

Organic & Biomolecular Chemistry

www.rsc.org/obc

Volume 11 | Number 31 | 21 August 2013 | Pages 5049–5196

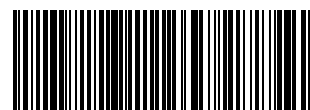


ISSN 1477-0520

RSC Publishing

PAPER

John S. Fossey *et al.*
Synthesis of azetidines and pyrrolidines *via* iodocyclisation of homoallyl amines and exploration of activity in a zebrafish embryo assay



1477-0520 (2013) 11:31;1-9

Synthesis of azetidines and pyrrolidines *via* iodocyclisation of homoallyl amines and exploration of activity in a zebrafish embryo assay†

Antonio Feula,^{a,b} Sundeep S. Dhillon,^c Rama Byravan,^b Mandeep Sangha,^b Ronald Ebanks,^b Mariwan A. Hama Salih,^b Neil Spencer,^d Louise Male,^e Istvan Magyary,^f Wei-Ping Deng,^a Ferenc Müller^c and John S. Fossey^{*b}

Room temperature iodocyclisation of homoallylamines stereoselectively delivers functionalised 2-(iodomethyl)azetidine derivatives in high yield. Increasing reaction temperature from 20 °C to 50 °C switches the reaction outcome to realise the stereoselective formation of functionalised 3-iodopyrrolidine derivatives. It was shown that these pyrrolidines are formed *via* thermal isomerisation of the aforementioned azetidines. Primary and secondary amines could be reacted with iodomethyl azetidine derivatives to deliver stable methylamino azetidine derivatives. With subtle changes to the reaction sequences homoallyl amines could be stereoselectively converted to either *cis*- or *trans*-substituted 3-amino pyrrolidine derivatives at will. The stereochemical divergent synthesis of *cis* and *trans* substituted pyrrolidines supports an ion pair, aziridinium, isomerisation pathway for azetidine to pyrrolidine isomerisation. Six azetidine derivatives were probed in a zebrafish embryo developmental assay to detect potential biological effects through the analysis of morphology and motility behaviour phenotypes. The range of effects across the probed molecules demonstrates the suitability of this assay for screening azetidine derivatives. One of the probed molecules, *rac*-(((*cis*)-1-benzyl-4-phenylazetidin-2-yl)methyl)piperidine, exhibited particularly interesting effects in the developmental assay presenting with hypopigmentation and reduced circulation amongst others. This shows that the zebrafish embryo provides a fast, sensitive and effective way to screen new compounds and in the future in combination with existing *in vivo* and *in vitro* assays it will become an integral part in drug discovery and development.

Received 29th April 2013,
Accepted 18th June 2013

DOI: 10.1039/c3ob41007b

www.rsc.org/obc

Introduction

Four and five membered saturated nitrogen containing heterocycles, azetidines and pyrrolidines respectively, are important

agrochemical and pharmacological motifs. Whilst pyrrolidines are a mainstay of the pharmaceutical and natural products arenas far fewer molecules containing azetidines have been reported, despite their intriguing biological activities.¹ Naturally occurring azetidine containing compounds include the siderophore mugenic acid^{2,3} and a marine sponge extract penaresidin A.^{4–6} Azetidines are often compared to pyrrolidines in terms of various biological assays, for example nicotine binds less effectively than its azetidine analogue, Fig. 1, to acetylcholine receptors.⁷ This coupled with studies into the psychotropic potency,⁸ potential CNS-focused leads in drug-discovery⁹ and antibacterial activity¹⁰ demonstrates that azetidine derivatives are attractive biologically active motifs.

Results and discussion

Synthesis

In an earlier report we detailed an iodine mediated cyclisation and isomerisation protocol that delivered pyrrolidines *via*

^aSchool of Pharmacy, East China University of Science and Technology, 130 Meilong Road, Shanghai 200237, China

^bSchool of Chemistry, University of Birmingham, Birmingham, West Midlands B15 2TT, UK. E-mail: j.s.fossey@bham.ac.uk

^cSchool of Clinical and Experimental Medicine, College of Medical and Dental Sciences, University of Birmingham, Birmingham, West Midlands B15 2TT, UK

^dSchool of Chemistry, NMR Spectroscopy Facility, University of Birmingham, Birmingham, West Midlands B15 2TT, UK

^eSchool of Chemistry, X-Ray Crystallography Facility, University of Birmingham, Birmingham, West Midlands B15 2TT, UK

^fUniversity of Kaposvar, Faculty of Animal Science, Department of Nature Protection, H7400 Kaposvar Guba S. u. 40, Hungary

†Electronic supplementary information (ESI) available: General and experimental chemical procedures, biological procedures and statistics and X-ray crystallographic information are available. CCDC 936733. For ESI and crystallographic data in CIF or other electronic format see DOI: 10.1039/c3ob41007b



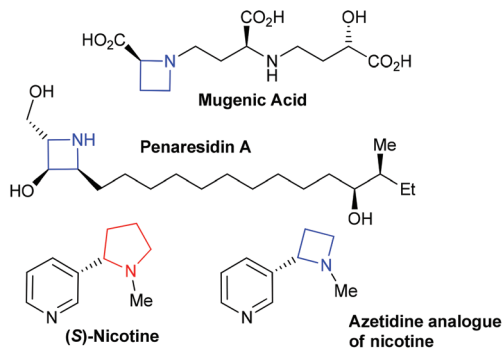
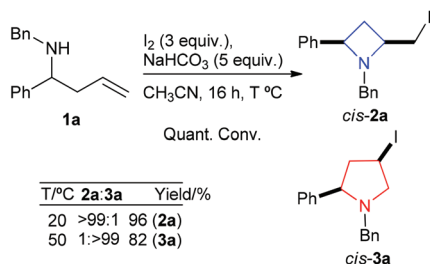


Fig. 1 Examples of azetidine containing molecules alongside pyrrolidine containing nicotine.



Scheme 1 Temperature dependent product distribution in the iodine mediated cyclisation of homoallyl amine **1a**.

unstable azetidine intermediates.¹¹ Herein new observations and understanding are presented, such as streamlined access to pyrrolidines, and exploiting the observation that simply carefully controlling reaction temperature allows direct access to either azetidines **2** or pyrrolidines **3**. For example reaction of *N*-benzyl-1-phenylbut-3-en-1-amine (**1a**) with iodine and sodium hydrogen carbonate in acetonitrile at 20 °C, delivered *cis*-azetidine **2a** in 96% yield (which is prone to isomerisation to *cis*-pyrrolidines), whereas running this cyclisation reaction at a slightly elevated temperature (50 °C) cleanly delivers *cis*-pyrrolidine **3a** as the only detectable product (82% isolated yield), Scheme 1. It should be noted that the temperature should be closely controlled through manipulations since azetidines **2** were found to readily isomerise under solvent removal conditions above 20 °C.

Azetidines

Having established the general synthetic protocol for the synthesis of 2,4-*cis*-azetidines from homoallyl amines, *i.e.* treatment with iodine and sodium hydrogen carbonate in acetonitrile at room temperature,¹¹ the effect of varying the base was probed. Since iodine containing azetidines **2** are prone to isomerisation with time the post-workup mixture obtained from the reaction was used to determine conversion (proton NMR spectroscopy), post work-up isolates had already been shown by us to be excellent starting points for subsequent derivatisation.¹¹ Table 1 (entries 1 to 12) details the effect of varying the base under the standard conditions of a

Table 1 Optimisation of reaction conditions to form *cis*-azetidine **2a** from **1a** via iodine mediate cyclisation

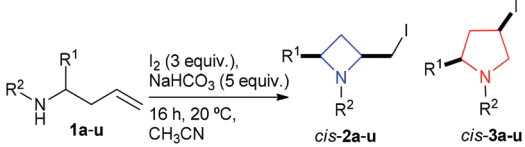
Reaction scheme: **1a** + I₂ (3 equiv.), Base (5 equiv.), 16 h, 20 °C, Solvent → *cis*-**2a**, *cis*-**3a**, **4a**, **5a**.

