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Combretazets: Enantiomeric β −Lactams for the Treatment of Breast Cancer

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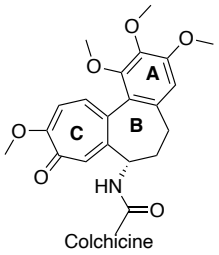
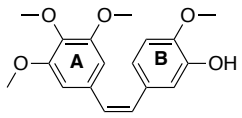
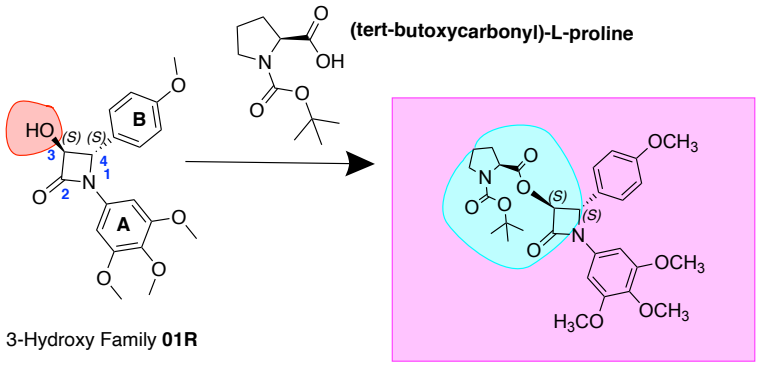
Combretazets: Enantiomeric β −Lactams for the Treatment of Breast Cancer







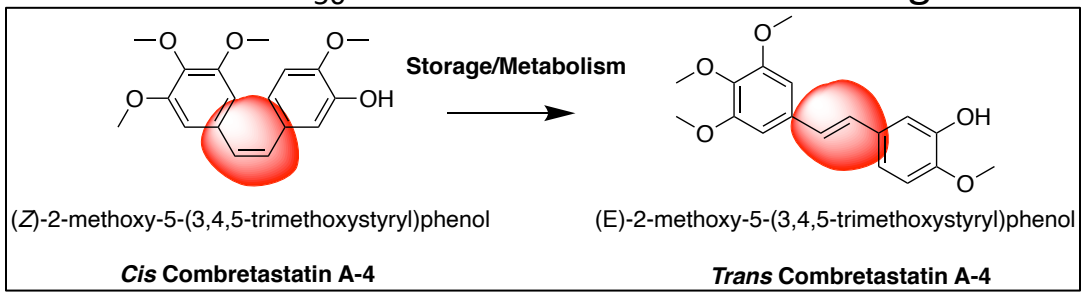
Abstract:

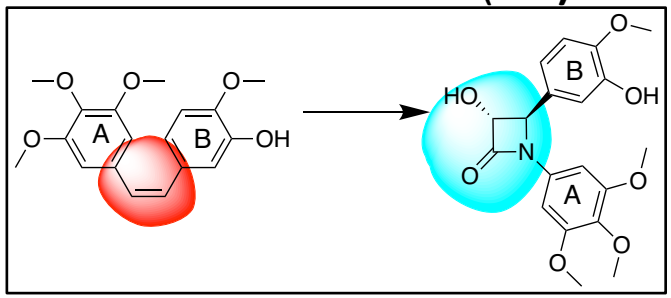
The combretastatins are diaryl stilbenoid natural products isolated from the bark of the South African willow tree *Combretum caffrum*. CA-4 **(Figure 1)** is a potent anticancer agent, which inhibits cancer cell proliferation and microtubule polymerisation by binding at the colchicine-binding site of tubulin. Only the *cis* configuration of CA-4 possesses anticancer bioactivity. It readily isomerizes *in vivo* during metabolism and upon storage into the more thermodynamically stable but significantly less active *trans* isomer. Our group has reported extensive series of antiproliferative, tubulin-binding β-lactam compounds – the 'Combretazets' – over the last decade with aim of overcoming this undesirable *in vivo* conversion to the inactive *trans* form. Substituting the ethylene bridge with a 1,4-diaryl-2-azetidinone ring (**01**, Figure 1) allows similar structural arrangement between CA-4's two aromatic rings and overcomes*cis*/*trans* isomerization. The racemic azetidinone **01** has an IC_{50} value of 4 nM in MCF-7 cells. It is essential to distinguish the eutomer from the distomers. Here, we describe the synthesis, resolution, and biochemical activity of the enantiomers of **01.**

Keywords: tubulin, anti-tubulin, tubulin poylmerisation inhibitors, colchicine, CA-4

Introduction: CA-4 and The Meegan family of β −Lactam derivatives

- CA-4 is the most potent anti-cancer molecule of a family of anti-cancer di-aryl stilbenoid molecules, isolated from the bark of the South African willow tree *Combretum Caffrum* **(1).** *(IC_{50} in MCF-7 cells of 5.2nM)* **(2)**
- However, it's active *cis* isomeric form is rapidly inactivated *in vivo* and during storage due to conversion towards the more stable, yet inactive *trans* isomeric species.
- The Meegan group over the last decade has inserted a β −Lactam structure in place of CA-4's *cis* double bond to create a family of extremely potent anti-cancer racemic mixtures over the last decade known as the **Combretazets**, with IC_{50} values in low nanomolar range over a host of cancer cell lines. **(3-6)**



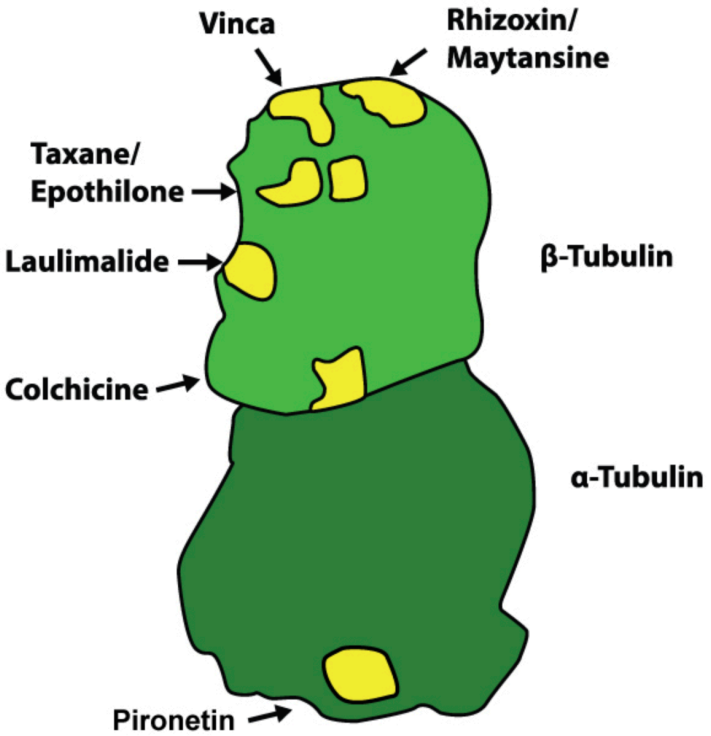


Microtubules and the Colchicine Binding site as the target of anti-tubulin agents: CA-4, Colchicine and the Combretazets.

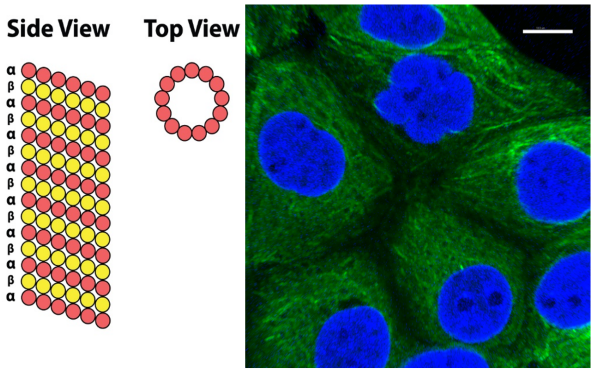
- Microtubules are major components of the cellular cytoskeleton.
- In the context of anti-cancer small molecule drug development, they are responsible for chromosomal separation during mitosis, the process where one cell splits into two daughter cells. **(9)**
- Microtubules are dynamic polymers alernating between periods of growth and shrinkage.
- **Tubulin, a heterodimer protein,** is the principle building block of microtubules.
- Tubulin heterodimers formed from α and β monomers,the binding site of many Microtubule targeting agents (MTAs).
- Popular clinically used MTAs include the Taxanes and the Vinca alkaloids for the treatment of cancer.
- When cells enter mitosis, the rate of microtubule growth and shortening increases 100 fold, making them attractive targets for many microtubule targeting agents incuding the Combretazet family.

