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Design and Structural Analysis of Aromatic Inhibitors of Type II Dehydroquinase from *Mycobacterium tuberculosis*

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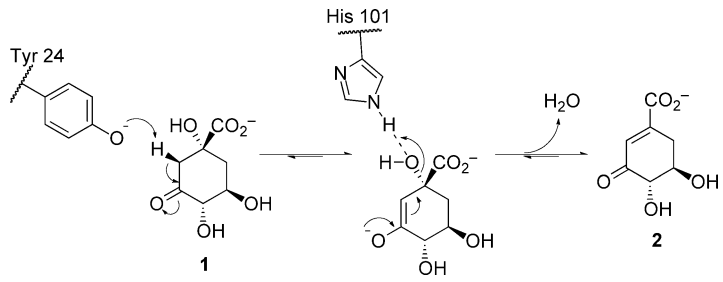
3-Dehydroquinase, the third enzyme in the shikimate pathway, is a potential target for drugs against tuberculosis. Whilst a number of potent inhibitors of the *Mycobacterium tuberculosis* enzyme based on a 3-dehydroquinate core have been identified, they generally show little or no in vivo activity, and were synthetically complex to prepare. This report describes studies to develop tractable and drug-like aromatic analogues of the most potent inhibitors. A range of carbon–carbon linked biaryl analogues were prepared to investigate the effect of hydrogen bond acceptor and donor patterns on inhibition. These exhibited inhibitory activity in the high-micromolar range. The addition of flexible linkers in the compounds led to the identification of more potent 3-nitrobenzylgallate- and 5-aminoisophthalate-based analogues.

Introduction

The shikimate pathway is the essential sequential seven-step enzymatic pathway that converts erythrose-4-phosphate and phosphoenol pyruvate into chorismate, the branch point in the biosynthesis of the aromatic amino acids and other aromatic metabolites in plants, fungi, and bacteria.[1–3] The lack of this pathway in mammals makes it an attractive target for the development of new antimicrobials.[1,4] Dehydroquinase (3-dehydroquinate dehydratase, EC 4.2.1.10), the third enzyme in the pathway, catalyzes the conversion of 3-dehydroquinate (**1**) into 3-dehydroshikimate (**2**) (Scheme 1).

There are two forms of the enzyme that catalyze the same reaction, but by differing mechanisms.[5] Type II dehydroquinases are homododecameric enzymes that catalyze the *anti*-elimination of water via an enol intermediate (Scheme 1).[6–9] These are structurally and mechanistically very different from type I dehydroquinases, which are dimeric and catalyze the *syn*-elimination of water via a Schiff base formed with a conserved lysine residue.[10,11] The type II enzyme has been identified in several pathogenic bacteria, including *Mycobacterium tuberculosis*[12,13] and *Helicobacter pylori* (gastritis, stomach ulcers),[14] that are responsible for enormous mortality and economic loss across the world, especially in developing countries. Consequently, this enzyme has become an important target for rational inhibitor design in the past few years.[15–22]



Scheme 1. Catalytic mechanism of the type II 3-dehydroquinase from *Mycobacterium tuberculosis*.

Early studies with substrate analogues **3** and **4** (Figure 1), that have K_i values of 600 and 30 μM, respectively, against the *Streptomyces coelicolor* enzyme,[15] demonstrated that the flattening of the six-membered ring in going from quinate to anhydroquinate was beneficial to binding. Elaboration of the anhydroquinate core from the 3-position resulted in potent (nanomolar) and selective inhibitors of type II dehydroquinase, with K_i values against the *M. tuberculosis* form of the enzyme of 940 nM for the dicarboxylate **5**,[18] 54 nM for the 3-nitrophenyl analogue **6**,[17] 250 nM for the 2-thienyl analogue **7**,[22] 490 nM for the biaryl analogue **8**,[20] 11 μM for the benzophenone analogue **9**,[20] and 140 nM for the alkenyl analogue **10**,[21] (Figure 1). However, the use of these compounds as antimicrobials is not straightforward. Their hydrophilicity and charge hinder bioavailability, making them unlikely to pass though the *M. tuberculosis* cell wall efficiently.

Attempts to improve the bioavailability of such compounds by use of propyl ester-based prodrugs shows promise.[23] However, the most potent compounds involve multistep syntheses from quinic acid, requiring several protection and deprotection steps. The cost of goods suggests this may not be a way for-

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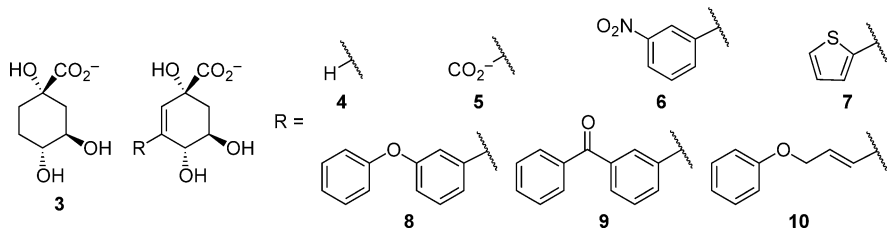


Figure 1. Examples of previously published 3-dehydroquinase inhibitors.[15,17,18,20–22]

ward for antibiotics intended for use in developing countries. In this study, we explored new avenues of inhibitor design by investigating the impact of flattening the core anhydroquinate ring further into aromatic structures. The use of simple aromatic structures as a substitute for the complex shikimate ring in the synthesis of potent inhibitors of 5-enolpyruvoylshikimate-3-phosphate synthase, the sixth enzyme on the shikimate pathway, has been previously demonstrated by Miller et al.[24]

Studies on 5-deoxy- and 4,5-dideoxydehydroquinate have suggested that the C5 hydroxy group of the natural substrate forms key hydrogen bond interactions to the enzyme upon binding, whereas attempts to define the role played by the C4 hydroxy were inconclusive.[25] As a result, replacement of the quinate core moiety with analogues based on gallate **11**, protocatechuate **12**, 3,5-dihydroxybenzoate **13**, 5-hydroxyisophthalate **14**, citrazinate **15**, 6-hydroxynicotinate **16**, and 5-aminoisophthalate **17** were investigated in order to further study the role that the patterns of hydrogen bond donors and acceptors have on binding and inhibition (Figure 2).

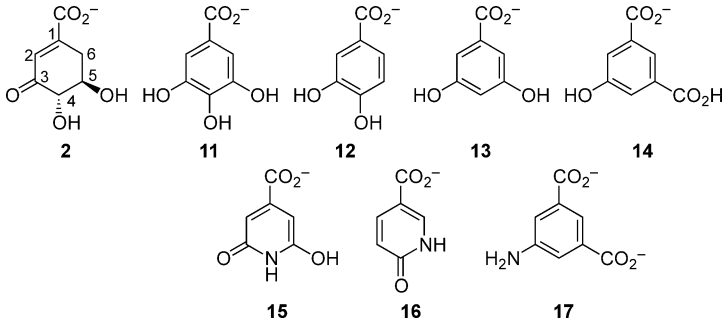


Figure 2. 3-Dehydroshikimate **2** and potential aromatic core mimetics **11**–**17**.

The rationale for each series is as follows: analogues of gallate **11** with side chains attached at the C3-hydroxy position have hydrogen bond donors at both the C4 and C5 positions, the oxygen atom at C3 becomes a hydrogen bond acceptor, and replacement of the C3 oxygen with an amine changes that position to a hydrogen bond donor. Procatechuate **12** and 3,5-dihydroxybenzoate **13** analogues were chosen to remove a hydrogen bond donor at the C4 and C5 positions, respectively. Isophthalates **14** and **17** were chosen to introduce a functional group at the C5 position, where its hydrogen bonding oxygen atoms can move away from the planarity of the core moiety, potentially allowing the carboxylate group greater freedom to adopt favorable hydrogen bond interactions rather than requiring the core unit to be conformationally ideal. The isophthalates differ in that the C5 oxygen atom of **14** will act as a hydrogen bond acceptor upon alkylation, whilst the amine of **17** will still act as a hydrogen bond donor.

Citrazinate **15** and 6-hydroxynicotinate **16** are structurally related compounds that can exist as lactam/lactim tautomers which have reversible hydrogen bond donor and acceptor arrangements at the 1- and 6-positions (Figure 3). They differ from the relative position of the

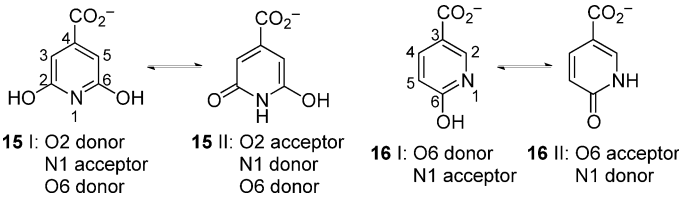


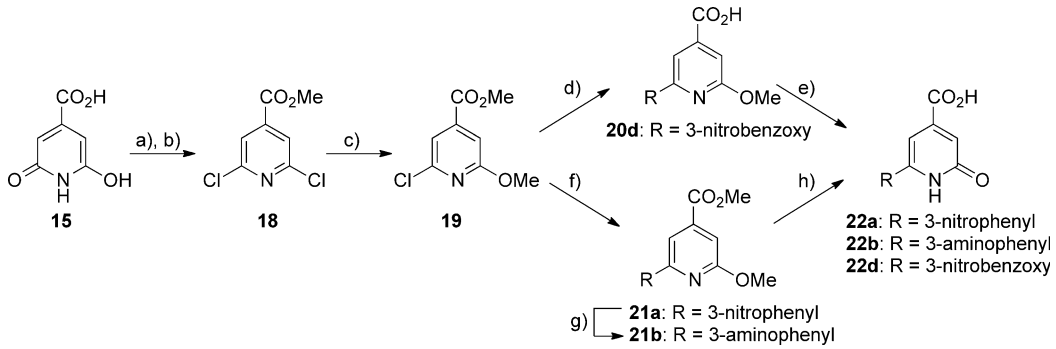
Figure 3. Tautomers of citrazinate **15** and 6-hydroxynicotinate **16**. Lactim (I) and lactam (II) (based on atom arrangement at the 1- and 6-positions).

carboxylate to the ring nitrogen, *para* in the case of citrazinate **15** and *meta* for 6-hydroxynicotinate **16**. The lactam tautomer (II) is expected to be the predominant species in aqueous solution, but it is possible that the compound can bind as the lactim (I) if that hydrogen bond arrangement were more favorable.

Results and Discussion

Synthesis

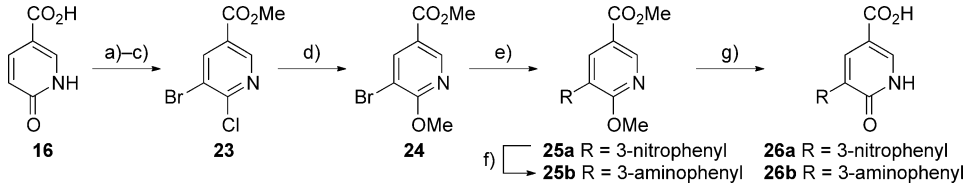
The synthesis of analogues incorporating a citrazinate core moiety is shown in Scheme 2. Methyl 2-chloro-6-methoxyisonicotinate **19** was prepared from citrazinic acid **15** by heating with phosphorous oxychloride and tetramethylammonium chloride,[26] followed by methanol and sulfuric acid to give methyl 2,6-dichloroisonicotinate **18** in 50% yield for the two steps. Displacement of one of the chlorines was achieved by heating with sodium methoxide at 80 °C to give **19** in 52% yield. The copper-catalyzed Ullmann reaction,[27] between methyl 2-chloro-6-methoxyisonicotinate **19** and 3-nitrobenzyl alcohol generated the free acid **20d** rather than the corresponding methyl ester in a modest 7% yield. Presumably the ester was cleaved during the aqueous workup, which may also explain the low yield. The methyl ether of **20d** was deprotected by treatment with iodotrimethylsilane, formed in situ with chlorotrimethylsilane and sodium iodide, to give the 3-nitrobenoxy analogue **22d** in 82% yield. The 3-nitrophenyl side chain was attached by palladium-catalyzed Suzuki coupling[28] between



Scheme 2. *Reagents and conditions*: a) $POCl_3$, Me_4NCl, 130 °C; b) MeOH, H_2SO_4, reflux; c) NaOMe, MeOH, reflux; d) 3-nitrobenzyl alcohol, Cu, CuI, K_2CO_3, pyridine, 100 °C; e) TMSCl, NaI, MeCN, 65 °C; f) 3-nitrophenylboronic acid, $Pd(Ph_3)_4$, Et_3N, DMF, 100 °C; g) H_2, Pd/C, EtOAc, RT; h) HBr, AcOH, reflux. See Experimental Section for reaction times and yields.

methyl 2-chloro-6-methoxyisonicotinate **19** and 3-nitrophenylboronate to give compound **21a** in 33% yield. Palladium-catalyzed hydrogenation of **21a** gave the 3-amino analogue **21b** in quantitative yield. Both 3-nitro and 3-amino analogues **21a** and **21b** were deprotected in one step by heating at reflux in hydrobromic acid and acetic acid to give **22a** and **22b** as the hydrobromide salts in quantitative and 71% yields, respectively.

The synthesis of analogues incorporating a 6-hydroxynicotinate core was carried out as shown in Scheme 3. Synthetic routes to the 2-pyridone derivatives were based on published procedures.[29] Starting from 6-hydroxynicotinic acid **16**, methyl 5-bromo-6-chloronicotinate **23** was synthesized in 66% yield over three steps. In contrast to the published procedure,[28] it was found that the introduction of the methoxy group by aromatic nucleophilic substitution of **23** gave low yields at room temperature if methanol was used as the solvent (27% of the desired product **24** and 54% of recovered starting material). A slightly better yield of **24** (38%) was obtained by heating **23** with two equivalents of sodium methoxide in 1,4-dioxane at reflux. Suzuki coupling of the bromide **24** with 3-nitrophenylboronic acid formed the desired product **25a**. This was difficult to separate from decomposition products of the boronic acid and was consequently obtained in a rather low yield of 32%, despite the same reaction with phenylboronic acid having been described in 86% yield.[29] Hydrogenation with palladium on charcoal of the nitro group in **25a** afforded the amine derivative **25b**. Deprotection of both the methyl ether and methyl esters of **25a** and **25b** was achieved by heating with hydrobromic acid in acetic acid at reflux to give **26a** and **26b** in 94 and 71% yields, respectively.

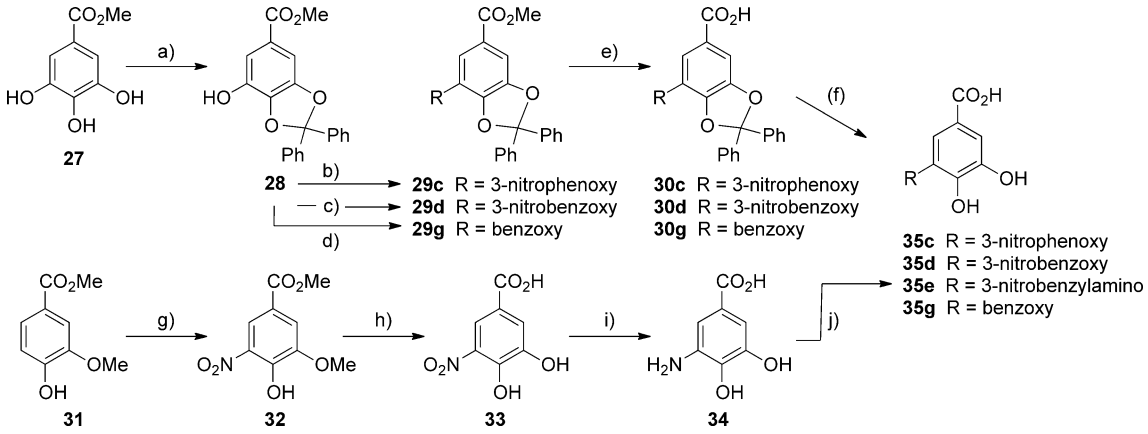


Scheme 3. *Reagents and conditions*: a) Br_2, AcOH, 50 °C; b) PCl_5, $POCl_3$, reflux; c) MeOH, CH_2Cl_2, reflux; d) NaOMe, MeOH, 1,4-dioxane, reflux; e) 3-nitrophenyl boronic acid, $Pd(OAc)_2$, PPh_3, Et_3N, DMF, 100 °C; f) H_2, Pd/C, EtOAc, RT; g) HBr, AcOH, reflux. See Experimental Section for reaction times and yields.

