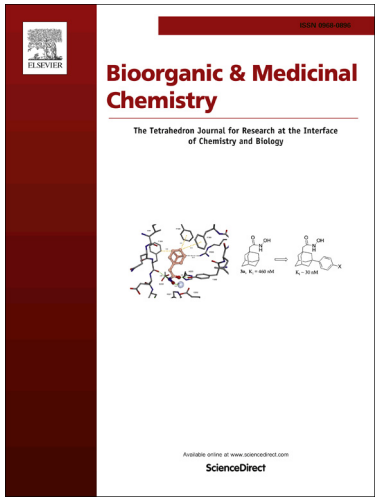
Accepted Manuscript



Initial development of a cytotoxic amino-*seco* -CBI warhead for delivery by prodrug systems

Elvis A. Twum, Amit Nathubhai, Pauline J. Wood, Matthew D. Lloyd, Andrew S. Thompson, Michael D. Threadgill

PII: S0968-0896(15)00332-6
DOI: http://dx.doi.org/10.1016/j.bmc.2015.04.034
Reference: BMC 12246

To appear in: *Bioorganic & Medicinal Chemistry*

Received Date: 2 February 2015
Revised Date: 2 April 2015
Accepted Date: 10 April 2015

Please cite this article as: Twum, E.A., Nathubhai, A., Wood, P.J., Lloyd, M.D., Thompson, A.S., Threadgill, M.D., Initial development of a cytotoxic amino-*seco* -CBI warhead for delivery by prodrug systems, *Bioorganic & Medicinal Chemistry* (2015), doi: http://dx.doi.org/10.1016/j.bmc.2015.04.034

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

ACCEPTED MANUSCRIPT

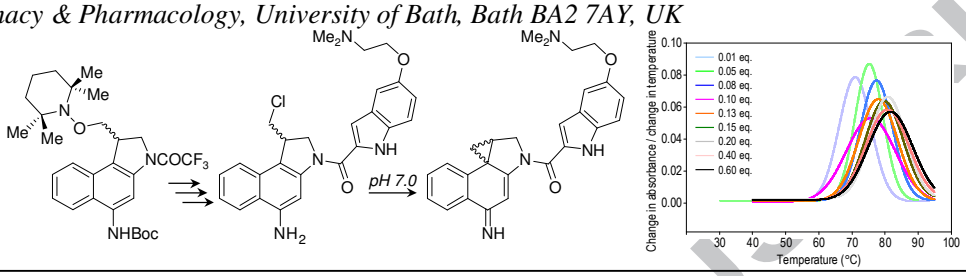
Graphical Abstract

To create your abstract, type over the instructions in the template box below.
Fonts or abstract dimensions should not be changed or altered.

Initial development of a cytotoxic amino-*seco*-CBI warhead for delivery by prodrug systems

Leave this area blank for abstract info.

E. A. Twum, A. Nathubhai, P. J. Wood, M. D. Lloyd, A. S. Thompson, M. D. Threadgill
Dept. Pharmacy & Pharmacology, University of Bath, Bath BA2 7AY, UK



ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

Bioorganic & Medicinal Chemistry
journal homepage: www.elsevier.com

Initial development of a cytotoxic amino-*seco*-CBI warhead for delivery by prodrug systems

Elvis A. Twum, Amit Nathubhai, Pauline J. Wood, Matthew D. Lloyd, Andrew S. Thompson and Michael D. Threadgill*

Medicinal Chemistry, Department of Pharmacy & Pharmacology, University of Bath, Claverton Down, Bath BA2 7AY, UK
Telephone +44 1225 386840; FAX +44 1225 386114; e-mail m.d.threadgill@bath.ac.uk

ARTICLE INFO

Article history:
Received
Received in revised form
Accepted
Available online

Keywords:
CBI
Prodrug
TEMPO
Amino-CBI
Cytotoxin

ABSTRACT

Cyclopropabenzaindoles (CBIs) are exquisitely potent cytotoxins which bind and alkylate in the minor groove of DNA. They are not selective for cancer cells, so prodrugs are required. CBIs can be formed at physiological pH by Winstein cyclisation of 1-chloromethyl-3-substituted-5-hydroxy-2,3-dihydrobenzo[*e*]indoles (5-OH-*seco*-CBIs). Corresponding 5-NH_2-*seco*-CBIs should also undergo Winstein cyclisation similarly. A key triply orthogonally protected intermediate on the route to 5-NH_2-*seco*-CBIs has been synthesised, *via* selective monotrifluoroacetylation of naphthalene-1,3-diamine, Boc protection, electrophilic iodination, selective allylation at the trifluoroacetamide and 5-*exo* radical ring-closure with TEMPO. This intermediate has potential for introduction of peptide prodrug masking units (deactivating the Winstein cyclisation and cytotoxicity), addition of diverse indole-amide side-chains (enhancing non-covalent binding prior to alkylation) and use of different leaving groups (replacing the usual chlorine, allowing tuning of the rate of Winstein cyclisation). This key intermediate was elaborated into a simple model 5-NH_2-*seco*-CBI with a dimethylaminoethoxyindole side-chain. Conversion to a bioreactive entity and the bioactivity of this system were confirmed through DNA-melting studies (ΔT_m = 13 deg. C) and cytotoxicity against LNCaP human prostate cancer cells (IC_{50} = 18 nM).

2009 Elsevier Ltd. All rights reserved.

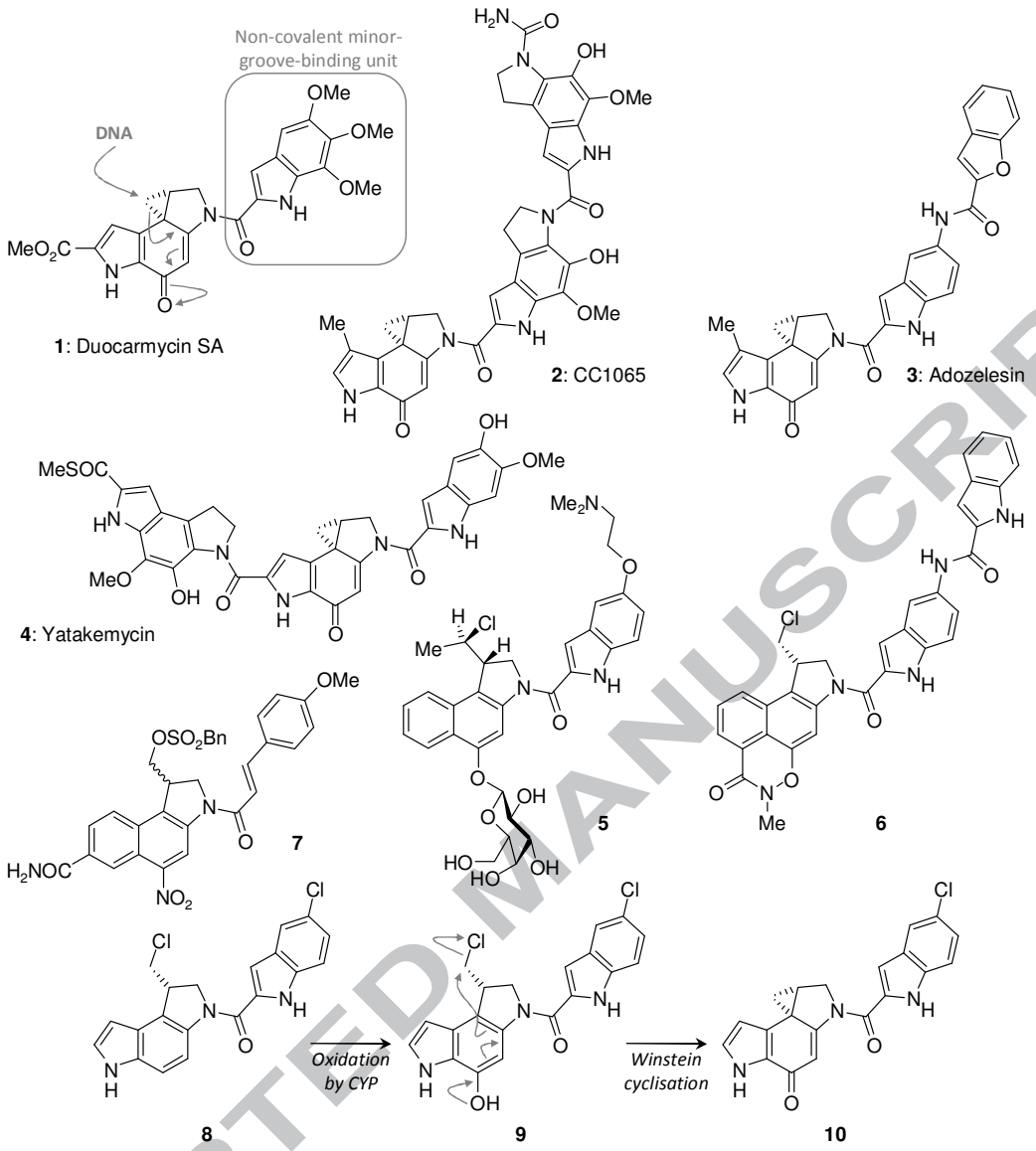
1. Introduction

Duocarmycin SA **1** (Scheme 1) is a natural product of the cyclopropapyrroloindole (CPI) class with exquisitely potent cytotoxicity, having IC_{50} = 10 pM in L1210 cells.[1] It acts by binding in the minor groove of DNA, with consequent alkylation by the strained spirocyclopropane ring. In the structure of the related natural product CPI **2** (CC1065), the side-chain, which binds non-covalently in the minor groove of DNA prior to covalent reaction, is extended to interact over a longer section of the groove. This also provides high cytotoxic potency for this compound (IC_{50} = 24 pM in L1210 cells).[2] The semisynthetic experimental drug adozelesin **3** carries the same CPI war-head as does **2** but the side-chain is simplified; the binding of this molecule into the minor groove as a π-stacked dimer has been studied in depth.[3] The non-covalently-binding units can also be displayed on each side of the CPI, as in yatakemycin **4**,[4,5] increasing potency yet further (IC_{50} = 5 pM in L1210 cells). However, this extreme cytotoxicity is not selective for tumour cells; compounds such as **2** also cause a delayed lethality in mice and rabbits.[6,7]

This lack of selectivity has led to research on the development of prodrugs designed to release the potent cytotoxins selectively in tumour tissue. Many of these prodrugs release the cyclopropabenzaindole (CBI) alkylating warhead, rather than CPI; these alkylating units have similar potency but improved stability and synthetic accessibility.[8,9] Tietze has disclosed a prodrug **5**, in which the exocyclic OH of the *seco* compound is masked as a β-D-galactoside.[10,11] This prodrug is essentially non-toxic until the β-D-galactoside is cleaved by a β-D-galactosidase in the context of ADEPT; Winstein cyclisation then gives the active CBI. The exocyclic oxygen is masked as a cyclic hydroxamate ester in **6**; bioreductive cleavage of the N–O bond exposes the phenol, triggering the required Winstein cyclisation.[12] Bioreduction of the nitro group to amino in hypoxic tumour cells is also required for activation of the nitro-prodrug **7**.[13] The amine then promotes Winstein cyclisation, with a sulfonate as the leaving group; **7** shows ~1000-fold selectivity for killing hypoxic HT29 human colon cancer cells *vs.* oxic HT29 cells *in vitro*. Oxidative activation is required for *seco*-CPI prodrugs such as **8**, developed by the Bradford group.[14] Metabolic oxidation, by CYP1A1 or CYP2W1 (over-expressed in some tumours) gives **9**, from which Winstein cyclisation leads to the active cytotoxin **10** (Scheme 1).