Introduction: CA-4 as a Colchicine Binding Site inhibitor

- Microtubule-targeting agents (MTAs) bind to β tubulin in the α-β heterodimer and suppress microtubule dynamics. **(3).**
- MTAs interact with tubulin at a number of different binding sites **(4)** (*shown on right)*
- The Colchicine binding site (CBS) lies at the interface of α and β subunits.**(5)** CA-4 and it's β −Lactam derivatives also bind here.
- Colchicine, CA-4 and 'The Combretazets' are **anti-tubulin agents**
- They are tubulin destabilizers and cause tubulin depolymerisation through binding at this α β interface . **(6)(7)(8)(8)**



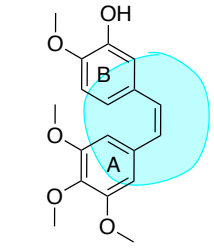
Anti-tubulin agents: Mode of Action of CA-4, Colchicine and The Meegan Group's Combretazet Family



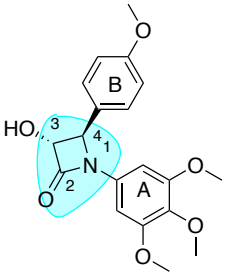
- Colchicine derivatives will bind at the CBS and induce GTPase activity at the GTP cap on microtubules.
- GTPase activity will promote loss of the microtubules GTP cap and cause tubulin dissasebly and de-polymerisation.
- Colchicines toxicity limits it's clinical use at higher doses for the treatment of cancer. Currently it is used in low doses for acute treatment of gout only. It's toxicity also limits its use for the prevention of gout. **(9,10)**
- The phosphate prodrug of CA-4, Fosbretabulin is currently undergoing clinical trials. **(11-14)**
- To date no MTA targeting the Colchicine binding site is in routine clinical use.

Microtubule structure from side (left) and top perspectives (right). Side: Long, linear protofilaments consisting of αβ-tubulin heterodimers associate laterally. Top: Association of 13 protofilaments forms the microtubule, a long hollow cylinder. Right: Microtubule network in MCF-7 breast cancer cell in interphase ; cells were stained with mouse monoclonal anti-α-tubulin–FITC antibody (clone DM1A) (tubulin, green) Alexa Fluor 488 dye and counterstained with DAPI (nuclei, blue). Scale bar: 10 µM

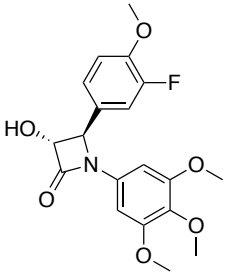
Modifications to prevent *cis* → *trans* isomerization by the Meegan Group



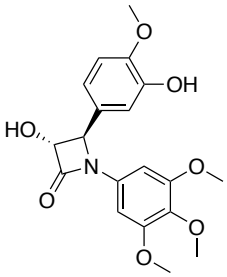
Combretastatin A-4



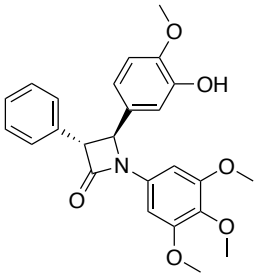
01R



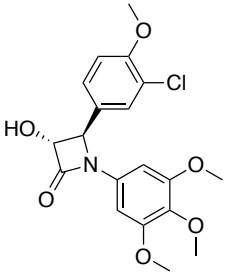
02R



03R



04R



05R

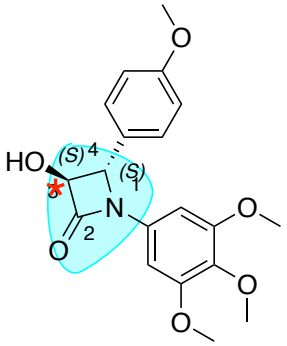
Most potent analogues selected for isolation as single enantiomer derivatives *via* chiral resolution (*one enantiomer shown)*

Compound	IC_{50} in MCF7 Cells
CA-4	5.2 nM
01R	4 nM
02R	22 nM
03R	3 nM
04R	4 nM
05R	12 nM
06R	5 nM

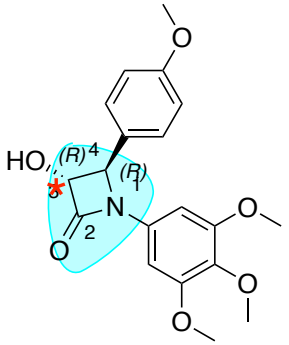
- Replace **double bond of *Cis* CA-4** with **β-Lactam ring**
- **Enables *cis* restriction of A and B rings**
- **β-Lactam *Cis* Restricted Analogues of CA-4 –** 'the combretazets',
  - **Promising analogues –** greater **OR** comparable tubulin depolymerization potency with respect to CA-4
- Rigid β-Lactam ring scaffold permits similar spatial arrangement between the two aromatic rings of *cis* CA-4
  - overcoming issue of isomerization to inactive *trans* derivative.
- Potency **enhanced for analogues shown** with respect to CA-4 in human breast cancer cell lines.
- Potential treatment for triple negative breast cancer.
- All analogues illustrated to date are racemic mixtures.
- Isolation of individual enantiomers from respective racemic mixtures with potential to provide analogues of CA-4 with sub nanomolar IC_{50} values for the treatment of breast cancer.

Lessons on Stereochemistry of organic compounds: Significance of chirality in Biochemical Environments

- Stereoisomers are identical molecular species in both atomic constitution and bonding, differing only in three- dimensional spatial arrangement of atoms.
- Stereoisomers with similar **asymmetric centres** which are mirror images of each other are termed enantiomers (seen for **01R below)**
- **Enantiomers are physically identical (same melting point, boiling point, ^{1} H NMR etc.) with the exception of their optical rotation of plane polarized light.**
- Chemical properties of chiral compounds are identical in **achiral** environments only.
- However, properties of enantiomers can be vastly different in chiral environments including biological systems.
    - Drug receptors are 3D proteins which are made up of chiral amino acids.
    - Enantiomeric pairs may have greater or less affinity for receptors **OR** may be metabolised to a greater or lesser extent.

