Pergamon

PII: S0031-9422(97)00965-5

Phytochemistry, Vol. 48, No. 4, pp. 619–624, 1998

Printed in Great Britain
0031-9422/98 $19.00+0.00

SUBSTRATE ANALOGUES AND INHIBITION OF ACC OXIDASE: CONVERSION OF *D*-VALINE TO *ISO*-BUTANAL

EDWARD J. GIBSON, ZHIHONG ZHANG, JACK E. BALDWIN and CHRISTOPHER J. SCHOFIELD*

The Dyson Perrins Laboratory and the Oxford Centre of Molecular Sciences, South Parks Road, Oxford OX1 3QY, U.K.

(*Received in revised form* 30 *September* 1997)

Key Word Index—ethylene biosynthesis; oxidase; oxygenase; 1-aminocyclopropane-1-carboxylic acid.

Abstract—Amino acids and their hydroxamates were studied as inhibitors of purified recombinant 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase (tomato). Neither *L*-valine, *L*-alanine, *L*- nor *D*-isoleucine displayed inhibition, but *D*-valine and *D*- alanine were weak inhibitors of ACC oxidase catalysed conversion of ACC to ethylene. GC-MS analysis showed that *D*-valine was converted to *iso*-butanal. In contrast to studies using native ACC oxidase (apple), inhibition of recombinant ACC oxidase (tomato) by amino acid hydroxamates appears to result (at least predominantly) from chelation of free iron in solution.

INTRODUCTION

1-Aminocyclopropane-1-carboxylate (ACC) oxidase catalyses the final step in the biosynthesis of the plant signalling molecule ethylene. It is a member of the non-haem ferrous dependent family of oxygenases and oxidases and, uniquely for known members of this family, requires ascorbate (as opposed to 2-oxoglutarate) as a co-substrate and carbon dioxide (or bicarbonate) as an essential activator (Fig. 1) [1].

Inhibition and substrate-analogue studies on ACC oxidase utilising ACC, ascorbate and 2-oxo acids as templates have led to some insights into its active site, and to mechanistic proposals [2, 3]. In particular, the use of coronamic and *allo*-coronamic acids (i.e. 2-ethyl-ACCs) as ACC analogues using ACC oxidases from different sources (apple, mung bean, tomato) [4–7], have demonstrated the following order of substrate selectivity for the possible stereoisomers: $1R,2S \gg 1S,2R > 1S,2S > 1R,2R$.

It has also been reported that a series of amino acid hydroxamates inhibit native ACC oxidase (apple) [2]. It was proposed that the amino acid hydroxamates inhibit ACC oxidase by monodentate chelation to the active-site bound iron and the results were used to propose a crude active site topology for ACC oxidase.

Previous studies have demonstrated that *L*- but not *D*-amino acids inhibit ethylene production [8, 9]. Since crude whole cell extracts were used in these studies it was not possible to consider the effects on ACC oxidase independent of other enzymes which may utilise ACC or potential inhibitors.

It is known that *D*-amino acids are competitive with ACC with respect to its malonylation and subsequent breakdown, whereas *L*-amino acids are not [8]. Addition of *D*-amino acids to crude extracts causes inhibition of this ACC breakdown pathway and a consequent accumulation of ACC in cell extracts. Thus, experiments in which equal concentrations of *D*- or *L*- amino acids are added to crude whole plant cell extracts may give markedly different results with respect to ethylene production compared with conducting the same experiments using purified ACC oxidase.

Herein, we report a study on the inhibition of highly purified recombinant ACC oxidase (tomato) by amino acids and amino acid hydroxamates. The results contrast with those previously reported for native ACC oxidase (apple) [2, 8]. We also demonstrate that *D*-valine, but not *L*-valine, acts as a substrate for ACC oxidase, undergoing a two electron oxidation to yield *iso*-butanal.