Many of these prodrugs are activated by reduction or oxidation, with only **5** requiring enzyme-catalysed hydrolysis. As part

ACCEPTED MANUSCRIPT



Scheme 1. Structures of CPI extreme cytotoxins duocarmycin SA **1**, CC1065 **2**, adozelesin **3** and yatakemycin **4** and of *seco*-CBI prodrugs **5-7**; also metabolic oxidation of *seco*-CPI prodrug **8** by CYP1A1 and CYP2W1, leading to insertion of the phenol in **9** and Winstein cyclisation to the active CPI cytotoxin **10**.

of our studies towards prodrugs where *seco*-CBIs are released by tumour-specific peptidases, we sought an appropriate *seco*-CBI carrying an amine at the 5-position for linkage to the peptide through an amide bond.

2. Results & discussion

2.1. Design of a protected *seco*-CBI

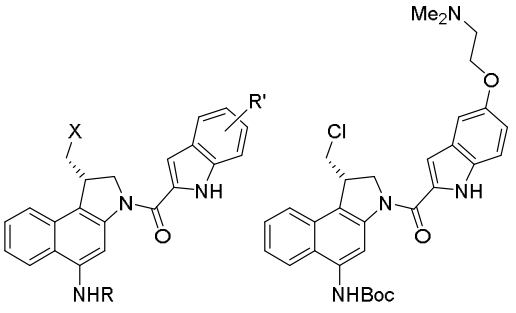
Figure 1 shows our general design of target 5-amino-*seco*-CBIs for later linkage to masking peptides. 5-Hydroxy-*seco*-CBIs would be inappropriate, as the link to the peptides would have to be aryl esters, which may be chemically labile in biofluids. Figure 1 shows the general design of the target 5-amino-*seco*-CBIs as structure **11**. In this structure, X represents a suitable leaving group (tuned to provide a balance of reactivity and stability), R represents a suitable acid-labile protecting group (or H), R' represents substitution on the indole side-chain. This substitution may be the OH or OMe groups found in natural CPIs (contributing to non-covalent binding in the minor groove), additional indole or benzofuran units (also contributing to binding) or water-solubilising entities. Structure **12** is the specific target for the present work. Here, the leaving group X is simply a chloride (as in many *seco*-CPIs and *seco*-CBIs) and the side-chain extension is the water-solubilising dimethylaminoethoxy group used by Tietze *et al.*[10] in their galactosidase-activated prodrug **5**.

2.2. Chemical synthesis

The key step in assembling the target *seco*-CBI-warhead **12** is cyclisation of a suitable 1,2,4-trisubstituted naphthalene. Two cyclisations were investigated: intramolecular 5-*exo* reaction of a chiral 1-metallo-2-oxiranylmethylaminonaphthalene (to give enantiopure material) and radical 5-*exo* cyclisation of a 1-iodo-2-allylaminonaphthalene (giving a racemate).

Both routes required an orthogonally protected 1-iodonaphthalene-2,4-diamine. Scheme 2 shows the routes investigated thereto. Firstly, attempted nitration of 4-aminonaphthalen-1-ol **13** towards 4-amino-2-nitronaphthalen-1-ol failed, giving only naphtho-1,4-quinone **14** by oxidation. Martius Yellow **15** is a convenient starting material, with the correct pattern of functionalities on the naphthalene ring, although the nitro groups need to be discriminated. Selective reduction of the 4-nitro group with tin(II)

ACCEPTED MANUSCRIPT

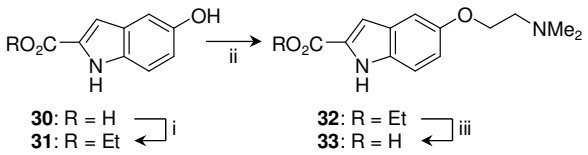


11 12

Figure 1. General structure of target 5-amino-*seco*-CBIs **11** and structure of synthetic target **12**. R = H or protecting group; R' = water-solubilising group or extension providing additional non-covalent binding; X = leaving group.

chloride, in a modification of the literature method,[15] afforded **16**. Attempted protection of the aniline under basic conditions gave only the carbonate **17**, arising from reaction at the more nucleophilic phenoxide anion. Under neutral conditions, the amine of **16** is more nucleophilic towards Boc_2O, giving **18** in excellent yield. The phenol was readily triflated but the attempted replacement of OTf in **19** with iodine to give **20** failed; this had previously been successful in the conversion of **23** to **24**.[16] An attempt to remove the triflyloxy group reductively (aiming for N-Boc 3-nitronaphthalene-1-amine) served only to cleave the O–S bond to regenerate **18**. With that avenue closed, attempts were made to reduce the nitro group of **18** to the corresponding amine, before orthogonal protection and introduction of iodine. Hydrogenolysis proceeded well in the presence of Pd but all attempts to isolate the very electron-rich naphthalene product failed, giving the N-Boc naphthoquinoneimine **21**. Reduction, followed by trifluoroacetylation, gave orthogonally protected **22** in moderate yield but no routes could be found to elaborate this towards **29**.

An alternative route was investigated, in which discrimination between the nitrogens was achieved by selective protection of the



Scheme 3. Preparation of side-chain unit **33**. *Reagents & conditions*: i, EtOH, HCl, reflux; ii, $Me_2N(CH_2)_2Cl.HCl$, K_2CO_3, H_2O, $CHCl_3$, reflux; iii, Cs_2CO_3, MeOH, H_2O, reflux.

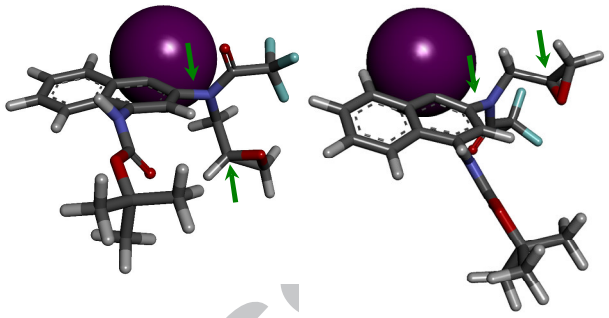
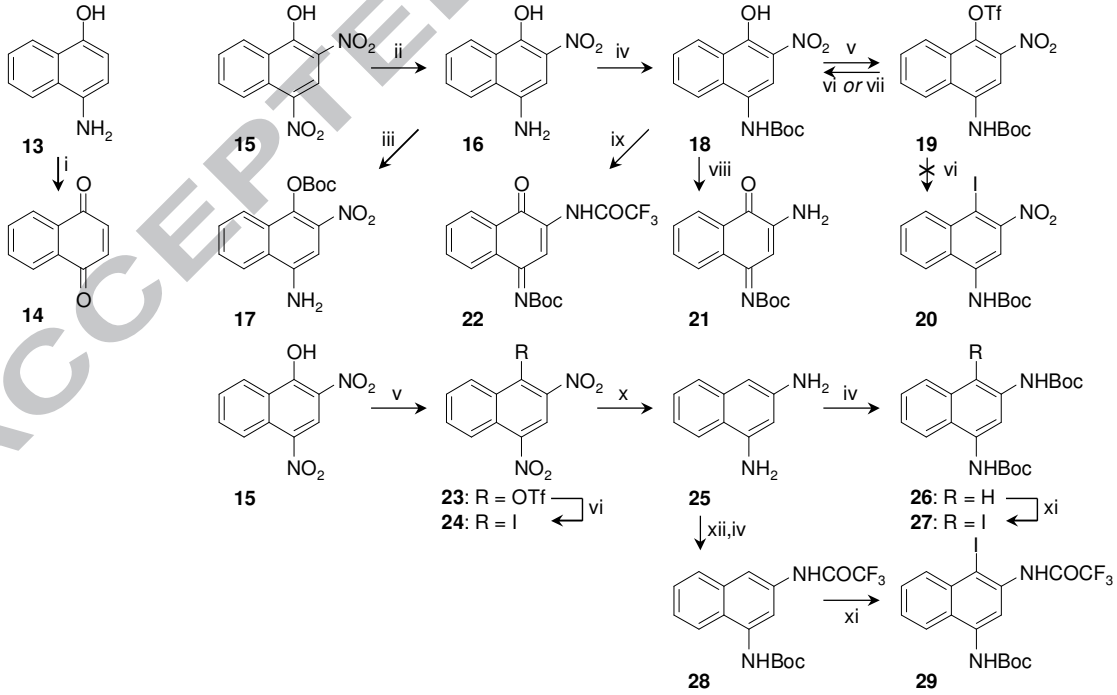


