Inhibition of chorismate-utilising enzymes by 2-amino-4-carboxypyridine and 4-carboxypyridone and 5-carboxypyridone analogues†

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Several 2-amino-4-carboxypyridine, 4- and 5-carboxypyridone-based compounds were prepared and tested against three members of the chorismate-utilising enzyme family, anthranilate synthase, isochorismate synthase and salicylate synthase. Most compounds exhibited low micromolar inhibition of these three enzymes. The most potent inhibitor was a 4-carboxypyridone analogue bearing a lactate side chain on the pyridyl nitrogen which exhibited inhibition constants of 5, 91 and 54 μM against anthranilate synthase, isochorismate synthase and salicylate synthase respectively.

Introduction

The shikimate biosynthetic pathway is utilised by algae, higher plants, bacteria, fungi and apicomplexan parasites. The pathway consists of seven enzyme-catalysed steps which converts phosphoenolpyruvate and erythrose-4-phosphate into chorismate.[1-4] Chorismate serves as the branchpoint for the synthesis of a diverse range of aromatic compounds including the amino acids tryptophan, tyrosine and phenylalanine, and essential vitamins including the folate coenzymes, benzoid and naphthenoid quinones.[5-7]

The first committed step in the biosynthesis of these metabolites is catalysed by a family of chorismate-utilising enzymes (Scheme 1). For example, one member of this enzyme family, isochorismate synthase catalyses the reversible conversion of chorismate (**1**) into isochorismate (**2**).[5] *Escherichia coli* possesses two isochorismate synthases, EntC and MenF, which represent the first committed steps of two distinct biosynthetic pathways.[8,9] The EntC isochorismate synthase is required for the biosynthesis

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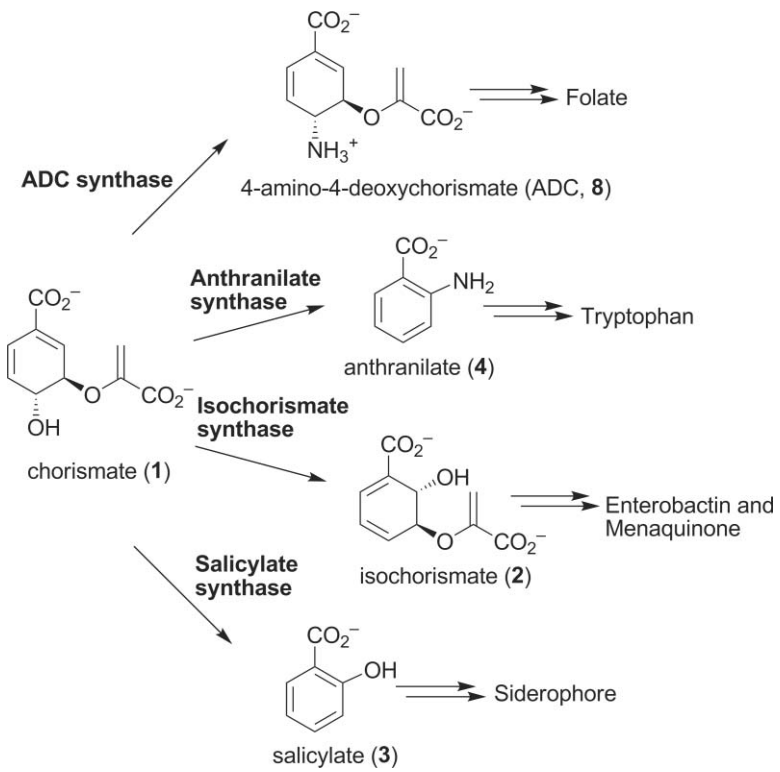
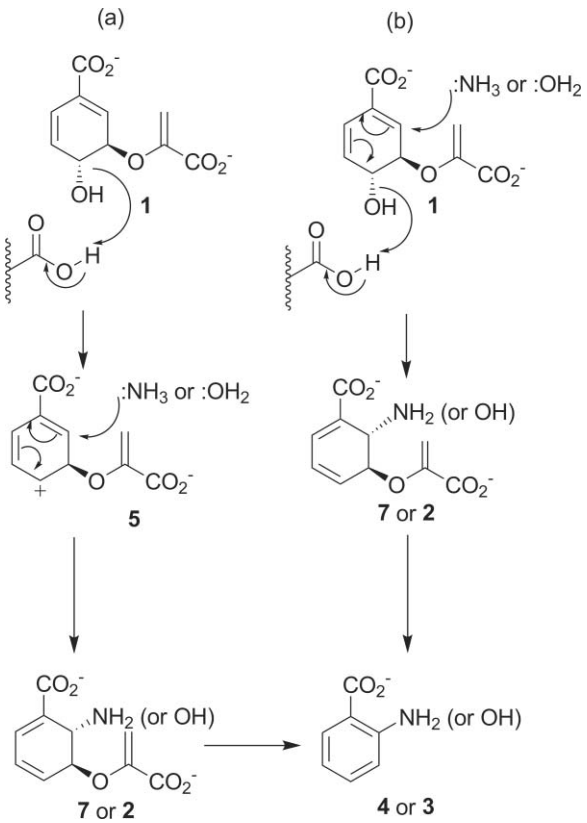


Scheme 1 Chorismate-utilising enzymes

of the siderophore enterobactin,[10,11] whilst MenF is involved in a separate pathway leading to the biosynthesis of menaquinone (Vitamin K).[12-14] A related enzyme, salicylate synthase catalyses the conversion of chorismate into salicylate (**3**) and represents the first commited step in the biosynthesis of iron chelating siderophores in bacteria (Scheme 1).[15] Recent evidence from HPLC and NMR analyses suggest that this enzyme carries out the conversion *via* an isochorismate (**2**) intermediate.[16-18] Anthranilate synthase catalyses the conversion of chorismate (**1**) to anthranilate (**4**), and represents the first commited step in tryptophan biosynthesis.[6] Anthranilate synthase is a multifunctional enzyme composed of a glutamine amidotransferase subunit, TrpG, and a chorismate binding subunit, TrpE.[19] TrpG is responsible for the conversion of glutamine to glutamate and delivers the ammonia generated in the process to the chorismate binding site in the TrpE domain. The TrpE subunit converts chorismate (**1**) to the intermediate 2-amino-2-deoxyisochorismate (ADIC) and then eliminates the enol-pyruvyl side chain to form anthranilate.[19,20]

These three chorismate-utilising enzymes are of particular interest due to their sequence similarity, and the similarity in the reaction each catalyses. This has led to the suggestion that there may be some unifying features in their mechanisms.[21-23] There are several feasible mechanisms for this initial step. One possibility is that the reaction proceeds *via* an S_N1 type mechanism where the C-4 hydroxyl of chorismate (**1**) is first eliminated to form a carbocation (**5**) at C-4 (mechanism (a), Scheme 2). The hydroxyl is thought to be protonated by a nearby glutamate residue in the active site of these enzymes to promote its elimination. Attack at C-2 by either ammonia or water would then proceed to give ADIC (**7**, in anthranilate synthase) and isochorismate (**2**, in isochorismate synthase and salicylate synthase) respectively.[11] A second possible, and perhaps more likely, mechanism involves the direct S_N2'' attack at C-2 by ammonia or water, with concomitant loss of the C-4 hydroxyl of chorismate to yield ADIC (**7**) or isochorismate (**2**) in one step (mechanism b, Scheme 2). Indirect evidence for this type of mechanism has been gained both in our laboratory[23,24] and that of Toney[22] using the structurally related 4-amino-4-deoxychorismate (ADC) synthase, responsible for the conversion of chorismate (**1**) to ADC (**8**) in the folate biosynthetic pathway (Scheme 1). For anthranilate synthase and salicylate synthase there is an additional step in which the the C-3 enol-pyruvyl side chain is cleaved with concomitant aromatisation, to afford the enzymatic products anthranilate (**4**) and salicylate (**3**) respectively. Recent evidence suggests that this reaction may proceed *via* a pericyclic rearrangement, a relatively rare enzymatic transformation.[18,25,26]



Scheme 2 Two proposed mechanisms for anthranilate synthase, isochorismate synthase and salicylate synthase, (a) two step mechanism (b) S_N2'' mechanism.