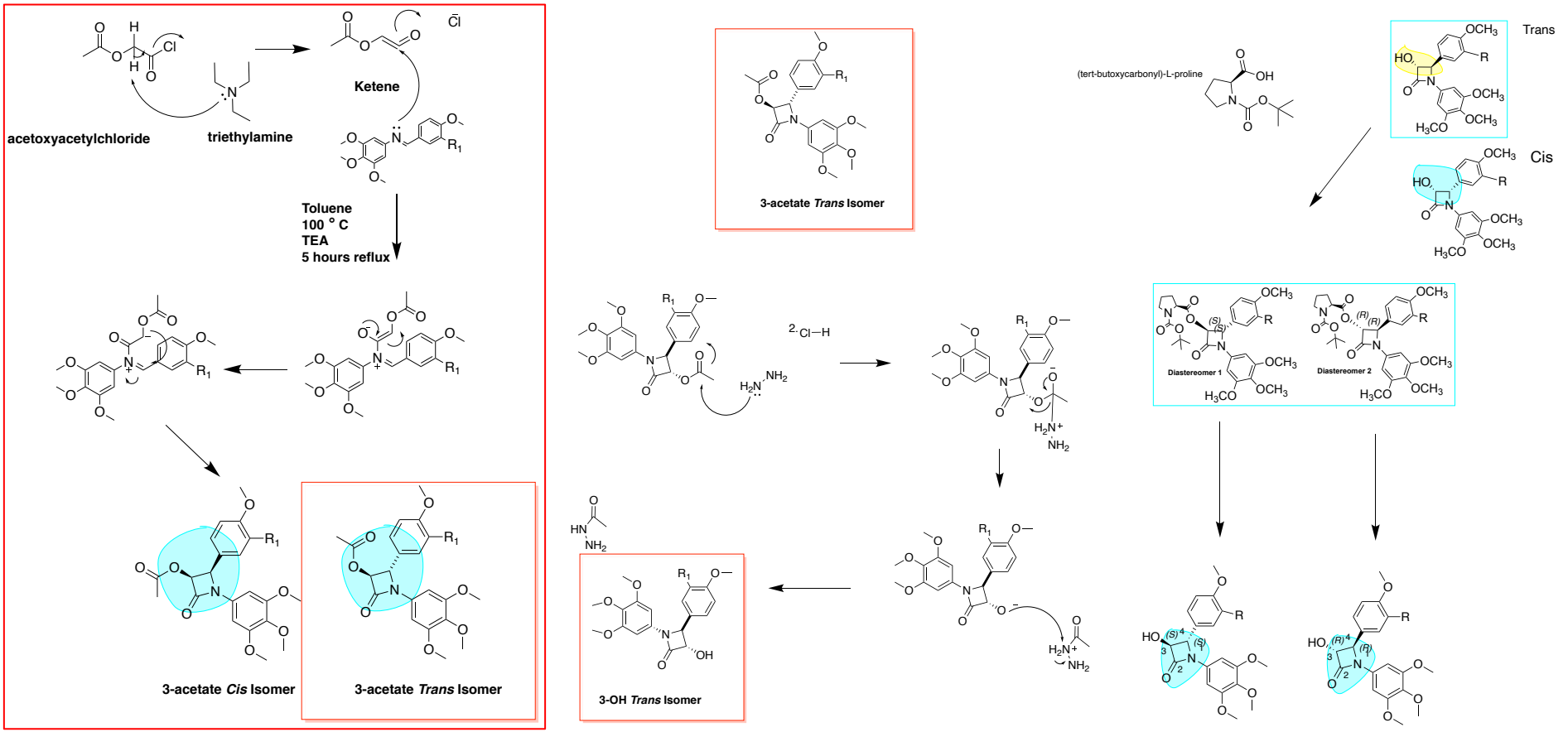


01En1



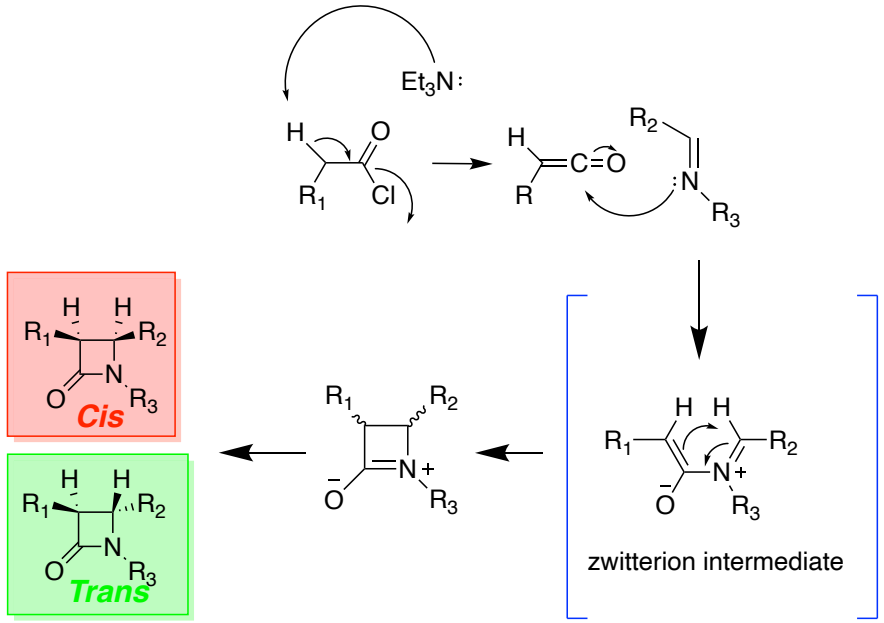
01En2

Chemistry: Synthesis of Racemic mixtures of 'The Combretezet Family' followed by Isolation of Enantiomers using Chiral Resolving agent



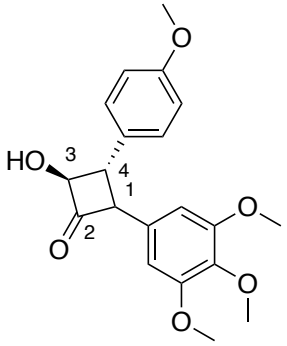
Synthesis of Racemic Mixtures: Staudinger Reaction

- Staudinger reaction is a simple reaction between three reagents
  - Imine
  - Acid chloride
  - Weak base
- Isomerization is **not possible** once the ring has already cyclised.
- Relative ratios of ***cis:trans*** are determined during the reaction procedure (by order of addition of reagents and substituents on Imine precursors)
- For the 3-Hydroxy ***β*** –lactam family, without optimisation a large proportion of *cis* isomer is present prior to reaction optimisation



Synthesis of Racemic Mixtures: Staudinger Reaction

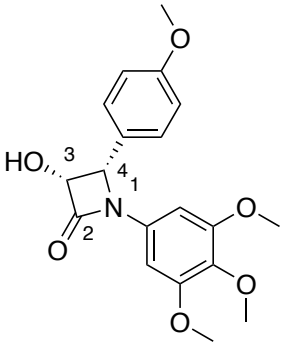
- Important to isolate only the ***trans*** isomer. ***Cis*** isomer is significantly less active.



01R

IC_{50} MCF-7 Cells (2020)
average of three replicates

Trans 10.79 nM ±3.07



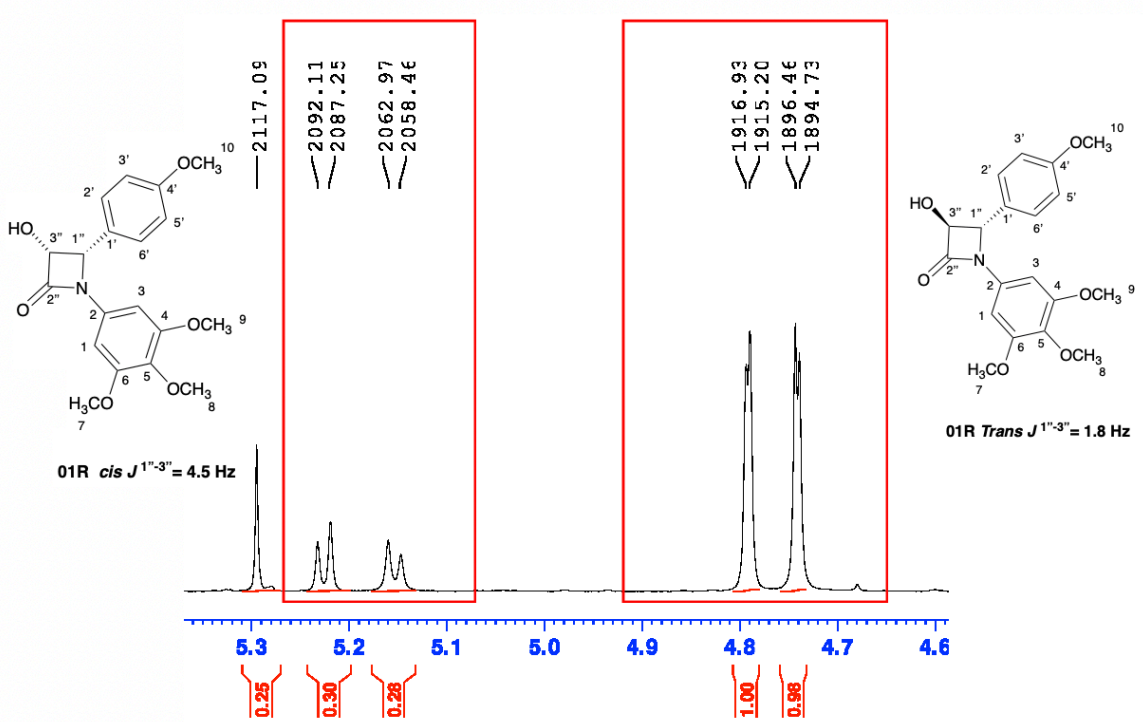
01C

Cis 43.73 nM ± 13

Preliminary Synthesis of 3-Hydroxy-β-Lactams

Mixed isomers present

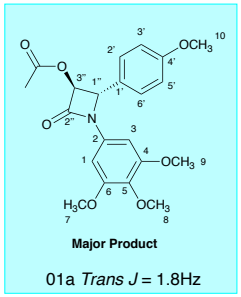
- Evidence:
  - Duplication of peaks
  - β-Lactam hydrogen doublets on ^{1} H NMR duplicated. ($H_{1\&3}$)

