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Substitution of the phosphonic acid and hydroxamic acid functionalities of the DXR inhibitor FR900098: An attempt to improve the activity against *Mycobacterium tuberculosis*

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ABSTRACT

Two series of FR900098/fosmidomycin analogs were synthesized and evaluated for *Mt*DXR inhibition and *Mycobacterium tuberculosis* whole-cell activity. The design rationale of these compounds involved the exchange of either the phosphonic acid or the hydroxamic acid part for alternative acidic and metal-coordinating functionalities. The best inhibitors provided IC_{50} values in the micromolar range, with a best value of 41 μM.

Tuberculosis (TB) is a great public health problem causing death, illness and poverty. The World Health Organization has estimated that a third of the world's population today is infected with the pathogenic bacteria *Mycobacterium tuberculosis* (*Mt*), and that there were around 9.4 million new active TB cases and 1.3 million TB deaths among HIV-negative people in 2009.[1] The disease has a huge impact not only on the patients and their families but also on entire countries.

Two major factors have during the last decades increased the complexity of the TB problem; the susceptibility of people infected with the acquired immune deficiency syndrome (AIDS), which augments the risk of developing TB 100-fold and the increase in resistant strains of *Mt*, leading to multidrug-resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB).[1] The alarming development of resistant strains of *Mt* is to a large extent caused by poor compliance due to the lengthy, disagreeable treatment (a minimum of 4 months) and associated unpleasant side-effects, in combination with underfunded and underdeveloped healthcare. When standard pharmacotherapies fail, second-line TB drugs are used, but these drugs have a far lower efficacy and require even longer treatment periods (18–24 months) associated with higher cost, higher rates of adverse effects, and low cure rates.[1] Since the WHO's declaration of TB as a public health emergency in 1993, serious efforts have been implemented to combat the TB epidemic. After peaking at 143 cases per 100,000 people in 2004, the TB incidence level has slowly decreased.[1] A new and more effective TB drug could greatly help these efforts provided that it could bring down costs and make TB-treatment more available. The need for new TB drugs is perhaps greatest in the battle against MDR-TB and XDR-TB for which the pharmacotherapy is expensive or non-existent. New TB drugs, for which MDR-TB strains are not resistant, can be discovered by inhibiting essential *Mt*-enzymes that are not targeted by any of the currently used TB-drugs. One such potential target is the metal-containing enzyme 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR).

DXR is involved in the nonmevalonate (DOXP/MEP) pathway of isoprenoid biosynthesis, which leads to isopentenyl diphosphate, an important isoprenoid precursor.[2] Since the DOXP/MEP pathway is absent in humans it is believed to be an excellent drug discovery target for the development of both anti-malaria and anti-TB drugs. Fosmidomycin, and the close analog FR900098, resemble DXR's natural substrate 1-deoxy-D-xylulose 5-phosphate (DXP), and are known *Mt*DXR inhibitors (Fig. 1). In addition, the structure of *Mt*DXR in complex with fosmidomycin is available.[3] However, neither fosmidomycin nor FR900098 affect the growth or viability of

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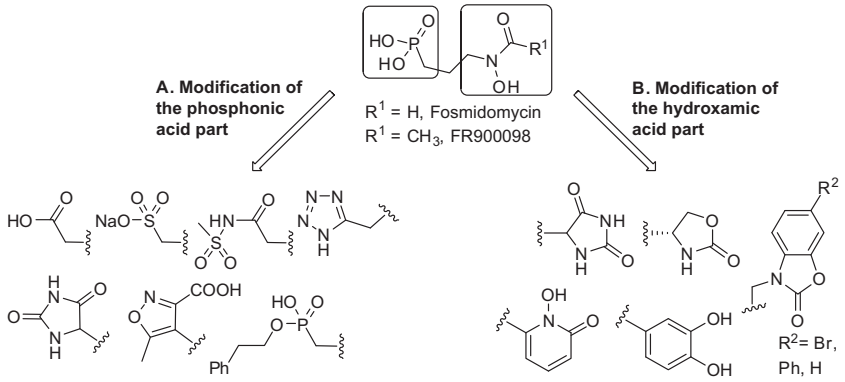
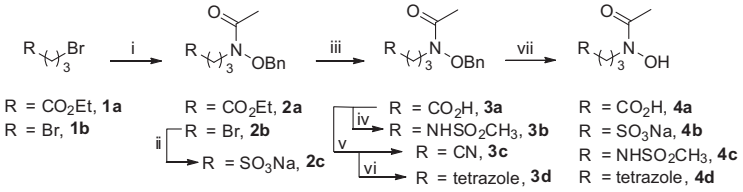
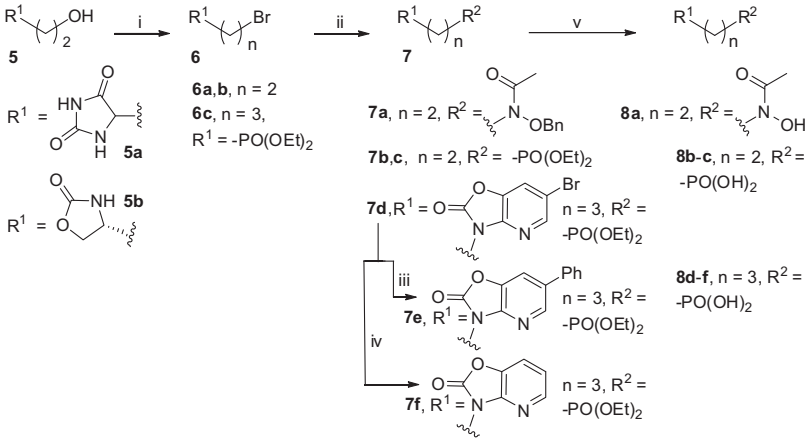