Entry	Base	Solvent	1a ^a	2a ^a	3a ^a	4a ^a	5a ^a
1	—	CH ₃ CN	56.0	44.0	—	—	—
2	Li ₂ CO ₃	CH ₃ CN	25.0	75.0	—	—	—
3	Na ₂ CO ₃	CH ₃ CN	—	61.0	39.0	—	—
4	K ₂ CO ₃	CH ₃ CN	—	35.5	—	64.5	—
5	Cs ₂ CO ₃	CH ₃ CN	—	—	—	82	18
6	LiOH	CH ₃ CN	48.5	33.0	18.5	—	—
7	NaHCO ₃	CH ₃ CN	—	>99.5	—	—	—
8	NaOH	CH ₃ CN	—	60.0	40.0	—	—
9	KOH	CH ₃ CN	—	53.0	47.0	—	—
10	LiOAc	CH ₃ CN	—	73.0	27.0	—	—
11	NaOAc	CH ₃ CN	—	68.5	31.5	—	—
12	KOAc	CH ₃ CN	—	50.0	20.0	30.0	—
13	NaHCO ₃	THF	63.0	37.0	—	—	—
14	NaHCO ₃	MeOH	—	50.0	21.0	29.0	—
15	NaHCO ₃	CH ₂ Cl ₂	58.0	42.0	—	—	—
16	NaHCO ₃	DMSO	—	12.0	55.0	33.0	—

^a Ratio determined by comparison of ¹H NMR integrations in post work-up NMR spectra.

16 h room temperature reaction of homoallyl amine **1a** with three equivalents of iodine in acetonitrile. Whilst initially selected sodium hydrogen carbonate proved to be the superior base for azetidine formation, providing **2a** as the sole product with no detectable starting material or by-products (Table 1, entry 7), the use of no base at all (Table 1, entry 1) permits the formation of the desired azetidine product in a modest 44% yield. Use of lithium carbonate as base improves the conversion of amine **1a** to azetidine **2a** (Table 1, entry 2) a little (75%). Lithium, sodium and potassium hydroxide (Table 1, entries 6, 8 and 9 respectively) gave 50–100% conversion of amine **1a** to mixtures of azetidine (**2a**) and pyrrolidine (**3a**), as did lithium and sodium acetate (Table 1, entries 10 and 11 respectively). Potassium acetate (Table 1, entry 12) showed evidence of oxidation of **1a** with a product distribution that contained 50% of the desired azetidine **2a**, 20% of the corresponding pyrrolidine **3a** accompanied by imine **4a** (30%). Potassium carbonate (Table 1, entry 4) gave complete conversion of **1a** to a mixture of azetidine **2a** and imine **4a** (~1:2 ratio). Somewhat surprisingly cesium carbonate gave only imine containing products, both the aforementioned oxidation product **4a** (from oxidation of **1a**) but also imine **5a** (the condensation product of benzaldehyde and benzylamine).^{12,13} After confirming sodium hydrogen carbonate was the most effective base for azetidine formation THF, methanol, dichloromethane and DMSO (Table 1, entries 13–16 respectively) were tried as solvent. Whilst none of the solvents tried proved to be superior to acetonitrile in azetidine formation, it is noteworthy



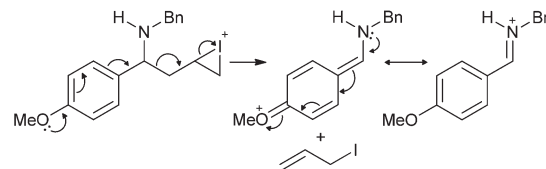
Table 2 Substrate scope for the iodine mediated cyclisation of homoallyl amines **1**


Entry		R ¹	R ²	2 + 3/% ^a	2 : 3 ^b
1	a	Ph	Bn	>99	>99 : 1
2	b	Ph	PMB	>99	>99 : 1
3	c	Ph	PTB	>99	>99 : 1
4	d	3-Py	Bn	>99	5 : 1
5	e	4-Py	Bn	>99	6 : 1
6	f	PNP	Bn	>99	3 : 1
7	g	2-Br-Ph	Bn	>99	3 : 1
8	h	<i>t</i> -Bu	Bn	>99	>99 : 1
9	i	3-OMe-Ph	Bn	>99	6 : 1
10	j	3-Furyl	Bn	>99	>99 : 1
11	k	Cy	Bn	>99	>99 : 1
12	l	Ph	PNB	>99	7 : 1 ^c
13	m	ONP	PMB	>99	3 : 1
14	n	Fc	Bn	>99	>99 : 1
15	o	Ph	Cy	38	1 : >99 ^c
16	p	Ph	<i>n</i> -Pr	33	1 : >99 ^c
17	q ^d	4-OMe-Ph	Bn	—	—
18	r	1-Naph	Bn	80	>99 : 1
19	s	Ph	3-Py	>99	>99 : 1

PMB = *para*-methoxybenzyl. PTB = *para*-tolylbenzyl. PNP = *para*-nitrophenyl. PNB = *para*-nitrobenzyl. ONP = *ortho*-nitrophenyl. Fc = ferrocenyl. ^a 2 + 3 by ¹H NMR spectroscopy. ^b *cis*-(2 : 3) Ratio obtained by inspection of the ¹H NMR spectrum post aqueous work-up. ^c Sodium carbonate was used as base. ^d **1r** suffered loss of allyl group and oxidation to corresponding imine.

that both methanol and DMSO did show evidence of amine oxidation to imine **4a** (~30% in both cases).

Next substrate scope was investigated, Table 2 shows the effect of varying the substituents labelled R¹ and R² in starting amines **1a–t**. Under conditions optimised for iodoazetidine formation conversion of starting amine to cyclised products was generally high and showed no evidence for *trans* substitution about the azetidine (or pyrrolidine where detected) rings, the ratio of azetidine to pyrrolidine was good to excellent for benzylic R² substituents. Aromatic substituents bearing electron donating groups in R¹ (e.g. 3- and 4-OMe, Table 2, entries 9 and 17 respectively) were employed, and whilst the 3-methoxy derivative gave complete conversion to a 3 : 1 mixture of the corresponding azetidine and pyrrolidine the 4-methoxy derivative failed to give any cyclised product, rather an apparent loss of the allyl group and oxidation of **1r** was observed. Perhaps, after initial activation of the alkene by iodine, allyl iodide might be expelled assisted by the resonance stabilization of the generated cation, as proposed in Scheme 2. Electron withdrawing groups in R¹ 4- and 2-nitro phenyl (Table 2, entries 6 and 13) gave 3 : 1 mixtures of azetidine to pyrrolidine. Heteroaromatic 3- and 4-pyridyl and 3-furyl (Table 2, entries 4, 5 and 10) gave complete conversion to cyclised product with good to excellent azetidine to pyrrolidine ratios.

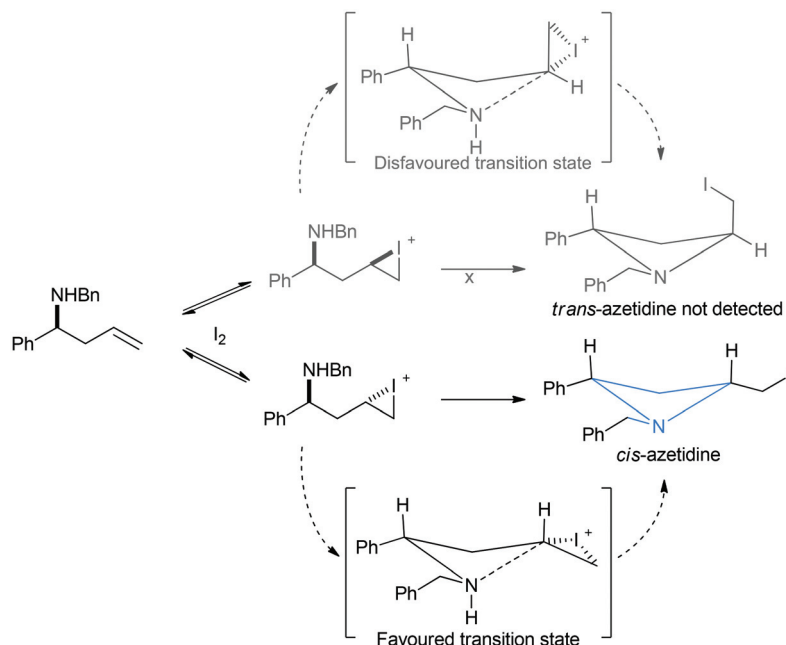
**Scheme 2** Proposed mechanism for the deallylation and imine formation observed in the reaction of **1r** under typical cyclisation conditions.

