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Research paper

3-(Benzodioxan-2-ylmethoxy)-2,6-difluorobenzamides bearing hydrophobic substituents at the 7-position of the benzodioxane nucleus potently inhibit methicillin-resistant *Sa* and *Mtb* cell division

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ABSTRACT

Lipophilic substituents at benzodioxane C (7) of 3-(benzodioxan-2-ylmethoxy)-2,6-difluorobenzamide improve the antibacterial activity against methicillin-resistant *Staphylococcus aureus* strains to MIC values in the range of 0.2–2.5 μg/mL, whereas hydrophilic substituents at the same position and modifications at the benzodioxane substructure, excepting for replacement with 2-cromanyl, are deleterious. Some of the lead compounds also exhibit good activity against *Mtb*. Parallel SARs to those of 3-(2-benzothiazol-2-ylmethoxy)-2,6-difluorobenzamide, well known FtsZ inhibitor, and cells alterations typical of FtsZ inhibition indicate such a protein as the target of these potent antibacterial benzodioxane-benzamides.

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1. Introduction

Filamentous temperature-sensitive protein Z (FtsZ) is a highly conserved and ubiquitous bacterial protein. When bacteria divide, FtsZ polymerizes in a GTP-dependent manner to form the Z-ring at the mid-point of the cell under the membrane and, after recruiting other cell division proteins, such a cytokinetic structure contracts resulting in septum closure and formation of two daughter cells [1]. Because of its important role in bacterial cell division, FtsZ is a promising target for agents able of altering its proper assembly and thus of exerting antibiotic activity by inhibition of cell division. Among the FtsZ-targeting compounds, the benzamide derivative PC190723, developed from 2,6-difluoro-3-nonyloxybenzamide (DFNB) [2], displays a potent antistaphylococcal activity (1 μg/mL MIC) and it is one of the most attractive and studied antibacterial agent [3,4]. In the successive optimization of PC190723 [5,6], the best option proved to be replacement of thiazolopyridine with a phenyl substituted oxazole and hydroxymethyl substitution at the pseudo-benzylic position of the linker, this latter modification allowing both advantageous chiral switch to eutomer (*R*)-**1** (0.12 μg/mL MIC against *Staphylococcus aureus*) and esterification to succinate prodrug (*R*)-**2** [6]. Successively to PC190723 discovery and contemporarily with its recent optimization to oxazole-difluorobenzamide (*R*)-**1**, we have reported that the chloro-substituted benzodioxane-difluorobenzamide (*S*)-**4** is a potent bacterial cell division inhibitor **1** (0.25 μg/mL MIC against *S. aureus*) causing cell volume increase, as potent as *rac*-**1**, with which it is thought to share the same FtsZ-targeting mechanism proper of

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heteroarylmethyl ethers of 2,6-difluoro-5-hydroxybenzamide [7]. We have found that **4**, as a racemate, is ten-fold more potent than the unsubstituted benzodioxane-difluorobenzamide **3** and its *S* enantiomer, the eutomer, is twenty-fold more potent than **3** (Chart 1).

In a continuation of our effort to further develop benzodioxane-difluorobenzamides as antibacterial agents and to consolidate their SAR analysis, we have designed a number of analogues of **3** through a series of isosteric, positional or substituent modifications and we have extended the biological evaluation to strains of gram-positive bacterial clinical isolates, with different drug-resistance, and to *Mycobacterium tuberculosis*. Here we report the synthesis of difluorobenzamides **5**–**23**, the antibacterial activity and the SAR study of **3**–**22**, some of which showed <1 μg/mL antistaphylococcal and ~10 μg/mL antitubercular activity, associated to bacterial swelling and, as revealed by electron microscopy, to inhibition of septum formation (Chart 2).

1.1. Chemistry

The syntheses of compounds **5**–**10** are shown in Scheme 1. The chromane derivatives **5** and **6** and the tetrahydronaphthalene analogue **7** were prepared by mesylation of 2-hydroxymethylchromane [8], 3-hydroxymethylchromane [8] and 2-hydroxymethyl-1,2,3,4-tetrahydronaphthalene [8], respectively, and etherification of 2,6-difluoro-3-oxybenzamide [9] with the resulting mesylates **24**–**26**. Compound **8**, in which the difluorobenzamide portion is linked through the oxymethylene bridge to benzodioxane benzene, was prepared by *O*-alkylating 2,6-difluoro-3-oxybenzamide with 1,2-ethylenedioxy-4-bromomethylbenzene [10]. The glycerol 1,3-diphenyl ether **9** was synthesized from 1-benzyloxy-3-phenoxy-2-propanol [11], which was mesylated (**27**), reacted with 2,6-difluoro-3-oxybenzamide (**28**) and debenzylated. To prepare the 6,7-dichlorobenzodioxane **10**, 3,4-dichlorophenol was converted into acetate **29**, submitted to Fries transposition (**30**), condensed with epibromohydrin (**31**), Baeyer–Villiger-oxidized to phenyl acetate **32**, cyclized to 6,7-dichloro-2-hydroxymethyl-1,4-benzodioxane **33**, mesylated (**34**) and then used to *O*-alkylate 2,6-difluoro-3-oxybenzamide.

The syntheses of 7-substituted benzodioxane derivatives **11**–**19** are shown in Scheme 2. The 7-bromo substituted benzodioxane **11** was synthesized from 2-acetyl-4-bromophenol in five steps: (1) etherification with epichlorohydrin (**35**), (2) Baeyer–Villiger oxidation to phenyl acetate **36**, (3) ester hydrolysis followed by intramolecular cyclization (**37**), (4) mesylation (**38**), (5) *O*-alkylation of 2,6-difluoro-3-oxybenzamide with the mesylate of 2-hydroxymethl-7-bromo-1,4-benzodioxane **38**. By the same steps (**39**–**42**), the 7-trifluoromethyl analogue **18** was prepared from 2-hydroxy-5-trifluoromethylbenzaldeyde. The 7-fluoro derivative **13** was obtained from 2-hydroxymethyl-7-fluoro-1,4-benzodioxane, the synthesis of which has been previously reported [12], by mesylation (**43**) and *O*- alkylation of 2,6-difluoro-3-oxybenzamide with the resultant mesylate. The 2-hydroxymethl-7-bromo-1,4-benzodioxane intermediate **37** was used as an acetate (**44**) to prepare both the 7-vinyl and the 7-ethynyl derivative, **15** and **14**. Palladium catalysed cross-coupling reaction with potassium vinyltrifluoroborate provided the 7-vinyl-1,4-benzodioxane intermediate **45**, whose acetate function was hydrolyzed. The resultant 2-hydroxymethyl-7-vinyl-1,4-benzodioxane **46** was mesylated (**47**) and used to *O*-alkylate 2,6-difluoro-3-oxybenzamide to yield **15**. On the other hand, Sonogashira reaction with trimethylsilylacetylene gave the 7-trimethylsilylethynyl-1,4-benzodioxane intermediate **48**, which was converted into 7-ethynyl-2-hydroxymethyl-1,4-benzodioxane **49** and then into **14** by the same two last steps as those used to obtain **15**. Reaction of 4-nitrocatechol with epibromohydrin yielded 7-nitro-2-hydroxymethyl-1,4-benzodioxane (**51**), which was tosylated (**52**) and reacted with 2,6-difluoro-3-oxybenzamide to give the 7-nitro derivative **16**. Reduction with $SnCl_2$ converted **16** into the 7-amino derivative **17**, which was transformed into the corresponding methanesulfonamide **19** by treatment with mesyl chloride. 7-Nitro-2-hydroxymethyl-1,4-benzodioxane (**51**) was used to synthesize the 7-iododerivative **12** through acetylation (**53**), reduction of nitro to amino (**54**), diazotation and treatment with NaI (**55**), acetate hydrolysis (**56**), mesylation (**57**) and reaction with 2,6-difluoro-3-oxybenzamide.

The syntheses of compounds **20**–**23** are shown in Scheme 3. The synthesis of 7-methoxycarbonyl substituted **21** started from methyl 3,4-dihydroxybenzoate which was reacted with epibromohydrin to give 7-methoxycarbonyl substituted 2-hydroxymethyl-1,4-benzodioxane containing 15% of the 6-methoxycarbonyl regioisomer. After conversion into the corresponding benzyl ethers mixture and methyl ester hydrolysis, double crystallization from toluene allowed the undesired regioisomer to be removed. The 7-carboxy intermediate **58** was reconverted into methyl ester (**59**), debenzylated (**60**) and condensed with 2,6-difluoro-3-oxybenzamide to **21** by Mitsunobu reaction or, alternatively, it was converted into primary amide **61** or *N*-butylamide **64**, debenzylated (**62** or **65**), tosylated (**63**) or mesylated (**66**) and reacted

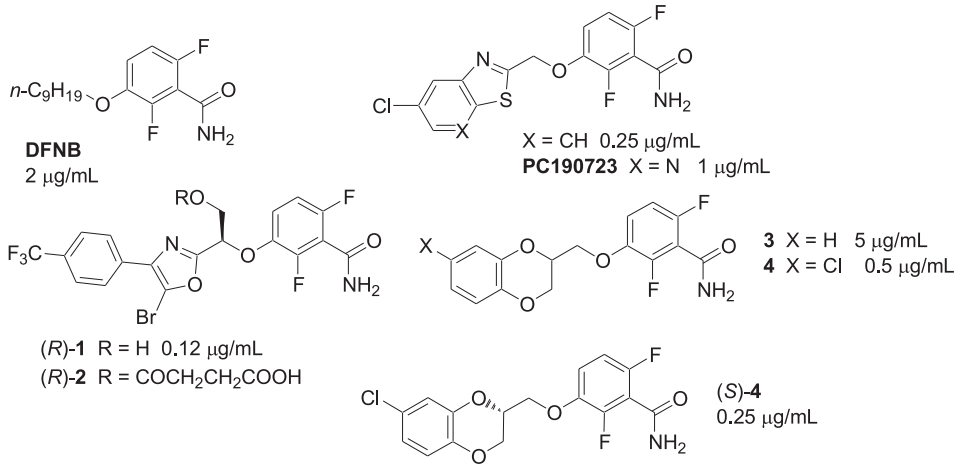


Chart 1. Chemical structures of DFNB, PC190723, (*R*)-**1**, (*R*)-**2**, **3** and (*S*)-**4**.

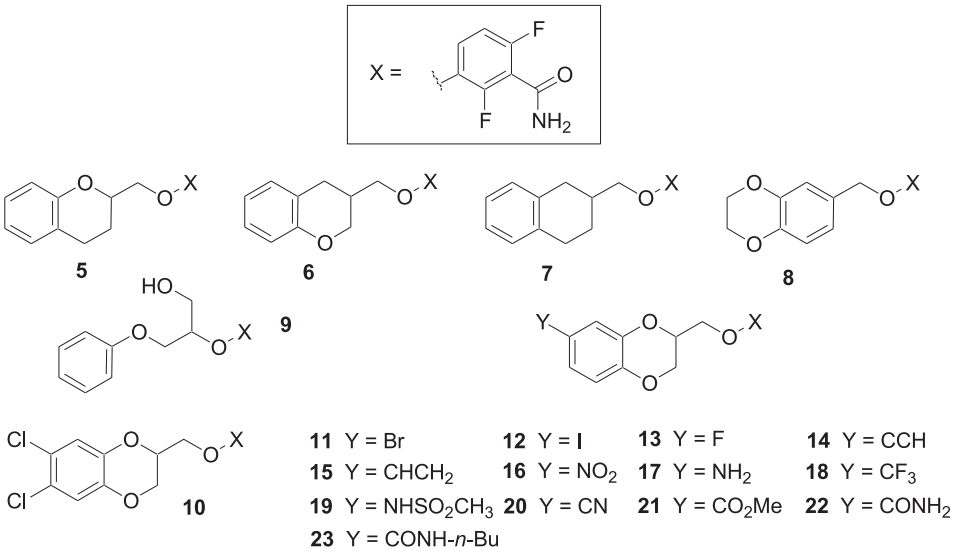
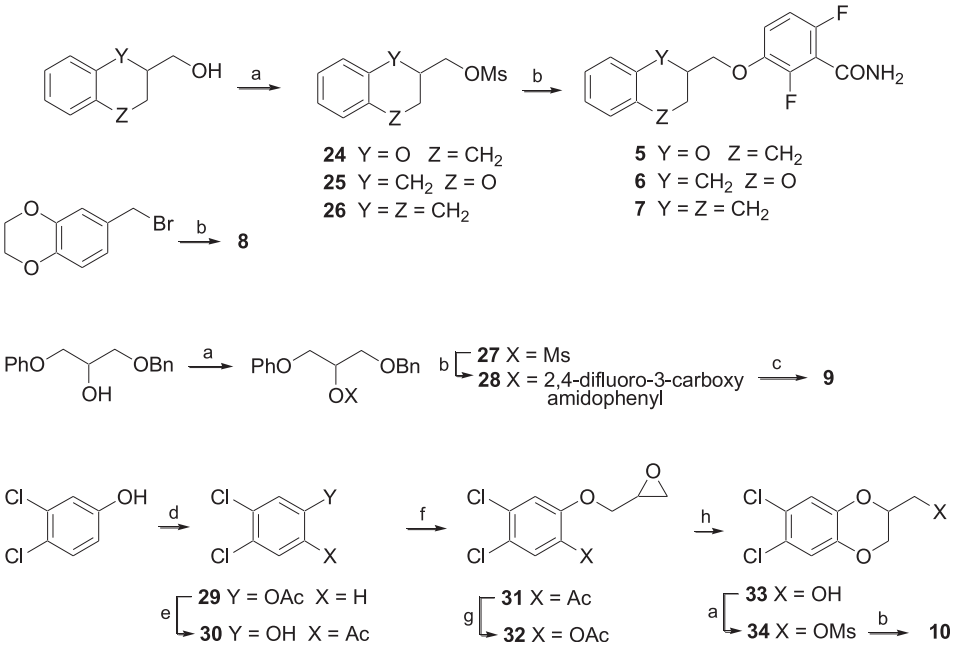


Chart 2. Chemical structures of compounds **5**–**23**.



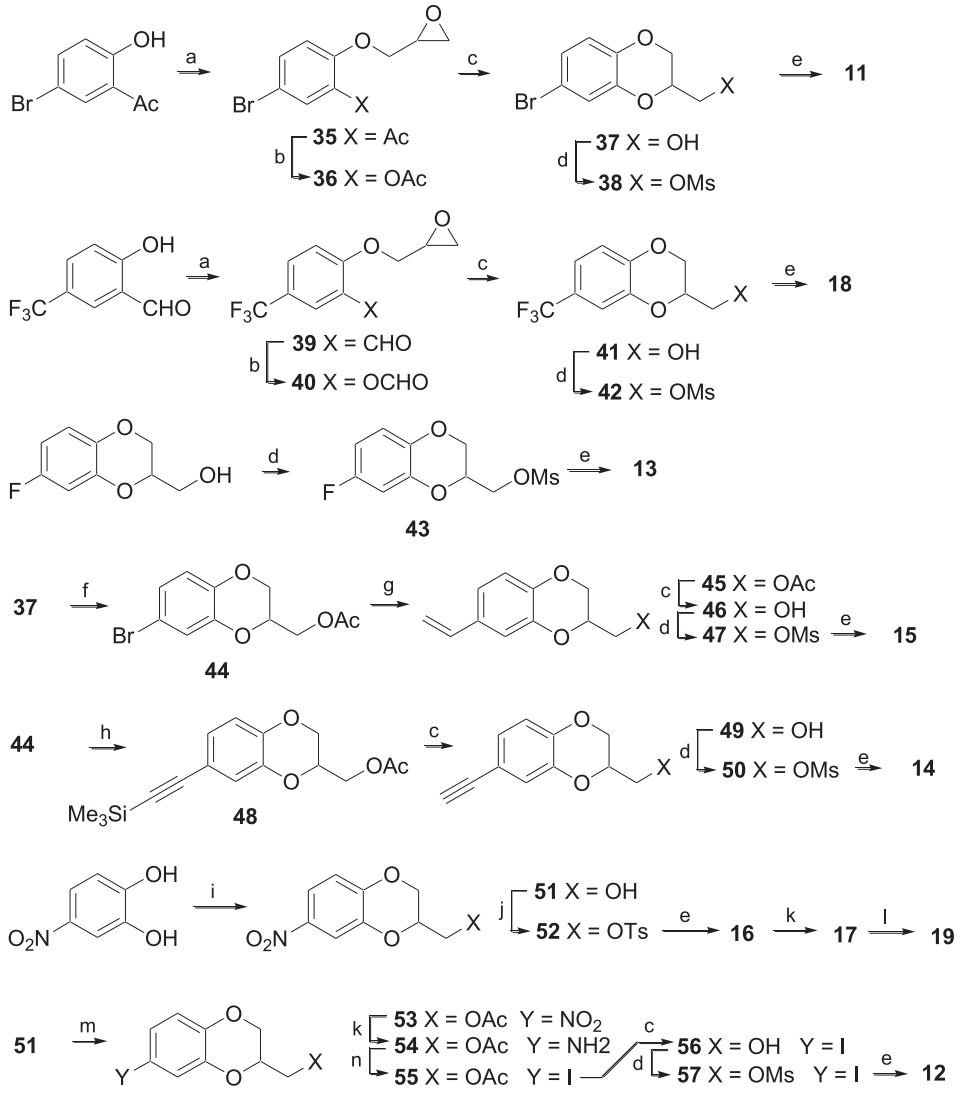
Scheme 1. Syntheses of compounds **5**–**10** (a) Mesyl chloride, TEA, DCM. (b) 2,6-Difluoro-3-oxybenzamide, K_2CO_3, DMF. (c) H2, 5% Pd/C, methanol. (d) AcCl, TEA, DCM. (e) $AlCl_3$, DCM. (f) Epibromohydrin, K_2CO_3, DMF. (g) m-Chloroperbenzoic acid, DCM. (h) NaOH aq, methanol.

with 2,6-difluoro-3-oxybenzamide to **22** or **23**. The cyano derivative **20** was obtained by dehydration of 7-aminocarbonyl-2-benzyloxymethyl-1,4-benzodioxane (**67**), followed by mesylation (**68**) and condensation with 2,6-difluoro-3-oxybenzamide.

