d, 1H, J = 8.8 Hz, Ar-H), 7.85 (d, 1H, J = 9.0 Hz, Ar-H), 8.74 (s, 1H, CH=N). ^{13}C NMR (100 MHz, $CDCl_3$): δ 55.96 (O–CH_3), 60.82 (O–CH_3), 60.87 (O–CH_3), 61.96 (O–CH_3), 98.15, 107.71, 122.45, 123.57, 135.96, 141.64, 148.61, 153.38, 154.47, 155.49 (Aromatic C), 155.25 (CH=N),. Elemental Analysis: Found: C, 63.06; H, 6.44; N, 3.82. $C_{19}H_{23}NO_6$ requires C, 63.10; H, 6.40; N, 3.90%.

4.1.5. (3,4-Dimethoxybenzylidene)-(3,4,5-trimethoxyphenyl)amine (***11j***)

Preparation as described above from 3,4-dimethoxybenzaldehyde (10 mmol, 1.34 g) and 3,4,5-trimethoxyaniline (10 mmol, 1.37 g). Yield 73%, yellow crystals, m.p. 102–104 °C. IR ν_{max} (KBr) cm^{-1}: 1606.9 cm^{-1} (C=N). ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.87 (s, 3H, O–CH_3), 3.90 (s, 6H, 2× O–CH_3), 3.95 (s, 3H, O–CH_3), 3.99 (s, 3H, O–CH_3), 6.48 (s, 2H, Ar-H), 6.93 (d, 1H, J = 8.4 Hz, Ar-H), 7.31–7.33 (m, 1H, Ar-H), 7.60 (s(br), 1H, Ar-H), 8.39 (s, 1H, –CH=N). ^{13}C NMR (100 MHz, $CDCl_3$): δ 55.89 (O–CH_3), 55.97 (O–CH_3), 60.90 (O–CH_3), 97.97, 108.35, 110.35, 124.31, 129.25, 135.97, 148.05, 151.92, 153.42 (Aromatic C) 159.13 (CH=N). HRMS: Calculated for $C_{21}H_{22}NO_5$: 332.1498; Found: 332.1485, (M^+ + H).

4.1.6. Thiophen-2-ylmethylene-(3,4,5-trimethoxyphenyl)amine (***11k***)

Preparation as described above from thiophene-2-carbaldehyde (10 mmol, 1.12 g) and 3,4,5-trimethoxyaniline (10 mmol, 1.37 g). Yield 81%, pale yellow crystals, m.p. 169 °C. IR ν_{max} (KBr) cm^{-1}:

1617.8 (C═N), 1584.53 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 3.88 (s, 3H, OCH_3), 3.92 (s, 6H, 2× OCH_3), 6.52 (s, 2H, Ar-H), 7.16–7.18 (m, 1H, Ar-H), 7.52–7.55 (m, 2H, Ar-H), 8.61 (s, 1H, HC═N). ^{13}C NMR (100 MHz, $CDCl_3$): δ 55.65 (O–CH_3), 60.56 (O–CH_3), 97.82, 127.38, 129.90, 131.83, 135.96, 142.17, 146.81, 153.30 (Aromatic C), 151.95 (CH═N). HRMS Calculated for $C_{14}H_{16}NO_3S$: 278.0851; Found: 278.0852, ($M^+ + H$). 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%.

4.1.7. (6-Methoxynaphthalen-2-ylmethylene)-(3,4,5-trimethoxyphenyl)amine (**11l**)

Preparation as described above from 6-methoxynaphthaldehyde (10 mmol, 1.86 g) and 3,4,5-trimethoxyaniline (10 mmol, 1.37 g). Yield 80%, yellow crystals, m.p. 40 °C. IR ν_{max} (KBr) cm^{-1}: 1616.4 cm^{-1} (C═N). ^1H NMR (400 MHz, $CDCl_3$): δ 3.88 (s, 3H, O–CH_3), 3.91 (s, 6H, 2× O–CH_3), 3.93 (s, 3H, O–CH_3), 6.53 (s, 2H, Ar-H), 7.17 (m, 2H, Ar-H), 7.82 (m, 2H, Ar-H), 8.08–8.12 (m, 2H, Ar-H), 8.58 (s, 1H, N═CH). ^{13}C NMR (100 MHz, $CDCl_3$): δ 54.95 (O–CH_3), 55.67 (O–CH_3), 60.60 (O–CH_3), 97.72, 105.61, 119.02, 124.08, 127.09, 128.00, 129.89, 130.57, 131.32, 135.82, 136.09, 147.72, 153.14, 158.63 (Aromatic C), 159.36 (N═CH). HRMS: Calculated for $C_{21}H_{22}NO_4$: 352.1549; Found: 352.1549 ($M^+ + H$).

4.1.8. [3-(tert-Butyldimethylsilanyloxy)(4-methoxybenzylidene)-(3,4,5-trimethoxyphenyl)amine (**11b**)

To a suspension of 2-methoxy-5-[(3,4,5-trimethoxyphenylamino) methyl]phenol (**11a**) (0.02 mol, 6.34 g) and dimethyl-*tert*-butylchlorosilane (0.024 mol) in dry DCM (60 mL) was added 1,8-diazobicyclo[5.4.0] undec-7-ene (DBU) (0.032 mol). The resulting mixture was stirred at room temperature until complete on thin layer chromatography. The solution was then diluted with DCM (80 mL) and washed with water (60 mL), 0.1 M HCl (60 mL) and finally with saturated aqueous $NaHCO_3$ (60 mL). The organic layer was removed and dried using anhydrous sodium sulphate. Evaporation of the solvent afforded the product which was recrystallised from ethanol. Yield 91%, yellow crystals, m.p. 88–92 °C, IR ν_{max} (KBr) cm^{-1}: 1618.8 cm^{-1} (C═N). ^1H NMR (400 MHz, DMSO): δ 0.21 (s, 6H, CH_3–Si–CH_3), 1.04 (s, 9H, Si–C–$(CH_3)_3$), 3.74 (s, 3H, O–CH_3), 3.87 (s, 6H, 2× O–CH_3), 3.93 (s, 3H, O–CH_3), 6.58 (s, 2H, Ar-H), 7.12 (d, 1H, J = 8.2 Hz, Ar-H), 7.48–7.55 (m, 2H, Ar-H), 8.51 (s, 1H, CH═N). ^{13}C NMR (100 MHz, DMSO): δ −5.78 (CH_3–Si–CH_3), 17.71 (CH_3–C–CH_3), 24.74 (C–($\underline{CH_3}$)$_3$), 55.00 (O–CH_3), 59.25 (O–CH_3), 98.04, 111.22, 121.27, 125.43, 129.50, 135.95, 144.68, 147.73, 153.32 (Aromatic C), 158.27 (CH═N). HRMS: Calculated for $C_{23}H_{34}O_5Si$: 432.2216; Found: 432.2213 ($M^+ + H$).

4.1.9. 3-(tert-Butyldimethylsilanyloxy)-(4-methoxyphenyl)-(3,4,5-trimethoxybenzylidene)amine (**11d**)

Preparation as described above from 2-methoxy-5-[(3,4,5-trimethoxybenzylidene)amino]phenol (**11c**) (0.02 mol, 6.34 g). Yield 71%, yellow crystals, m.p.108–109 °C. IR ν_{max} (KBr) cm^{-1}: 1614.7 cm^{-1} (C═N). ^1H NMR (400 MHz, DMSO): δ 0.20 (s, 6H, CH_3–Si–CH_3), 1.03 (s, 9H, Si–C–$(CH_3)_3$), 3.84 (s, 3H, O–CH_3), 3.93 (s, 3H, O–CH_3), 3.96 (s, 6H, 2× O–CH_3), 6.84–6.87 (m, 3H, Ar-H), 7.15 (2H, s, Ar-H), 8.36 (s, 1H, CH═N). ^{13}C NMR (100 MHz, DMSO): δ −5.05 (CH_3–Si–CH_3), 18.02 (CH_3–C–CH_3), 25.30 (C–($\underline{CH_3}$)$_3$), 55.24 (O–CH_3), 55.78 (O–CH_3), 60.53 (O–CH_3), 105.04, 111.72, 113.62, 113.82, 131.52, 140.16, 144.65, 144.95, 149.33, 153.02 (Aromatic C), 157.53 (CH═N). HRMS: Calculated for $C_{23}H_{34}NO_5Si$: 432.2206; Found: 432.2216, ($M^+ + H$).