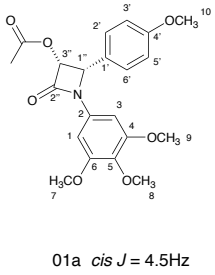


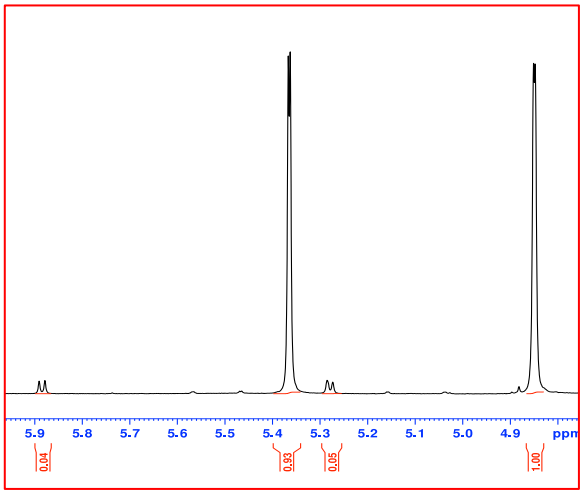
Ratio of ***cis:trans*** can be seen as 1:4 with an integration of 0.3 *cis* :1.01 *trans. (*$H_{1''}$ and $H_{3''}$ doublets for *cis and trans of* ***01R)***

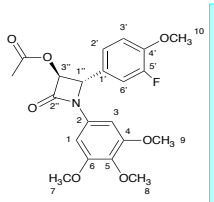
Optimised Synthesis of 3-Hydroxy-β-Lactams yielding > 95% *Trans* isomer

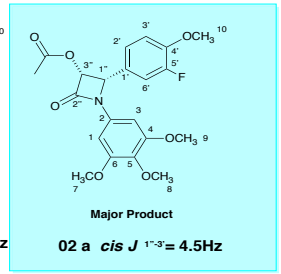
Reaction	Reaction Conditions	Ratio of Trans: Cis as per crude ^{1}H NMR of Staudinger
1	Imine and acid chloride (acetoxyacetyl chloride) allowed to stir for 20 mins at reflux prior to dropwise addition of base TEA.	95:5
2	Imine, TEA and acetoxyacetyl chloride added to solvent at 0 degrees and heated to reflux immediately	20:80