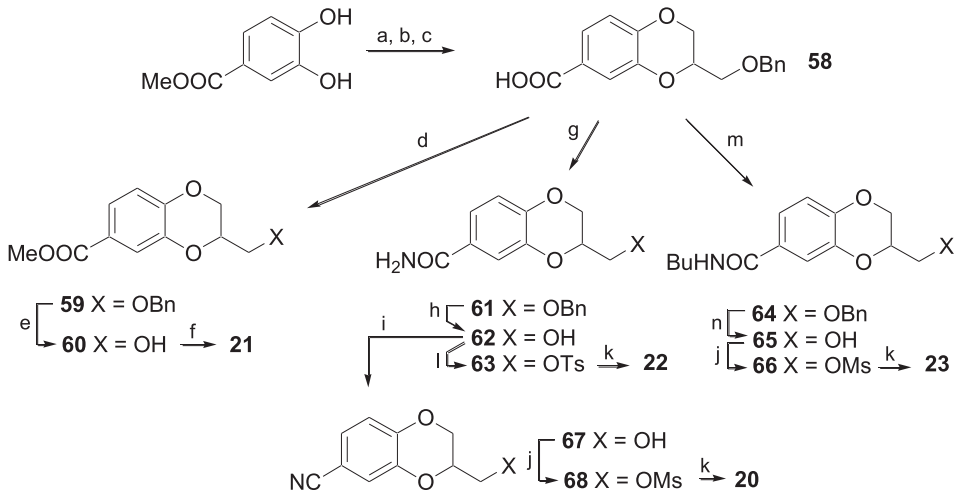
2. Results and discussion

To test whether compounds **5**–**23** could inhibit bacterial growth, both Gram-positive and Gram-negative bacteria were used. In particular, the minimal inhibiting concentration (MIC), i.e. the lowest compound dose (μg/mL) at which growth is inhibited, and the minimal bactericidal concentration (MBC), i.e. the minimal dose (μg/mL) at which cell growth is inhibited after compound removal, were determined. The compounds with <1 μg/mL MIC were also evaluated for cytotoxicity on human MRC-5 cells and the doses (μg/mL) reducing the viability of these cells by 90% (TD_{90}) are reported. The therapeutic index (TI) was also determined and defined as the ratio between TD_{90} and MBC values.

When tested on an extended-spectrum beta-lactamase-positive (ESBL) *Escherichia coli*, no compound was able to inhibit cell growth at the highest dose of 100 μg/mL (data not shown). Conversely, as listed in Table 1, different levels of inhibition were found when they were tested against a methicillin-resistant *S. aureus* strain (MRSA, ATCC 29213). Six compounds (**5**, **10**, **13**, **16**, **18** and **20**) showed a MIC ranging from 5 to 1.25 μg/mL and five (**11**, **12**, **14**, **15** and **21**) were very active with MICs lower than 1 μg/mL. The other eight



Scheme 2. Syntheses of compounds **11**–**19**. (a) Epichlorohydrin, K_2CO_3, DMF. (b) *m*-Chloroperbenzoic acid, DCM. (c) NaOH aq, methanol. (d) Mesyl chloride, TEA, DCM. (e) 2,6-Difluoro-3-oxybenzamide, K_2CO_3, DMF. (f) AcCl, TEA, DCM. (g) Potassium vinyltrifluoroborate, $PdCl_2(dppe)\cdot CH_2Cl_2$, *t*-butylamine, *i*-PrOH, water. (h) Ethynyltrimethylsilane, $PdCl_2$, CuI, PPh_3, TEA. (i) Epibromohydrin, $NaHCO_3$, DMF. (j) TsCl, Py. (k) $SnCl_2$, EtOAc. (l) Mesyl chloride, TEA, EtOAc. (m) AcCl, TEA, EtOAc. (n) $NaNO_2$, H_2SO_4, NaI.



Scheme 3. Syntheses of compounds **20**–**23**. (a) Epibromohydrin, K_2CO_3, DMF. (b) BnBr, NaH, THF. (c) NaOH aq, methanol. (d) Methanol, H_2SO_4, trimethyl orthoformate. (e) H_2, 10% Pd/C, ethanol. (f) 2,6-Difluoro-3-oxybenzamide, DIAD, PPh_3, THF. (g) Thionyl chloride and then aq NH_3 (30%), DCM. (h) H_2, 5% Pd/C, acetone. (i) $(CF_3CO)_2O$, Py, dioxane. (j) Mesyl chloride, TEA, DCM. (k) 2,6-Difluoro-3-oxybenzamide, K_2CO_3, DMF. (l) TsCl, Py. (m) Thionyl chloride and then *n*-$BuNH_2$, DCM. (n) H_2, 10% Pd/C, methanol.

Table 1
Inhibitory activity against a methicillin-resistant *S. aureus* strain (MRSA, ATCC 29213) and cytotoxicity of DFNB[a] and compounds **5**–**23**.

Compd	*S. aureus*			MRC-5 toxicity	Compd	*S. aureus*			MRC-5 toxicity
	MIC (μg/mL)	MBC (μg/mL)	TI	TD_{90} (μg/ml)		MIC (μg/mL)	MBC (μg/mL)	TI	TD_{90} (μg/ml)
DFNB	1	1	>200	>200	**14**	0.313	0.625	640	400
5	5	5			**15**	0.625	1.25	>640	>800
6	100	100			**16**	1.25	2.5		
7	>100				**17**	100	100		
8	100	100			**18**	2.5	2.5		
9	>100				**19**	>100			
10	2.5	5			**20**	1.25	2.5		
11	0.625	0.625	>1280	>800	**21**	0.625	0.625	>1280	>1280
12	0.313	0.625	>1280	>800	**22**	>100			
13	2.5	2.5			**23**	>100			

[a] *S. aureus* MIC of DFNB is 2 μg/mL in Ref. [2]. MIC, minimal inhibitory concentration; MBC, minimal bactericidal concentration; TD_{90}, compound concentration reducing MRC-5 viability of 90%; TI, Therapeutic Index (ratio between TD_{90} and MBC values).

compounds did not inhibit *S. aureus* growth up to 100 μg/mL (compounds **7**, **9**, **19**, **22** and **23**) or showed a modest 100 μg/mL MIC (compounds **6**, **8** and **17**). Identical MICs and MBCs (0.625 μg/mL) were determined for **11** and **21**, whereas MBCs were found higher than MICs for the other three very active compounds, namely **12** and **14** (0.625 vs 0.313 μg/mL) and **15** (1.25 vs 0.625 μg/mL). All of the five most active compounds showed a low cytotoxicity on mammalian cells and a favourable TI. In particular, compounds **11**, **12** and **21** had a TI higher than 1280, while **14** and **15** higher than 640. To obtain cytotoxicity in 90% of the cells (TD_{90}), **11**, **12**, **15** and **21** had to be used at a concentration higher than 800 μg/mL and **14** higher than 400 μg/mL.

The investigation on the antimicrobial activity was successively extended to clinical isolates of Gram-positive bacteria by using a panel of 30 strains including 10 sensitive *S. aureus* (MSSA) and 10 methicillin-resistant *S. aureus* (MRSA), 5 vancomycin-sensitive *Enterococcus faecalis* (VSE) and 5 vancomycin-resistant *E. faecalis* (VRE). DFNB, **3**, (*S*)-**4**, **10** and **12** were selected for testing: MICs and MCBs are reported in Table 2. All the compounds exhibited from moderate to high antistaphylococcal activity against both MSSA and MRSA and the MIC_{90} and MBC_{90} values are consistent with the MICs and MBCs reported in Table 1 (**10** and **12**) and with those we have previously published (**3** and (*S*)-**4**). In particular, the reported potent submicromolar activity of the lead compound (*S*)-**4** against MSSA (0.25 μg/mL; 0.7 μM) was confirmed (0.19 μg/mL; 0.5 μM) and now found to be almost equally exerted also against clinical isolates of MRSA (0.39 μg/mL; 1 μM). Within this group of compounds, high anti-MSSA and anti-MRSA activities, almost identical to those of (*S*)-**4**, were shown by **12**. Inhibition of VSE and VRE growth was observed at higher concentrations (6–25 μg/mL) and it is noteworthy that **12**, potent antistaphylococcal agent, exhibits also a moderate anti-VSE and anti-VRE activity.

DFNB, **3**, (*S*)-**4** and **10** were tested also as antitubercular agents by evaluating their activity against *Mtb* H37Rv. The antitubercular activities are indicated by the MIC values reported in Table 2, where the *S* enantiomer of **4**, endowed with the highest antistaphylococcal activity, appears as the most active compound also against *Mtb*, showing a good 8 μg/mL MIC.

The study of the compounds **5**–**8** was aimed at understanding the role of the benzodioxane substructure. Previous researches on 2,6-difluorobenzamides 3-substituted with different benzoheteroaryl methoxy groups had shown very different antistaphylococcal activities depending on the benzo-condensed heterocycle. Consistently, the present results indicate that the benzodioxane substructure of our compounds is not a mere scaffold correctly positioning pharmacophoric groups, but also a target-interacting part of the molecule, in particular, through its O(1). In fact, the chromane **5** maintains unaltered the good activity of the previously reported unsubstituted benzodioxane **3** (5 μg/mL MIC against *S. aureus*), whereas the chromane **6**, lacking of O(1), the tetrahydronaphthalene **7**, lacking of both oxygens, and the 'reversed' benzodioxane **8** are modestly active or inactive. Neither can the phenoxyethyl residue efficaciously replace the 2-chromanylmethyl and 2-benzodioxanylmethyl substructures, although the oxygen position is not changed: compound **9**, unlike **3** and **5**, is inactive. This can be ascribed to the flexibility of the phenoxyethyl residue or to the interference with correct interaction by intramolecular hydrogen bonding between its oxygen and the hydroxymethyl substituent, beta positioned on the ethylene bridge to resemble **1** where the hydroxymethyl group has the same position relative to oxazole heteroatoms.

After investigating the role of the benzodioxane oxygen atoms, we considered a series of *meta*-substituents relative to the benzodioxane O(1), since introducing chlorine at the 7-position of benzodioxane had been a breakthrough in our previous search for new antibacterial 2,6-difluorobenzamides. Compound **4** (0.5 μg/mL

Table 2
In vitro susceptibilities of *S. aureus* methicillin-susceptible (MSSA), -resistant (MRSA), and *E. faecalis* vancomycin-susceptible (VSE) or –resistant (VRE) isolates and of *Mtb* H37Rv to DFNB and compounds **3**, (*S*)-**4**, **10** and **12**.[a]

Compd	*S. aureus* MSSA		*S. aureus* MRSA		*E. faecalis* VSE		*E. faecalis* VRE		*Mtb* H37Rv
	MIC_{90} (μg/mL)	MBC_{90} (μg/mL)	MIC_{90} (μg/mL)	MBC_{90} (μg/mL)	MIC_{90} (μg/mL)	MBC_{90} (μg/mL)	MIC_{90} (μg/mL)	MBC_{90} (μg/mL)	MIC (μg/mL)
DFNB	0.19	0.19	0.39	0.78	6.25	12.5	12.5	25	16
3	3.12	12.5	3.12	6.25	25	50	25	50	16
(*S*)-4	0.19	0.39	0.39	0.78	25	50	25	50	8
10	3.12	6.25	3.12	6.25	6.25	50	12.5	50	16
12	0.78	3.12	0.39	1.56	6.25	12.5	6.25	25	

[a] As determined by the CLSI M27-A3 broth microdilution method [13]. MIC, minimal inhibitory concentration; MBC, minimal bactericidal concentration.

MIC) is ten-fold more potent than its des-chloro analogue **3** as a racemate and twenty-fold as *S* enantiomer (0.25 μg/mL MIC). Based on these results, we initially selected alternative substituents having positive σ_m like chlorine and from negligible to high lipophilicity such as CO_2Me, CN, NO_2, F, CF_3, Br, I, ethinyl and vinyl. The bromo- and iodo-analogues, **11** and **12**, the methoxycarbonyl analogue **21** and the alkinyl and alkenyl substituted compounds **14** and **15** showed high potency, similar to that of **4**, with <1 μg/mL, antistaphylococcal MICs, closely followed behind by the fluoro, nitro, trifluoromethyl and cyano derivatives, **13**, **16**, **18** and **20**, with antistaphylococcal MICs ranging between 1.25 and 2.5 μg/mL. The higher activity of the 7-susbtituted analogues relative to unsusbstituted **3** was confirmed also against clinical isolates of MSSA and MRSA, which were found sensible to (*S*)-**4** and **12** at submicromolar concentrations, 4–16-fold lower than those of **3**. The significant influence of the 7-substitution and the preference for hydrophobic substituents at this position were confirmed by the dramatic loss of activity resulting from their replacement with hydrophilic and hydrogen bond donor substituents, either electron-releasing or attracting, such as NH_2, $NHSO_2Me$ and $CONH_2$ (compounds **17**, **19** and **22**): a detrimental effect which could not be overcome neither by N-alkylation as indicated by the inactivity of the N-butyl derivative **23**. An almost identical trend in antistaphylococcal MICs can be observed for the substitutions at the five position of benzothiazole in the benzothiazole analogue of PC190723: chlorine, bromine, CF_3 and COOMe increase the antistaphylococcal activity from 2 μg/mL to 0.1–1 μg/mL, whereas NH_2 and $CONH_2$ annul it. The PC190723 carba analogue and **3** have identical structures except for the benzoheterocycle substructure, benzothiazole and benzodioxane respectively, and the benzothiazole C(5) of the latter is superimposable to the benzodioxane C(7). Therefore, the observed parallel shifts in antibacterial activity resulting from parallel substitutions at these two carbons strongly suggests that **3** and the PC190723 carba analogue exert their antibacterial activity interacting in a similar fashion with the same biological target, that has been proven to be, in the case of PC190723, the bacterial cell division protein FtsZ. Such a statement, based on the shared 3-benzoheteroarylmethoxy-2,6-difluorobenzamide molecular structure and on very similar SARs, is consistent with the results of the morphometric analysis, which reveals that bacteria treated with some of our most potent compounds show alterations indicative of the inhibition of FtsZ assembly and cell division. By light microscopy, an increase in cell volume of *S. aureus* cultured in the presence of (*S*)-**4**, **14** and **21** was observed (Fig. 1A, B, C), which was comparable to the effect of DFNB (Fig. 1E). Bacterial swelling was also accompanied by cytocidal activity as indicated by cell desegregation (Fig. 1B, C, E). As expected, no morphological change could be instead observed in untreated *S. aureus* (Fig. 1D). These results were confirmed by transmission electron microscopy, which visualized alterations in the septum formation in most bacteria exposed to (*S*)-**4** and DFNB (Fig. 2A, D) and complete bacterial desegregation (ghosts) with total loss of the cytoplasmatic content upon treatment with **21** (Fig. 2B). On the other hand, normal cell morphology and septum formation were observed in the untreated negative control (Fig. 2C). Significantly altered bacterial morphology was also visible upon exposure of *Mycobacterium smegmatis* at subMIC concentrations of DFNB and (*S*)-**4** (Fig. 3B and C), as shown by the presence of elongated cells with an enlarged bacterial pole. These changes are compatible with the perturbation of the FtsZ polymerization. A similar, though less evident pattern was also observed when *M. tuberculosis* was subjected to subMIC concentrations of (*S*)-**4** (Fig. 3F) and DFNB (Fig. 3E).

3. Conclusions

We have tested a series of 3-(benzodioxan-2-yl)-2,6-difluorobenzamides substituted at benzodioxane C(7) or modified at the benzodioxane substructure for the activity against a strain of methicillin resistant *S. aureus* and, after selecting the most active compounds, against clinical isolates of either antibiotic sensitive or antibiotic resistant gram-positive bacteria (*S. aureus*, *E. faecalis*) and against *Mtb* H37Rv. Among the compounds modified at the bicyclic scaffold, only the 2-cromanyl analogue **5** maintains the good activity of the parent unsubstituted benzodioxane-difluorobenzamide **3** thus indicating the importance of benzodioxane O(1) for target-activity. Within the series of the 7-substituted analogues, high antibacterial activity, much higher than that of **3**, against MRSA and *Mtb* is showed by the derivatives bearing a lipophilic substituent at that position, whereas hydrophilic substituents have a highly detrimental effect. These results are consistent with the previously observed enhancement in antibacterial activity resulting from chlorination of benzodioxane C(7)

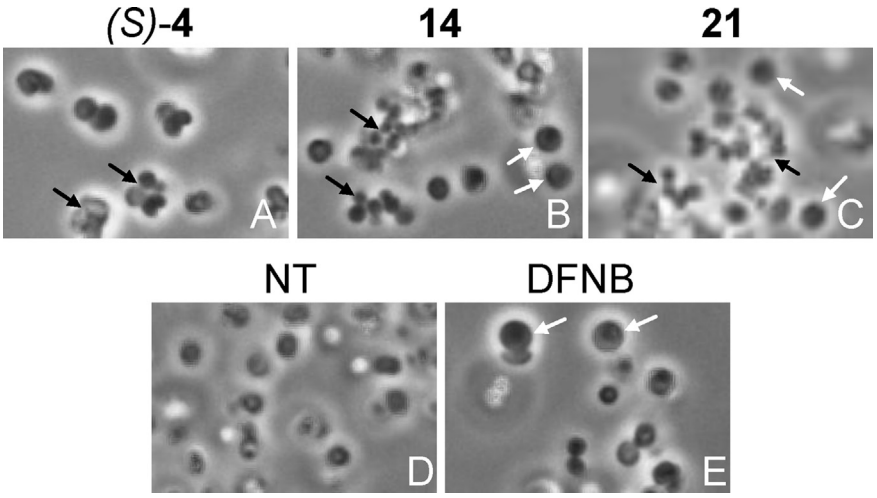


Fig. 1. Phase-contrast light microscopy of *S. aureus* after over night incubation in the presence of compounds (*S*)-**4** (0.5 μg/mL), **14** (0.5 μg/mL), **21** (1 μg/mL). Bacteria with no compound (NT) or in the presence of DFNB (0.25 μg/mL) were used as negative and positive control respectively. All picture were taken at 630 × optical enlargement. Desegregations: black arrows; bacterial swelling: white arrows.