4.1.10. 4-[3-(tert-Butyldimethylsilanyloxy)-4-methoxyphenyl]-1-(3,4,5-trimethoxyphenyl)azetidin-2-one (**12a**)

To a suspension of zinc dust (0.9 g, 13.8 mmol) in benzene (20 mL) under nitrogen was added trimethylchlorosilane (0.65 mL, 5 mmol) and the resulting mixture was stirred at room temperature for 15 min and then under reflux for a further 2 min. The suspension was cooled and (3-(*tert*-butyldimethylsilanyloxy)-4-methoxybenzylidene)-(3,4,5-trimethoxyphenyl)amine (**11b**) (7 mmol, 3.017 g) and ethylbromoacetate (1.33 mL, 12 mmol) were successively added. The reaction mixture was refluxed under nitrogen for 8 h and then cooled in an ice-water bath. It was then poured over 20 mL of saturated NH_4Cl and 20 mL of 25% NH_4OH. The mixture was extracted with dichloromethane (20 mL) and the organic layer was further washed with HCl (20 mL, 0.1 N) and water (20 mL). The organic layer was separated and dried over anhydrous sodium sulphate. The solvent was evaporated under reduced pressure, and the β-lactam was purified by flash chromatography over silica gel (eluent: dichloromethane–ethyl acetate; 19:1) Yield 21%, yellow crystals, m.p. 90–91 °C. IR ν_{max} (KBr) cm^{-1}: 1748.1 cm^{-1} (C═O). ^1H NMR (400 MHz, $CDCl_3$): δ 0.15 (s, 3H, CH_3–Si–CH_3), 0.16(s, 3H, CH_3–Si–CH_3), 0.88 (s, 9H, C–$(CH_3)_3$), 2.91 (dd, 1H, J = 2.0 Hz, 15.0 Hz, H-3), 3.49 (dd, 1H, J = 5.4 Hz, 15.4 Hz, H-3), 3.70 (s, 6H, 2× O–CH_3), 3.75 (s, 3H, O–CH_3), 3.79 (s, 3H, O–CH_3), 4.86, (dd, 1H, J = 2.0 Hz, J = 5.4 Hz, H-4), 6.54 (s, 2H, Ar-H), 6.81–6.83 (m, 2H, Ar-H), 6.92–6.95 (m, 1H, Ar-H). ^{13}C NMR (100 MHz, $CDCl_3$): δ −5.13 (CH_3–Si–CH_3), 13.65 (CH_3–C–CH_3), 25.20 (C–$(CH_3)_3$), 46.39 (CH_2), 53.57 (CH), 54.91 (O–CH_3), 55.41 (O–CH_3), 55.69 (O–CH_3), 94.04, 111.20, 111.81, 115.26, 119.05, 129.99, 133.57, 143.98, 150.72, 152.72 (Aromatic C), 164.08 (C═O). HRMS: Calculated for $C_{25}H_{36}NO_6Si$: 474.2311; Found: 474.2312 ($M^+ + H$).

4.1.11. 4-([3-tert-Butyldimethylsilanyloxy]-4-methoxyphenyl)-3-methyl–1-(3,4,5-trimethoxy phenyl)azetidin-2-one (**12b**)

Preparation as described above from 3-(*tert*-butyldimethylsilanyloxy)-(4-methoxyphenyl)-(3,4,5-trimethoxybenzylidene)amine (**11a**) (10 mmol, 1.8312 g) and ethyl-2-bromopropionate (12 mmol, 1.55 mL) and isolated as mixture of diastereomers. Yield 61%, brown solid which was used without further purification. IR ν_{max} (film) cm^{-1}: 1745.6 cm^{-1} (C═O, β-lactam). ^1H NMR (400 MHz, $CDCl_3$): δ 0.04 (s, 3H, Si–CH_3), 0.05 (s, 3H, Si–CH_3), 0.91 (s, 9H, C–$(CH_3)_3$), 1.17–1.21 (m, 2H, –CH_3), 1.26–1.30 (m, 1H, –CH_3), 3.05–3.12 (m, 0.4H, H-3), 3.55–3.58 (m, 0.6H, H-3), 3.68 (s, 6H, 2× O–CH_3), 3.73 (s, 3H, O–CH_3), 3.78 (s, 3H, O–CH_3), 4.44 (bs, 0.4H, H-4), 5.05 (d, 0.6H, J = 5.5 Hz, H-4), 6.51 (s, 1H, Ar-H), 6.52 (s, 1H, Ar-H), 6.54 (s (br), 1H, Ar-H), 6.67–6.82 (m, 2H, Ar-H). ^{13}C NMR (100 MHz, $CDCl_3$): δ −5.38 to −5.30 (CH_3–Si–CH_3), 9.05 (CH_3), 12.52 (CH_3), 17.87 (C–($\underline{CH_3}$)$_3$), 28.73 (C–$(CH_3)_3$), 48.61 (CH), 54.50 (O–CH_3), 54.87 (O–CH_3), 54.93 (O–CH_3), 55.36 (CH), δ 55.39 (O–CH_3), 60.32 (CH), 62.10 (CH), 94.10, 94.32, 111.48, 111.76, 110.17, 120.08, 127.78, 133.53, 133.61, 144.55, 150.39, 152.90 (Aromatic C), 167.80 (C═O), 167.94 (C═O).

4.1.12. 4-([3-tert-Butyldimethylsilanyloxy]-4-methoxyphenyl)-3,3-dimethyl-1-(3,4,5-trimethoxyphenyl)azetidin-2-one (**12c**)

Preparation as described above from [3-(*tert*-butyldimethylsilanyloxy)-(4-methoxyphenyl]-(3,4,5-trimethoxybenzylidene) amine (**11a**) (10 mmol, 1.831 g) and ethyl-2-bromoisobutyrate (12 mmol, 1.77 mL). Yield 80%, orange solid, which was used without further purification. IR ν_{max} (film) cm^{-1}: 1751.2 cm^{-1} (C═O, β-lactam). ^1H NMR (400 MHz, $CDCl_3$): δ 0.03 (s, 3H, Si–CH_3), 0.07 (s, 3H, Si–CH_3), 0.88 (s, 3H, –CH_3), 0.93 (s, 9H, C–$(CH_3)_3$), 1.50 (s, 3H, –CH_3), 3.72 (s, 6H, 2× O–CH_3), 3.77 (s, 3H, O–CH_3), 3.88 (s, 3H, O–CH_3), 4.69 (s, 1H, H-4), 6.57 (s, 2H, Ar-H), 6.67 (d, 1H, J = 8.5 Hz, Ar-H), 6.77–6.80 (m, 1H, Ar-H), 6.83 (d, 1H, J = 2.5 Hz, Ar-H). ^{13}C NMR (100 MHz, $CDCl_3$): δ −4.97 (CH_3–Si–CH_3), 17.60 (C–$\underline{CH_3}$)$_3$), 18.28 (C–($\underline{C}$)–C), 22.64 (–CH_3), 25.46 (–CH_3), 55.19 (CH_3–$\underline{C}$–CH_3, C_3), 55.30 (O–CH_3), 55.82 (O–CH_3), 60.78 (O–CH_3), 66.25 (CH), 94.84, 111.85, 119.28, 120.10, 127.47, 133.87, 134.01, 144.97, 150.74, 153.30 (Aromatic C), 171.34 (C═O).