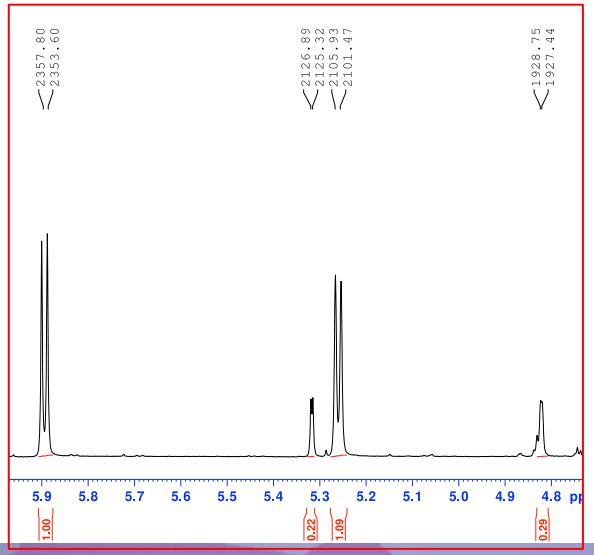




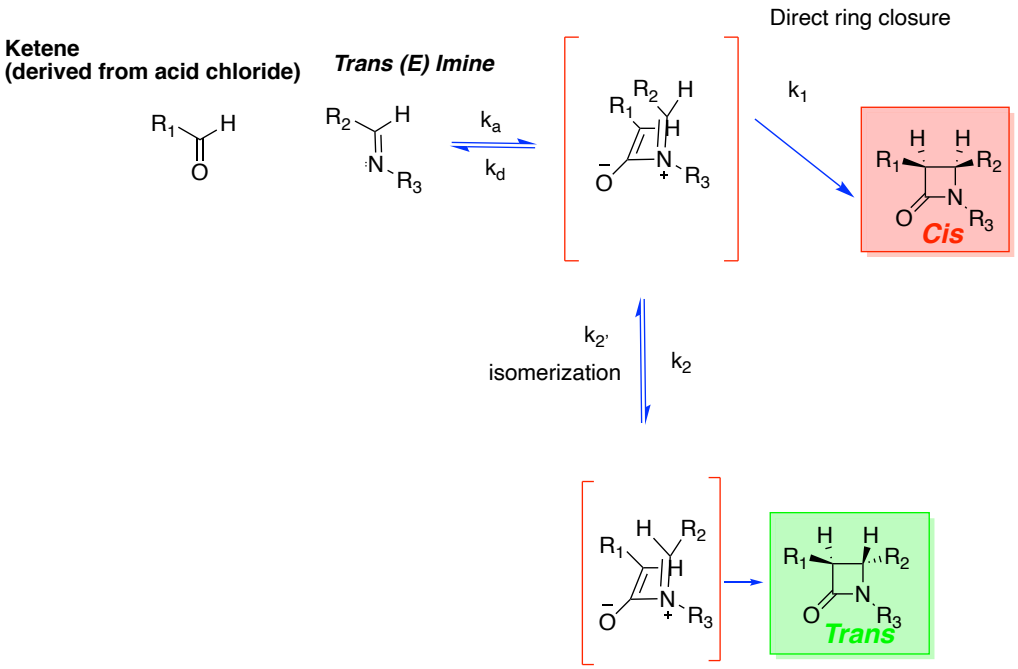


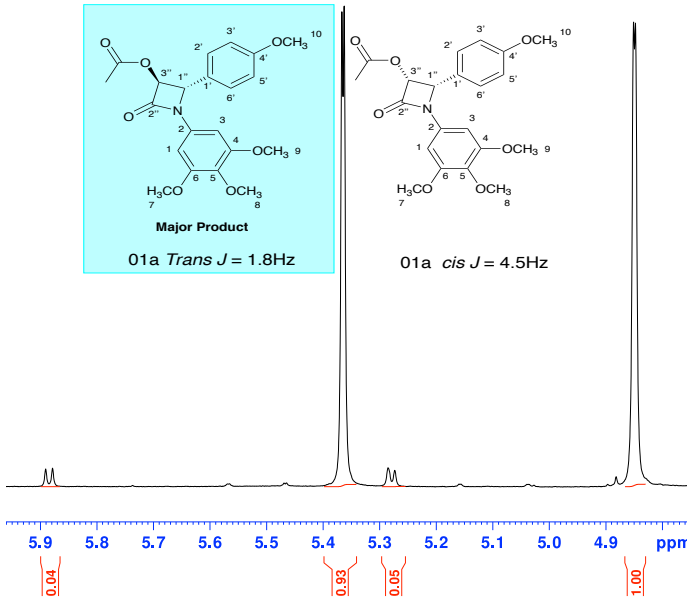






Optimised Synthesis of 3-Hydroxy-β-Lactams yielding > 95% *Trans* isomer



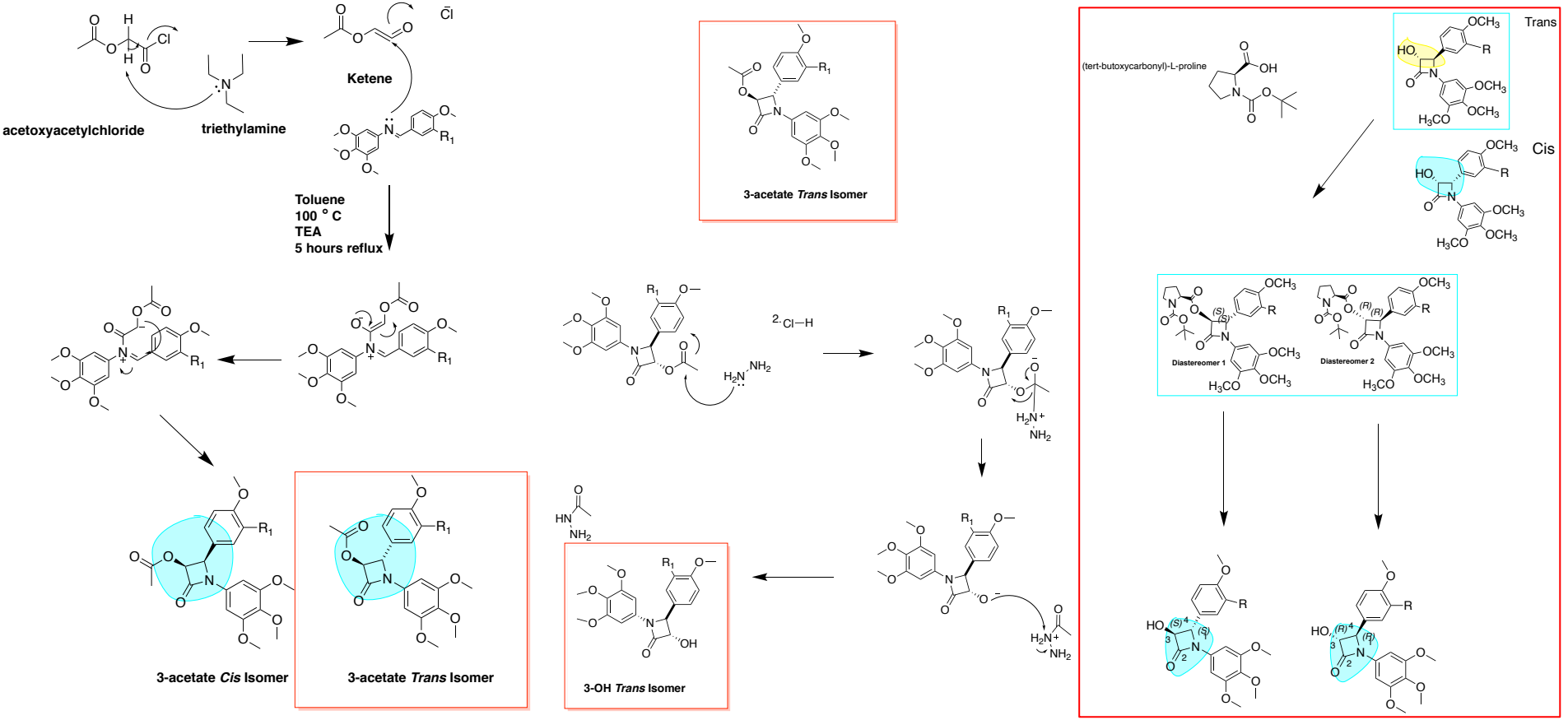


Clipping of ^{1}H NMR for Optimised Staudinger showing *Trans* 01a as major product

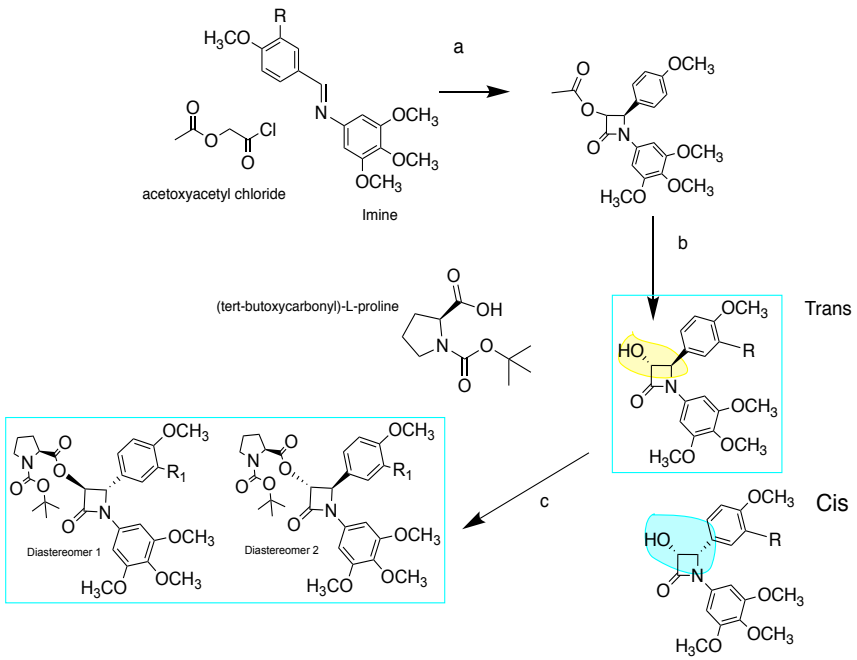
Conclusions:

Heating to reflux conditions prior to addition of tertiary base - appears to allow isomerization (k_2) by allowing reaction of imine with acid chloride

Chemistry: Synthesis of Racemic mixtures of 'The Combretezet Family' followed by Isolation of Enantiomers using Chiral Resolving agent



Procedure for isolating enatiomeric β −Lactam of the Combretezet Family: Chiral Resolution



Chiral resolution using N-(*tert*-butoxycarbonyl)-**L**-Proline is used to obtain enantiomerically pure β-lactams (illustrated on top left) obtained by esterification of the 3-OH.

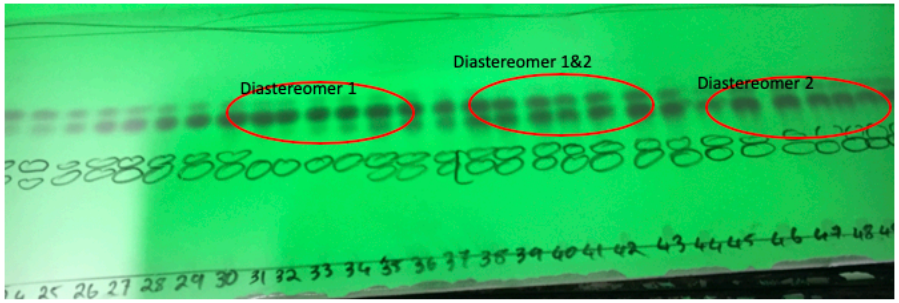
This is followed by diastereomer separation using **gravity** column chromatography and a slow gradient elution (*n*-hexane: MTBE 80:20 – 33:66).

The eluent was optimised by visualising diastereomer separation on TLC.

Gradual separation is essential and thus the decision to separate using **elution under gravity** versus **elution under flash pressure.**

Removal of the amino acid affords free and optically pure enantiomers for biological testing.

TLC plates showing separation of 01DS1&2



TLC plates illustrating diastereomer separation. TLC plates were developed three times to visualise clear separation of diastereomer bands in 2:1 TBME: *n*-hexane.

(Racemic as impurity sitting on top of diasteromer 1 (highest Rf) on this TLC)

Choice of Chiral Resolving agent appears to be significant

- **N-(*Tert*-butoxycarbonyl)-L-Proline** is the only amino acid which functions as a chiral resolving agent resulting in separation on TLC and subsequently on polar silica during column chromatography.
- The eluent was optimized for the Resolution Procedure using TLC.

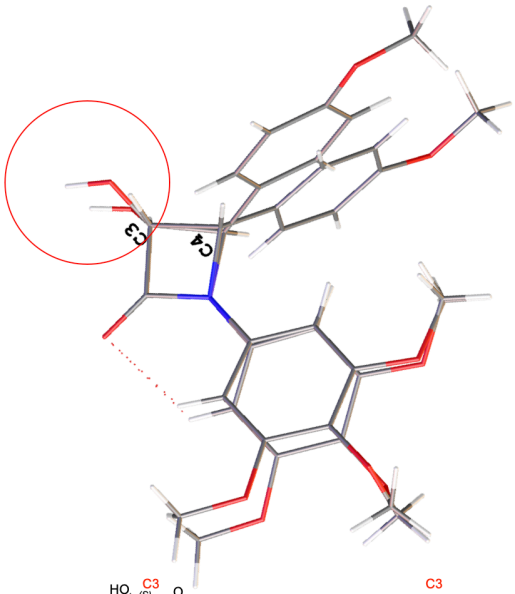
- **N-(*Tert*-butoxycarbonyl)-D-Proline** trialled with **no** success.