Alkyl *tert*-butyl and cyclohexyl groups at R¹ (Table 2, entries 8 and 11) gave complete conversion to azetidine products. An organometallic fragment in R¹ ferrocenyl (Table 2, entry 14) also gave only azetidine product in quantitative consumption of homoallylamine starting material. In the variation of R² *para*-methoxybenzyl, *para*-tolylbenzyl, *para*-nitrobenzyl and CH₂-3-pyridyl (Table 2, entries 2, 3, 12 and 20) respectively all gave quantitative conversion with only the *para*-nitrobenzyl derivative providing anything less than complete selectivity in favour of azetidine products. Alkyl groups in R² proved troublesome cyclohexyl and *n*-propyl (Table 2, entries 15 and 16) gave low conversion to pyrrolidine products (with sodium carbonate as base), and CH₂C(CH₃)₃ gave slightly higher conversion but the product distribution was a 1 : 1 mixture of pyrrolidine and azetidine (under standard conditions).

The observation that the iodine mediated cyclisation consistently delivers *cis* relative stereochemistry can be ascribed to the suggested differences in transition state geometries that would be required to deliver *cis* or *trans* products. Addition of iodine to the carbon–carbon double bond of a homoallyl amine, such as **1a**, could form two diastereomeric iodonium species (Scheme 3). In order for cyclisation to a four-membered azetidine to occur two possible transition states are possible. The most disfavoured of the two transition states would increase a pseudo 1,3-diaxial interaction, whereas the transition state reasoned to be more favoured positions protons in the pseudo axial positions (Scheme 3: upper and lower transition state respectively).

Since it had been already demonstrated that iodoazetidines of the general class *cis*-2 were highly prone to isomerisation and that the *trick* of rapidly reacting the crude, post work-up azetidine with a nucleophile to replace iodine could deliver stable *cis*-azetidine derivatives (*cis*-6), a range of amine derivatives of *cis*-azetidines **2** were prepared in 50 to 87% isolated yield over two steps from readily accessible homoallyl amines **1**, Table 3. It was vital that liquid amines were used neat (as solvent) in the iodine displacement step, when co-solvents were used isomerisation to the corresponding pyrrolidines (**3**) became problematic, however this feature can be exploited (as discussed later) in pyrrolidine synthesis. Yields of amine bearing azetidines **6** ranged from 50 to 82% over two steps from starting homoallyl amines **1**. Primary and secondary amines could be used in the derivatisation, linear and branch aliphatic, benzylic, and heteroatom containing substituents in the derivatising amine part could all be accommodated.





Scheme 3 Proposed transition state argument for the *cis*-selectivity observed in the iodine mediated cyclisation of homoallyl amines.

The nucleophilic displacement of iodine from *cis*-iodoazetidines was employed in an azide to triazole so-called *click reaction* sequence. Homoallyl amines **1a** and **1d** were reacted under standard cyclisation conditions and worked-up as before, the iodoazetidines **2a** and **2d** were treated with sodium azide in DMF at room temperature and stirred overnight (16 h), all the while ensuring the temperature did not exceed 20 °C, the reactions were monitored by TLC (silica EtOAc–Hex), which indicated that although the displacement of iodide by azide was incomplete the iodides **2a** and **2b** were showing evidence of a small level of isomerisation to the corresponding pyrrolidines (as judged by TLC). In order to minimise contamination from products of reaction after azetidine isomerisation a terminal alkyne (phenyl acetylene or pent-1-yne) was added at that point with copper(i) iodide and the reaction stirred for a further 24 h. Triazole appended azetidines **7aa**, **7ab** and **7da** were obtained in approximately 50% yield over the three synthetic steps from homoallyl amines **1a** or **1d** (Scheme 4).

The cyclisation, iodide displacement sequence was applied to the synthesis of single enantiomer azetidines (*S,R*)-**6ab**, (*S,R*)-**6tb**, (*S,R*)-**6ua** and (*S,R*)-**6ue**.¹⁴ The synthesis of (*S,R*)-**6ab** is shown in Scheme 1 which describes the use of single enantiomer **1a**, no loss of enantiopurity is witnessed and (*S,R*)-**6ab** was isolated in 84% yield. Single enantiomer **1a** was obtained utilising a stereoselective allylation of the corresponding imine¹⁵ via an Ellman auxiliary protocol (Scheme 5).^{16–19}

Pyrrolidines

Whilst azetidines represent an attractive synthetic target pyrrolidines, the side product of our azetidine formation protocol, are highly desirable heterocyclic constructs in the organic

synthesis arena.^{20–23} It was shown that pyrrolidine *cis*-**3a** could be quantitatively formed upon reaction of homoallyl amines with iodine and a base with mild heating. It was also previously shown (by us in an earlier report¹¹) that iodine containing azetidine derivative **2d** stereospecifically isomerized, with time, to the corresponding pyrrolidine *cis*-**3d**. In order to confirm isomerisation of iodoazetidines to iodopyrrolidines, azetidines **2c–e** were separately heated at 50 °C in acetonitrile for 4 hours (Scheme 6), in each case ≥95% of the corresponding pyrrolidine **3c–e** was isolated. In this report evidence of a dissociative isomerisation mechanism is revealed, which corroborates hypotheses of Couty and others in the area (see later).^{24,25}

Paulmier, Outurquin and co-workers reported selenium induced cyclisation of homoallyl amines to azetidines and pyrrolidines.^{26,27} Bott *et al.* reported a one carbon ring expansion of azetidines via ammonium ylide shifts.²⁸ Couty and co-workers have studied a number of ring enlargement reactions that deliver pyrrolidines from azetidines, such as fluoride,²⁹ chloride²⁵ and bromide derivatives,³⁰ alkene derivatives³¹ and hydroxy derivatives.²⁵ Van Brabandt *et al.* also utilised ring expanded chloride and bromide bearing azetidines.³² Amongst this catalogue of work Couty and coworkers reported a computational study where ring expansion of 2-(chloromethyl)-1-isopropylazetidine was discussed,²⁴ and noted that a polar solvent (DMSO) reduced the barrier to isomerisation and the reaction proceeds via two transition states through a strained intermediate, Scheme 7.