RESULTS AND DISCUSSION

Various amino acids were tested as inhibitors of purified ACC oxidase (tomato). Most of the amino acids tested (*L*-alanine, *L*-valine, *D*- and *L*-isoleucine, *D*- and *L-allo*-isoleucine, *D*- and *L*-phenylalanine, *L*-

* Author to whom correspondence should be addressed. Tel: +44-1865-275-625. Fax: +44-1865-275-674. E-mail: christopher.schofield@chem.ox.ac.uk

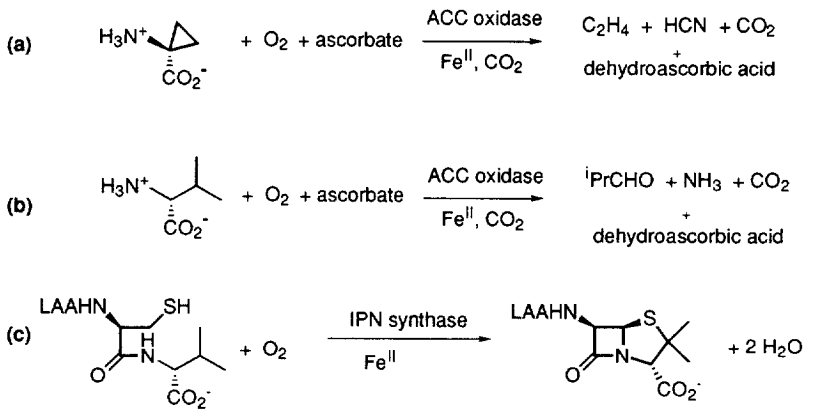


Fig. 1. (a) The ACC oxidase catalysed conversion of ACC into ethylene; (b) The ACC oxidase catalysed conversion of *D*-valine into *iso*-butanal; (c) The isopenicillin N synthase (IPNS) catalysed conversion of *L*-δ-(α-aminoadipoyl)*L*-cysteinyl-*D*-valine into isopenicillin N (IPN).

glutamic acid, glycine, *N*-acetyl-glycine, *N*-acetyl-*L*-alanine, *N*-acetyl-*D*-alanine, *N*-acetyl-*L*-valine, *N*-acetyl-*D*-valine, 1-aminocyclobutane-1-carboxylic acid, 1-aminocyclopentane-1-carboxylic acid, 1-aminocyclohexane-1-carboxylic acid) gave insignificant inhibition of ACC oxidase activity (IC_{50} values > 100 mM). Two *D*-amino acids with hydrophobic side chains, *D*-valine and *D*-alanine, displayed weak but clearly discernible inhibition of ACC oxidase activity, with IC_{50} values of 60.1 ± 3.1 mM and 37.7 ± 3.6 mM, respectively.

The recombinant ACC oxidase used in the present study was highly purified and active. However, the lability of even highly purified ACC oxidase under *in vitro* catalytic conditions ($t_{1/2}$ < 10 min in the absence of dithiothreitol (DTT), BSA and catalase) [10, 11] makes analysis of kinetic data difficult, since the period of linear activity is short. Thus, determination of accurate kinetic parameters was precluded by the weak nature of the inhibition and the lability of ACC oxidase under the assay conditions. Both *D*- and *L*-cysteine also inhibited ethylene production under *in vitro* conditions. The inhibition, however, could be fully overcome by the addition of excess iron (II) sulphate to the incubation mixture (data not shown). Inhibition by *D*- and *L*-cysteine was thus assumed to be due, at least predominantly, to iron chelation in free solution.

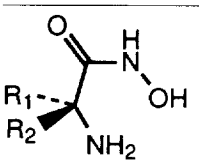
The observation that *D*-valine and *D*-alanine, but not their *L*-enantiomers are weak inhibitors of ACC oxidase, may seem surprising since ACC itself is achiral. Since the inhibition of ACC oxidase by *D*-amino acids is weak, its effect on crude cell extracts may be disguised. The high concentration of *D*-amino acids required for substantial inhibition of ACC oxidase together with the normally low *in vivo* concentrations of *D*-amino acids probably render inhibition by *D*-amino acids unimportant in plant tissues.

Assays for substrate-analogue turnover were conducted in the absence of ACC and volatile components analysed by GC-MS after heating. In the case of *D*-valine a single product, identical to authentic *iso*-butanal by comparison of GC retention times and by mass spectral analysis, was observed (Fig. 1b). Although *iso*-butanal was the only detected product other non-volatile products, not detectable by the GC-MS assay, may have been produced. Quantitative time course analysis of *iso*-butanal production by GC-FID proved unviable due to the low concentrations of *iso*-butanal produced. It should be noted that the yield of *iso*-butanal was low and it cannot be ruled out that *D*-valine reacts to give other products.