4.1.13. 1-[3-(tert-Butyldimethylsilanyloxy)-4-methoxyphenyl]-4-(3,4,5-trimethoxyphenyl)azetidin-2-one (**12g**)

Preparation as described above from [3-(*tert*-butyldimethylsilanyloxy)-4-methoxyphenyl]-(3,4,5-trimethoxybenzylidene)amine (**11d**) (10 mmol, 4.310 g). Yield 47%, orange crystals, m.p. 112 °C. IR ν_{max} (KBr) cm^{-1}: 1747.6 cm^{-1} (C═O). ^1H NMR (400 MHz, $CDCl_3$): δ 0.21 (s, 6H, CH_3–Si–CH_3), 0.87 (s, 9H, C–$(CH_3)_3$), 2.84 (dd, 1H, J = 2.0 Hz, 15.0 Hz, H-3), 3.40 (dd, 1H, J = 5.4 Hz, 15.0 Hz, H-3), 3.82 (s, 3H, O–CH_3), 3.85 (s, 9H, 3× O–CH_3), 4.79 (dd, 1H, J = 2.4 Hz, J = 5.0 Hz H-4), 6.53 (s, 2H, Ar-H), 6.57 (d, 1H, J = 3.2 Hz, Ar-H), 6.62 (d, 1H, J = 3.4 Hz, Ar-H), 6.72 (d, 1H, J = 8.8 Hz, Ar-H). ^{13}C NMR (100 MHz, $CDCl_3$): δ −5.14 (CH_3–Si–CH_3), 17.97 (CH_3–C–CH_3), 25.24 (C–$(CH_3)_3$), 43.10 (C_3, CH_2), 55.34 (O–CH_3), 60.35 (O–CH_3), 70.04 (CH), 102.03, 111.82, 113.36, 136.76, 138.00, 152.80 (Aromatic C), 167.08 (C═O). HRMS: Calculated for $C_{25}H_{35}NO_6SiNa$: 496.2131; Found 496.2131 (M^+ + Na).

4.1.14. 1-[(3-tert-Butyldimethylsilanyloxy)-4-methoxyphenyl]-3-methyl-4-(3,4,5-trimethoxyphenyl)azetidin-2-one (**12h**)

Preparation as described above from [(3-*tert*-butyldimethylsilanyloxy)-4-methoxyphenyl]-(3,4,5-trimethoxybenzylidene)amine (**11d**) (5 mmol, 2.158 g) and ethyl-2-bromopropionate (6 mmol, 0.78 mL) and isolated as mixture of diastereomers. Yield 10%, orange gel. IR ν_{max} (film) cm^{-1}: 1749.1 cm^{-1} (C═O, β-lactam). ^1H NMR (400 MHz, $CDCl_3$): δ 0.01–0.03 (m, 3H, Si–CH_3), 0.05–0.06 (m, 3H, Si–CH_3), 0.90 (s, 9H, C–$(CH_3)_3$), 0.96 (d, 2H, J = 7.6 Hz, CH_3), 1.46 (d, 1H, J = 7.0 Hz, CH_3), 3.14 (m, 0.4H, H-3), 3.65–3.68 (m, 0.6H, H-3), 3.75–3.78 (m, 6H, 2× O–CH_3), 3.81–3.84 (m, 6H, 2× O–CH_3), 4.42 (bs, 0.4H, H-4), 5.03 (d, 0.6H, J = 5.5 Hz, H-4), 6.42 (s, 1H, Ar-H), 6.55 (s, 1H, Ar-H), 6.65–6.66 (m, 1H, Ar-H), 6.74–6.76 (m, 1H, Ar-H), 6.99–7.02 (m, 1H, Ar-H). ^{13}C NMR (100 MHz, $CDCl_3$):δ −5.32 (CH_3–Si–CH_3), 9.17 (−CH_3), 12.63 (−CH_3), 17.93 (C–$(CH_3)_3$), 25.14 (C–$(CH_3)_3$), 25.28 (C–$(CH_3)_3$), 48.80 (C_3,CH), 54.73 (C_3,CH), 55.38 (O–CH_3), 55.42 (O–CH_3), 55.59 (O–CH_3), 55.64 (O–CH_3), 55.69 (O–CH_3), 60.33 (CH), 60.37 (O–CH_3), 62.81 (CH), 102.13, 103.44, 109.71, 110.71, 110.15, 110.18, 112.13, 112.23, 130.27, 130.88, 131.04, 131.04, 137.05, 137.38, 147.19, 147.23, 153.03, 153.42 (Aromatic C), 167.38 (C═O), 167.51 (C═O). HRMS: Calculated for $C_{26}H_{37}NO_6SiNa$: 510.2288; Found: 510.2281 (M^+ + Na).

4.1.15. 1-[(3-tert-Butyldimethylsilanyloxy)-4-methoxyphenyl]-3,3-dimethyl-4-(3,4,5-trimethoxyphenyl)azetidin-2-one (**12i**)

Preparation as described above from [(3-*tert*-butyldimethylsilanyloxy)-4-methoxyphenyl]-(3,4,5-trimethoxybenzylidene)amine (**11d**) (5 mmol, 2.158 g) and ethyl-2-bromoisobutyrate (6 mmol, 0.88 mL). Yield 18%, orange solid, m.p. 95 °C. IR ν_{max} (film) cm^{-1}: 1747.5 cm^{-1} (C═O, β-lactam). ^1H NMR (400 MHz, $CDCl_3$): δ −0.17 (s, 3H, Si–CH_3), −0.12 (s, 3H, Si–CH_3), 0.72 (s 9H, C–$(CH_3)_3$), 0.74 (s, 3H, −CH_3), 1.32 (s, 3H, −CH_3), 3.57 (s, 3H, O–CH_3), 3.58 (s, 6H, 2× O–CH_3), 3.65 (s, 3H, O–CH_3), 4.46 (s, 1H, H-4), 6.21 (s, 2H, Ar-H), 6.57 (m, 2H, Ar-H), 6.81 (dd, 1H, J = 2.5 Hz, 8.5 Hz, Ar-H). ^{13}C NMR (100 MHz, $CDCl_3$): δ −5.37 (CH_3–Si–CH_3), 17.23 (C–C–C), 22.36 (C–$(CH_3)_3$), 25.11 (−CH_3), 25.25 (−CH_3), 54.92 (CH_3–C–CH_3), 55.60 (O–CH_3), 55.69 (O–CH_3), 60.32 (O–CH_3), 66.47 (CH), 94.84, 111.85, 119.28, 120.10, 127.47, 133.87, 134.01, 144.96, 150.74, 153.30 (Aromatic C), 171.33 (C═O). HRMS: Calculated for $C_{27}H_{39}NO_6SiNa$: 524.2444; Found 524.2427 (M^+ + Na).

4.1.16. 4-(4-Fluorophenyl)-1-(3,4,5-trimethoxyphenyl)azetidin-2-one (**12m**)

Preparation as described above from (4-fluorobenzylidene)-3,4,5-trimethoxyphenylamine (**11e**) (10 mmol, 2.89 g). Yield 53%, pale orange crystals, m.p. 96 °C. IR ν_{max} (KBr) cm^{-1}: 1739.8 cm^{-1} (C═O, β-lactam). ^1H NMR (400 MHz, $CDCl_3$): δ 2.72 (dd, 1H, J = 2.5 Hz, 15.5 Hz, H-3), 3.34 (dd, 1H, J = 5.5 Hz, 15.0 Hz, H-3), 3.48 (s, 6H, 2× O–CH_3), 3.54 (s, 3H, O–CH_3), 4.78 (dd, 1H, J = 2.0 Hz, 5.5 Hz, H-4), 6.34 (s, 2H, Ar-H), 6.83–6.87 (m, 2H, Ar-H), 7.17–7.21 (m, 2H, Ar-H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 46.42 (CH_2), 53.23 (CH), 55.45 (O–CH_3), 60.44 (O–CH_3), 90.90, 115.58, 127.22, 133.36, 133.52, 133.84, 153.00 (Aromatic C), 160.94 (C–F), 163.40 (C═O). HRMS: Calculated for $C_{18}H_{18}FNO_4Na$: 354.1118; Found 354.1132 (M^+ + Na).