As mammals do not possess any of the chorismate-utilising enzymes, the design of highly specific inhibitors against this family of enzymes may lead to the development of selective therapeutic agents including antibacterials. In particular isochorismate synthase and salicylate synthase, which are critical for the biosynthesis of siderophores, could provide effective drug targets.[7,27] Siderophores are low molecular weight organic chelators designed to sequester ferric iron. Iron is essential for growth of pathogenic bacteria and during infection bacteria must obtain iron from host stores to support their normal metabolism. For many pathogenic bacteria the efficiency of iron-uptake is directly related to virulence.[28-30]

Previous inhibitor studies include the work of Kozlowski *et al.* who designed several competitive inhibitors mimicking proposed transition states for isochorismate synthase and anthranilate synthase.[21] The most potent of these (**9**) exhibited a K_I of 0.053 and 0.62 μM against isochorismate synthase and anthranilate synthase respectively (Fig. 1). We have recently reported the synthesis of 2,5-dihydrochorismate-[31] and benzoic acid-based[32-34] inhibitors of the chorismate-utilising enzymes. However, these compounds proved to be less potent than **9** against the family of enzymes.

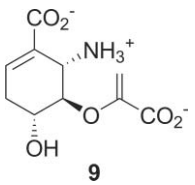


Fig. 1 Chorismate-utilising enzyme inhibitor designed by Kozlowski *et al.*[21]

As a tool to investigate the mechanism of these enzymes and to develop potentially useful inhibitors, a variety of 2-amino-4-carboxypyridine (**10**, **11**), 5-carboxypyridone (**12–14**) and 4-carboxypyridone (**15**, **16**) analogues were considered (Fig. 2). The compounds were designed as competitive reversible inhibitors of the chorismate-utilising enzymes. The pyridyl nitrogen ($pK_a \approx 7$) of the 2-amino-4-carboxypyridine compounds (**10**, **11**) would be partially protonated under the assay conditions and, as such, would serve as a mimic of the positive charge at C-4 of chorismate

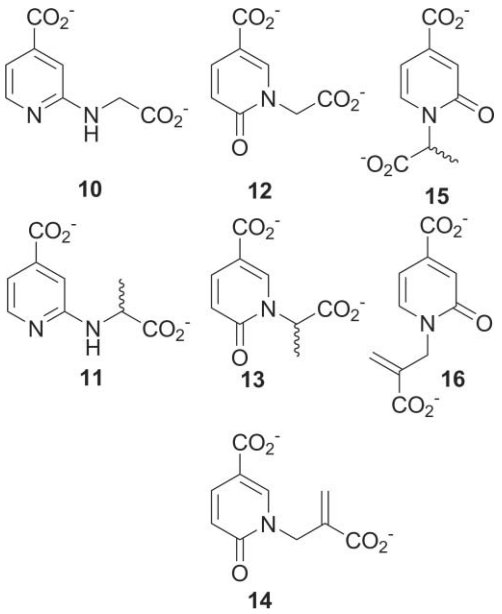


Fig. 2 Proposed 2-amino-4-carboxypyridine (**10**, **11**) and 5-carboxypyridone (**12–14**) and 4-carboxypyridone (**15**, **16**) inhibitors.

in the transition state of one of the proposed mechanisms (Scheme 2a). The 4- and 5-carboxypyridone compounds (**12–16**) were designed as analogues of the substrate chorismate (**1**). A variety of side chains were incorporated in **10–17** to probe the enol-pyruvyl binding pocket of the enzymes.

Results and Discussion

Docking Studies

Analogues **10–16** containing glycol, lactate and acrylate side chains were prepared for modelling using SYBYL7.1.[35] These compounds were then docked into the active site of *Serratia marcescens* anthranilate synthase (AS), *E. coli* isochorismate synthase (EntC) and *Yersinia enterocolitica* salicylate synthase (Irp9) using GOLD to assess their suitability as inhibitors of the three chorismate-utilising enzymes.[36,37] Interestingly, the compounds exhibited two distinct binding modes when docked against the three chorismate-utilising enzymes (see ESI†). In addition, these same two binding modes were observed when inhibitor **9** (the most potent chorismate-utilising enzyme inhibitor prepared to date) was docked against the three enzymes (see ESI†).

The first of these binding modes has been observed in previous docking studies with aromatic chorismate analogues.[32] In this mode the compounds are oriented with the C-1 carboxylate bound to the magnesium ion. The side chain carboxylate forms an ionic interaction with a conserved arginine residue on the opposite side of the active site, corresponding to Arg469, Arg391 and Arg347 in AS, Irp9 and EntC, respectively. Compound (*R*)-**11** docked in this binding mode into the active sites of AS and Irp9 (Fig. 3a and b). In the second binding mode, as observed when (*R*)-**11** was docked into the active site of EntC, the compound is flipped over so that the side chain carboxylate interacts with the magnesium ion and the C-1 carboxylate binds to the conserved arginine (Fig. 3c).[33] For Irp9 the majority of compounds docked in the first binding mode, while for AS and EntC the second mode was predominate