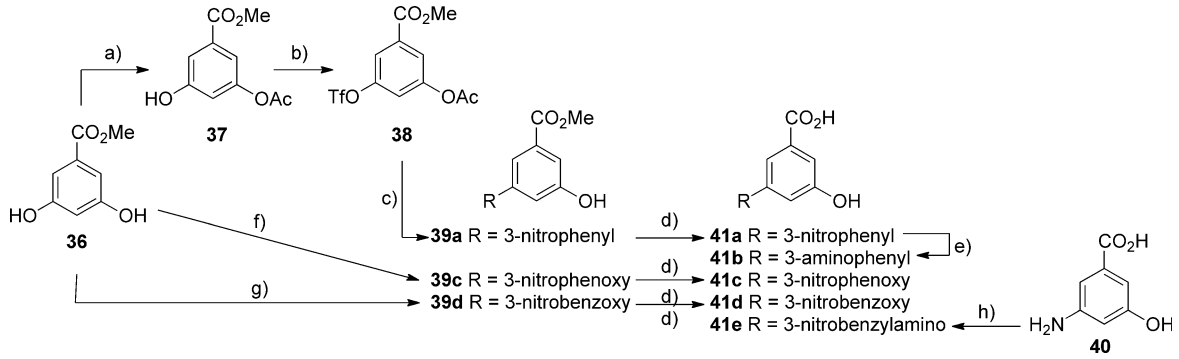
Analogues based on a gallate core were synthesized as outlined in Scheme 4. Two of the three aromatic hydroxy groups of the commercially available methyl gallate **27** were protected as the diphenyl acetonide **28** by treatment with α,α-dichlorodiphenylmethane in 46% yield. Treatment of protected gallate **28** with 1-fluoro-3-nitrobenzene, trimethylsilyl diethylamine and the strong phosphazene base *t*Bu-P4 gave the diaryl ether **29c** in 26% yield using the method of Ebisawa et al.[30] The benzylic side chains were attached by reaction of the protected gallate **28** with 3-nitrobenzyl- or benzyl bromide to give the intermediates **29d** and **29g** in 41 and 51% yields, respectively. The methyl esters **29c**, **29d**, and **29g** were deprotected by treatment with aqueous base and worked up to give the free acids **30c**, **30d**, and **30g** in yields between 30 and 77%. Subsequent deprotection of the diphenyl acetonide with trifluoroacetic acid gave final compounds **35c**, **35d**, and **35g** in 73–96% yields. It was necessary to conduct the deprotection in this order, as removal of the diphenyl acetonide results in methyl esters that are stabilized by electron donation from the gallate *para*-hydroxy group, and could not be cleaved by any standard chemistry that would not also cleave the ether-linked side chains.[31]

The 5-amino analogue **35e** was prepared from methyl vanillate **31** in four steps. Nitration at the 5-position of **31** with a mixture of nitric acid and acetic acid afforded **32** in 58% yield. Both the methyl ester and methyl ether of **32** were deprotected by heating in 48% aqueous hydrogen bromide solution to give free acid **33** in 45% yield. The nitro group of **33** was reduced by palladium-catalyzed hydrogenation to give amine **34** in 98% yield. Reductive amination of **34** with 3-nitrobenzaldehyde afforded product **35e** in 91% yield.

The synthesis of 3-hydroxy-5-substituted benzoic acids is given in Scheme 5. Compounds **41a** and **41b** were prepared



Scheme 4. *Reagents and conditions*: a) Cl_2CPh_2, Cs_2CO_3, MeCN, RT; b) 1-fluoro-3-nitrobenzene, *t*Bu-P4, $TMSNEt_2$, DMF, 100 °C; c) 3-nitrobenzyl bromide, Cs_2CO_3, DMF; d) benzyl bromide, Cs_2CO_3, DMF, RT; e) NaOH, H_2O, THF, RT; f) TFA, CH_2Cl_2, RT; g) AcOH, HNO_3, 0 °C→RT; h) 48% $HBr_{(aq)}$, reflux; i) H_2, Pd/C, MeOH, RT; j) 1. 3-nitrobenzaldehyde, AcOH, DMF, 3 Å molecular sieves, 2. $NaBH_3CN$, RT. See Experimental Section for reaction times and yields.

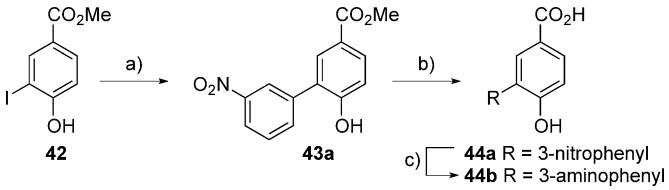


Scheme 5. *Reagents and conditions*: a) Ac_2O, RT; b) Tf_2O, pyridine, 0 °C; c) 3-(nitrophenyl)boronic acid, $Pd(PPh_3)_4$, $Na_2CO_{3(aq)}$, DME, reflux; d) KOH, H_2O, THF, RT; e) H_2, Pd/C, EtOH; f) 1-fluoro-3-nitrobenzene, *t*Bu-P4, TMS-NEt_2, DMF, 100 °C; g) 3-nitrobenzyl bromide, NaH, THF, 0 °C→RT; h) 1. 3-nitrobenzaldehyde, AcOH, DMF, 3 Å molecular sieves, 2. $NaBH_3CN$, RT. See Experimental Section for reaction times and yields.

from methyl 3,5-dihydroxybenzoic acid **36**, which was converted into the triflate **38**, to be used as a substrate in Suzuki couplings. Treatment of **36** with one equivalent of acetyl chloride was non-selective, and gave an approximate 1:1:1 mixture of the mono- and di-acylated products and the starting material, which were separable by column chromatography. Trifluoromethanesulfonic anhydride was used to convert the mono-protected compound **37** into triflate **38**, with pyridine acting as both base and nucleophilic catalyst. Suzuki coupling was then used to couple **38** with 3-nitrophenylboronic acid. The acyl-protecting group was removed under the mildly basic conditions of the coupling reaction to give **39a**. A further hydrolysis step gave the free acid **41a**. The 3-nitro compound **41a** was then converted by catalytic hydrogenation into the corresponding amine **41b**.

The 5-(3-nitrophenoxy)-3-hydroxy analogue **41c** was synthesized from methyl 3,5-dihydroxybenzoate **36** in 45% yield by similar methodology used for **31c** (Scheme 4). The 5-(3-nitrobenzoxy)-3-hydroxybenzoic acid analogue **41d** was synthesized from methyl 3,5-dihydroxybenzoic acid **36** by treatment with one equivalent of sodium hydride then 3-nitrobenzyl bromide to give intermediate **39d**, followed by hydrolysis to give **41d**. The 5-amino analogue **41e** was prepared by reductive amination between 3-amino-5-hydroxybenzoic acid **40** and 3-nitrobenzaldehyde in 93% yield without the need for protection/deprotection steps.

Scheme 6 shows the synthesis of analogues incorporating a 3-aryl-4-hydroxybenzoate core. It was originally proposed that **44a** and **44b** could be synthesized by following the same scheme used for the *meta*-hydroxy compound **41a**, using 3,4-dihydroxybenzoic acid as a starting point. This strategy was



Scheme 6. *Reagents and conditions*: a) 3-nitrophenylboronic acid, $Pd(PPh_3)_4$, $Na_2CO_{3(aq)}$, DME, reflux; b) KOH, H_2O, THF, RT; c) H_2, Pd/C, EtOH, RT. See Experimental Section for reaction times and yields.

abandoned, however, as the acylation protection step was considered insufficiently selective. Consequently the commercially available 4-hydroxy-3-iodobenzoic acid methyl ester **42** was used as a Suzuki coupling partner with 3-nitrophenylboronic acid to give product **43a** in 46% yield. Hydrolysis of the methyl ester in **43a** gave the free acid **44a**. The 3-nitro group of **44a** was reduced by hydrogenation to give the aromatic amine **44b**.

Analogues incorporating an isophthalate core were synthesized as illustrated in Scheme 7. The ether-linked isophthalate

Scheme 7. *Reagents and conditions*: a) 3-nitrobenzyl bromide, Cs_2CO_3, DMF, RT; b) 1. 3-nitrobenzaldehyde, toluene, 130 °C (Dean–Stark), 2. $NaBH_3CN$, AcOH, MeOH, RT; c) KOH, H_2O, THF, 60 °C; d) 1. 4-nitrobenzaldehyde, AcOH, DMF, 3 Å molecular sieves, 2. $NaBH_3CN$, RT. See Experimental Section for reaction times and yields.

49d was prepared from dimethyl 5-hydroxyisophthlate **45** by alkylation with 3-nitrobenzyl bromide to give **47d** in 79% yield, followed by saponification of the methyl ester with aqueous base to give **49d** in 64% yield. The 3-nitrobenzyl amino-linked isophthalic acid **49e** was prepared from dimethyl 5-aminoisophthalate **46**. Amine **46** and 3-nitrobenzaldehyde were heated under Dean–Stark conditions, and the resultant imine was reduced with sodium cyanoborohydride to give the secondary amine **47e** in 65% yield. Saponification of **47e** with aqueous base gave **49e** in quantitative yield. A shorter synthetic route avoiding the need for protecting groups was investigated for the synthesis of the 4-nitrobenzyl amino analogue **49f**. Reductive amination between the free di-acid **48** and 4-nitrobenzaldehyde afforded the **49f** in one step and 95% yield.

Protein production, crystallization, and data collection

Type II dehydroquinase from *M. tuberculosis* (MtDHQase) was expressed, purified and crystallized according to Dias et al.[32] X-ray crystallographic data were collected using various synchrotron X-ray sources. MtDHQase structures were solved by molecular replacement using MOLREP[33] or Phaser[34] implemented in CCP4.[35] The MtDHQase dodecamers (PDB: 3N7A) and (PDB: 3N76) were used as probes for the $P2_1$ and F23 crystals, respectively. Refinement was carried out using the REFMAC 5.2[36] also implemented in CCP4.[35] Coot 0.3.1 was used for visual analysis, manual inspection and to add water molecules.[37] The figures were prepared using PyMOL.[38]

MtDHQase assays

M. tuberculosis type II dehydroquinase was assayed either by direct enzyme assay, monitoring the increase in absorbance at λ 234 nm due to the formation of the enone–carboxylate chromophore of the product 3-dehydroshikimate **2**, or by a coupled assay with *E. coli* shikimate dehydrogenase, monitoring the decrease in absorption of NADPH at λ 340 nm (see the Experimental Section below for details). All compounds were initially tested at a concentration of 200 μM with a substrate concentration at K_M (30 μM), and the percentage inhibition measured (Table 1). Further testing was performed to determine the K_i values of those compounds that exhibited inhibition above 40% under these conditions (Table 1). All experiments were performed in duplicate.

Comparison of the percentage inhibition (Table 1), by the corresponding pairs of 3-nitro and 3-amino compounds **22a** (15%) and **22b** (13%), **26a** (18%) and **26b** (32%), **41a** (32%) and **41b** (13%), and **44a** (25%) and **44b** (12%) showed that they have similar potencies, although the 3-nitro compounds were often marginally more potent than their 3-amino analogues. A comparison of the effect of C3 and C4 hydroxy substitution on inhibition between corresponding pairs of compounds, **41a** (32%) and **44a** (25%), **41b** (13%) and **44b** (12%) (Table 1) showed little difference between the respective pairs. This suggests that binding from both the C3 and C4 hydroxy groups of the inhibitors is important.

Analyses of the compounds designed using different flat quinate analogues show that both the citrazinate analogue **22a** and 6-hydroxynicotinate analogue **26a** are less potent inhibitors than their corresponding 3-hydroxy **41a** and 4-hydroxy **44a** benzoate analogues, and all four compounds less than citrazinate **15** itself (K_i = 300 μM). Citrazinate **15** has three possible binding motifs in the active site of the enzyme (Figure 4): in the first, the compound has the O2 oxygen as a carbonyl directed toward the "active site" pocket and both the ring nitrogen N1 and the O6 oxygen are protonated (Figure 4a); secondly, both oxygen atoms are protonated, and the ring nitrogen is deprotonated (Figure 4b); and thirdly, the O6 oxygen is a carbonyl and both ring nitrogen N1 and O2 oxygen are protonated (Figure 4c).

In compound **22a**, the attachment of a six-membered ring side chain to the C6 position and the subsequent binding of that side chain in the "active site" pocket means that the binding motif adopted can only be that shown in either Figure 4b or 4c. It is proposed that citrazinate **15** binds in the manner shown in Figure 4a, where the compound is a hydrogen bond donor at the 3- and 4-positions to both Asp88 and His81. The loss of inhibition observed in compound **22a** would result from the inability to adopt this two-hydrogen-bond-donor arrangement, as compound **22a** is forced to adopt either of the hydrogen bond donor/acceptor arrangements (Figure 4b and 4c).

For compound **26a**, this can adopt one of the two one-donor/one-acceptor hydrogen bonding arrangements (Figure 5), albeit with the functional groups at the 1- and 6-po-

Table 1. Inhibition of type II 3-dehydroquinase from *M. tuberculosis*.

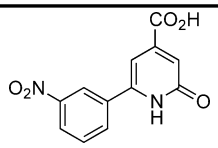
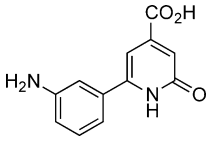
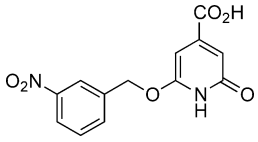
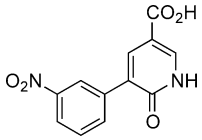
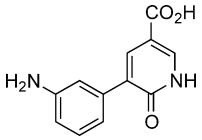
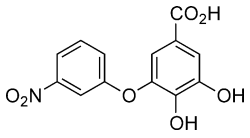
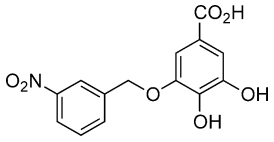
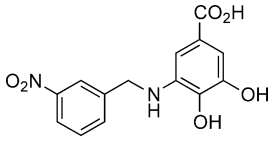
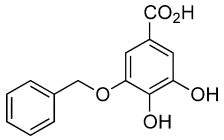
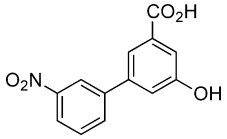
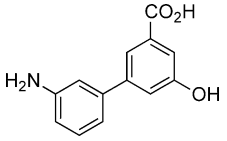
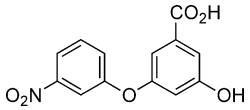
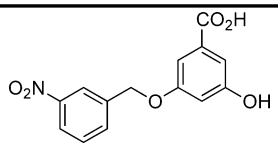
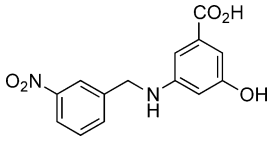
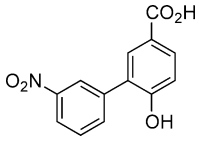
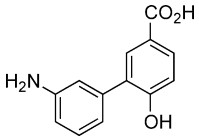
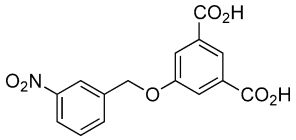
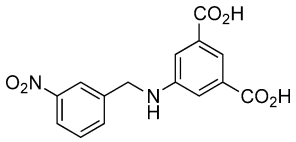
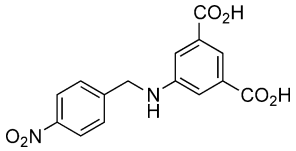
Compd		Inhib. [%][a]	K_i [μM][b]
22a		15	ND
22b		13	ND
22d		51	190 ± 10
26a		18	ND
26b		32	ND
35c		90	50 ± 4
35d		94	10 ± 1
35e		91	20 ± 4
35g		47	240 ± 20
41a		32	ND
41b		13	ND
41c		61	170 ± 10

Table 1. (Continued)

Compd		Inhib. [%][a]	K_i [μM][b]
41d		66	130 ± 10
41e		92	20 ± 3
44a		25	ND
44b		12	ND
49d		62	140 ± 10
49e		99	7.5 ± 1
49f		92	20 ± 5

[a] Percent inhibition caused by test compound at 200 μM, with a substrate concentration of 30 μM (K_M). [b] K_i values were determined for compounds with inhibition >40% (ND: not determined).

sitions reversed relative to the carboxylate and side chain of citrazinate-based compound **22a**. The similarity in levels of inhibition between **22a** and **26a** suggest neither configuration gives an advantage over the other, and if both compounds bind with the generally favorable lactam tautomer, there is little difference in whether the compounds form a hydrogen bond to Asp88 (Figure 4c) or His81 (Figure 5a). In addition, the observation that these compounds result in less potent inhibitors than a single hydrogen bond donor alone, the corresponding 3-hydroxybenzoate **41a** and 4-hydroxybenzoate **44a** suggest that the presence of hydrogen bond donors at both the C3 and C4 positions (benzoate numbering) is advantageous for binding and inhibition, and hydrogen bond acceptors at these positions are unfavorable. This is backed up by the observation that the 3,4-dihydroxy-5-(3-nitrophenoxy)benzoate **35c** (K_i = 49 μM) is more potent than the 3-hydroxy-5-(3-nitrophenoxy)benzoate **41c** (K_i = 165 μM), and that 3,4-dihy-

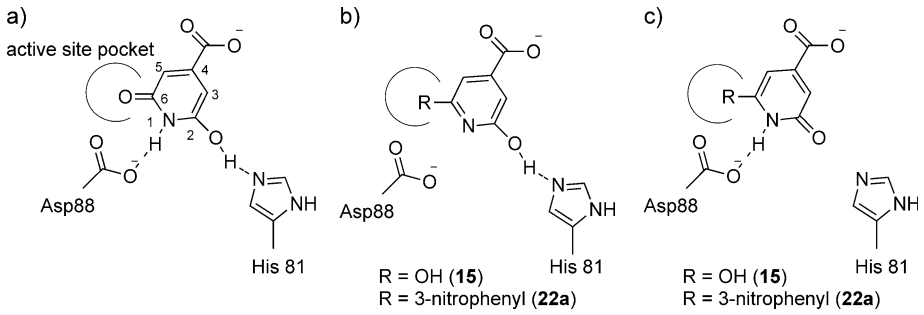


Figure 4. a) Binding of citrazinate **15**-lactam tautomer with carbonyl directed toward the "active site" pocket. b) Binding of citrazinate **15** and inhibitor **22a**-lactim tautomers. c) Binding of citrazinate **15** and inhibitor **22a**-lactam tautomers with carbonyl directed toward His 81.

