

MedChemComm

Accepted Manuscript

This article can be cited before page numbers have been issued, to do this please use: V. Vinader, M. Sadiq, M. Sutherland, M. Huang, P. Loadman, L. Elsalem, S. Shnyder, H. Cui, K. Afarinkia, M. Searcey, L. H. Patterson and K. Pors, *Med. Chem. Commun.*, 2014, DOI: 10.1039/C4MD00411F.



This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the [Information for Authors](#).

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard [Terms & Conditions](#) and the [Ethical guidelines](#) still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

COMMUNICATION

Probing cytochrome P450-mediated activation with a truncated azinomycin analogue

Cite this: DOI: 10.1039/x0xx00000x

Victoria Vinader,^a Maria Sadiq,^a Mark Sutherland,^a Mengying Huang,^b Paul M. Loadman,^a Lina Elsalem,^a Steven D. Shnyder,^a Hongjuan Cui,^b Kamyar Afarinkia,^a Mark Searcey,^{*c} Laurence H. Patterson,^a and Klaus Pors^{*a}Received 00th January 2012,
Accepted 00th January 2012

DOI: 10.1039/x0xx00000x

www.rsc.org/

A deactivated alkene precursor ($IC_{50} = 81 \mu M$) to the azinomycin epoxide natural product can be bioactivated by several cytochromes P450 (CYP) to generate antiproliferative metabolites with increased potency ($IC_{50} = 1-30 \mu M$) in CHOwt cells. CYP1A1 and 3A4 were shown to generate exclusively the unnatural and the natural-configured azinomycin epoxide diastereoisomer respectively, while CYP1B1 produced both epoxides in a 3:1 mixture. The antiproliferative activity is linked to DNA damage as demonstrated using the comet assay.

The azinomycins A (1), B (2) and the azinomycin epoxide (3) are natural products isolated from *Streptomyces griseofuscus* (strain S42227) that have been extensively studied due to their potent antitumour activity, their novel structures and their mode of action.¹⁻⁴ The complete azinomycin A and B structures 1 and 2 bind to the major groove of DNA and form interstrand crosslinks through alkylation by the epoxide and aziridine moieties.^{5, 6} The truncated epoxide structure 3 has been shown to alkylate at the N7 of guanine and promote a depurination reaction that leads to single strand breaks.⁷ The naphthalene moiety is important in 3 due to an ability to intercalate into the DNA duplex following alkylation.⁸

^aInstitute of Cancer Therapeutics, University of Bradford, West Yorkshire BD7 1DP, U.K.; *k.pors1@bradford.ac.uk

^bState Key Laboratory of Silkworm Genome Biology, Southwest University, Chongqing, 400715, China.

^cSchool of Pharmacy, University of East Anglia, Norwich Research Park, Norwich NR4 7TJ, UK; *M.Searcey@uea.ac.uk

† Electronic Supplementary Information (ESI) available: Experimental details for representative compounds synthesised and description of biological assays. This material is available free of charge via the Internet at <http://pubs.acs.org>. See DOI: 10.1039/b000000x/

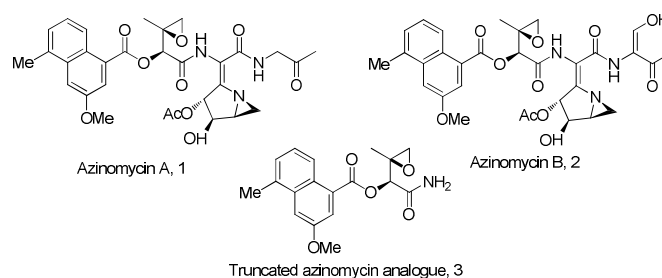


Figure 1. Structure of azinomycins A (1) and B (2) and the azinomycin epoxide (3).