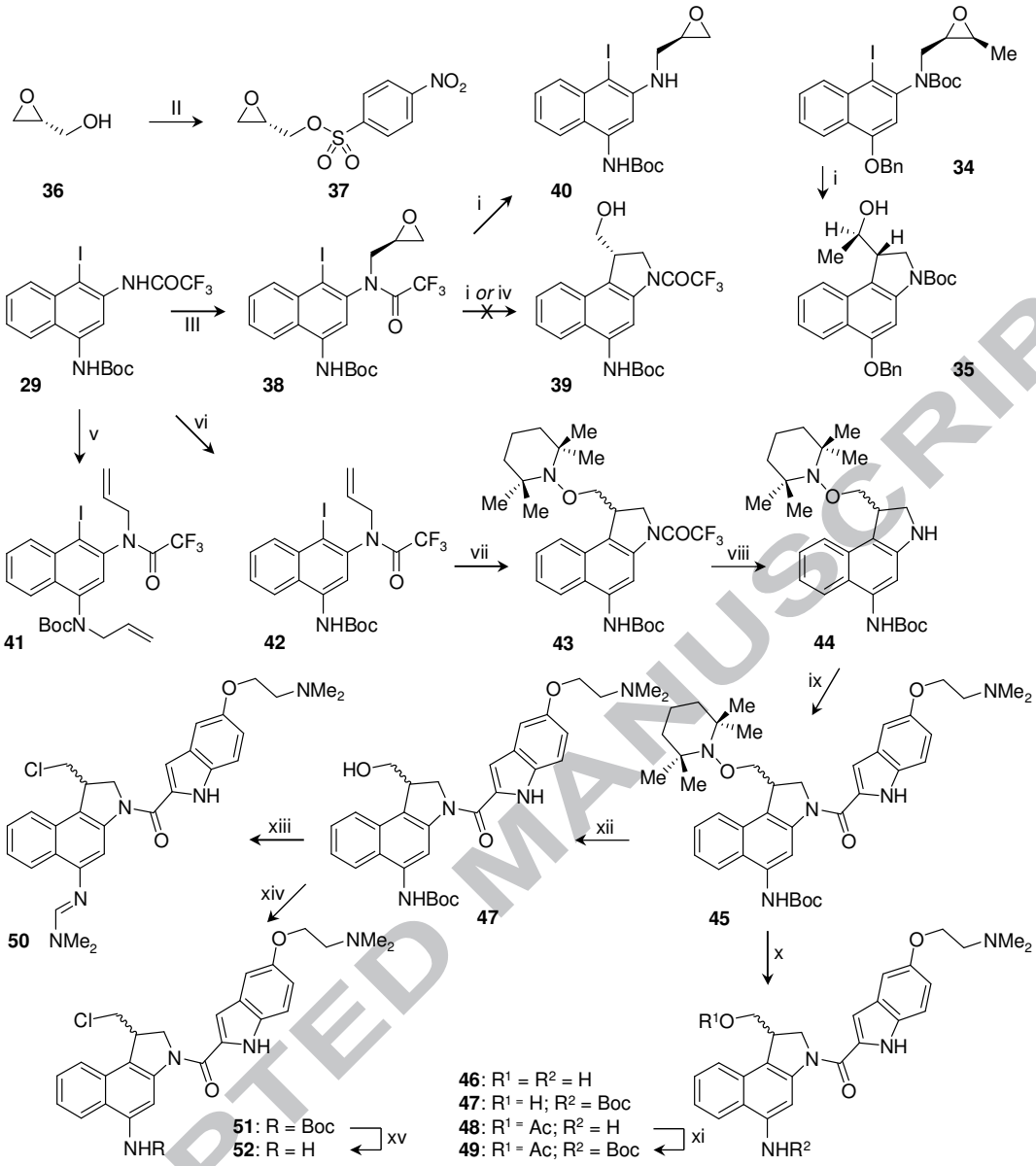
Figure 2. Structures of non-interconverting atropisomers of **38**. Chiral centres are indicated by green arrows. The iodine is shown in CPK mode to emphasise the steric bulk.

diamine **25**. As reported previously,[16] the phenolic OH of **15** was triflated to give **23** and S_NAr reaction with iodide ion furnished **24**. From here, reduction of the nitro groups also caused loss of the iodine, affording naphthalene-1,3-diamine **25**. This electron-rich naphthalene is unstable to autoxidation, so it was protected as the diBoc derivative **26**. The iodine was restored electrophilically with electrophilic iodine generated from N-iodosuccinimide and toluenesulfonic acid, affording **27**. However, all attempts to remove one Boc group selectively from **27** failed, with many acidic conditions also causing loss of the iodine, through a Wheland intermediate which was observed by NMR.[16] Changing the point at which selectivity is achieved to the protection step, rather than deprotection, treatment of **25** with trifluoroacetic an-



Scheme 2. Synthetic approaches to the key orthogonally protected 1-iodonaphthalene-2,4-diamine **29** *Reagents & conditions*: i, KNO_3, CF_3CO_2H, -20°C; ii, $SnCl_2$, aq. HCl, EtOH, <35°C; iii, Boc_2O, DMAP, CH_2Cl_2; iv, Boc_2O, THF; v, Tf_2O, pyridine, 0°C; vi, NaI, DMF, 80°C; vii, $Pd(OAc)_2$, Ph_3P, HCO_2H, 65°C; viii, H_2, Pd/C, MeOH, then exposure to air; ix, K_2CO_3, $Na_2S_2O_4$, H_2O, CH_2Cl_2 then $(F_3CCO)_2O$, Pr^i_2NEt, CH_2Cl_2; x, $SnCl_2$, EtOAc; xi, N-iodosuccinimide, TsOH, THF; xii, $(F_3CCO)_2O$, Pr^i_2NEt, THF.

ACCEPTED MANUSCRIPT



Scheme 4. The ring-closure **34→35** developed by Tietze *et al*.[17] and assembly of target 5-amino-*seco*-CBIs **47** and **48**, *via* radical ring-closure. *Reagents & conditions*: i, MeLi, CuCN, Et_2O, THF, N_2, -78°C; ii, 4-nitrobenzenesulfonyl chloride, Et_3N, toluene; iii, **37**, K_2CO_3, acetone, 50°C; iv, Bu^tLi, $ZnCl_2$, THF, -78°C; v, $H_2C{=}CHCH_2Br$, K_2CO_3, acetone, 50°C; vi, $H_2C{=}CHCH_2Br$, $KOBu^t$, THF; vii, TEMPO, Bu_3SnH, benzene, 60°C; viii, NaOH, H_2O, THF; ix, **33**, DIC, DMF, 40°C; x, Zn, THF / AcOH / H_2O (1 : 3 : 1), 70°C; xi, Boc_2O, THF, reflux; xii, Zn, THF / AcOH / H_2O (3 : 1 : 1), 70°C; xiii, MsCl (3 eq.), Et_3N, DMF, 0°C, then LiCl; xiv, MsCl (1.4 eq.), Et_3N, pyridine, 0°C then LiCl; xv, HCl, 1,4-dioxane.

hydride under very mild conditions led to reaction at the less-sterically-encumbered 3-NH_2; subsequent protection of the 1-NH_2 with Boc led to the orthogonally protected diamine **28** in modest yield. As for **26→27**, **28** was iodinated electrophilically to provide the key intermediate **29**.

Scheme 3 shows the preparation of the non-covalently binding and water-solubilising indole side-chain. The carboxylic acid of **30** was protected as the ethyl ester **31** before the corresponding phenoxide was alkylated with N-(2-chloroethyl)dimethylamine to afford **32**. Hydrolysis of the ester with caesium carbonate in aqueous methanol gave the required carboxylic acid **33**.

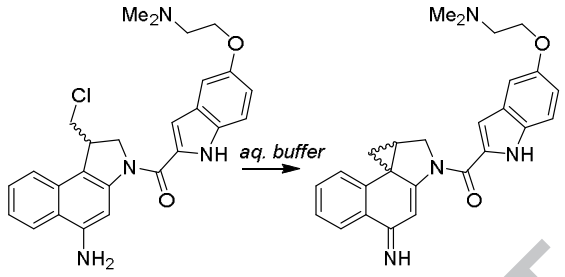
With these building blocks in hand, methods were investigated to build the target *seco*-CBIs. Initial attempts sought to exploit the ring-closure developed by Tietze *et al*.,[17] in which the protected iodo-(oxiranylmethylamino)naphthalene **34** is metallated with a higher-order lithium cuprate (replacing the iodine); attack of the aryl-copper on the oxirane then proceeds with chelation-controlled regioselectivity and stereoselectivity to form **35** (Scheme 4). The 4-nitrobenzenesulfonate (nosylate) ester **37** of *R*-glycidol **36** was formed in the usual way; this leaving group was selected as this and similar oxiranylmethyl nosylates react with nucleophiles strictly in an S_N2 manner,[17] retaining the configuration at the oxirane. The F_3CCON–H is the more acidic in **29** and selective alkylation of the corresponding anion with **37** afforded **38** in good yield. Interestingly, **38** was formed as a mixture of chromatographically separable diastereomeric atropisomers **38A** and **38B**. The bulky iodine twists the N-oxiranylmethyltrifluoroacetamide out of the plane of the naphthalene by rotation about the naphthalene–N bond, generating a chiral centre. Interconversion of the atropisomers by rotation about this C–N bond is so severely impeded that it does not occur at ambient temperature.

ACCEPTED MANUSCRIPT

Owing to the presence of the conventional *R*-chiral centre in the oxirane, the atropisomers are diastereomeric. ^{1}H, ^{13}C and ^{19}F NMR analysis of atropisomer **38A** also showed a 3:2 mixture of rotamers; this probably reflects restricted rotation of the tertiary amide carbonyl–N bond in the trifluoroacetamide. No similar rotamers were evident in the corresponding spectra of **38B**, suggesting that one rotamer about the amide bond predominates greatly in this diastereoisomer. Figure 2 shows the proposed structures of these atropisomers, using structures minimised in Sybyl and rendered with Accelrys DS.

Unfortunately, the metalation / cyclisation reaction developed by Tietze *et al*.[17] failed to form **39** from a mixture of diastereoisomers of **38** The only isolable product (in high yield) was **40**, derived from loss of the F_3CCO protection (Scheme 4). We speculate that the failure to cyclise was due to the presence of the BocN–H, which may have quenched the cuprate before it could engage in metallation. Nucleophilic ^{-}CN may then have cleaved the trifluoroacetamide. The NMR spectra of **40** showed the presence of the diastereomeric atropisomers, which were not separable in this case. Furuyama *et al*. reported that 4-iodophenol (carrying an acidic proton) is readily metallated with $Bu^{t}_{4}ZnLi_2$ and the arylzinc reacts with allyl bromide to give exclusively 4-allylphenol.[18] This process also failed to cyclise **38** to **40**, giving only unreacted **38**. The door was closed on this potential route to homochiral CBIs carrying NHBoc.