Whilst the carboxylate and 3-hydroxy groups of the two compounds overlap, and can form the same hydrogen bond interactions, the vector at which the C–C bond linking the aromatic moiety of **9** and the quinate core leaves the puckered six-membered ring is ~30° away from the vector to which the C–O bond of the citrazinate points. In the case of the former, the result is that the aromatic

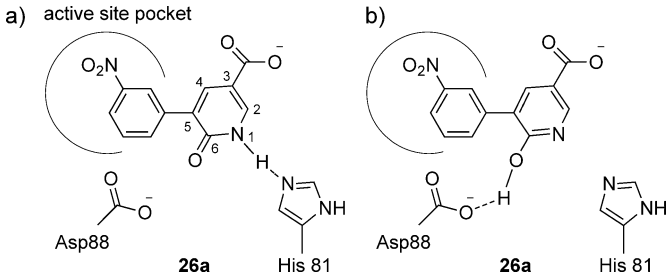


Figure 5. a) Binding of inhibitor **26a**-lactam tautomer. b) Binding of inhibitor **26a**-lactim tautomer.

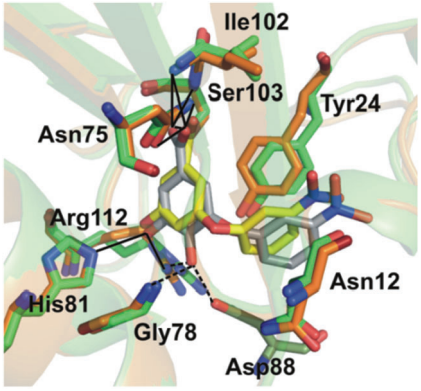


Figure 6. Superposition of MtDHQase–**35c** (ribbon diagram of protein in orange and molecular structure of compound in grey) and MtDHQase–**41c** (ribbon diagram of protein in green and molecular structure of compound in yellow). The molecular structures of the active site residues correspond to the MtDHQase–**41c** complex. The key hydrogen bond/electrostatic interactions between the central gallate core of compound **35c** and protein are shown as black lines, and dashed lines indicate the interactions lost if an inhibitor lacks a hydroxy group at the 4-position.

droxy-5-(3-nitrobenzoxy)benzoate **35d** (K_i = 10 μM) is more potent than both the 3-hydroxy-5-(3-nitrobenzoxy)benzoate **41d** (K_i = 130 μM) and 3-hydroxy-5-(3-nitrophenoxy)citrazinate **22d** (K_i = 186 μM).

To get a better understanding of the roles of the 4- and 5-hydroxy groups, crystals of MtDHQase in complex with compounds **35c**, **35d**, and **41c** were obtained and their structures solved (PDB codes 4KIJ, 4KIM, and 4KI7, respectively). An overlay of the crystal structures of MtDHQase in complex with **41c** (PDB: 4KI7) and **35c** (PDB: 4KIJ) (Figure 6) reveals that three of the key hydrogen bond/electrostatic interactions to the C4 hydroxy group of the gallate moiety of **35c**, and presumably to the C4 hydroxy group of the quinate core of inhibitors **4**–**10** as well, are lost when 3-hydroxy-5-benzoate is used as a scaffold. As a result, compound **41c**, lacking the C4 hydroxy group, is displaced ~0.5 Å in comparison with **35c** (PDB: 4KIJ) (Figure 6) and also **35d** (PDB: 4KIM); consequently the orientation of the nitrophenyl group is slightly changed.

The C–C linked biaryl compounds **22a**, **22b**, **26a**, **26b**, **41a**, **41b**, **44a**, and **44b** proved to be relatively poor inhibitors, the best **26b** and **41a** resulting in only 32% inhibition at 200 μM. During the course of this investigation, the crystal structures of citrazinate **15** and inhibitor **9** bound in the active site of MtDHQase were obtained (PDB codes 3N8K and 3N87).[33] An overlay of compounds **9** and **15** (Figure 7) gives a possible explanation for the poor inhibition for the biaryl compounds (N.B.: the protein structure used is from the complex with **9**, wherein the side chain of residue Arg 18 is displaced away from the active site by the biaryl side chain of the inhibitor).

moiety sits in a pocket formed by the active site loop, whilst for the latter, the vector is directed into the main body of the enzyme. Therefore, it is postulated that the C–C linked citrazinate analogues cannot bind with the pyridinyl moiety bound in the same configuration as citrazinate itself, which in turn results in the loss of key hydrogen bond interactions and lower potency. The addition of ether linkages improved potency: **41c** (K_i = 165 μM) and **35c** (K_i = 49 μM), whilst extension of the linker with a methylene unit increased potency even further: **41d** (K_i = 130 μM) and **35d** (K_i = 10 μM), respectively. Furthermore, superposition of the MtDHQase–**35c** and MtDHQase–**35d** (Figure 8) shows that the different linkages affect the relative orientation of the rings of these compounds and consequently different interactions are observed. Despite the gallate ring occupying the same position as the quinate, conserving most of the interactions, the nitrophenyl rings are displaced by ~0.7 Å, and some of the interactions observed in the MtDHQase–**35d** complex are absent in the MtDHQase–**35c** complex. Due to the extra methylene carbon in the linkage, **35d** is more flexible, and the rings may be able to optimize their interactions with the active site of the enzyme. Thus the

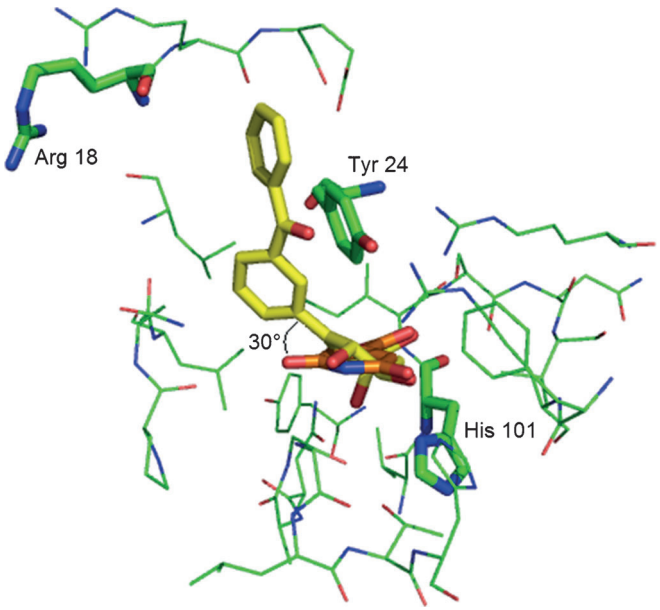


Figure 7. Overlay of citrazinate **15** (orange) and inhibitor **9** (yellow) bound to 3-dehydroquinase with residues Arg 18, Tyr 24, and His 101 highlighted. The angle shown is defined by atoms O6 (**15**)–C3 (**9**)–C1′ (**9**), and is an estimate of the difference in vector trajectory into the side chain pocket of the C6–C1′ bond of **9** and C6–O6 bond of **15**.

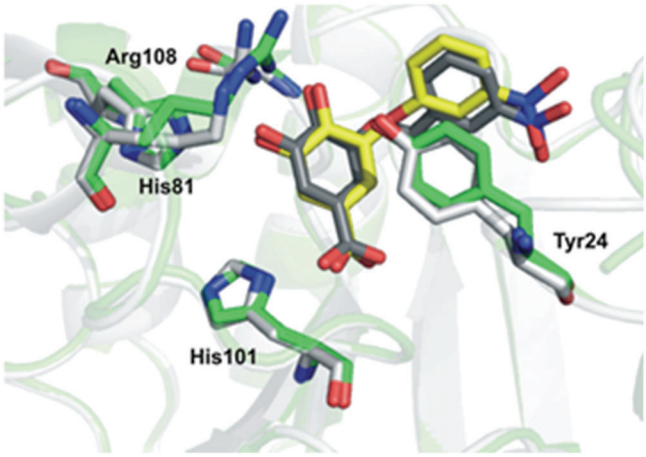


Figure 8. Superposition of MtDHQase–**35 c** (protein is green and compound in yellow) and MtDHQase–**35 d** (protein in white and compound in grey), showing the effect the additional methylene group in **35 d** has on the location of the 3-nitrophenyl ring and active site residue Tyr 24.

linkage of **35 c** is more distended than **35 d**, and it conserves the key interactions of the quinate binding site and the π interaction with Tyr 24.

The presence of an amino group at the 5-position has a marked effect on potency in compounds lacking a non-hydrogen substituent at the 4-position. Both 5-amino-3-hydroxybenzoate **41 e** (K_i = 17 μM) and 5-amino-isophthalate **49 e** (K_i = 7.5 μM) are an order of magnitude more potent than their corresponding ether-linked analogues **41 d** (K_i = 130 μM) and **49 d** (K_i = 136 μM). However, with a hydroxy group at the 4-position the amino analogue **35 e** (K_i = 19 μM) has similar potency to gallate **35 d** (K_i = 10 μM). The crystal structures of amino-linked isophthalate **49 e** (Figure 7, PDB: 4KIW) and ether-linked isophthalate **49 d** (PDB: 4KIU) bound in the active site of MtDHQase were solved. In these structures, both the 5-amino and 5-ether groups from the linkage form a hydrogen bond network with N^{δ}-Asn 12 and the β-carboxylate side chain from Asp 88 of the neighboring protomer via a water molecule (Figure 9). This water molecule has been observed in other MtDHQase–inhibitor complexes.[21]

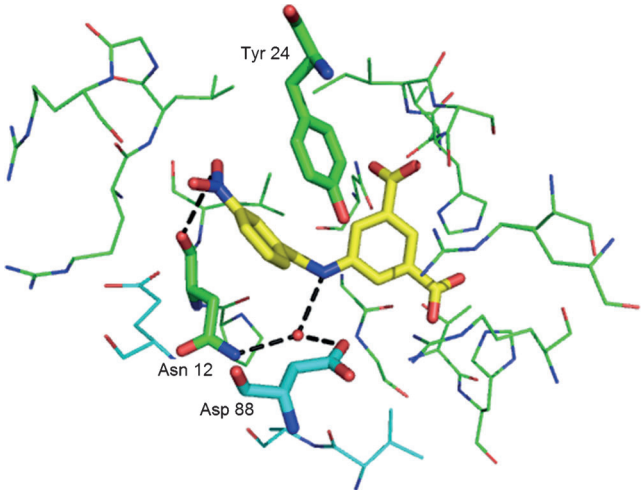


Figure 9. Crystal structure of **49 e** bound in the 3-dehydroquinase active site, showing key electrostatic interactions. Residues Asn 12 and Tyr 24 from chain 1 (green), and Asp 88 from chain 2 (cyan) are highlighted, with water shown as a red sphere.

The affinity of compound **49 e** (K_i = 7.5 μM) is significantly higher than **49 d** (K_i = 136 μM). The oxygen of the ether linkage in **49 e** forms an angle of 109°, compared with 118° for the nitrogen of amino linkage in **49 d**. This difference affects the positions of the nitrophenyl and isophthalate rings (Figure 10).

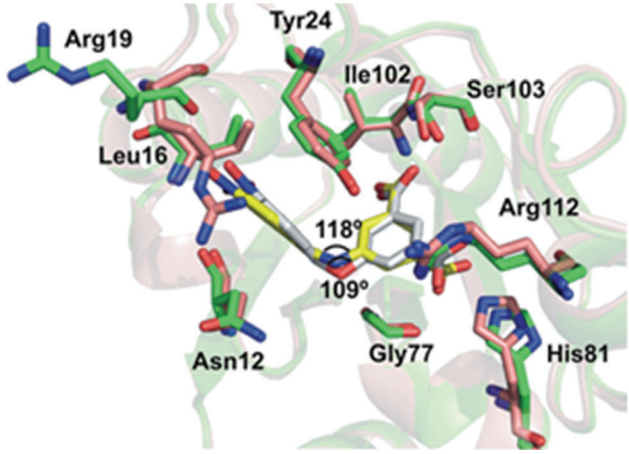


Figure 10. Superposition of MtDHQase–**49 d** (protein in salmon and compound in white) and MtDHQase–**49 e** (protein in green and compound in yellow). The image shows the different angles of the two compounds **49 d** and **49 e** and their effects on the orientation of the nitrophenyl groups.

The nitrophenyl ring of **49e** is displaced ~0.7 Å in relation to **49d** forward to the flexible loop region, and a network of five hydrogen bond interactions with the protein is formed. In comparison, **49d** has only one hydrogen bond interaction in the same region. The isophthalate is identically located in both structures.

The significance of the 3-nitro group was investigated by first preparing the benzyl analogue **35g** of the most potent gallate-based inhibitor **35d** ($K_i = 10\ \mu M$). This analogue was found to be considerably less potent ($K_i = 240\ \mu M$). Second, the 4-nitro analogue **49f** of the most potent isophthalate inhibitor **49e** ($K_i = 7.5\ \mu M$) was prepared. This analogue was found to have slightly lower potency ($K_i = 19\ \mu M$), which suggests that substitution at the 3-position is preferable. It is known that in general, the presence of electron-withdrawing groups on the aromatic side chain improves potency of the anhydroquinate-core-based inhibitors,[17] and it appears that it is the same for inhibitors with an aromatic core.

Conclusions

The simple flattening of the anhydroquinate core of the known highly potent inhibitors of 3-dehydroiquinase to a number of aromatic systems (the C–C linked range of inhibitors) was found to lead to a loss of potency. The crystal structures suggest that the rigidity of, and the conformations adopted by, such compounds are such that they cannot occupy the "side chain" pocket and the substrate binding pocket at the same time in a manner that maximizes hydrogen bond interactions in the same way as the potent quinate-based inhibitors. Attempts to increase flexibility between the aromatic rings in the inhibitors to allow greater conformational freedom generally resulted in increased potency due to the aromatic "side chains" being able to maximize π-stacking interactions with Tyr24 of the flexible active site loop, whilst the central aromatic core maintains favorable hydrogen bond interactions. It was observed that electron-withdrawing side chains were favored, and that hydrogen bond donors in the 3-, 4-, and 5-positions are all favorable. The present studies identified a number of ways in which the potency of these synthetically tractable series can be improved.

Experimental Section

Chemistry

All non-aqueous reactions were carried out in pre-dried glassware under an inert atmosphere (N_2 or Ar). All starting materials and reagents were commercially available and used without further purification. Organic solvents were freshly distilled prior to use, and Milli-Q deionized H_2O was used for all biochemical work. Unless otherwise stated, petroleum ether (PE) refers to the fraction collected between 40 and 60 °C. Analytical thin-layer chromatography (TLC) was carried out on commercial silica gel 60 0.25 mm plates using UV absorption, potassium permanganate stain (3 g $KMnO_4$, 20 g K_2CO_3, 5 mL 5% NaOH, 300 mL H_2O), or cerium molybdate stain (2 g ammonium cerium(IV) sulfate, 5 g ammonium heptamolybdate, 12 mL 98% H_2SO_4, 188 mL H_2O) for visualization. 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 or on a Biotage Isolera 1 (using KP-SIL cartridges: size and gradient used as described). Where quoted, high-performance liquid chromatography (HPLC) was performed on a Gilson HPLC system fitted with a Gilson 118 UV/Vis detector set at λ 220 nm. HPLC purification was performed with either: A) a semi-preparative Phenomenex Luna 5 μM $C_{18}(2)$ 10×250 mm column (flow rate: 4.6 mL min^{-1}), B) a semi-preparative Hi-Chrom Kromasil 100 5 μM C_{18} 10×250 mm column (4.0 mL min^{-1}), C) a preparative Hi-Chrom Kromasil 100 5 μM C_{18} 21.2×250 mm column (14 mL min^{-1}), or D) a preparative Macherey–Nagel Nucleosil 100 5 μM C_{18} 21.2×250 mm column (21.2 mL min^{-1}). The columns were eluted with MeCN in H_2O (containing 0.1% trifluoroacetic acid (TFA)) with gradients as described. Amberlite IR-120 (H^+) (cation exchanger) was washed alternately with Milli-Q H_2O, 10% HCl, and finally Milli-Q H_2O before use. Infrared spectra were recorded on a PerkinElmer Spectrum One FTIR spectrometer using attenuated transmittance reflectance (ATR); ν_{max} values are quoted in wavenumbers (cm^{-1}). ^{1}H NMR spectra were recorded on a Bruker DPX-400 spectrometer or a Bruker Avance 500 spectrometer in deuterated solvents, as indicated. ^{13}C NMR spectra were recorded on a Bruker DPX-400 spectrometer or a Bruker Avance 500 spectrometer linked to a Bruker 5 mm dual Cryoprobe (operating at 100 and 125 MHz, respectively). ^{19}F NMR spectra were recorded on a Bruker Avance 400 QNP Ultrashield spectrometer (operating at 376 MHz). All chemical shifts (δ) are quoted in parts per million (ppm) referenced with residual solvent peaks.[39] Coupling constants (J) are assigned where possible and are given in Hz. Yields of water-soluble compounds were determined by ^{1}H NMR spectroscopy and 2,2,3,4-tetradeutero-3-(trimethylsilyl)propionic acid sodium salt (TSP) as an internal standard. Liquid chromatography–mass spectrometry (LC–MS) was carried out using an Alliance HT Waters 2795 Separations Module fitted with a Waters Atlantis dC_{18} 4.6×30 mm, 3 μm column and coupled to a photodiode array detector and a Waters Micromass ZQ Quadrapole Mass Analyzer (using electrospray ionization, ES). Samples were run with an isocratic elution of 10 mM NH_4OAc containing 0.1% formic acid for 0.7 min, followed by a gradient over a period of 3.5 min to reach 95% MeCN with 0.05% formic acid, which was then run under isocratic conditions for 3.5 min before going back to the initial eluent over 0.3 min (flow rate: 1 mL min^{-1}). Low- and high-resolution mass spectrometry (LRMS and HRMS, respectively) were performed at the EPSRC National Mass Spectrometry Service Centre (Swansea, UK). Electron ionization (EI) LRMS data were collected on a Micromass Quattro quadrupole instrument. Accurate masses were recorded using either a Finnigan MAT 900 XLT or a Finnigan MAT 95 XP instrument. Perfluorotributylamine was used as the reference compound for EI, and polyethylenamine for electrospray ionization (ES).