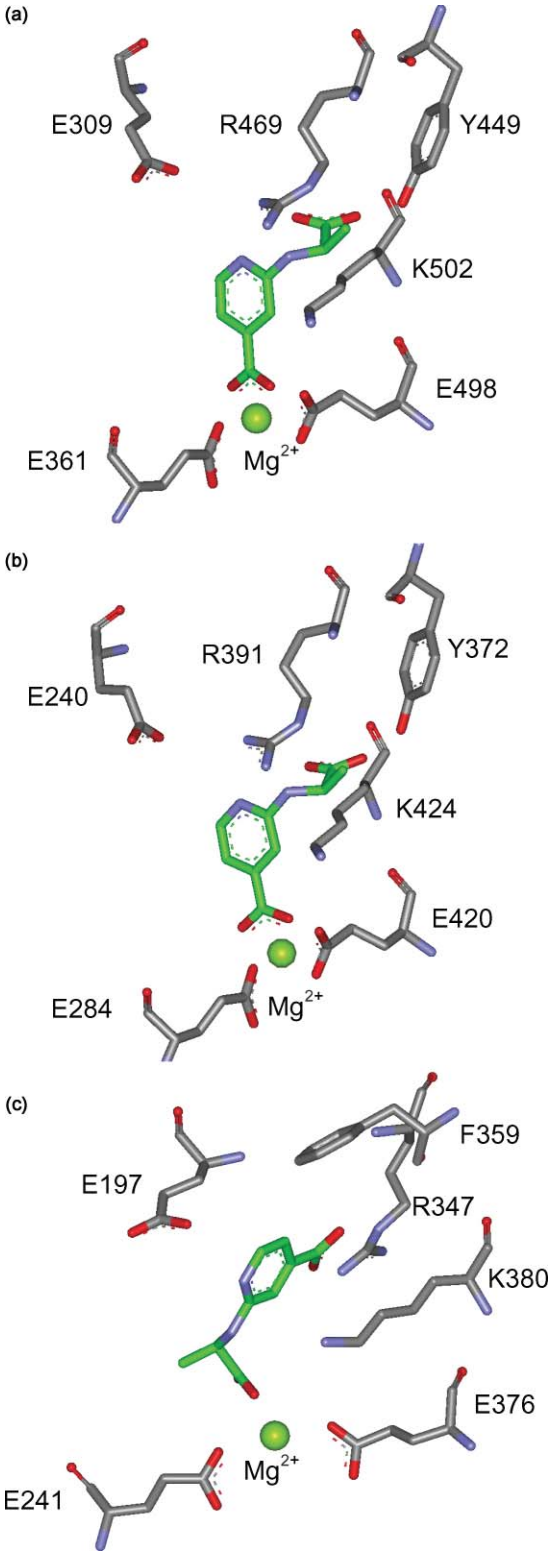
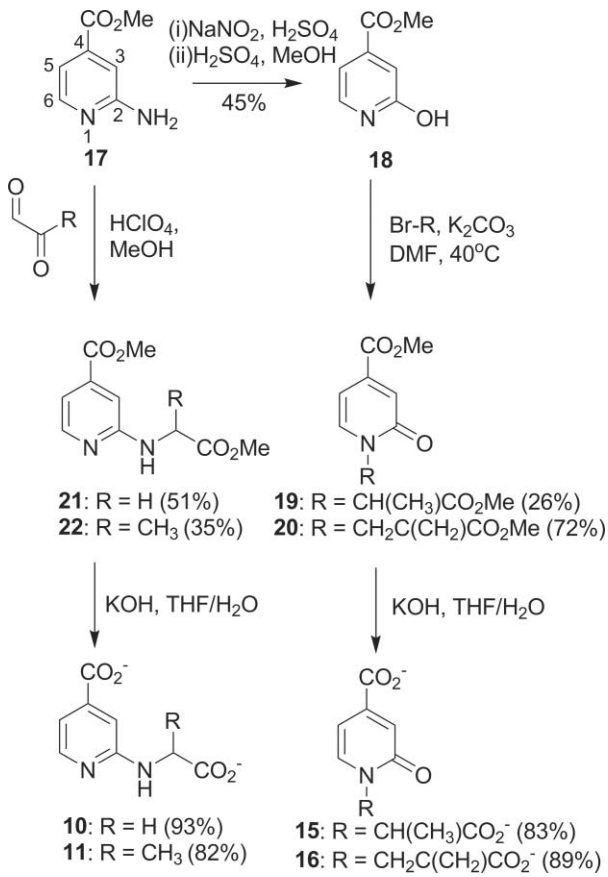


Fig. 3 Dockings of (*R*)-**11** into the active sites of (a), *S. marcescens* anthranilate synthase (AS) (b) *Yersinia enterocolitica* salicylate synthase (Irp9) (c) *E. coli* isochorismate synthase (EntC).

(See ESI†). It was anticipated that these variations in binding mode would be reflected in different specificities of the compounds against the three chorismate-utilising enzymes.

Synthesis

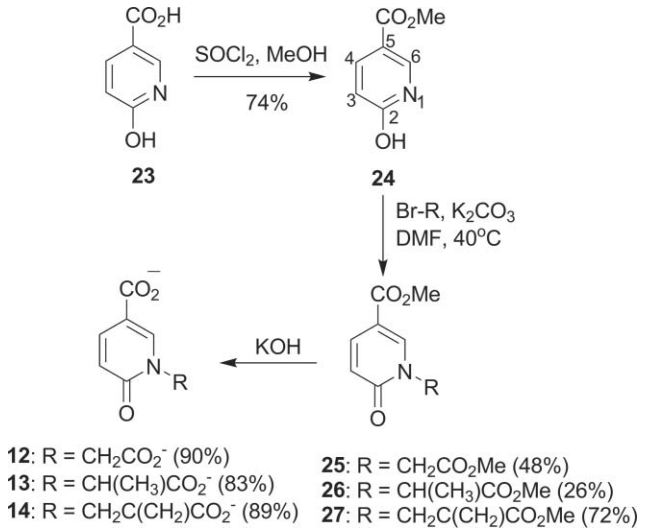
Synthesis of the 4-carboxypyridone analogues began from commercially available methyl-2-amino-isonicotinic acid **17** (Scheme 3). Initial diazatisation of **17** with sodium nitrite and sulfuric acid produced the desired 2-hydroxy functionality, however the strongly acidic conditions also hydrolysed the methyl ester. The methyl ester was reformed using concentrated sulfuric acid in refluxing methanol to give **18** in 45% yield over the two steps. Treatment of **18** with methyl-2-bromopropionate and methyl-2-(bromomethyl)acrylate afforded the desired *N*-alkylated products **19** and **20** in 26% and 72% yields respectively. Their regiochemistry was confirmed by nOe analysis. Finally, methyl ester hydrolysis produced the pyridone dicarboxylates **15** and **16** in good yields.



Scheme 3 Synthesis of 2-amino-4-carboxypyridine (**10**, **11**) and 4-carboxypyrididone inhibitors (**15**, **16**).

Synthesis of the 2-amino-4-carboxypyridine analogues also began from methyl-2-amino-isonicotinic acid **17**. It is well documented that alkylation of 2-amino pyridines usually results in alkylation at the pyridine nitrogen, giving the related imidazo[1,2-*a*] derivatives through the corresponding intermediate pyridone imines.[38] A study by Alcaide *et al.* reported that reaction of 2-aminopyridines, 2-aminopyrimidine and 2-aminothiazole with various glyoxal derivatives in boiling alcohol in the presence of aqueous 60% perchloric acid, forms regiospecifically *N*-(2-heteroaryl)-α-amino esters in good yields.[39] This was the approach adopted for the synthesis of the desired compounds as other alkylation methods indeed produced adducts where alkylation had only occurred on the ring nitrogen. Alkylation using the above conditions with glyoxal and pyruvic aldehyde gave the desired 2-amino alkylated products **21** and **22** in 51% and 35% yields respectively. Finally, base hydrolysis of the methyl esters proceeded smoothly to afford the desired dicarboxylates **10** and **11**.

Synthesis of the 5-carboxypyridone analogues began from 6-hydroxynicotinic acid **23** and involved initial methyl ester formation (Scheme 4). Alkylation with methylbromoacetate, methyl-2-bromopropionate and methyl-(2-bromomethyl) acrylate produced diesters **25–27** in low to moderate yields. The regiochemistry for **25–27** was confirmed by nOe studies. Finally, methyl ester hydrolysis with aqueous potassium hydroxide gave the desired pyridone dicarboxylates **12–14** in excellent yields.



Scheme 4 Synthesis of 5-carboxypyridone inhibitors **12–14**.