4.1.17. 4-(2,4,5-Trimethoxyphenyl)-1-(3,4,5-trimethoxyphenyl) azetidin-2-one (**12n**)

Preparation as described above from (2,4,5-trimethoxybenzylidene)-3,4,5-trimethoxyphenylamine (**11f**) (10 mmol, 3.614 g). Yield 28%, orange gel, IR ν_{max} (film) cm^{-1}: 1746.9 cm^{-1} (C═O, β-lactam). ^1H NMR (400 MHz, $CDCl_3$): δ 3.01 (dd, 1H, J = 2.0 Hz, 15.0 Hz, H-3), 3.51 (dd, 1H, J = 6.0 Hz, 15.1 Hz, H-3), 3.73 (s, 6H, 2× O–CH_3), 3.74 (s, 3H, O–CH_3), 3.75 (s, 3H, O–CH_3), 3.77 (s, 6H, 2× O–CH_3), 5.24 (dd, 1H, J = 2.5, 5.7 Hz, H-4), 5.91 (s, 2H, Ar-H), 6.59 (s, 1H, Ar-H), 6.78 (s,1H, Ar-H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 44.88 (CH_2), 48.02 (CH), 51.70 (O–CH_3), 55.49 (O–CH_3), 56.24 (O–CH_3), 60.45 (O–CH_3), 60.58 (O–CH_3), 92.05, 109.79, 118.39, 133.64, 133.71, 142.68, 145.07, 149.39, 151.32, 152.96, (Aromatic C), 164.68 (C═O). HRMS: Calculated for $C_{21}H_{25}NO_7Na$: 426.1529; Found 426.1534 (M^+ + Na).

4.1.18. 4-(4-Methoxyphenyl)-1-(3,4,5-trimethoxyphenyl)azetidin-2-one (**12o**)

Preparation as described above from (4-methoxybenzylidene)-3,4,5-trimethoxyphenylamine (**11g**) (5 mmol, 1.506 g). Yield 43%, green crystals, m.p. 70–71 °C. IR ν_{max} (film): 1747.5 cm^{-1} (C═O, β-lactam). ^1H NMR (400 MHz, $CDCl_3$): δ 2.85 (dd, 1H, J = 2.5 Hz, 15.0 Hz, H-3), 3.48 (dd, 1H, J = 5.5 Hz, J = 15.1 Hz, H-3), 3.65 (s, 6H, 2× O–CH_3), 3.70 (s, 3H, O–CH_3), 3.73 (s, 3H, O–CH_3), 4.88 (dd, J = 2.6 Hz, 5.5 Hz, 1H, H-4), 6.50 (s, 2H, Ar-H), 6.86 (d, 2H, J = 8.6 Hz, Ar-H), 7.26 (d, 2H, J = 8.6 Hz, Ar-H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 46.36 (CH_2), 53.56 (CH), 54.78 (O–CH_3), 55.49 (O–CH_3), 60.36 (O–CH_3), 93.92, 113.58, 126.83, 129.48, 133.62, 133.68, 152.94, 159.29 (Aromatic C), 164.14 (C═O). HRMS: Calculated for $C_{19}H_{21}NO_5Na$: 366.1317; Found: 366.1330 (M^+ + Na).

4.1.19. 4-(4-Methoxyphenyl)-3-methyl-1-(3,4,5-trimethoxyphenyl) azetidin-2-one (**12p**)

Preparation as described above from (4-methoxybenzylidene)-(3,4,5-trimethoxyphenyl)amine (**11g**) (5 mmol, 1.506 g) and ethyl-2-bromopropionate (6 mmol, 0.78 mL) and isolated as mixture of diastereomers. Yield 83%, dark orange gel. IR ν_{max} (film): 1725.5 cm^{-1} (C═O, β-lactam). ^1H NMR (400 MHz, $CDCl_3$): δ 0.75 (d, 2H, J = 7.5 Hz, CH_3), 1.33 (d, 1H, J = 7.5 Hz, CH_3), 3.54 (q(br), 0.66H, J = 7.5 Hz, H-3), 3.94 (q(br), 0.34H, J = 7.5 Hz, H-3), 3.57 (3H, s, O–CH_3), 3.58 (3H, s, O–CH_3), 3.62 (s, 1.2H, O–CH_3), 3.64 (s, 2.8H, O–CH_3), 4.42 (bs, 0.34H, H-4), 5.01 (d, 0.66H, J = 6.0 Hz, H-4), 6.44 (s, 1.3H, Ar-H), 6.46 (s, 0.8H, Ar-H), 6.76 (m, 2H, Ar-H), 7.06 (d, 1.33H, J = 8.0 Hz, Ar-H), 7.20 (d, 0.67H, J = 8.5 Hz, Ar-H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 9.16 (−CH_3), 12.43(CH_3),48.65 (CH), 55.38 (CH), 54.52 (O–CH_3), 54.58 (O–CH_3), 54.65 (O–CH_3), 55.32 (O–CH_3), 57.76 (CH), 60.25 (CH), 62.09 (O–CH_3), 93.98, 94.24, 113.51, 113.93, 126.05, 126.77, 127.67, 129.14, 133.47, 152.90, 158.90 (Aromatic C), 167.78 (C═O), 167.93 (C═O). HRMS: Calculated for $C_{20}H_{23}NO_5Na$: 380.1473; Found 380.1473 (M^+ + Na).

4.1.20. 4-(4-Methoxyphenyl)-3,3-dimethyl-1-(3,4,5-trimethoxyphenyl)azetidin-2-one (**12q**)

Preparation as described above from (4-methoxybenzylidene)-(3,4,5-trimethoxyphenyl)amine (**11g**) (5 mmol, 1.507 g) and ethyl-2-bromoisobutyrate (6 mmol, 0.88 mL). Yield 68%, yellow powder, m.p. 110 °C. IR ν_{max} (KBr): 1747.6 cm^{-1} (C═O, β-lactam). ^1H NMR (400 MHz, $CDCl_3$): δ 0.86 (s, 3H, −CH_3), 1.52 (s, 3H, −CH_3), 3.72 (s,

6H, 2× O–CH_3), 3.78 (s, 3H, O–CH_3), 3.81 (s, 3H, O–CH_3), 4.73 (s, 1H, H-4), 6.57 (s, 2H, Ar-H), 6.87 (d, 2H, $J = 8.5$ Hz, Ar-H), 7.14 (d, 2H, $J = 8.5$ Hz, Ar-H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 17.43 (–CH_3), 22.31 (–CH_3), 54.79 (O–CH_3), 54.93 (C-3), 55.57 (O–CH_3), 60.51 (O–CH_3), 66.11 (CH), 94.50, 113.61, 126.79, 127.39, 133.68, 153.00, 158.92 (Aromatic C), 171.11 (C=O). HRMS: Calculated for $C_{21}H_{25}NO_5Na$: 394.1631; Found: 394.1620 ($M^+ + Na$).

4.1.21. 1-(3,4,5-Trimethoxyphenyl)-4-(3,4,5-trimethoxyphenyl) azetidin-2-one (**12r**)

Preparation as described above from (3,4,5-trimethoxybenzylidene)-(3,4,5-trimethoxyphenyl)amine (**11h**) (5 mmol, 1.807 g). Yield 68%, orange gel. IR ν_{max} (film): 1746.9 cm^{-1} (C=O). ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.88 (dd, 1H, $J = 2.5$ Hz, 15.5 Hz, H-3), 3.49 (dd, 1H, $J = 5.5$ Hz, $J = 15.1$ Hz, H-3), 3.64 (s, 3H, O–CH_3), 3.66 (s, 6H, 2× O–CH_3), 3.76 (s, 9H, 3× O–CH_3), 4.82 (dd, $J = 5.5$ Hz, 2.5 Hz, 1H, H-4), 6.55 (s, 2H, Ar-H), 6.59 (s, 2H, Ar-H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 46.25 (CH_2), 55.19 (CH), 55.53 (O–CH_3), 55.68 (O–CH_3), 60.22 (O–CH_3), 60.41 (O–CH_3), 90.86, 102.22, 129.58, 133.41, 137.46, 138.00, 152.93, 153.36 (Aromatic C), 164.14 (C=O). HRMS: Calculated for $C_{21}H_{25}NO_7Na$: 426.1529; Found 426.1530 ($M^+ + Na$).