Methyl 2,6-dichloroisonicotinate (18): Citrazinic acid **15** (2.50 g, 16.1 mmol) and Me_4NCl (1.84 g, 16.8 mmol) were suspended in $POCl_3$ (7.4 g, 4.5 mL, 47.7 mmol) and heated at 130 °C. When the internal temperature reached 75 °C, the solids dissolved to yield a clear brown solution. After 18 h, the temperature was increased to 145 °C for 2 h. After cooling to 25 °C, the mixture was poured onto ice (30 g) and stirred for 2 h. The resulting solids were filtered, dried, and then stirred with 25 mL EtOAc. Insoluble material (mostly citrazinic acid) was removed by filtration. The organic solution was dried over Na_2SO_4, and the solvent was removed to yield 2,6-dichloroisonicotinic acid as a light-brown solid (1.77 g, 57%). ^{1}H NMR (400 MHz, [D$_6$]DMSO): $\delta = 7.81$ ppm (s, 2H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): $\delta = 163.7$, 150.1, 144.6, 122.9 ppm; IR (ATR): $\tilde{\nu}_{max} = 3078$, 3000–2400, 1722 cm^{-1}; LCMS-ES m/z 214 $[M+Na]^+$,

192 $[M+H]^+$, 190 $[M-H]^-$. These data are in agreement with those previously reported by Henegar et al.[26]

2,6-Dichloroisonicotinic acid (1.0 g, 5.2 mmol) was dissolved in freshly distilled MeOH (25 mL), and concentrated H_2SO_4 (1 mL) was added dropwise. The mixture was heated at reflux for 2 h. After cooling to 25 °C, the solvent was removed under reduced pressure, and the residue was partitioned between H_2O (30 mL) and CH_2Cl_2 (30 mL). The organic phase was washed with saturated aqueous $NaHCO_3$ (2×30 mL) and brine (30 mL), dried (Na_2SO_4), and the solvent was removed under reduced pressure. The residue was purified by silica gel column chromatography (eluent: 3:17 *v/v* EtOAc/PE) to afford the desired methyl ester **18** as white needles (0.94 g, 88%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.80 (s, 2H), 3.97 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 163.4, 151.7, 142.5, 122.8, 53.4 ppm; IR (ATR): $\tilde{\nu}_{max}$ = 3090, 2960, 1730 cm^{-1}; LCMS-ES *m/z*: 206 $[M+H]^+$; HRMS-ES *m/z* $[M^+]$ calcd for $C_7H_5Cl_2NO_2$ 204.9692, found 204.9690.

Methyl 2-chloro-6-methoxyisonicotinate (19): Methyl 2,6-dichloroisonicotinate **18** (0.50 g, 2.4 mmol) was dissolved in freshly distilled MeOH (15 mL) and NaOMe (0.45 g, 8.4 mmol) was added slowly. The mixture was heated at reflux for 5 h with a $CaCl_2$ guard. After cooling to 25 °C, the mixture was neutralized with glacial AcOH (~0.5 mL) and concentrated to dryness. The residue was partitioned between CH_2Cl_2 (50 mL) and saturated $NaHCO_3$ solution (50 mL). The organic layer was dried over $MgSO_4$, and the solvent was removed under reduced pressure to afford the title compound **19** as a white solid (250 mg, 52%); mp: 101–102 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.43 (d, *J* = 1.1 Hz, 1H), 7.21 (d, *J* = 1.1 Hz, 1H), 3.97 (s, 3H), 3.93 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 164.4, 149.2, 142.3, 115.7, 109.7, 54.5, 52.8 ppm; IR (ATR): $\tilde{\nu}_{max}$ = 3110, 3090, 2960, 1736 cm^{-1}; LCMS-ES *m/z* 202 $[M+H]^+$; HRMS-ES *m/z* $[M+H]^+$ calcd for $C_8H_9ClNO_3$ 202.0265, found 202.0264.

2-Methoxy-6-((3-nitrobenzyl)oxy)isonicotinate (20d): 3-Nitrobenzyl alcohol (456 mg, 2.98 mmol), methyl 2-chloro-6-methoxyisonicotinate **19** (300 mg, 1.49 mmol), K_2CO_3 (414 mg, 3.00 mmol), copper (7.2 mg, 0.11 mmol), and copper(I) iodide (6 mg, 0.03 mmol) were suspended in anhydrous pyridine (15 mL) under N_2 and heated at 100 °C for 24 h. The reaction was allowed to cool to RT, and partitioned between water (150 mL)and EtOAc (2× 150 mL). The combined organic layers were washed with saturated brine (150 mL), dried over Na_2SO_4, and the solvent was removed under reduced pressure. The residue was purified by silica gel column chromatography (eluent 1:9 *v/v* EtOAc/hexane then 1:3 *v/v* EtOAc/hexane) to give the title product **20d** as a white solid (23 mg, 7%). ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 11.26 (br s, 1H), 8.30 (t, *J* = 2.3 Hz, 1H), 8.18 (ddd, *J* = 0.9, 2.3, 8.2 Hz, 1H), 7.92 (ddd, *J* = 0.8, 2.3, 8.1 Hz, 1H), 7.69 (t, *J* = 8.0 Hz, 1H), 6.75 (br s, 1H), 6.67 (d, *J* = 1.1 Hz, 1H), 5.46 (s, 2H), 3.85 ppm (s, 3H); ^{13}C NMR (100 MHz, [D_6]DMSO): δ = 169.7, 168.1, 167.2, 152.7, 147.7, 144.2, 139.1, 134.8, 127.6, 127.0, 105.8, 104.6, 70.9, 57.5 ppm; IR (ATR): $\tilde{\nu}_{max}$ = 2957, 1722, 1660, 1618, 1527 cm^{-1}; LCMS-ES *m/z* 305 $[M+H]^+$, 303 $[M-H]^-$; HRMS-ES *m/z* $[M+H]^+$ calcd for $C_{14}H_{13}N_2O_6$ 305.0774, found 305.0784.

Methyl 2-methoxy-6-(3′-nitrophenyl)isonicotinate (21a): Methyl 2-chloro-6-methoxyisonicotinate **19** (140 mg, 0.69 mmol), 3-nitrophenylboronic acid (173 mg, 1.04 mmol), Et_3N (0.3 mL, 2.07 mmol), and tetrakis(triphenylphosphine)palladium (40 mg, 35 μmol) were heated at 100 °C in dry DMF (2.8 mL) under argon for 21 h. After cooling to 25 °C, the solvent was removed under reduced pressure. The residue was dissolved in CH_2Cl_2 (30 mL) and washed with saturated $NaHCO_3$ solution (3×30 mL), saturated brine (30 mL), dried over Na_2SO_4, and the solvent was removed under reduced pressure. The residue was purified by column chromatography, eluting with 3:17 *v/v* EtOAc/PE, to afford the title compound **21a** as a white solid (65 mg, 33%) and 48% of the starting chloride **19**. R_f = 0.31 (3:17 *v/v* EtOAc/PE); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.92 (t, *J* = 2.0 Hz, 1H), 8.40 (ddd, *J* = 1.1, 2.0, 8.0 Hz, 1H), 8.25 (ddd, *J* = 1.1, 2.0, 8.0 Hz, 1H), 7.93 (d, *J* = 1.1 Hz, 1H), 7.64 (t, *J* = 8.0 Hz, 1H), 7.32 (d, *J* = 1.1 Hz, 1H), 4.09 (s, 3H), 3.98 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 165.4, 164.8, 153.0, 149.0, 141.5, 140.2, 132.6, 129.8, 124.0, 121.8, 112.4, 111.3, 54.1, 52.9 ppm; IR (ATR): $\tilde{\nu}_{max}$ = 3094, 2949, 1723 cm^{-1}; LCMS-ES *m/z* 289 $[M+H]^+$; HRMS-ES *m/z* $[M+H]^+$ calcd for $C_{14}H_{13}N_2O_5$ 289.0819, found 289.0820.

Methyl 6-(3′-aminophenyl)-2-methoxyisonicotinate (21b): Methyl 2-methoxy-6-(3′-nitrophenyl)isonicotinate **21a** (24 mg, 83 μmol) and Pd/C (2.5 mg, 10 wt% Pd) were stirred in EtOAc (1.5 mL) under a balloon of H_2 for 30 min. The reaction mixture was then filtered through Celite, and the solvent removed under reduced pressure. The residue was purified by silica gel chromatography, eluting with 2:3 *v/v* EtOAc/PE to afford the title compound **21b** as a yellow oil (21 mg, quant). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.85 (d, *J* = 1.0 Hz, 1H), 7.44–7.48 (m, 2H), 7.23–7.28 (m, 2H), 6.75 (ddd, *J* = 1.0, 2.2, 7.5 Hz, 1H), 4.06 (s, 3H), 3.96 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 165.9, 164.4, 156.0, 146.9, 140.9, 139.5, 129.8, 117.4, 116.3, 113.6, 112.3, 109.4, 53.9, 52.7 ppm; IR (ATR): $\tilde{\nu}_{max}$ = 3461, 3376, 3225, 2949, 1723 cm^{-1}; LCMS-ES *m/z* 259 $[M+H]^+$; HRMS-ES *m/z* $[M+H]^+$ calcd for $C_{14}H_{15}N_2O_3$ 259.1077, found 259.1081.

2-Hydroxy-6-(3-nitrophenyl)isonicotinic acid (22a): Methyl 2-methoxy-6-(3′-nitrophenyl)isonicotinate **21a** (25 mg, 87 μmol) was dissolved in 48% HBr solution (0.6 mL) and glacial AcOH (0.6 mL), and the mixture was heated at reflux for 1 h. After cooling to 25 °C, the solvent was removed under reduced pressure. The resulting solid was collected by filtration, washed with Et_2O (1 mL) to extract an orange impurity, and dried to give the desired product **22a** as a yellow solid (23 mg, quant). ^{1}H NMR (500 MHz, D_2O + NaOD): δ = 8.54 (t, *J* = 2.0 Hz, 1H), 8.09–8.15 (m, 2H), 7.56 (t, *J* = 8.0 Hz, 1H), 7.12 (d, *J* = 1.0 Hz, 1H), 6.62 ppm (d, *J* = 1.0 Hz, 1H); ^{13}C NMR (125 MHz, D_2O + NaOD): δ = 174.8, 172.1, 153.7, 148.0, 141.2, 133.4, 129.6, 123.0, 121.7, 112.5, 107.4 ppm; IR (ATR): $\tilde{\nu}_{max}$ = 3094, 3000, 2450, 1681, 1621 cm^{-1}; LCMS-ES *m/z* 261 $[M+H]^+$, 259 $[M-H]^-$; HRMS-ES *m/z* $[M-H]^-$ calcd for $C_{12}H_7N_2O_5$: 259.0360, found 259.0358.

3-(4-Carboxy-6-hydroxypyridin-2-yl)benzenaminium bromide (22b): Methyl 6-(3′-aminophenyl)-2-methoxyisonicotinate **21b** (14 mg, 54 μmol) was dissolved in 48% HBr solution (0.6 mL) and glacial AcOH (0.6 mL), and the mixture was heated at reflux for 30 min. After cooling to 25 °C, the solvent was removed under reduced pressure. The resulting solid was collected by filtration, washed with Et_2O (1 mL) to extract an orange impurity, and dried to give the desired product **22b** as a pale-yellow solid (12 mg, 71%). ^{1}H NMR (400 MHz, D_2O + NaOD): δ = 7.28–7.34 (m, 3H), 7.15 (d, *J* = 2.0 Hz, 1H), 6.88 (m,1H), 6.67 ppm (d, *J* = 2.0 Hz, 1H); ^{13}C NMR (125 MHz, D_2O + NaOD): δ = 175.1, 172.0, 156.1, 147.8, 146.3, 141.0, 129.6, 118.1, 116.5, 114.7, 111.4, 107.0 ppm; IR (ATR): $\tilde{\nu}_{max}$ = 3195, 3144, 3089, 2849, 2617, 2544, 1711 cm^{-1}; LCMS-ES *m/z* 231 $[M+H]^+$, 229 $[M-H]^-$; HRMS-ES *m/z* $[M+H]^+$ calcd for $C_{12}H_{11}N_2O_3$ 231.0764, found 231.0761.

2-Hydroxy-6-((3-nitrobenzyl)oxy)isonicotinic acid (22d): 2-Methoxy-6-((3-nitrobenzyl)oxy)isonicotinate **20d** (23 mg, 0.072 mmol) and NaI (17 mg, 0.113 mmol) were suspended in anhydrous MeCN (4 mL) in the dark under N_2. Chlorotrimethylsilane (14 μL, 12 mg, 0.11 mmol) was added, and the mixture heated at 65 °C for 7 h. The reaction was allowed to cool to RT, 1:1 *v/v* 5% sodium sulfite/

saturated brine (5 mL) was added and extracted with EtOAc (3× 5 mL). The combined organics were dried over Na_2SO_4, and solvent concentrated under reduced pressure. The residue was purified by silica gel column chromatography (eluent 1:9 *v/v* MeOH/CH_2Cl_2) to give the title product **22 d** as a white solid (15 mg, 82%). ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 8.30 (t, J = 1.9 Hz, 1H), 8.18 (dd, J = 2.3, 8.4 Hz, 1H), 7.92 (td, J = 1.3, 7.7 Hz, 1H), 7.69 (t, J = 7.9 Hz, 1H), 6.72 (d, J = 1.0 Hz, 1H), 6.66 (d, J = 1.1 Hz, 1H), 5.46 ppm (s, 2H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 165.9, 163.3, 162.3, 147.9, 144.4, 139.5, 134.4, 130.1, 122.8, 122.3, 101.2, 99.8, 66.1 ppm; IR (ATR): $\tilde{\nu}_{max}$ = 3210, 2919, 1675, 1628, 1529, 1464, 1346 cm^{-1}; LCMS-ES *m/z* 291 $[M+H]^+$, 289 $[M-H]^-$; HRMS-ES *m/z* $[M+H]^+$ calcd for $C_{13}H_{11}N_2O_6$ 291.0617, found 291.0636.