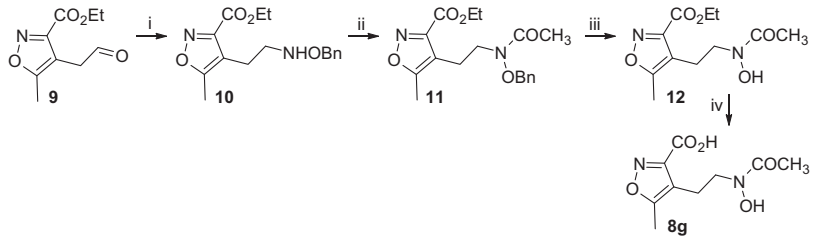
Figure 1. Design rationale of novel FR900098/fosmidomycin analogs.



Scheme 1. Synthetic route for the compounds **4a**–**4d**. Reagents and conditions: (i) $BnONHCOCH_3$, NaH, DMF, 50 °C; (ii) Na_2SO_3, H_2O, 120 °C; (iii) NaOH (1 M), MeOH, 60 °C; (iv) CDI, DBU, methanesulfonamide, 60 °C; (v) (a) CDI, NH_3(g), THF, rt; (b) TEA, TFAA, DCM, rt; (vi). NaN_3, NH_4Cl, DMF, MW 140 °C, 2 h; (vii) H_2, 10% Pd/C, rt.

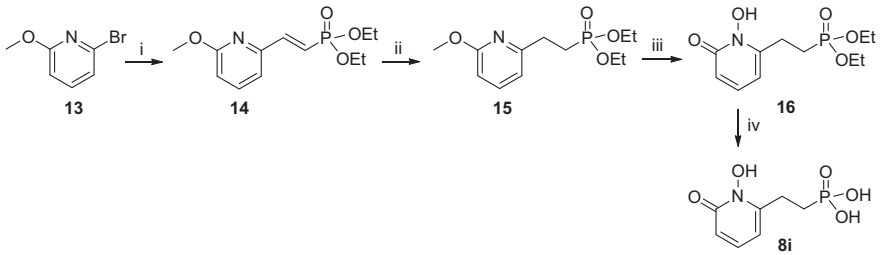


Scheme 2. Synthesis route for the compounds **8a**–**8f**. Reagents and conditions: (i) **6a**: HBr 47%, Δ; **6b**; (a) TEA, MsCl, DCM, rt; (b) LiBr, THF, rt; (ii) **7a**: $BnONHCOCH_3$, NaH, DMF, 50 °C; **7b** and **7c**: TEP, Δ; **7d**: 6-bromo-oxazolo[4,5-b]pyridin-2(3H)-one, NaOEt, DMF, rt; (iii) phenylboronic acid, $Pd(PPh_3)_4$, TEA, DMF, MW, 120 °C; (iv) H_2, 10% Pd/C, MeOH, rt; (v) **8a**: H_2, 10% Pd/C, MeOH, rt; **8b**–**8f**: TMSBr, DCM, rt.