4.1.22. 4-(2,3,4-Trimethoxyphenyl)-1-(3,4,5-trimethoxyphenyl) azetidin-2-one (**12s**)

Preparation as described above from (2,3,4-trimethoxybenzylidene)-(3,4,5-trimethoxyphenyl)amine (**11i**) (5 mmol, 1.807 g). Yield 16%, yellow gel. IR ν_{max} (film): 1751.7 cm^{-1} (C=O). ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.99 (dd, 1H, $J = 2.5$ Hz, 15.1 Hz, H-3), 3.50 (dd, 1H, $J = 5.5$ Hz, $J = 15.1$ Hz, H-3), 3.73 (s, 6H, 2× O–CH_3), 3.75 (s, 3H, O–CH_3), 3.83 (s, 3H, O–CH_3), 3.86 (s, 3H, O–CH_3), 3.92 (s, 3H, O–CH_3), 5.21 (dd, 1H, $J = 2.5$ Hz, 6.0 Hz, H-4), 6.57 (s, 2H, Ar-H), 6.64 (d, 1H, $J = 8.5$ Hz, Ar-H), 6.97 (d, 1H, $J = 8.5$ Hz, Ar-H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 45.34 (CH_2), 48.66 (CH), 55.50 (O–CH_3), 55.56 (O–CH_3), 59.80 (O–CH_3), 60.33 (O–CH_3), 60.95 (O–CH_3), 93.84, 107.10, 120.99, 122.69, 133.64, 139.03, 141.66, 151.42, 153.38 (Aromatic C), 164.38 (C=O). HRMS: Calculated for $C_{21}H_{25}NO_7Na$: 426.1529; Found: 426.1549 ($M^+ + Na$).

4.1.23. 4-(3,4-Dimethoxyphenyl)-1-(3,4,5-trimethoxyphenyl) azetidin-2-one (**12t**)

Preparation as described above from (3,4-dimethoxybenzylidene)-(3,4,5-trimethoxyphenyl)amine (**11j**) (5 mmol, 1.655 g). Yield 15%, orange crystals, m.p. 99 °C. IR ν_{max} (KBr): 1745.9 cm^{-1} (C=O). ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.86 (dd, 1H, $J = 2.5$ Hz, 15.1 Hz, H-3), 3.48 (dd, 1H, $J = 5.5$ Hz, $J = 15.1$ Hz, H-3), 3.64 (s, 6H, 2× O–CH_3), 3.67 (s, 3H, O–CH_3), 3.69 (s, 6H, 2× O–CH_3), 4.83 (dd, 1H, $J = 2.5$ Hz, 5.5 Hz, H-4), 5.84 (s(br), 2H, Ar-H), 6.50 (s, 2H, Ar-H), 6.91 (dd, 1H, $J = 2$ Hz, 8.0 Hz, Ar-H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 46.37 (CH_2), 54.16 (O–CH_3), 55.27 (O–CH_3), 55.48 (O–CH_3), 60.52 (CH), 90.91, 105.19, 118.60, 124.46, 127.70, 128.28, 128.63, 133.59, 153.25, 157.90 (Aromatic C), 164.16 (C=O). HRMS: Calculated for $C_{20}H_{23}NO_6Na$: 396.1423; Found: 396.1408 ($M^+ + Na$).

4.1.24. 4-(3,4-Dimethoxyphenyl)-3-methyl-1-(3,4,5-trimethoxyphenyl)azetidin-2-one (**12u**)

Preparation as described above from (3,4-dimethoxybenzylidene)-(3,4,5-trimethoxyphenyl)amine (**11j**) (5 mmol, 1.657 g) and ethyl-2-bromopropionate (6 mmol, 0.78 mL) and isolated as mixture of diastereomers. Yield 10%, brown gel. IR ν_{max} (film): 1744.6 cm^{-1} (C=O). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.94 (d, 2H, $J = 7.5$ Hz, –CH_3), 1.48 (d, 1H, $J = 7.5$ Hz, –CH_3), 3.16 (m, 0.34H, H-3), 3.69 (m, 0.66H, H-3), 3.72 (s, 3H, O–CH_3), 3.76 (s, 3H, O–CH_3), 3.80 (s, 9H, 3× O–CH_3), 4.50 (d, 0.34H, $J = 2.0$ Hz, H-4), 5.11 (d, 0.66H, $J = 6.0$ Hz, H-4), 6.56 (s, 0.6H, Ar-H), 6.59 (s, 1.4H, Ar-H), 6.72 (d, 1H, $J = 1.5$ Hz, Ar-H), 6.72–6.85 (dd, 1H, $J = 1.65$ Hz, 8.1 Hz, Ar-H), 6.88 (d, 1H, $J = 8.0$ Hz, Ar-H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 9.18 (–CH_3), 12.62 (–CH_3), 48.84 (CH), 54.59 (CH), 55.40 (O–CH_3), 55.49 (O–CH_3), 55.51 (O–CH_3), 55.54 (O–CH_3), 55.58 (O–CH_3), 58.18 (CH), 60.49(O–CH_3), 60.60 (O–CH_3), 62.65 (CH), 92.06, 94.64, 107.90, 109.31, 110.67, 110.89, 118.28, 119.04, 126.68, 130.08, 133.55, 142.61, 152.98, 153.36 (Aromatic C), 168.00 (C=O), 168.13 (C=O). HRMS: Calculated for $C_{21}H_{25}NO_6Na$: 410.1580; Found: 410.1567 ($M^+ + Na$).

4.1.25. 4-(3,4-Dimethoxyphenyl)-3,3-dimethyl-1-(3,4,5-trimethoxyphenyl)azetidin-2-one (**12v**)

Preparation as described above using method 2 from (3,4-dimethoxybenzylidene)-(3,4,5-trimethoxyphenyl)amine (**11j**) (5 mmol, 1.657 g) and ethyl-2-bromoisobutyrate (6 mmol, 0.88 mL) Yield 10%, green powder, m.p. 102 °C. IR ν_{max} (film): 1745.9 cm^{-1} (C=O). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.90 (s, 3H, –CH_3), 1.52 (s, 3H, –CH_3), 3.73 (s, 6H, 2× O–CH_3), 3.79 (s, 3H, O–CH_3), 3.84 (s, 3H, O–CH_3), 3.89 (s, 3H, O–CH_3), 4.73 (s, 1H, H-4), 6.60 (s, 2H, Ar-H), 6.70 (d, 1H, $J = 2.0$ Hz, Ar-H), 6.81 (dd, 1H, $J = 2.0$ Hz, 8.5 Hz, Ar-H), 6.88 (d, 1H, $J = 8.5$ Hz, Ar-H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 17.29 (–CH_3), 22.35 (–CH_3), 30.48 (C-3), 54.99 (O–CH_3), 55.39 (O–CH_3), 55.56 (O–CH_3), 55.58 (O–CH_3), 66.39 (CH), 94.47, 109.01, 110.67, 118.69, 127.33, 133.71, 133.77, 148.32, 148.65, 152.99 (Aromatic C), 171.12 (C=O). HRMS: Calculated for $C_{22}H_{27}NO_6Na$: 424.1736; Found 424.1726 ($M^+ + Na$).

4.1.26. 4-Thiophen-2-yl-1-(3,4,5-trimethoxyphenyl)azetidin-2-one (**12w**)

Preparation as described above from thiophen-2-ylmethylene-(3,4,5-trimethoxyphenyl)amine (**11k**) (5 mmol, 1.387 g). Yield 27%, dark brown gel. IR ν_{max} (film): 1735.5 cm^{-1} (C=O). ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.95 (dd, 1H, $J = 15.1$ Hz, $J = 2.9$ Hz, H-3), 3.61 (dd, 1H, $J = 15.1$ Hz, $J = 5.8$ Hz, H-3), 3.74 (s, 6H, 2× O–CH_3), 3.76 (s, 3H, O–CH_3), 5.05, (dd, 1H, $J = 2.9$ Hz, 5.5 Hz, H-4), 6.61 (s, 2H, Ar-H), 7.00 (m, 1H, thiophene H), 7.17 (d, 1H, $J = 4.1$ Hz, thiophene H), 7.31 (d, 1H, $J = 5.2$ Hz, thiophene H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 47.52 (CH_2), 51.40 (CH), 55.68 (O–CH_3), 55.84 (O–CH_3), 60.85 (O–CH_3), 94.26, 123.82, 125.81, 127.05, 133.67, 134.24, 141.93, 153.31 (Aromatic C), 163.86 (C=O). HRMS: Calculated for $C_{16}H_{18}NO_4S$: 320.0957; Found 320.1003 ($M^+ + H$).