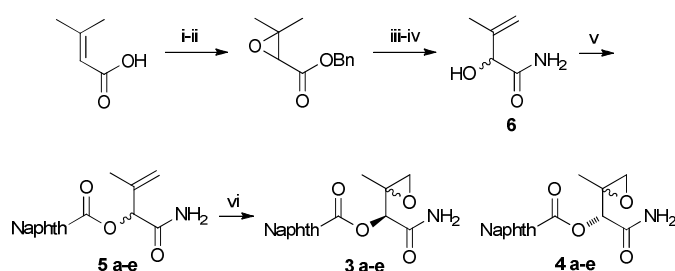
Although the azinomycins possess potent cytotoxicity *in vitro*, they have variable *in vivo* activity, most likely as a consequence of the metabolic instability of the epoxide and aziridine moieties. In studies to define the biosynthetic pathways to the azinomycins, Watanabe and co-workers have demonstrated that valine is the precursor to the epoxide.⁹ This amino acid undergoes a series of transformations to generate 3-methyl-2-oxobutenoic acid, which is predicted to be reduced to the alcohol and incorporated into the azinomycin structure. Enzymatic epoxidation of the alkene amide also occurs in this sequence of events. This incorporation of an alkene is similar to the synthetic sequence described previously and adapted by us to generate all four diastereoisomers of 3.⁷ We hypothesised that the alkene derivative has utility as a bioprecursor that could increase the therapeutic utility of the azinomycins in malignant tissues that (over)express enzymes capable of epoxidising xenobiotics notably the cytochrome P450 (CYP) monooxygenases. Several drug metabolising CYPs have been shown to be expressed at high frequency in cancer tissues¹⁰ and we have focused our attention on several CYP1 and 2 family members as tumour selective prodrug activating enzymes. We developed AQ4N, a Phase II clinical trial candidate, to undergo bioreductive activation by CYP1A1 and 3A4

selectively in hypoxic tumours.^{11, 12} More recently we demonstrated that bioprecursors of the duocarmycins can be activated selectively to ultrapotent anti-proliferative metabolites by tumours expressing CYP1A1¹³ and 2W1¹⁴ and we are proposing this approach to address the failure of the duocarmycins in the clinic due to unacceptable normal tissue toxicity. Similarly, despite the promise of the azinomycins as cancer chemotherapeutics, they have thus far failed to progress clinically.

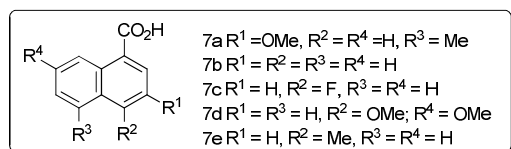
Towards the aim of developing a class of chemotherapeutics based on the azinomycins we describe the synthesis and biological evaluation of a library of azinomycin epoxide bioprecursors with potential for CYP bioactivation

We previously⁷ adopted the stereoselective synthesis of the epoxide **4** using Shipman's methodology (i.e. a Sharpless dihydroxylation) in order to set the stereochemistry of the subsequent alkene (in this case, compound **6**). In this study, we reasoned that as a proof-of-concept, synthesis of the racemic alkene is appropriate, as it is not predictable as to whether the stereochemistry of the naphthoate ester amide fragment has an influence on enzymic oxidation of the alkene by selected CYPs. The benzyl ester of commercially available 3,3-dimethylacrylic acid was epoxidised directly using *m*-CPBA to give an excellent yield (93%) of the racemic compound, a reaction which can be carried out on a multigram scale. Acid catalysed ring opening to give the alkene was followed by direct treatment with ammonia to give the amide **6**.

It has previously been shown that changes in substitution on the naphthalene ring maintain antitumour activity.¹⁵ In this study we wanted to establish whether the substitution pattern influenced CYP mediated epoxidation of the alkene. To facilitate this structure activity relationship (SAR) for CYP-catalysed epoxidation, we introduced a number of different analogues of the naphthalene moiety alongside the natural product structure. The aromatic substituents were introduced via DCC mediated coupling with fragment **6**. Subsequent chemical oxidation of the alkenes gave a mixture of diastereoisomers that were separable into the racemic mixtures. The relative stereochemistry was assigned through reference to the H2 proton (-proton) in the 1H NMR which was shifted upfield (5.32 from 5.21 ppm) in the configuration opposite to that found in the natural azinomycin analogue.