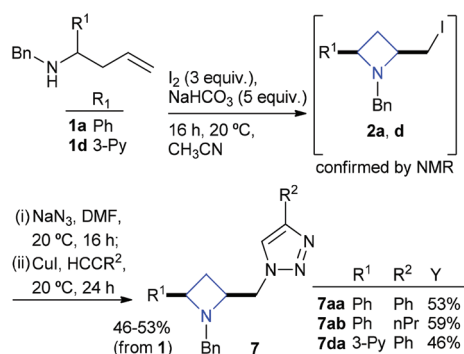
It is also important to point out that a five (pyrrolidine) to six (piperidine) membered ring expansion reaction that exploits the nucleophilic displacement of a beta iodide by Davies *et al.* supports an ion pair aziridinium salt hypothesis



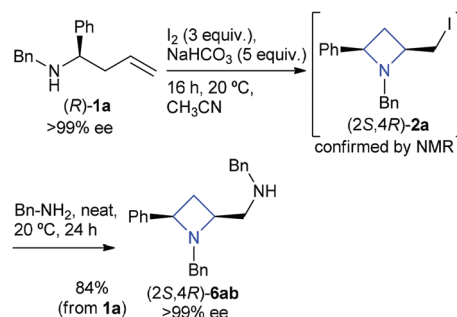
Table 3 Aminoazetidines synthesised in two steps from homoallylamines **1**

$$\begin{array}{c}
 \text{R}^1 \\
 | \\
 \text{R}^2\text{NH}-\text{CH}-\text{CH}_2-\text{CH}=\text{CH}_2 \\
 \text{1}
 \end{array}
 \xrightarrow[\text{(2 steps)}]{\begin{array}{l} \text{(i) I}_2 \text{ (3 equiv.), NaHCO}_3 \text{ (5 equiv.), 16 h, 20 }^\circ\text{C, CH}_3\text{CN;} \\ \text{(ii) 1}^\circ \text{ or 2}^\circ \text{ amine } \text{R}^3\text{NH}-\text{R}^4, \text{ neat, 24 h, 20 }^\circ\text{C} \end{array}}
 \begin{array}{c}
 \text{R}^3 \\
 | \\
 \text{R}^1-\text{CH}_2-\text{CH}-\text{CH}_2-\text{N}-\text{R}^4 \\
 | \\
 \text{R}^2 \\
 \text{cis-6aa-sb}
 \end{array}$$

	cis-6aa	86%		cis-6ab	87%		cis-6ac	83%		cis-6ad	69%		cis-6ae	83%
	cis-6af	71%		cis-6ag	81%		cis-6ah	78%		cis-6bb	74%		cis-6ca	75%
	cis-6da	72%		cis-6di	82%		cis-6dj	76%		cis-6ea	71%		cis-6fa	76%
	cis-6ga	65%		cis-6ha	86%		cis-6ib	85%		cis-6ja	79%		cis-6kb	81%
	cis-6lb	82%		cis-6mb	69%		cis-6ra	50%		cis-6sa	81%		cis-6sb	65%

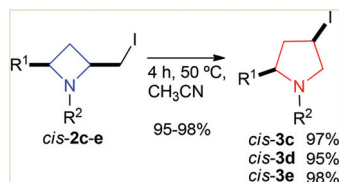
**Scheme 4** Synthesis of triazole appended azetidines **7** in ~50% yield over three steps.

since they were able to isolate (and characterise by single crystal X-ray diffraction) the tetrafluoroborate salt **13** of their intermediate (Scheme 8), which further reacts with acetate to give a 55 : 45 mixture of compounds **14** : **15**.³³

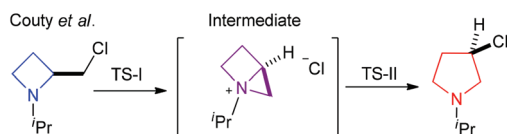
**Scheme 5** Non-racemic synthesis of amino azetidine (2*S*,4*R*)-**6ab** in 84% yield.

Using the knowledge that the isomerisation of 2,4-*cis*-azetidines **2** to 2,4-*cis*-pyrrolidines **3** is facile and our knowledge that nucleophilically displacing iodine from azetidine products delivers stable amino azetidines we chose to run a similar protocol for generation of *trans*-pyrrolidine (whilst never observed it was speculated that iodopyrrolidines might be prone to

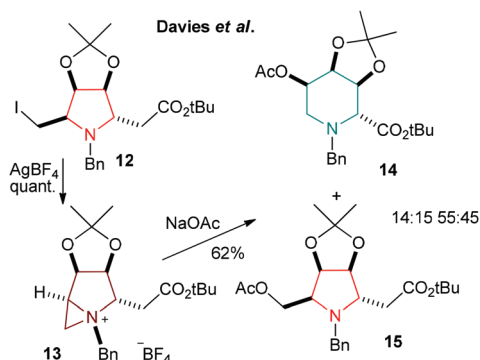




Scheme 6 Thermal isomerisation of *cis*-azetidines **2c–e** to *cis*-pyrrolidines **3c–e**.

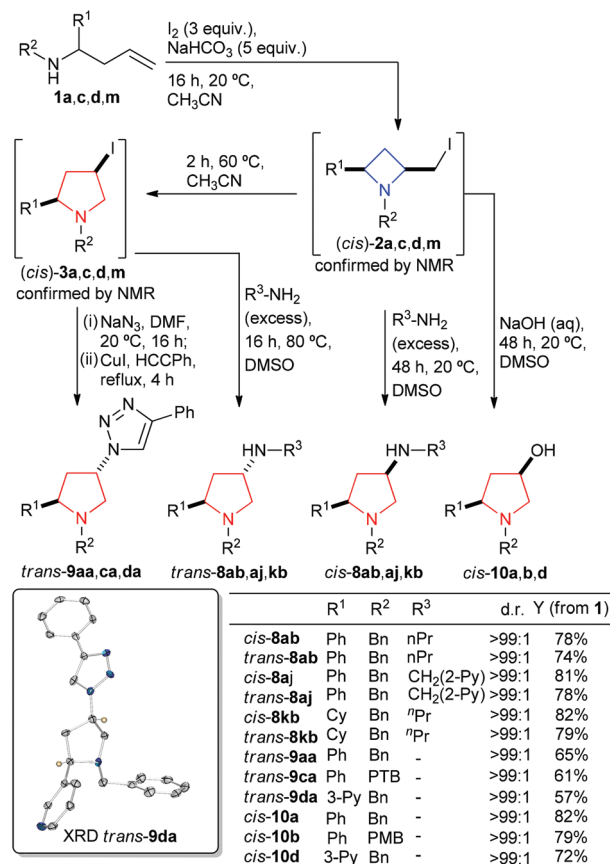


Scheme 7 Computationally determined reaction sequence via a strained intermediate of Couty *et al.*²⁴



Scheme 8 Ring expansion of **12** through aziridinium formation (**13**) to piperidine **14** by Davies *et al.*³³

polymerisation *via* N-quaternisation or other side reactions so reaction of the iodide directly was deemed appropriate). It was envisaged that, in one pot, a homoallyl amine (**1**) could be cyclised to a 2,4-*cis*-azetidine (**2**), which in turn could be isomerised by action of heat to a 2,4-*cis*-pyrrolidine (**3**). The *cis*-pyrrolidine thus formed upon treatment with a nucleophile would deliver a *trans*-substituted pyrrolidine. Indeed, for the case of homoallyl amines **1a**, **c**, **d** and **m** the corresponding *cis*-azetidines (**2a**, **c**, **d** and **m**) and then *cis*-pyrrolidines (**3a**, **c**, **d** and **m**) were identified by proton NMR spectroscopy and treated with a primary amine to give 2,4-*trans*-pyrrolidines **8ab**, **aj** and **kb** (>73% yield over three steps) or with sodium azide followed by a terminal alkyne and a copper(I) catalyst to give 2,4-*trans*-pyrrolidines **9aa**, **ca** and **da** as single diastereoisomers in good isolated yields (57–65% yield over four steps), Scheme 9 – left side. An X-ray crystal structure of *trans*-**9da** helped confirm the relative *trans*-stereochemistry (inset Scheme 9). To our surprise (and later delight) when *cis*-azetidines **2a** and **2k** (confirmed by proton NMR spectroscopy) where stirred at room temperature in DMSO with primary amine nucleophiles not only were pyrrolidines the major