In contrast to *D*-valine, incubation of *L*-valine under the same conditions used for the turnover of *D*-valine did not result in the production of any detected volatile products. Reaction of both *L*- and *D*-valine with hydrogen peroxide, bicarbonate and iron (II) in a buffered solution, conditions previously demonstrated to effect the non-enzyme catalysed conversion of ACC into ethylene [12], yielded no detectable volatile products by GC-MS.

Assuming that binding of the α-amino acid units is conserved, the observation that *D*-alanine and *D*-valine are substrates/inhibitors of ACC oxidase is consistent with the observed preferential turnover of the 1*R*,2*S* diastereomer of coronamic acid [4, 5, 13]. One interpretation of the observed stereoselectivity is that there is a binding pocket within the ACC oxidase active site which orientates the productive substrate-analogues towards an intermediate oxidising species (such as —Fe=O) generated by prior reaction of the active site bound iron with dioxygen. The preferential reaction of *D*- rather than *L*-amino acids is interesting since isopenicillin N synthase (IPNS), with which ACC oxidase shows significant sequence similarity [14], catalyses the conversion of *L*-δ-(α-aminoadipoyl)-*L*-cysteinyl-*D*-valine to isopenicillin N, but does not accept *L*-δ(α-aminoadipoyl)-*L*-cysteinyl-*L*-valine as a substrate [15]. It is possible that *D*-valine binds to ACC oxidase in a similar manner to which the *D*-

Table 1. IC_{50} values for the inhibition of ACC oxidase (tomato) by amino acid hydroxamates ± standard error of the mean



R_1	R_2	IC_{50}/mM −DTT	IC_{50}/mM +DTT
H	H	$2.9 \pm 6.0 \times 10^{-2}$	>10
H	Me	$4.3 \pm 7.1 \times 10^{-2}$	>10
Me	H	$3.6 \pm 6.3 \times 10^{-2}$	>10
Me	Me	$2.8 \pm 7.5 \times 10^{-2}$	>10
H	Me_2CH—	$3.3 \pm 6.3 \times 10^{-2}$	>10
Me_2CH—	H	$3.0 \pm 6.6 \times 10^{-2}$	>10

valine of *L*-δ-(α-aminoadipoyl)-*L*-cysteinyl-*D*-valine is bound in the case of IPNS. However, prediction of the precise substrate binding orientations within the ACC oxidase active site based on the IPNS structure is, problematic due to the relative complexity of the reaction catalysed by ACC oxidase and small size of its cofactors/substrates. Thus, it may be that, rather than an ACC binding pocket which can accommodate *D*- but not *L*- amino acids (and preferentially the 1*R*,2*S* isomer of coronamic acid), the productive binding of *L*-amino acids and the other coronamic acid isomers is hindered because of (a) disfavourable interaction(s). However, it might be considered likely that an interaction which prevents *L*-alanine binding may also make ACC binding disfavourable.

In the absence of DTT, the amino acid hydroxamates tested displayed a similar level of inhibition of ACC oxidase (tomato) regardless of stereochemistry and nature of the side chain (Table 1). Inhibition was completely reversed upon increasing the amount of iron in the assay above half the concentration of the hydroxamate present (Fig. 2) or by the addition of 2 mM DTT to the assay mixture. Kinetic analysis of glycine hydroxamate-mediated inhibition of ACC oxidase (tomato) showed it to be non-competitive with respect to ACC, carbon dioxide or ascorbate (data not shown). Incubation of *L*- and *D*-valine hydroxamic acids (at 10 mM final concentration) under the standard assay conditions in the absence of ACC failed to demonstrate conversion to volatile products: specifically formation of *iso*-butanal was not observed.

The results suggest that the most plausible inhibition mode for the hydroxamates of the ACC oxidase (tomato) is via chelation of iron (III) in free solution. The observation that hydroxamate mediated inhibition occurs at iron concentrations up to half the amino acid hydroxamate concentration and is reversed by the addition of the reducing agent DTT implies the formation of a complex with a stoichiometry of two amino acid hydroxamate molecules: one ferric ion, as anticipated for the formation for a ferric hydroxamate complex in free solution [16].