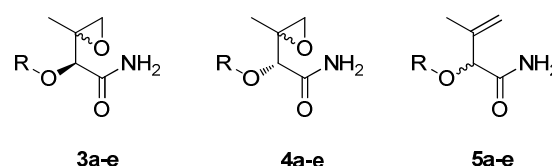
i) BnCl, Aq NaOH, CH₂Cl₂, reflux, 15 h; ii) *m*CPBA, CH₂Cl₂, rt, 2-3 days; iii) TsOH, Toluene, reflux, 6 h; iv) Aq ammonia, rt, 24 h; v) 1-NaphthCO₂H (**7a-e**), DCC, CH₂Cl₂, rt, 15 h; vi) *m*CPBA, CH₂Cl₂, r.t., 15 h.



Scheme 1. Synthesis of compounds **3-6**

To determine the cytotoxicity of the truncated azinomycin precursors, chemosensitivity studies were carried out using a Chinese Hamster Ovary (CHO) cell line +/- transfected with CYP1A1. The anti-proliferative activity of the various naphthalene derivatives against this isogenic pair of CHO cell lines was assessed using the MTT cell viability assay (Table 1). The substitution pattern of the naphthalene moiety in the epoxyamide compounds **3** and **4** has little effect on the biological activity, with IC₅₀ values between 2-10 μM for all compounds tested. However, incubation of the alkenes **5a-e** with the cell lines demonstrated that only the natural product naphthalene structure (**5a**) was bioactivated in the CYP1A1 expressing cell line, indicating that the substitution pattern on the naphthalene moiety affects the binding mode with CYP1A1.

Table 1. Antiproliferative activity of azinomycin analogues in wild-type and CYP1A1-transfected CHO cell lines



Compound ID	Structure of R group	IC ₅₀ CHO (μM)	IC ₅₀ CHO(1A1) (μM)
3a		2.8	3.2
4a		5.7	6.0
5a		85	6.3
3b		10	5
4b		10	9
5b		>100	>100
3c		7	3
4c		7	2
5c		>100	>100
3d		6	3
4d		3	9
5d		>100	>100
3e		6	3
4e		5	9
5e		>100	>100

Table 2. Growth inhibition of bioprecursor **5a** and CYP bacosomes generated metabolite fractions of **5a** against CHO cell line

5a	5a + CYP1A1		5a + CYP1A2		5a + CYP1B1		5a + CYP2D6		5a + CYP3A4	
	IC ₅₀ *	IC ₅₀	PF**	IC ₅₀	PF**	IC ₅₀	PF**	IC ₅₀	PF**	IC ₅₀
81.61 ± 4.23	14.15 ± 3.07	5.8	20.30 ±	4.0	11.75 ±	7.0	28.31 ±	2.9	3.34 ±	24.4

*IC₅₀ = μM; **Potentiation Factor (IC₅₀ of **5a** in CHO cells/IC₅₀ of **5a** + CYP isoform metabolites)

Since the naphthoate substitution pattern of compound **5a** is that of the natural azinomycin we explored whether other CYPs important in drug metabolism could influence its cytotoxicity. Bioactivation by CYP1A1 was compared to CYP1B1, known to be overexpressed in many tumour types¹⁶ and three hepatic CYPs (1A2, 2D6 and 3A4). NADPH dependent CYP/CYP reductase expressing bacosomes were incubated for 1 h with **5a**. Subsequently the incubation extract was added to CHO wild type cells which were subjected to the MTT antiproliferative assay. This revealed that all CYPs could significantly (3-24 fold) potentiate **5a** activity. Potentiation by CYP3A4 (24.4-fold) was much greater than CYP1B1 (7-fold) or CYP1A1 (5.8-fold), Table 2. The bioactivation of the CYP bacosomes brings the potency of the inactive azinomycin precursor to the low micromolar region, which is similar to what we have observed for the four diastereoisomers of compound **3** in the NCI 60 cell line panel.⁷ The CYP-mediated potentiation observed here suggests the possible formation of an azinomycin epoxide metabolite capable of causing DNA damage. We therefore explored the nature of this activation further.