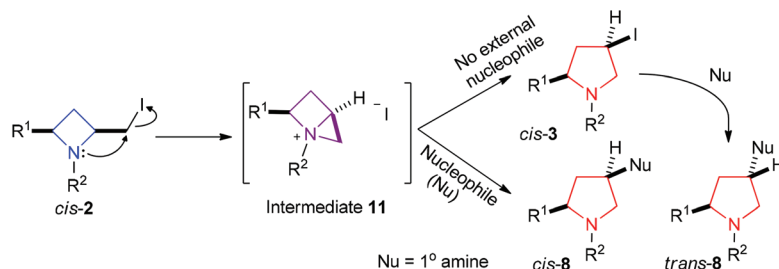


Scheme 9 The synthesis of 2,4-*cis*- and *trans*-pyrrolidines (**8**, **9** and **10**) from homoallyl amines (**1**). Inset: X-ray crystal structure of *trans*-**9da**, only pyrrolidine and triazole C–H protons shown for clarity, nitrogen atoms are coloured blue (Ortep plot 30% probability ellipsoids), for full details see ESI.†

products (as indicated earlier in the azetidine section, use of a solvent rather than neat amine gave some pyrrolidine product) but these products were the *cis*-diastereoisomer **8ab**, **aj** and **kb** in greater than 77% isolated yield from the corresponding homoallylamines, Scheme 9 – right side. Thus it is possible to synthesise both diastereoisomers of the 2,4-pyrrolidines from the same starting material in good yield and high diastereoselectivity simply by adjusting the addition sequence and reaction temperature. Alongside this finding it was also possible to prepare *cis*-2,4-hydroxy functionalised pyrrolidines (although it was not possible to obtain the *trans* isomer) by using hydroxide as the nucleophile in 72–89% yield in the cases tried (including a pyrrolidine bearing a 3-pyridyl functionality in R¹ offering access to functionalised analogues of a nicotine core).

The observation that both *cis*- and *trans*-pyrrolidines **8** are readily accessible from the same starting materials by simply subtly changing the reaction conditions (namely adding nucleophile before or after isomerisation of azetidine to pyrrolidine) leads to the conclusion that the isomerisation is likely to proceed *via* intermediate **11**, Scheme 10. In the absence of any external nucleophile the *cis*-azetidine isomerises to the corresponding *cis*-pyrrolidines **3**, which can then undergo an S_N2 reaction with a nucleophile, such as a primary amine, to





Scheme 10 Divergent conversion of *cis*-azetidines **2** via intermediate **11** to both *cis*- and *trans*-pyrrolidines **8**.

give *trans*-substituted pyrrolidines **8**. However, intermediate **11** ought to be stabilised by polar solvent (according to calculations for a related system by Couty *et al.*), so if it forms in the presence of an external nucleophile (*e.g.* primary amine) it is intercepted to give *cis*-**8**.

Preliminary biological activity

Zebrafish embryo development can be used as an assay to assess potential drug-like behaviour of molecules in a complex organism; certain developmental manifestations can help to provide early indications of possible utility in therapeutic regimes and also toxic side-effects that could develop.^{34–41}

Zebrafish are a suitable vertebrate model for drug screening due to their ease of access, low maintenance costs, optical transparency, fast development and relatedness to humans in terms of physiology, genetics and toxic responses.⁴⁰ In comparison, rodent models are more expensive to maintain, their development is slow and occurs *in utero* making drug testing difficult and the number of embryos that can be obtained is also small reducing the scale of the experiments.⁴² Additionally, in comparison to *in vitro* cell culture models, the zebrafish embryo enables the response of the organism as a whole to a compound to be observed, such as cell–cell and cell–matrix interactions, as well as enabling the efficiency of uptake, metabolism and excretion of a compound to be determined; information that cannot be obtained from *in vitro* cell culture models.⁴² Zebrafish screens require small amounts of compound, which saves money and the compound can also be added directly to the bathing solution, so injection is not required for drug delivery. Zebrafish screens can also be automated enabling high throughput screening to be carried out where thousands of compounds can be tested at one time. The first chemical screen in zebrafish was carried out by Peterson and colleagues in 2000 where compounds from a library were screened in zebrafish to see what phenotypes they evoked, some of the phenotypes observed included effects on pigmentation and ear development.⁴³ These phenotype based screens can help to uncover the mechanism by which compounds may be acting. For example when fertilised zebrafish embryos are exposed to a certain concentration of probe molecule and the morphological changes are monitored, reduced pigmentation can be an indicator that the probe molecule may be acting on thyroid hormone signaling and therefore warrants further investigation as an agent for treating thyroid disorders.⁴⁴ In a

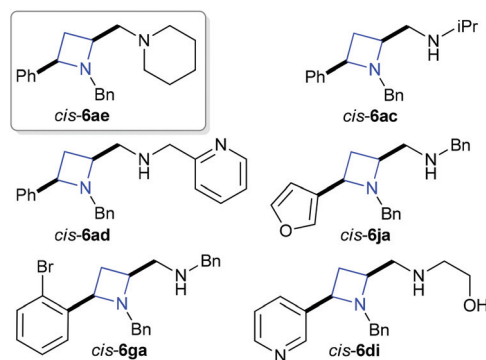


Fig. 2 Azetidines selected for preliminary biological screening.

forward chemical screen in zebrafish carried out by Das *et al.* a retinoid analogue which caused specific cardiovascular defects in zebrafish (heart tube deformations) was identified, indicating potential usefulness against conditions such as acute promyelocytic leukaemia.⁴⁵

For our investigation of biological activity using zebrafish embryo development as a screening technique six azetidine derivatives were selected. The azetidines selected, racemic *cis*-**6ae**, **6ac**, **6ad**, **6ja**, **6ga** and **6di** represent a cross section of the azetidines synthesized and provide an interesting comparison in terms of the chemical space they explore, Fig. 2. Comparisons between the structures selected could be made along the following lines tertiary amine *versus* secondary amine; aliphatic *versus* benzylic; heteroaromatic *versus* aromatic; *etc.*

Zebrafish embryo developmental morphology assay

Table 4 shows a summary of the morphological defects observed after five days following treatment of zebrafish embryos at 75%-epiboly stage with the six selected azetidines at a concentration of 25 μM. Azetidines were added as DMSO solutions against a control and a DMSO blank. Most compounds had minimal effects on overall morphology (see Table S1, ESI†). However, compound *cis*-**6ae** was found to cause severe morphological effects over the whole exposure period showing increased severity of phenotypes at higher doses (see Fig. S1 to S8, ESI†), *cis*-**6ga** was also found to cause morphological effects at later time points (see Fig. S9, ESI†).

Compound *cis*-**6ae**, which contains a piperidine fragment, shows effects in all of the nine morphological defect indicators. This suggests *cis*-**6ae** has high biological activity in a



Table 4 Summary of effects of chosen azetidines (25 μ M) on zebrafish embryos after 5 days

Additive	Morphological defects								
	Curved tails	Abnormal jaw	Delayed growth	Lack of pigmentation	Slowed heart rate	Tail necrosis	Cardiac oedema	Brain oedema	Reduced circulation/blood clots
<i>cis</i> - 6ae	++++	++++	++++	++++	++++	++++	++++	+++	++++
<i>cis</i> - 6ja	+	—	+	—	++	—	++	—	++
<i>cis</i> - 6ga	+++	—	+	—	+++	—	+++	—	+++
<i>cis</i> - 6ac	+	—	+/-	—	+	—	+	—	+
<i>cis</i> - 6di	+/-	—	—	—	+/-	—	+/-	—	+/-
<i>cis</i> - 6ad	+/-	+/-	+/-	—	+	—	+	—	+
Control	+/-	—	+/-	—	+/-	—	+/-	—	+/-
Blank	—	—	—	—	+/-	—	+/-	—	+/-

++++ = very severe effect (75–100%); +++ = severe effect (50–75%); ++ = moderate effect (25–50%); + = minimal effect (5–25%); +/- = either minimal or no effect (0–5%); — = no effect (0%).