There are several possible reasons for the difference in the hydroxamate inhibition results observed for the native ACC oxidase (apple) [2] and the present study

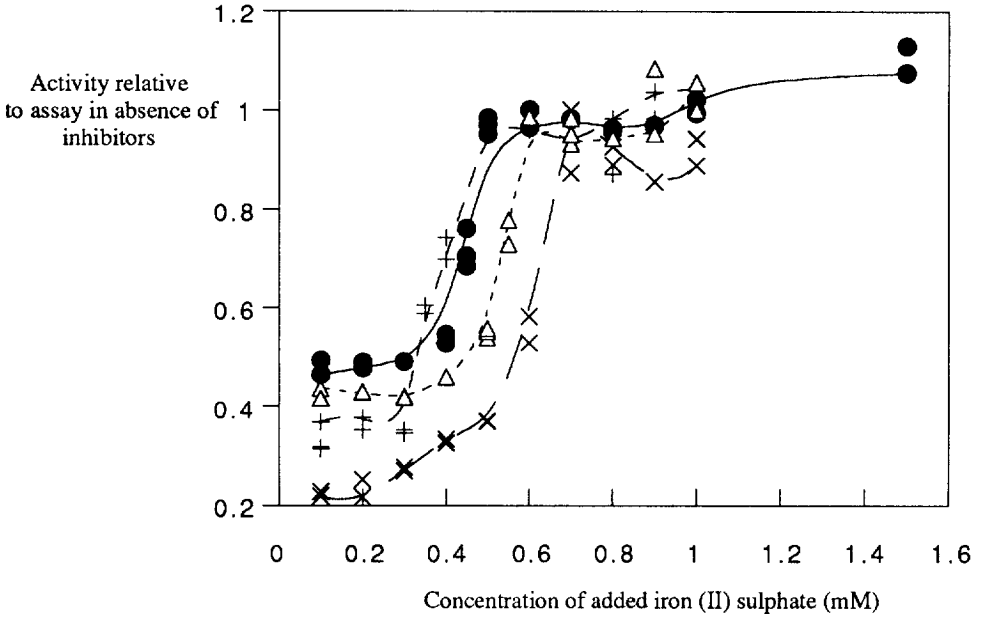


Fig. 2. Effect of iron (supplied as ferrous sulphate) concentration on the inhibition of ACC oxidase (tomato) by amino acid hydroxamates. -●- 1 mM glycine hydroxamate, -×- 1 mM aminoisobutyric acid hydroxamate, -+- 1 mM *D*-valine hydroxamate and -Δ- *L*-valine hydroxamate.