Methyl 5-bromo-6-chloronicotinate (23):[29,40] Bromine (1.5 mL, 30 mmol) was added to a suspension of 6-hydroxynicotinic acid **15** (2.78 g, 20 mmol) in glacial AcOH (5 mL) with stirring. The mixture was heated at 50 °C for 18 h then concentrated to dryness under reduced pressure. $POCl_3$ (5 mL) and PCl_5 (8.4 g, 40 mmol) were added to the crude residue, and the mixture was heated at reflux for 12 h. Excess $POCl_3$ was distilled off under reduced pressure (the bath temperature was kept < 50 °C). The resulting crude brown oil was dissolved in CH_2Cl_2 (25 mL) and MeOH (10 mL) and heated at reflux for 2 h. The solvents were removed under reduced pressure, and the residue was dissolved in Et_2O (40 mL) and washed with saturated aqueous $NaHCO_3$ solution (3×15 mL). The Et_2O layer was dried over $MgSO_4$, and the solvent was removed under reduced pressure. The residue was purified by column chromatography, eluting with 3:17 *v/v* EtOAc/PE, to afford the title compound **23** as a white crystalline solid (3.32 g, 66%); mp: 76–77 °C (lit.:[41] 76–77 °C); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.90 (d, J = 2.0 Hz, 1H), 8.50 (d, J = 2.0 Hz, 1H), 3.95 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 163.9, 155.1, 149.0, 143.0, 126.2, 120.4, 53.0 ppm; IR (ATR): $\tilde{\nu}_{max}$ = 3067, 2954, 1722 cm^{-1}; LCMS-ES *m/z* 250, 252 $[M+H]^+$. These data are in agreement with those previously reported.[29,40]

Methyl 5-bromo-6-methoxynicotinate (24): NaOMe (288 mg, 5.28 mmol) in MeOH (5 mL), was added to a solution of methyl 5-bromo-6-chloronicotinate **23** (0.66 g, 2.65 mmol) in 1,4-dioxane (7 mL). The mixture was heated at reflux for 16 h under a $CaCl_2$ guard. The reaction mixture was neutralized with glacial AcOH (0.3 mL). After removal of the solvent under reduced pressure, the residue was dissolved in CH_2Cl_2 (15 mL) and washed with saturated $NaHCO_3$ solution (5 mL). The organic extract was dried over $MgSO_4$, and the solvent was removed under reduced pressure. The residual solid was recrystallized from Et_2O/hexane to afford the title compound **24** as a white crystalline solid (0.25 g, 37%); mp: 104–105 °C (Et_2O/hexane; lit.:[29] 104–106 °C); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.74 (d, J = 2.1 Hz, 1H), 8.39 (d, J = 2.1 Hz, 1H), 4.08 (s, 3H), 3.92 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 164.9, 162.9, 148.2, 142.4, 121.1, 106.8, 55.3, 52.4 ppm; IR (ATR): $\tilde{\nu}_{max}$ = 3066, 3022, 2954, 1719 cm^{-1}; LCMS-ES *m/z* 246, 248 $[M+H]^+$. These data are in agreement with those previously reported.[29]

Methyl 6-methoxy-5-(3-nitrophenyl)nicotinate (25 a): Methyl 5-bromo-6-methoxynicotinate **24** (369 mg, 1.5 mmol), 3-nitrophenylboronic acid (376 mg, 2.25 mmol), Et_3N (0.63 mL, 4.5 mmol), $Pd(OAc)_2$ (10 mg, 45 μmol), and PPh_3 (24 mg, 93 μmol) in DMF (6 mL) were heated at 100 °C under argon for 24 h. Additional 3-nitrophenylboronic acid (84 mg, 0.5 mmol), $Pd(OAc)_2$ (10 mg, 45 μmol) and PPh_3 (24 mg, 93 μmol) were added and the heating was continued for 4 h. After cooling to 25 °C, the solvent was removed under reduced pressure (over a water bath at 60 °C). The residue was partitioned between CH_2Cl_2 (50 mL) and saturated $NaHCO_3$ solution (50 mL). The organic layer was washed with saturated $NaHCO_3$ solution (4×50 mL), saturated brine (50 mL), dried (Na_2SO_4), and the solvent was removed under reduced pressure. The residue was purified by column chromatography, eluting with 3:17 *v/v* EtOAc/PE followed by 1:1 *v/v* EtOAc/PE, and recrystallized from $CHCl_3$/EtOAc to afford the title compound **25 a** as a yellow solid (137 mg, 32%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.86 (d, J = 2.1 Hz, 1H), 8.43 (t, J = 2.0 Hz, 1H), 8.24 (d, J = 2.1 Hz, 1H), 8.21 (ddd, J = 1.1, 2.0, 7.9 Hz, 1H), 7.89 (ddd, J = 1.1, 2.0, 7.9 Hz, 1H), 7.60 (t, J = 7.9 Hz, 1H), 4.05 (s, 3H), 3.93 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 165.6, 163.3, 149.6, 148.3, 139.4, 137.3, 135.1, 129.3, 124.2, 122.8, 121.8, 120.3, 54.6, 52.2 ppm; IR ATR): $\tilde{\nu}_{max}$ = 3029, 2961, 2917, 2849, 1720 cm^{-1}; LCMS-ES *m/z* 289 $[M+H]^+$; HRMS-ES *m/z* $[M+H]^+$ calcd for $C_{14}H_{13}N_2O_5$ 289.0819, found 289.0820.

Methyl 6-methoxy-5-(3-aminophenyl)nicotinate (25 b): Methyl 6-methoxy-5-(3-nitrophenyl)nicotinate **25 a** (10 mg, 35 μmol) and Pd/C (2.5 mg, 10 wt% Pd) were stirred in EtOAc (1.5 mL) under a balloon of H_2 for 1 h. The reaction mixture was then filtered through Celite and the solvent removed under reduced pressure. The residue was purified by column chromatography, eluting with 6:4 *v/v* EtOAc/PE, to afford the desired amine **25 b** as a yellow solid (9 mg, quant). ^{1}H NMR (400 MHz, CD_3OD): δ = 8.74 (d, J = 2.3 Hz, 1H), 8.15 (d, J = 2.3 Hz, 1H), 7.17 (t, J = 7.8 Hz, 1H), 6.93 (t, J = 2.0 Hz, 1H), 6.86 (ddd, J = 0.9, 2.0, 7.8 Hz, 1H), 6.76 (ddd, J = 0.9, 2.0, 7.8 Hz, 1H), 4.01 (s, 3H), 3.93 ppm (s, 3H); ^{13}C NMR (100 MHz, CD_3OD): δ = 167.4, 165.1, 149.0, 148.6, 140.0, 137.7, 130.0, 126.4, 121.3, 120.1, 117.3, 116.3, 54.6, 52.6 ppm; IR (ATR): $\tilde{\nu}_{max}$ = 3427, 3321, 3220, 3013, 2954, 1718 cm^{-1}; LCMS-ES *m/z* 259 $[M+H]^+$; HRMS-ES *m/z* $[M+H]^+$ calcd for $C_{14}H_{15}N_2O_3$ 259.1077, found 259.1075.

5-(3-Nitrophenyl)-6-oxo-1,6-dihydropyridine-3-carboxylic acid (26 a): Methyl 6-methoxy-5-(3-nitrophenyl)nicotinate **25 a** (31 mg, 0.11 mmol) was dissolved in 48% HBr solution (0.6 mL) and glacial AcOH (0.6 mL) and the mixture was heated at reflux for 1 h. After cooling to 25 °C, the solvent was removed under reduced pressure. The resulting solid was collected by filtration, washed with Et_2O (1 mL) to extract an orange impurity and dried to afford the desired pyridone **26 a** as an off-white solid (27 mg, 94%). ^{1}H NMR (500 MHz, [D$_6$]DMSO): δ = 8.66 (t, J = 2.0 Hz, 1H), 8.21 (ddd, J = 1.0, 2.0, 8.0 Hz, 1H), 8.17 (ddd, J = 1.0, 2.0, 8.0 Hz, 1H), 8.12 (d, J = 2.5 Hz, 1H), 8.09 (br, 1H), 7.71 ppm (t, J = 8.0 Hz, 1H); ^{13}C NMR (125 MHz, [D$_6$]DMSO): δ = 165.3, 161.3, 147.6, 140.4, 138.3, 137.3, 134.6, 129.8, 126.7, 122.9, 122.6, 109.7 ppm; IR (ATR): $\tilde{\nu}_{max}$ = 3088, 2511, 1685, 1638 cm^{-1}; LCMS-ES *m/z* 261 $[M+H]^+$, 259 $[M-H]^-$; HRMS-ES *m/z* $[M+H]^+$ calcd for $C_{12}H_9N_2O_5$ 261.0506, found 261.0505.

3-(5-carboxy-2-oxo-1,2-dihydropyridin-3-yl)benzenaminium bromide (26 b): Methyl 6-methoxy-5-(3-aminophenyl)nicotinate **25 b** (7.0 mg, 27 μmol) was dissolved in 48% HBr solution (0.6 mL) and glacial AcOH (0.6 mL) and the mixture was heated at reflux for 1 h. After cooling to 25 °C, the solvent was removed under reduced pressure. The resulting solid was collected by filtration, washed with Et_2O (1 mL) to extract an orange impurity, and dried to give the desired pyridone **26 b** was obtained as a light-brown solid (6.0 mg, 71%). ^{1}H NMR (400 MHz, D_2O + NaOD): δ = 8.35 (d, J = 2.5 Hz, 1H), 7.86 (d, J = 2.5 Hz, 1H), 7.26 (t, J = 7.8 Hz, 1H), 6.96–7.01 (m, 2H), 6.79 ppm (br d, J = 7.8 Hz, 1H); ^{13}C NMR (125 MHz, D_2O + NaOD): δ = 175.1, 170.6, 149.1, 145.8, 140.6, 138.8, 129.2, 125.2, 120.2, 117.8, 116.8, 114.9 ppm; IR (ATR): $\tilde{\nu}_{max}$ = 3421, 3368, 3200, 2500, 1721, 1636 cm^{-1}; LCMS-ES *m/z* 231 $[M+H]^+$, 229 $[M-H]^-$; HRMS-ES *m/z* $[M+H]^+$ calcd for $C_{12}H_{10}N_2O_3$ 231.0764, found 231.0761.

Methyl 7-hydroxy-2,2-diphenylbenzo[*d*][1,3]dioxole-5-carboxylate (28): Methyl 3,4,5-trihydroxybenzoate **27** (2.00 g, 10.86 mmol), α,α-dichlorodiphenylmethane (3.35 g, 14.12 mmol), and K_2CO_3 (4.50 g, 32.58 mmol) in anhydrous MeCN (30 mL) were stirred at room temperature under N_2 for 24 h. The mixture was diluted with saturated NH_4Cl (75 mL) and H_2O (75 mL), then extracted with EtOAc (2×100 mL). The combined organics were washed with saturated brine (50 mL), dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified by flash silica chromatography (Biotage Isolera). Column 1, 50 g column, 40 mL min^{-1}, gradient: 5% EtOAc/hexane 1 cv; 5→25% EtOAc 5 cv; 25% EtOAc/hexane 10 cv; Column 2, 50 g column, 40 mL min^{-1}, gradient: 1% MeOH/CH_2Cl_2 1 cv; 1→10% MeOH 10 cv, 10% MeOH/CH_2Cl_2 2 cv, to give product **28**, a white solid (1.74 g, 46%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.50–7.55 (m, 4H), 7.41 (d, J = 1.5 Hz, 1H), 7.26–7.31 (m, 6H), 7.18 (d, J = 1.5 Hz, 1H), 3.77 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 167.4, 148.8, 139.9, 139.6, 138.7, 129.8, 128.7, 126.7, 124.5, 119.1, 114.6, 103.7, 52.7 ppm; IR (ATR): $\tilde{\nu}_{max}$ = 3670, 3387, 2988, 1697, 1643 cm^{-1}; LCMS-ES m/z 349 $[M+H]^+$, 347 $[M-H]^-$; HRMS-ES m/z $[M+H]^+$ calcd for $C_{21}H_{17}O_5$ 349.1076, found 349.1083.

Methyl 7-(3-nitrophenoxy)-2,2-diphenylbenzo[*d*][1,3]dioxole-5-carboxylate (29c): Methyl 7-hydroxy-2,2-diphenylbenzo[*d*]-[1,3]dioxole-5-carboxylate **28** (125 mg, 0.359 mmol), and 1-fluoro-3-nitrobenzene (46 μL, 0.432 mmol) were dissolved in anhydrous DMF (1 mL) under argon. Trimethylsilyl diethylamine (165 μL, 0.871 mmol) was added, followed by *t*Bu-P4 phosphazene base (1 M in hexane; 45 μL, 0.045 mmol) dropwise over a period of 1 min (N.B. phosphazene solution warmed to 45 °C prior to use). The reaction was heated at 100 °C for 17 h, then allowed to cool to room temperature. The reaction was diluted with saturated NH_4Cl (10 mL) and H_2O (10 mL), and extracted with EtOAc (2×10 mL). The combined organics were washed with H_2O (20 mL), saturated brine (20 mL), dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified by flash silica chromatography (Biotage Isolera), 10 g column, 12 mL min^{-1}, gradient: 5% EtOAc/hexane 1 cv; 5→40% EtOAc 10 cv, 40% EtOAc/hexane 3 cv, to give the product **29c** as a yellow solid (13 mg, 26% based on recovery of 88 mg starting material **28**). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.11 (t, J = 1.0 Hz, 1H), 7.97 (ddd, J = 0.9, 2.1, 8.1 Hz, 1H), 7.65 (t, J = 8.4 Hz, 1H), 7.55–7.60 (m, 5H), 7.35–7.42 (m, 7H), 7.22 (d, J = 1.5 Hz, 1H), 3.87 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 168.0, 165.8, 157.7, 149.6, 139.0, 137.0, 130.4, 129.7, 129.4, 128.4, 126.4, 126.3, 125.3, 123.3, 118.1, 112.1, 110.0, 107.3, 52.4 ppm; IR (ATR): $\tilde{\nu}_{max}$ = 3385, 1696, 1643, 1610 cm^{-1}; LCMS-ES m/z 470 $[M+H]^+$; HRMS-ES m/z $[M+H]^+$ calcd for $C_{27}H_{20}NO_7$ 470.1240, found 470.1254.

Methyl 7-(3-nitrobenzyloxy)-2,2-diphenylbenzo[*d*][1,3]dioxole-5-carboxylate (29d): NaH (60% in mineral oil; 60 mg, 1.5 mmol) was washed with hexane (4 mL) under N_2. A solution of methyl 7-hydroxy-2,2-diphenylbenzo[*d*][1,3]dioxole-5-carboxylate **28** (400 mg, 1.448 mmol) in anhydrous THF (7.5 mL) was added and stirred under N_2 at room temperature for 5 min. A solution of 3-nitrobenzyl bromide (312 mg, 1.444 mmol) in anhydrous THF (7.5 mL) was added dropwise over a period of 3 min. The reaction was stirred at room temperature for 7 h, then 50 °C for 16 h. The reaction was cooled to room temperature, diluted with saturated brine (100 mL) and H_2O (100 mL), and extracted with EtOAc (2×100 mL). The combined organics were washed with saturated brine (150 mL), dried over Na_2SO_4, filtered through Celite, and concentrated under reduced pressure. The residue was purified by flash silica chromatography (Biotage Isolera), 25 g column, 25 mL min^{-1}, gradient: 2% MeOH/CH_2Cl_2 1 cv, 2→20% MeOH 10 cv, 20% MeOH/CH_2Cl_2 2 cv, to give product **29c** as a white solid (355 mg, 51%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.32 (s, 1H), 8.16 (m, 1H), 7.75 (dd, J = 0.4, 7.6 Hz, dd), 7.54–7.59 (m, 5H), 7.36–7.42 (m, 7H), 7.30 (d, J = 1.4 Hz, 1H), 5.32 (s, 2H), 3.86 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 166.6, 149.1, 148.8, 141.8, 140.1, 139.8, 133.7, 130.0, 129.9, 128.7, 126.7, 124.9, 123.5, 122.8, 119.2, 113.3, 105.1, 70.9 ppm; IR (ATR): $\tilde{\nu}_{max}$ = 3675, 2988, 2901, 1716, 1632 cm^{-1}; LCMS-ES m/z 484 $[M+H]^+$; HRMS-ES m/z $[M+H]^+$ calcd for $C_{28}H_{22}NO_7$ 484.1391, found 484.1382.

Methyl 7-(benzyloxy)-2,2-diphenylbenzo[*d*][1,3]dioxole-5-carboxylate (29g): Methyl 7-hydroxy-2,2-diphenylbenzo[*d*][1,3]dioxole-5-carboxylate **28** (107 mg, 0.307 mmol), benzyl bromide (37 μL, 0.311 mmol), and Cs_2CO_3 (200 mg, 0.614 mmol) in anhydrous DMF (3 mL) were stirred at room temperature under N_2 for 18 h. The mixture was diluted with saturated NH_4Cl (10 mL) and H_2O (10 mL), and extracted with EtOAc (2×15 mL). The combined organics were dried over Na_2SO_4, filtered, and concentrated under reduced pressure to ~1.5 mL. The sample was purified by flash silica chromatography (Biotage Isolera). Column, 10 g, 12 mL min^{-1}, gradient: 5% EtOAc/hexane 1 cv, 5→80% EtOAc 10 cv, 80% EtOAc/hexane 2 cv, to give the product **29g** as a white solid (56 mg, 41%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.57–7.61 (m, 4H), 7.45 (m, 2H), 7.37–7.41 (m, 8H), 7.33–7.36 (m, 2H), 7.29 (d, J = 1.5 Hz, 1H), 5.25 (s, 2H), 3.86 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 166.8, 148.9, 142.5, 140.1, 140.0, 136.9, 129.7, 129.0, 128.7, 128.6, 128.1, 126.8, 124.7, 118.9, 113.2, 104.6, 72.1, 52.5 ppm; IR (ATR): $\tilde{\nu}_{max}$ = 3670, 2989, 2901, 1710, 1634 cm^{-1};LCMS-ES m/z 439 $[M+H]^+$; HRMS-ES m/z $[M+H]^+$ calcd for $C_{28}H_{23}O_5$ 439.1545, found 439.1555.

7-(3-Nitrophenoxy)-2,2-diphenylbenzo[*d*][1,3]dioxole-5-carboxylic acid (30c): Methyl 7-(3-nitrophenoxy)-2,2-diphenylbenzo[*d*]-[1,3]dioxole-5-carboxylate **29c** (22 mg, 0.047 mmol) was dissolved in 2.5 M NaOH solution (1 mL) and THF (5 mL), and heated at 40 °C for 24 h. The reaction was allowed to cool to room temperature, and THF removed under reduced pressure. The remaining solution was diluted with H_2O (10 mL), and extracted with EtOAc (2× 10 mL). The combined organics were dried over Na_2SO_4, filtered and concentrated under reduced pressure. The residue was purified by flash silica chromatography (Biotage Isolera), 10 g column, 15 mL min^{-1}, gradient: 1% MeOH/CH_2Cl_2 1 cv, 1→15% MeOH 10 cv, 15% MeOH/CH_2Cl_2 2 cv, to give the product **30c** as a white solid (13 mg, 75% based on recovery of 4 mg starting material). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.98 (ddd, J = 0.8, 2.0, 8.1 Hz, 1H), 7.77 (t, J = 2.3 Hz, 1H), 7.58 (m, 1H), 7.43–7.47 (m, 5H), 7.35–7.40 (m, 7H), 7.22 ppm (d, J = 1.5 Hz, 1H); ^{13}C NMR (100 MHz, [D_6]DMSO): δ = 167.3, 157.5, 149.5, 148.2, 143.6, 139.9, 138.5, 137.6, 132.1, 130.5, 130.2, 129.3, 126.5, 119.6, 119.2, 118.4, 117.9, 112.4, 110.5, 107.5, 104.0 ppm; IR (ATR): $\tilde{\nu}_{max}$ = 2932, 1684, 1631, 1608 cm^{-1}; LCMS-ES m/z 456 $[M+H]^+$, 454 $[M-H]^-$; HRMS-ES m/z $[M+H]^+$ calcd for $C_{26}H_{18}NO_7$ 456.1083, found 456.1086.