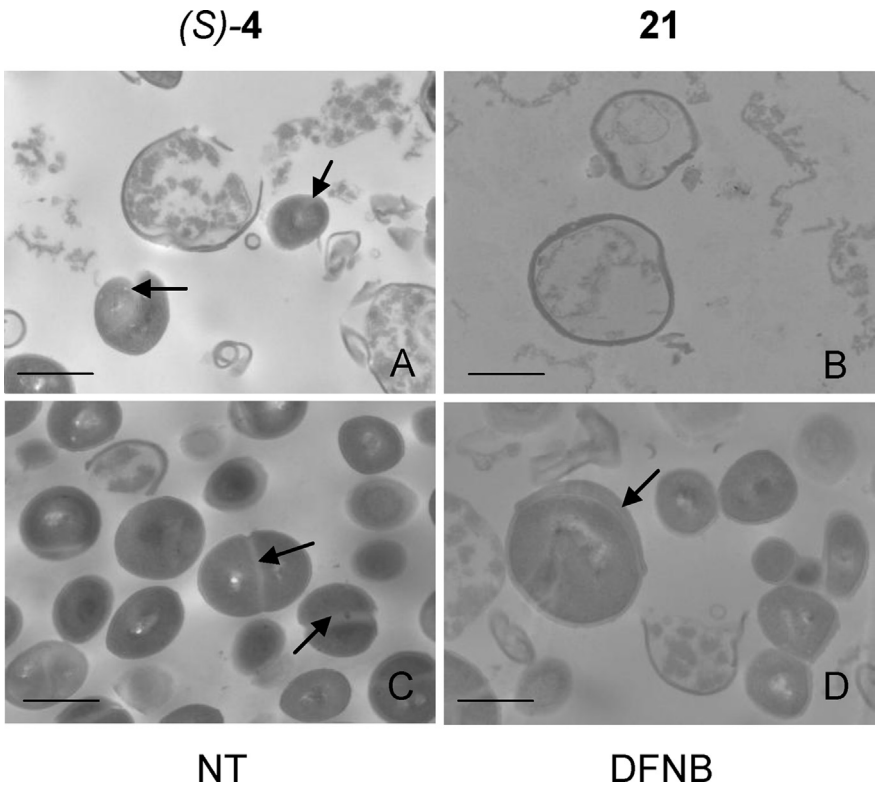


Fig. 2. Ultrastructural analysis of *S. aureus* cultured in the presence of (*S*)-**4** (0.5 μg/mL) and **21** (1 μg/mL). Bacteria with no compound (NT) or in the presence of DFNB (0.25 μg/mL) were used as negative and positive control respectively. Cells were fixed and processed after overnight growth, and alterations were examined at the ultrastructural level by transmission electron microscopy. Arrows indicate septum formation. Scale bar: 0.5 μ.

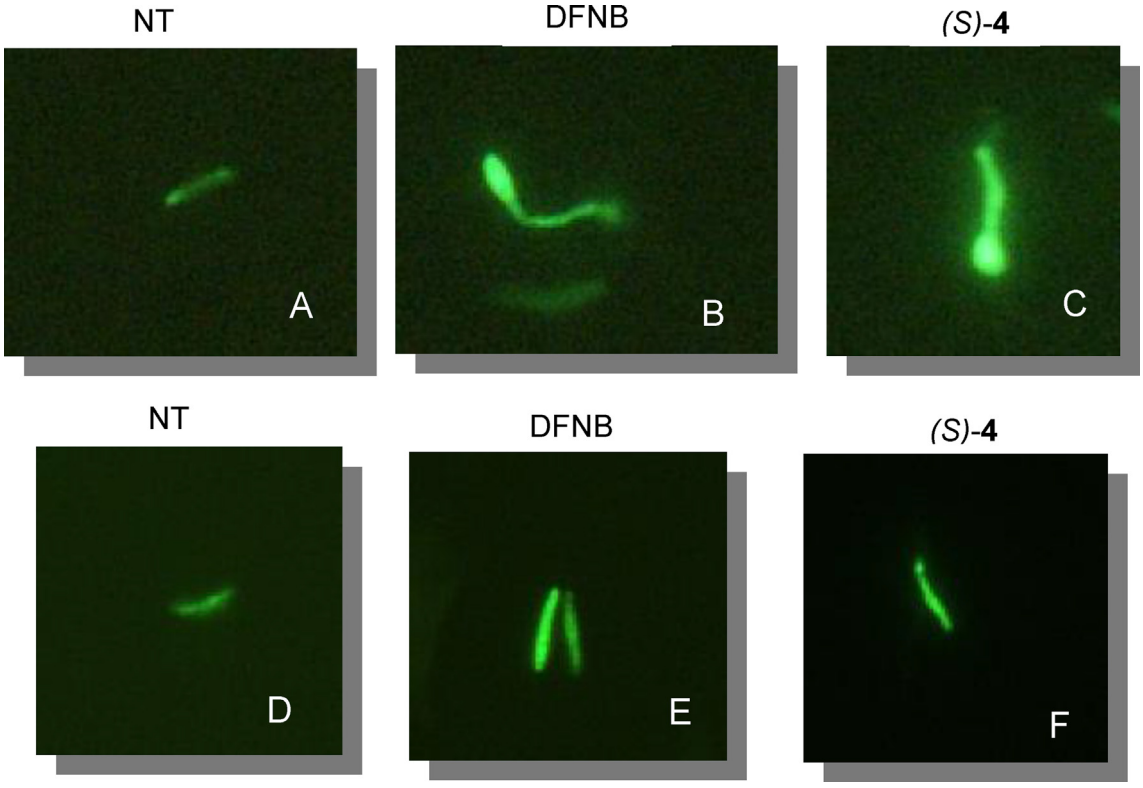


Fig. 3. Pictures of *M. smegmatis* (A, B, C) and *M. tuberculosis* (D, E, F) expressing GFP when untreated (A and D) or exposed at subMIC concentrations of (*S*)-**4** (C and F: 4 μg/mL) and DFNB (B: 0.5 μg/mL; E: 8 μg/mL).

of **3** and suggest that this is a crucial position for hydrophobic substituents as C(5) in the benzothiazole analogues of PC190723, a well-known FtsZ inhibiting difluorobenzamide. The common mechanism of action of these two classes of compounds, benzodioxane-difluorobenzamides and benzothiazole-difluorobenzamides, is then supported by morphometric analyses of *Sa* and *Mtb* treated with (*S*)-**4**, **14** and **21** by optical and electron microscopy, which show cell alterations typical of FtsZ inhibition.

4. Experimental

4.1. Chemistry

Melting points were determined by DSC analysis and correspond to the peak maximum. ^{1}H NMR spectra were recorded operating at 300 MHz and ^{13}C NMR at 75 MHz. Chemical shifts are reported in ppm relative to residual solvent ($CHCl_3$ or DMSO) as internal standard. Signal multiplicity is designed according to the following abbreviations: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = doublet of doublets, dq = doublet of quartets, qd = quartet of doublets, dt = doublet of triplets, td = triplet of doublets, ddd 0 doublet of doublet of doublets, ddt = doublet of doublet of triplets, br s = broad singlet. Coupling constants are reported in Hertz (Hz). Optical rotations were determined by a Perkin–Elmer 241 Polarimeter at 25 °C. Elemental analyses (C, H, N, Br, Cl, I, S) of the new substances are within 0.40% of theoretical values. Purifications were performed by flash chromatography using silica gel (particle size 40–63 μm, Merck).

2-Mesyloxymethylchromane (24). Mesyl chloride (0.44 mL, 5.77 mmol) was added dropwise to a solution of 2-hydroxymethylchromane (0.63 g, 3.84 mmol) and TEA (0.80 mL, 5.77 mmol) in DCM (10 mL) at 0 °C. The mixture was stirred at room temperature for 30 min, cooled at 4 °C, and diluted with water (20 mL) and DCM (10 mL). The organic layer was separated, washed with 10% HCl (10 mL), saturated aqueous $NaHCO_3$ (10 mL), and brine (10 mL), dried over Na_2SO_4, and concentrated to give 866 mg of **24** (93.1%) as a light brown oil: ^{1}H NMR ($CDCl_3$) δ 7.09 (m, 2 H), 6.84 (m, 2 H), 4.42 (m, 2 H), 4.30 (m, 1 H), 3.10 (s, 3 H), 2.90 (m, 1 H), 2.78 (m, 1 H), 2.05 (m, 1 H), 1.91 (m, 1 H).

3-(Chroman-2-yl)methoxy-2,6-difluorobenzamide (5). Potassium carbonate (304 mg, 2.20 mmol) was added to a solution of 2,6-difluoro-3-hydroxybenzamide (380 mg, 2.20 mmol) in DMF (6 mL). After stirring at room temperature for 15 min, a solution of **23** (485 mg, 2 mmol) in DMF (3 mL) was added. The reaction mixture was stirred at 80 °C for 2 h, concentrated, and diluted with water and ethyl acetate. The organic phase was separated, washed with brine, dried over Na_2SO_4, and concentrated. The resulting residue was purified by flash chromatography on silica gel. Elution with 55/45 cyclohexane/ethyl acetate gave 386 mg (60.5%) of **5** as a white solid: mp 159.1 °C; ^{1}H NMR ($CDCl_3$) δ 7.13 (dd, 1 H, *J* = 9.3, 5.3 Hz), 7.08 (m, 2 H), 6.91 (dd, 1 H, *J* = 9.0, 2.1 Hz), 6.84 (m, 2 H), 5.96 (br s, 2 H), 4.40 (ddd, 1 H, *J* = 5.4, 5.1, 2.4 Hz), 4.27 (dd, 1 H, *J* = 10.0, 5.4 Hz), 4.18 (dd, 1 H, *J* = 10.0, 5.1 Hz), 2.88 (m, 2 H), 2.16 (m, 1 H), 1.99–1.82 (m, 1H). ^{13}C NMR ($CDCl_3$) δ 162.2, 154.4, 154.1 (dd, *J* = 245.9, 5.4 Hz), 150.6 (dd, *J* = 253.2, 7.0), 144.0 (dd, *J* = 11.3, 3.6 Hz), 129.8, 127.6, 121.9, 120.8, 118.5 (d, *J* = 8.3 Hz), 117.0, 111.6 (m), 111.3 (m), 74.2, 73.0, 24.41, 24.38. Anal. Calcd for $C_{17}H_{15}F_2NO_3$ (319.30).

3-Mesyloxymethylchromane (25). Mesyl chloride (0.44 mL, 5.77 mmol) was added dropwise to a solution of 3-hydroxymethylchromane (0.79 g, 4.81 mmol) and TEA (0.90 mL, 6.25 mmol) in DCM (15 mL) at 4 °C. The mixture was stirred at 4 °C for 15 min and then at room temperature for 30 min, cooled at 4 °C, and diluted with water (20 mL) and DCM (10 mL). The organic layer was separated, washed with 10% HCl (20 mL), saturated aqueous $NaHCO_3$ (20 mL), and brine (20 mL), dried over Na_2SO_4, and concentrated. The resulting residue was purified by flash chromatography on silica gel. Elution with 70/30 cyclohexane/ethyl acetate gave 1.03 g (88.8%) of **25** as a colourless oil: ^{1}H NMR ($CDCl_3$) δ 7.15 (m, 2 H), 6.85 (m, 2 H), 4.24 (m, 3 H), 4.08 (dd, 1 H, *J* = 11.0, 6.3 Hz), 2.99 (m, 4 H), 2.65 (dd, 1 H, *J* = 16.5, 6.3 Hz), 2.54 (m, 1 H).

3-(Chroman-3-yl)methoxy-2,6-difluorobenzamide (6). Potassium carbonate (700 mg, 5.05 mmol) was added to a solution of 2,6-difluoro-3-hydroxybenzamide (800 mg, 4.63 mmol) in DMF (7 mL). After stirring at room temperature for 15 min, a solution of **25** (1.02 g, 4.63 mmol) in DMF (7 mL) was added. The reaction mixture was stirred at 60 °C for 6 h, concentrated, and diluted with water. The aqueous phase was extracted with ethyl acetate (3 × 20 mL). The combined organic phases were washed with 1 M NaOH (40 mL), and brine (40 mL), dried over Na_2SO_4, and concentrated. The resulting solid residue was crystallized from DCM to give 650 mg (48.5%) of **5** as a white solid: mp 124.5 °C; ^{1}H NMR ($CDCl_3$) δ 7.11 (m, 3 H), 6.85 (m, 3 H), 6.18 (br s, 1 H), 6.02 (br s, 1 H), 4.33 (dd, 1 H, *J* = 10.7, 2.7 Hz), 4.14 (dd, 1 H, *J* = 11.0, 6.6 Hz), 4.03 (d, 1 H, *J* = 6.6 Hz), 3.00 (dd, 1 H, *J* = 16.5, 5.0 Hz), 2.72 (dd, 1 H, *J* = 16.5, 7.2 Hz), 2.61 (m, 2 H). ^{13}C NMR (DMSO-*d*) δ 162.5), 154.6, 153.8 (dd, *J* = 245.4, 5.2 Hz, 150.4 (dd, *J* = 253.1, 6.8 Hz), 144.0 (dd, *J* = 11.1, 3.4 Hz), 130.3, 127.7, 120.9, 120.6, 117.6 (d, *J* = 9.7 Hz), 116.9, 114.5 (d, *J* = 16.5 Hz), 111.4 (d, *J* = 23.6 Hz), 70.9, 67.4, 32.7, 27.5. Anal. Calcd for $C_{17}H_{15}F_2NO_3$ (319.30).

2-Mesyloxymethyl-1,2,3,4-tetrahydronaphthalene (26). Mesyl chloride (0.47 mL, 6.05 mmol) was added dropwise to a solution of 2-hydroxymethyl-1,2,3,4-tetrahydronaphthalene (0.88 g, 5.42 mmol) and TEA (0.99 mL, 7.05 mmol) in DCM (15 mL) at 4 °C. The mixture was stirred at 4 °C for 15 min and then at room temperature for 30 min, cooled at 4 °C, and diluted with water (20 mL) and DCM (10 mL). The organic layer was separated, washed with 10% HCl (15 mL), saturated aqueous $NaHCO_3$ (15 mL), and brine (15 mL), dried over Na_2SO_4, and concentrated to give 1.31 g of **26** (99.2%) as a yellow oil: ^{1}H NMR ($CDCl_3$) δ 7.11 (m, 4 H), 4.21 (m, 2 H), 3.04 (s, 3 H), 2.88 (m, 3 H), 2.58 (dd, 1 H, *J* = 16.5, 10.5 Hz), 2.24 (m, 1 H), 2.03 (m, 1 H).

2,6-Difluoro-3-(1,2,3,4-tetrahydronaphthalen-2-yl)methoxybenzamide (7). Potassium carbonate (460 mg, 2.50 mmol) was added to a solution of 2,6-difluoro-3-hydroxybenzamide (530 mg, 3.06 mmol) in DMF (4 mL). After stirring at room temperature for 15 min, a solution of **26** (670 mg, 2.78 mmol) in DMF (5 mL) was added. The reaction mixture was stirred at 70 °C for 7 h, concentrated, and diluted with water. The aqueous phase was extracted with ethyl acetate (3 × 20 mL). The combined organic phases were washed with 1 M NaOH (30 mL), and brine (30 mL), dried over Na_2SO_4, and concentrated. The resulting solid residue was crystallized from 70/30 cyclohexane/ethyl acetate to give 330 mg (37.5%) of **7** as a white solid: mp 165.1 °C; ^{1}H NMR ($CDCl_3$) δ 7.06 (m, 5 H), 6.88 (dt, 1 H, *J* = 9.1, 1.9 Hz), 5.98 (br s, 2 H), 3.98 (d, 2 H, *J* = 6.5 Hz), 3.00 (dd, 1 H, *J* = 17.9, 5.7 Hz), 2.87 (dd, 1 H, *J* = 8.1, 4.7 Hz), 2.65 (dd, 1 H, *J* = 16.3, 10.4 Hz), 2.34 (m, 1 H), 2.11 (m, 1 H), 1.60 (m, 2 H). ^{13}C NMR (DMSO-*d*) δ 162.0, 152.4 (dd, *J* = 239.7, 6.9 Hz), 148.6 (dd, *J* = 246.4, 8.6 Hz), 144.0 (dd, *J* = 10.6, 2.9 Hz), 136.9, 136.0, 129.7, 129.4, 126.3, 117.3 (dd, *J* = 24.7, 20.5 Hz), 116.2 (d, *J* = 9.4 Hz), 111.6 (d, *J* = 9.1 Hz), 74.4, 34.4, 32.8, 32.4, 28.6, 26.1. Anal. Calcd for $C_{18}H_{17}F_2NO_2$ (317.33).

3-(1,4-Benzodioxan-6-ylmethoxy)-2,6-difluorobenzamide (8). Potassium carbonate (362 mg, 2.62 mmol) was added to a solution of 2,6-difluoro-3-hydroxybenzamide (453 mg, 2.62 mmol) in DMF (3 mL). After stirring at room temperature for 15 min, a solution of 1,2-ethylenedioxy-4-bromomethylbenzene (500 mg, 2.18 mmol) in DMF (3 mL) was added. The reaction mixture was stirred at 100 °C for 1 h, cooled to room temperature and then poured into iced water (80 mL). A precipitate was formed which

was collected by filtration, washed with water, dried under vacuum and crystallized from $CHCl_3$ to give 426 mg (60.8%) of **8** as a pale pink solid: mp 130.0 °C; ^{1}H NMR (DMSO-*d*) δ 8.11 (br s, 1 H), 7.83 (br s, 1 H), 7.25 (dd, 1 H, *J* = 9.2, 5.4 Hz), 7.03 (dd, 1 H, *J* = 9.2, 1.7 Hz), 6.88 (m, 3 H), 5.03 (s, 2 H), 4.22 (s, 4 H). ^{13}C NMR (DMSO-*d*) δ 162.0, 152.5 (dd, *J* = 239.7, 6.8 Hz), 148.7 (dd, *J* = 247.0, 8.4 Hz), 144.1, 143.9, 143.4 (dd, *J* = 10.8, 3.1 Hz), 129.9, 121.8, 117.7 (d, *J* = 4.3 Hz), 117.3, 117.0, 116.4 (dd, *J* = 9.4, 2.3 Hz), 111.5 (dd, *J* = 23.0, 3.8 Hz), 71.1, 64.73, 64.71. Anal. Calcd for $C_{16}H_{13}F_2NO_4$ (321.28).

1-Benzyloxy-2-mesyloxy-3-phenoxypropane (27). Mesyl chloride (0.21 mL, 2.71 mmol) was added dropwise to a solution of 1-benzyloxy-2-hydroxy-3-phenoxypropane (580 mg, 2.25 mmol) and TEA (0.4 mL, 2.87 mmol) in DCM (6 mL) at 4 °C. The mixture was stirred at 4 °C for 15 min and then at room temperature for 30 min, cooled at 4 °C, and diluted with water (15 mL) and DCM (10 mL). The organic layer was separated, washed with 10% HCl (10 mL), saturated aqueous $NaHCO_3$ (10 mL), and brine (10 mL), dried over Na_2SO_4, and concentrated to give 670 mg (88.5%) of **27** as a colourless oil: ^{1}H NMR ($CDCl_3$) δ 7.41–7.28 (m, 7 H), 6.98 (t, 1 H, *J* = 7.2 Hz), 6.89 (d, 2 H, *J* = 7.2 Hz), 5.08 (m, 1 H), 4.62 (d, 1 H, *J* = 9.1 Hz), 4.58 (d, 1 H, *J* = 9.1 Hz), 4.24 (d, 2 H, *J* = 6.5 Hz), 3.82 (d, 2 H, *J* = 6.5 Hz), 3.09 (s, 3 H).