number of tissues, resembling many effects seen when zebrafish embryos are exposed to piperidines, effects such as hypopigmentation and cardiac edema.⁴⁶ Next most active is compound *cis*-**6ga**, which bears an *ortho*-bromophenyl substituent in the 2-position of the of the azetidine ring. Compound *cis*-**6ga** resulted in curved tails, slowed heart rate, cardiac edema and reduced circulation in more than 50% of embryos. The 3-furyl derivative **6ja** showed a similar but less extensive spread of defects. Compounds *cis*-**6ae**, *cis*-**6ga** and *cis*-**6ja** all produced effects similar to those seen with nicotine;⁴⁷ however, they showed varying degrees of severity. An additional feature observed with *cis*-**6ae** not included in the morphological defect scoring was the development of an atrioventricular block, specifically a 2 : 1 type (data not shown), which is often observed when zebrafish embryos are treated with QT prolonging drugs,⁴⁸ suggesting that this compound in particular may also be affecting cardiac ion channels. Additionally, somites were found to be u-shaped and melanophores were found to be small and granulated, as well as reduced in number. U-shaped somites are characteristic features observed when there are defects in slow muscle development caused by disruption to the Hedgehog signaling pathway.⁴⁹ The pigmentation effects seen may have been caused by reduced cyclic adenosine monophosphate (cAMP) levels and disruption of microtubule function, as both are required for aggregating and dispersing melanin.⁵⁰ Additional disruption to xanthophores may have also contributed to the pigmentation phenotype at later stages.⁵¹ Embryos treated with compound *cis*-**6ae** were also unable to hatch, which could have been due to the hatching enzyme chorionase being inhibited, osmotic disturbances or due to weakened muscular movements.⁵² The other compounds tested in this preliminary screen did not show any marked defects, which could be due to the small concentration range tested, as different compounds are likely to have different effective concentrations. However, it is clear that among even this small sample set various important pharmacological effects can be seen and small structure variation have wide impact on activity, boding well for future investigations. Since these compounds have not been previously tested in zebrafish, correlation to a similar molecule can provide important

benchmarking, a small ring nitrogen-containing heterocycle which has been extensively studied is nicotine, which is a naturally occurring alkaloid that acts on nACh receptors. Nicotine in is extremely toxic to humans and is a known human teratogen.⁵³ In zebrafish embryos, nicotine has been found to reduce notochord length, cause apoptotic death of brain cells,⁵⁴ lead to underdeveloped tails, reduced number of somites, increased muscular bends in spine and cause complications in jaw formation.⁵³ Many of these effects correlate with those seen with some of the azetidines screened here, particularly compound *cis*-**6ae**. The muscular bends, which are common features of exposure to compound *cis*-**6ae** and *cis*-**6ga** are thought to occur due to slow muscle fibres becoming disorganised and the nACh receptors becoming desensitised, which explains the short stature of the fish and the bent tails.⁵⁵ With compound *cis*-**6ae** reduced locomotion was observed when 6 dpf larval zebrafish were treated at a concentration of 20 μ M, which is similar to that which occurs when zebrafish are treated with high concentrations of nicotine.⁵⁶ This is because at low concentrations, nicotine causes stimulation of nACh receptors in the CNS, whereas at higher concentrations nicotine will bind more preferentially to nACh receptors in muscles (particularly those involved in movement), which can have negative depressant effects reducing locomotion.

Since compound *cis*-**6ae** was shown to elicit morphological defects across the nine probed criteria it was selected for a more detailed investigation. Specific characterisation (detailed phenotypic analysis) of compound **6ae** to look for further effects undetectable by morphology analysis alone was embarked upon (Fig. 3). Analysis of locomotion by assessing motility of zebrafish larvae through measurement of pixel change over time, overall blood vessel development through fluorescence visualisation of vascular endothelial cells using the Tg(*fli-1:EGFP*) transgenic zebrafish line and blood circulation through fluorescence visualisation of red blood cells using Tg(*gata-1:dsRed*); Tg(*fli-1:EGFP*) transgenic zebrafish line revealed that compound *cis*-**6ae** reduced locomotion, did not affect gross blood vessel development and reduced circulation with formation of blood clots. Compound *cis*-**6ae** at a concentration of 20 μ M was found to significantly reduce movement



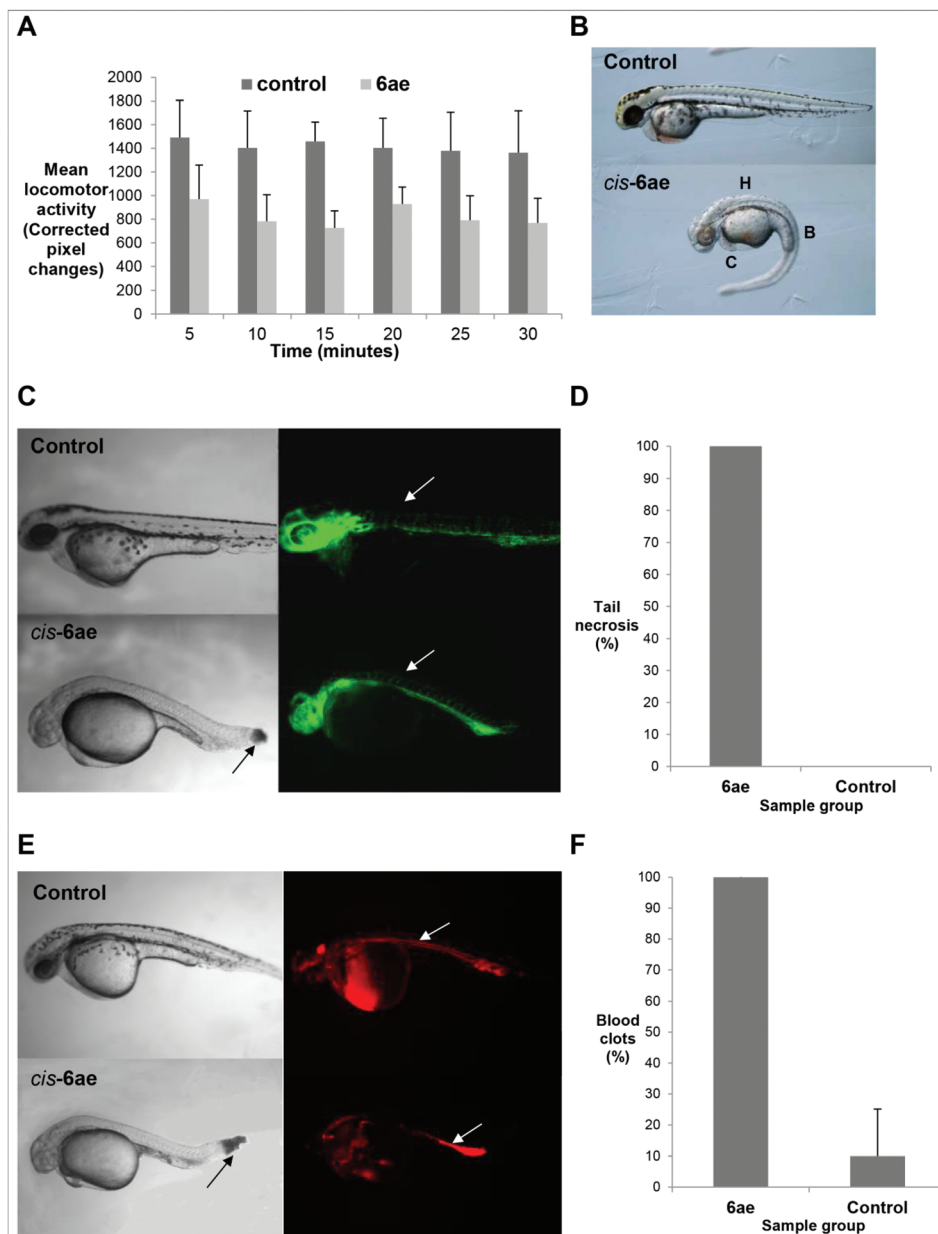


Fig. 3 Effects of compound *cis*-6ae: (A) bar chart comparing movement/locomotion of *cis*-6ae-treated 6 dpf zebrafish larvae compared to controls; (B) bright field lateral view of a *cis*-6ae-treated 2 dpf embryo presenting with morphological defects (H = hypopigmentation, B = bent spine, C = cardiac edema) compared to control; (C) bright field lateral view of a *cis*-6ae-treated 36 hpf zebrafish embryo compared to control (left) with corresponding fluorescence images of vasculature through *fli-1:EGFP* transgene activity (right) where individual blood vessels can be seen (arrows) and the formation of a blood clot in the *cis*-6ae-treated embryo; (D) bar chart comparing frequency of tail necrosis in *cis*-6ae-treated 36 hpf embryos compared to controls; (E) bright field lateral view of a *cis*-6ae-treated 36 hpf zebrafish embryo compared to control (left) with corresponding fluorescence images of blood circulation through *gata-1:dsRed* transgene activity (right) where erythrocytes can be seen (arrows) and the formation of a blood clot in the *cis*-6ae-treated embryo; (F) bar chart comparing frequency of blood clots in *cis*-6ae-treated 36 hpf embryos compared to controls.