7-(3-Nitrobenzyloxy)-2,2-diphenylbenzo[*d*][1,3]dioxole-5-carboxylate (30d): Methyl 7-(3-nitrobenzyloxy)-2,2-diphenylbenzo[*d*]-[1,3]dioxole-5-carboxylate **29d** (77 mg, 0.159 mmol) was dissolved in 2.5 M NaOH solution (1 mL) and THF (7 mL), and heated at 40 °C for 16 h. The reaction was allowed to cool to room temperature, acidified with 1 N HCl (9 mL), and extracted with EtOAc (2×25 mL). The combined organics were washed with saturated brine (25 mL), dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified by flash silica chromatography (Biotage Isolera), 10 g column, 12 mL min^{-1}, gradient: 5% EtOAc/hexane 1 cv, 5→40% EtOAc 10 cv, 40% EtOAc/hexane 3 cv, then 5→20%

MeOH/CH_2Cl_2 5 cv, to give product **30 d** as a white solid (8 mg, 30% based on recovery of 50 mg starting material). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.33 (s, 1H), 8.18 (d, J = 8.1 Hz, 1H), 7.76 (d, J = 7.5 Hz, 1H), 7.53–7.59 (m, 5H), 7.33–7.46 (m, 8H), 5.33 ppm (s, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 167.0, 148.4, 148.1, 141.2, 139.4, 139.2, 138.6, 133.2, 129.4, 129.1, 128.1, 126.0, 124.3, 122.7, 122.0, 118.4, 112.9, 104.6, 70.2 ppm; IR (ATR): $\tilde{\nu}_{max}$ = 3677, 2919, 1683, 1636 cm^{-1};LCMS-ES m/z 470 $[M+H]^+$, 468 $[M-H]^-$; HRMS-ES m/z $[M-H]^-$ calcd for $C_{27}H_{18}NO_7$ 468.1089, found 468.1104.

7-(Benzyloxy)-2,2-diphenylbenzo[*d*][1,3]dioxole-5-carboxylate (30 g): Methyl 7-(benzyloxy)-2,2-diphenylbenzo[*d*][1,3]dioxole-5-carboxylate **29 g** (56 mg, 0.128 mmol) was suspended in 100 mM KOH (5.12 mL, 0.512 mmol) and THF (3 mL), and heated at 60 °C for 6 h. The reaction was allowed to cool to room temperature, acidified with 3 N HCl (10 mL), and extracted with EtOAc (2×20 mL). The combined organics were dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified by flash silica chromatography (Biotage Isolera), 10 g column, 12 mL min^{-1}, gradient: 1% MeOH/CH_2Cl_2 1 cv; 1→15% MeOH 10 cv, 15% MeOH/CH_2Cl_2 2 cv, to give the product **30 g** (15 mg, 65% based on recovery of 32 mg starting material). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.54–7.62 (m, 4H), 7.42–7.48 (m, 3H), 7.38 (m, 8H), 7.33 (m, 2H), 5.25 ppm (s, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 166.3, 158.0, 157.4, 148.8, 142.7, 140.2, 139.8, 139.5, 130.4, 130.3, 129.8, 128.8, 126.5, 125.4, 123.8, 122.8, 119.3, 118.7, 118.5, 118.1, 113.3, 104.3, 71.2 ppm; IR (ATR): $\tilde{\nu}_{max}$ = 3488, 2524, 2159, 2030, 1679, 1628 cm^{-1}; LCMS-ES m/z 425 $[M+H]^+$, 423 $[M-H]^-$; HRMS-ES m/z $[M+H]^+$ calcd for $C_{27}H_{21}O_5$ 425.1389, found 425.1401.

Methyl 4-hydroxy-3-methoxy-5-nitrobenzoate (32): Methyl vanillate **31** (1.0 g, 5.5 mmol) was dissolved in AcOH (5 mL) at 0 °C. A mixture of fuming nitric acid (0.4 mL) and AcOH (2 mL) was added slowly. The reaction mixture was allowed to warm to room temperature and stirred for 3 h. The resulted precipitate was filtered and recrystallized from EtOAc to give the product **32** as a yellow solid (725 mg, 58%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.39 (s, 1H) 7.71 (s, 1H), 3.95 (s, 3H), 3.81 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 165.4, 150.4, 150.0, 133.8, 121.7, 118.8, 117.7, 57.0, 53.2 ppm; IR (ATR): $\tilde{\nu}_{max}$ = 3263, 3079, 3007, 2952, 2928, 1715, 1617, 1539, 1439, 1375 cm^{-1}; LCMS-ES m/z 228 $[M+H]^+$; HRMS-ES m/z $[M+H]^+$ calcd for $C_9H_{10}NO_6$ 228.0503, found 228.0518.

3,4-Dihydroxy-5-nitrobenzoic acid (33): Methyl 4-hydroxy-3-methoxy-5-nitrobenzoate **32** (500 mg, 2.2 mmol) was dissolved in 48% HBr aqueous solution (20 mL) and heated at reflux until a clear solution was observed. After complete demethylation (TLC monitoring), the solution was cooled on ice, and the resulted precipitate was filtered, washed with H_2O and dried to give the crude product **33** as a yellow solid (195 mg, 45%). ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 8.01 (s, 1H), 7.83 (s, 1H), 5.31 ppm (s, 2H); ^{13}C NMR (100 MHz, [D_6]DMSO): δ = 168.7, 149.6, 146.0, 139.5, 125.1, 119.3, 118.3 ppm; IR (ATR): $\tilde{\nu}_{max}$ = 3537, 3088, 2822, 2648, 2523, 1791, 1682, 1616, 1588, 1542, 1455, 1407 cm^{-1}; LCMS-ES m/z 200 $[M+H]^+$; HRMS-ES m/z $[M+H]^+$ calcd for $C_7H_6NO_6$ 200.0190, found 200.0203.

3-Amino-4,5-dihydroxybenzoic acid (34): Crude 3,4-dihydroxy-5-nitrobenzoic acid **33** (100 mg, 0.5 mmol) was dissolved in MeOH (1 mL) and Pd/C (20 mg, 10 wt% Pd) was added. The mixture was stirred under hydrogen for 18 h. The mixture was filtered, and the filtrate was dried in vacuo to give the crude product **34** as a grey solid (83 mg, 98%). ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 10.56 (br, 1H), 7.49 (s, 1H), 7.45 (s, 1H), 3.99 (s, 2H), 3.76 ppm (s, 2H); ^{13}C NMR (100 MHz, [D_6]DMSO): δ = 166.7, 146.1, 144.0, 121.7, 119.2, 116.3, 116.0 ppm; IR (ATR): $\tilde{\nu}_{max}$ = 3020, 1778, 1694, 1614, 1573, 1534, 1489, 1450, 1392 cm^{-1}; LCMS-ES m/z 168 $[M-H]^-$; HRMS-ES m/z $[M+H]^+$ calcd for $C_7H_8NO_4$ 170.0453, found 170.0462.

3,4-Dihydroxy-5-(3-nitrophenoxy)benzoic acid (35 c): 7-(3-Nitrophenoxy)-2,2-diphenylbenzo[*d*][1,3]dioxole-5-carboxylic acid **30 c** (13 mg, 0.028 mmol) was dissolved in CH_2Cl_2 (1 mL) and TFA (1 mL), and stirred for 3 h. The reaction was concentrated under reduced pressure, and the residue was purified by HPLC, column D, solvent A: H_2O, solvent B: MeCN, gradient: 5% B 1 cv, 5→95% B 6 cv, 95% B 1 cv, 95→5% B 1 cv, 5% B 2 cv. The sample containing fractions were pooled, flash-frozen in liquid N_2, and lyophilized to give the product **31 c** as a white solid (6 mg, 73%). ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 10.04 (br s, 1H), 9.72 (br s, 1H), 7.91 (ddd, J = 0.8, 2.2, 8.2 Hz, 1H), 7.62 (t, J = 8.2 Hz, 1H), 7.56 (t, J = 2.3 Hz, 1H), 7.38 (ddd, J = 0.8, 2.2, 8.2 Hz, 1H), 7.36 (d, J = 2.0 Hz, 1H), 7.13 ppm (d, J = 2.3 Hz, 1H); ^{13}C NMR (125 MHz, [D_6]DMSO): δ = 166.7, 158.4, 148.8, 147.0, 142.6, 141.6, 131.2, 123.0, 121.5, 117.1, 114.5, 113.9, 110.3 ppm; IR (ATR): $\tilde{\nu}_{max}$ = 3280, 1665, 1605, 1521 cm^{-1}; LCMS-ES m/z 290 $[M-H]^-$; HRMS-ES m/z $[M-H]^-$ calcd for $C_{13}H_8NO_7$ 290.0295, found 290.0300.

3,4-Dihydroxy-5-((3-nitrobenzyl)oxy)benzoic acid (35 d): 7-(3-Nitrobenzyloxy)-2,2-diphenylbenzo[*d*][1,3]dioxole-5-carboxylate **30 d** (8 mg, 0.017 mmol) was dissolved in CH_2Cl_2 (1 mL) and TFA (1 mL) and stirred at room temperature for 3 h. The solvents were removed under reduced pressure, and the residue was purified by HPLC, column D, solvent A: H_2O, solvent B: MeCN, gradient: 5% B 1 cv, 5→95% B 6 cv, 95% B 1 cv, 95→5% B 1 cv, 5% B 2 cv, to give product **31 d** as a white solid (5 mg, 96%). ^{1}H NMR (400 MHz, CD_3OD): δ = 8.42 (s, 1H), 8.21 (d, J = 8.1 Hz, 1H), 7.91 (d, J = 7.6 Hz, 1H), 7.66 (d, J = 8.0 Hz, 1H), 7.24 (d, J = 1.9 Hz, 1H), 7.23 (d, J = 1.8 Hz, 1H), 5.32 ppm (s, 2H); ^{13}C NMR (100 MHz, [D_6]DMSO): δ = 167.6, 148.2, 146.7, 146.0, 140.1, 140.0, 134.3, 130.2, 122.9, 122.2, 120.9, 111.5, 107.3, 69.3 ppm; IR (ATR): $\tilde{\nu}_{max}$ = 3205, 1685, 1606 cm^{-1}; LCMS-ES m/z 304 $[M-H]^-$; HRMS-ES m/z $[M-H]^-$ calcd for $C_{14}H_{10}NO_7$ 304.0463, found 304.0474.

3,4-Dihydroxy-5-((3-nitrobenzyl)amino)benzoic acid (35 e): 3-Nitrobenzaldehyde (67 mg, 0.44 mmol) and crude 3-amino-4,5-dihydroxybenzoic acid **34** (100 mg, 0.59 mmol) were stirred in anhydrous DMF (1 mL) containing 1% AcOH and molecular sieves (3 Å) under nitrogen for 18 h. $NaBH_3CN$ (80 mg, 1.27 mmol) was added, and reaction stirred for 1 h then quenched by the addition of H_2O (100 μL). The molecular sieves were removed by filtration, and the filtrate evaporated in vacuo to dryness. The crude product was purified by flash silica chromatography ($CHCl_3$/MeOH/AcOH/H_2O, 120:15:3:2 *v/v/v/v*) to give the product **35 e** as a brown solid (120 mg, 91%). ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 11.98 (br s, 1H) 8.21 (s, 1H), 8.16 (d, J = 1.2 Hz, 1H), 7.81 (d, J = 1.1 Hz, 1H), 7.61 (t, J = 2.3 Hz, 1H), 6.83 (s, 1H), 6.52 (s, 1H), 5.78 (br s, 2H), 4.48 (s, 2H), 4.12 ppm (br s, 1H); ^{13}C NMR (100 MHz, [D_6]DMSO): δ = 172.3, 168.7, 128.2, 145.6, 144.2, 136.1, 134.0, 130.1, 122.0, 107.2, 103.8, 46.1 ppm; IR (ATR): $\tilde{\nu}_{max}$ = 3455, 3441, 3273, 3097, 1675, 1621, 1595, 1523, 1458, 1427, 1382 cm^{-1}; LCMS-ES m/z 305 $[M+H]^+$; HRMS-ES m/z $[M+H]^+$ calcd for $C_{14}H_{13}N_2O_6$ 305.0774, found 305.0798.

3-(Benzyloxy)-4,5-dihydroxybenzoic acid (35 g): 7-(Benzyloxy)-2,2-diphenylbenzo[*d*][1,3]dioxole-5-carboxylate **30 g** (15 mg, 0.035 mmol) was dissolved in CH_2Cl_2 (1.5 mL), and TFA (50 μL) was added. The reaction was stirred at room temperature for 2 h. Additional TFA (50 μL) was added, and the reaction was stirred for a further 2 h. The solvents were removed under reduced pressure, and the residue purified by flash silica chromatography (Biotage Isolera), 10 g column, 12 mL min^{-1}, gradient: 2% MeOH/CH_2Cl_2 1 cv;

2→20% MeOH 10 cv, 20% MeOH/CH_2Cl_2 2 cv, to give product **31 g** as a white solid (8 mg, 88%). ^{1}H NMR (400 MHz, CD_3OD): δ = 7.46–7.53 (m, 2H), 7.35–7.42 (m, 2H), 7.32 (d, J = 7.2 Hz, 1H), 7.24 (d, J = 1.9 Hz, 1H), 7.20 (d, J = 1.9 Hz, 1H), 5.19 ppm (s, 2H); ^{13}C NMR (100 MHz, [D_6]DMSO): δ = 167.6, 147.1, 145.8, 139.9, 137.6, 128.7, 128.1, 127.8, 120.9, 111.1, 107.2, 70.4 ppm; IR (ATR): $\tilde{\nu}_{max}$ = 3398, 2926, 1648, 1614 cm^{-1}; LCMS-ES *m/z* 259 [*M*−H]$^-$; HRMS-ES *m/z* [*M*−H]$^-$ calcd for $C_{14}H_{11}O_5$ 259.0612, found 259.0619.

Methyl 3-acetoxy-5-hydroxybenzoate (37):[24] Acetyl chloride (1.10 mL, 15.2 mmol) was added dropwise to a cooled solution of methyl 3,5-dihydroxybenzoate **36** (2.56 g, 15.2 mmol) in dry pyridine (10 mL) at 0 °C. The reaction mixture was stirred for 1 h at 0 °C and then at 25 °C for a further 1 h. The reaction mixture was diluted with EtOAc (30 mL) before being washed with saturated NH_4Cl solution (2×20 mL), brine (20 mL), and dried ($MgSO_4$). The solvent was removed under reduced pressure to give the crude mixture of the mono- and di-acylated methyl ester as a white solid. The mono-acylated product was purified by flash column chromatography (initially eluting with 4:1 *v/v* PE/EtOAc; switching to progressively more polar solvent systems as each product was collected). The mono-acylated product **37** was collected as a white solid (0.86 g, 27%). Some di-acylated product was collected as a white solid (1.00 g, 24%). ^{1}H NMR (400 MHz, CD_3OD): δ = 7.30 (dd, J = 1.5, 2.3 Hz, 1H), 7.19 (dd, J = 1.5, 2.3 Hz, 1H), 6.77 (t, J = 2.3 Hz, 1H), 3.87 (s, 3H), 2.26 ppm (s, 3H); ^{13}C NMR (100 MHz, CD_3OD): δ = 170.9, 167.7, 159.8, 153.1, 133.2, 114.8, 114.7, 52.8, 20.9 ppm; IR (ATR): $\tilde{\nu}_{max}$ = 3283, 3128, 3087, 2962, 1757, 1695, 1601, 1547, 1500 cm^{-1}; LCMS-ES *m/z* 209 [*M*−H]$^-$; HRMS-ES *m/z* [*M*+NH_4]$^+$ calcd for $C_{10}H_{14}NO_5$ 228.0866, found 228.0866.

Methyl 3-acetoxy-5-trifluromethanesulfonyloxybenzoate (38): Methyl 3-acetoxy-5-hydroxybenzoate **37** (0.65 g, 3.0 mmol) and pyridine (0.74 mL, 9.3 mmol) were dissolved in THF (15 mL) under argon, and the colorless solution cooled to 0 °C. Trifluoromethanesulfonic anhydride (0.66 mL, 3.9 mmol) was added dropwise over 1 min and the reaction mixture stirred for a further 10 min which gave a yellow solution and a white precipitate. The precipitate was filtered off under reduced pressure, washed on the filter with THF (15 mL) and discarded. THF was removed from the filtrate under reduced pressure and the crude product was re-dissolved in EtOAc (40 mL) and washed consecutively with saturated $NaHCO_3$ solution (40 mL), 1 N HCl solution (40 mL), saturated $NaHCO_3$ solution (40 mL), saturated brine solution (40 mL), and dried over $MgSO_4$. The solvent was removed under reduced pressure to give the crude product as an off-white solid, which was then purified by column chromatography, eluting with 3:1 then 1:1 *v/v* PE/EtOAc. The triflate **38** was collected as a yellow oil (0.40 g, 64%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.84 (dd, J = 1.3, 2.3 Hz, 1H), 7.81 (dd, J = 1.3, 2.3 Hz, 1H), 7.31 (t, J = 2.3 Hz, 1H), 3.95 (s, 3H), 2.34 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 168.3, 164.3, 151.4, 149.2, 133.2, 123.0, 119.8, 118.7 (q, $^1J_{CF}$ = 323 Hz, *C*F_3), 52.9, 20.9 ppm; ^{19}F NMR (376 MHz, $CDCl_3$): δ = −72.9 ppm; IR (ATR): $\tilde{\nu}_{max}$ = 3093, 3026, 2958, 2845, 1777, 1730, 1615, 1595, 1464, 1424, 1370, 1185 cm^{-1}; LCMS-ES *m/z* 341 [*M*−H]$^-$; HRMS-ES *m/z* [*M*+NH_4]$^+$ calcd for $C_{11}H_{13}F_3NO_7S$ 360.0359, found 360.0359.