4.1.27. 4-(7-Methoxynaphthalen-2-yl)-1-(3,4,5-trimethoxyphenyl) azetidin-2-one (**12x**)

Preparation as described above from (6-methoxynaphthalen-2-ylmethylene)-(3,4,5-trimethoxyphenyl)amine (**11l**) (5 mmol, 1.757 g). Yield 20%, orange gel. IR ν_{max} (film): 1736.4 cm^{-1} (C=O). ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.02 (dd, 1H, $J = 2.5$ Hz, 12.1 Hz, H-3), 3.61 (dd, 1H, $J = 5.5$ Hz, $J = 15.3$ Hz, H-3), 3.69 (s, 6H, 2× O–CH_3), 3.76 (s, 3H, O–CH_3), 3.89 (s, 3H, O–CH_3), 5.09, (dd, 1H, $J = 2.5$ Hz, $J = 5.5$ Hz, H-4), 5.89 (s, 2H, Ar-H), 6.62 (s, 1H, Ar-H), 7.10–7.14 (m, 2H, Ar-H), 7.46–7.48 (dd, 1H, Ar-H), 7.69–7.78 (m, 2H, Ar-H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 47.52 (CH_2), 51.40 (CH), 55.68 (O–CH_3), 55.84 (O–CH_3), 60.85 (O–CH_3), 90.90, 94.26, 105.31, 118.60, 123.82, 125.81, 127.05, 128.43, 128.85, 129.62, 133.67, 134.24, 141.93, 153.31 (Aromatic C), 163.86 (C=O).

4.1.28. 4-(3-Hydroxy-4-methoxyphenyl)-1-(3,4,5-trimethoxyphenyl)azetidin-2-one (**12d**)

To a suspension of 4-[3-(*tert*-butyldimethylsilanyloxy)-4-methoxyphenyl]-1-(3,4,5-trimethoxyphenyl)azetidin-2-one (**12a**) (0.634 mmol, 0.30 g) in THF (20 mL) was added of 1 M *tetra-n*-butylammonium fluoride (1.5 equiv). The solution was stirred in an ice bath for 15 min to avoid decomposition of the β-lactam ring. The reaction mixture was then diluted with ethyl acetate (100 mL) and quenched with 10% HCl (100 mL). The layers are separated and the aqueous layer was extracted with ethyl acetate (2 × 50 mL). The

organic layer was then washed with water (100 mL) and brine (100 mL) and dried with sodium sulphate. The crude product was purified by flash chromatography over silica gel (eluent: dichloromethane). Yield 62%, yellow gel. IR ν_{max} (film): 1746.0 (C═O), 3404.2 cm^{-1} (OH). ^1H NMR (400 MHz, $CDCl_3$): δ 2.91 (dd, 1H, $J = 2.4$ Hz, 15.6 Hz, H-3), 3.48 (dd, 1H, $J = 5.5$ Hz, 15.0 Hz, H-3), 3.70 (s, 6H, 2× O−CH_3), 3.74 (s, 3H, O−CH_3), 3.85 (s, 3H, O−CH_3), 4.86 (dd, 1H, $J = 2.5$ Hz, $J = 5.5$ Hz, H-4), 6.54 (s, 2H, Ar-H), 6.81–6.94 (m, 3H, Ar-H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 30.47 (CH_2), 46.31 (CH), 53.66 (O−CH_3), 55.53 (O−CH_3), 60.44 (O−CH_3), 94.01, 110.13, 111.60, 117.35, 130.64, 133.57, 143.21, 145.88, 146.44, 152.94, 164.21 (C═O). HRMS: Calculated for $C_{19}H_{22}NO_6$: 360.1447; Found 360.1454 ($M^+ + H$).

4.1.29. 4-(3-Hydroxy-4-methoxyphenyl)-3-methyl−1-(3,4,5-trimethoxyphenyl)azetidin-2-one (**12e**)

Preparation as described above from 4-([3-*tert*-butyldimethylsilanyloxy]-4-methoxyphenyl)-3-methyl−1-(3,4,5-trimethoxyphenyl)azetidin-2-one (**12b**) (8 mmol, 3.901 g) and isolated as mixture of diastereomers. Yield 45%, brown gel. IR ν_{max} (KBr): 1724.2, (C═O), 3240.2 cm^{-1} (OH). ^1H NMR (400 MHz, $CDCl_3$): δ 0.91 (d, 2H, $J = 7.5$ Hz, −CH_3), 1.44 (d, 1H, $J = 7.5$ Hz, −CH_3), 3.09–3.14 (dq, 0.33H, $J = 2.2$ Hz, 7.5 Hz, H-3), 3.59–3.67 (dq, 0.67H, $J = 7.5$ Hz, 6.0 Hz, H-3), 3.72 (s(br), 6H, 2× O−CH_3), 3.76 (s, 1H, O−CH_3), 3.78 (s, 2H, O−CH_3), 3.89 (s, 3H, O−CH_3), 4.44 (d, 0.33H, $J = 2.0$ Hz, H-4), 5.07 (d, 0.67H, $J = 5.6$ Hz, H-4), 5.81 (bs, 1H, OH), 6.55 (s, 0.67H, Ar-H), 6.57 (s, 1.33H, Ar-H), 6.70–6.85 (m, 3H, Ar-H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 9.15 (−CH_3), 12.62 (−CH_3), 45.51 (CH), 54.61 (CH), 55.47 (O−CH_3), 55.56 (O−CH_3), 56.62 (O−CH_3), 57.90 (CH), 62.31 (CH), 94.14, 94.37, 110.17, 110.49, 112.71, 118.24, 127.36, 130.48, 133.64, 145.33, 146.29, 152.98 (Aromatic C), 167.94 (C═O), 168.10 (C═O). HRMS: Calculated for $C_{20}H_{23}NO_6Na$: 396.1423; Found: 396.1419 ($M^+ + Na$).

4.1.30. 4-(3-Hydroxy-4-methoxyphenyl)-3,3-dimethyl−1-(3,4,5-trimethoxyphenyl)azetidin-2-one (**12f**)

Preparation as described above from 4-([3-*tert*-butyldimethylsilanyloxy]-4-methoxyphenyl)-3,3-dimethyl-1-(3,4,5-trimethoxyphenyl)azetidin-2-one (**12c**) (8 mmol, 4.01 g). Yield 53%, pale brown powder. IR ν_{max} (KBr): 1747.4 (C═O), 3460.1 cm^{-1} (OH). ^1H NMR (400 MHz, $CDCl_3$): δ 0.79 (s, 3H, −CH_3), 1.40 (s, 3H, −CH_3), 3.63 (s, 6H, 2× O−CH_3), 3.69 (s, 3H, O−CH_3), 3.79 (s, 3H, O−CH_3), 4.60 (s, 1H, H-4), 6.50 (s, 2H, Ar-H), 6.61 (dd, 1H, $J = 2.0$ Hz, 8.5 Hz, Ar-H), 6.71 (d, 1H, $J = 2.0$ Hz, Ar-H), 6.76 (d, 1H, $J = 8.5$ Hz, Ar-H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 17.24 (−CH_3), 22.27 (−CH_3), 54.89 (C-3), 55.53 (O−CH_3), 55.56 (O−CH_3), 60.46 (O−CH_3), 66.06 (CH), 94.54, 110.24, 112.45, 117.80, 127.87, 133.63, 133.73, 145.38, 146.12, 152.96, 153.17 (Aromatic C), 171.56 (C═O). HRMS: Calculated for $C_{21}H_{25}NO_6Na$: 410.1580; Found: 410.1588 ($M^+ + Na$).