3-(1-Benzyloxy-3-phenoxy-2-propyloxy)-2,6-difluorobenzamide (28). Potassium carbonate (275 mg, 2.00 mmol) was added to a solution of 2,6-difluoro-3-hydroxybenzamide (345 mg, 2.00 mmol) in DMF (5 mL). After stirring at room temperature for 15 min, a solution of **27** (345 mg, 2.00 mmol) in DMF (5 mL) was added. The reaction mixture was stirred at 100 °C for 7 h, concentrated, and diluted with water (15 mL). The aqueous phase was extracted with ethyl acetate (3 × 20 mL). The combined organic phases were washed with 1 M NaOH (30 mL), and brine (30 mL), dried over Na_2SO_4, and concentrated. The resulting residue was purified by flash chromatography on silica gel. Elution with 80/20 toluene/ethyl acetate gave 420 mg (50.8%) of **28** as a colourless oil: ^{1}H NMR ($CDCl_3$) δ 7.39–7.18 (m, 8 H), 6.95 (t, 1 H, *J* = 7.2 Hz), 6.85 (m, 3 H), 5.90 (br s, 2 H), 4.64 (m, 1 H), 4.60 (s, 2 H), 4.23 (d, 2 H, *J* = 6.8 Hz), 3.83 (d, 2 H, *J* = 6.8 Hz).

2,6-Difluoro-3-(1-hydroxy-3-phenoxy-2-propyloxy)benzamide (9). A solution of **28** (260 mg, 0.63 mmol) in methanol (7 mL) was added with 5% Pd/C (50 mg) and vigorously shaken under hydrogen at room temperature for 3 h. The catalyst was removed by filtration and the filtrate concentrated to give 194 mg (95.2%) of **9** as a yellow oil: ^{1}H NMR (DMSO-*d*) δ 8.08 (br s, 1H), 7.79 (br s, 1H), 7.36–7.32 (m, 3H), 7.03 (dt, 1H, *J* = 9.1, 1.8 Hz), 6.92 (m, 3H), 5.03 (t, 1 H, *J* = 5.5 Hz), 4.6 (m, 1H), 4.23 (dd, 1 H, *J* = 10.5, 3.6 Hz), 4.14 (dd, 1 H, *J* = 10.5, 5.2 Hz), 3.69 (m, 2 H). ^{13}C NMR (DMSO-*d*) δ 161.8, 158.8, 152.6 (dd, *J* = 239.9, 6.3 Hz), 149.3 (dd, *J* = 247.4, 9.1 Hz), 143.0 (dd, *J* = 11.4, 3.4 Hz), 130.0, 121.3, 118.8 (d, *J* = 9.1 Hz), 117.1 (dd, *J* = 25.2, 20.6 Hz), 114.9, 111.4 (dd, *J* = 22.9, 3.4 Hz), 80.4, 67.6, 60.3. Anal. Calcd for $C_{16}H_{15}F_2NO_4$ (323.29).

3,4-Dichlorophenyl acetate (29). Acetyl chloride (0.89 mL, 12.5 mmol) was added dropwise to a solution of 3,4-dichlorophenol (1.63 g, 10 mmol) and triethylamine (1.74 mL, 12.5 mmol) in DCM (15 mL) at 0 °C. The mixture was stirred at room temperature for 20 h, diluted with DCM (15 mL), washed with 10% HCl (10 mL), saturated aqueous $NaHCO_3$ (10 mL), and brine (10 mL), dried over Na_2SO_4, and concentrated to give 2.05 g (~100%) of **29** as a yellow oil: ^{1}H NMR ($CDCl_3$) δ 7.42 (d, 1 H, *J* = 8.7 Hz), 7.24 (d, 1 H, *J* = 2.6 Hz), 6.96 (dd, 1 H, *J* = 8.7, 2.6 Hz), 2.27 (s, 3 H).

2-Acetyl-4,5-dichlorophenol (30). Aluminium trichloride (1.56 g, 11.7 mmol) was added portion wise to **29** (2 g, 9.75 mmol) in DCM (2 mL) at room temperature under nitrogen atmosphere. The mixture was stirred at 120 °C for 3 h, cooled to room temperature, and diluted with DCM (20 mL). 10% HCl (20 mL) was slowly added at 0 °C. After stirring at 0 °C for 30 min, the organic phase was separated and the aqueous phase was extracted with DCM (30 mL). The organic extracts were combined, washed with brine, dried over Na_2SO_4, and concentrated to give 1.87 g (93.4%) of **30** as a light pink solid: ^{1}H NMR ($CDCl_3$) δ 12.17 (s, 1 H), 7.79 (s, 1 H), 7.12 (s, 1 H), 2.62 (s, 3 H).

3-(2-Acetyl-4,5-dichlorophenoxy)-1,2-epoxypropane (31). Potassium carbonate (1.45 g, 10.53 mmol) was added to a solution of **30** (1.89 g, 8.78 mmol) in DMF (20 mL). After stirring at room temperature for 30 min, epibromohydrin (1.44 g, 10.53 mmol) was added. The mixture was heated at 40 °C for 18 h, cooled to room temperature, and concentrated under reduced pressure. Ethyl acetate and brine were added to the resulting residue. The organic phase was separated, washed with brine twice, dried over Na_2SO_4, and concentrated. The resultant solid residue was purified by flash chromatography on silica gel. Elution with 70/30 cyclohexane/ethyl acetate gave 1.52 g (66.2%) of **31** as a light yellow solid: ^{1}H NMR ($CDCl_3$) δ 7.84 (s, 1 H), 7.07 (s, 1 H), 4.40 (dd, 1 H, *J* = 11.0, 2.5 Hz), 3.95 (dd, 1 H, *J* = 11.0, 6.3 Hz), 3.42–3.38 (m, 1 H), 2.96 (t, 1 H, *J* = 4.5 Hz), 2.77 (dd, 1 H, *J* = 4.7, 2.8 Hz), 2.63 (s, 3 H).

3-(2-Acetyloxy-4,5-dichlorophenoxy)-1,2-epoxypropane (32). *m*-Chloroperbenzoic acid (4.8 g, 21.45 mmol) was added portion wise to a solution of **31** (1.40 g, 5.36 mmol) in DCM (14 mL) at room temperature. The mixture was refluxed for 18 h, cooled to room temperature and then to 0 °C. After 2 h, the precipitate of 3-chlorobenzoic acid was removed by filtration and washed on the filter with ethyl acetate. The whole filtrate was washed with 10% aqueous $NaHCO_3$, dried over Na_2SO_4 and concentrated to give 1.48 g (~100%) of **32** as a yellow oil: ^{1}H NMR ($CDCl_3$) δ 7.15 (s, 1 H), 7.08 (s, 1 H), 4.26 (dd, 1 H, *J* = 11.3, 2.8 Hz), 3.91 (dd, 1 H, *J* = 11.0, 5.8 Hz), 3.31–3.28 (m, 1 H), 2.88 (t, 1 H, *J* = 4.5 Hz), 2.71 (dd, 1 H, *J* = 5.0, 2.8 Hz), 2.30 (s, 3 H).

6,7-Dichloro-2-hydroxymethyl-1,4-benzodioxane (33). Water (3 mL) and 2.5 M NaOH (4 mL) were added to a solution of **32** (1.4 g, 5.05 mmol) in methanol (15 mL). The mixture was stirred at room temperature for 3 h and concentrated. Ethyl acetate and 2.5 M NaOH were added to the residue. The organic phase was separated, washed with brine, dried over Na_2SO_4 and concentrated. The residue was crystallized from DCM to give 776 mg (65.4%) of **33** as a white solid:: mp 97.0 °C; ^{1}H NMR ($CDCl_3$) δ 6.99 (s, 1 H), 6.98 (s, 1 H), 4.30 (dd, 1 H, *J* = 11.3, 2.2 Hz), 4.27–4.20 (m, 1 H), 4.15–4.06 (m, 2 H), 3.93–3.80 (m, 2 H).

6,7-Dichloro-2-mesyloxymethyl-1,4-benzodioxane (34). Mesyl chloride (0.35 mL, 4.47 mmol) was added dropwise to a solution of **33** (700 mg, 2.98 mmol) and TEA (0.62 mL, 4.47 mmol) in DCM (7 mL) at 0 °C. The mixture was stirred at room temperature for 2 h, cooled at 4 °C, and diluted with 10% HCl (10 mL) and DCM (10 mL). The organic layer was separated, washed with saturated aqueous $NaHCO_3$ (10 mL), and brine (10 mL), dried over Na_2SO_4, and concentrated to give 887 mg (95.0%) of **34** as a yellow oil: ^{1}H NMR ($CDCl_3$) δ 7.01 (s, 2 H), 4.49–4.41 (m, 3 H), 4.32 (dd, 1 H, *J* = 11.6, 2.3 Hz), 4.11 (dd, 1 H, *J* = 11.6, 6.3 Hz), 3.09 (s, 3 H).

3-(6,7-Dichloro-1,4-benzodioxan-2-yl)methoxy-2,6-difluorobenzamide (10). Potassium carbonate (388 mg, 2.81 mmol) was added to a solution of 2,6-difluoro-3-hydroxybenzamide (486.5 mg, 2.81 mmol) in DMF (4 mL). After stirring at room temperature for 15 min, a solution of **34** (800 mg, 2.55 mmol) in DMF (4 mL) was added. The reaction mixture was stirred at 70 °C for 18 h, concentrated under vacuum, and diluted with water and ethyl acetate. The organic phase was washed with brine, dried over Na_2SO_4, and concentrated to give a residue which was purified by flash chromatography on silica gel. Elution with 40/60 cyclohexane/ethyl acetate gave **10** as a white solid, which was further purified by crystallization from chloroform yielding 299 mg (30.0%) of the desired product: mp 176.0 °C; ^{1}H NMR (DMSO-*d*)

δ 8.11 (br s, 1 H), 7.83 (br s, 1 H), 7.27 (dt, 1 H, $J = 8.9$, 5.3 Hz), 7.22 (s, 1 H), 7.21 (s, 1 H), 7.06 (dt, 1 H, $J = 8.9$, 1.9 Hz), 4.64 (m 1 H), 4.46 (dd, 1H, $J = 11.7$, 2.4 Hz), 4.32 (m, 2 H), 4.17 (dd, 1 H, $J = 11.7$, 6.9 Hz). ^{13}C NMR (DMSO-*d*) δ 161.9 (d, $J = 0.8$ Hz), 152.9 (dd, $J = 240.6$, 6.8 Hz), 148.7 (dd, $J = 247.5$, 8.4 Hz), 143.3 (dd, $J = 11.0$, 3.0 Hz), 143.3, 143.1, 123.6, 123.4, 119.1, 119.0, 117.4 (dd, $J = 25.1$, 20.2 Hz), 116.8 (m), 111.7 (d, $J = 21.6$ Hz), 72.3, 68.8, 65.2. Anal. Calcd for $C_{16}H_{11}Cl_2F_2NO_4$ (390.17).

3-(2-Acetyl-4-bromophenoxy)-1,2-epoxypropane (35). Potassium carbonate (1.41 g, 10,2 mmol) and epichlorohydrin (1.46 mL, 18.6 mmol) were added to a solution of 2-acetyl-4-bromophenol (2 g, 9.3 mmol) in DMF (22 mL). After stirring at 40 °C for 64 h, the mixture was poured into water (60 mL) and extracted with ethyl acetate (3 × 30 mL). The combined organic extracts were washed with 1 M NaOH (30 mL) and brine (30 mL), dried over Na_2SO_4, and concentrated to give a brownish oil, which was purified by flash chromatography. Elution with 70/30 cyclohexane/ethyl acetate gave 1.21 g (48.0%) of **35** as a white wax: ^{1}H NMR ($CDCl_3$) δ 7.84 (d, 1 H, $J = 2.6$ Hz), 7.53 (dd, 1 H, $J = 8.8$, 2.6 Hz), 6.85 (d, 1 H, $J = 8.8$ Hz), 4.37 (dd, 1 H, $J = 10.9$, 2.7 Hz), 3.97 (dd, 1 H, $J = 10.9$, 6.0 Hz), 3.39 (m, 1 H), 2.95 (t, 1 H, $J = 4.5$ Hz), 2.76 (dd, 1 H, $J = 4.7$, 2.6 Hz), 2.64 (s, 3 H).

3-(2-Acetyloxy-4-bromophenoxy)-1,2-epoxypropane (36). *m*-Chloroperbenzoic acid (2.58 g, 11.52 mmol) was added portion wise to a solution of **35** (1.21 g, 5.76 mmol) in DCM (13 mL) at room temperature. The mixture was refluxed overnight, cooled to room temperature and then to 4 °C. Saturated aqueous $Na_2S_2O_5$ (20 mL) was added. The mixture was filtered to remove a white precipitate. The organic phase was diluted with DCM (20 mL), washed with saturated aqueous $NaHCO_3$, dried over Na_2SO_4 and concentrated to give 1.55 g (94.0%) of **36** as a yellow oil: ^{1}H NMR ($CDCl_3$) δ 7.30 (dd, 1 H, $J = 8.7$, 2.4 Hz), 7.20 (d, 1 H, $J = 2.4$ Hz), 6.88 (d, 1 H, $J = 8.7$ Hz), 4.25 (dd, 1 H, $J = 11.2$, 2.9 Hz), 3.95 (dd, 1 H, $J = 11.2$, 5.6 Hz), 3.30 (ddd, 1 H, $J = 5.6$, 2.8, 1.4 Hz), 2.88 (dd, 1 H, $J = 4.9$, 4.2 Hz), 2.71 (dd, 1 H, $J = 4.9$, 2.6 Hz), 2.32 (s, 3 H).

7-Bromo-2-hydroxymethyl-1,4-benzodioxane (37). 2.5 M NaOH (2.9 mL) was added dropwise to a solution of **36** (1.55 g, 5.4 mmol) in methanol (16 mL). After stirring at room temperature for 15 min, the reaction mixture was heated at 50 °C for 2.5 h, concentrated under vacuum, and diluted with water (15 mL) and ethyl acetate (15 mL). The aqueous phase was separated and extracted with ethyl acetate (2 × 15 mL). The combined organic phases were washed with 1 M NaOH (30 mL), brine (2 × 30 mL), dried over $NaSO_4$, and concentrated under vacuum to give 1.1 g (83.1%) of **37** as a yellow oil: ^{1}H NMR ($CDCl_3$) δ 7.05 (d, 1 H, $J = 2.3$ Hz), 6.95 (dd, 1 H, $J = 8.6$, 2.3 Hz), 6.76 (d, 1 H, $J = 8.6$ Hz), 4.26 (m, 2 H), 4.11 (m, 1 H), 3.86 (m, 2 H), 1.87 (br s, 1 H).

7-Bromo-2-mesyloxymethyl-1,4-benzodioxane (38). Mesyl chloride (0.22 mL, 2.82 mmol) was added dropwise to a solution of **37** (550 mg, 2.24 mmol) and TEA (0.43 mL, 3.04 mmol) in DCM (8 mL) at 4 °C. The mixture was stirred at 4 °C for 15 min and then at room temperature for 30 min. After cooling to 4 °C, water (10 mL) and DCM (10 mL) were added. The organic layer was separated, washed with 10% HCl (10 mL), saturated aqueous $NaHCO_3$ (10 mL), and brine (10 mL), dried over Na_2SO_4, and concentrated to give 700 mg (97.2%) of **38** as a colourless oil: ^{1}H NMR ($CDCl_3$) δ 7.05 (d, 1 H, $J = 2.3$ Hz), 6.98 (dd, 1 H, $J = 8.6$, 2.3 Hz), 6.78 (d, 1 H, $J = 8.6$ Hz), 4.46 (m, 3 H), 4.31 (dd, 1 H, $J = 11.7$, 2.3 Hz), 4.11 (dd, 1 H, $J = 11.7$, 6.2 Hz), 3.09 (s, 3 H).

3-(7-bromo-1,4-benzodioxan-2-yl)methoxy-2,6-difluorobenzamide (11). Potassium carbonate (330 mg, 2.40 mmol) was added to a solution of 2,6-difluoro-3-hydroxybenzamide (419 mg, 2.40 mmol) in DMF (5 mL). After stirring at room temperature for 15 min, a solution of **38** (700 mg, 2.17 mmol) in DMF (5 mL) was added. The reaction mixture was stirred at 60 °C for 18 h, concentrated under vacuum, and diluted with water (15 mL) and ethyl acetate (15 mL). The aqueous phase was separated and extracted with ethyl acetate (2 × 15 mL). The combined organic phases were washed with 1 M NaOH (15 mL), and brine (15 mL), dried over Na_2SO_4, and concentrated to give a residue which was purified by flash chromatography on silica gel. Elution with 60/40 cyclohexane/ethyl acetate gave 540 mg (62.1%) of **11** as a white solid: mp 114.5 °C; ^{1}H NMR ($CDCl_3$) δ 7.06 (m, 2 H), 6.93 (m, 2 H), 6.77 (d, 1 H, $J = 8.6$ Hz), 6.02 (br s, 2 H), 4.55 (m, 1 H), 4.38 (dd, 1 H, $J = 11.6$, 2.4 Hz), 4.23 (m, 3 H). ^{13}C NMR ($CDCl_3$) δ 162.9, 154.3 (dd, $J = 246.5$, 5.4 Hz), 150.6 (dd, $J = 253.7$, 7.1 Hz), 143.6, 143.1 (dd, $J = 11.7$, 3.7 Hz), 142.6, 124.8, 120.6, 118.8; 118.5 (d, $J = 8.8$ Hz), 114.5 (dd, $J = 20.8$, 16.3 Hz), 113.5, 111.6 (d, $J = 23.6$ Hz), 71.6, 69.0, 65.0. Anal. Calcd for $C_{16}H_{12}BrF_2NO_4$ (400.17).