- **N-(*Tert*-butoxycarbonyl)-L-Valine** trialled with no success.

- **N-(*Tert*-butoxycarbonyl)-L-Phenylalanine –** trialled tried with no success.

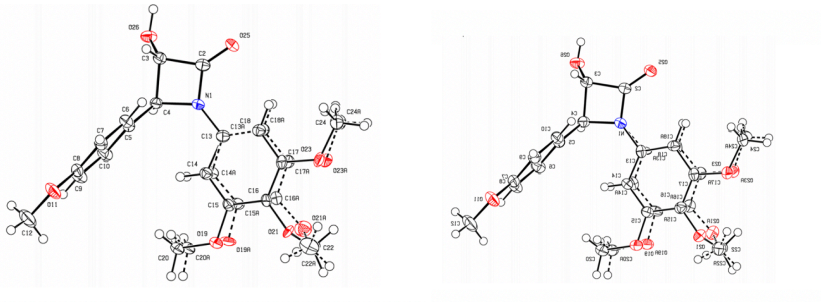
Confirmation of Chiral Purity of Enantiomers

1. X-ray Crystallography providing absolute configuration
2. Chiral HPLC to determine % enantiomeric excess



01En1 $[\alpha]^{20}_D$: +25.14

01En2 $[\alpha]^{20}_D$: -25.38

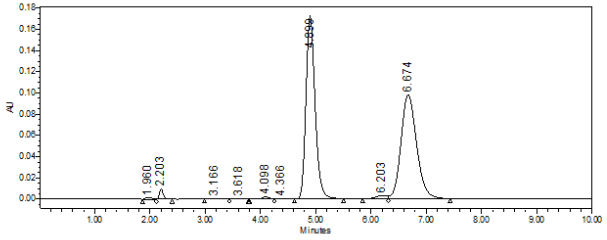


X-ray Crystallography for 01En1 & 01En2 confirming Absolute Stereochemistry and Chiral Purity

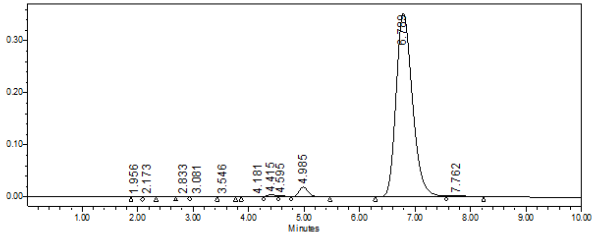
- Chiral resolution has been achieved as seen on the superimposed X-ray crystal structures for **01 3-*S*,4-*S*** and **01 3-*R*,4-*R*** at the C3 and C4 positions. X ray crystallography confirms the absolute configuration for **01En1** as ***S*** and **01En2** As ***R*** at the C3 position. ***(highlighted by the red circle)***

Chiral HPLC for 01 Combretazet Family

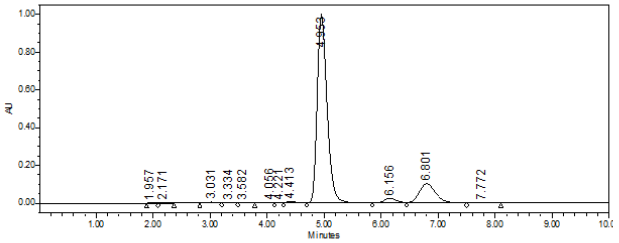
Chiral HPLC data for 01R



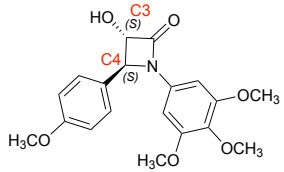
Chiral HPLC data for 01En1



Chiral HPLC data for 01En2

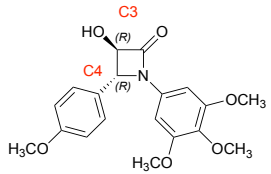


	Major Peak %	Minor Peak %	Major-Minor	Total % Enantiomeric Excess(ee%)
01 En1 (S,S)	96.96	3.04	96.96-3.04	93.93%
01 En2(R,R)	85.39	14.61	85.39-14.61	70.78%



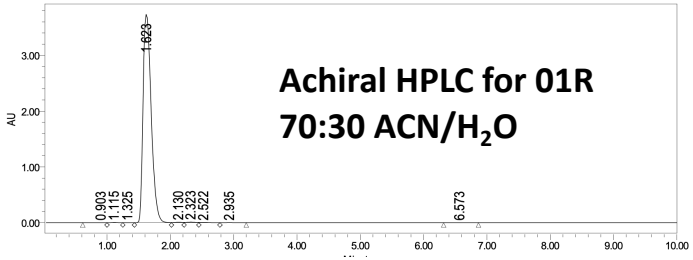
(3S,4S)-3-hydroxy-4-(4-methoxyphenyl)-1-(3,4,5-trimethoxyphenyl)azetidin-2-one

01En1



01En2

(3R,4R)-3-hydroxy-4-(4-methoxyphenyl)-1-(3,4,5-trimethoxyphenyl)azetidin-2-one



Achiral HPLC for 01R
70:30 ACN/H_2O

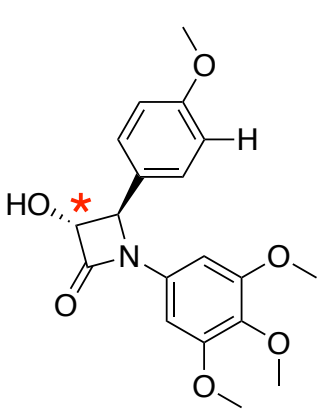
General method

- Chrompak-IH-3 150 x 4.6 mm supplied by Chiral Technologies Europe with a Chiral- IH-3 guard column was used. This column utilised an immobilized chiral polysaccharide stationary phase, *tris(S)*-a-*methylbenzylcarbamate*
- injection of 5 µL of sample
- 1 mg/mL
- 10 minute Run time
- using n-hexane:propan-2-ol, 50:50 as mobile phase.

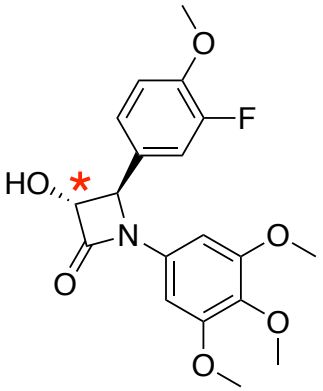
Biochemical Data in MCF-7 Breast Cancer Cells for 01 and 02 Combretazets

- 3-*S*, 4-*S* enantiomer appears superior in both cases (01En1 and 02En1)
- 02En1 appears extremely potent with an IC_{50} in sub nanomolar range in MCF-7 cells
- Compounds with greater activity than CA-4 in MCF-7 cells.

	IC_{50} MCF7 Cells (nM)	SD
01R	10.8	3.06
01En1	7.9	3.33
01En2	823	177
02R	11.8	5.96
02En1	0.87	1.03
02En2	68.3	6.9
CA-4 (positive control)	10.3	10.3