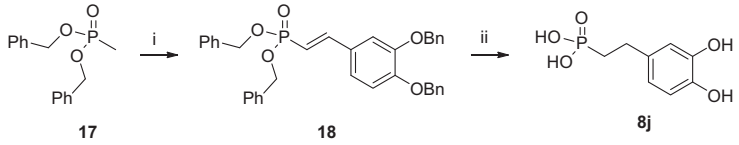


Scheme 3. Synthesis route for inhibitor **8g**. Reagents and conditions: (i) (a) *O*-Benzylhydroxylamine hydrochloride, pyridine, EtOH, rt; (b) $NaCNBH_3$, MeOH, HCl (12 M), rt; (ii) TEA, AcCl, DCM, rt; (iii) H_2, 10% Pd/C, MeOH; (iv) NaOH (10 M), MeOH, rt.

the whole-cell *Mt* bacteria because of the lack of uptake through the highly complex mycobacterial cell wall. This is probably due to the inherent polar and charged nature of this class of phosphonic acid/hydroxamic acid inhibitors.[4]



Scheme 4. Synthesis route for the inhibitor **8i**. Reagents and conditions: (i) diethyl vinylphosphonate, $Pd(OAc)_2$, *t*-Bu_4NOAc, KCl, K_2CO_3, DMF, 90 °C; (ii) H_2, 10% Pd/C, MeOH, rt; (iii) (a) mCBPA, DCM, rt; (b) AcCl, MeOH, rt; (iv) TMSBr, DCM, rt.



Scheme 5. Synthesis route for the inhibitor **8j**. Reagents and conditions: (i) *n*-BuLi, THF, −78 °C, 1 h, 3,4-dibenzyloxybenzaldehyde, −78 °C-rt, 4 h, *t*-BuONa, −78 °C, 10 min; (ii) H_2, 10% Pd/C, MeOH/cat. AcOH, rt.

In a drug discovery program aiming to identify less polar *Mt*DXR inhibitors that can penetrate the *Mt* cell wall and yield whole-cell minimum inhibition concentration (MIC) activity, we decided to prepare and test FR900098 analogs carrying only one of the original phosphonic acid or the hydroxamic acid groups. Thus, the design rationale of these compounds involved the substitution of either the phosphonic acid (strategy **A**) or the hydroxamic acid (strategy **B**) of the FR900098 scaffold with alternative acidic or metal-coordinating functionalities (Fig. 1). Several bioisosters of the phosphonate group and the hydroxamate group have been previously evaluated for activity on DXR.[5–7] In order to investigate both strategies we re-synthesized previously reported DXR inhibitors **4a**, **4b**, **8d**, **8e**, **8f**, **8h**, **8j** and developed new synthetic routes to obtain the novel structures **4c**, **4d**, **8a**, **8b**, **8c**, **8g**, **8i** for evaluation in the *Mt*DXR inhibition assay and to measure the in vitro anti-*Mt* whole-cell activity.

The synthetic route used for the preparation of the first set of compounds **4a–4d** is outlined in Scheme 1. Treatment of ethyl 4-bromoacetate (**1a**) with *N*-(benzyloxy)acetamide[8] in the presence of sodium hydride as base and DMF as solvent led to the formation of ethyl 4-(*N*-(benzyloxy)acetamido)butanoate (**2a**). The generation of *N*-(benzyloxy)-*N*-(3-bromopropyl)acetamide (**2b**) was performed in two steps according to the procedure previously reported in the literature.[9] The sodium 3-(*N*-(benzyloxy)acetamido)propane-1-sulfonate compound (**2c**) was obtained as the sodium salt by nucleophilic substitution of the corresponding bromide **2b** with sodium sulfite and basic work-up.[10] Next, hydrolysis of ester **2a** with 1 M sodium hydroxide yielded the expected carboxylic acid (**3a**). The latter was activated by 1,1′-carbonyldiimidazole (CDI) in THF at 60 °C, followed by reaction with methanesulfonamide to achieve the corresponding acyl sulfonamide (**3b**). Treatment of **3a** with gaseous ammonia and CDI at 0 °C furnished the primary amide, which was subsequently reacted with triethylamine (TEA) and trifluoroacetic acid (TFAA) to afford the corresponding nitrile compound **3c**. Tetrazole **3d** was synthesized according to the procedure previously reported in the literature, employing sodium azide and microwave heating.[11] The final inhibitors were obtained by catalytic hydrogenation of the intermediates **2c**, **3a**, **3b** and **3d**, providing a test set of phosphonic acid-free compounds **4a–4d**.