3-(2-Formyl-4-trifluoromethylphenoxy)-1,2-epoxypropane (39). Potassium carbonate (1.66 g, 12.0 mmol) and epibromohydrin (1.04 mL, 12.0 mmol) were added to a solution of 2-hydroxy-5-trifluoromethylbenzaldehyde (1.90 g, 10.0 mmol) in DMF (30 mL). The reaction mixture was stirred at 40 °C overnight, concentrated, diluted with water (20 mL) and ethyl acetate (20 mL). The aqueous phase was separated and extracted with ethyl acetate (2 × 20 mL). The combined organic phases were washed with 1 M NaOH (40 mL), and brine (40 mL), dried over Na_2SO_4, concentrated to give a residue which was purified by flash chromatography on silica gel. Elution with 70/30 cyclohexane/ethyl acetate gave 1.80 g (73.2%) of **39** as a colourless oil: ^{1}H NMR ($CDCl_3$) δ 10.52 (s, 1 H), 8.13 (d, 1 H, $J = 2.5$ Hz), 7.79 (d, 1 H, $J = 8.8$, 2.5 Hz), 7.12 (d, 1 H, $J = 8.8$ Hz), 4.50 (dd, 1 H, $J = 11.2$, 2.7 Hz), 4.11 (dd, 1 H, $J = 11.2$, 5.9 Hz), 3.43 (ddt, 1 H, $J = 5.9$, 4.2, 2.6 Hz), 2.98 (dd, 1 H, $J = 4.7$, 4.2 Hz), 2.81 (dd, 1 H, $J = 4.8$, 2.6 Hz).

3-(2-Formyloxy-4-trifluoromethylphenoxy)-1,2-epoxypropane (40). *m*-Chloroperbenzoic acid (3.61 g, 14.65 mmol) was added portion wise to a solution of **39** (1.80 g, 7.31 mmol) in DCM (18 mL) at room temperature. The mixture was refluxed overnight, and cooled to 4 °C. Saturated aqueous $Na_2S_2O_5$ (30 mL) was added. The mixture was filtered to remove a white precipitate. The organic phase was diluted with ethyl acetate (30 mL), washed with saturated aqueous $NaHCO_3$ (4 × 30 mL), dried over Na_2SO_4 and concentrated to give 1.90 g (98.9%) of **40** as a yellow oil: ^{1}H NMR ($CDCl_3$) δ 7.95 (m, 1 H), 7.19 (d, 1 H, $J = 2.2$ Hz), 7.11 (m, 1 H), 6.93 (d, 1 H, $J = 8.4$ Hz), 4.39 (dd, 1 H, $J = 11.4$, 2.6 Hz), 4.04 (dd, 1 H, $J = 11.4$, 5.9 Hz), 3.40 (m, 1 H), 2.97 (m, 1 H), 2.81 (dd, 1 H, $J = 4.7$, 2.7 Hz).

2-Hydroxymethyl-7-trifluoromethyl-1,4-benzodioxane (41). 2.5 M NaOH was added dropwise to a solution of **40** (1.90 g, 7.25 mmol) in methanol (20 mL). After stirring at room temperature for 15 min, the reaction mixture was heated at 60 °C for 2 h, concentrated under vacuum, and diluted with water (20 mL) and ethyl acetate (20 mL). the aqueous phase was extracted with ethyl acetate (2 × 20 mL). The combined organic phases were washed with 1 M NaOH (20 mL), and brine (2 × 20 mL), dried over Na_2SO_4, and concentrated to yield a residue, which was purified by flash chromatography on silica gel. Elution with 70/30 cyclohexane/ethyl acetate gave 700 mg (41.2%) of **41** as a colourless liquid: ^{1}H NMR ($CDCl_3$) δ 7.17 (d, 1 H, $J = 2.1$ Hz), 7.11 (m, 1 H), 6.95 (d, 1 H, $J = 7.9$ Hz), 4.36 (dd, 1 H, $J = 12.1$, 4.6 Hz), 4.28 (m, 1 H), 4.14 (m, 2 H), 3.90 (qd, 1 H, $J = 12.1$, 4.6 Hz), 1.83 (br s, 1 H).

2-Mesyloxymethyl-7-trifluoromethyl-1,4-benzodioxane (42). Mesyl chloride (0.28 mL, 3.59 mmol) was added dropwise to a solution of **41** (700 mg, 2.99 mmol) and TEA (0.55 mL, 3.89 mmol) in DCM (10 mL) at 4 °C. The mixture was stirred at 4 °C for 15 min and then at room temperature for 30 min. After cooling to 4 °C, water (10 mL) and DCM (10 mL) were added. The organic layer was separated, washed with 10% HCl (10 mL), saturated aqueous $NaHCO_3$ (10 mL), and brine (10 mL), dried over Na_2SO_4, and

concentrated to yield a residue which was purified by flash chromatography on silica gel. Elution with 70/30 cyclohexane/ethyl acetate gave 710 mg (76.3%) of **42** as a white wax: ^{1}H NMR ($CDCl_3$) δ 7.15 (m, 2 H), 6.98 (d, 1 H, J = 8.3 Hz), 4.50 (m, 3 H), 4.38 (dd, 1 H, J = 11.7, 2.3 Hz), 4.17 (dd, 1 H, J = 11.7, 6.5 Hz), 3.10 (s, 3 H).

2,6-Difluoro-3-(7-trifluoromethyl-1,4-benzodioxan-2-yl) methoxybenzamide (18). Potassium carbonate (345 mg, 2.50 mmol) was added to a solution of 2,6-difluoro-3-hydroxybenzamide (433 mg, 2.50 mmol) in DMF (5 mL). After stirring at room temperature for 15 min, a solution of **42** (710 mg, 2.27 mmol) in DMF (5 mL) was added. The reaction mixture was stirred at 65 °C for 7 h, concentrated under vacuum, and diluted with water (15 mL) and ethyl acetate (15 mL). The aqueous phase was separated and extracted with ethyl acetate (2 × 15 mL). The combined organic phases were washed with 1 M NaOH (20 mL), and brine (15 mL), dried over Na_2SO_4, and concentrated to give a residue which was purified by flash chromatography on silica gel. Elution with 60/40 cyclohexane/ethyl acetate gave 500 mg (56.6%) of **18** as a white solid: mp 125.8 °C; ^{1}H NMR (DMSO-*d*) δ 8.12 (br s, 1 H), 7.84 (br s, 1 H), 7.29 (dt, 1 H, J = 9.4, 5.4 Hz), 7.24 (s, 1 H), 7.19 (dd, 1 H, J = 8.7, 1.5 Hz), 7.10 (s, 1 H), 7.06 (dt, 1 H, J = 9.4, 2.1 Hz), 4.67 (m, 1 H), 4.51 (dd, 1 H, J = 11.7, 2.1 Hz), 4.39–4.28 (m, 2 H), 4.22 (dd, 1 H, J = 11.7, 6.9 Hz). ^{13}C NMR (DMSO-*d*) δ 16.7, 152.6 (dd, J = 240.5, 6.8 Hz), 148.4 (dd, J = 247.4, 9.2 Hz), 146.5, 143.3, 143.1 (dd, J = 10.3, 3.4 Hz), 124.5 (q, J = 270.0 Hz), 122.7 (q, J = 31.4 Hz), 118.9 (q, J = 4.3 Hz), 118.3, 117.4 (dd, J = 25.1, 20.4 Hz), 116.4 (d, J = 9.1 Hz), 114.8 (q, J = 3.4 Hz), 111.5 (dd, J = 22.9, 4.6 Hz), 72.0, 68.6, 65.1. Anal. Calcd for $C_{17}H_{12}F5NO_4$ (389.27).

7-Fluoro-2-mesyloxymethyl-1,4-benzodioxane (43). Mesyl chloride (0.40 mL, 5.15 mmol) was added dropwise to a solution of 7-fluoro-2-hydroxymethyl-1,4-benzodioxane (790 mg, 4.29 mmol) and TEA (0.785 mL, 5.58 mmol) in DCM (10 mL) at 4 °C. The mixture was stirred at 4 °C for 15 min and then at room temperature for 90 min. After cooling to 4 °C, water (10 mL) and DCM (10 mL) were added. The organic layer was separated, washed with 10% HCl (10 mL), saturated aqueous $NaHCO_3$ (10 mL), and brine (10 mL), dried over Na_2SO_4, and concentrated to yield a residue which was purified by flash chromatography on silica gel. Elution with 70/30 cyclohexane/ethyl acetate gave 860 mg (76.4%) of **43** as a colourless oil: ^{1}H NMR ($CDCl_3$) δ 6.83 (dd, 1 H, J = 8.9, 5.4 Hz), 6.61 (m, 2 H), 4.61 (m, 3 H), 4.10 (dd, 1 H, J = 11.7, 6.1 Hz), 3.09 (s, 3 H).

2,6-Difluoro-3-(7-fluoro-1,4-benzodioxan-2-yl)methoxybenzamide (13). Potassium carbonate (498 mg, 3.61 mmol) was added to a solution of 2,6-difluoro-3-hydroxybenzamide (625 mg, 3.28 mmol) in DMF (10 mL). After stirring at room temperature for 15 min, a solution of **43** (860 mg, 3.28 mmol) in DMF (5 mL) was added. The reaction mixture was stirred at 75 °C for 4 h, concentrated under vacuum, and diluted with water (20 mL) and ethyl acetate (20 mL). The aqueous phase was separated and extracted with ethyl acetate (2 × 20 mL). The combined organic phases were washed with 1 M NaOH (30 mL), and brine (30 mL), dried over Na_2SO_4, and concentrated to give a residue which was purified by flash chromatography on silica gel. Elution with 1/1 cyclohexane/ethyl acetate gave 620 mg (55.7%) of **13** as a white solid: mp 120.1 °C; ^{1}H NMR ($CDCl_3$) δ 7.07 (dt, 1 H, J = 9.1, 5.1 Hz), 6.90 (dt, 1 H, J = 9.1, 2.0 Hz), 6.83 (dd, 1 H, J = 8.9, 5.4 Hz), 6.60 (m, 2 H), 5.96 (br s, 2 H), 4.57 (dd, 1 H, J = 11.2, 2.4 Hz), 4.36 (dd, 1 H, J = 11.2, 2.4 Hz), 4.23 (m, 3 H). ^{13}C NMR($CDCl_3$) δ 162.1, 157.6 (d, J = 238.0 Hz), 154.3 (dd, 246.5, 5.2 Hz), 150.6 (dd, J = 253.6, 7.1 Hz), 143.5 (d, J = 7.9 Hz), 143.1 (d, J = 12.0 Hz), 139.5, 118.4 (d, J = 8.0 Hz), 117.8 (d, J = 9.4 Hz), 114.4 (dd, J = 19.9, 16.3 Hz), 111.6 (d, J = 23.6 Hz), 108.4 (d, J = 23.3 Hz), 104.8 (d, J = 26.5 Hz), 71.7, 69.0, 64.9. Anal. Calcd for $C_{16}H_{12}F3NO_4$ (339.27).

2-Acetyloxymethyl-7-bromo-1,4-benzodioxane (44). Acetyl chloride (0.20 mL, 2.71 mmol) was slowly added to a solution of **37** (530 mg, 2.17 mmol) and TEA (0.42 mL, 2.93 mmol) in DCM (10 mL) at 4 °C. The mixture was stirred at 4 °C for 15 min and then at room temperature for 1 h. After cooling to 4 °C, water (15 mL) and DCM (10 mL). The organic layer was separated, washed with 10% HCl (15 mL), saturated aqueous $NaHCO_3$ (15 mL), and brine (15 mL), dried over Na_2SO_4, and concentrated to yield a residue which was purified by flash chromatography on silica gel. Elution with 80/20 cyclohexane/ethyl acetate gave 540 mg (87.1%) of **44** as a colourless oil: ^{1}H NMR ($CDCl_3$) δ 7.06 (d, 1 H, J = 2.2 Hz), 6.96 (dd, 1 H, J = 8.6, 2.2 Hz), 6.76 (d, 1 H, J = 8.6 Hz), 4.39 (m, 1 H), 4.28 (m, 3 H), 4.04 (dd, 1 H, J = 11.5, 6.8 Hz), 2.12 (s, 3 H).

2-Acetyloxymethyl-7-vinyl-1,4-benzodioxane (45). Potassium vinyl trifluoborate (400 mg, 2.98 mmol), $PdCl_2$(dppe)·CH_2Cl_2 (170 mg, 0.3 mmol), *t*-butylamine (0.94 mL, 8.94 mmol) were added to a solution of **44** (590 mg, 2.98 mmol) in 2-propanol (16 mL) and water (10 mL) under nitrogen. The mixture was refluxed overnight, concentrated under vacuum, and diluted with water (10 mL) and ethyl acetate (10 mL). The aqueous layer was separated, extracted with ethyl acetate (2 × 10 mL). The combined organic phases were washed with 10% HCl (10 mL), and brine (3 × 10 mL), dried over Na_2SO_4, and concentrated to give 400 mg (57.3%) of **45** as a green oil: ^{1}H NMR ($CDCl_3$) δ 6.96 (m, 3 H), 6.60 (dd, 1 H, J = 17.6, 10.8 Hz), 5.59 (dd, 1 H, J = 17.5, 0.8 Hz), 5.14 (dd, 1 H, J = 10.8, 0.8 Hz), 4.27 (m, 2 H), 4.10 (m, 1 H), 3.88 (m, 2 H), 2.12 (s, 3 H).

2-Hydroxymethyl-7-vinyl-1,4-benzodioxane (46). 2.5 M NaOH (1.0 mL) and water (3.0 mL) were added to a solution of **45** (400 mg, 2.01 mmol) in methanol (5 mL). The reaction mixture was stirred at room temperature for 30 min, concentrated, and diluted with water (10 mL) and ethyl acetate (10 mL). The aqueous layer was separated and extracted with ethyl acetate (2 × 10 mL). The combined organic phases were washed with brine (2 × 10 mL), dried over Na_2SO_4, and concentrated to yield a residue which was purified by flash chromatography on silica gel. Elution with 80/20 cyclohexane/ethyl acetate gave 140 mg (36.2%) of **46** as a colourless wax: ^{1}H NMR ($CDCl_3$) δ 6.89 (m, 3 H), 6.60 (dd, 1 H, J = 17.6, 10.8 Hz), 5.59 (dd, 1 H, J = 17.5, 0.9 Hz), 5.14 (dd, 1 H, J = 10.8, 0.9 Hz), 4.27 (m, 2 H), 4.12 (m, 1 H), 3.88 (m, 2 H), 1.90 (br s, 1 H).

2-Mesyloxymethyl-7-vinyl-1,4-benzodioxane (47). Mesyl chloride (0.09 mL, 1.17 mmol) was added dropwise to a solution of **46** (140 mg, 0.73 mmol) and TEA (0.17 mL, 1.17 mmol) in DCM (5 mL) at 4 °C. The mixture was stirred at 4 °C for 15 min and then at room temperature for 30 min. After cooling to 4 °C, water (10 mL) and DCM (10 mL) were added. The organic layer was separated, washed with 10% HCl (10 mL), saturated aqueous $NaHCO_3$ (10 mL), and brine (10 mL), dried over Na_2SO_4, and concentrated to yield a residue which was purified by flash chromatography on silica gel. Elution with 80/20 cyclohexane/ethyl acetate gave 110 mg (55.7%) of **47** as a colourless wax: ^{1}H NMR ($CDCl_3$) δ 6.91 (m, 3 H), 6.59 (dd, 1 H, J = 17.6, 10.8 Hz), 5.60 (dd, 1 H, J = 17.5, 0.9 Hz), 5.15 (dd, 1 H, J = 10.8, 0.9 Hz), 4.47 (m, 3 H), 4.31 (dd, 1 H, J = 11.6, 2.2 Hz), 4.13 (dd, 1 H, J = 11.6, 6.1 Hz), 3.09 (s, 3 H).

2,6-Difluoro-3-(7-vinyl-1,4-benzodioxan-2-yl)methoxybenzamide (15). Potassium carbonate (62 mg, 0.45 mmol) was added to a solution of 2,6-difluoro-3-hydroxybenzamide (78 mg, 0.45 mmol) in DMF (2 mL). After stirring at room temperature for 15 min, a solution of **47** (110 mg, 0.41 mmol) in DMF (3 mL) was added. The reaction mixture was stirred at 70 °C for 6.5 h, concentrated under vacuum, and diluted with water (10 mL) and ethyl acetate (10 mL). The aqueous phase was separated and extracted with ethyl acetate (2 × 10 mL). The combined organic phases were washed with 1 M NaOH (20 mL), and brine (20 mL), dried over Na_2SO_4, and concentrated to give a residue which was purified by flash chromatography on silica gel. Elution with 60/40 cyclohexane/ethyl acetate gave 100 mg (69.9%) of **15** as a white wax: ^{1}H NMR ($CDCl_3$) δ 7.06 (m, 1 H), 6.90 (m, 3 H), 6.59 (dd, 1 H,

J = 17.5, 10.9 Hz), 6.00 (br s, 2 H), 5.59 (d, 1 H, J = 17.6 Hz), 5.14 (d, 1 H, J = 10.9 Hz), 4.56 (m, 1 H), 4.39 (dd, 1 H, J = 11.5, 2.4 Hz), 4.25 (m, 4 H). ^{13}C NMR ($CDCl_3$) 162.1, 154.3 (dd, J = 247.0, 5.7 Hz), 150.6 (dd, J = 253.4, 7.0 Hz), 143.6 (dd, J = 11.4, 3.6 Hz), 143.1, 142.8, 136.2, 132.2, 122.1 (d, J = 13.9 Hz), 120.2, 118.6, 117.5, 115.0 (dd, J = 16.8, 12.0 Hz), 112.8, 111.5 (dd, J = 23.8, 2.1 Hz), 71.5, 69.3, 65.1. Anal. Calcd for $C_{18}H_{15}F_2NO_4$ (347.31).

2-Acetyloxymethyl-7-trimethylsilylethynyl-1,4-benzodioxane (48). Ethynyltrimethylsilane (0.33 mL, 2.33 mmol), $PdCl_2$ (20 mg, 0.1 mmol), CuI (15 mg, 0.07 mmol), and PPh_3 (50 mg, 0.19 mmol) were added to a solution of **44** (540 mg, 1.88 mmol) in TEA (2.5 mL). The mixture was heated to 55 °C overnight. After cooling to room temperature, ethyl acetate (20 mL) and water (15 mL) were added. The organic layer was separated, washed with 10% HCl (15 mL) and brine (3 × 15 mL), dried over Na_2SO_4, and concentrated to give a residue which was purified by flash chromatography on silica gel. Elution with 90/10 cyclohexane/ethyl acetate provided 320 mg (55.9%) of **48** as a red oil: ^{1}H NMR ($CDCl_3$) δ 7.05 (d, 1 H, J = 2.1 Hz), 6.95 (dd, 1 H, J = 8.5, 2.2 Hz), 6.76 (d, 1 H, J = 8.5 Hz), 4.35 (m, 4 H), 4.04 (dd, 1 H, J = 11.2, 6.6 Hz), 2.12 (s, 3 H), 0.23 (s, 9 H).