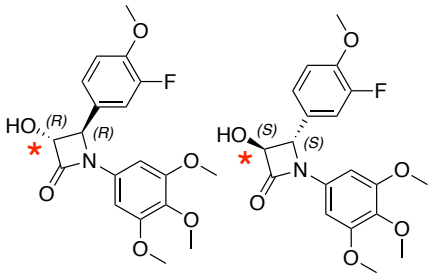


01



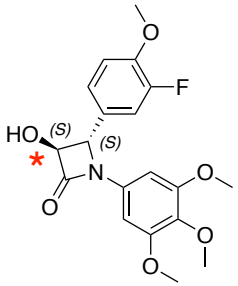
02

Cell Viability Data for 02R, 02En1 and 02En2 in MCF-7 Breast Cancer Cells



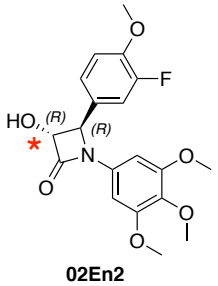
02R
50:50

IC_{50}
11.87
nM



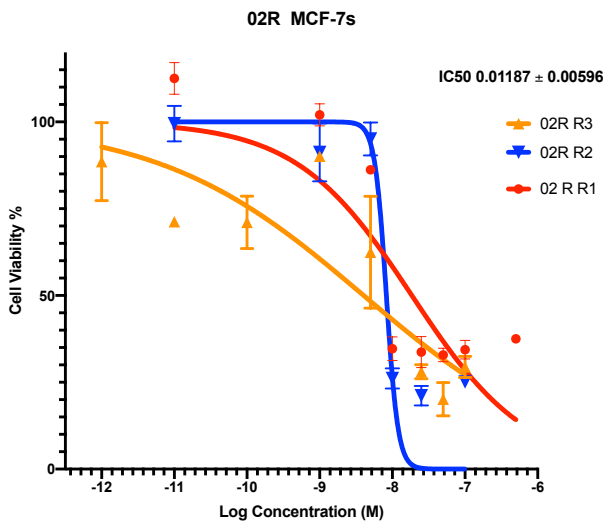
02En1

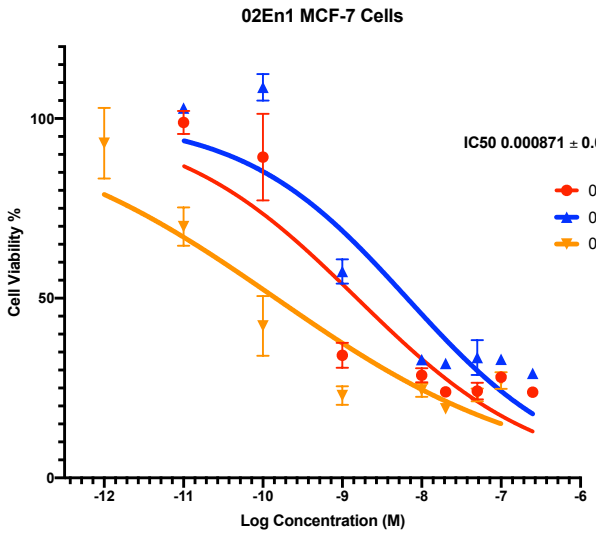
IC_{50}
0.871
nM

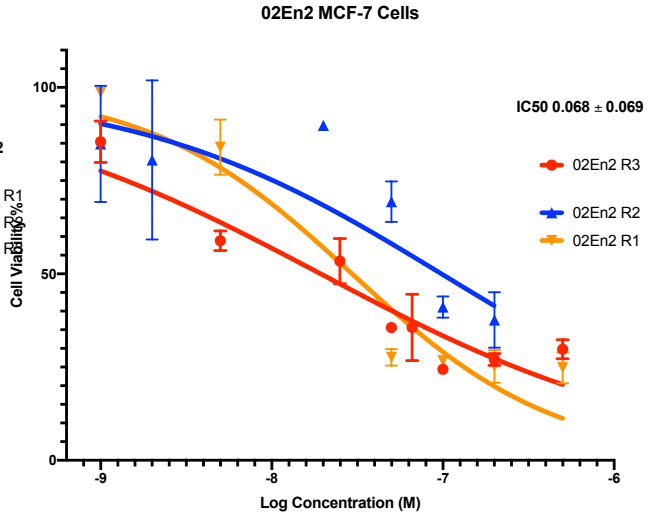


02En2

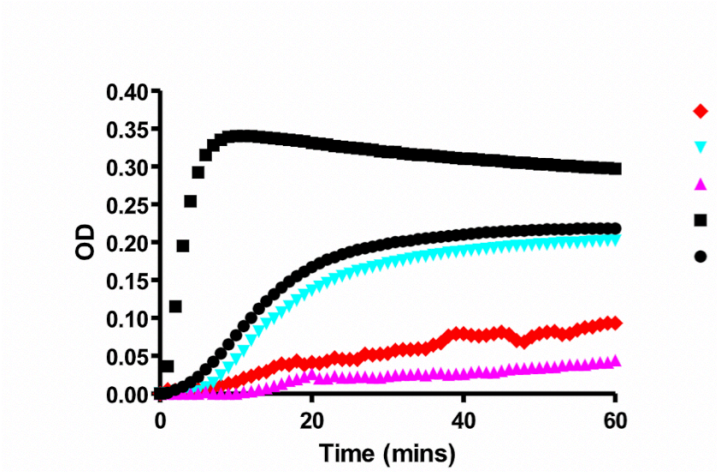
IC_{50}
68 nM



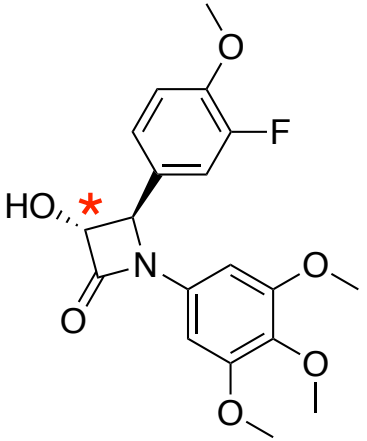




Preliminary Tubulin Polymerisation Assay for 02R, 02En2 and 02En2



- 02R (red diamond)
- 02En2 (light blue triangle)
- 02En1 (pink triangle)
- Paclitaxel (positive control – black square)
- Vehicle DMSO (black circle)



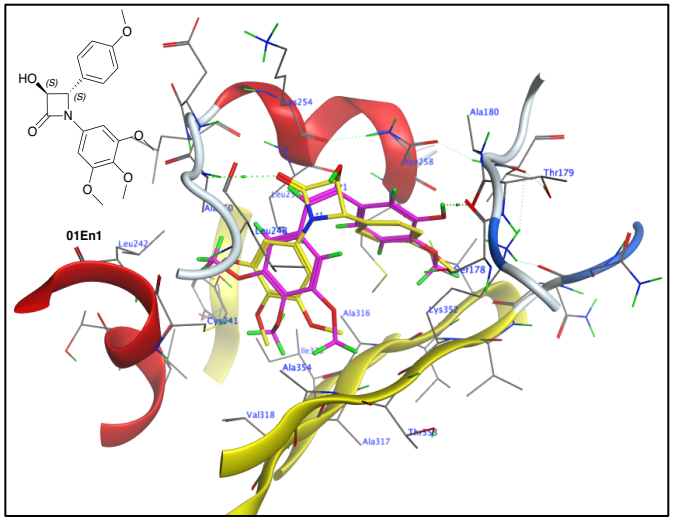
02

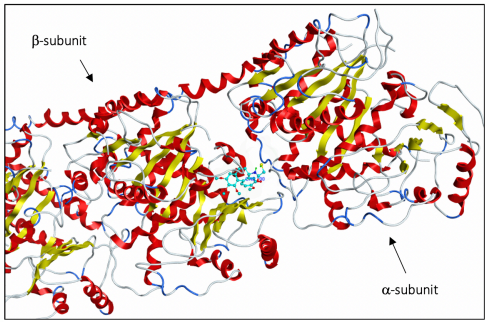
Preliminary Tubulin Polymerisation Assay illustrating 02R in red as an anti-tubulin agent, 02En1 in pink as a superior anti-tubulin agent.

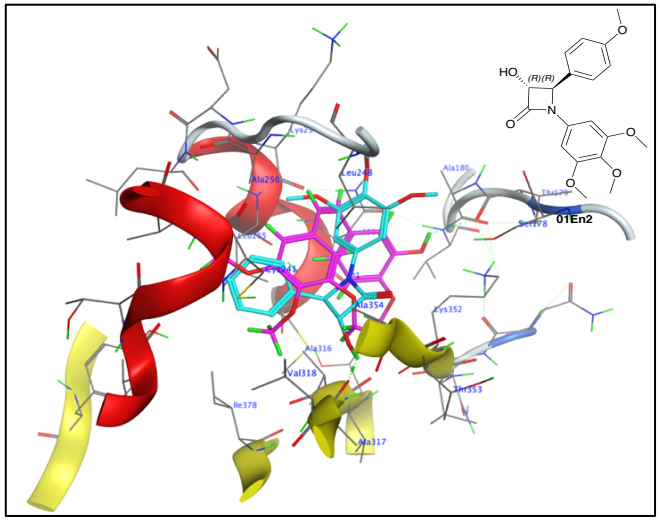
02En2 although a potent Anti-proliferative analogue, does not appear to have anti-tubulin effects.