Enzyme Assays

The 2-amino-4-carboxypyridine (**10**, **11**), 5-carboxypyridone (**12–14**) and 4-carboxypyridone (**15**, **16**) inhibitors were tested against three different chorismate-utilising enzymes, *S. marcescens* anthranilate synthase (AS), *E. coli* isochorismate synthase (EntC) and *Y. enterocolitica* salicylate synthase (Irp9). The assays were carried out in 96 well plates at 25 °C using fluorescence detection. AS was assayed by detecting the formation of anthranilate (ex. 313 nm, em. 390 nm).[40] EntC and Irp9 were assayed by detecting the formation of salicylate (ex. 305 nm, em. 440 nm).[34,41,42] EntC was assayed with a coupling enzyme, *Pseudomonas aeroginosa* isochorismate pyruvate lyase (PchB), in order to convert isochorismate into salicylate which could be detected fluorimetrically.‡

The inhibition constants of **10–16** against the three chorismate-utilising enzymes are shown in Table 1. All compounds proved to be competitive reversible inhibitors of the enzymes with respect to the substrate chorismate. Unfortunately, compounds

‡ Although the isochorismate pyruvate lyase was used in a coupling concentration in the assay (2.4 equivalents) it was assumed that the analogues may also inhibit this enzyme. Therefore when discussing the inhibition results, the analogues are described as inhibiting the entire enzymatic system rather than only isochorismate synthase

Table 1 Inhibition constants of 2-aminopyridine and 4- and 5-pyridone analogues against *S. marcescens* anthranilate synthase (AS), *E. coli* isochorismate synthase/*P.aeroginosa* isochorismate pyruvate lyase (EntC/PchB) and *Y. enterocolitica* salicylate synthase (Irp9)[a]

Compounds	AS K_I/μM	EntC/PchB K_I/μM	Irp9 K_I/μM
10	94 ± 19	150 ± 18	90 ± 18
11	50 ± 7	210 ± 17	170 ± 38
12	100 ± 18	120 ± 29	48 ± 12
13	5.3 ± 1.0	91 ± 10	54 ± 10
14	120 ± 16	170 ± 17	73 ± 13
15	41± 8	nd	nd
16	59 ± 10	nd	nd

[a] Kinetic parameters of the chorismate-utilising enzymes: AS: $K_M = 3.7 \pm 0.5$ μM, $k_{cat} = 5.6\ s^{-1}$; EntC: $K_M = 15 \pm 2.4$ μM, $k_{cat} = 13s^{-1}$; Irp9: $K_M = 4.2 \pm 0.9$ μM, $k_{cat} = 0.1s^{-1}$; nd, not determined.

15 and **16** could not be assayed against EntC/PchB or Irp9. The highly fluorescent nature of these compounds under the assay conditions resulted in the spectrophotometer operating out of the linear range of accuracy even at low inhibitor concentrations (50 μM).

The 2-amino-4-carboxypyridine inhibitors (**10** and **11**) were generally less potent than the corresponding 5-carboxypyridone counterparts (**12** and **13**). This effect was especially evident in analogue **13** containing a lactate side chain, which was significantly more potent against all three chorismate-utilising enzymes. These results suggest that any positive charge on the pyridyl nitrogen does not aid in binding to the enzymes.

Compounds containing a lactyl side chain were more potent against AS than analogues containing the alternative glycol or acrylate side chains. This result is consistent with previous inhibition results with aromatic analogues tested against the chorismate-utilising enzymes.[31-34] Side chain specificity does not appear to be as obvious in the case of EntC/PchB and Irp9. Compounds containing glycol (**10** and **12**) or lactate (**11** and **13**) side chains exhibited very similar inhibition constants against these two enzymes. Analogue **14** containing an acrylate side chain was two fold less potent against EntC/PchB and Irp9. Docking studies suggest that this may be a result of the side chain being too large to be accommodated into the enol-pyruvyl pocket in the active site of these enzymes.

It is interesting to note that compound **16**, containing an acrylate side chain exhibited an inhibition constant of 59 μM against AS, two fold more potent than 5-carboxypyridone analogue **14** containing the identical side chain. This result is consistent with the molecular dockings, where an extended acrylate side chain in **16** would allow for more favourable electrostatic interactions with the magnesium ion, if the inhibitor bound to the enzyme in this conformation. This trend was reversed for analogues containing a lactate side chain where 4-carboxypyridone analogue **15** was ten fold less potent against AS than 5-carboxypyridone analogue **13**. Variation in the binding modes of these compounds, as observed in the dockings, may explain the observed trends in the inhibition data. Future work in our laboratory will involve crystallisation studies of the inhibitors with the chorismate-utilising enzymes to quantify their binding mode which should aid in the design of more potent inhibitors of this family of enzymes.

Conclusions

In summary, we have designed and synthesised a novel series of chorismate-utilising enzyme inhibitors based on 2-amino-4-carboxypyridine and 4- and 5-carboxypyridone scaffolds. These were screened against *S. marcescens* anthranilate synthase (AS), *E. coli* isochorismate synthase (EntC)/*P. aeroginosa* isochorismate pyruvate lyase (PchB) and *Y. enterocolitica* salicylate synthase (Irp9). The compounds proved to be low- to mid-micromolar inhibitors of the enzymes and will now serve as lead structures for the design of second generation inhibitors.

Experimental

Molecular modelling

All ligands and the receptors (TrpE, Irp9 and EntC) were prepared using SYBYL7.1 and used as MOL2 files.[35] The ligands were prepared as carboxylate anions and their structures were energy minimised using the Tripos force-field. Gasteiger-Huckel charges were calculated prior to docking. Each ligand was docked using GOLD2.1 in 25 independent genetic algorithm (GA) runs.[36,37] For each of these, a maximum number of 100 000 GA operations was performed on a single population of 50 individuals. Operator weights for crossover, mutation and migration in the entry box were used as default parameters (95, 95 and 10 respectively), as well as the hydrogen bonding (4.0 Å) and van der Waals (2.5 Å) parameters. The position of the active site was introduced and the radius was set to 10 Å, with the automatic active-site detection selected.

Biological

Over-expression and purification of His_6-Irp9, His_6-EntC, His_6-PchB and His_6-AS. Preparation of the expression vectors pET-28a/*irp9*,[16] pET-28a/*entC*,[16] pET-28a/*pchB*,[31] and mini-pRSETA/*trpEG*[32] has been described previously. An overnight culture (100 ml or 10 ml for His_6-AS) of *E. coli* C41(DE3) cells transformed with the appropriate expression was added to 1 l of fresh 2YT media supplemented with 30 μg ml^{-1} kanamycin (or 50 μg ml^{-1} ampicillin for His_6-AS) and divided into 2 × 500 ml cultures. Expression of His_6-Irp9, His_6-EntC, His_6-PchB or His_6-AS was induced at an OD_{600} of 0.6 by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. After 4 h shaking at 37 °C, the IPTG-induced cells were harvested by centrifuging at 6,000 × *g* for 15 min at 4 °C. Cell pellets were stored at –20 °C.

All purification steps were carried out at 4 °C. Harvested cells were suspended in 0.02 M potassium phosphate, 0.5 M NaCl, 0.02 M imidazole, pH 7.4 (buffer A) containing lysozyme (0.35 mg ml^{-1}) and stirred at room temperature for 30 min. For His_6-AS, cell pellets were only resuspended in buffer N1 (50 mM sodium phosphate buffer pH 8.0, 300 mM NaCl, 10 mM imidazole, 1 mM β-mercaptoethanol). The suspension was homogenized for 20 min by sonication on ice. The crude extract was then centrifuged for 30 min at 39,000 × *g*. The cell-free lysate was applied to a 5 ml Ni-NTA (nitriloacetic acid) column that had been pre-equilibrated with buffer A or buffer N1 for His_6-AS. After washing with buffer A or buffer N2 (buffer N1 with 50 mM imidazole) for His_6-AS, the protein was eluted using a

linear gradient of 20–500 mM imidazole in 100 ml buffer A or 4 – 5 column volumes of buffer N3 (buffer N1 with 250 mM imidazole) for His_6-AS. The elution was followed *via* SDS-PAGE and fractions containing the protein of interest were concentrated using a protein centrifugal concentrator. The concentrated protein sample was then dialyzed against 100 mM potassium phosphate, pH 7.0 or 50 mM Tris.HCl pH 7.5, 1 mM DTT, 0.1 mM EDTA for His_6-AS using a PD-10 column containing Sephadex G-25 resin (Amersham Biosciences). The purity of the protein was judged to be ≥ 95% by SDS-PAGE. Enzyme concentrations were evaluated by measuring the absorbance at 280 nm and using the conversion factor calculated using the software Vector NTI (version 6; Invitrogen). Aliquots of pure His_6-tag recombinant proteins were frozen with liquid nitrogen and stored at –80 °C until use.