of 6 dpf zebrafish compared to controls (Fig. 3, A) ($n = 60$, $P < 0.05$). Morphological defects observed after 48 hours following treatment with 25 μM *cis*-6ae at 75%-epiboly stage included bent tails, hypopigmentation, severely delayed development, brain and cardiac edema (Fig. 3, B). Analysis of 2 dpf *Tg(fli-1:EGFP)* zebrafish treated at 75%-epiboly with 50 μM *cis*-6ae (Fig. 3, C) under the green channel (which marks blood vessels) showed severely delayed growth with the tip of the tail

not fully formed and tail necrosis was evident with no blood vessels present in the necrotic part of the tail. Tail necrosis was found to occur in all embryos treated with 50 μM *cis*-6ae (Fig. 3, D) ($n = 40$, $P < 0.05$). However, overall vascular development was unperturbed. Analysis of 2 dpf *Tg(gata-1:dsRed)*; *Tg(fli-1:EGFP)* zebrafish treated at 75%-epiboly with 50 μM *cis*-6ae (Fig. 3, E) under the red channel (which labels circulating blood cells) showed reduced circulation and the formation of



blood clots. The formation of blood clots was found to occur in all embryos treated with 50 μ M *cis*-6ae with aggregation of erythrocytes evident at the end of the tail due to reduced blood circulation (Fig. 3, F) ($n = 40$; $P < 0.05$).

Exposure to compound *cis*-6ae resulted in numerous morphological defects in zebrafish embryos, suggesting that it was able to act on many tissues. Many similarities to nicotine toxicity were also observed suggesting that it may have a similar mode of action or operate *via* a similar route. Compound *cis*-6ae displayed similar effects to those which have been documented in zebrafish embryos following treatment with different piperidines, such as 2-methylpiperidine,⁴⁶ such compounds are often used as insecticides.⁵⁷ Recent studies with pyrethroid insecticides also showed similar morphological defects, such as jaw abnormalities, curvature of body axis and pericardial edema.⁵⁸ Additionally, the pigmentation defects observed with *cis*-6ae are similar to those seen with anti-thyroid agents such as methimazole,⁵⁹ suggesting there might be an effect on thyroid hormone signalling. However, there are many ways in which pigmentation can be reduced (*e.g.* disturbances to non-skeletal neural crest or melanophore formation), therefore further investigation would be required to ascertain the precise cause in this case. Overall, *cis*-6ae shows high toxicity at the concentrations that were used in this study and would be detrimental particularly to aquatic habitats if it were to be released into such an environment. Further studies to investigate mechanism of action and dose ranging may reveal potential therapeutic benefits (*e.g.* treatment for overactive thyroid/lice/scabies) or industrial applications (*e.g.* use as an agricultural insecticide).

From the selection of compounds tested at the specified concentrations, compound *cis*-6ae, as mentioned previously, has detrimental effects on zebrafish embryo development, making this compound particularly toxic to aquatic environments. Further studies would be necessary to elucidate its mechanism of action and metabolism in zebrafish. This study however shows that the zebrafish embryo is a suitable vertebrate model to investigate lead compounds for toxic effects due to its ease of accessibility, optical transparency, contribution to 3Rs (replacement, reduction and refinement) of animals in research, fast development, low cost of maintenance and high similarity in toxic responses when compared to humans and rodents. These advantages make the zebrafish embryo an attractive model for testing during pre-clinical drug development before tests are performed in rodents or higher mammals, saving time and money by filtering out any lead compounds with safety liabilities before they reach the next step in the drug development pipeline.

Conclusions

A synthetically versatile protocol for the stereospecific cyclisation of homoallyl amine derivatives ultimately delivered *cis*-azetidine and *cis* or *trans* pyrrolidine derivatives. Importantly, it was the subtle change of temperature and reaction sequence

in this iodine and base mediated cyclisation and subsequent halide displacement that gave selective access to these three groups of compound. A zebrafish embryo developmental morphology assay was demonstrated as a useful tool in assessing the biological activity of a sample of azetidine derivatives and a compound bearing an azetidine and a piperidine to elicit morphological responses across the panel of indicators of potential activity in a variety of settings. This demonstrates both the utility of the fertilised zebrafish egg morphology screening technique and the potential for the underlying azetidine scaffold discussed to be probed for biological activity using advanced high throughput techniques employing zebrafish embryos. With the increasing number of transgenic lines available and the latest technological advances the zebrafish will provide a platform for drug screening in the years to come.

Acknowledgements

The authors thank the University of Birmingham and ERDF AWM II for support. AF thanks The School of Chemistry, University of Birmingham for a studentship and the EPSRC for underpinning support. MAHS thanks the HCDP - Ministry of Higher Education-Kurdistan Region for financial support. JSF is extremely grateful to the Leverhulme Trust (F/00351/P and F/00094/BC) for early funding, East China University of Science and Technology for a visiting professorship, the Natural Science Foundation of China for a research fellowship (no. 21050110426) and the Royal Society Industry Fellowship scheme (2012/R1) for on-going support. FM and SSD thank the Marie Curie ITN project BOLD (Biology of liver and pancreatic development and disease) and "ZF-Health" Integrating projects in the Framework 7 programme of the European Commission for their financial support. The EPSRC UK National Crystallography Service at the University of Southampton are thanked for the collection of the crystallographic data.⁶⁰

References

- 1 G. Cardillo, L. Gentilucci and A. Tolomelli, in *Asymmetric Synthesis of Nitrogen Heterocycles*, ed. J. Royer, Wiley-VCH Verlag GmbH & Co. KGaA, 2009, pp. 1–50.
- 2 J. F. Ma and K. Nomoto, *Plant Physiol.*, 1993, **102**, 373–378.
- 3 T. Takemoto, K. Nomoto, S. Fushiya, R. Ouchi, G. Kusano, H. Hikino, S. I. Takagi, Y. Matsuura and M. Kakudo, *Proc. Jpn. Acad., Ser. B*, 1978, **54**, 469–473.
- 4 S. Knapp and Y. H. Dong, *Tetrahedron Lett.*, 1997, **38**, 3813–3816.
- 5 H. Takikawa, T. Maeda and K. Mori, *Tetrahedron Lett.*, 1995, **36**, 7689–7692.
- 6 H. Yoda, T. Uemura and K. Takabe, *Tetrahedron Lett.*, 2003, **44**, 977–979.
- 7 J. D. Schmitt, *Curr. Med. Chem.*, 2000, **7**, 749–800.
- 8 D. X. Wang, H. Booth, N. Lerner-Marmarosh, T. S. Osdene and L. G. Abood, *Drug Dev. Res.*, 1998, **45**, 10–16.