To investigate if the antiproliferative activity could be correlated with DNA damage, CHO cells were treated with bioprecursor **5a** or the metabolite fraction generated from **5a** incubation with the CYP3A4 bacosomes. After 1 h exposure to cells, DNA damage was assessed using the comet assay. EO9, camptothecin and melphalan were used as positive controls for DNA single strand breaks (SSBs), DNA double strand breaks (DSBs) and DNA interstrand cross-links (ICLs), respectively. The results showed that CYP3A4-generated metabolites were capable of producing DNA damage primarily through SSBs (Figure 2) with no evidence of DSBs as measured by increase in mean tail moment (MTM) (Figure S3).

The results also indicated that a small amount of ICLs were generated. This is intriguing because it would require two sites of metabolic activation to generate the requisite proximate crosslinking alkylation sites. Furthermore, **5a** alone produced no DNA damage confirming that CYP-activation is required for the generation of DNA damage.

CYP2W1 has been reported to be expressed in both primary¹⁷ and metastatic¹⁸ colon cancer tissue but not healthy tissues and we are engaged with discovering new pharmacophores that are bioactivated by CYP2W1. In this context, we explored the potential for **5a** to be bioactivated by CYP2W1 using our isogenic SW480 +/- CYP2W1 cell line pair, but we did not observe any significant antiproliferative activity in the CYP2W1-transfected cells (Figure S4). However, **5a** displayed a dose-dependent growth inhibition against two human gastric cancer cell lines, SGC-7901 and MKN-45, which expressed CYP1A1 and 3A4 at both gene and protein level (Figure 3).

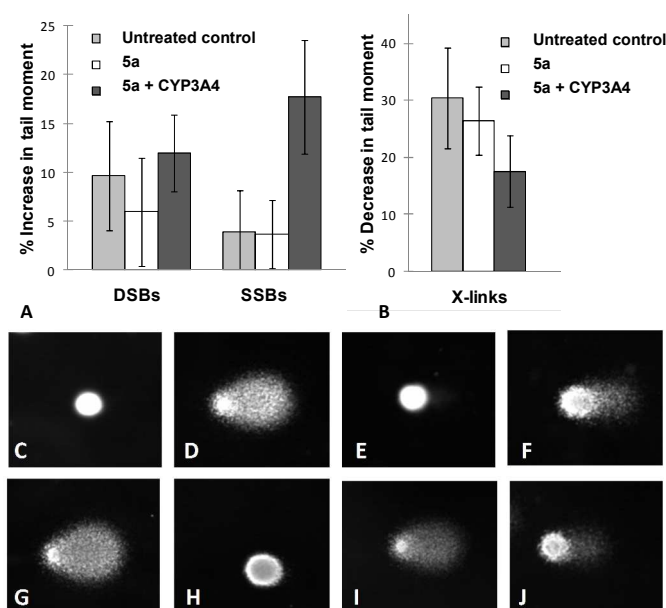


Figure 2. DNA damage caused by **5a** metabolite generated from incubation with CYP3A4. DSBs and SSBs were measured by increase in MTM (A) and ICLs by a decrease in MTM (B). Typical comet images show SSBs with no drug (C), 20 μM EO9 (D), 50 μM **5a** (E) and 50 μM CYP3A4-metabolised **5a** (F). DNA ICLs with no drug (G), 20 μM melphalan (H), 50 μM **5a** (I) and 50 μM CYP3A4-metabolised **5a** (J). The results are the mean ± SD of at least three independent assays.