Therefore, a route was developed in which cyclisation of an achiral starting material would give a *seco*-CBI in racemic form. Boger and McKie carried out a 5-exo radical cyclisation of 2-(N-allyl-BocN)-4-benzyloxy-1-iodonaphthalene with TEMPO and Bu_3SnH to afford racemic 5-BnO-3-Boc-1-(2,2,6,6-tetramethylpiperidin-1-yloxy)methyl)-2,3-dihydrobenzo[*e*]indol-5-yl)carbamate.[19] Similar cyclisations have been disclosed by Stevenson *et al*.[13] Alkylation of **29** with allyl bromide in the presence of excess K_2CO_3 (Scheme 4) introduced two allyl groups (forming **41**) but selectivity for the more acidic N–H was achieved using one equivalent of $KOBu^{t}$ to form the monoanion, leading to **42**. As for **38**, **42** formed two separable but non-interconvertible conformers, in a 4:1 ratio. Clearly, one of the centres of atropisomerism is the naphthalene–N(allyl)$COCF_3$ bond which is restricted by the adjacent iodine but one may only speculate that the other source of atropisomerism may be the naphthalene–NHBoc bond, the rotation of which might be impeded by the *peri* 5-H. Cyclisation of the atropisomeric mixture to **43** was achieved in 60% yield using TEMPO and Bu_3SnH in boiling benzene. Tricycle **43** contains the three groups protected with completely mutually orthogonal masking groups. Selective basic hydrolysis of the trifluoroacetyl protection revealed the secondary amine in **44**, to which the 5-(2-dimethylaminoethoxy)indole-2-carbonyl side-chain was affixed in a carbodiimide coupling, affording **45**. The O-(2,2,6,6-tetramethylpiperidin-1-yl) protection was removed reductively, after much optimisation. Reaction of **45** with zinc powder in THF / AcOH / H_2O (1 : 3 : 1) gave a mixture of **46-49**, in which the N-Boc had been removed in **46** and **48** and the exposed alcohol had been esterified in **48** and **49**. Zinc acetate is a known Lewis acid catalyst for acetylation of primary alcohols.[20] The amine **48** was reprotected to give **49**. Reduction of **45** under less acidic conditions (THF / AcOH / H_2O (3 : 1 : 1)) allowed uneventful cleavage of the N–O bond to provide **47** in good yield. It only remained for the OH to be converted into the chloride and for the N-Boc to be removed. Reaction of **47** with mesyl chloride in DMF in the presence of Et_3N aimed to form the mesylate, which would be displaced by chloride ion from added excess LiCl. These conditions introduced the required chlorine but reaction of the MsCl with solvent DMF generated a Vilsmeier reagent, which cleaved the N–Boc and formed the dimethylformamidine in **50**. Use of stoichiometric MsCl in pyridine obviated this side-reaction to generate the required protected *seco*-CBI **51**. Finally the Boc protection was acidolysed to form **52** as the stable dihydrochloride, with the cation being unable to trigger Winstein cyclisation.



52 53

Scheme 5. Proposed Winstein cyclisation of **52** in aq. buffer (pH 7.0).

2.3. Biochemical and cell-biological evaluation

The CBIs act by binding and alkylating in the minor groove of DNA. When they do so, they cause an increase in the temperature at which the strands of double-stranded DNA separate, the so-called melting temperature (T_m). Indeed, the CPI CC-1065 **2** causes an increase in T_m of up to 31 deg. C.[2] Thus, to confirm that the *seco*-CBI **52** did form a DNA-reactive entity (presumably the CBI **53** formed by Winstein cyclisation (Scheme 5)) in aqueous buffer at pH 7.0 and that the expected **53** did bind and react with double-stranded DNA, a DNA-melting study was carried out. Firstly, to ensure that the putative electrophile **53** was fully formed in the buffer, the dihydrochloride salt of **52** was incubated with double-stranded calf-thymus DNA in phosphate buffer at pH 7.0 for 1 h and for 24 h and the increase in melting temperature (ΔT_m) was determined for both incubations. The identical values demonstrated that the naphthylamine was present as the free base at pH 7.0 and that formation of an electrophilic product (putatively **53**) was complete within 1 h. Thus the full DNA-melting study was conducted with an incubation time of 1 h (Figure 3). Panel A shows the primary changes in UV absorption at 256 nm with temperature, using nine different molar ratios of **52/53** to DNA (calculated per base-pair), whereas panel B shows the first derivative of these primary curves, allowing the T_m to be determined as the temperature at the apex of the derivative curve. In panel C, ΔT_m is plotted against the molar ratio of **52/53** per DNA base-pair. The data confirm that generation of the electrophile (putatively **53**) takes place rapidly at pH 7.0 and that it does bind to and alkylate double-stranded DNA, giving a maximum ΔT_m of 13 deg. C. This maximum is achieved with *ca*. 0.2 drug molecules per base-pair. Of course, **52** and **53** are racemates. It is known that only one enantiomer of CBIs binds strongly to DNA, the other having a shape which does not match the curve of the minor groove. Indeed, (-)-duocarmycin SA **1** alkylates DNA at least ten-times more efficiently than does its (+)-enantiomer.[21] Thus it may be argued that the appropriate ratio to consider is the *ca*. 0.1 ratio of the correct enantiomer per base-pair.

The cytotoxicity of **52** (presumably forming **53**) towards LNCaP human prostate carcinoma cells was measured using the MTS assay. This cell line produces Prostate-Specific Antigen (PSA) and its proliferation is sensitive to androgens.[22] An IC_{50} of 18 ± 3 nM was observed for the antiproliferative effect on these cells. To demonstrate that masking the naphthylamine with a carbonyl (in the Boc-protected precursor **51**) does diminish the cytotoxic potency, a similar assay was conducted for **51**. This compound indeed proved to be markedly less active, with IC_{50} = 218 ± 47 nM. In each case, only one of the enantiomers is likely to be potent.

ACCEPTED MANUSCRIPT

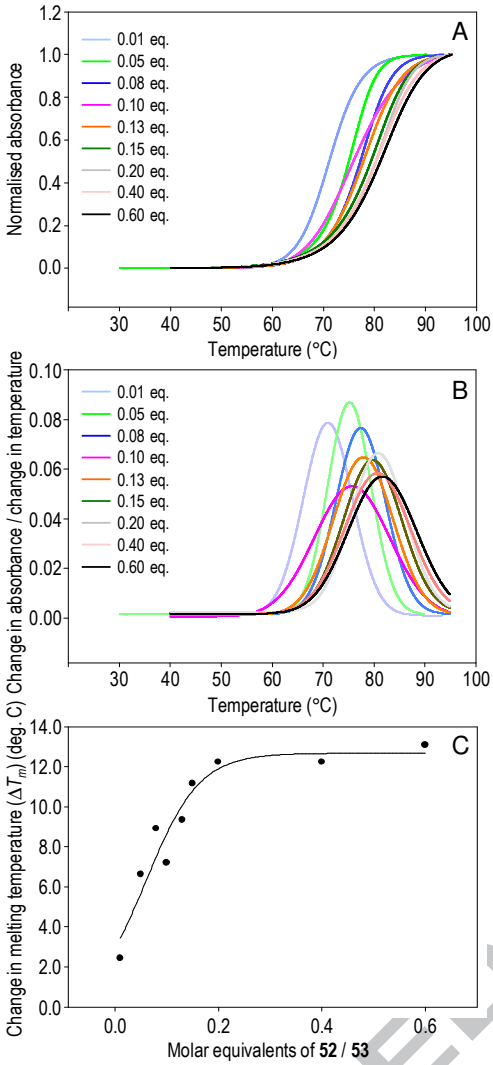


Figure 3. DNA-melting studies on **52/53**. Equivalents / molar equivalents refer to the number of equivalents of **52/53** per base-pair. **Panel A:** Graphs of absorbance (256 nm) *vs*. temperature for different molar ratios of **52/53**. **Panel B:** First derivative graphs from which the melting temperature (T_m) values were measured. **Panel C:** Graph of T_m *vs*. molar equivalents of **52/53**.

3. Conclusions

In this paper, the challenge of assembling a *seco*-CBI carrying a protected amine at the 5-position has been overcome. The key steps were preparation of the orthogonally protected 1-iodonaphthalene-2,4-diamine **29**, allowing selective allylation at the 2-trifluoroacetamido group, and radical 5-*exo* ring-closure of the N-allyl compound **42** to the *seco*-CBI core in **43**. This cyclisation uses an achiral educt and achiral reagents, leading to racemic **43**. The biological activities of CBIs (binding in the minor groove of double-stranded DNA, cytotoxicity) reside in one enantiomer only; the development of a stereoselective radical ring-closure will be the subject of a later publication. This route has the benefit that **43** contains three functionalities with completely orthogonal protection; the OH is protected by the O-tetramethylpiperidine (removable by reduction), the secondary amine site for introduction of the non-covalently bonding side-chain is protected as the base-labile trifluoroacetamide and the primary aniline (later to trigger Winstein cyclisation) is masked by the acid-labile Boc group. These benefits were exploited by firstly exposing the pyrrolidine nitrogen and attaching the indole side-chain in **45**. The OH was revealed and converted into the required leaving group in **51**. The target amino-*seco*-CBI **52** was generated by simple rapid acidolysis of the Boc protection. The mutual orthogonality of the protection in **43** means that this intermediate is potentially highly versatile, with opportunities to modify the order in which the subsequent steps are carried out, leading to access to diverse amino-*seco*-CBIs and to the potential for attaching the deactivating prodrug linker to the 5-NH_2 prior to "arming" the *seco*-CBI warhead by installing the leaving group.

Confirmation that the amino-*seco*-CBI system is a useful warhead for release / delivery by peptide prodrugs was provided by allowing **52** to undergo Winstein cyclisation in aqueous buffer at pH 7.0 to provide the CBI **53**. This was demonstrated to be complete in < 1 h by the equivalence of DNA-binding at 1 h and 24 h time-points. DNA-melting studies showed the generated **53** to bind to and react with double-stranded DNA in the stoichiometry of 0.2 molecules per base-pair (0.1 molecules if only the active enantiomer is considered). The delivered CBI is also a highly potent cytotoxin towards LNCaP human tumour cells, with IC_{50} in the nanomolar range. Further studies optimising the potency of this warhead towards the picomolar range of **1-4** by modifying the non-covalently-binding side-chain will be published later.