4.1.31. 1-(3-Hydroxy-4-methoxyphenyl)]-4-(3,4,5-trimethoxyphenyl)azetidin-2-one (**12j**)

Preparation as described above from 1-[3-(*tert*-butyldimethylsilanyloxy)-4-methoxyphenyl]-4-(3,4,5-trimethoxyphenyl) azetidin-2-one (**12g**) (1.05 mmol, 0.497 g). Yield 39%, orange gel. IR ν_{max} (film): 1731.9 (C═O), 3500.0 cm^{-1} (OH). ^1H NMR (400 MHz, $CDCl_3$): δ 2.90 (dd, 1H, $J = 2.7$ Hz, 15.0 Hz, H-3), 3.50 (dd, 1H, $J = 5.5$ Hz, 15.3 Hz, H-3), 3.82 (s, 3H, O−CH_3), 3.85 (s, 9H, 3× O−CH_3), 4.86 (dd, 1H, $J = 2.7$ Hz, 5.4 Hz, H-4), 6.55 (s, 2H, Ar-H), 6.73 (d, 1H, $J = 8.9$ Hz, Ar-H), 6.82 (dd, 1H, $J = 2.1$ Hz, 8.8 Hz, Ar-H), 6.95 (d, 1H, $J = 2.7$ Hz, Ar-H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 46.43 (CH_2), 54.07 (CH), 55.56 (O−CH_3), 55.63 (O−CH_3), 60.34 (O−CH_3), 102.05, 103.92, 107.98, 110.64, 131.38, 133.49, 143.05, 145.56, 152.76, 153.33 (Aromatic C), 164.01 (C═O). HRMS: Calculated for $C_{19}H_{22}NO_6$: 360.1447; Found: 360.1449 (M^++H).

4.1.32. 1-(3-Hydroxy-4-methoxyphenyl)]-3-methyl-4-(3,4,5-trimethoxyphenyl)azetidin-2-one (**12k**)

Preparation as described above from 1-[3-(*tert*-butyldimethylsilanyloxy)-4-methoxyphenyl]-3-methyl-4-(3,4,5-trimethoxyphenyl)azetidin-2-one (**12h**) (0.149 mmol, 0.072 g) and isolated as mixture of diastereomers. Yield 82%, brown solid, m.p. 165 °C; ν_{max} (film): 1739.6 (C═O), 3409.0 cm^{-1} (OH). ^1H NMR (400 MHz, $CDCl_3$): δ 0.93 (d, 2H, $J = 7.6$ Hz, −CH_3), 1.40 (d, 1H, $J = 7.8$ Hz, −CH_3), 3.15 (dq, 0.36H, $J = 2.0$ Hz, 7.6 Hz, H-3), 3.64 (dq, 0.64H, $J = 7.5$ Hz, 5.2 Hz, H-3), 3.79 (s, 3H, O−CH_3), 3.83 (m, 9H, 3× O−CH_3), 4.43 (d, 0.36H, $J = 1.9$ Hz, H-4), 5.05 (d, 0.64H, $J = 5.5$ Hz, H-4), 5.81 (bs, 1H, OH), 6.41 (s, 0.89H, Ar-H), 6.53 (s, 1.10H, Ar-H), 6.74 (2× d, overlapping, 1H, $J = 8.5$ Hz, 8.6 Hz, Ar-H), 6.84–6.90 (2× dd overlapping, 1H, $J = 2.5$ Hz, 8.8 Hz, Ar-H), 6.92 (d, 0.5H, $J = 2.5$ Hz), 6.95 (d, 0.5H, $J = 2.3$ Hz, Ar-H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 9.16 (−CH_3), 12.66 (−CH_3), 48.86 (CH), 54.81 (CH), 55.70 (O−CH_3), 55.73 (O−CH_3), 58.41 (CH), 60.42 (O−CH_3), 62.80 (CH), 102.01, 103.34, 103.81, 103.90, 108.28, 108.39, 110.51, 110.51, 130.23, 131.46, 133.22, 142.73, 142.79, 145.41, 153.02–153.39 (Aromatic C), 167.69 (C═O). HRMS: Calculated for $C_{20}H_{23}NO_6Na$: 396.1423; Found: 396.1443 ($M^+ + Na$).

4.1.33. 1-(3-Hydroxy-4-methoxyphenyl)]-3,3-dimethyl-4-(3,4,5-trimethoxyphenyl)-azetidin-2-one (**12l**)

Preparation as described above from 1-[3-(*tert*-butyldimethylsilanyloxy)-4-methoxyphenyl]-3,3-dimethyl-4-(3,4,5-trimethoxyphenyl)azetidin-2-one (**12i**) (0.7 mmol, 0.352 g). Yield 59%, colourless powder, m.p. 174–178 °C. IR ν_{max} (film): 1724.5 (C═O), 3367.7 cm^{-1} (OH). ^1H NMR (400 MHz, $CDCl_3$): δ 0.91 (s, 3H, −CH_3), 1.51 (s, 3H, −CH_3), 3.78 (s, 6H, 2× O−CH_3), 3.83 (s, 3H, O−CH_3), 3.85 (s, 3H, O−CH_3), 4.67 (s, 1H, H-4), 6.39 (s, 2H, Ar-H), 6.74 (d, 1H, $J = 8.5$ Hz, Ar-H), 6.80 (dd, 1H, $J = 2.5$ Hz, 8.8 Hz, Ar-H), 7.08 (d, 1H, $J = 2.5$ Hz, Ar-H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 17.20 (−CH_3), 22.34 (−CH_3), 54.91 (C-3), 55.66 (O−CH_3), 55.69 (O−CH_3), 60.38 (O−CH_3), 66.60 (C-4), 103.06, 104.37, 108.27, 110.62, 130.79, 131.44, 137.05, 143.00, 145.60, 153.00, 170.98 (C═O). HRMS: Calculated for $C_{21}H_{25}NO_6Na$: 410.1580; Found 410.1591 ($M^+ + Na$).

4.1.34. 4-(4-Methoxyphenyl)-1-(3,4,5-trimethoxyphenyl)azetidin-2-thione (**13a**)

4-(4-Methoxyphenyl)-1-(3,4,5-trimethoxyphenyl)azetidin-2-one (**12o**) (0.206 g, 0.6 mmol) and Lawesson's reagent (0.4 mmol, 0.162 g) (0.68 equiv) were refluxed in toluene (5 mL) at 135 °C under N_2 for 3 h. The solvent was evaporated and the brown solid residue was purified by flash column chromatography (eluent: dichloromethane) to afford the product as a yellow solid. Yield 47.5%, R_f 0.89 (CH_2Cl_2: MeOH; 19:1), m.p. 84 °C. IR: NaCl film ν_{max}: 1595.7 cm^{-1} (C═S). ^1H NMR (400 MHz, $CDCl_3$): δ 3.00 (dd, 1H, $J = 15.6$ Hz, $J = 2.0$ Hz, H-3), 3.50 (dd, 1H, $J = 15.6$ Hz, $J = 5.0$ Hz, H-3), 3.72 (s, 6H, 2× O−CH_3), 3.78 (s, 3H, O−CH_3), 3.80 (s, 3H, O−CH_3), 5.43 (dd, $J = 2.0$ Hz, $J = 5.0$ Hz, 1H, H-4), 6.92 (d, 2H, $J = 8.5$ Hz, Ar-H), 7.19 (s, 2H, Ar-H), 7.30–7.32 (d, 2H, $J = 9.0$ Hz, Ar-H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 49.30 (CH_2), 54.90 (O−CH_3), 55.58 (O−CH_3), 60.43 (O−CH_3), 62.13 (CH), 95.26, 114.21, 127.04, 128.29, 134.11, 135.17, 152.63, 159.65 (Aromatic C), 196.01 (C═S). HRMS, Calculated for $C_{19}H_{21}NO_4SNa$: 382.1089; Found: 382.1076 ($M^+ + Na$).

