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A Series of 2(*Z*)-2-Benzylidene-6,7-dihydroxybenzofuran-3[2H]-ones as Inhibitors of Chorismate Synthase

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Abstract—A series of 2(*Z*)-2-benzylidene-6,7-dihydroxybenzofuran-3[2H]-ones was identified as potent inhibitors of bacterial chorismate synthase. The 2′-hydroxy-4′-pentoxy analogue **33** is a potent inhibitor of *Streptococcus pneumoniae* chorismate synthase.

Introduction

The emergence of bacterial resistance to established antibiotics in community and hospital-acquired infections is an ever-growing concern. In particular, the 'big three' Gram-positive species, staphylococcus, enterococcus and streptococcus, have all evolved resistant strains over the last few years: methicillin-resistant *Staphylococcus aureus* (MRSA) strains now account for over 40% of *S. aureus* infections in hospitals;[1] vancomycin-resistant enterococcal (VRE) infections have also increased substantially, with some strains resistant to all established antibiotics;[2] *Streptococcus pneumoniae* is the most common cause of community-acquired pneumoniae and is increasingly resistant to β-lactams and macrolides. For these reasons, there is an urgent need for new antibacterial agents, particularly those that act on novel targets.

The shikimic acid pathway is essential for the synthesis of aromatic amino acids in bacteria. Because the pathway is absent in man, it is an attractive target for potential antibiotics. The pathway has been validated as an antimicrobial target by Monsanto's herbicide glyphosate **1** (Roundup®)[3] which acts on EPSP (5-enolpyruvyl shikimate-3-phosphate) synthase, and the Zeneca compound 6(*S*)-fluoroshikimate **2**,[4] which, after conversion to 6(*S*)-fluoro-EPSP, is a substrate for chorismate synthase (Fig. 1).

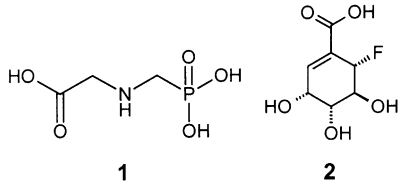
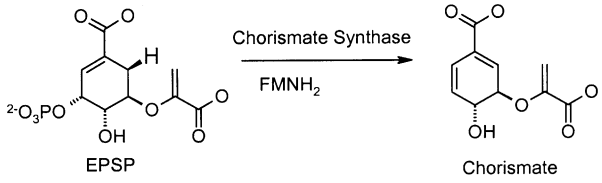


Figure 1. Structures of glyphosate **1** and 6(*S*)-fluoroshikimate **2**.

Chorismate synthase (CS) is a key enzyme in the pathway and converts EPSP to chorismate via a 1,4-*trans* elimination of phosphate[5] (Scheme 1). The reaction is unusual in that there is an absolute requirement for reduced FMN as a cofactor, although the reaction does not involve a net redox change.

The *aroC* gene from *S. pneumoniae* was amplified by PCR and cloned into a pET-16b expression vector system (Novagen). The N-terminally His-tagged protein was over-expressed in *Escherichia coli* by induction with IPTG, and purified to homogeneity in three chromatographic steps.



Scheme 1. Conversion of EPSP to chorismate.

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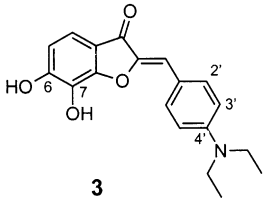
S. pneumoniae CS (SpCS) was assayed using a phosphate release detection system.[6] Briefly; CS, FMN, EPSP and the compound of interest were incubated together under anaerobic conditions. The reaction was initiated by addition of dithionite to reduce FMN. Phosphate was detected continuously by coupling its production to the cleavage of 2-amino-6-mercapto-7-methylpurine ribonucleoside by purine nucleoside phosphorylase and measuring the increase in absorbance at 360 nm. For screening purposes, the enzymic solution was overlaid with mineral oil prior to the addition of sodium dithionite.

An extensive library of chemically diverse small molecules was screened for inhibitors of SpCS. These included the benzofuran-3[2H]-one **3**. The compound was moderately potent, with an IC_{50} of 8 μM. Kinetic studies demonstrated that **3** was a competitive inhibitor with respect to EPSP (K_i 0.65 μM), and non-competitive with respect to the cofactor FMN (αK_i 1.7 μM), suggesting that the compound was binding in the active site of the enzyme. The K_m values for EPSP and FMN are 30 and 0.08 μM, respectively.

We initiated a chemistry programme to improve CS potency. The 2-benzylidene group was modified, whilst the catechol was left unchanged in order to retain solubility.