Thus we have identified **43** as an important core intermediate for development of potential warheads of extreme potency for selective delivery through peptide prodrug systems.

4. Experimental section

4.1. General

^{1}H and ^{13}C NMR spectra were recorded at 400.04 or 500.13 MHz for ^{1}H NMR and 100.59 or 125.76 MHz for ^{13}C NMR using $CDCl_3$, containing $SiMe_4$, unless otherwise noted. ^{13}C NMR spectra were assigned using HSQC and HMBC. MS data were obtained using electrospray ionisation using a microTOF instrument (Bruker Daltonics, Bremen, Germany), calibrated using sodium formate. Mps were obtained using a hot-stage microscope (Reichert-Jung). Experiments were conducted at ambient temperature, unless otherwise noted. Solutions in organic solvents were dried with $MgSO_4$. Synthetic methods for **14, 16-19, 21, 22, 31-33, 37, 38, 40, 41** are reported in the Supplementary Information; syntheses of **23-29** were reported previously.[16]

4.2. 1,1-Dimethylethyl N-(1-iodo-2-(N-(prop-2-enyl)-2,2,2-trifluoroacetamido)naphthalen-4-yl)carbamate (42A & 42B)

Compound **29** (1.22 g, 2.5 mmol) was stirred with KOBu^t (318 mg, 2.8 mmol) in THF (10 mL) under N_2 for 30 min, followed by addition of 3-bromopropene (973 mg, 8.04 mmol). The mixture was stirred for 2 h and heated to 50ºC for 16 h. Sat. aq. $NaHCO_3$ was added. Extraction (EtOAc), drying, evaporation and chromatography (petroleum ether → petroleum ether / EtOAc 19:1) gave **42A** (200 mg, 15%) as a yellow solid: mp 146-147°C; ^{1}H NMR (NOESY) δ 1.56 (9 H, s, Bu^t), 3.95 (1 H, dd, *J* = 14.2, 7.4 Hz, propenyl 1-H), 4.84 (1 H, dd, *J* = 14.2, 6.1 Hz, propenyl 1-H), 5.12 (1 H, dd, *J* = 17.0, 1.3 Hz, propenyl 3-H), 5.21 (1 H, dd, *J* = 10.1, 1.1 Hz, propenyl 3-H), 5.94 (1 H, m, propenyl 2-H), 7.27 (1 H, s, NH), 7.51 (1 H, dd, *J* = 8.2, 6.9, 1.2 Hz, 6-H), 7.60 (1 H, dd, *J* = 8.3, 6.9, 1.3 Hz, 7-H), 7.67 (1 H, d, *J* = 8.3 Hz, 5-H), 8.17 (1 H, d, *J* = 8.1 Hz, 8-H), 8.27 (1 H, s, 3-H); ^{13}C NMR δ 27.30 (C*Me*$_3$), 53.30 (propenyl 1-C), 80.76 (*C*Me$_3$), 91.92 (1-C), 115.16 (q, *J* = 288.6 Hz, CF_3), 119.84 (3-C), 120.03 (propenyl 3-C), 121.93 (5-C), 125.63 (6-C), 126.43 (4a-C), 127.97 (7-C), 129.49 (propenyl 2-C), 131.56 (8-C), 134.31 (4-C), 135.26 (8a-C), 136.78 (2-C), 151.32 (Boc C=O), 156.35 (q, *J* = 36.3 Hz, CF_3*C*=O); ^{19}F NMR δ -68.20 (CF_3); MS *m/z* 559.0149 (M + K) ($C_{20}H_{20}F_3IN_2KO_3$ requires 559.0107); 543.0371 (M +

ACCEPTED MANUSCRIPT

Na) ($C_{20}H_{20}F_3IN_2NaO_3$ requires 543.0368); 538.0828 (M + NH_4^+) ($C_{20}H_{24}F_3IN_3O_3$ requires 538.0809). Further elution gave **42B** (750 mg, 57%) as a yellow oil: ^{1}H NMR (NOESY) δ 1.55 (9 H, s, Bu^t), 3.82 (1 H, dd, *J* = 14.5, 7.6 Hz, propenyl 1-H), 4.96 (1 H, dd, *J* = 14.5, 5.6 Hz, propenyl 1-H), 5.18 (1 H, dq, *J* = 18.0, 1.3 Hz, propenyl 3-H) 5.22 (1 H, dq, *J* = 11.0, 1.0 Hz, propenyl 3-H), 5.95 (1 H, m, propenyl 2-H), 7.01 (1 H, s, NH), 7.61-7.67 (2 H, m, 6,7-H), 7.85 (1 H, m, 5-H), 7.93 (1 H, s, 3-H), 8.30 (1 H, m, 8-H); ^{13}C NMR δ 28.27 (C*Me*$_3$), 54.00 (propenyl 1-C), 81.56 (*C*Me$_3$), 115.89 (q, *J* = 288.7 Hz, CF_3), 118.90 (3-C), 120.59 (propenyl 3-C), 120.66 (5-C), 127.92 (6-C or 7-C), 128.67 (7-C or 6-C), 130.30 (propenyl 3-C), 134.38 (8-C), 134.70 (8a-C), 135.39 (4-C), 140.14 (2-C), 152.45 (Boc C=O), 156.53 (q, *J* = 36.5 Hz, F_3C*C*=O); ^{19}F NMR δ -68.55 (s, CF_3); MS *m/z* 559.0154 (M + K) ($C_{20}H_{20}F_3IN_2KO_3$ requires 559.0107); 543.0437 (M + $Na)^+$ ($C_{20}H_{20}F_3IN_2NaO_3$ requires 543.0368); 521.0516 (M + $H)^+$ ($C_{20}H_{21}F_3IN_2O_3$ requires 521.0549); 486.9814 [(M - Bu^t) + Na)]$^+$ ($C_{16}H_{12}F_3IN_2NaO_3$ requires 486.9742).

4.3. 1,1-Dimethylethyl N-(1-(2,2,6,6-tetramethylpiperidin-1-yloxy)methyl)-3-(trifluoroacetyl-2,3-dihydrobenzo[*e*]indol-5-yl)carbamate (43)

TEMPO (35.7 mg, 0.23 mmol) and Bu_3SnH (21 mg, 73 μmol) were stirred with **42** (38.1 mg, 73 μmol) in benzene (2.0 mL) at 60°C for 135 min. Additional Bu_3SnH (21 mg, 73 μmol) was added at the 30 min, 60 min and 90 min time-points. Evaporation and chromatography (petroleum ether / EtOAc 49:1 → 19:1) gave **43** (24.1 mg, 60%) as a yellow solid: mp 160-161°C; IR ν_{max} 3364, 3129, 3266, 1700 cm^{-1}; ^{1}H NMR (NOESY) δ 0.90 (3 H, s, piperidine-Me), 1.00 (3 H, s, piperidine-Me), 1.07 (3 H, s, piperidine-Me), 1.20 (3 H, s, piperidine-Me), 1.25, (1 H, m, piperidine 4-H), 1.29 (1 H, m, piperidine 3-H or 5-H), 1.35 (1 H, m, piperidine 3-H or 5-H), 1.42 (1 H, m, piperidine 3-H or 5-H), 1.54 (1 H, m, piperidine 4-H), 1.54 (9 H, s, Bu^t), 1.66 (1 H, m, piperidine 3-H or 5-H), 3.83 (1 H, t, *J* = 8.8 Hz, NOCH), 3.99 (1 H, m, 1-H), 4.08 (1 H, dd, *J* = 8.9, 4.3 Hz, NOCH), 4.30 (1 H, t, *J* = 8.7 Hz, 2-H), 4.61 (1 H, d, *J* = 10.9 Hz, 2-H), 6.10 (1 H, s, NH), 7.47 (1 H, t, *J* = 7.8 Hz, 7-H), 7.53 (1 H, t, *J* = 7.6 Hz, 8-H), 7.81 (1 H, d, *J* = 8.3 Hz, 9-H), 7.90 (1 H, d, *J* = 8.4 Hz, 6-H), 8.79 (1 H, s, 4-H); ^{13}C NMR δ 16.97 (piperidine 4-C), 19.97 (piperidine-Me), 20.05 (piperidine-Me), 28.30 (C*Me*$_3$), 32.92 (piperidine-Me), 32.96 (piperidine-Me), 39.52 (piperidine 3-C or 5-C), 39.60 (piperidine 5-C or 3-C), 39.86 (1-C), , 52.42 (2-C), 59.88 (piperidine 2,6-C2), 77.26 ($NOCH_2$), 81.05 (*C*Me$_3$), 111.17 (4-C), 116.20 (q, *J* = 288.1 Hz, CF_3), 122.15 (6-C), 123.13 (9b-C), 124.01 (9-C), 125.36 (7-C), 125.52 (5a-C), 127.06 (8-C), 129.77 (9a-C), 134.19 (5-C), 139.56 (3a-C), 153.34 (Boc C=O), 154.28 (q, *J* = 37.5 Hz, CF_3*C*=O); ^{19}F NMR δ -72.21 (s, CF_3); MS *m/z* 572.2804 (M + $Na)^+$ ($C_{29}H_{38}F_3N_3NaO_4$ requires 572.2712), 550.2937 (M + $H)^+$ ($C_{29}H_{39}F_3N_3O_4$ requires 550.2893), 516.2095 (M – 2-methylpropene + $Na)^+$ ($C_{25}H_{30}F_3N_3NaO$ requires 516.2086).