Methyl 5-hydroxy-3′-nitro-[1,1′-biphenyl]-3-carboxylate (39 a): Methyl 3-acetoxy-5-trifluromethanesulfonyloxybenzoate **35** (200 mg, 0.58 mmol) and 3-nitrophenylboronic acid (167 mg, 1.0 mmol), 2 M Na_2CO_3 solution (0.45 mL, 0.90 mmol) and tetrakis(-triphenylphosphine)palladium (19 mg, 16 μmol) were dissolved sequentially in 1,2-dimethoxyethane (2.2 mL) under argon. The reaction mixture was heated under reflux for 19 h before cooling to 25 °C and H_2O (20 mL) was added. The reaction mixture was extracted with EtOAc (4×20 mL) and the combined organic phases were washed with saturated $NaHCO_3$ solution (80 mL), saturated brine (80 mL), and dried over $MgSO_4$. The product **39 a** was purified by column chromatography, eluting with 2:1 *v/v* PE/EtOAc, and collected as an off-white solid (160 mg, 88%). ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 8.39 (t, J = 2.0 Hz, 1H), 8.25 (ddd, J = 1.0, 2.0, 8.0 Hz, 1H), 8.12 (ddd, J = 1.0, 2.0, 8.0 Hz, 1H), 7.77 (t, J = 8.0 Hz, 1H), 7.71 (t, J = 1.6 Hz, 1H), 7.43 (dd, J = 1.6, 2.4 Hz, 1H), 7.39 (dd, J = 1.6, 2.4 Hz, 1H), 3.88 ppm (s, 3H); ^{13}C NMR (100 MHz, [D_6]DMSO): δ = 165.8, 158.2, 148.3, 140.7, 139.7, 133.3, 131.7, 130.5, 122.5, 121.1, 118.5, 118.2, 115.7, 52.2 ppm; IR (ATR): $\tilde{\nu}_{max}$ = 3358, 2953, 2855, 1686, 1600, 1526, 1485, 1455, 1433, 1413, 1332, 1314 cm^{-1}; LCMS-ES *m/z* 274 [*M*+H]$^+$, 272 [*M*−H]$^-$; HRMS-ES *m/z* [*M*]$^+$ calcd for $C_{14}H_{11}NO_5$ 273.0632, found 273.0629.

Methyl 3-hydroxy-5-(3-nitrophenoxy)benzoate (39 c): Methyl 3,5-dihydroxybenzoate **36** (250 mg, 1.49 mmol) was dissolved in anhydrous DMF (2 mL) under argon. 1-Fluoro-3-nitrobenzene (160 μL, 1.50 mmol) was added, followed by trimethylsilyl diethylamine (570 μL, 3.01 mmol), and *t*Bu-P4 phosphazene base (1 M in hexane; 150 μL, 0.15 mmol) dropwise over a period of 1 min (N.B. phosphazene solution warmed to 45 °C prior to use). The reaction was heated at 100 °C for 17 h, then allowed to cool to room temperature. The reaction was diluted with saturated NH_4Cl (10 mL) and H_2O (10 mL), and extracted with EtOAc (2×10 mL). The combined organics were washed with H_2O (20 mL), saturated brine (20 mL), dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified by flash silica chromatography (Biotage Isolera), 25 g column, 25 mL min^{-1}, gradient: 2% MeOH/CH_2Cl_2 1 cv, 2→20% MeOH 10 cv, 20% MeOH/CH_2Cl_2 2 cv, to give the product **39 c** as a yellow solid (53 mg, 12%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.99 (ddd, J = 0.9, 2.1, 8.2 Hz, 1H), 7.82 (t, J = 2.3 Hz, 1H), 7.52 (t, J = 8.2 Hz, 1H), 7.36 (m, 2H), 7.26 (m, 1H), 6.77 (t, J = 2.3 Hz, 1H), 3.90 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 163.5, 159.5, 158.3, 157.0, 149.6, 132.0, 130.8, 124.8, 118.3, 113.6, 112.3, 108.7, 108.0, 52.4 ppm; IR (ATR): $\tilde{\nu}_{max}$ = 3098, 1718, 1585 cm^{-1}; LCMS-ES *m/z* 288 [*M*−H]$^-$

5-Methyl 3-hydroxy-5-((3-nitrobenzyl)oxy)benzoate (39 d): Methyl 3,5-dihydroxybenzoate **36** (250 mg, 1.49 mmol), and NaH (60% in mineral oil; 60 mg, 1.49 mmol) were cooled to 0 °C under N_2. Anhydrous THF (5 mL) was added and mixture stirred for 10 min. A solution of 3-nitrobenzyl bromide (322 mg, 1.49 mmol) in anhydrous THF (5 mL) was added dropwise over a period of 5 min. The mixture was stirred at 0 °C for 15 min, then allowed to warm to room temperature and stirred for 17 h. Very little reaction was observed by TLC. An additional 60 mg of NaH (60% in mineral oil) was added, and reaction stirred for a further 8 h. The reaction was acidified with 1 N HCl (15 mL), and extracted with EtOAc (2× 15 mL). The combined organics were washed with saturated brine (15 mL), dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified by flash silica chromatography (Biotage Isolera), 25 g column, 25 mL min^{-1}, gradient: 1% MeOH/CH_2Cl_2 1 cv; 1→20% MeOH 10 cv; 20% MeOH/CH_2Cl_2 3 cv, to give the product **39 d** as a yellow solid (21 mg, 6% based on recovery of 56 mg starting material). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.32 (s, 1H), 8.20 (dd, J = 1.3, 8.1 Hz, 1H), 7.76 (d, J = 7.6 Hz, 1H), 7.58 (t, J = 7.9 Hz, 1H), 7.24 (dd, J = 1.3, 2.3 Hz, 1H), 7.17 (dd, J = 1.3, 2.3 Hz, 1H), 6.70 (t, J = 2.3 Hz, 1H), 5.17 (s, 2H), 3.91 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 166.2, 158.4, 156.9, 147.5, 137.9, 132.3, 131.2, 128.7, 122.1, 121.2, 109.4, 106.7, 106.4, 67.9, 51.5 ppm; IR (ATR): $\tilde{\nu}_{max}$ = 3678, 3075, 2924, 1722 cm^{-1}; LCMS-ES *m/z* 304 [*M*+H]$^+$, 302 [*M*−H]$^-$; HRMS-ES *m/z* [*M*−H]$^-$ calcd for $C_{15}H_{12}NO_6$ 302.0670, found 302.0679.

Hydroxy-3′-nitro-[1,1′-biphenyl]-3-carboxylic acid (41 a): KOH (38 mg, 0.68 mmol) in H_2O (1 mL) was added dropwise to a solution of methyl 5-hydroxy-3′-nitro-[1,1′-biphenyl]-3-carboxylate **39 a** (75 mg, 0.27 mmol) in THF (1 mL). The reaction mixture was stirred for 19 h at 25 °C. The reaction mixture was diluted with 1 M NaOH (10 mL) and washed with EtOAc (3×10 mL) before the aqueous phase was acidified by careful addition of 1 N HCl, and then extracted with EtOAc (3×70 mL). The organic phase was then washed with brine (200 mL), and dried over $MgSO_4$, before the solvent was removed under reduced pressure. Product **41 a** was obtained as a white solid (61 mg, 89%). ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 8.38 (t, J = 2.0 Hz, 1H), 8.24 (ddd, J = 1.0, 2.0, 8.1 Hz, 1H), 8.13 (ddd, J = 1.0, 2.0, 8.1 Hz, 1H), 7.77 (t, J = 8.1 Hz, 1H), 7.70 (t, J = 1.6 Hz, 1H), 7.42 (dd, J = 1.6, 2.4 Hz, 1H), 7.36 ppm (dd, J = 1.6, 2.4 Hz, 1H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 166.9, 158.1, 148.3, 140.9, 139.4, 133.2, 133.0, 130.5, 122.4, 121.1, 118.4, 118.0, 115.9 ppm; IR (ATR): $\tilde{\nu}_{max}$ = 3169, 3078, 2848, 2651, 1715, 1616, 1602, 1532, 1505, 1476, 1451, 1431, 1341 cm^{-1}; LCMS-ES m/z 277 $[M+NH_4]^+$, 258 $[M-H]^-$; HRMS-ES m/z $[M-H]^-$ calcd for $C_{13}H_8NO_5$ 258.0408, found 258.0412.

3′-Amino-5-hydroxy-[1,1′-biphenyl]-3-carboxylic acid (41 b): 5-Hydroxy-3′-nitro-[1,1′-biphenyl]-3-carboxylic acid **41 a** (5.0 mg, 19 μmol) and Pd/C (2.5 mg, 10 wt% Pd) were stirred in EtOH (1.5 mL) under a balloon of H_2 for 1 h. The reaction mixture was then filtered through Celite and the solvent removed under reduced pressure. The residue was purified by HPLC, column B, gradient: elution with 5% MeCN for 2.5 min then linear increase to 40% MeCN over 15 min, t_R = 13.4 min; to afford the title compound **41 b** as a white solid (3.7 mg, 85%). ^{1}H NMR (500 MHz, CD_3OD): δ = 7.74 (t, J = 1.4 Hz, 1H), 7.60 (dt, J = 1.4, 7.8 Hz, 1H), 7.55 (t, J = 7.8 Hz, 1H), 7.49 (t J = 2.0 Hz, 1H), 7.47 (dd, J = 1.4, 2.0 Hz, 1H), 7.27–7.29 ppm (m, 2H); ^{13}C NMR (125 MHz, CD_3OD): δ = 169.6, 159.5, 143.7, 142.6, 135.9, 134.0, 131.7, 126.7, 121.9, 121.6, 120.4, 119.4, 117.0 ppm; IR (ATR): $\tilde{\nu}_{max}$ = 3049, 2886, 2707, 2608, 1691, 1655, 1595, 1513, 1490, 1422, 1383, 1318, 1307 cm^{-1}; LCMS-ES m/z 230 $[M+H]^+$, 228 $[M-H]^-$; HRMS-ES m/z $[M+H]^+$ calcd for $C_{13}H_{12}NO_3$ 230.0812, found 230.0809.

3-Hydroxy-5-(3-nitrophenoxy)benzoic acid (41 c): Methyl 3-hydroxy-5-(3-nitrophenoxy)benzoate **39 c** (53 mg, 0.183 mmol) was dissolved in THF (4 mL) and 100 mM KOH (3 mL), and heated at 70 °C for 8 h. The reaction was allowed to cool to room temperature, acidified with 1 N HCl (15 mL), and extracted with EtOAc (2× 15 mL). The combined organics were washed with saturated brine (15 mL), dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified by flash silica chromatography (Biotage Isolera), 10 g column, 12 mL min^{-1}, gradient: 4% MeOH/CH_2Cl_2 1 cv; 4→20% MeOH 9 cv; 20→30% MeOH, 2 cv; 30% MeOH/CH_2Cl_2 6 cv, to give the product **41 c** as an off-white solid (15 mg, 45% based on recovery of 18 mg starting material). ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 7.98 (dd, J = 2.1, 8.0 Hz, 1H), 7.73 (t, J = 2.3 Hz, 1H), 7.67 (t, J = 8.2 Hz, 1H), 7.50 (dd, J = 2.4, 8.0 Hz, 1H), 7.26 (t, J = 1.7 Hz, 1H), 7.00 (t, J = 1.8 Hz, 1H), 6.68 ppm (t, J = 2.3 Hz, 1H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 159.4, 157.8, 156.5, 149.2, 131.7, 125.4, 118.5, 114.5, 113.2, 113.1, 110.6, 110.0, 104.5 ppm; IR (ATR): $\tilde{\nu}_{max}$ = 3255, 1716, 1605, 1585 cm^{-1}; LCMS-ES m/z 274 $[M-H]^-$; HRMS-ES m/z $[M-H]^-$ calcd for $C_{13}H_8NO_6$ 274.0357, found 274.0367.

3-Hydroxy-5-((3-nitrobenzyl)oxy)benzoic acid (41 d): Methyl 3-hydroxy-5-((3-nitrobenzyl)oxy)benzoate **39 d** (21 mg, 0.07 mmol) was dissolved in 100 mM KOH (2 mL) and THF (2 mL), and heated at 60 °C for 6 h. The reaction was allowed to cool to room temperature, acidified with 3 N HCl (20 mL), and extracted with EtOAc (2× 20 mL). The combined organics were washed with saturated brine (20 mL), dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified by flash silica chromatography (Biotage Isolera), 10 g column, 12 mL min^{-1}, gradient: 10% MeOH/CH_2Cl_2 1 cv; 1→20% MeOH 10 cv; 20% MeOH/CH_2Cl_2 1 cv; 20→30% MeOH, 1 cv; 30% MeOH/CH_2Cl_2 3 cv, to give the product **41 d** as a white solid (11 mg, 55%). ^{1}H NMR (400 MHz, CD_3OD): δ = 8.33 (s, 1H), 8.19 (dd, J = 1.4, 8.2 Hz, 1H), 7.85 (d, J = 7.6 Hz, 1H), 7.63 (t, J = 7.9 Hz, 1H), 7.15 (d, J = 2.1 Hz, 1H), 7.09 (d, J = 1.9 Hz, 1H), 6.66 (t, J = 2.2 Hz, 1H), 5.21 ppm (s, 2H); ^{13}C NMR (100 MHz, [D$_6$]acetone): δ = 167.3, 160.0, 159.0, 148.8, 140.0, 133.9, 133.2, 130.2, 123.0, 122.2, 110.1, 107.2, 107.1, 68.8 ppm; IR (ATR): $\tilde{\nu}_{max}$ = 3162, 1719, 1616 cm^{-1}; LCMS-ES m/z 288 $[M-H]^-$; HRMS-ES m/z calcd for $C_{14}H_{10}NO_6$ $[M-H]^-$ 288.0514, found 288.0527.

3-Hydroxy-5-((3-nitrobenzyl)amino)benzoic acid (41 e): 3-Nitrobenzaldehyde (79 mg, 0.52 mmol) and 3-amino-5-hydroxybenzoic acid **40** (90 mg, 0.59 mmol) were stirred in anhydrous DMF (1 mL) containing 1% AcOH and molecular sieves (3 Å) under nitrogen for 17 h. $NaBH_3CN$ (80 mg, 1.27 mmol) was added and reaction stirred for 1 h, then quenched by addition of H_2O (100 μL). The molecular sieves were removed by filtration, and the filtrate evaporated in vacuo to dryness. The crude product was purified by flash silica chromatography ($CHCl_3$/MeOH/AcOH/H_2O, 120:15:3:2 *v/v/v/v*) to give the product (41e) as a yellow solid, (138 mg, 93%). ^{1}H NMR (400 MHz, CD_3OD): δ = 8.39 (s, 1H), 8.18 (d, J = 2.4 Hz, 1H), 7.86 (1d, J = 2.4 Hz, 1H), 7.54 (t, J = 3.1 Hz, 1H), 6.87 (s, 1H), 6.72 (s, 1H), 6.28 (s, 1H), 4.86 ppm (s, 2H); ^{13}C NMR (100 MHz, CD_3OD): δ = 169.4, 158.5, 151.1, 147.7, 143.5, 134.6, 133.4, 129.6, 121.8 (×2), 106.2, 105.6, 104.0, 46.7 ppm; IR (ATR): $\tilde{\nu}_{max}$ = 3303, 3289, 3121, 3085, 1696, 1669, 1616, 1595, 1531, 1504, 1440, 1372 cm^{-1}; HRMS-ES m/z $[M+H]^+$ calcd for $C_{14}H_{13}N_2O_5$ 289.0819, found 289.0835.