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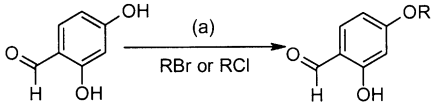
Chemistry

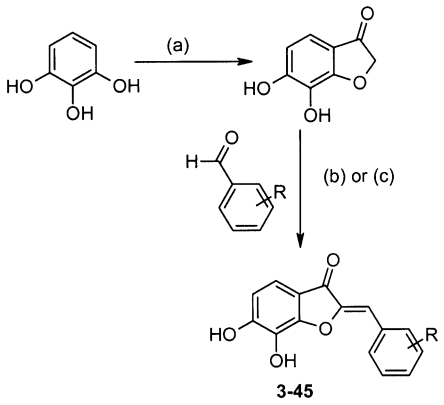
2-Hydroxy-4-alkoxy benzaldehydes were synthesized by alkylation of 2,4-dihydroxybenzaldehyde in refluxing DMF, with sodium bicarbonate as base[7] (Scheme 2).

2-Benzylidenebenzofuran-3[2H]-ones were synthesized according to Scheme 3. 6,7-Dihydroxybenzofuran-3[2H]-one was synthesized from pyrogallol by treatment with chloroacetic acid and phosphorus oxychloride to generate the chloroacetophenone. This was cyclised using sodium acetate[8] in refluxing ethanol.

The condensation of 6,7-dihydroxybenzofuran-3[2H]-one with variously substituted benzaldehydes was carried out in refluxing ethanol, in the presence of either HCl or NaOH. Condensations gave single isomers, as shown by both LC–MS and NMR experiments. Literature precedent[9] suggested that the *Z*-isomer would be obtained, and this was confirmed by crystallography.



Scheme 2. Reagents and conditions: (a) $NaHCO_3$, DMF, reflux, 28–55%.



Scheme 3. Reagents and conditions: (a) (i) chloroacetic acid, phosphorus oxychloride, 70 °C; (ii) sodium acetate, ethanol, reflux, 28%; (b) HCl, ethanol, reflux, 50–95%; (c) NaOH, ethanol, reflux, 50–75%.

Biology

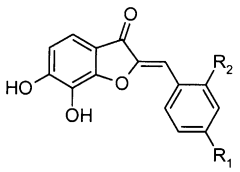
Modification of the substituents on the benzylidene group of **3** led to more potent analogues. Based on the hypothesis that the nitrogen of the 4′-diethylamino group was acting as a hydrogen bond acceptor, while the ethyl groups were filling a hydrophobic pocket, the effect of varying the dialkylamino group was examined (Table 1).

The dimethylamino analogue **4** showed a 3-fold lower potency than **3**. However, the pyrrolidine analogue **5** was slightly more potent, whilst the morpholine analogue **6** was significantly less so. This indicated that there was limited space for further substitutions around the 4′-position.

As changes to the *N*-alkyl substituents led to limited improvement in potency, alternative 4′-substituents were examined (Table 2). Surprisingly, the unsubstituted analogue **9** had an IC_{50} of 3.5 μM, suggesting that the 4′-amino group did not contribute favorably to binding.

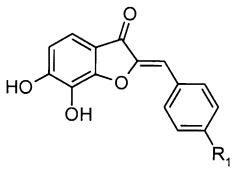
Of the 4′-substituted analogues, methyl **10**, nitrile **11**, carboxylate **12** and fluoro **16** proved inactive, whilst moderate potency was seen for trifluoromethyl **13**, nitro **14**, hydroxy **17** and alkoxy analogues **18**–**20**. In this latter

Table 1. Effect of changes to the 4′-amino group on inhibition of *Streptococcus pneumoniae* chorismate synthase, compounds **3**–**8**



Compd	R_1	R_2	Activity versus SpCS IC_{50}, μM
3	$N(CH_2CH_3)_2$	H	8.5
4	$N(CH_3)_2$	H	25.9
5	Pyrrolidino	H	5.5
6	Morpholino	H	>50
7	$N(CH_2CH_3)_2$	OH	5.0
8	$N(CH_2CH_3)(CH_2)_2OH$	Me	3.4

Table 2. Effect of 4′-substituents on inhibition of *Streptococcus pneumoniae* chorismate synthase, compounds **9**–**20**



Compd	R_1	Activity versus SpCS IC_{50}, μM[a]
9	H	3.5
10	CH_3	n/a
11	CN	n/a
12	COOH	24
13	CF_3	7
14	NO_2	4.8
15	*N*-Imidazoyl	5.1
16	F	>60
17	OH	15.0
18	OCH_3	14.3
19	OCH_2CH_3	5.8
20	$O(CH_2)_3CH_3$	1.5

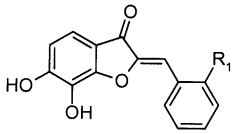
[a]n/a, inactive compounds.

group, we observed increasing potency with longer chain lengths, with the 4′-butoxy compound having an IC_{50} of 1.5 μM.