4.4. 1,1-Dimethylethyl N-(1-((2,2,6,6-tetramethylpiperidin-1-yloxy)methyl)-2,3-dihydrobenzo[*e*]indol-5-yl)carbamate (44)

Compound **43** (70.4 mg, 0.13 mmol) in THF (4.0 mL) was stirred with aq. NaOH (5.0 M, 4.0 mL) for 105 min. The mixture was diluted with water and extracted with EtOAc. Washing (brine), drying and evaporation gave **44** (60.7 mg, quant.) as a yellow oil: IR ν_{max} 3376, 1715 cm^{-1}; ^{1}H NMR (NOESY) δ 1.06 (3 H, s, piperidine-Me), 1.14 (6 H, s, 2 × piperidine-Me), 1.16 (3 H, s, piperidine-Me), 1.16-1.42 (6 H, m, piperidine 3,4,5-H_6), 1.54 (9 H, s, Bu^t), 3.70-3.75 (2 H, m, 2-H_2), 3.80-3.90 (2 H, m, 1-H + NOCH), 4.00 (1 H, dd, *J* = 7.6, 4.2 Hz, NOCH), 6.86 (1 H, s, Boc NH), 7.20 (1 H, t, *J* = 7.5 Hz, 7-H or 8-H), 7.38 (1 H, t, *J* = 7.1 Hz, 8-H or 7-H), 7.51 (1 H, s, 4-H), 7.69 (2 H, m, 6,9-H_2); ^{13}C NMR δ 17.07 (piperidine 4-C), 20.07 (piperidine-Me), 20.26 (piperidine-Me), 28.33 (C*Me*$_3$), 32.93 (piperidine-Me), 33.34 (piperidine-Me), 39.45 (piperidine 3-C or 5-C), 39.59 (piperidine 5-C or 3-C), 41.05 (1-C), 51.29 (2-C), 59.63 (piperidine 2-C or 6-C), 59.72 (piperidine 6-C or 2-C), 76.68-77.32 ($NOCH_2$), 80.54 (*C*Me$_3$), 105.52 (4-C), 116.77 (5a,9b-C_2), 120.98 (6-C or 9-C), 121.52 (7-C or 8-C), 123.23 (9-C or 6-C), 126.29 (8-C or 7-C), 131.41 (9a-C), 133.52 (5-C), 149.40 (3a-C), 153.34 (C=O); MS *m/z* 476.2924 (M + $Na)^+$ ($C_{27}H_{39}N_3NaO_3$ requires 476.2810), 454.3107 (M + $H)^+$ ($C_{27}H_{40}N_3O_3$ requires 454.3070).

4.5. 1,1-Dimethylethyl N-(3-(5-(2-(dimethylaminoethoxy)-indole-2-carbonyl)-1-((2,2,6,6-tetramethylpiperidin-1-yloxy)-methyl)-2,3-dihydrobenzo[*e*]indol-5-yl)carbamate (45)

N,N'-Diisopropylcarbodiimide (106 mg, 0.81 mmol) was stirred with **33** (101 mg, 0.41 mmol) and HOBt (125 mg, 0.81 mmol) in dry DMF (10 mL) at 0°C under N_2 for 1 h. Compound **44** (203 mg, 0.37 mmol) in dry DMF (10 mL) was added. The mixture was allowed to warm slowly to 20°C during 16 h and was heated at 40°C for 2 h. Sat. aq. $NaHCO_3$ was added and the mixture was extracted (EtOAc). The extract was washed (water, brine). Drying, evaporation and chromatography (EtOAc → EtOAc / MeOH / Et_3N 900:100:1) gave **45** (163 mg, 64%) as a yellow oil: IR ν_{max} 3447, 1727, 1693 cm^{-1}; ^{1}H NMR (NOESY) δ 0.88 (3 H, s, piperidine-Me), 1.00 (3 H, s, piperidine-Me), 1.04 (3 H, s, piperidine-Me), 1.17 (3 H, s, piperidine-Me), 1.24-1.50 (6 H, m, piperidine 3,4,5-H_6), 1.54 (9 H, s, Bu^t), 2.45 (6 H, s, NMe_2), 2.88 (2 H, t, *J* = 5.5 Hz, C$H_2$$NMe_2$), 3.87 (1 H, t, *J* = 8.8 Hz, NOCH), 4.05 (1 H, m, 1-H), 4.13 (1 H, dd, *J* = 9.0, 4.8 Hz, NOCH), 4.17 (2 H, t, *J* = 5.6 Hz, OCH_2), 4.60 (1 H, t, *J* = 9.1 Hz, 2-H), 4.81 (1 H, dd, *J* = 10.2, 1.2 Hz, 2-H), 6.90 (1 H, s, Boc NH), 7.00 (1 H, s, indole 7-H), 7.02 (1 H, dd, *J* = 8.9, 2.4 Hz, indole 6-H), 7.13 (1 H, d, *J* = 2.1 Hz, indole 3-H), 7.36 (1 H, d, *J* = 8.9 Hz, indole 7-H), 7.42 (1 H, t, *J* = 7.5 Hz, 7-H), 7.50 (1 H, t, *J* = 7.2 Hz, 8-H), 7.84 (1 H, d, *J* = 8.2 Hz, 9-H), 7.89 (1 H, d, *J* = 8.5 Hz, 6-H), 8.90 (1 H, s, 4-H), 9.52 (1 H, s, indole NH); ^{13}C NMR δ 16.94 piperidine 4-C), 20.01 (piperidine-Me), 20.16 (piperidine-Me), 28.30 (C*Me*$_3$), 33.02 (piperidine-Me), 33.07 (piperidine-Me), 39.48 (piperidine 3-C or 5-C), 39.53 (piperidine 5-C or 3-C), 40.20 (1-C), 45.51 (NMe_2), 54.66 (2-C), 58.06 (OCH_2*C*$H_2$$NMe_2$), 59.77 (piperidine 2-C or 6-C), 59.82 (piperidine 6-C or 2-C), 66.14 (OCH_2), 77.64 ($NOCH_2$), 80.69 (*C*Me$_3$), 103.71 (indole 3-C), 105.58 (indole 4-C), 111.95 (4-C), 112.61 (indole 7-C), 116.79 (indole 6-C), 122.13 (6-C), 122.62 (9b-C), 123.91 (9-C), 124.47 (7-C), 126.60 (8-C), 128.31 (indole 3a-C), 130.07 (9a-C), 130.99 (5a-C), 131.26 (indole 7a-C), 133.71 (5-C), 141.39 (3a-C), 153.50 (indole 5-C + indole C=O), 160.23 (Boc C=O); MS *m/z* 706.3970 (M + $Na)^+$ ($C_{40}H_{53}N_5NaO_5$ requires 706.3944).

4.6. 1,1-Dimethylethyl N-(3-(5-(2-dimethylaminoethoxy)-indole-2-carbonyl)-1-hydroxymethyl-2,3-dihydrobenzo[*e*]-indol-5-yl)carbamate (47)

Water (1.0 mL), Zn dust (934 mg, 14.4 mmol) and AcOH (1.0 mL) were added sequentially to **45** (123 mg, 0.18 mmol) in THF (3.0 mL). The mixture was heated at 70°C in a sealed pressure tube for 16 h. The cooled mixture was filtered (Celite®) and the solvents were evaporated. Sat aq. $NaHCO_3$ was added and the suspension was extracted (EtOAc). The extract was washed (water, brine). Drying, evaporation and chromatography (EtOAc → EtOAc / MeOH / Et_3N 900:100:1) gave **47** as an off-white solid (69.3 mg, 71%): mp 219-220°C; IR ν_{max} 3567, 3423, 3293, 1684 cm^{-1}; 1H NMR ($(CD_3)_2SO$) (NOESY) δ 1.56 (9 H, s, Bu^t), 2.36 (6 H, s, NMe_2), 2.79 (2 H, t, *J* = 5.7 Hz, C$H_2$$NMe_2$), 3.23 (1

ACCEPTED MANUSCRIPT

H, m, C*H*OH), 3.85 (1 H, m, C*H*OH), 4.02 (1 H, m, 1-H), 4.15 (2 H, t, *J* = 5.8 Hz, OCH_2CH$_2$), 4.74 (2 H, m, 2-H), 5.11 (1 H, br, OH), 6.98 (1 H, dd, *J* = 8.9, 2.4 Hz, indole 6-H), 7.18 (1 H, d, *J* = 1.7 Hz, indole 3-H), 7.24 (1 H, d, *J* = 2.3 Hz, indole 4-H), 7.46 (1 H, d, *J* = 8.8 Hz, indole 7-H), 7.48 (1 H, t, *J* = 8.2 Hz, 7-H), 7.8 (1 H, t, *J* = 7.8 Hz, 8-H), 7.96 (1 H, d, *J* = 8.3 Hz, 9-H), 8.06 (1 H, d, *J* = 8.4 Hz, 6-H), 8.58 (1 H, s, 4-H), 9.29 (1 H, s, Boc NH), 11.66 (1 H, s, indole NH); ^{13}C NMR ((CD$_3$)$_2$SO) δ 28.16 (C*Me*$_3$), 42.81 (1-C), 45.35 (NMe$_2$), 54 (2-C), 57.65 (*C*H$_2$NMe$_2$), 62.81 (CH$_2$OH), 65.95 (OCH_2CH$_2$), 78.95 (*C*Me$_3$), 103.31 (indole 4-C), 105.35 (indole 3-C), 113.13 (indole 7-C), 113.61 (4-C), 115.78 (indole 6-C), 123.38 (9b-C), 123.65 (9-C), 123.88 (6-C), 124.01 (7-C), 125.78 (5a-C), 126.62 (indole 2-C), 129.72 (9a-C), 131.08 (indole 3a-C), 131.66 (indole 7a-C), 134.20 (5-C), 141.01 (3a-C), 152.90 (indole 5-C), 154.07 (Boc C=O), 160.26 (indole C=O); MS *m/z* 567.2608 (M + Na) ($C_{31}H_{36}N_4NaO_5$ requires 567.258340), 545.2829 (M + H) ($C_{31}H_{37}N_4O_5$ requires 545.2764).