- 9 J. T. Lowe, M. D. Lee, L. B. Akella, E. Davoine, E. J. Donckele, L. Durak, J. R. Duvall, B. Gerard, E. B. Holson, A. Joliton, S. Kesavan, B. C. Lemercier, H. Liu, J.-C. Marié, C. A. Mulrooney, G. Muncipinto, M. Welzel-O'Shea, L. M. Panko, A. Rowley, B.-C. Suh, M. Thomas, F. F. Wagner, J. Wei, M. A. Foley and L. A. Marcaurelle, *J. Org. Chem.*, 2012, **77**, 7187–7211.
- 10 J. Frigola, J. Pares, J. Corbera, D. Vano, R. Merce, A. Torrens, J. Mas and E. Valenti, *J. Med. Chem.*, 1993, **36**, 801–810.
- 11 A. Feula, L. Male and J. S. Fossey, *Org. Lett.*, 2010, **12**, 5044–5047.
- 12 The unexpected oxidation, deallylation, sequence witnessed when using cesium carbonate as base was examined in detail in a parallel report, see next reference.
- 13 A. Feula and J. S. Fossey, *RSC Adv.*, 2013, **3**, 5370.
- 14 See ESI† for details.
- 15 S. Kobayashi, Y. Mori, J. S. Fossey and M. M. Salter, *Chem. Rev.*, 2011, **111**, 2626–2704.
- 16 G. C. Liu, D. A. Cogan and J. A. Ellman, *J. Am. Chem. Soc.*, 1997, **119**, 9913–9914.
- 17 G. C. Liu, D. A. Cogan, T. D. Owens, T. P. Tang and J. A. Ellman, *J. Org. Chem.*, 1999, **64**, 1278–1284.
- 18 D. A. Cogan, G. C. Liu and J. Ellman, *Tetrahedron*, 1999, **55**, 8883–8904.
- 19 J. A. Ellman, T. D. Owens and T. P. Tang, *Acc. Chem. Res.*, 2002, **35**, 984–995.
- 20 T. Harrison, *Contemp. Org. Synth.*, 1995, **2**, 209–224.
- 21 M. Wang, Z. Wang, Y.-H. Shi, X.-X. Shi, J. S. Fossey and W.-P. Deng, *Angew. Chem., Int. Ed.*, 2011, **50**, 4897–4900.
- 22 M. P. Patil, A. K. Sharma and R. B. Sunoj, *J. Org. Chem.*, 2010, **75**, 7310–7321.
- 23 G. Barker, J. L. McGrath, A. Klapars, D. Stead, G. Zhou, K. R. Campos and P. O'Brien, *J. Org. Chem.*, 2011, **76**, 5936–5953.
- 24 F. Couty and M. Kletschii, *J. Mol. Struct. (THEOCHEM)*, 2009, **908**, 26–30.
- 25 F. Durrat, M. V. Sanchez, F. Couty, G. Evano and J. Marrot, *Eur. J. Org. Chem.*, 2008, 3286–3297.
- 26 B. Berthe, F. Outurquin and C. Paulmier, *Tetrahedron Lett.*, 1997, **38**, 1393–1396.
- 27 F. Outurquin, X. Pannecoucke, B. Berthe and C. Paulmier, *Eur. J. Org. Chem.*, 2002, 1007–1014.
- 28 T. M. Bott, J. A. Vanecko and F. G. West, *J. Org. Chem.*, 2009, **74**, 2832–2836.
- 29 B. Drouillat, F. Couty, O. David, G. Evano and J. Marrot, *Synlett*, 2008, 1348.
- 30 F. Couty, F. Durrat and D. Prim, *Tetrahedron Lett.*, 2003, **44**, 5209–5212.
- 31 F. Couty, F. Durrat, G. Evano and J. Marrot, *Eur. J. Org. Chem.*, 2006, 4214–4223.
- 32 W. Van Brabant, R. Van Landeghem and N. De Kimpe, *Org. Lett.*, 2006, **8**, 1105–1108.
- 33 S. G. Davies, R. L. Nicholson, P. D. Price, P. M. Roberts, A. J. Russell, E. D. Savory, A. D. Smith and J. E. Thomson, *Tetrahedron: Asymmetry*, 2009, **20**, 758–772.
- 34 C. Delvecchio, J. Tiefenbach and H. M. Krause, *Assay Drug Dev. Technol.*, 2011, **9**, 354–361.
- 35 C. A. Macrae, *Expert Opin. Drug Discovery*, 2010, **5**, 619–632.
- 36 T. V. Bowman and L. I. Zon, *ACS Chem. Biol.*, 2010, **5**, 159–161.
- 37 C. Chakraborty, C. H. Hsu, Z. H. Wen, C. S. Lin and G. Agoramoorthy, *Curr. Drug Metab.*, 2009, **10**, 116–124.
- 38 T. P. Barros, W. K. Alderton, H. M. Reynolds, A. G. Roach and S. Berghmans, *Br. J. Pharmacol.*, 2008, **154**, 1400–1413.
- 39 L. I. Zon and R. T. Peterson, *Nat. Rev. Drug Discovery*, 2005, **4**, 35–44.
- 40 G. J. Lieschke and P. D. Currie, *Nat. Rev. Genet.*, 2007, **8**, 353–367.
- 41 R. T. Peterson and M. C. Fishman, *Methods Cell Biol.*, 2011, **105**, 525–541.
- 42 G. N. Wheeler and A. W. Brandli, *Dev. Dyn.*, 2009, **238**, 1287–1308.
- 43 R. T. Peterson, B. A. Link, J. E. Dowling and S. L. Schreiber, *Proc. Natl. Acad. Sci. U. S. A.*, 2000, **97**, 12965–12969.
- 44 O. A. Elsalini and K. B. Rohr, *Dev. Genes Evol.*, 2003, **212**, 593–598.
- 45 B. C. Das, K. McCartin, T. C. Liu, R. T. Peterson and T. Evans, *PLoS One*, 2010, **5**, e10004.
- 46 C. Schulte and R. Nagel, *ATLA, Altern. Lab. Anim.*, 1994, **22**, 12–19.
- 47 L. T. Thomas, L. Welsh, F. Galvez and K. R. Svoboda, *Toxicol. Appl. Pharmacol.*, 2009, **239**, 1–12.
- 48 U. Langheinrich, G. Vacun and T. Wagner, *Toxicol. Appl. Pharmacol.*, 2003, **193**, 370–382.
- 49 I. G. Woods and W. S. Talbot, *PLoS Biol.*, 2005, **3**, e66.
- 50 D. W. Logan, S. F. Burn and I. J. Jackson, *Pigm. Cell Res.*, 2006, **19**, 206–213.
- 51 J. Odenthal, K. Rossnagel, P. Haffter, R. N. Kelsh, E. Vogelsang, M. Brand, F. J. van Eeden, M. Furutani-Seiki, M. Granato, M. Hammerschmidt, C. P. Heisenberg, Y. J. Jiang, D. A. Kane, M. C. Mullins and C. Nusslein-Volhard, *Development*, 1996, **123**, 391–398.
- 52 Q. He, K. Liu, S. Wang, H. Hou, Y. Yuan and X. Wang, *Drug Chem. Toxicol.*, 2012, **35**, 149–154.
- 53 E. W. Klee, J. O. Ebbert, H. Schneider, R. D. Hurt and S. C. Ekker, *Nicotine Tob. Res.*, 2011, **13**, 301–312.
- 54 B. Parker and V. P. Connaughton, *Zebrafish*, 2007, **4**, 59–68.
- 55 L. Welsh, R. L. Tanguay and K. R. Svoboda, *Toxicol. Appl. Pharmacol.*, 2009, **237**, 29–40.
- 56 A. M. Petzold, D. Balciunas, S. Sivasubbu, K. J. Clark, V. M. Bedell, S. E. Westcot, S. R. Myers, G. L. Moulder, M. J. Thomas and S. C. Ekker, *Proc. Natl. Acad. Sci. U. S. A.*, 2009, **106**, 18662–18667.
- 57 K. A. Bandara, V. Kumar, U. Jacobsson and L. P. Molleyres, *Phytochemistry*, 2000, **54**, 29–32.
- 58 A. DeMicco, K. R. Cooper, J. R. Richardson and L. A. White, *Toxicol. Sci.*, 2010, **113**, 177–186.
- 59 O. A. Elsalini and K. B. Rohr, *Dev. Genes Evol.*, 2003, **212**, 593–598.
- 60 S. J. Coles and P. A. Gale, *Chem. Sci.*, 2012, **3**, 683–689.