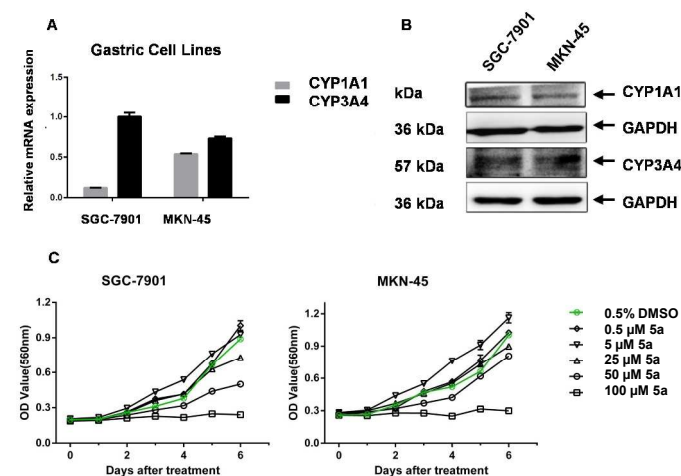


Figure 3. CYP1A1 and 3A4 expression and antiproliferative activity in gastric cell lines SGC-7901 and MKN-45. CYP1A1 and 3A4 expression was measured by qRT-PCR (A) and Western blot (B). The growth inhibition was measured using the MTT assay (C).

To identify the active metabolites likely to be responsible for the DNA damage and CYP-associated antiproliferative activity, metabolite fractions from CYP1A1, 1B1 and 3A4 were analysed by LC/MS/MS. All three CYPs oxidised the alkene to the epoxide, the anticipated alkylating species of these truncated azinomycins. However the epoxide stereochemistry generated by different CYP isoforms was not the same. CYP1A1 and 3A4 were shown to generate exclusively the ‘unnatural’ diastereoisomer epoxide **3a** and the naturally-configured epoxide **4a** respectively, while CYP1B1 produced both epoxides in a 3:1 mixture as identified by co-elution with the respective authentic truncated azinomycins (Figure 3). The IC₅₀ data and our CYP-metabolism data from this study are consistent with the high potency of the naturally-configured diastereoisomer epoxides previously observed.⁷

Conclusions

We have demonstrated that the putative bioprecursor **5a** to the natural azinomycin product **3** can be activated in cells expressing a number of CYPs known to be responsible for drug metabolism. Certain isoforms, notably CYP1A1, 1B1 and 3A4 are identified as highly expressed in certain tumours¹⁰ indicating the value of truncated azinomycin analogues as potential bioprecursors with chemotherapeutic efficacy. We have demonstrated proof of principle with bioprecursors of the duocarmycin natural products targeting CYP1A1^{19, 20} and 2W1^{14, 21} enzymes. Uniquely, we have demonstrated that CYP selective bioactivation *in vivo* can be obtained with no overt toxicity to normal tissues including the liver.^{13, 14} Further development of such a bioprecursor approach could provide a strategy to exploit the azinomycins for therapeutic use. While CYP1A1 and 1B1 provide selective cancer drug targets, the hepatic CYP3A4 enzyme could be exploited for liver specific treatment of hepatitis B, hepatitis C and hepatocellular carcinoma²² or with the reemergence of the oncolytic virus, a ‘suicide’ GDEPT approach also exists as a therapeutic strategy.^{23, 24}

Acknowledgements

The authors wish to gratefully acknowledge financial support from Yorkshire Cancer Research for Medicines Discovery Programme grant number B209 and EPSRC Science Bridges China for grant number EP/G042365/1. We thank Miss Gemma Marston and Miss Komal Nadeem for their contribution to generation of experimental data and the EPSRC NMSSC for HRMS data