7-Ethynyl-2-hydroxymethyl-1,4-benzodioxane (49). 2.5 M NaOH (0.51 mL) was added to a solution of **48** (320 mg, 1.05 mmol) in methanol (4 mL). After stirring at room temperature for 1 h, the reaction mixture was concentrated and diluted with water (10 mL) and ethyl acetate (10 mL). The aqueous layer was separated and extracted with ethyl acetate (3 × 10 mL). The combined organic phases were washed with brine (30 mL), dried over Na_2SO_4, and concentrated to give 198 mg (99.1%) of **49** as a brownish oil: ^{1}H NMR ($CDCl_3$) δ 7.02 (m, 2 H), 6.81 (d, 1 H, J = 8.3 Hz), 4.28 (m, 2 H), 4.12 (dd, 1 H, J = 11.6, 7.2 Hz), 3.88 (m, 2 H), 2.97 (s, 1 H), 1.98 (br s, 1 H).

7-Ethynyl-2-mesyloxymethyl-1,4-benzodioxane (50). Mesyl chloride (0.10 mL, 1.26 mmol) was added dropwise to a solution of **49** (198 mg, 1.05 mmol) and TEA (0.20 mL, 1.36 mmol) in DCM (6 mL) at 4 °C. The mixture was stirred at 4 °C for 15 min and then at room temperature for 30 min. After cooling to 4 °C, water (10 mL) and DCM (10 mL) were added. The organic layer was separated, washed with 10% HCl (10 mL), saturated aqueous $NaHCO_3$ (10 mL), and brine (10 mL), dried over Na_2SO_4, and concentrated to give 279 mg (98.1%) of **50** as a brownish oil: ^{1}H NMR ($CDCl_3$) δ 7.02 (m, 2 H), 6.83 (d, 1 H, J = 8.0 Hz), 4.44 (m, 3 H), 4.33 (m, 1 H), 4.13 (dd, 1 H, J = 11.5, 6.1 Hz), 3.09 (s, 3 H), 2.98 (s, 1 H).

2,6-Difluoro-3-(7-ethynyl-1,4-benzodioxan-2-yl)methoxybenzamide (14). Potassium carbonate (160 mg, 1.16 mmol) was added to a solution of 2,6-difluoro-3-hydroxybenzamide (201 mg, 1.16 mmol) in DMF (3 mL). After stirring at room temperature for 15 min, a solution of **50** (279 mg, 1.04 mmol) in DMF (3 mL) was added. The reaction mixture was stirred at 60 °C for 20 h, concentrated under vacuum, and diluted with water (10 mL) and ethyl acetate (10 mL). The aqueous phase was separated and extracted with ethyl acetate (2 × 10 mL). The combined organic phases were washed with 1 M NaOH (20 mL), and brine (20 mL), dried over Na_2SO_4, and concentrated to give a residue which was purified by flash chromatography on silica gel. Elution with 60/40 cyclohexane/ethyl acetate yielded 220 mg (60.8%) of **14** as a pale yellow wax: ^{1}H NMR ($CDCl_3$) δ 7.05 (m, 3 H), 6.85 (m, 2 H), 6.14 (br s, 1 H), 6.03 (br s, 1 H), 4.55 (m, 1 H), 4.41 (dd, 1 H, J = 11.5, 2.3 Hz), 4.24 (m, 3 H), 2.97 (s, 1 H). ^{13}C NMR (DMSO-*d*) δ 161.9, 152.8 (dd, J = 240.5, 6.7 Hz), 148.6 (dd, J = 247.3, 8.4 Hz), 144.4, 143.4 (dd, J = 3.3, 10.9 Hz), 143.1, 126.0, 121.0, 118.1, 117.3 (dd, J = 24.9, 20.2 Hz), 116.6 (d, J = 9.1 Hz), 115.4, 111.7 (dd, J = 22.8, 3.8 Hz), 83.9, 79.9, 72.1, 68.8, 65.3. Anal. Calcd for $C_{18}H_{13}F_2NO_4$ (345.08).

2-Hydroxymethyl-7-nitro-1,4-benzodioxane (51). $NaHCO_3$ (1.16 g, 13.5 mmol) was added to a solution of 4-nitrocatechol (2.0 g, 12.89 mmol) in DMF (28 mL). After cooling to 4 °C, epibromohydrin (1.4 mL, 16.37 mmol) was added dropwise. The mixture was stirred at 4 °C for 15 min and then at 80 °C for 8 h. Water (100 mL) was added at 4 °C and extraction was accomplished by ethyl acetate (3 × 30 mL). The combined organic extracts were washed with 1 M NaOH (30 mL), and with brine (2 × 50 mL), dried over Na_2SO_4, and concentrated. The resulting residue (2.28 g) was crystallized twice from DCM to give 0.45 g (16.5%) of **51** as a white solid: mp 136.0 °C; ^{1}H NMR ($CDCl_3$) δ 7.80 (m, 2 H), 6.96 (d, 1 H, J = 8.6 Hz), 4.43 (dd, 1 H, J = 11.1, 1.9 Hz), 4.27 (m, 1 H), 3.93 (qd, 2 H, J = 12.2, 4.4 Hz), 1.78 (br s, 1 H).

7-Nitro-2-tosyloxymethyl-1,4-benzodioxane (52). TsCl (0.30 g, 1.52 mmol) was added to a solution of **51** (0.32 g, 1.52 mmol) in pyridine (3 mL) at 4 °C. The mixture was stirred at room temperature for 3 h, diluted with water (20 mL) and DCM (20 mL). The organic phase was separated, washed with 10% HCl (20 mL), saturated aqueous $NaHCO_3$ (20 mL), and brine (20 mL), dried over Na_2SO_4, and concentrated. The oily residue was purified by flash chromatography on silica gel. Elution with 80/20 cyclohexane/ethyl acetate gave 270 mg (48.6%) of **52** as a white solid: mp 101.2 °C; ^{1}H NMR ($CDCl_3$) δ 7.78 (m, 3 H), 7.64 (d, 1 H, J = 2.6 Hz), 7.37 (d, 2 H, J = 8.0 Hz), 6.93 (d, 1 H, J = 8.8 Hz), 4.42 (m, 2 H), 4.26 (m, 2 H), 4.14 (dd, 1 H, J = 11.7, 6.8 Hz), 2.46 (s, 3 H).

2,6-Difluoro-3-(7-nitro-1,4-benzodioxan-2-yl)methoxybenzamide (16). Potassium carbonate (110 mg, 0.81 mmol) was added to a solution of 2,6-difluoro-3-hydroxybenzamide (140 mg, 0.81 mmol) in DMF (2 mL). After stirring at room temperature for 15 min, a solution of **52** (270 mg, 0.74 mmol) in DMF (2 mL) was added. The reaction mixture was stirred at 75 °C for 4 h, concentrated under vacuum, and diluted with water (10 mL) and ethyl acetate (10 mL). The aqueous phase was separated and extracted with ethyl acetate (2 × 10 mL). The combined organic phases were washed with 1 M NaOH (15 mL), and brine (15 mL), dried over Na_2SO_4, and concentrated to give a residue which was purified by flash chromatography on silica gel. Elution with 1/1 cyclohexane/ethyl acetate yielded 160 mg (59.0%) of **16** as a white solid: mp 172.2 °C; ^{1}H NMR (DMSO-*d*) δ 8.11 (br s, 1H), 7.83 (br s, 1H), 7.78 (dd, 1 H, J = 9.1, 2.8 Hz), 7.70 (d, 1 H, J = 2.8 Hz), 7.29 (dt, 1 H, J = 9.3, 5.2 Hz), 7.10 (d, 1H, J = 9.1 Hz), 7.07 (dt, 1 H, J = 9.3, 1.8 Hz), 4.71 (m, 1H), 4.59 (dd, 1 H, J = 11.5, 2.5 Hz), 4.41–4.26 (m, 3 H). ^{13}C NMR (DMSO-*d*) δ 161.4, 152.4 (dd, J = 240.4, 7.0 Hz), 149.2, 148.2 (dd, J = 247.3, 8.4 Hz), 142.8 (dd, J = 10.8, 3.2 Hz), 142.7, 141.5, 117.77, 117.70, 116.9 (dd, J = 25.4, 20.5 Hz), 116.2 (d, J = 9.4 Hz), 112.9, 111.2 (dd, J = 23.2, 3.8 Hz), 71.8, 68.3, 65.2. Anal. Calcd for $C_{16}H_{12}F_2N_2O_6$ (366.27).

3-(7-Amino-1,4-benzodioxan-2-yl)methoxy-2,6-difluorobenzamide (17). $SnCl_2$ (430 mg, 2.29 mmol) was added to a solution of **16** (210 mg, 0.57 mmol) in ethyl acetate (5 mL). The mixture was stirred at room temperature for 15 min, refluxed for 2 h, cooled to room temperature and poured into an ice-cooled saturated aqueous $NaHCO_3$ (20 mL). The resulting suspension was filtered and the filtrate was extracted with ethyl acetate (3 × 20 mL). The combined organic extracts were dried over Na_2SO_4 and concentrated to give a residue which was purified by flash chromatography on silica gel. Elution with 60/40 toluene/ethyl acetate provided 190 mg (99.1%) of **17** as a white solid: mp 141.3 °C; ^{1}H NMR (DMSO-*d*) δ 8.09 (br s, 1H), 7.81 (br s, 1H), 7.25 (dt, 1 H, J = 9.1, 5.3 Hz), 7.04 (dt, 1 H, J = 9.1, 1.8 Hz), 6.50 (d, 1 H, J = 8.4 Hz), 6.10 (d, 1H, J = 2.4 Hz), 6.05 (dd, 1 H, J = 8.4, 2.4 Hz), 4.62 (br s, 2H), 4.47–4.59 (m, 1 H), 4.25–4.19 (m, 3 H), 3.98 (dd, 1 H, J = 11.4, 6.6 Hz). ^{13}C NMR (DMSO-*d*) δ 161.7, 152.5 (dd, J = 240.5, 6.9 Hz), 148.4 (dd, J = 247.4, 8.0 Hz), 143.9, 143.2 (dd, J = 11.5, 4.6 Hz), 143.1, 134.1, 117.4, 117.1 (d, J = 4.6 Hz), 116.3 (d, J = 6.9 Hz), 111.4 (dd, J = 22.9, 4.6 Hz), 108.0, 102.8, 71.9, 68.8, 64.6. Anal. Calcd for $C_{16}H_{14}F_2N_2O_4$ (336.29).

2,6-Difluoro-3-(7-methanesulfonamido-1,4-benzodioxan-2-yl)methoxybenzamide (19). Mesyl chloride (0.05 mL, 0.67 mmol) was added dropwise to a solution of **17** (190 mg, 0.56 mmol) and TEA (0.1 mL, 0.73 mmol) in ethyl acetate (8 mL) at 4 °C. The mixture was refluxed for 30 min. After cooling to 4 °C, 10% HCl was added. The aqueous phase was separated and extracted with ethyl acetate (3 × 15 mL). The combined organic phases were dried over Na_2SO_4, and concentrated. The resulting residue was purified by flash chromatography on silica gel. Elution with 1/1 toluene/ethyl acetate gave 100 mg (43.1%) of **19** as a colourless wax: ^{1}H NMR (DMSO-*d*) δ 9.40 (br s, 1H), 8.11 (br s, 1H), 7.84 (br s, 1H), 7.27 (dt, 1 H, *J* = 9.0, 5.2 Hz), 7.06 (dt, 1 H, *J* = 9.0, 1.5 Hz), 6.9 (d, 1 H, *J* = 8.9 Hz), 6.8 (d, 1 H, *J* = 2.5 Hz), 6.70 (dd, 1 H, *J* = 8.9, 2.5 Hz), 4.57 (m, 1H), 4.39 (dd, 1 H, *J* = 11.6, 2.5 Hz), 4.31 (m, 2H), 4.10 (dd, 1 H, *J* = 11.6, 7.2 Hz), 2.88 (s, 3 H). ^{13}C NMR (DMSO-*d*) δ 161.9, 152.8 (dd, *J* = 240.3, 6.8 Hz), 148.6 (dd, *J* = 247.1, 8.3 Hz), 143.4 (dd, *J* = 10.6, 3.2 Hz), 143.3, 140.6, 132.7, 118.1, 117.5 (dd, *J* = 24.7, 20.5 Hz), 116.5 (d, *J* = 9.1 Hz), 115.1, 111.7 (dd, *J* = 23.1, 4.0 Hz), 110.8, 71.5, 69.0, 65.1. The methyl signal is obscured by the overlap with the DMSO signal. Anal. Calcd for $C_{17}H_{16}F_2N_2O_4S$ (414.38).

2-Acetyloxymethyl-7-nitro-1,4-benzodioxane (53). TEA (0.36 mL, 2.56 mL) was added to a solution of **51** (0.45 g, 2.13 mmol) in ethyl acetate (5 mL). After cooling to 4 °C, acetyl chloride (0.18 mL, 2.56 mmol) was added dropwise. The mixture was stirred at 4 °C for 15 min and diluted with water (15 mL) and ethyl acetate (20 mL). The organic layer was separated, washed with 10% HCl (20 mL), saturated aqueous $NaHCO_3$ (20 mL), and brine (20 mL), dried over Na_2SO_4, and concentrated to give 0.51 g (94.6%) of **53** as a colourless oil: ^{1}H NMR ($CDCl_3$) δ 7.80 (m, 2 H), 6.96 (d, 1 H, *J* = 8.8 Hz), 4.40 (m, 4 H), 4.14 (m, 1 H), 2.12 (s, 3 H).

7-Amino-2-acetyloxymethyl-1,4-benzodioxane (54). $SnCl_2$ (1.53 g, 8.06 mmol) was added to a solution of **53** (0.51 g, 2.01 mmol) in ethyl acetate (8 mL). The mixture was refluxed for 2 h. Saturated aqueous $NaHCO_3$ (25 mL) was added and the resulting suspension was filtered. The filtrate was extracted with ethyl acetate (3 × 20 mL). The combined extracts were dried over Na_2SO_4, and concentrated. The residue was purified by flash chromatography on silica gel. Elution with 1/1 cyclohexane/ethyl acetate gave 0.28 g (62.4%) of **54** as a pale yellow oil: ^{1}H NMR ($CDCl_3$) δ 6.68 (d, 1 H, *J* = 8.6 Hz), 6.27 (d, 1 H, *J* = 2.7 Hz), 6.22 (dd, 1 H, *J* = 8.6, 2.7 Hz), 4.31 (m, 3 H), 3.97 (dd, 1 H, *J* = 11.5, 6.9 Hz), 3.06 (br s, 2 H), 2.13 (s, 3 H).

2-Acetyloxymethyl-7-iodo-1,4-benzodioxane (55). A solution of $NaNO_2$ (95 mg, 1.38 mmol) in water (2 mL) was slowly added to a solution of **54** (0.28 g, 1.25 mmol) in 0.29 M H_2SO_4 (4.68 mL) at −5 °C. After 15 min, a solution of NaI (320 mg, 2.13 mmol) in water (2 mL) was added and the mixture was stirred at room temperature overnight. Saturated aqueous $Na_2S_2O_5$ (10 mL) was added and extraction was accomplished with ethyl acetate (3 × 20 mL). The combined organic extracts were dried over Na_2SO_4 and concentrated to give a residue which was purified by flash chromatography on silica gel. Elution with 80/20 cyclohexane/ethyl acetate gave 0.24 g (57.5%) of **55** as a colourless oil: ^{1}H NMR ($CDCl_3$) δ 7.23 (d, 1 H, *J* = 2.1 Hz), 7.14 (dd, 1 H, *J* = 8.5, 2.1 Hz), 6.63 (d, 1 H, *J* = 8.5 Hz), 4.37 (m, 1 H), 4.28 (m, 3 H), 4.03 (dd, 1 H, *J* = 11.5, 6.9 Hz), 2.07 (s, 3 H).

2-Hydroxymethyl-7-iodo-1,4-benzodioxane (56). Water (3 mL) and 2.5 M NaOH (0.37 mL) were added to a solution of **55** (0.24 g, 0.72 mmol) in methanol (3 mL). The reaction mixture was stirred at room temperature for 1 h, concentrated, diluted with water (10 mL), and extracted with ethyl acetate (3 × 10 mL). The organic extracts were washed with brine (20 mL), dried over Na_2SO_4, and concentrated to give 0.18 g (85.6%) of **56** as a brownish oil: ^{1}H NMR ($CDCl_3$) δ 7.22 (d, 1 H, *J* = 2.1 Hz), 7.13 (dd, 1 H, *J* = 8.5, 2.1 Hz), 6.63 (d, 1 H, *J* = 8.5 Hz), 4.25 (m, 2 H), 4.11 (m, 1 H), 3.86 (qd, 2 H, *J* = 12.1, 4.7 Hz), 1.47 (br s, 1 H).

7-Iodo-2-mesyloxymethyl-1,4-benzodioxane (57). Mesyl chloride (0.06 mL, 0.74 mmol) was slowly added to a solution of **56** (0.18 g, 0.62 mmol) and TEA (0.11 mL, 0.81 mmol) in DCM (5 mL) at 4 °C. The mixture was stirred for 15 min at 4 °C and for 30 min at room temperature. After cooling to 4 °C, water (10 mL) and DCM (10 mL) were added. The organic phase was separated, washed with 10% HCl (10 mL), saturated aqueous $NaHCO_3$ (10 mL), and brine (10 mL), dried over Na_2SO_4, and concentrated. The resulting residue was purified by flash chromatography on silica gel. Elution with 70/30 cyclohexane/ethyl acetate gave 0.19 g (82.8%) of **57** as a colourless oil: ^{1}H NMR ($CDCl_3$) δ 7.23 (d, 1 H, *J* = 2.1 Hz), 7.16 (dd, 1 H, *J* = 8.6, 2.1 Hz), 6.65 (d, 1 H, *J* = 8.5 Hz), 4.45 (m, 3 H), 4.30 (dd, 1 H, *J* = 11.7, 2.3 Hz), 4.11 (dd, 1 H, *J* = 11.7, 6.2 Hz), 3.09 (s, 3 H).