Next, the effects of changes at the *ortho* position of the benzylidene group were investigated (Table 3). A number of functional groups were tolerated in the 2′-position. Methyl, chloro, nitro and oxyacetic acid analogues (**22**, **24**, **26** and **28**, respectively) all had similar IC_{50}'s to the unsubstituted benzylidene compund, whereas the carboxylate derivative **27** was less potent and the methoxy compound **23** was inactive. The best inhibitor was the 2′-hydroxy analogue **21**, with an IC_{50} of 0.8 μM. Comparison of compounds **21** (2′-hydroxy) and **23** (2′-methoxy) suggested that the hydroxyl group was acting as a hydrogen bond donor.

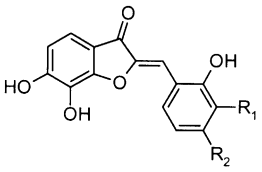
Lastly, compounds were synthesized that combined the *ortho*-hydroxy and *para*-alkoxy substituents on the benzylidene ring (Table 4). Potency improved with increasing alkoxy chain length, reaching a maximum with

Table 3. Effect of 2′-substituents on inhibition of *Streptococcus pneumoniae* chorismate synthase, compounds **21**–**28**



Compd	R_1	Activity versus SpCS IC_{50}, μM
21	OH	0.8
22	CH_3	4.0
23	OCH_3	>60
24	Cl	5.2
25	CF_3	17.3
26	NO_2	2.0
27	COOH	10.7
28	OCH_2COOH	1.8

Table 4. Inhibition of *Streptococcus pneumoniae* chorismate synthase for compounds **21** and **29**–**45**, 2′-hydroxy substituted analogues



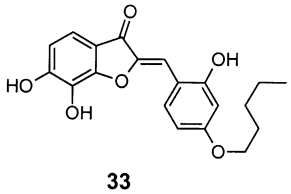
Compd	R_1	R_2	Activity versus SpCS IC_{50}, μM
21	H	H	0.80
29	OCH_3	H	1.7
30	H	OCH_3	0.70
31	H	$O(CH_2)_2CH_3$	0.51
32	H	$O(CH_2)_3CH_3$	0.45
33	H	$O(CH_2)_4CH_3$	0.22
34	H	$O(CH_2)_5CH_3$	0.32
35	H	$OCH(CH_3)_2$	1.0
36	H	$OCH_2CH(CH_3)_2$	0.58
37	H	$OCH_2C_6H_5$	1.0
38	H	$O(CH_2)_3OH$	2.5
39	H	$O(CH_2)_4OH$	0.86
40	H	$O(CH_2)_6OH$	1.6
41	H	OCH_2COOEt	10.3
42	H	OCH_2COOH	2.6
43	H	$O(CH_2)_3COOEt$	0.65
44	H	$O(CH_2)_3COOH$	1.1
45	H	$O(CH_2)_3CN$	1.1

the pentoxy-analogue **33** at 0.22 μM, and decreasing thereafter with the hexyloxy analogue **34**. This represented a 40-fold improvement in potency over the initial lead.

The effect of chain branching was investigated. The 4′-*iso*propoxy analogue **35** showed a 2-fold lower potency than the *n*-propoxy compound **31**, and the *iso*-butoxy **36** was less potent than the *n*-butoxy derivative **32**. The introduction of an *O*-benzyl substituent was also detrimental to potency.

In order to compare the binding of this series of analogues with the lead compound **3**, kinetic studies were performed on compound **30**. This analogue was shown to be competitive with EPSP (K_i 0.10 μM) and non-competitive with FMN (αK_i 1.4 μM), indicating that these compounds were binding in the same way as **3**.

Attempts to improve activity further, by incorporation of functionality into the alkoxy group, met with limited success. The acids **42** and **44** were reasonable inhibitors, with IC_{50}'s of 2.6 and 1.1 μM, respectively. Both the alcohol **39** and the ester **43** were sub-micromolar inhibitors and were more soluble than the unfunctionalized analogues, a factor that may be important for later compounds in the series.



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Conclusion

A total of 70 analogues retaining the 6,7-dihydroxy functionality were synthesized as inhibitors of CS. This led to the identification of the 2′-hydroxy-4′-pentoxy analogue **33** which was forty times more potent than the original lead. Due to the metabolic vulnerability and potential toxicity of the 6,7-dihydroxy functionality, and also in an effort to increase potency, further synthetic efforts have focused on replacing or removing this group. These results will be reported subsequently.

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