References

- G. T. Kelly, C. Liu, R. Smith, 3rd, R. S. Coleman and C. M. Watanabe, *Chem Biol*, 2006, **13**, 485-492.
- S. Ishizeki, M. Ohtsuka, K. Irinoda, K. Kukita, K. Nagaoka and T. Nakashima, *J Antibiot (Tokyo)*, 1987, **40**, 60-65.
- K. Nagaoka, M. Matsumoto, J. Oono, K. Yokoi, S. Ishizeki and T. Nakashima, *J Antibiot (Tokyo)*, 1986, **39**, 1527-1532.
- K. Yokoi, K. Nagaoka and T. Nakashima, *Chem Pharm Bull (Tokyo)*, 1986, **34**, 4554-4561.
- R. S. Coleman, R. J. Perez, C. H. Burk and A. Navarro, *J Am Chem Soc*, 2002, **124**, 13008-13017.
- R. S. Coleman, R. L. Woodward, A. M. Hayes, E. A. Crane, A. Artese, F. Ortuso and S. Alcaro, *Org Lett*, 2007, **9**, 1891-1894.
- M. H. David-Cordonnier, M. Casely-Hayford, M. Kouach, G. Briand, L. H. Patterson, C. Bailly and M. Searcey, *Chembiochem*, 2006, **7**, 1658-1661.
- M. A. Casely-Hayford, K. Pors, L. H. Patterson, C. Gerner, S. Neidle and M. Searcey, *Bioorg Med Chem Lett*, 2005, **15**, 653-656.
- V. Sharma, G. T. Kelly and C. M. Watanabe, *Org Lett*, 2008, **10**, 4815-4818.
- C. Rodriguez-Antona and M. Ingelman-Sundberg, *Oncogene*, 2006, **25**, 1679-1691.
- A. Yakkundi, V. McErlane, M. Murray, H. O. McCarthy, C. Ward, C. M. Hughes, L. H. Patterson, D. G. Hirst, S. R. McKeown and T. Robson, *Cancer gene therapy*, 2006, **13**, 598-605.
- L. H. Patterson, S. R. McKeown, T. Robson, R. Gallagher, S. M. Raleigh and S. Orr, *Anticancer Drug Des*, 1999, **14**, 473-486.
- M. Sutherland, J. H. Gill, P. M. Loadman, J. P. Laye, H. M. Sheldrake, N. A. Illingworth, M. N. Alandas, P. A. Cooper, M. Searcey, K. Pors, S. D. Shnyder and L. H. Patterson, *Molecular cancer therapeutics*, 2011.

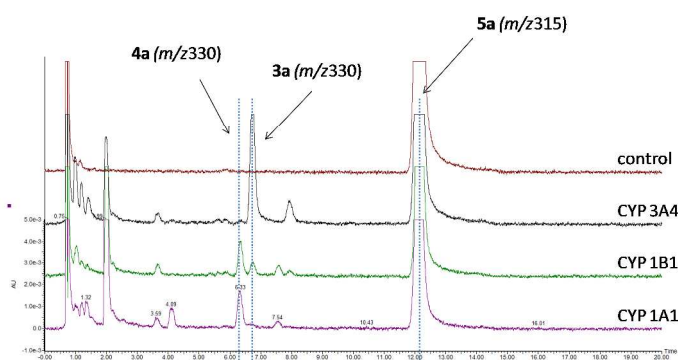
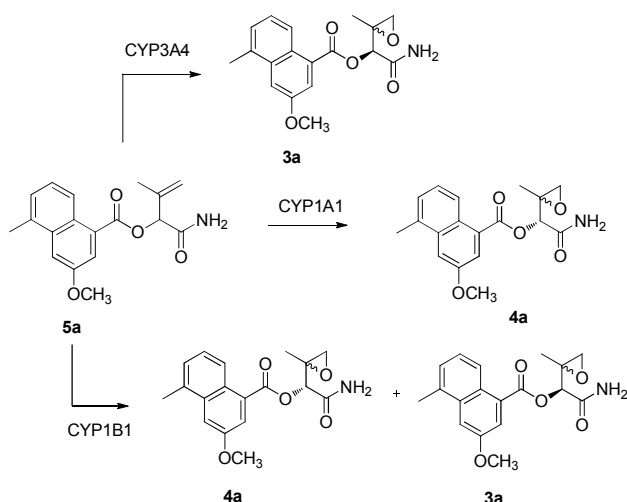