Molecular Modelling Data for 01 Combretazet Family

- Molecular modelling demonstrates **favourable interactions of the 3-*S*, 4-*S* enantiomer.** Docking studies were carried out **in triplicate** to confirm suggested pattern od binding orientation.
- Findings suggest that the 3-*S*, 4-*S* enantiomer docks consistently favouring the binding orientation of CA-4.
- The 3-*R*, 4-*R* enantiomer varies in docking orientation with each docking simulation.
- The β-lactam carbonyl points out of the Colchicine Binding Pocket for **01En2** abolishing critical hydrogen bonding interactions between Ala β250 as seen for **01En1.**
- The trimethoxyphenyl moiety docks in a backwards orientation for **01En2** abolishing known critical interactions for both CA-4 and Colchicine, the interactions with Cys β241 and Val β318 (**15)**





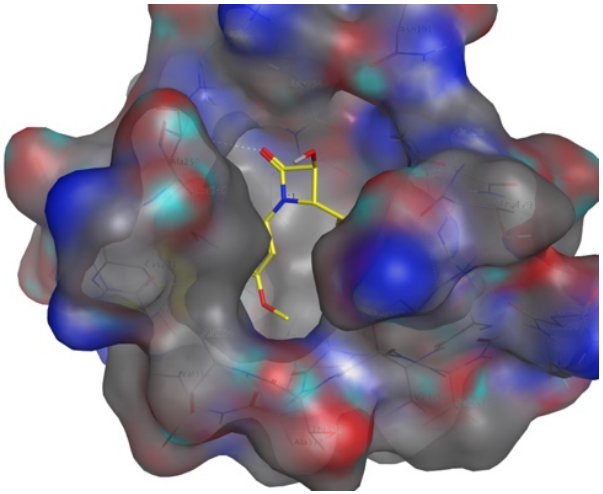


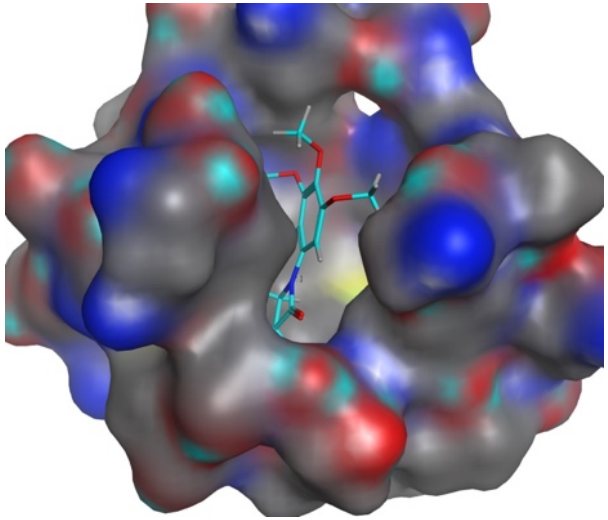
01En1 (yellow) in colchicine-binding site superimposed on CA-4 (pink). Atom colours: Red: Oxygen, Blue: Nitrogen, Green: Hydrogen. 01En2 (blue) in colchicine-binding site superimposed on CA-4 (pink). ***Location of Colchicine binding site at the $\alpha\beta$ interface shown below***

Molecular Modelling Data for 01 Combretazet Family

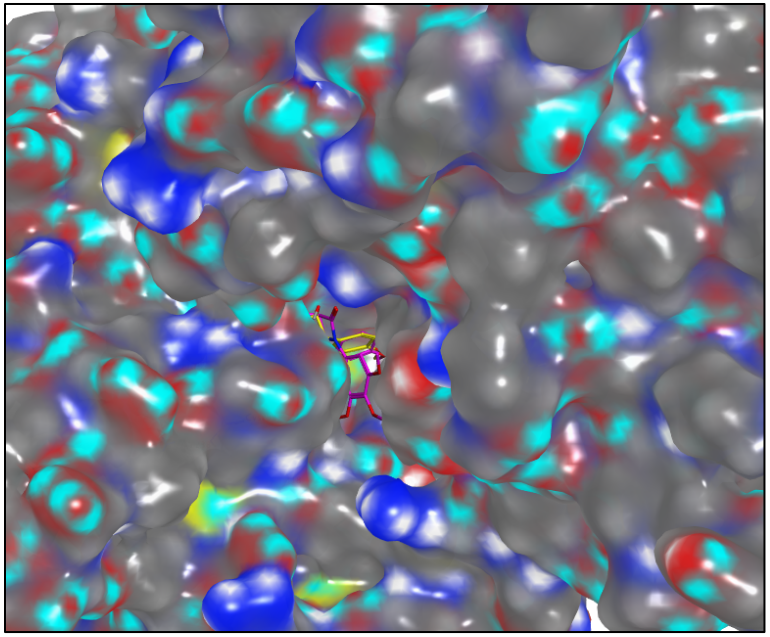
01En1 (yellow), **01En2** (cyan) in the Colchicine binding pocket. Red: Hydrophilic pocket interactions. Blue: hydrophobic pocket interactions. Black: neutral pocket interactions.

- **01En1 (**yellow) can be visualised with carbonyl hydrogen bonding to Alaβ250 and 3-OH pointing out of Colchicine binding pocket.
- **01En2** (cyan) in contrast is seen with trimethoxyphenyl ring and carbonyl pointing out of pocket without the critical Alaβ250 interaction.

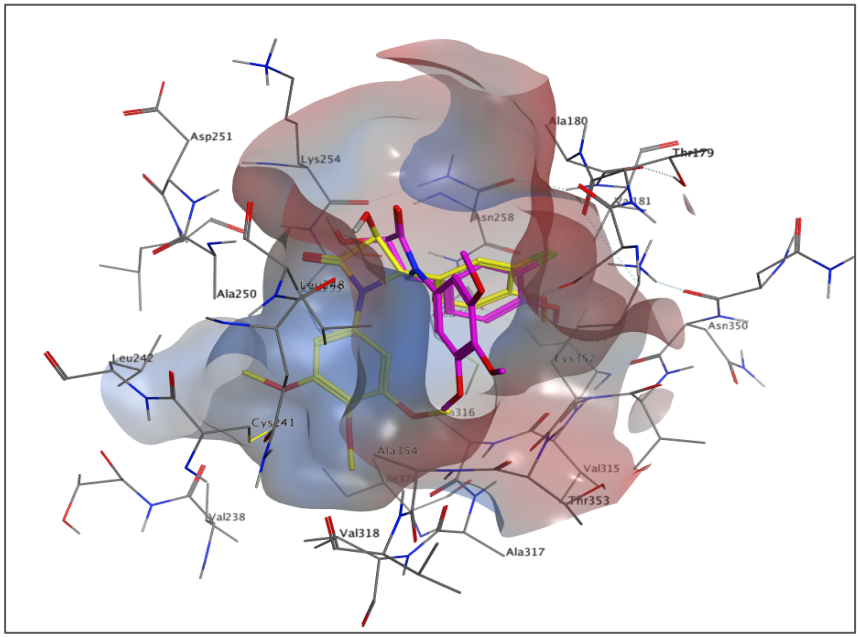




Molecular Modelling Data for 02 Combretazet Family



- Colchicine binding site opening in 1SA0 receptor illustrating trimethoxyphenyl ring of **02En2** (pink) emerging and β-lactam carbonyl pointing out of pocket for **02En2** (pink).
- **02En1** in yellow seen buried deep within the binding pocket (Blue: hydrophobic pocket interactions. Black: neutral pocket interactions)



- **02En1** (yellow) and **02En2** (pink) docked at the colchicine-binding site. B rings display similar interactions while the β-lactam ring points to opposite sides of the pocket.
- The trimethoxyphenyl rings are at a 180° angle to one another with **02En2** failing to interact with the key residues Cysβ241, Valβ238 and Valβ318

Conclusions & Future Work

- The 3-*S*, 4-*S* enantiomer holds more biological activity relative to the 3-*R*,4-*R*. Relative contribution of each enantiomer towards the racemic IC_{50} value must be further investigated.
- Full panel of Combretezets to be screened in both MCF-7 cells (estrogen receptor positive breast cancer cells) and MDA-MB-231 cells (triple negative cells)
- Tubulin Polymerisation Assays for each enantiomer.
- Prodrug synthesis for most promising lead analogue.
- Co-crystallisaton of analogues in Tubulin as a follow up to molecular modelling.

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The University of Dublin

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