Enzyme and inhibition assays. Steady-state kinetic experiments were performed spectrophotometrically on a microplate reader by monitoring the products anthranilate **4** or salicylate **3** by fluorescence (**4**: excitation 313 nm, emission 390 nm; **3**: excitation 305 nm, emission 440 nm) as described previously.[34,41,42] The assays for AS containing 1 μM enzyme were performed in duplicate at 25 °C. The reaction of EntC with chorismate was measured using a coupled assay with PchB, an isochorismate pyruvate lyase from *P. aeruginosa*, as described previously by Gaille *et al.*[41] Reaction mixtures contained, in a total volume of 0.2 ml, 100 mM potassium phosphate, pH 7.0, 10 mM $MgCl_2$, PchB (220 nM), EntC (90 nM) and various amounts of chorismate (22.5–90 μM) and were conducted at 25 °C. Irp9 assays were initiated by the addition of 1.7 μM His_6-Irp9 and were monitored for 5 min by the salicylate fluorescence assay.

Initial rates for various concentrations of chorismic acid at a number of inhibitor concentrations were measured to characterise the mechanism of inhibition. Kinetic data were fitted to the appropriate equations using GraFit 5.0.10. (Erithacus software).[43]

Synthetic

General. All organic solvents were freshly distilled prior to use. Milli-Q deionised water was used for all biochemical work. Analytical thin layer chromatography was carried out on commercial silica gel 60 0.25 mm plates using either UV absorption or potassium permanganate stain for visualisation. R_F values are quoted with respect to the solvent system used to develop the plate. Column chromatography was carried out using 230–400 mesh silica gel 60. Petroleum ether refers to the fraction distilled between 40–60 °C. ^{1}H NMR spectra were recorded on a Bruker AM-400 spectrometer or a Bruker Avance 500 spectrometer in deuterated solvents, as indicated. ^{13}C NMR spectra were recorded on a Bruker AM-400 spectrometer operating at 100 MHz or a Bruker Avance 500 spectrometer operating at 125 MHz in deuterated solvents as indicated. All chemical shifts are quoted in parts per million (ppm). Coupling constants for ^{1}H NMR spectroscopy are assigned where possible and are given in Hz. Yields of final inhibitors **10–16** were calculated by using ^{1}H NMR studies. Samples were dissolved in D_2O with a known molarity of an internal standard (3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid, sodium salt). Infrared spectra were recorded on a Perkin Elmer Spectrum One FTIR spectrometer using attenuated transmittance reflectance (ATR). High resolution mass spectrometry was carried out using a Micromass Quadrapole-Time of Flight (Q-Tof) spectrometer.

2-Hydroxy-isonicotinic acid methyl ester (18). Methyl-2-amino-isonicotinic acid (1.00 g, 6.57 mmol) was dissolved in sulfuric acid (40 ml of a 2 M solution). Dropwise addition of concentrated sulfuric acid (6 ml) was followed by cooling to 0 °C. A solution of sodium nitrite (0.75 g, 0.01 mol) in water (12 ml) was added dropwise at 0 °C (**caution:** elimination of N_2). The reaction was stirred for a further 3 h at 22 °C and then boiled for one minute. The solution was neutralised with saturated aqueous sodium bicarbonate solution at which point the carboxylic acid precipitated and was isolated as a pale brown solid. After extensive drying of the acid under high vacuum for 16 h, the acid was dissolved in methanol (8 ml). Concentrated sulfuric acid (1 ml) was added dropwise and the reaction was stirred at reflux for 15 h. The reaction was allowed to cool to 22 °C before neutralising with saturated $NaHCO_3$ solution. The methyl ester was extracted into ethyl acetate (2 × 20 ml), the organic fractions were dried ($MgSO_4$) and the solvent removed under reduced pressure to afford the desired alcohol (**18**) as a pale yellow solid. (0.45 g, 45%).

$\nu_{max.}$ (ATR): 2833 (OH stretch) 3033, 2959 (Ar C–H stretch), 1728 (C=O, str), 1656, 1618, 1540 (C=C, ar) cm^{-1}; ^{1}H NMR (DMSO) δ 3.81 (3H, s, CO_2Me), 6.48 (1H, dd, *J* 6.7, 1.7 Hz, H-5), 6.78 (1H, dd, *J* 0.6, 1.7 Hz, H-3), 7.48 (1H, dd, *J* 0.6, 6.7 Hz, H-6), 11.80 (1H, br s, OH); ^{13}C NMR (DMSO) δ 53.1, 103.4, 121.5, 137.2, 141.6, 162.5, 165.2; HRMS calcd for $C_7H_8NO_3$: MH^+, 154.0515. Found: MH^+ 154.0504.

1-(1-Methoxycarbonyl-ethyl)-2-oxo-1,2-dihydro-pyridine-4-carboxylic acid methyl ester (19). Methyl-2-bromopropionate (0.85 mmol, 95 μl) was added dropwise to a solution of **18** (100 mg, 0.65 mmol) and anhydrous potassium carbonate (0.13 mmol, 18 mg) in dry DMF (5 ml) and the reaction was heated to 40 °C for 16 h. The reaction was allowed to cool to 22 °C before dilution with ethyl acetate (20 ml). The reaction mixture was washed with water (5 × 20 ml), dried ($MgSO_4$) and the solvent removed *in vacuo*. Purification by column chromatography (eluent 6 : 1 v/v ethyl acetate–hexane) gave **19** as a colourless oil. (40 mg, 26%).

R_F [6 : 1 v/v ethyl acetate–hexane] = 0.50; $\nu_{max.}$ (ATR): 3082 (Ar C–H stretch), 1730, 1665 (C=O, str), 1592, 1538 (C=C, ar) cm^{-1}; ^{1}H NMR (CD_3OD) δ 1.66 (3H, d, *J* 7.2 Hz, CH_3), 3.71 (3H, s, CO_2Me), 3.90 (3H, s, CO_2Me), 5.24 (1H, q, *J* 7.2 Hz, CH), 6.80 (1H, dd, *J* 7.1, 1.8 Hz, H-5), 7.06 (1H, d, *J* 1.8 Hz, H-3), 7.76 (1H, d, *J* 7.1 Hz, H-6); nOe: Irradiation of the doublet at 1.66 ppm caused enhancement of the doublet at 7.76 ppm (H-6), thus confirming the regiochemistry of the product; ^{13}C NMR (CD_3OD) δ 16.3, 53.6, 53.8, 58.7, 106.9, 122.4, 139.3, 143.5, 164.4, 166.4, 172.1; HRMS calcd for $C_{11}H_{13}NO_5$: M^+, 239.0794. Found: M^+, 239.0787.

1-(1-Carboxy-ethyl)-2-oxo-1,2-dihydro-pyridine-4-carboxylate (15). Potassium hydroxide (33 mg, 0.59 mmol) in milliQ water (2 ml) was added dropwise to a solution of **19** (35 mg, 0.15 mmol) in freshly distilled THF (2 ml) and the reaction was heated to 40 °C for 3 h. The reaction was allowed to cool to 22 °C before dilution with water (3 ml). The aqueous fraction was washed with dichloromethane (5 ml) before acidifying to pH 7.5 with 0.1 M HCl. The water was lyophilised to give the desired product (**15**) as a white solid. (0.13 mmol, 83%).