Figure 3. Metabolism profile of **5a** incubated with CYP1A1, 1B1 or 3A4

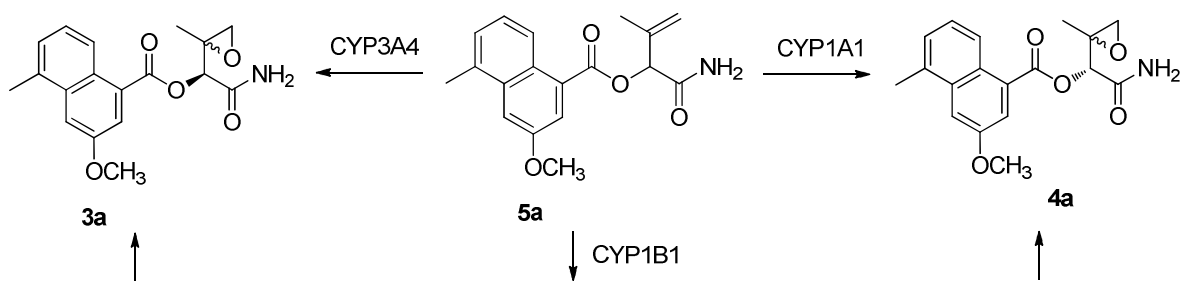
The identification of a *m/z* 330 metabolite, consistent with epoxide formation (Scheme 2), and DNA SSBs following CYP-oxidation indicates the potential for bioactivation of the precursor azinomycins prepared in this study.



Scheme 2. Generation of metabolites by CYP1A1, 1B1 and 3A4.

14. S. Travica, K. Pors, P. M. Loadman, S. D. Shnyder, I. Johansson, M. N. Alandas, H. M. Sheldrake, S. Mkrtchian, L. H. Patterson and M. Ingelman-Sundberg, *Clin Cancer Res*, 2013.
15. T. J. Hodgkinson, L. R. Kelland, M. Shipman and F. Suzenet, *Bioorg Med Chem Lett*, 2000, **10**, 239-241.
16. M. C. McFadyen and G. I. Murray, *Future oncology*, 2005, **1**, 259-263.
17. K. Stenstedt, M. Hallstrom, I. Johansson, M. Ingelman-Sundberg, P. Ragnhammar and D. Edler, *Anticancer Res*, 2012, **32**, 3869-3874.
18. K. Stenstedt, M. Hallstrom, F. Ledel, P. Ragnhammar, M. Ingelman-Sundberg, I. Johansson and D. Edler, *Acta oncologica*, 2014.
19. K. Pors, P. M. Loadman, S. D. Shnyder, M. Sutherland, H. M. Sheldrake, M. Guino, K. Kiakos, J. A. Hartley, M. Searcey and L. H. Patterson, *Chem Commun (Camb)*, 2011, **47**, 12062-12064.
20. M. Sutherland, J. H. Gill, P. M. Loadman, J. P. Laye, H. M. Sheldrake, N. A. Illingworth, M. N. Alandas, P. A. Cooper, M. Searcey, K. Pors, S. D. Shnyder and L. H. Patterson, *Mol Cancer Ther*, 2013, **12**, 27-37.
21. H. M. Sheldrake, S. Travica, I. Johansson, P. M. Loadman, M. Sutherland, L. Elsalem, N. Illingworth, A. J. Cresswell, T. Reuillon, S. D. Shnyder, S. Mkrtchian, M. Searcey, M. Ingelman-Sundberg, L. H. Patterson and K. Pors, *J Med Chem*, 2013, **56**, 6273-6277.
22. M. D. Erion, K. R. Reddy, S. H. Boyer, M. C. Matelich, J. Gomez-Galeno, R. H. Lemus, B. G. Ugarkar, T. J. Colby, J. Schanzer and P. D. Van Poelje, *J Am Chem Soc*, 2004, **126**, 5154-5163.
23. H. Lu, C. S. Chen and D. J. Waxman, *Cancer gene therapy*, 2009, **16**, 393-404.
24. H. O. McCarthy, A. Yakkundi, V. McErlane, C. M. Hughes, G. Keilty, M. Murray, L. H. Patterson, D. G. Hirst, S. R. McKeown and T. Robson, *Cancer gene therapy*, 2003, **10**, 40-48.

Graphical TOC



Selective cytochrome P450 bioactivation of truncated azinomycin