4.1.35. 4-(4-Methoxyphenyl)-3-methyl-1-(3,4,5-trimethoxyphenyl) azetidin-2-thione (**13b**)

Preparation as described above from 4-(4-methoxyphenyl)-3-methyl-1-(3,4,5-trimethoxyphenyl)azetidin-2-one (**12p**) (0.214 g, 0.6 mmol). Evaporation of the solvent yielded a brown solid residue which was purified using by flash column chromatography over silica gel (eluent: dichloromethane) to afford the diastereomeric product as a yellow gel. Yield 63.8%, R_f 0.86 (CH_2Cl_2: MeOH; 19:1). IR: NaCl film ν_{max}: 1591.11 cm^{-1} (C═S). ^1H NMR (400 MHz, $CDCl_3$):

δ 0.96 (d, 2H, J = 7.5 Hz, CH_3), 1.47 (d, 1H, J = 7.5 Hz, $-CH_3$), 2.99–3.04 (m, 0.33H, H-3), 3.46–3.49 (m, 0.67H, H-3), 3.71 (s, 6H, 2× O–CH_3), 3.77 (s, 1H, O–CH_3), 3.80 (s, 5H, O–CH_3), 5.02 (d, J = 1.8 Hz, 0.33H, H-4), 5.67 (d, 0.67H, J = 5.5 Hz, H-4), 6.88–6.90 (m, 2H, Ar-H), 6.92–7.31 (m, 4H, Ar-H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 11.31 ($-CH_3$), 14.59 ($-CH_3$), 50.66 (CH), 54.80 (O–CH_3), 54.89 (O–CH_3), 55.56 (O–CH_3), 55.61 (O–CH_3), 56.06 (CH), 60.43 (O–CH_3), 66.31 (CH), 70.38 (CH), 95.50, 95.61, 113.73, 114.16, 125.21, 127.06, 127.77, 133.93, 135.07, 152.61, 158.19, 159.59 (Aromatic C), 201.72, 202.21 (C=S). HRMS: Calculated for $C_{20}H_{23}NO_4SNa$: 396.1245; Found: 396.1235 (M^+ + Na).

4.1.36. 4-(4-Methoxyphenyl)-3,3-dimethyl-1-(3,4,5-trimethoxyphenyl)azetidin-2-thione (**13c**)

Preparation as described above from 4-(4-methoxyphenyl)-3,3-dimethyl-1-(3,4,5-trimethoxyphenyl)azetidin-2-one (**12q**) (1.065 g, 2.868 mmol). Evaporation of the solvent yielded a brown solid residue which was purified using flash column chromatography over silica gel (eluent: dichloromethane) to afford the product as a yellow solid. Yield 46.7%, R_f 0.85 (CH_2Cl_2: MeOH; 19:1), m.p. 94 °C. IR: NaCl film ν_{max}: 1595.2 cm^{-1} (C=S). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.88 (s, 3H, $-CH_3$), 1.48 (s, 3H, $-CH_3$), 3.71 (s, 6H, 2× O–CH_3), 3.79 (s, 3H, O–CH_3), 3.795 (s, 3H, O–CH_3), 5.29 (s, 1H, H-4), 6.89 (d, 2H, J = 8.6 Hz, Ar-H), 7.1 (d, 1H, J = 8.5 Hz, Ar-H), 7.23 (s, 2H, Ar-H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 19.37 ($-CH_3$), 24.34 ($-CH_3$), 54.79 (O–CH_3), 51.14 (C-3), 56.60 (O–CH_3), 60.42 (O–CH_3), δ 73.85 (C-4), 95.91, 113.69, 125.85, 127.46, 133.99, 135.03, 152.62, 159.11 (Aromatic C), 206.80 (C=S). HRMS: Calculated for $C_{21}H_{26}NO_4S$: 388.1583; Found: 388.1586 (M^+ + Na).

4.2. Biochemical evaluation of activity

4.2.1. Antiproliferation studies

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, 2 mM 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, 2 mM L-glutamine and 100 μg/mL penicillin/streptomycin (complete medium). Cells were trypsinised and seeded at a density of 2.5 × 10^4 cells/mL in a 96-well plate and incubated at 37 °C, 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 37 °C. 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 and from these, graphs of percentage cell viability versus concentration of subject compound added were drawn.

4.2.2. Cell cycle analysis

Flow cytometry : The MDA-MB-231 cells were seeded at a density of 18 × 10^4 cells/mL in 5 mL of medium (900,000 cells per flask). After 24 h, cells were treated with either 50 μL of ethanol (1% v/v) as vehicle control or selected compound ranging from 10 nM to 10 μM (final concentration). They were incubated for 72 h. Following incubation, the cells were removed from the bottom of the flask by scraping and the medium placed in a 20 mL sterilin. Cells were then centrifuged for 10 min at 600 × g. The supernatant was decanted and the pellet resuspended in 1 mL of ice-cold phosphate buffered saline (PBS); cells were again centrifuged for 10 min at 600 × g. The supernatant was decanted and the pellet resuspended in 200 μL of ice-cold (PBS). Subsequently ice-cold 70% ethanol (2 mL) was slowly added to the tube as it was gently vortexed. The cells were kept at −20 °C for at least 1 h. After the fixation 5 μL of FBS was added to the samples. The cells were harvested by centrifugation at 600 × g for 10 min. The ethanol was carefully removed and the pellet resuspended in 400 μL of PBS and transferred to FACS microtubes. A 25 μL aliquot of RNase A (1 mg/mL) and 75 μL of propidium iodide (PI) 1 mg/mL, a DNA binding fluorescent dye, was added to each tube. The samples were wrapped in aluminium foil and incubated for a minimum of 30 min at 37 °C. The samples were read at 488 nM using a FACscalibur flow cytometer from Becton Dickinson. The FACS data for 10,000 cells was analysed using the Macintosh-based application Cellquest and the data was stored as frequency histograms.

4.2.3. Tubulin polymerisation assay

The effect of compounds on the assembly of purified bovine brain tubulin was determined spectrophotometrically by monitoring the change in turbidity. This assay used a 96-well plate format with 300 μg of >99% purified bovine brain tubulin in each well. Lyophilised tubulin (1 mg, Cytoskeleton, Denver, CO) was resuspended on ice in 300 μL in ice-cold G-PEM buffer (80 mM PIPES pH 6.9, 0.5 mM $MgCl_2$, 1 mM EGTA, 1 mM guanidine triphosphate (GTP), 10.2% (v/v glycerol)) and was left on ice for 1 min to allow for complete resuspension. 10 μL of 10× strength of each compound tested was pipetted into a half area 96-well plate prewarmed to 37 °C. A 100 μL volume of tubulin was then pipetted into the prewarmed plate. Samples were mixed well and tubulin assembly was monitored at an absorbance of A340 nm at 30 s intervals for 60 min at 37 °C in a Spectromax 340PC spectrophotometer (Molecular Devices).

4.3. Computational procedure

For ligand preparation, all compounds were drawn using ACD/Chemsketch v10 and SMILES strings generated. A single conformer was generated using Corinav3.4 and ensuring Omegav2.2.1 was subsequently employed to generate a maximum of 1000 conformations of each compound. For the receptor preparation, the PDB entries 1SA0 and 1SA1 were downloaded from the Protein Data Bank (PDB). All waters were retained in both isoforms. Addition and optimization 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) [42], possible binding orientations ligands were probed with the docking program FREDv2.2.3 (Openeye Scientific Software) [43]. Docking was carried out using FREDv2.2.3 in conjunction with Chemgauss3. 3-D ligand conformations of compound **12d** were enumerated using CORINAv3.4 (Molecular Networks GMBH) [44] for ligands followed by generation of multiple conformations using OMEGAv2.2.1 (Openeye Scientific Software) [45]. Each conformation was subsequently docked and scored with Chemgauss3 as outlined previously [46]. The top binding poses were refined using the LigX procedure (MOE – Chemical Computing Group) [47] together with Postdock analysis (SVL script; MOE) of the docked ligand poses.

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