2,6-Difluoro-3-(7-iodo-1,4-benzodioxan-2-yl)methoxybenzamide (12). Potassium carbonate (77 mg, 0.56 mmol) was added to a solution of 2,6-difluoro-3-hydroxybenzamide (97 mg, 0.56 mmol) in DMF (2 mL). After stirring at room temperature for 15 min, a solution of **57** (190 mg, 0.51 mmol) in DMF (2 mL) was added. The reaction mixture was stirred at 75 °C for 4 h, concentrated under vacuum, and diluted with water (10 mL) and ethyl acetate (10 mL). The aqueous phase was separated and extracted with ethyl acetate (2 × 10 mL). The combined organic phases were washed with 1 M NaOH (15 mL), and brine (15 mL), dried over Na_2SO_4, and concentrated to give a residue which was purified by flash chromatography on silica gel. Elution with 1/1 cyclohexane/ethyl acetate yielded 120 mg (52.6%) of **12** as a white wax: ^{1}H NMR ($CDCl_3$) δ 7.22 (d, 1 H, *J* = 2.2 Hz), 7.15 (dd, 1 H, *J* = 8.5, 2.2 Hz), 7.06 (dt, 1 H, *J* = 9.1, 5.1 Hz), 6.90 (dt, 1 H, *J* = 9.1, 2.0 Hz), 6.65 (d, 1 H, *J* = 8.5 Hz), 6.00 (br s, 2 H), 4.55 (m, 1 H), 4.38 (dd, 1 H, *J* = 11.6, 2.4 Hz), 4.23 (m, 3 H). ^{13}C NMR (DMSO-*d*) δ 161.9, 154.4 (dd, *J* = 246.5, 5.4 Hz), 150.6 (dd, *J* = 253.8, 7.0 Hz), 143.8, 143.5 (dd, *J* = 11.4, 3.8 Hz), 143.4, 130.8, 126.4, 123.3, 119.4; 114.4 (dd, *J* = 20.2, 16.3 Hz), 114.5 (dd, *J* = 20.8, 16.3 Hz), 111.5 (dd, *J* = 19.4, 4.3 Hz), 71.5, 69.0, 65.1. Anal. Calcd for $C_{16}H_{12}F_2INO_4$ (447.17). Anal. Calcd for $C_{16}H_{12}F_2INO_4$ (447.17).

2-Benzyloxymethyl-7-carboxy-1,4-benzodioxane (58). Potassium carbonate (5.45 g, 39.4 mmol) and epibromohydrin (3.37 mL, 39.4 mmol) were added to a solution of methyl 3,4-dihydroxybenzoate (5.52 g, 32.8 mmol). The mixture was stirred at room temperature for 15 min and at 60 °C overnight, and then concentrated. Water (100 mL) and ethyl acetate (100 mL) were added. The aqueous layer was separated and extracted with ethyl acetate (2 × 100 mL). The combined organic phases were washed with 1 M NaOH (50 mL) and with brine (2 × 50 mL), dried over Na_2SO_4 and concentrated. The TLC analysis (silica, 60/40 cyclohexane/ethyl acetate) of the residue indicated the presence of a major spot with R_f 0.25, which was isolated, as a colourless oil, in amount of 5.19 g by flash chromatography on silica gel with the same eluent. ^{1}H NMR spectrum showed that it was a mixture of two regioisomers: desired 2-hydroxymethyl-7-methoxycarbonyl-1,4-benzodioxane (~85%) and, likely, undesired 2-hydroxymethyl-6-methoxycarbonyl-1,4-benzodioxane (~15%). Such a mixture was dissolved in dry THF (10 mL) and dropped into a suspension of NaH (610 mg, 25.47 mmol) in dry THF (10 mL) under nitrogen. After stirring at room temperature for 30 min, a solution of benzyl bromide (3.03 mL, 25.47 mmol) in dry THF (10 mL) was added. After 15 min at room temperature, the mixture was refluxed for 3 h, and then cooled to room temperature. Ethyl acetate (30 mL) and 10% HCl (30 mL) were added. The organic phase was separated, washed with brine (2 × 30 mL), dried over Na_2SO_4, and concentrated. The residue was purified by flash chromatography on silica gel. Elution with 90/10 cyclohexane/ethyl acetate gave 4.30 g of colourless oil, corresponding to a TLC spot faster running than the starting alcohols mixture (silica, 60/40 cyclohexane/ethyl acetate, R_f 0.58) and

analogously composed of 2-benzyloxymethyl-7-methoxycarbonyl-1,4-benzodioxane (~85%) and its undesired 6-methoxycarbonyl regioisomer (~15%) as inferable from the ^{1}H NMR spectrum. Such a benzyl ethers mixture was dissolved in methanol (40 mL); 2.5 M NaOH (6.9 mL) and water (10 mL) were added dropwise. The mixture was stirred at room temperature for 15 min and at 50 °C for 2 h, and concentrated. Ethyl acetate (20 mL) and 10% HCl (15 mL) were added to the residue. The aqueous layer was separated and extracted with ethyl acetate (2 × 20 mL). The combined organic phases were washed with brine (2 × 20 mL), dried over Na_2SO_4 and concentrated. The resulting solid (4.43 g) was twice crystallized from toluene to give 1.64 g (16.6% of starting methyl 3,4-dihydroxybenzoate) of **58** as a white solid: mp 132.3 °C; ^{1}H NMR ($CDCl_3$) δ 7.63 (m, 2 H), 7.33 (m, 5 H), 6.92 (d, 1 H, J = 8.5 Hz), 4.61 (s, 2 H), 4.39 (m, 2 H), 4.17 (dd, 1 H, J = 11.8, 7.7 Hz), 3.72 (m, 2 H).

2-Benzyloxymethyl-7-methoxycarbonyl-1,4-benzodioxane (59). Trimethyl orthoformate (0.12 mL, 1.07 mmol) and concentrated sulfuric acid (0.1 mL) were added to a solution of **58** (320 mg, 1.07 mmol) in methanol (6 mL). The mixture was heated at 65 °C overnight. After cooling to room temperature, DCM (10 mL) and saturated aqueous $NaHCO_3$ (10 mL) were added. The organic phase was separated, washed with saturated aqueous NaCl (2 × 10 mL), dried over Na_2SO_4 and concentrated to give 290 mg (86.3%) of **59** as an oil: ^{1}H NMR ($CDCl_3$) δ 7.56 (m, 2 H), 7.32 (m, 5 H), 6.89 (d, 1 H, J = 6.6 Hz), 4.60 (m, 2 H), 4.37 (m, 2 H), 4.15 (m, 1 H), 3.86 (s, 3 H), 3.71 (m, 2 H).

2-Hydroxymethyl-7-methoxycarbonyl-1,4-benzodioxane (60). A solution of **59** (290 mg, 0.92 mmol) in ethanol (10 mL) was added with 10% Pd/C (50 mg) and vigorously shaken under hydrogen at room temperature for 6 h. The catalyst was removed by filtration and the filtrate concentrated to give 200 mg (97.0%) of **60** as an oil: ^{1}H NMR ($CDCl_3$) δ 7.58 (m, 2 H), 6.90 (d, 1 H, J = 8.2 Hz), 4.39 (m, 4 H), 4.13 (m, 1 H), 3.86 (s, 3 H).

2,6-Difluoro-3-(7-methoxycarbonyl-1,4-benzodioxan-2-yl) methoxybenzamide (21). Triphenylphosphine (351 mg, 1.34 mmol) and 2,6-difluoro-3-hydroxybenzamide (154 mg, 0.89 mmol) were added to a solution of **60** (200 mg, 0.89 mmol) in THF (3 mL). A solution of DIAD (0.263 mL, 1.34 mmol) in THF (5 mL) was added dropwise at 0 °C. After stirring overnight at rt, the mixture was concentrated and water (30 mL) and ethyl acetate (30 mL) were added to the residue. The aqueous layer was separated and extracted with ethyl acetate (3 × 20 mL). The combined organic phases were dried over Na_2SO_4 and concentrated. The residue was purified by chromatography on silica gel; elution with 1/1 cyclohexane/ethyl acetate and successive crystallization from methanol gave 90 mg (26.6%) of **21** as a white solid: mp 154.7 °C; ^{1}H NMR (DMSO-d) δ 8.09 (br s, 1H), 7.81 (br s, 1H), 7.46 (dd, 1 H, J = 8.4, 2.4 Hz), 7.40 (d, 1 H, J = 2.4 Hz), 7.28 (dt, 1 H, J = 9.1, 5.4 Hz), 7.05 (dt, 1H, J = 9.1, 1.9 Hz), 7.00 (d, 1 H, J = 8.4 Hz), 4.66–4.60 (m, 1H), 4.50 (dd, 1 H, J = 11.6, 2.3 Hz), 4.38–4.27 (m, 2H), 4.21 (dd, 1 H, J = 11.9, 7.0 Hz), 3.8 (s, 3H). ^{13}C NMR (DMSO-d) δ 166.0, 161.6, 152.6 (dd, J = 240.5, 6.9 Hz), 148.4 (dd, J = 247.3, 8.0 Hz), 147.6, 143.1 (dd, J = 10.8, 2.9 Hz), 142.8, 123.5, 118.56, 117.71, 117.69, 117.1 (dd, J = 24.0, 20.6 Hz), 116.5 (dd, J = 9.1, 2.3 Hz), 111.5 (dd, J = 22.4, 4.1 Hz), 71.8, 66.8, 65.2, 52.4. Anal. Calcd for $C_{18}H_{15}F_2NO_6$ (379.31).

7-Aminocarbonyl-2-benzyloxymethyl-1,4-benzodioxane (61). The carboxylic acid **58** (1.26 g, 4.19 mmol) was added to $SOCl_2$ (12 mL). The mixture was stirred at room temperature for 15 min and then at 50 °C for 2 h. After concentrating under vacuum, the residue was dissolved into DCM (15 mL) and the resulting solution was cooled to 4 °C and added with 30% ammonia (7 mL). After stirring at room temperature overnight, water (15 mL) and DCM (15 mL) were added. The organic layer was separated, washed with 10% HCl (15 mL), saturated aqueous $NaHCO_3$ (15 mL), and brine (15 mL), dried over Na_2SO_4 and concentrated. The residue was purified by flash chromatography on silica gel. Elution with 1/1 cyclohexane/ethyl acetate gave 650 mg (52.0%) of **61** as a white wax: ^{1}H NMR ($CDCl_3$) δ 7.33 (m, 7 H), 6.91 (d, 1 H, J = 8.5 Hz), 5.75 (br s, 2 H), 4.60 (s, 2 H), 4.35 (m, 2 H), 4.13 (dd, 1 H, J = 11.8, 7.7 Hz), 3.71 (m, 2 H).

7-Aminocarbonyl-2-hydroxymethyl-1,4-benzodioxane (62). A solution of **61** (650 mg, 2.17 mmol) in acetone (10 mL) was added with 5% Pd/C (70 mg) and vigorously shaken under hydrogen at room temperature for 8 h. The catalyst was removed by filtration and the filtrate concentrated to give 430 mg (93.5%) of **62** as a white wax: ^{1}H NMR (DMSO-d_6) δ 7.78 (br s, 1 H), 7.37 (m, 2 H), 7.18 (br s, 1 H), 6.87 (d, 1 H, J = 8.2 Hz), 5.05 (t, 1 H, J = 5.8 Hz), 4.35 (dd, 1 H, J = 11.3, 2.2 Hz), 4.16 (m, 1 H), 4.03 (dd, 1 H, J = 11.3, 7.2 Hz), 3.61 (m, 2 H).

7-Aminocarbonyl-2-tosyloxymethyl-1,4-benzodioxane (63). Tosyl chloride (501 mg, 2.63 mmol) was added to a solution of **62** (500 mg, 2.39 mmol) in pyridine (2 mL). After stirring at 0 °C for 2 h, 10% HCl (30 mL) and AcOEt (30 mL) were added. The aqueous layer was separated and extracted with ethyl acetate (20 mL). The combined organic phases were washed with 10% $NaHCO_3$ and brine, then dried over Na_2SO_4 and concentrated. The residue was crystallized from 1/1 2-propanol/butanone to give 230 mg (26.5%) of **63** as a white solid: mp 180.2 °C; ^{1}H NMR ($CDCl_3$) δ 7.78 (m, 1 H), 7.36 (m, 5 H), 6.87 (d, 1 H, J = 8.5 Hz), 5.57 (br s, 2 H), 4.46 (m, 1 H), 4.33 (m, 3 H), 4.19 (m, 1 H), 2.49 (s, 3 H).

2,6-Difluoro-3-(7-aminocarbonyl-1,4-benzodioxan-2-yl) methoxybenzamide (22). Potassium carbonate (105 mg, 0.76 mmol) was added to a solution of 2,6-difluoro-3-hydroxybenzamide (120 mg, 0.70 mmol) in DMF (3 mL). After stirring at room temperature for 15 min, a solution of **63** (230 mg, 0.63 mmol) in DMF (7 mL) was added. The reaction mixture was stirred at 75 °C for 3 h, then concentrated under vacuum, and diluted with water (20 mL) and ethyl acetate (20 mL). The aqueous phase was separated and extracted with ethyl acetate (2 × 20 mL). The combined organic phases were washed with 1 M NaOH (20 mL), and brine (2 × 20 mL), dried over Na_2SO_4, and concentrated to give **22** as a white solid: mp 218.8 °C; ^{1}H NMR (DMSO-d_6) δ 8.09 (br s, 1 H), 7.81 (br s, 1 H), 7.78 (br s, 1 H), 7.4 (d,1 H, J = 2.1 Hz), 7.39 (dd, 1 H, J = 8.3, 2.2 Hz), 7.27 (dt, 1 H, J = 9.0, 5.2 Hz), 7.17 (br s, 1 H), 7.05 (dt, 1 H, J = 9.0, 1.7 Hz), 6.90 (d, 1 H, J = 8.4 Hz), 4.61 (m, 1 H), 4.46 (dd, 1 H, J = 11.8, 2.5 Hz), 4.33 (m, 2 H), 4.17 (dd, 1 H, J = 11.5, 7.2 Hz). ^{13}C NMR (DMSO-d) δ 167.4, 161.7, 152.6 (dd, J = 240.5, 6.9 Hz), 148.4 (dd, J = 247.4, 8.0 Hz), 145.8, 143.2 (dd, J = 10.3, 3.4 Hz), 142.4, 128.2, 121.7, 117.1, 117.1 (dd, J = 25.3, 20.4 Hz), 117.0, 116.4 (dd, J = 10.3, 2.3 Hz), 111.5 (dd, J = 22.9, 3.5 Hz), 71.8, 68.7, 65.1. Anal. Calcd for $C_{17}H_{14}F_2N_2O_5$ (364.30).

7-Cyano-2-hydroxymethyl-1,4-benzodioxane (67). Trifluoroacetic anhydride (1.6 mL, 11.5 mmol) was added dropwise to a solution of **62** (550 mg, 2.63 mmol) in in 4/1 dioxane/pyridine (10 mL) at 4 °C. The mixture was stirred at room temperature for 1 h, and then diluted with water (20 mL) and ethyl acetate (20 mL). The organic layer was separated, washed with 10% HCl (20 mL), saturated aqueous $NaHCO_3$ (20 mL), and brine (20 mL), dried over Na_2SO_4 and concentrated. The residue was purified by flash chromatography on silica gel. Elution with 70/30 cyclohexane/ethyl acetate gave 350 mg (46.4%) of **67** as a white wax: ^{1}H NMR ($CDCl_3$) δ 7.17 (m, 2 H), 6.93 (d, 1 H, J = 8.2 Hz), 4.38 (dd, 1 H, J = 11.0, 1.9 Hz), 4.19 (m, 2 H), 3.90 (dq, 2 H, J = 12.1, 4.1 Hz), 1.59 (br s, 1 H).

7-Cyano-2-mesyloxymethyl-1,4-benzodioxane (68). Mesyl chloride (0.17 mL, 2.20 mmol) was slowly added to a solution of **67** (350 mg, 1.83 mmol) and TEA (0.34 mL, 2.38 mmol) in DCM (6 mL) at 4 °C. The mixture was stirred for 15 min at 4 °C and for 30 min at room temperature. After cooling to 4 °C, water (10 mL) and DCM (10 mL) were added. The organic phase was separated, washed with 10% HCl (10 mL), saturated aqueous $NaHCO_3$ (10 mL), and brine

(10 mL), dried over Na_2SO_4, and concentrated to give 480 mg (97.4%) of **68** as a colourless oil: ^{1}H NMR ($CDCl_3$) δ 7.22 (m, 2 H), 6.96 (d, 1 H, J = 8.5 Hz), 4.44 (m, 4 H), 4.17 (dd, 1 H, J = 11.3, 6.6 Hz), 3.14 (s, 3 H).

3-(7-Cyano-1,4-benzodioxan-2-yl)methoxy-2,6-difluoro-benzamide (20). Potassium carbonate (296 mg, 2.14 mmol) was added to a solution of 2,6-difluoro-3-hydroxybenzamide (370 mg, 2.14 mmol) in DMF (5 mL). After stirring at room temperature for 15 min, a solution of **68** (480 mg, 1.78 mmol) in DMF (5 mL) was added. The reaction mixture was stirred at 75 °C for 6 h, concentrated under vacuum, and diluted with water (20 mL) and ethyl acetate (20 mL). The aqueous phase was separated and extracted with ethyl acetate (2 × 20 mL). The combined organic phases were washed with 1 M NaOH (15 mL), and brine (15 mL), dried over Na_2SO_4 and concentrated. Crystallization of the solid residue from 1/1 toluene/ethyl acetate gave 175 mg (30.8%) of **20** as a white solid: mp 158.2 °C; ^{1}H NMR ($CDCl_3$) δ 8.10 (br s, 1 H), 7.82 (br s, 1 H), 7.40 (d, 1 H, J = 1.9 Hz), 7.32 (dd, 1 H, J = 8.4, 2.1 Hz), 7.26 (dt, 1 H, J = 9.3, 5.1 Hz), 7.07 (dt, 1 H, J = 9.3, 2.0 Hz), 7.1 (d, 1H, J = 8.4 Hz), 4.67 (m, 1 H), 4.52 (dd, 1 H, J = 11.6, 2.5 Hz), 4.27 (m, 3 H). ^{13}C NMR (DMSO-*d*) δ 161.9, 152.9 (dd, J = 241.1, 7.0 Hz), 148.6 (dd, J = 247.9, 8.3 Hz), 147.9, 143.7, 143.3 (d, J = 11.1 Hz), 126.8, 121.7, 119.4, 119.0, 117.4 (dd, J = 24.7, 20.2 Hz), 116.6 (d, J = 9.1 Hz), 111.7 (d, J = 22.8 Hz), 104.4, 72.2, 68.8, 65.5. Anal. Calcd for $C_{17}H_{12}F_2N_2O_4$ (346.29).