$\nu_{max.}$ (ATR): 3330 (br, acid OH stretch), 1610, 1558 (C=O, str), 1396, 1337 (C=C, ar) cm^{-1}; ^1H NMR (D_2O) δ 1.56 (3H, d, J 7.4 Hz, CH_3), 5.11 (1H, q, J 7.4 Hz, CH), 6.75 (1H, dd, J 7.1, 1.6 Hz, H-5), 7.06 (1H, d, J 1.6 Hz, H-3), 7.76 (1H, d, J 7.1 Hz, H-6); ^{13}C NMR (CD_3OD) δ 16.9, 58.4, 108.1, 118.2, 136.8, 149.7, 164.7, 173.0, 177.8; HRMS calcd for $C_9H_7NO_5NaK_2$: MNaK$_2^+$, 309.9496. Found: MNaK$_2^+$ 309.9510.

1-(2-Methoxycarbonylallyl)-2-oxo-1,2-dihydro-pyridine-4-carboxylic acid methyl ester (20). Methyl-(2-bromomethyl)acrylate (81 µl, 0.68 mmol) was added dropwise to a solution of **18** (80 mg, 0.52 mmol) and anhydrous potassium carbonate (14 mg, 0.10 mmol) in dry DMF (4 ml) and the reaction was heated to 40 °C for 16 h. The reaction was allowed to cool to 22 °C before dilution with ethyl acetate (20 ml). The reaction mixture was washed with water (5 × 20 ml), dried ($MgSO_4$) and the solvent removed *in vacuo*. Purification by column chromatography (eluent 6 : 1 v/v ethyl acetate–hexane) gave **20** as a colourless oil. (94 mg, 72%).

R_F [6 : 1 v/v ethyl acetate–hexane] = 0.50; $\nu_{max.}$ (ATR): 3128 (Ar C–H stretch), 1731, 1706 (C=O, str), 1667, 1639, 1581, 1540 (C=C, ar), 1437 (C=C) cm^{-1}; ^1H NMR (CD_3OD) δ 3.76 (3H, s, CO_2Me), 3.90 (3H, s, CO_2Me), 4.83 (2H, s, CH_2), 5.62 (1H, s, C*H*H), 6.35 (1H, s, CH*H*), 6.77 (1H, dd, J 7.1, 1.8 Hz, H-5), 7.08 (1H, d, J 1.8 Hz, H-3), 7.74 (1H, d, J 7.1 Hz, H-6); ^{13}C NMR (CD_3OD) δ 51.4, 53.1, 53.8, 106.7, 122.6, 129.3, 136.5, 141.5, 143.5, 164.6, 166.4, 167.5; HRMS calcd for $C_{12}H_{13}NO_5Na$: MNa$^+$, 274.0691. Found: MNa$^+$ 274.0683.

1-(2-carboxy-allyl)-2-oxo-1,2-dihydro-pyridine-4-carboxylate (16). Potassium hydroxide (84 mg, 1.50 mmol) in milliQ water (2 ml) was added dropwise to a solution of **20** (94 mg, 0.38 mmol) in freshly distilled THF (2 ml) and the reaction was heated to 40 °C for 3 h. The reaction was allowed to cool to 22 °C before dilution with water (5 ml). The aqueous fraction was washed with dichloromethane (5 ml) before acidifying to pH 7.5 with 0.1 M HCl. The water was lyophilised to give the desired product (**16**) as a white solid (0.34 mmol, 89%).

$\nu_{max.}$ (ATR): 3342 (br, acid OH stretch), 1661, 1567 (C=O, str), 1527, 1474 (C=C, ar) cm^{-1}; ^1H NMR (D_2O) δ 4.77 (2H, s, CH_2), 5.01 (1H, s, C*H*H), 5.91 (1H, s, CH*H*), 6.77 (1H, dd, J 6.9, 1.8 Hz, H-5), 6.90 (1H, d, J 1.8 Hz, H-3), 7.62 (1H, d, J 6.9 Hz, H-6); ^{13}C NMR (D_2O) δ 51.4, 108.6, 118.7, 122.7, 139.7, 140.1, 150.4, 164.7, 172.7, 173.3; HRMS calcd for $C_{10}H_7NO_5NaK_2$: MNaK$_2^+$, 321.9496. Found: MNaK$_2^+$ 321.9482.

2-(1-Methoxycarbonylmethyl-amino)-isonicotinic acid methyl ester (21). A solution of glyoxal (300 µl of a 40% wt solution in water, 1.97 mmol) in methanol (3 ml) was added in one portion over a slurry of methyl-2-aminopyridine-4-carboxylate (300 mg, 1.97 mmol) in 60% perchloric acid (0.6 ml). The resulting solution was heated to reflux for 48 h. The reaction was allowed to cool to 22 °C before neutralising by the dropwise addition of saturated aqueous sodium bicarbonate solution. The solution was extracted with ethyl acetate (2 × 10 ml), the combined organic fractions washed with brine (20 ml), dried ($MgSO_4$) and the solvent removed *in vacuo*. Purification by column chromatography (eluent 1 : 1 v/v petroleum ether/ethyl acetate) gave **21** as a pale yellow solid. (224 mg, 51%).

R_F [1 : 1 v/v petroleum ether/ethyl acetate] = 0.57; $\nu_{max.}$ (ATR): 3390 (N–H stretch), 1748, 1726 (C=O, str), 1620, 1560, 1521 (C=C, ar) cm^{-1}; ^1H NMR ($CDCl_3$) δ 3.71 (3H, s, CO_2Me), 3.85 (3H, s, CO_2Me), 4.13 (2H, d, J 5.6 Hz, CH_2), 5.32 (1H, t, J 5.6 Hz, NH), 7.00 (1H, m, ArH), 7.06 (1H, dd, J 1.3, 5.3 Hz, ArH), 8.13 (1H, dd, J 0.5, 5.3 Hz, ArH); ^{13}C NMR ($CDCl_3$) δ 45.8, 54.5, 54.8, 110.9, 114.7, 140.9, 151.0, 160.5, 168.4, 174.0; HRMS calcd for $C_{10}H_{12}N_2O_4Na$: MNa$^+$, 247.0695. Found: MNa$^+$ 247.0696.

2-(Carboxymethyl-amino)-isonicotinate (10). Potassium hydroxide (84 mg, 1.50 mmol) in milliQ water (6 ml) was added dropwise to a solution of **21** (112 mg, 0.50 mmol) in freshly distilled THF (6 ml) and the reaction was heated to 40 °C for 3 h. The reaction was allowed to cool to 22 °C before dilution with water (7 ml). The aqueous fraction was washed with dichloromethane (14 ml) before acidifying to pH 7.5 with 0.1 M HCl. The water was lyophilised to give **10** as a yellow/brown solid. (0.47 mmol, 93%)

$\nu_{max.}$ (ATR): 3275 (br, acid OH stretch), 1595, 1545 (C=O, str), 1502, 1388 (C=C, ar) cm^{-1}; ^1H NMR (D_2O) δ 3.80 (2H, s, CH_2), 6.81 (1H, s, H-3), 6.89 (1H, d, J 5.4 Hz, ArH), 7.91 (1H, d, J 5.4 Hz, ArH); ^{13}C NMR (D_2O) δ 46.1, 108.4, 112.5, 147.0, 147.3, 159.2, 174.3, 179.0; HRMS calcd for $C_8H_6N_2O_4NaK_2$: MNaK$_2^+$, 294.9499. Found: MNaK$_2^+$ 294.9486.