4.7. 1,1-Dimethylethyl N-(1-acetoxymethyl-3-(5-(2-dimethylaminoethoxy)indole-2-carbonyl)-2,3-dihydrobenzo[*e*]indol-5-yl)carbamate (49)

Zn dust (354 mg, 5.4 mmol) was added to **45** (123 mg, 0.18 mmol) in THF (2.0 mL), AcOH (6.0 mL) and H_2O (2.0 mL) in a pressure tube. The tube was closed and the mixture was heated for 70°C for 2 h. Further activated Zn dust (354 mg, 5.4 mmol) was added, the tube was resealed and the mixture was heated at 70°C for 21 h, then cooled to 20°C. The mixture was diluted with THF and filtered (Celite®). Evaporation and chromatography (EtOAc → EtOAc / MeOH / Et$_3$N 950:50:1) gave two fractions, each comprising two compounds. The first fraction (14.0 mg) consisted of **46** (MS *m/z* 587.2945 (M + H) ($C_{33}H_{39}N_4O_6$ requires 587.2870)) and **47** (MS *m/z* 487.2432 (M + H) ($C_{28}H_{31}N_4O_4$ requires 487.2345)), whilst the second fraction (41.3 mg) consisted of **48** (MS *m/ z* 445.2328 (M + H) ($C_{26}H_{29}N_4O_3$ requires 445.2240)) and **49** (MS *m/z* 545.2815 (M + H) ($C_{31}H_{37}N_4O_5$ requires 545.2764)). The first fraction (**46** + **47**) was boiled under reflux with Boc$_2$O (63 mg, 0.29 mmol) in dry THF (10 mL) under N_2 for 16 h. Evaporation and chromatography (EtOAc → EtOAc / MeOH / Et$_3$N 900:100:1) gave **49** as a pale buff solid (14.5 mg, 14%): mp 170-171°C; ^{1}H NMR (CD$_3$OD) (NOESY) δ 1.61 (9 H, s, Bu^t), 2.03 (3 H, s, COMe), 2.46 (6 H, s, NMe$_2$), 2.88 (2 H, t, *J* = 5.4 Hz, CH_2NMe$_2$), 4.10 (1 H, dd, *J* = 10.8, 8.0 Hz, C*H*OAc), 4.19 (2 H, t, *J* = 5.4 Hz, OCH_2CH$_2$NMe$_2$), 4.19 (1 H, m, 1-H), 4.59 (1 H, dd, *J* = 10.8, 3.6 Hz, C*H*OAc), 4.68 (2 H, d, *J* = 4.4 Hz, 2-H), 7.04 (1 H, dd, *J* = 9.0, 2.4 Hz, indole 6-H), 7.11 (1 H, s, indole 3-H), 7.23 (1 H, d, *J* = 2.1 Hz, 4-H), 7.45 (1 H, d, *J* = 8.9 Hz, indole 7-H), 7.51 (1 H, ddd , *J* = 8.2, 6.8, 1.0 Hz, 7-H), 7.60 (1 H, ddd, *J* = 8.0, 6.8, 1.0 Hz, 8-H), 8.01 (1 H. d, *J* = 8.3 Hz, 9-H), 8.08 (1 H, d, *J* = 8.5 Hz, 6-H); ^{13}C NMR (CD$_3$OD) δ 20.73 (CO*Me*), 28.78 (C*Me*$_3$), 41.13 (1-C), 45.82 (NMe$_2$), 56.45 (2-C), 59.27 (*C*H$_2$NMe$_2$), 66.48 (*C*H$_2$OAc), 67.12 (OCH$_2$CH$_2$NMe$_2$), 81.30 (*C*Me$_3$), 104.65 (indole 4-C), 107.35 (indole 3-C), 114.01 (indole 7-C), 115.05 (4-C), 117.67 (indole 6-C), 123.93 (9b-C), 124.64 (6-C, or 9-C), 124.71 (9-C or 6-C), 125.87 (7-C), 127.90 (5a-C), 128.21 (8-C), 129.37 (indole 3a-C), 131.54 (9a-C), 132.06 (indole 2-C), 133.70 (indole 7a-C), 135.96 (indole 5-C), 142.52 (indole 3a-C), 154.91 (indole 5-C), 156.70 (Boc C=O), 162.89 (indole C=O), 172.77 (OAc C=O); MS *m/z* 587.2945 (M + H) ($C_{33}H_{39}N_4O_6$ requires 587.2870).

4.8. 1-Chloromethyl-3-(5-(2-dimethylaminoethoxy)indole-2-carbonyl)-5-(N,N-dimethylformamidino)-2,3-dihydrobenzo-[*e*]indole (50)

Et$_3$N (28 mg, 0.27 mmol) was added to **47** (30 mg, 54 μmol) in dry DMF (0.4 mL) under N_2 at 0°C, followed by MsCl (19 mg, 0.16 mmol). The mixture was stirred at 0°C for 1 h. LiCl (134 mg, 3.2 mmol) was added and the mixture was stirred at 20°C for 3 d. Water (5 mL) was added and the solvents were evaporated (< 30°C). Chromatography (EtOAc → EtOAc / MeOH / Et$_3$N 950:50:1) gave **50** (21 mg, 76%) as a yellow solid: mp 229-230°C; ^{1}H NMR (NOESY) δ 2.42 (6 H, s, CH$_2$N*Me*$_2$), 2.84 (2 H, t, *J* = 5.6 Hz, CH_2NMe$_2$), 3.04 (3 H, br, formamidine-Me), 3.10 (3 H, br, formamidine-Me), 3.45 (1 H, t, *J* = 10.9 Hz, CHCl), 3.98 (1 H, dd, *J* = 11.4, 3.1 Hz, CHCl), 4.14 (1 H, m, 1-H), 4.16 (2 H, t, *J* = 5.6 Hz, OCH$_2$), 4.63 (1 H, t, *J* = 8.6 Hz, 2-H), 4.78 (1 H, dd, *J* = 9.3, 1.5 Hz, 2-H), 7.00 (1 H, d, *J* = 8.8 Hz, indole 6-H), 7.02 (1 H, d, *J* = 2.3 Hz, indole 3-H), 7.14 (1 H, d, *J* = 2.2 Hz, indole 4-H), 7.35 (1 H, d, *J* = 8.9 Hz, indole 7-H), 7.38 (1 H, t, *J* = 7.3 Hz, 7-H), 7.50 (1 H, dd, *J* = 8.0, 1.1 Hz, 8-H), 7.67-7.69 (2 H, m, 9-H + formamidine-H), 7.70 (1 H, s, 4-H), 8.47 (1 H, d, *J* = 8.3 Hz, 6-H), 9.59 (1 H, s, indole NH); ^{13}C NMR δ 35 (br, formamidine-Me), 40 (br, formamidine-Me), 43.30 (1-C), 45.63 (CH$_2$N*Me*$_2$), 46.08 (CH$_2$Cl), 55.05 (2-C), 58.21 (OCH$_2$*C*H$_2$N), 66.30 (OCH$_2$), 103.70 (indole 4-C), 104.68 (4-C), 105.97 (indole 3-C), 112.63 (indole 7-C), 117.10 (indole 6-C), 118.26 (9b-C), 121.97 (9-C), 123.64 (7-C), 125.76 (6-C), 127.26 (8-C), 127.81 (5a-C), 128.25 (indole 3a-C), 129.75 (9a-C), 130.81 (indole 7-C), 131.36 (indole 7a-C), 142.03 (3a-C), 150.91 (5-C), 153.03 (formamidine CH), 153.66 (indole 5-C), 160.49 (indole C=O); MS *m/z* 520.2375 (M + H)$^+$ ($C_{29}H_{32}{}^{37}ClN_5O_2$ requires 520.2293), 518.2341 (M + H)$^+$ ($C_{29}H_{32}{}^{35}ClN_5O_2$ requires 518.2323).

4.9. 1,1-Dimethylethyl N-(1-chloromethyl-3-(5-(2-dimethylaminoethoxy)indole-2-carbonyl)-2,3-dihydrobenzo[*e*]indol-5-yl)carbamate (51)

MsCl (7.0 mg, 61 μmol) was stirred with **47** (24 mg, 44 μmol) in dry pyridine (0.7 mL) under N_2 at 0°C for 1 h. LiCl (92 mg, 2.2 mmol) was added and the mixture was stirred at 20°C for 7 d. EtOAc was added, followed by sat. aq. NaHCO$_3$. The mixture was extracted with EtOAc. The extract was washed (water, brine). Drying, evaporation and chromatography (EtOAc → EtOAc / MeOH / Et$_3$N 980:20:1) gave **51** (16.3 mg, 66%) as a yellow solid: mp >250°C; ^{1}H NMR (COSY / NOESY) δ 1.55 (9 H, s, Bu^t), 2.37 (6 H, s, NMe$_2$), 3.47 (1 H, t, *J* = 10.7 Hz, ClCH), 3.96 (1 H, dd, *J* = 11.2, 2.9 Hz, ClCH), 4.13 (2 H, t, *J* = 5.8 Hz, OCH$_2$), 4.14 (1 H, m, 1-H), 4.66 (1 H, t, *J* = 8.6 Hz, 2-H), 4.82 (1 H, dd, *J* = 9.0, 1.7 Hz, 2-H), 6.91 (1 H, s, Boc NH), 7.01 (1 H, d, *J* = 1.4 Hz, indole 3-H), 7.04 (1 H, dd, *J* = 8.9, 2.4 Hz, indole 6-H), 7.14 (1 H, d, *J* = 2.2 Hz, indole 4-H), 7.35 (1 H, d, *J* = 8.9 Hz, indole 7-H), 7.45 (1 H, ddd, *J* = 8.3, 7.0, 1.1 Hz, 7-H), 7.55 (1 H, ddd, *J* = 7.9, 6.8, 0.9 Hz, 8-H), 7.77 (1 H, d, *J* = 8.2 Hz, 9-H), 7.90 (1 H, d, *J* = 8.4 Hz, 6-H), 8.92 (1 H, s, 4-H), 9.42 (1 H, s, indole NH); ^{13}C NMR δ 28.28 (C*Me*$_3$), 43.40 (1-C), 45.84 (NMe$_2$ + CH$_2$Cl), 54.94 (2-C), 58.37 (*C*H$_2$NMe$_2$), 66.59 (OCH$_2$), 80.92 (*C*Me$_3$), 103.64 (indole 4-C), 105.90 (indole 3-C), 111.46 (4-C), 112.59 (indole 7-C), 117.32 (indole 6-C), 120.77 (9b-C), 122.37 (6-C), 123.04 (9-C), 124.71 (5a,7-C$_2$), 127.23 (8-C), 128.24 (indole 3a-C), 129.68 (9a-C), 130.49 (indole 2-C), 131.32 (indole 7a-C), 134.75 (5-C), 141.66 (3a-C), 153.28 (Boc C=O), 153.86 (indole 5-C), 160.41 (indole C=O); MS *m/z* 565.2382 (M + H)$^+$ ($C_{31}H_{36}{}^{37}ClN_4O_4$ requires 565.2396), 563.2443 (M + H)$^+$ ($C_{31}H_{36}{}^{37}ClN_4O_4$ requires 563.2425), 427.2157 ((M – (Boc + Cl) + H)$^+$ ($C_{26}H_{27}N_4O_2$ requires 427.2134).