2-Benzyloxymethyl-7-*N*-butylaminocarbonyl-1,4-benzodioxane (64). The carboxylic acid **58** (1.00 g, 3.32 mmol) was added to $SOCl_2$ (10 mL). The mixture was stirred at room temperature for 15 min and then at 50 °C for 2 h. After concentrating under vacuum, the residue was dissolved into DCM (15 mL) and the resulting solution was cooled to 4 °C and added with a solution of *n*-butylamine (2.0 mL, 20.23 mmol) in DCM (10 mL). After stirring at room temperature overnight, water (15 mL) and DCM (15 mL) were added. The organic layer was separated, washed with 10% HCl (15 mL), saturated aqueous $NaHCO_3$ (15 mL), and brine (15 mL), dried over Na_2SO_4 and concentrated. The oily residue was purified by flash chromatography on silica gel. Elution with 60/40 cyclohexane/ethyl acetate gave 670 mg (56.8%) of **64** as a white wax: ^{1}H NMR ($CDCl_3$) δ 7.31 (m, 7 H), 6.88 (d, 1 H, J = 8.5 Hz), 5.96 (br s, 1 H), 4.60 (s, 2 H), 4.35 (m, 2 H), 4.12 (dd, 1 H, J = 11.8, 7.7 Hz), 3.70 (m, 2 H), 3.42 (t, 2 H, J = 6.9 Hz), 1.57 (m, 2 H), 1.41 (m, 2 H), 0.95 (t, 3 H, J = 7.2 Hz).

7-*N*-Butylaminocarbonyl-2-hydroxymethyl-1,4-benzodioxane (65). A solution of **64** (670 mg, 1.88 mmol) in methanol (10 mL) was added with 10% Pd/C (70 mg) and vigorously shaken under hydrogen at room temperature for 24 h. The catalyst was removed by filtration and the filtrate concentrated to give 460 mg (92.2%) of **65** as a white wax: ^{1}H NMR ($CDCl_3$) δ 7.34 (d, 1 H, J = 2.2 Hz), 7.24 (dd, 1 H, J = 8.5, 2.2 Hz), 6.89 (d, 1 H, J = 8.5 Hz), 6.06 (br s, 1 H), 4.34 (dd, 1 H, J = 11.3, 2.2 Hz), 4.25 (m, 1 H), 4.13 (dd, 1 H, J = 11.3, 7.4 Hz), 3.70 (m, 2 H), 3.41 (q, 2 H, J = 6.9 Hz), 1.89 (br s, 1 H), 1.56 (m, 2 H), 1.40 (m, 2 H), 0.95 (t, 3 H, J = 7.2 Hz).

7-*N*-Butylaminocarbonyl-2-mesyloxymethyl-1,4-benzodioxane (66). Mesyl chloride (0.16 mL, 2.08 mmol) was slowly added to a solution of **65** (460 mg, 1.73 mmol) and TEA (0.32 mL, 2.25 mmol) in DCM (10 mL) at 4 °C. The mixture was stirred for 15 min at 4 °C and for 30 min at room temperature. After cooling to 4 °C, water (10 mL) and DCM (10 mL) were added. The organic phase was separated, washed with 10% HCl (10 mL), saturated aqueous $NaHCO_3$ (10 mL), and brine (10 mL), dried over Na_2SO_4, and concentrated. The oily residue (680 mg) was purified by flash chromatography on silica gel. Elution with 1/1 cyclohexane/ethyl acetate gave 460 mg (77.4%) of **66** as a colourless wax: ^{1}H NMR ($CDCl_3$) δ 7.38 (d, 1 H, J = 2.2 Hz), 7.29 (dd, 1 H, J = 8.5, 2.2 Hz), 6.91 (d, 1 H, J = 8.5 Hz), 6.0 (br s, 1 H), 4.49 (m, 3 H), 4.35 (dd, 1 H, J = 11.3, 2.3 Hz), 4.14 (dd, 1 H, J = 11.3, 7.4 Hz), 3.45 (q, 2 H), J = 6.8 Hz), 3.09 (s, 3 H), 1.51 (m, 2 H), 1.40 (m, 2 H), 0.95 (t, 3 H, J = 7.1 Hz).

3-(7-*N*-Butylaminocarbonyl-1,4-benzodioxan-2-yl)methoxy-2,6-difluoro-benzamide (23). Potassium carbonate (221 mg, 1.60 mmol) was added to a solution of 2,6-difluoro-3-hydroxybenzamide (254 mg, 1.47 mmol) in DMF (3 mL). After stirring at room temperature for 15 min, a solution of **66** (460 mg, 1.34 mmol) in DMF (7 mL) was added. The reaction mixture was stirred at 75 °C for 6 h, concentrated under vacuum, and diluted with water (20 mL) and ethyl acetate (20 mL). The aqueous phase was separated and extracted with ethyl acetate (2 × 20 mL). The combined organic phases were concentrated and the resulting grey solid was crystallized from ethyl acetate to give 350 mg (63.6%) of **23** as a white solid: mp 173.5 °C; ^{1}H NMR (DMSO-d_6) δ 8.24 (t, 1 H, J = 5.5 Hz), 8.11 (br s, 1 H), 7.84 (br s, 1 H), 7.33 (m, 3 H), 7.07 (dt, 1 H, J = 8.8, 1.9 Hz), 6.94 (d, 1 H, J = 8.5 Hz), 4.61 (m, 1 H), 4.46 (dd, 1 H, 11.8, 2.5 Hz), 4.31 (m, 2 H), 4.17 (dd, 1 H, J = 11.5, 7.2 Hz), 3.20 (m, 2H), 1.51 (m, 2 H), 1.35 (m, 2 H), 0.87 (t, 3H, J = 7.2 Hz). ^{13}C NMR (DMSO-*d*) δ 165.5, 161.7, 152.6 (dd, J = 240.5, 6.9 Hz), 148.4 (dd, J = 247.3, 9.2 Hz), 145.6, 143.2 (dd, J = 11.4, 3.4 Hz), 142.4, 128.6, 121.3, 117.1 (dd, J = 25.8, 20.0 Hz), 117.1, 116.6, 116.4 (dd, J = 9.2, 2.3 Hz), 111.5 (dd, J = 22.9, 3.5 Hz), 71.8, 68.7, 65.1, 39.3, 31.7, 20.1, 14.1. Anal. Calcd for $C_{21}H_{22}F_2N_2O_5$ (420.41).

4.2. Cells

Normal human lung fibroblasts (MRC-5) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated calf serum, 100 U/ml penicillin and 100 mg/ml streptomycin in an incubator at 5% CO_2 atmosphere and 37 °C. The Gram-positive *S. aureus* (*S. aureus*) and the Gram-negative *E. coli* (*E. coli*) bacterial cells were grown in Luria–Bertani Broth (LB) medium at 37 °C under constant shaking at 300 rpm.

4.3. Antibacterial activity

The antibacterial activity of compounds **5**–**23** and DFNB was tested by using a methicillin-resistant *S. aureus* strain (MRSA, ATCC 29213), and an extended-spectrum beta-lactamase-positive (ESBL) *E. coli* clinical isolate. All of the compounds were dissolved at the final concentration of 20 mg/ml in dimethyl sulphoxide (DMSO) and serially diluted in LB. After incubation at 37 °C for 16 h in aerobic culture tubes, cell concentration was determined by optical density measurement at 600 nm (OD_{600}) in a SmartSpec™ 3000 spectrophotometer (Bio-Rad, Oceanside, CA, USA). Fresh cell cultures were used at 10^3 cells/ml in a final volume of 3 ml. Each bacterial sample was grown with different compound concentrations, that ranged from 100 to 0.1 μg/ml for the first screening and with intermediate concentrations thereafter for **11**, **12**, **14**, **15** and **21**, that were active up to 1 μg/ml but not at 0.1 μg/ml. After overnight incubation at 37 °C, an aliquot of each sample was harvested under sterile conditions and the OD_{600} was measured to determine the minimal inhibitory concentration (MIC), i.e. the lowest compound dose at which bacterial growth is inhibited. To also determine the minimal bactericidal concentration (MBC), i.e. the minimal dose at which cell growth is inhibited after removing the compound, the bacteria were then washed three times with LB, centrifuged at 900 × *g* for 10 min at 4 °C, and the pellet resuspended in fresh LB. After overnight incubation at 37 °C, the absence of growth was confirmed by OD measurement.

The antimicrobial activity of compounds **3**, (*S*)-**4**, **10** and **12** was determined against a panel of 30 strains of clinical isolates including 10 methicillin-sensitive *S. aureus* (MSSA) and 10 methicillin-resistant *S. aureus* (MRSA), 10 vancomycin-sensitive *E. faecalis* (VSE) and 10 vancomycin-resistant *E. faecalis* (VRE). The

minimal inhibitory concentration (MIC) for liquid growth inhibition assay was determined by standard procedures (CLSI) using serial dilutions of the compounds in a 96 well flat-bottom Microtiter® plate. The compounds were dissolved in appropriate buffer and then diluted in LB medium to reach a final concentration of 200 μg/mL. Logarithmic phase bacterial cultures were suspended in saline solution to a final concentration of 1-2 × 10^5 CFU/mL. The assay mixture contained 100 μL diluted compounds suspension in LB medium and 100 μL serial diluted bacterial suspension. The final concentration of the compounds ranged from 0.097 to 100 μg/mL. After 24 h of incubation at 37 °C, the MIC was defined as the lowest concentration of the compound that totally inhibited the growth. To determine the minimum bactericidal concentration (MBC), an aliquot (100 μL) of the wells with no visible microbial growth was plated onto Trypticase Soy agar 5% sheep blood plate (bioMérieux) and incubated at 37 °C for 18 h. MBC was defined as the lowest concentration of the compound at which more than 99.9% of the cells were killed compared with a non-treated control. All tests were performed in triplicate and for each series of experiments, both positive (no compounds) and negative (no bacteria) controls were included.

Activity against *M. smegmatis* and *M. tuberculosis* was carried out in 7H9 liquid media supplemented with ADC (Difco) and Tween 80 (0.05%) and MICs were determined by the microplate Alamar Blue assay [14]. *M. smegmatis* and *M. tuberculosis* expressing the green fluorescent protein (GFP) [15] were grown at subMIC concentration on glass coverslips, fixed with a phosphate buffered 4% paraformaldehyde solution and then analysed at the fluorescence microscope (100× magnification).

4.4. Optical microscopy

Cells were grown over night in the presence of increasing concentrations of the compounds showing an evident inhibitory activity at concentrations lower than 1 μg/ml, starting from the previously determined MIC. Data are reported for *S. aureus* cultured in the presence of (*S*)-**4** (0.5 μg/ml), **14** (0.5 μg/ml) and **21** (1.0 μg/ml). DFNB (0.25 μg/ml) was used as a reference effective (positive) control whereas untreated *S. aureus* cultures were used as negative control. Samples were analysed by phase contrast under a Zeiss Axioskop microscope.

4.5. Transmission electron microscopy

S. aureus (10^9 cells/ml) were cultured in the presence of (*S*)-**4** and **21** at the same concentrations used for optical microscopy and, after 16-h incubation at 37 °C, the cells were harvested and processed for transmission electron microscopy, as already described [16], with minor modifications. Untreated *S. aureus* and *S. aureus* treated with DFNB were used as negative and positive controls, respectively. Briefly, after centrifugation at 3100 × *g* for 5 min at room temperature, pelletted bacteria were fixed in 2.5% glutaraldehyde (Polysciences, Warrington, PA) in 0.1 M Na cacodylate buffer, pH 7.4, for 1 h at 4 °C, rinsed twice, and post-fixed in Na cacodylate-buffered 1% OsO_4, for 1 h at 4 °C. The samples were dehydrated through a series of graded ethanol solutions and propylene oxide, and embedded in Poly/Bed 812 resin mixture. Ultrathin sections were obtained using a Reichert-Jung ultramicrotome equipped with a diamond knife. Samples were then stained with water-saturated uranyl acetate and 0.4% lead citrate in 0.1 M NaOH. The specimens were viewed under a Philips CM10 electron microscope.

4.6. Thiazolyl blue tetrazolium bromide cytotoxicity assay

Compounds **11**, **12**, **14**, **15** and **21**, showing antibacterial activity at a concentration lower than 1 μg/ml, were serially diluted in DMEM and tested on MRC-5 cells by the thiazolyl blue tetrazolium bromide (MTT) cytotoxicity assay (Sigma, St Louis, MO, USA). Cells (10^4 cell/well) were tested in a 96-well plate using serially two-fold-diluted concentrations of the compound in 100 μl DMEM medium. After a 24-h incubation, the compound was removed and the cells were overlaid with 1 mg/ml MTT in 100 μl serum-free DMEM for 3 h at 37 °C. The MTT solution was then replaced with DMSO for 10 min, and the absorbance was measured at 570 nm. The percentage of cytotoxicity was calculated by the formula 100 − (sample OD/untreated cells OD) x 100. The compound concentration reducing cell viability by 50 or 90% was defined as the TD_{50} or TD_{90} toxic dose. The therapeutic index (TI) was also determined and defined as the ratio between TD_{90} and the minimal bactericidal concentration (MBC) values.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2016.03.068.

References

[1] H.P. Erickson, D.E. Anderson, M. Osawa, FtsZ in bacterial cytokinesis: cytoskeleton and force generator all in one, Microbiol. Mol. Biol. Rev. 74 (2010) 504–528.
[2] L.G. Czaplewski, I. Collins, E.A. Boyd, D. Brown, S.P. East, M. Gardiner, R. Fletcher, D.J. Haydon, V. Henstock, P. Ingram, C. Jones, C. Noula, L. Kennison, C. Rockley, V. Rose, H.B. Thomaides-Brears, R. Ure, M. Whittaker, N.R. Stokes, Antibacterial alkoxybenzamide inhibitors of the essential bacterial cell division protein FtsZ, Bioorg. Med. Chem. Lett. 19 (2009) 524–527.
[3] D.J. Haydon, N.R. Stokes, R. Ure, G. Galbraith, J.M. Bennett, D.R. Brown, P.J. Baker, V.V. Barynin, D.W. Rice, S.E. Sedelnikova, J.R. Heal, J.M. Sheridan, S.T. Aiwale, P.K. Chauhan, A. Srivastava, A. Taneja, I. Collins, J. Errington, L.G. Czaplewski, An inhibitor of FtsZ with potent and selective anti-staphylococcal activity, Science 321 (2008) 1673–1675.
[4] D.J. Haydon, J.M. Bennett, D. Brown, I. Collins, G. Galbraith, P. Lancett, R. Macdonald, N.R. Stokes, P.K. Chauhan, J.K. Sutariya, N. Nayal, A. Srivastava, J. Beanland, R. Hall, V. Henstock, C. Noula, C. Rockley, L. Czaplewski, Creating an antibacterial with in vivo efficacy: synthesis and characterization of potent inhibitors of the bacterial cell division protein FtsZ with improved pharmaceutical properties, J. Med. Chem. 53 (2010) 3927–3936.
[5] N.R. Stokes, N. Baker, J.M. Bennett, J. Berry, I. Collins, L.G. Czaplewski, A. Logan, R. Macdonald, R. MacLeod, H. Peasley, J.P. Mitchell, N. Nayal, A. Yadav, A. Srivastava, D.J. Haydon, An improved small-molecule inhibitor of FtsZ with superior in vitro potency, drug-like properties, and in vivo efficacy, Antimicrob. Agents Chemother. 57 (2013) 317–325.
[6] N.R. Stokes, N. Baker, J.M. Bennett, P.K. Chauhan, I. Collins, D.T. Davies, M. Gavade, D. Kumar, P. Lancett, R. Macdonald, L. MacLeod, A. Manhajan, J.P. Mitchell, N. Nayal, Y.N. Nayal, G.R.W. Pitt, M. Singh, A. Yadav, A. Srivastava, L.G. Czaplewski, D.J. Haydon, Design, synthesis and structure-activity relationships of substituted oxazole-benzamide antibacterial inhibitors of FtsZ, Bioorg. Med. Chem. Lett. 24 (2014) 353–359.
[7] G. Chiodini, M. Pallavicini, C. Zanotto, M. Bissa, A. Radaelli, V. Straniero, C. Bolchi, L. Fumagalli, P. Ruggeri, C. De Giuli Morghen, E. Valoti, Benzodioxane-benzamides as new bacterial cell division inhibitors, Eur. J. Med. Chem. 89 (2015) 252–265.
[8] L. Costantino, F. Gandolfi, C. Sorbi, S. Franchini, O. Prezzavento, F. Vittorio, G. Ronsisvalle, A. Leonardi, E. Poggesi, L. Brasili, Synthesis and structure-activity relationships of 1-aralkyl-4-benzylpiperidine and 1-aralkyl-4-benzylpiperazine derivatives as potent σ ligands, J. Med. Chem. 48 (2005) 266–273.
[9] N.A. Sorto, M.M. Olmstead, J.T. Shaw, Practical synthesis of PC190723, an inhibitor of the bacterial cell division protein FtsZ, J. Org. Chem. 75 (2010) 7946–7949.
[10] J.J. Marugan, W. Zheng, O. Motabar, N. Southall, E. Goldin, W. Westbroek, B.K. Stubblefield, E. Sidransky, R.A. Aungst, W.A. Lea, A. Simeonov, W. Leister, C.P. Austin, Evaluation of quinazoline analogues as glucocerebrosidase inhibitors with chaperone activity, J. Med. Chem. 54 (2011) 1033–1058.
[11] P.H. Carlsen, K.J. Aase, Nucleophilic addition to cyclic 1,2-sulfites, Acta Chem. Scand. 47 (1993) 617–619.
[12] R. Henning, R. Lattrell, H.J. Gerhards, M. Leven, Synthesis and neuroleptic activity of a series of 1-[1-(benzo-1,4-dioxan-2-ylmethyl)-4-piperidinyl]benzimidazolone derivatives, J. Med. Chem. 30 (1987) 814–819.

[13] Approved Standard-Ninth Edition CLSI document M07-A9. vol. 32 No. 2 replaces M07–A8 vol. 29 No. 2, CLSI Susceptibility Tests for Bacteria that Grow Aerobically, Clinical and Laboratory Standards Institute, Wayne, PA, 2012.
[14] L. Collins, S.G. Franzblau, Microplate alamar blue assay versus BACTEC 460 system for high-throughput screening of compounds against *Mycobacterium tuberculosis* and *Mycobacterium avium*, Antimicrob. Agents Chemother. 41 (1997) 1004–1009.
[15] G. Delogu, C. Pusceddu, A. Bua, G. Fadda, M.J. Brennan, S. Zanetti, Rv1818c-encoded PE_PGRS protein of *Mycobacterium tuberculosis* is surface exposed and influences bacterial cell structure, Mol. Microbiol. 52 (2004) 725–733.
[16] M. Bissa, C. Zanotto, S. Pacchioni, L. Volonté, A. Venuti, D. Lembo, C. De Giuli Morghen, A. Radaelli, The L1 protein of human papilloma virus 16 expressed by a fowlpox virus recombinant can assemble into virus-like particles in mammalian cell lines but elicits a non-neutralising humoral response, Antivir. Res. 116 (2015) 67–75.