2-(1-Methoxycarbonyl-ethylamino)-isonicotinic acid methyl ester (22). A solution of pyruvic aldehyde (355 µl of a 40% wt solution in water, 1.97 mmol) in methanol (2 ml) was added in one portion over a slurry of methyl-2-aminopyridine-4-carboxylate (300 mg, 1.97 mmol) in 60% perchloric acid. The resulting solution was heated to reflux for 36 h. The reaction was allowed to cool to 22 °C before neutralising by the dropwise addition of saturated sodium bicarbonate solution. The solution was extracted with ethyl acetate (2 × 10 ml), the combined organic fractions washed with brine (20 ml), dried ($MgSO_4$) and the solvent removed *in vacuo*. Purification by column chromatography (eluent 1 : 1 v/v petroleum ether/ethyl acetate) afforded **22** as a pale yellow solid. (160 mg, 35%).

R_F [1 : 1 v/v petroleum ether/ethyl acetate] = 0.67; $\nu_{max.}$ (ATR): 3387 (N–H stretch), 1747, 1715 (C=O, str), 1614, 1564, 1516 (C=C, ar) cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.44 (3H, d, J 7.1 Hz, CH_3), 3.70 (3H, s, CO_2Me), 3.85 (3H, s, CO_2Me), 4.60 (1H, dq, J 7.1, 7.6 Hz, CH), 5.23 (1H, d, J 7.6 Hz, NH), 6.99 (1H, d, J 1.3 Hz, H-3), 7.03 (1H, dd, J 1.3, 5.2 Hz, H-5), 8.12 (1H, d, J 5.2 Hz, H-6); ^{13}C NMR ($CDCl_3$) δ 18.8, 50.3, 52.6, 109.0, 112.6, 138.9, 149.1, 158.3, 166.4, 175.2, one undetected double-up; HRMS calcd for $C_{11}H_{14}N_2O_4Na$: MNa$^+$, 261.0851. Found: MNa$^+$ 261.0840.

2-(1-Carboxy-ethylamino)-isonicotinate (11). Potassium hydroxide (53 mg, 0.94 mmol) in milliQ water (4 ml) was added dropwise to a solution of **22** (75 mg, 0.32 mmol) in THF (4 ml) and the reaction was heated to 40 °C for 3 h. The reaction was allowed to cool to 22 °C before dilution with water (7 ml). The aqueous fraction was washed with dichloromethane (20 ml) before acidifying to pH 7.5 with 0.1 M HCl. The water was lyophilised to give **11** as a yellow solid. (0.26 mmol, 82%).

$\nu_{max.}$ (ATR): 3274 (br, acid OH stretch), 2946 (N–H stretch), 1586, 1549 (C=O, str), 1440, 1393 (C=C, ar) cm^{-1}; ^1H NMR (D_2O) δ 1.38 (3H, d, J 7.1 Hz, CH_3), 4.07 (1H, q, J 7.1 Hz CH), 6.83 (1H, s, H-3), 6.92 (1H, d, J 5.0 Hz, ArH), 7.93 (1H, d, J 5.5 Hz, ArH); ^{13}C NMR (D_2O) δ 18.6, 53.4, 108.7, 112.6, 147.1, 158.7, 174.2, 182.6, one undetected double-up; HRMS calcd for $C_9H_8N_2O_4NaK_2$: MNaK$_2^+$, 308.9656. Found: MNaK$_2^+$ 308.9632.

6-Hydroxy-nicotinic acid methyl ester (24). To a suspension of 6-hydroxynicotinic acid (5 g, 0.04 mol) in methanol (36 ml) was added dropwise concentrated sulfuric acid (3.6 ml). The solution was heated at reflux under nitrogen for 18 h. The reaction was allowed to cool to 22 °C before dilution with water (20 ml). Saturated aqueous sodium carbonate solution was added dropwise until the solution reached pH 12. The aqueous fraction was extracted with ethyl acetate (3 × 30 ml). The combined organic fractions were dried and the solvent removed *in vacuo* to afford **24** as a white crystalline solid. (4.1 g, 74%).

$\nu_{max.}$ (ATR): 3012 (OH stretch) 3083, 2957 (Ar C–H stretch), 1722, 1704 (C=O, str), 1677, 1639, 1590 (C=C, ar) cm^{-1}; ^1H NMR (MeOD) δ 3.82 (3H, s, CO_2Me), 6.50 (1H, d, *J* 9.7 Hz, H-3), 7.97 (1H, dd, *J* 2.6, 9.7 Hz, H-4), 8.12 (1H, d, *J* 2.6 Hz, H-6). ^{13}C NMR (MeOD) δ 52.4, 111.7, 120.3, 141.2, 141.8, 165.5, 165.9; HRMS calcd for $C_7H_7NO_3Na$: *M*Na^+, 176.0324. Found: MNa^+, 176.0325.

1-Methoxycarbonylmethyl-6-oxo-1,6-dihydro-pyridine-3-carboxylic acid methyl ester (25). Methylbromoacetate (0.34 ml, 3.59 mmol) was added dropwise to a solution of **24** (500 mg, 3.23 mmol) and potassium carbonate (90 mg, 0.65 mmol) in DMF (20 ml) and the reaction was heated to 40 °C for 18 h. The reaction was allowed to cool to 22 °C before dilution with ethyl acetate (50 ml). The organic fraction was washed with water (5 × 50 ml), dried ($MgSO_4$) and the solvent removed *in vacuo*. Purification was achieved by column chromatography (eluent 4 : 1 v/v ethyl acetate–hexane) to afford **25** as a colourless oil. (300 mg, 48%).

R_F [4 : 1 ethyl acetate–hexane] = 0.36; $\nu_{max.}$ (ATR): 3078, 2955 (Ar C–H stretch), 1749, 1716 (C=O, str), 1659, 1612, 1542 (C=C, ar) cm^{-1}; ^1H NMR (MeOD) δ 3.75 (3H, s, CO_2Me), 3.82 (3H, s, CO_2Me), 4.80 (2H, s, CH_2), 6.52 (1H, d, *J* 9.6 Hz, H-3), 7.94 (1H, dd, *J* 2.5, 9.6 Hz, H-4), 8.42 (1H, d, *J* 2.5 Hz, H-6); nOe: Irradiation of the singlet at 4.80 ppm caused enhancement of the doublet at 8.42 ppm (H-6), thus confirming the regiochemistry of the product; ^{13}C NMR (MeOD) δ 51.7, 52.4, 52.9, 111.5, 119.5, 140.8, 145.4, 164.1, 165.6, 169.2; HRMS calcd for $C_{10}H_{11}NO_5Na$: *M*Na^+, 248.0535. Found: MNa^+, 248.0525.

1-Carboxymethyl-6-oxo-1,6-dihydro-pyridine-3-carboxylate (12). Potassium hydroxide (79 mg, 1.41 mmol) in milliQ water (4 ml) was added dropwise to a solution **25** (80 mg, 0.35 mmol) in THF (4 ml) and the reaction was heated to 40 °C for 3 h. The reaction was allowed to cool to 22 °C before dilution with milliQ water (10 ml). The solution was washed with ethyl acetate (15 ml) before the aqueous phase was acidified to pH 8 by the dropwise addition of 0.1 M HCl. The solution was lyophilised to give **12** as a white solid (0.31 mmol, 90%).