4.10. 1-Chloromethyl-3-(5-(2-dimethylaminoethoxy)indole-2-carbonyl)-2,3-dihydrobenzo[*e*]indole-5-amine dihydrochloride (52)

Compound **51** (18.6 mg, 33 μmol) was stirred with HCl in 1,4-dioxane (4.0 M, 2.0 mL) for 2 h. The solvent and excess HCl were evaporated at 20°C. The residue was triturated with MeCN

ACCEPTED MANUSCRIPT

and Et_2O. Drying gave **52** (15.3 mg, quant.) as a pale buff solid: mp >250°C. A sample in EtOAc was washed (sat. aq. $NaHCO_3$, brine). Drying and evaporation gave the free base as a yellow solid: mp > 250°C; ^{1}H NMR ($(CD_3)_2SO$) (NOESY) δ 2.90 (6 H, s, NMe_2), 3.55 (2 H , t, J = 4.2 Hz, CH_2NMe_2), 3.77 (1 H, dd, J = 11.0, 8.2 Hz, ClCH), 3.99 (1 H, dd, J = 11.1, 3.1 Hz, ClCH), 4.15 (1 H, m, 1-H), 4.33 (2 H, t, J = 4.9 Hz, OCH_2), 4.52 (1 H, dd, J = 10.8, 1.5 Hz, 2-H), 4.75 (1 H, t, J = 10.7 Hz, 2-H), 7.00 (1 H, d, J = 8.8 Hz, indole 6-H), 7.10 (1 H, d, J = 1.7 Hz, indole 3-H), 7.26 (1 H, d, J = 1.9 Hz, indole 4-H), 7.32 (1 H, t, J = 7.9 Hz, 7-H), 7.45 (1 H, d, J = 8.9 Hz, indole 7-H), 7.48 (1 H, t, J = 7.4 Hz, 8-H), 7.75-7.80 (2 H, m, 4,9-H_2), 8.08 (1 H, d, J = 8.5 Hz, 6-H), 11.66 (1 H, s, indole NH); ^{13}C NMR ($(CD_3)_2SO$) δ 41.40 (1-C), 42.89 (NMe_2), 47.51 ($ClCH_2$), 55.04 (2-C), 55.67 (CH_2NMe_2), 62.70 (OCH_2), 99.50 (4-C), 104.01 (indole 4-C), 105.00 (indole 3-C), 113.30 (indole 7-C), 115.54 (indole 6-C), 117.55 (9b-C), 120.67 (4a-C), 122.46 (7-C), 123.05 (9-C), 123.37 (6-C), 126.91 (8-C), 127.11 (indole 2-C), 130.01 (9a-C), 131.52 (indole 3a-C), 131.95 (indole 7a-C), 142.78 (5-C), 151.97 (indole 5-C), 157.90 (3a-C), 158.17 (C=O); MS *m/z* 465.1910 (M + H) ($C_{26}H_{28}{}^{37}Cl$-N_4O_2 requires 465.1871), 463.1874 (M + H) ($C_{26}H_{28}{}^{35}ClN_4O_2$ requires 463.1901).

4.21. DNA-melting studies

The buffer was prepared by dissolving NaH_2PO_4 (1.17 g, 9.75 mM), ethylenediaminetetraacetic acid (EDTA) disodium salt dihydrate (372 mg, 1.0 mM) and NaCl (438 mg, 7.5 mM) in 18.2 MΩ MilliQ water (800 mL). The pH was adjusted to pH 7.0 using aq. NaOH (0.1 M) or aq. HCl (0.1 M) as required and Milli-Q water was added to 1.00 L. The calf thymus DNA stock solution was prepared by dissolving DNA in 9.75 mM phosphate buffer to a concentration of 1.0 mg mL^{-1}. The stock was diluted with buffer until an absorbance of 0.6 at 256 nm was obtained. This gave a concentration of *ca.* 32 μg mL^{-1}, using the Beer-Lambert Law with ε = 6600 M^{-1} cm^{-1}. Compound **52** (1.04 mg, 1.94 μmol) was dissolved in DMSO (1.00 mL) to make a stock solution. Different volumes of this solution were added to the DNA solution (3.00 mL) in the appropriate quartz cuvettes and the mixtures were incubated at room temperature for 1.0 h to allow alkylation of the DNA. Five parallel cuvettes were prepared: A: blank containing buffer and DMSO (40 μL); B,C: controls containing DNA, buffer and DMSO (40 μL); D,E: tests containing **52/53**, DNA, buffer and DMSO. All cuvettes contained the same total volume of DMSO (40 μL). The absorbance at 256 nm was monitored for each sample over the range of 40°C → 95°C with the temperature rising at a rate of 0.5 deg. C min^{-1}, using a complete PC-controlled spectrometer system (Perkin Elmer Lambda BioDNA Melt system including PTP-6 temperature-controlled programmer for simultaneous curves, UVTemplab software and in-cuvette temperature sensor). Both direct and first-derivative graphs were plotted and the T_m was taken as temperature at the maximum in the first-derivative plot.

4.22. Cytotoxicity assays

The human prostate cancer cell line LNCaP (Sigma-Aldrich) was cultured in Dulbecco's Modified Eagle's Medium (DMEM) with high glucose (4.5 g L^{-1}), foetal bovine serum (FBS, 20% (v/v)), penicillin (100 U mL^{-1}) and streptomycin sulfate (100 μg mL^{-1}) at 37°C in a humidified atmosphere containing 5% CO_2 (v/v). The MTS assay was conducted as described previously.[23]

Acknowledgements

We thank Dr. Timothy J. Woodman (University of Bath) for help with the NMR spectra and Prostate Cancer UK (Grant 110816) for generous funding. We also thank Malham Al-Muhammad, Tsz-Huen Pang and Mujeeb Zia (University of Bath) for help with the DNA-melting studies.

References

1. Ichimura, M.; Ogawa, T.; Takahashi, K.; Kobayashi, E.; Kawamoto, I.; Yasuzawa, T.; Takahashi, I.; Nakano, H. *J. Antibiot*. **1990**, *43*, 1037.
2. Li, L. H.; Swenson, D. H.; Schpok, S. L. F.; Kuentzel, S. L.; Dayton, B. D.; Krueger, W. C. *Cancer Res.* **1982,** *42*, 999.
3. Hopton, S. R.; Thompson, A. S. *Biochemistry* **2011**, *50*, 4143.
4. Tichenor, M. S.; Boger, D. L. *Nat. Prod. Rep*. **2008**, *25*, 220.
5. Igarashi, Y.; Futamata, K.; Fujita, T.; Sekine, A.; Senda, H.; Naoki, H.; Furumai, T. *J. Antibiot.* **2003**, *56*, 107.
6. McGovren, J. P.; Clarke, G. L.; Pratt, E. A.; Dekoning, T. F.. *J. Antibiot.* **1984**, *37*, 63.
7. Mohamadi, F.; Spees, M. M.; Staten, G. S.; Marder, P.; Kipka, J. K.; Johnson, D. A.; Boger, D. L.; Zarrinmayeh, H. *J. Med. Chem.* **1994**, *37*, 232.
8. Boger, D. L.; Ishizaki, T.; Sakya, S. M.; Munk, S. A.; Kitos, P. A.; Jin, Q.; Besterman, J. M. *Bioorg. Med. Chem. Lett*. **1991**, *1*, 115.
9. Atwell, G. J.; Tercel, M.; Boyd, M.; Wilson, W. R.; Denny, W. A. *J. Org. Chem.* **1998**, *63*, 9414.
10. Tietze, L. F.; Major, F.; Schuberth, I. *Angew. Chem. Int. Ed.* **2006**, *45*, 6574.
11. Tietze, L. F.; Major, F.; Schuberth, I.; Spiegl, D. A.; Krewer, B.; Maksimenka, K.; Bringmann, G.; Magull, J. *Chem. Eur. J.* **2007**, *13*, 4396.
12. Wolfe, A. L.; Duncan, K. K.; Parelkar, N. K.; Brown, D.; Vielhauer, G. A.; Boger, D. L. *J. Med. Chem.* **2013**, *56*, 4104-4115.
13. Stevenson, R. J.; Denny, W. A.; Tercel, M.; Pruijn, F. B.; Ashoorzadeh, A. *J. Med. Chem.* **2012**, *55*, 2780.
14. Sheldrake, H. M.; Travica, S.; Johansson, I.; Loadman, P. M.; Sutherland, M.; Elsalem, L.; Illingworth, N.; Cresswell, A. J.; Reuillon, T.; Shnyder, S. D.; Mkrtchian, S.; Searcey, M.; Ingelman-Sundberg, M.; Patterson, L. H.; Pors, K. *J. Med. Chem.* **2013**, *56*, 6273.
15. Hodgson, H. H.; Smith, E. W. *J. Chem. Soc.* **1935**, 671.
16. Twum, E. A.; Woodman, T. J.; Wang, W.; Threadgill, M. D. *Org. Biomol. Chem.* **2013**, *11*, 6208.
17. Tietze, L. F.; Schuster, H. J.; Hampel, S. M.; Rühl, S.; Pfoh, R. *Chem. Eur. J.* **2008**, *14*, 895.
18. Furuyama, T.; Yonehara, M.; Arimoto, S.; Kobayashi, M.; Matsumoto, Y.; Uchiyama, M. *Chem. Eur. J.* **2008**, *14*, 10348.
19. Boger, D. L.; McKie, J. A. *J. Org. Chem.* **1995**, *60*, 1271.
20. Threadgill, M. D. PhD thesis, University of Cambridge, **1981**.
21. Boger, D. L.; Johnson, D. S. *Angew. Chem. Int. Ed. Engl.* **1996**, *35*, 1438.
22. Reiter, R. J.; Tan, D. X.; Manchester, L. C.; Korkmaz, A.; Fuentes-Broto, L.; Hardman, W. E.; Rosales-Corral, S. A.; Qi, W. *Cancer Invest.* **2013**, *31*, 365.
23. Paine, H. A.; Nathubhai, A.; Woon, E. C. Y.; Sunderland, P. T.; Wood, P. J.; Mahon, M. F.; Lloyd, M. D.; Thompson, A. S.; Haikarainen, T.; Narwal, M.; Lehtiö, L.; Threadgill, M. D. *J. Med. Chem.*, submitted.

ACCEPTED MANUSCRIPT