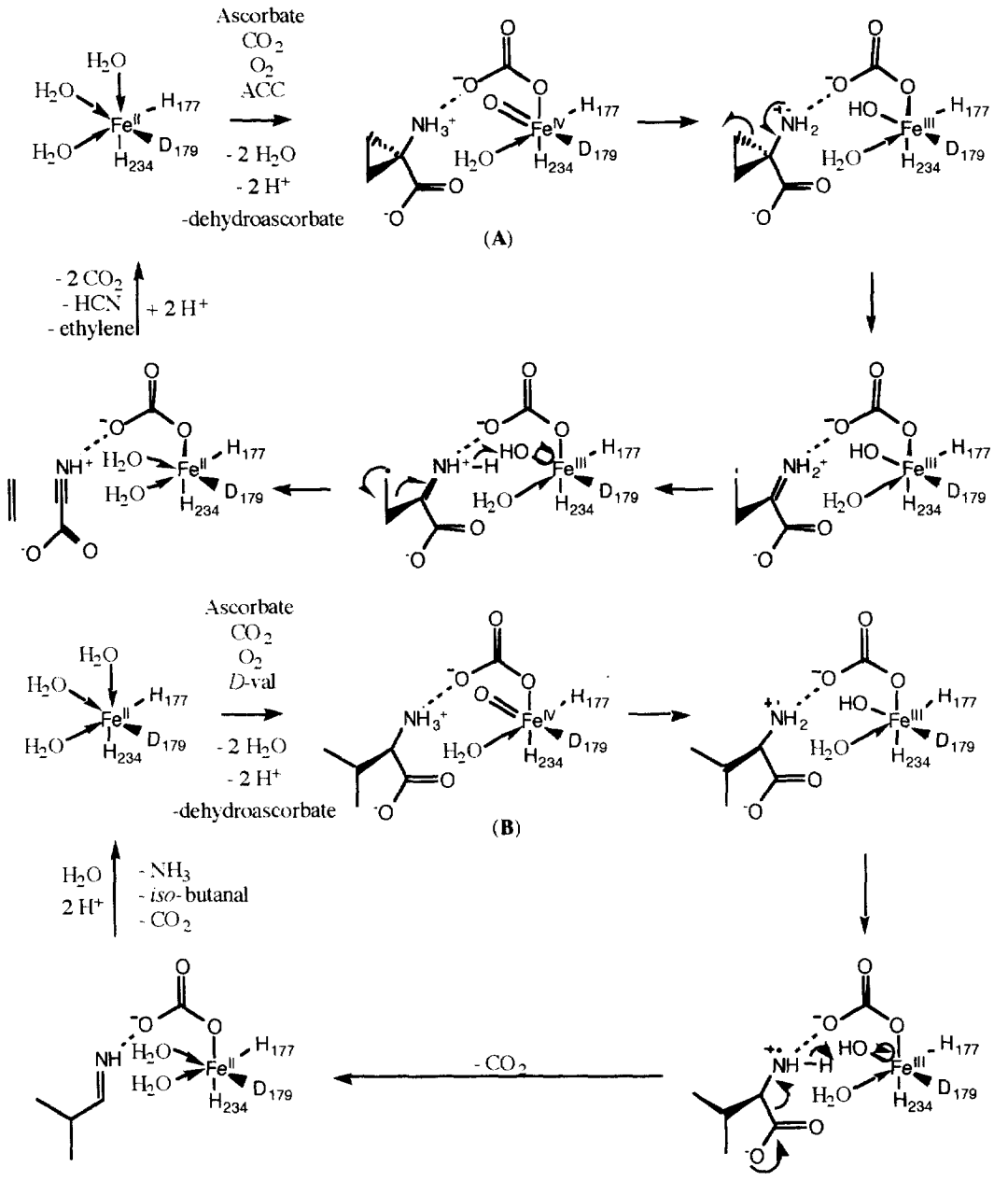


Fig. 3. Proposed mechanisms for ACC oxidase showing (a) reaction of ACC to give ethylene; (b) reaction of *D*-valine to give *iso*-butanal. It should be noted that dehydroascorbate may remain bound throughout.

using recombinant ACC oxidase (tomato). Although the two sequences are closely related it may be that there are significant differences within the active sites of the two enzymes. Alternatively it may be that the two studies were carried out on different conformational forms of ACC oxidase: there is some evidence for more than one conformationally active form of ACC oxidase [10].

An outline hypothesis for part of the mechanism of ACC oxidase is shown in Fig. 3a. In the first part of the catalytic cycle, ascorbate and dioxygen react with holo-ACC oxidase to generate a ferryl intermediate and dehydroascorbate. A bicarbonate ion is also shown ligating to the active site iron. The proposed co-ordination chemistry is, based in part on that proposed for the analogous ferryl intermediate in isopenicillin N synthase (IPNS) catalysis [17]. The three amino acid residues (H177, D179 and H234 corresponding to H214, D216 and H270 in the *Aspergillus nidulans* IPNS sequence [17]) proposed as ligands are conserved throughout the entire IPNS/ACC oxidase sub-family. The orientation of the ferryl is based upon the observe binding site of NO (acting as a dioxygen analogue to the holo-IPNS-substrate complex).

We also propose that the ACC carboxylate binds to the side chains of the conserved RXS motif which binds the carboxylate of the valine of *L*-δ-(α-aminoadipoyl)-*L*-cysteinyl-*D*-valine in IPNS catalysis. The observation that ACC oxidase reacts with *D*- but not *L*-valine supports this proposal. The carbon dioxide/bicarbonate activator is shown complexing to the iron opposite H234 in an analogous position to that of the thiol of *L*-δ-(α-aminoadipoyl)-*L*-cysteinyl-*D*-valine in the holo-IPNS-substrate structure. This is also the proposed ligation position of the 1-carboxylate group of 2-oxoglutarate in 2-oxoglutarate-dependent dioxygenases related to the IPNS/ACC oxidase sub-family [17]. Oxidative fragmentation of 2-oxoglutarate in these enzymes results in the conversion of the 1-carboxylate to carbon dioxide together with production of succinate and a (proposed) ferryl complex.