Methyl 6-hydroxy-3′-nitro-[1,1′-biphenyl]-3-carboxylate (43 a): Methyl 4-hydroxy-3-iodobenzoate **42** (200 mg, 0.72 mmol) and 3-nitrophenylboronic acid (170 mg, 1.0 mmol), 2 M Na_2CO_3 solution (0.45 mL, 0.90 mmol) and tetrakis(triphenylphosphine) palladium (19 mg, 16 μmol) were dissolved sequentially in 1,2-dimethoxyethane (2.2 mL) under argon. The reaction mixture was heated under reflux for 17 h before cooling to 25 °C and H_2O (20 mL) was added. The reaction mixture was extracted with EtOAc (4×20 mL) and the combined organic phases were washed with saturated $NaHCO_3$ solution (80 mL), brine (80 mL) and dried over $MgSO_4$. The residue was triturated and sonicated in PE (6×20 mL) to remove residual starting material before purification by column chromatography, eluting with 2:1 *v/v* PE/EtOAc. The product **43 a** was collected as an off-white solid (97 mg, 46%). ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 10.90 (br s, 1H), 8.40 (br t, J = 1.9 Hz, 1H), 8.21 (ddd, J = 1.0, 2.4, 8.0 Hz, 1H), 8.03 (ddd, J = 1.0, 1.8, 8.0 Hz, 1H), 7.94 (d, J = 2.2 Hz, 1H), 7.89 (dd, J = 2.2, 8.5 Hz, 1H), 7.73 (t, J = 8.0 Hz, 1H), 7.10 (d, J = 8.5 Hz, 1H), 3.82 ppm (s, 3H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 165.7, 158.9, 147.6, 138.7, 135.5, 131.7, 131.1, 129.7, 125.3, 123.4, 121.8, 120.9, 116.3, 51.7 ppm; IR (ATR): $\tilde{\nu}_{max}$ = 3390, 3079, 3048, 3007, 2961, 1688, 1603, 1517, 1508, 1477, 1449, 1428, 1409, 1345, 1312 cm^{-1}; LCMS-ES m/z 291 $[M+NH_4]^+$, 272 $[M-H]^-$; HRMS-EI m/z $[M]^-$ calcd for $C_{14}H_{11}NO_5$ 273.0643, found 273.0646.

6-Hydroxy-3′-nitro-[1,1′-biphenyl]-3-carboxylic acid (44 a): NaOH (17.6 mg, 0.40 mmol) in H_2O (0.5 mL) was added dropwise to a solution of methyl 6-hydroxy-3′-nitro-[1,1′-biphenyl]-3-carboxylate **43 a** (40 mg, 0.15 mmol) in THF (0.5 mL). The reaction mixture was stirred for 3 h at 70 °C. The reaction mixture was diluted with 1 M NaOH (10 mL) and washed with EtOAc (3×10 mL) before the aqueous phase was acidified by careful addition of 1 N HCl and then extracted with EtOAc (3×70 mL). The organic phase was then

washed with brine (220 mL) and dried ($MgSO_4$) before the solvent was removed under reduced pressure. The product **44a** was obtained as a white solid (37 mg, 98%). ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 12.62 (br s, 1H), 10.79 (br s, 1H), 8.40 (t, J = 2.0 Hz, 1H), 8.20 (ddd, J = 1.5, 2.0, 8.0 Hz, 1H), 8.03 (ddd, J = 1.5, 2.0, 8.0 Hz, 1H), 7.93 (d, J = 2.0 Hz, 1H), 7.85 (dd, J = 2.0, 8.5 Hz, 1H), 7.73 (t, J = 8.0 Hz, 1H), 7.08 ppm (d, J = 8.5 Hz, 1H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 166.8, 158.5, 147.6, 139.0, 135.5, 131.9, 131.3, 129.7, 125.0, 123.4, 122.1, 121.8, 116.1 ppm; IR (ATR): $\tilde{\nu}_{max}$ = 3397, 2824, 2548, 1683, 1609, 1592, 1572, 1527, 1509, 1479, 1454, 1426, 1401, 1349, 1314 cm^{-1}; LCMS-ES m/z 258 $[M-H]^-$; HRMS-EI m/z $[M]^-$ calcd for $C_{13}H_9NO_5$ 259.0486, found 259.0480.

3′-Amino-6-hydroxy-[1,1′-biphenyl]-3-carboxylic acid (44b): 6-Hydroxy-3′-nitro-[1,1′-biphenyl]-3-carboxylic acid **44a** (3.0 mg, 12 μmol) and Pd/C (2.5 mg, 10 wt% Pd) were stirred in EtOH (1.5 mL) under a balloon of H_2 for 1 h. The reaction mixture was then filtered through Celite, and the solvent removed under reduced pressure. The residue was purified by HPLC, column B, gradient: elution with 10% MeCN for 2.5 min then linear increase to 40% MeCN over 8 min, t_R = 9.8 min, to afford the desired product **44b** as a white solid (2.4 mg, 79%). ^{1}H NMR (500 MHz, CD_3OD): δ = 7.99 (d, J = 2.2 Hz, 1H), 7.90 (dd, J = 2.2, 8.5 Hz, 1H), 7.59–7.61 (m, 2H), 7.54 (t, J = 8.2 Hz, 1H), 7.27 (ddd, J = 1.0, 2.2, 8.2 Hz, 1H), 6.98 ppm (d, J = 8.5 Hz, 1H); ^{13}C NMR (125 MHz, CD_3OD): δ = 169.8, 160.3, 141.5, 134.0, 133.7, 132.5, 130.9, 129.6, 128.1, 124.0, 123.4, 121.6, 116.9 ppm; LCMS-ES m/z 230 $[M+H]^+$, 228 $[M-H]^-$; HRMS-ES m/z $[M+H]^+$ calcd for $C_{13}H_{12}NO_3$ 230.0812, found 230.0813.

Dimethyl 5-((3-nitrobenzyl)oxy)isophthalate (47d): 3-Nitrobenzyl bromide (103 mg, 0.477 mmol), dimethyl 5-hydroxyisophthalate **45** (100 mg, 0.476 mmol), and Cs_2CO_3 (308 mg, 0.945 mmol) were suspended in anhydrous DMF (5 mL) under N_2, and stirred at room temperature for 18 h. The reaction was diluted with saturated NH_4Cl (20 mL) and H_2O (20 mL), and extracted with EtOAc (2× 20 mL). The combined organics were washed with saturated brine (20 mL), dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified by flash silica chromatography (Biotage Isolera), 10 g column, 12 mL min^{-1}, gradient: 20% EtOAc/hexane 1 cv, 20→80% EtOAc10 cv, 80% EtOAc/hexane 2 cv, to give the product **47d** as a white solid (130 mg, 79%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.31 (s, 1H), 8.29 (s, 1H), 8.18 (d, J = 8.1 Hz, 1H), 7.81 (s, 2H), 7.77 (d, J = 7.6 Hz, 1H), 7.57 (t, J = 7.9 Hz, 1H), 5.22 (s, 2H), 3.92 ppm (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 166.3, 158.6, 148.9, 138.7, 133.5, 132.4, 130.1, 124.1, 123.6, 122.6, 120.4, 69.4, 52.9 ppm; IR (ATR): $\tilde{\nu}_{max}$ = 3094, 2959, 1719, 1594 cm^{-1}; LCMS-ES m/z 363 $[M+NH_4]^+$; HRMS-ES m/z $[M-H]^-$ calcd for $C_{17}H_{14}NO_7$344.0776, found 344.0787.

Dimethyl 5-((3-nitrobenzyl)amino)isophthalate (47e): 3-Nitrobenzaldehyde (166 mg, 1.10 mmol) and dimethyl 5-aminoisophthalate **46** (209 mg, 1.00 mmol) in anhydrous toluene (8 mL) were heated at 140 °C under N_2 in a flask with a Dean–Stark apparatus attached for 3 h. The solvent was removed under reduced pressure, $NaBH_3CN$ (69 mg, 1.10 mmol), AcOH (200 μL), and anhydrous THF (8 mL) were added, and the mixture was stirred under N_2 at room temperature for 15 h. The reaction was diluted with saturated $NaHCO_3$ solution (12.5 mL) and H_2O (12.5 mmol), and extracted with EtOAc (2×25 mL). The combined organics were dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified by flash silica chromatography (Biotage Isolera), 25 g column, 25 mL min^{-1}, gradient: 1% MeOH/CH_2Cl_2 1 cv, 1→10% MeOH 10 cv, 10→20% MeOH 2 cv, 20% MeOH/CH_2Cl_2 5.7 cv, to give the product **47e** as an off-white solid (245 mg, 65%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.22 (s, 1H), 8.12 (d, J = 8.2 Hz, 1H), 8.02 (t, J = 1.4 Hz, 1H), 7.69 (d, J = 7.7 Hz, 1H), 7.51 (d, J = 7.9 Hz, 1H), 7.45 (d, J = 1.4 Hz, 2H), 4.62 (br, 1H), 4.53 (s, 2H), 3.89 ppm (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 166.9, 149.0, 147.9, 141.3, 133.6, 132.0, 130.2, 123.0, 122.5, 120.6, 118.1, 52.7, 47.7 ppm; IR (ATR): $\tilde{\nu}_{max}$ = 3403, 2952, 1705, 1608 cm^{-1}; LCMS-ES m/z 345 $[M+H]^+$; HRMS-ES m/z $[M+H]^+$ calcd for $C_{17}H_{17}N_2O_6$ 345.1087, found 345.1086.

5-((3-Nitrobenzyl)oxy)isophthalic acid (49d): Dimethyl 5-((3-nitrobenzyl)oxy)isophthalate **47d** (51 mg, 0.148 mmol) was dissolved in THF (4 mL) and 100 mM KOH solution (4 mL), and heated at 60 °C for 18 h. The reaction was allowed to cool to room temperature, diluted with saturated NH_4Cl solution (10 mL) and H_2O (10 mL), and extracted with EtOAc (2×20 mL). The combined organics were dried over Na_2SO_4, filtered, and concentrated under reduced pressure to give the product **49d** as a white solid (30 mg, 64%). ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 8.36 (s, 1H), 8.21 (d, J = 8.2 Hz, 1H), 8.11 (t, J = 1.4 Hz, 1H), 7.96 (d, J = 7.9 Hz, 1H), 7.78 (d, J = 1.4 Hz, 2H), 7.72 (t, J = 8.0 Hz, 1H), 5.41 ppm (s, 2H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 166.7, 158.4, 148.2, 139.2, 134.2, 133.0, 130.3, 123.1, 123.1, 122.3, 119.8, 68.8 ppm; IR (ATR): $\tilde{\nu}_{max}$ = 2829, 2549, 1692, 1596 cm^{-1}; LCMS-ES m/z 316 $[M-H]^-$; HRMS-ES m/z $[M-H]^-$ calcd for $C_{15}H_{10}NO_7$ 316.0463, found 316.0472.

5-((3-Nitrobenzyl)amino)isophthalic acid (49e): Dimethyl 5-((3-nitrobenzyl)amino)isophthalate **47e** (210 mg, 0.610 mmol) was suspended in 2.5 M NaOH (5 mL), THF (5 mL) and MeOH (5 mL), and stirred at room temperature for 3 h. The reaction was acidified with 3 N HCl (20 mL) and extracted with EtOAc (2×50 mL). The combined organics were dried over Na_2SO_4, filtered, and concentrated under reduced pressure to give the product **49e** as a yellow solid (193 mg, quant). ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 12.87 (br, 2H), 8.23 (s, 1H), 8.11 (d, J = 8.2 Hz, 1H), 7.83 (d, J = 7.8 Hz, 1H), 7.70 (t, J = 1.4 Hz, 1H), 7.65 (t, J = 7.9 Hz, 1H), 7.37 (d, J = 1.4 Hz, 1H), 6.99 (t, J = 6.0 Hz, 1H), 4.52 ppm (d, J = 5.7 Hz, 2H); IR (ATR): $\tilde{\nu}_{max}$ = 3395, 3369, 2917, 2528, 1716, 1688 cm^{-1}; LCMS-ES m/z 317 $[M+H]^+$, 315 $[M-H]^-$; HRM-ES m/z $[M+H]^+$ calcd for $C_{15}H_{13}N_2O_6$ 317.0774, found 317.0776.

5-((4-Nitrobenzyl)amino)isophthalic acid (49f): 4-Nitrobenzaldehyde (347 mg, 2.30 mmol) and 5-aminoisophthalic acid **48** (500 mg, 2.76 mmol) were stirred in anhydrous DMF (3 mL) containing 1% AcOH and molecular sieves (3 Å) under nitrogen for 17 h. $NaBH_3CN$ (580 mg, 9.2 mmol) was added and reaction stirred for 1 h, then quenched by addition of H_2O (500 μL). The molecular sieves were removed by filtration and the filtrate evaporated in vacuo to dryness. The crude product was purified by flash silica chromatography ($CHCl_3$/MeOH/AcOH/H_2O, 120:15:3:2 *v/v/v/v*) to give the product **49f** as a brown solid (695 mg, 95%); ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 10.98 (br s, 2H), 8.23 (d, J = 2.0 Hz, 2H), 7.72 (s, 1H), 7.61 (d, J = 2.3 Hz, 2H), 7.36 (s, 2H), 4.52 (s, 1H), 4.51 ppm (s, 2H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 169.3, 168.5, 148.8, 147.5, 145.3, 133.6, 131.6, 129.3, 128.7, 125.8, 124.6, 121.7, 119.7, 118.8, 48.8 ppm; IR (ATR): $\tilde{\nu}_{max}$ = 3431, 3076, 2836, 2620, 1688, 1607, 1513, 1438, 1358 cm^{-1}; LCMS-ES m/z 317 $[M+H]^+$; HRMS-ES m/z $[M+H]^+$ calcd for $C_{15}H_{13}N_2O_6$ 317.0774, found 317.0770.

Synthesis of 3-dehydroquinate (1): 3-Dehydroquinate **1** was synthesized (as the potassium salt) from (−)-quinic acid using the method described by Le Sann et al.[41] Calibration of 3-dehydroquinate solutions (in H_2O) were determined from the absorbance difference at λ 234 nm resulting from the total conversion of an aliquot of 3-dehydroquinate to 3-dehydroshikimate by 1 μL of *S. coelicolor* type II dehydroquinase (5.1 mg mL^{-1}) using the kinetic assay A described below.

Enzyme purification

M. tuberculosis type II dehydroquinase was purified as described previously.[42]

E. coli shikimate dehydrogenase: The *aroE* gene (819 bp) from *E. coli* K12 was amplified by PCR and cloned into the *Nde*I/*Xho*I-digested pET-28a vector for expression in order to place the gene in-frame with an N-terminal $(His)_6$-tag sequence cleavable by thrombin. An overnight culture (100 mL) of *E. coli* C41(DE3) competent cells transformed with plasmid pET-28a/*aroE* was added to 1 L of fresh 2YT media supplemented with 30 $\mu g\,mL^{-1}$ kanamycin and divided into 2×500 mL cultures. Overexpression of $(His)_6$–AroE 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 $6084\times g$ for 15 min at 4 °C. Cell pellets were stored at −20 °C. The 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. The suspension was homogenized for 20 min by sonication on ice. The crude extract was then centrifuged for 30 min at $38\,724\times g$. The resulting supernatant was applied at a flow rate of $1.0\,mL\,min^{-1}$ to a 5 mL HiTrap chelating column (Amersham Biosciences) previously charged with 0.1 M $NiSO_4$ and equilibrated with buffer A at 4 °C. After washing with buffer A (10 bed volumes; $2.5\,mL\,min^{-1}$), the protein was eluted using a linear gradient of 20–500 mM imidazole in 100 mL buffer A ($2.5\,mL\,min^{-1}$). The concentrated protein sample was then dialyzed at 4 °C against 100 mM potassium phosphate, pH 7.0, using a PD-10 column containing Sephadex G-25 resin (Amersham Biosciences). The purity of the protein was determined to be ≥95% by SDS-PAGE. Enzyme concentrations were evaluated by measuring the absorbance at 280 nm and using the conversion factor ($OD_{280}=1.0$ corresponds to $1.74\,mg\,mL^{-1}$ for $(His)_6$-AroE) 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.

Kinetic assays for type II dehydroquinases

Two different assays for type II dehydroquinases were used in this work: a direct assay (A) and a coupled assay with *E. coli* shikimate dehydrogenase (B).

Assay A was previously described for *S. coelicolor* and *M. tuberculosis* type II dehydroquinases.[43] It consists of monitoring the increase in absorbance at λ 234 nm due to the formation of the enone–carboxylate chromophore of 3-dehydroshikimate (**2**). The assay was performed at 25 °C in Tris·HCl buffer (0.05 M, pH 7.0) using 90 nM *M. tuberculosis* dehydroquinase.

Assay B: Enzymes were assayed by coupling the formation of 3-dehydroshikimate to the oxidation of NADPH in the presence of *E. coli* shikimate dehydrogenase (EC 1.1.1.25). This assay was developed to overcome the problems caused by the absorption of some inhibitors at λ 234 nm. Moreover, due to their poor solubility in water, some of the compounds had to be assayed using DMSO, which also absorbs at λ 234 nm. The initial rate of the reaction was determined by monitoring the decrease in absorption of NADPH at λ 340 nm using a Bio-tek Powerwave XS multi-well plate spectrophotometer, thermostatically controlled at 28 °C. All coupled reactions were performed in 60 mM Tris·HCl buffer (pH 7.0), 0.5 mM NADPH, 1 μM *E. coli* shikimate dehydrogenase and included 90 nM *M. tuberculosis* dehydroquinase. It was ensured that the shikimate dehydrogenase reaction was not limiting, and that the K_M and k_{cat} of the enzymes were in agreement with published data.[44] There was no change in the measured rate when using twice as much shikimate dehydrogenase (2 μM) and the measured velocity was proportional to the amount of dehydroquinase (when doubled or trebled). Using a concentration of 0.5 mM NADPH allowed a linear consumption of NADPH vs. time over 5–10 min. Some of the assayed compounds were not soluble in water at high concentrations, hence stock solutions in DMSO had to be prepared previous to further dilution in buffer or water. DMSO (5%) was used in the assay in this case.

The inhibition kinetics data were obtained by measuring the initial rates of reaction over a range of inhibitor concentrations (between 4 and 7 different concentrations) at either 4 or 5 different substrate concentrations. The inhibition constants and standard deviations were obtained by least-squares fitting using the software GraFit.[45]

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