$\nu_{max.}$ (ATR): 3369 br. (acid O–H str.), 1660, 1625 (C=O, str), 1554 (C=C) cm^{-1}; ^1H NMR (D_2O) δ 4.42 (2H, s, CH_2), 6.44 (1H, d, *J* 9.3 Hz, H-5), 7.87 (1H, dd, *J* 2.3, 9.3 Hz, H-4), 7.99 (1H, d, *J* 2.3 Hz, H-2); ^{13}C NMR (D_2O) δ 54.5, 118.4, 142.3, 143.0, 164.7, 172.1, 181.8, 188.2; HRMS calcd for $C_8H_7NO_5K$: *M*K^+, 235.9956. Found: MK^+, 235.9955.

1-(1-Methoxycarbonyl-ethyl)-6-oxo-1,6-dihydro-pyridine-3-carboxylic acid methyl ester (26). Methyl-2-bromopropionate (0.40 ml, 3.59 mmol) was added dropwise to a solution of **24** (500 mg, 3.23 mmol) and potassium carbonate (90 mg, 0.65 mmol) in DMF (20 ml) and the reaction was heated to 40 °C for 18 h. The reaction was allowed to cool to 22 °C before dilution with ethyl acetate (50 ml). The organic fraction was washed with water (5 × 50 ml). The organic fraction was dried ($MgSO_4$) and the solvent removed *in vacuo*. Purification was achieved by column chromatography (eluent 1 : 1 v/v ethyl acetate–hexane) to afford the **26** as a colourless oil. (160 mg, 26%).

R_F [1 : 1 ethyl acetate–hexane] = 0.35; $\nu_{max.}$ (ATR): 3078, 2954 (Ar C–H stretch), 1744, 1717 (C=O, str), 1661, 1611, 1542 (C=C, ar) cm^{-1}; ^1H NMR (MeOD) δ 1.68 (3H, d, *J* 7.2 Hz, CH_3), 3.72 (3H, s, CO_2Me), 3.85 (3H, s, CO_2Me), 5.26 (1H, q, *J* 7.2 Hz, CH), 6.51 (1H, d, *J* 9.5 Hz, H-3), 7.93 (1H, dd, J 2.4, 9.5 Hz, H-4), 8.42 (1H, d, *J* 2.4 Hz, H-6); nOe: Irradiation of the singlet at 8.42 ppm (H-6), caused enhancement of the doublet at 1.68 ppm and the quartet at 5.26 ppm thus confirming the regiochemistry of the product; ^{13}C NMR (MeOD) δ 15.7, 52.3, 52.9, 58.4, 111.6, 119.7, 140.3, 143.0, 163.7, 165.6, 171.3; HRMS calcd for $C_{11}H_{13}NO_5Na$: *M*Na^+, 262.0691. Found: MNa^+, 262.0698.

1-(1-Carboxy-ethyl)-6-oxo-1,6-dihydro-pyridine-3-carboxylate (13). Potassium hydroxide (93 mg, 1.67 mol) in milliQ water (5 ml) was added dropwise to a solution of **26** (100 mg, 0.42 mmol) in THF (5 ml) and the reaction was heated to 40 °C for 3 h. The reaction was allowed to cool to 22 °C before dilution with milliQ water (10 ml). The solution was washed with ethyl acetate (15 ml) before the aqueous phase was acidified to pH 8 by the dropwise addition of 0.1 M HCl. The solution was lyophilised to give **13** as a pale yellow solid (0.35 mmol, 83%).

$\nu_{max.}$ (ATR): 3427 br. (acid O–H str.), 1651, 1609 (C=O, str), 1588, 1568 (C=C, ar) cm^{-1}; ^1H NMR (D_2O) δ 1.51 (3H, d, *J* 7.4 Hz, CH_3), 4.98 (1H, q, *J* 7.4 Hz, CH), 6.43 (1H, d, *J* 9.3 Hz, H-3), 7.84 (1H, dd, *J* 2.4, 9.3 Hz, H-4), 8.09 (1H, d, *J* 2.4 Hz, H-6); ^{13}C NMR (D_2O) δ 16.7, 58.8, 117.9, 139.7, 141.6, 164.7, 172.3, 177.8, 181.8; HRMS calcd for $C_9H_9NO_5K$: *M*K^+, 250.0112. Found: MK^+, 250.0113.

1-(2-Methoxycarbonyl-allyl)-6-oxo-1,6-dihydro-pyridine-4-carboxylic acid methyl ester (27). Methyl-(2-bromomethyl)acrylate (0.21 ml, 1.79 mmol) was added dropwise to a solution of **24** (250 mg, 1.63 mmol) and potassium carbonate (45 mg, 0.33 mmol) in DMF (10 ml) and the reaction was heated to 40 °C for 18 h. The reaction was allowed to cool to 22 °C before dilution with ethyl acetate (25 ml). The organic fraction was washed with water (5 × 25 ml), dried ($MgSO_4$) and the solvent removed *in vacuo*. Purification was achieved by column chromatography (eluent 4 : 1 v/v ethyl acetate–hexane) to **27** as a white solid. (180 mg, 44%).

R_F [4 : 1 ethyl acetate–hexane] = 0.37; $\nu_{max.}$ (ATR): 3038, 2955 (Ar C–H stretch), 1709 (C=O, str), 1661, 1644, 1612, 1538 (C=C, ar) cm^{-1}; ^1H NMR (MeOD) δ 3.77 (3H, s, CO_2Me), 3.83 (3H, s, CO_2Me), 4.86 (2H, s, CH_2), 5.70 (1H, s, C*H*H), 6.37 (1H, s, CH*H*), 6.51 (1H, d, *J* 9.5 Hz, H-3), 7.92 (1H, dd, *J* 2.5, 9.5 Hz, H-4), 8.48 (1H, d, *J* 2.5 Hz, H-6); nOe: Irradiation of the singlet at 8.48 ppm (H-6), caused enhancement of the singlet at 4.86 ppm thus confirming the regiochemistry of the product; ^{13}C NMR (MeOD) δ 15.7, 52.3, 52.9, 58.4, 111.6, 119.7, 140.3, 143.0, 163.7, 165.6, 171.3, one undetected double-up.

1-(2-Carboxy-allyl)-6-oxo-1,6-dihydro-pyridine-3-carboxylate (14). Potassium hydroxide (160 mg, 2.84 mol) in milliQ water (7 ml) was added dropwise to a solution of **27** (180 mg, 0.71 mmol) in THF (7 ml) and the reaction was heated to 40 °C for 3 h. The

reaction was allowed to cool to 22 °C before dilution with milliQ water (10 ml). The solution was washed with ethyl acetate (15 ml) before the aqueous phase was acidified to pH 8 by the dropwise addition of 0.1 M HCl. The solution was lyophilised to give **14** as a white solid. (0.63 mmol, 89%).

$\nu_{max.}$ (ATR): 3328 br. (acid O–H str.), 1674, 1606 (C=O, str) 1558, 1563 (C=C, ar) cm^{-1}; ^1H NMR (D_2O) δ 4.68 (2H, s, CH_2), 4.91 (1H, s, C=C*H*H), 5.80 (1H, s, C=CH*H*), 6.46 (1H, d, *J* 9.4 Hz, H-3), 7.96 (1H, dd, *J* 2.3, 9.4 Hz, H-4), 8.02 (1H, d, *J* 2.3 Hz, H-6); ^{13}C NMR (D_2O) δ 51.3, 117.7, 121.3, 140.0, 141.4, 141.7, 163.8, 171.1, 172.9, 181.0; HRMS calcd for $C_{10}H_9NO_5K$: MK^+, 262.0112. Found: MK^+, 262.0110.

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