The ferryl intermediate A effects a two electron oxidation of ACC to give ethylene, CO_2 and HCN, possibly via (a) radical intermediate(s) as shown in Fig. 3a [4]. HCN and CO_2 may be released from the enzyme as cyanoformic acid [18]. Assuming that the oxidation of *D*-valine follows a similar pathway to that proposed for ACC B, an intermediate analogous to A, will be formed (Fig. 3b). This intermediate cannot effect fragmentation of a valine derived intermediate via Cα-Cβ cleavage (as occurs for ACC in Fig. 3a). Instead oxidative decarboxylation to generate *iso*-butylimine, which is hydrolysed to yield *iso*-butanal and ammonia, occurs.

EXPERIMENTAL

Recombinant ACC oxidase was purified to near homogeneity as determined by SDS-PAGE analysis using a modified version of the procedure in Ref. [13]. ACC oxidase activities, in the presence or absence of inhibitors and DTT, were measured according to the method outlined in Ref. [13] and references therein. One unit of activity is defined under these conditions 1 μmol C_2H_4 produced per mg enzyme per min over a 15 min incubation period. The ACC oxidase used in this study had a minimum sp. ac. of 1.2 units.

Attempted conversions of valine and valine hydroxamates into *iso*-butanal were carried out by incubation of the potential substrates (10 mM final conc.) in the presence of 0.1 mM iron (II) sulphate, 2 mM DTT (Lancaster Synthesis Ltd), 3 mM Na_2 ascorbate, 10 mM (BDH Ltd) and 100 μg BSA in 1M HEPES containing 10% glycerol (Avocado Research Chemicals Ltd) at pH 7.1 for 45 min at 27° with vigorous shaking. The sealed sample vials were immersed in a water bath at 65–70° for 10 min and a sample (0.5 ml) of the head space gas taken. This sample was immediately assayed by GC-MS in CI+mode using NH_3. The mass spectra and retention times of volatile components were compared with that of an *iso*-butanal standard incubated in buffer at pH 7.1 at 27° for 45 min then heated to 65–70° and assayed by GC-MS.

Non-enzymatic oxidation of amino acid derivatives was carried out by incubation in the presence of 10 mM NH_4HCO_3, 0.1 mM iron (II) sulphate and 0.03% H_2O_2 in Tris-HCl buffered at pH 8.0 for 45 min at 27° according to the method of Ref. [12]. Reaction products were monitored by GC-MS analysis as described above.

Optically active amino acid hydroxamates were synthesised according to the method of Ref. [2]. The synthetic amino acid hydroxamates were identical by spectroscopic {^{1}H, ^{13}C NMR, IR and C-IMS} analysis to commercially available samples of the racemic amino acid hydroxamates, except *L*-valine hydroxamate: $[\alpha]_D^{25}$ (6 M HCl, $c = 1.2$) +4.3; *D*-valine hydroxamate: $[\alpha]_D^{25}$ (6 *N* HCl, $c = 1.2$) −4.1; *L*-alanine hydroxamate: $[\alpha]_D^{25}$ (6 M HCl, $c = 1.1$) +3.8; *D*-alanine hydroxamate: $[\alpha]_D^{25}\omega$ (6*N* HCl, $c = 1.1$) −3.8. Glycine hydroxamate, *L*-valine, *D*-valine, amino-isobutyric acid hydroxamate and other reagents were purchased from Sigma-Aldrich unless otherwise stated.

Acknowledgements—We thank Dr R. Aplin, for assistance with mass spectrometry, Dr J. Barlow, for providing purified ACC oxidase, Dr A. Prescott, Prof. M. Pirrung and Dr J. Thirkettle and Prof. P. John for helpful discussions. We thank the MRC, EPSRC, BBSRC and Zeneca for funding via the DTI LINK Scheme (Z.Z.) and the BBSRC for funding (E.J.G.).

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