The next part of the project concerned the synthesis of inhibitor structures **8a–8f**, representing one compound (**8a**) in which the phosphonic acid has been replaced and five compounds in which the hydroxamic acid moiety has been replaced by alternative metal-binding functionalities (**8b–8f**) (Scheme 2). Reflux of **5a**[12] in 47% HBr gave direct formation of the alkyl bromide **6a**. It is noteworthy that the bromination of the related compound **5b**[13] required a two-step procedure: mesylation of the hydroxy functionality followed by substitution using LiBr. The attachment of the hydroxamic acid part to the alkyl chain of **6a**, affording **7a**, was achieved by the addition of sodium hydride and *N*-(benzyloxy)acetamide in DMF. The synthesis of the phosphonate compounds **7b** and **7c** was performed by refluxing **6a** and **6b** in excess triethyl phosphite (TEP). Furthermore, *N*-alkylation of 6-bromo-oxazolo[4,5-b]pyridin-2(3*H*)-one[14] with diethyl (3-bromopropyl)phosphonate (**6c**) yielded compound **7d**, which was transformed into compound **7e** by a microwave-assisted Suzuki reaction employing phenylboronic acid as the coupling partner.[15–17] Classical hydrogenation of compounds **7a** and **7d** led to

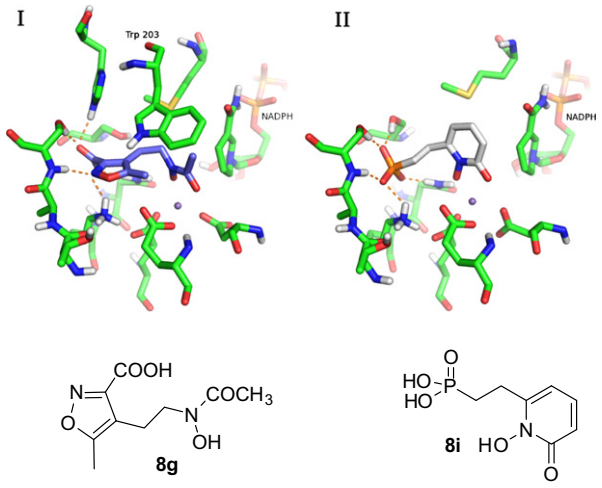


Figure 2. (I) Docking pose of compound **8g** (in blue) bound to *Mt*DXR (2JCZ). (II) Docking pose of compound **8i** (in grey) docked to *Mt*DXR (2Y1F). The absence of Trp203 gives greater room for ligand **8i**. In both images *Mt*DXR and NADPH are shown in green, the metal ion in purple, and hydrogen bond interactions in orange.

compounds **8a** and **7f**. The TMSBr-mediated hydrolysis of the phosphonate ester in **7b**–**7f** furnished the corresponding phosphonic acid products **8b**–**8f**.

Next series of FR900098 analogs prepared was **8g**–**8j**. Inhibitor **8h** was essentially synthesized according to the procedure reported by Perruchon et al.[5] In contrast, the novel compounds **8g** and **8i** required the development of the synthetic routes presented in Schemes 3 and 4. Regarding isoxazole carboxylic acid, **8g**, we decided to start the synthesis with aldehyde **9**.[18] Treatment of **9** with *O*-benzylhydroxylamine hydrochloride followed by reduction with sodium cyanoborohydride gave **10**. As expected, acetylation of *O*-benzylhydroxylamine **10** with acetyl chloride in the presence of triethylamine yielded acetamide **11**. Finally, catalytic hydrogenation of **11** followed by alkaline hydrolysis afforded test compound **8g**.

For *N*-hydroxy-pyridinone-containing **8i**, the synthetic procedure started with the Heck reaction of 2-bromo-6-methoxypyridine with diethyl vinylphosphonate under phase-transfer conditions (Scheme 4).[18,19] The arylated olefin, **14**, was hydrogenated with Pd/C in methanol to give the saturated product, **15**. Oxidation of the pyridine ring in **15** was conducted with *m*CBPA in DCM. Subsequent treatment of the intermediate with acetyl chloride followed by addition of methanol led to the formation of the pyridinone *N*-hydroxy ring system. Hydrolysis of compound **16** gave the desired inhibitor **8i**.

Finally, it was of interest to replace the hydroxamate functionality with a catechol group.[7] Thus, compound **8j** was prepared by the condensation[20] of dibenzyl methylphosphonate and 3,4-dibenzyloxybenzaldehyde followed by the hydrogenation using a Pd/C catalyst (Scheme 5).

Possible binding modes of the synthesized compounds were predicted using the docking software Glide.[21] Two different *Mt*DXR crystal structures were used; one with fosmidomycin (2JCZ)[3] and one with the recently reported *Mt*DXR inhibitor α-3,4-dichlorophenyl-fosmidomycin bound to the active site (2Y1F).[22,23] Both crystal structures were used in the docking study as the change in position of especially tryptophan 203[22] allowed differently sized inhibitors to fit into the enzyme.

All synthesized compounds retain either the phosphonic acid or hydroxamic acid part of the fosmidomycin molecule. This simplifies the evaluation as one can directly compare the docking poses with the binding mode of fosmidomycin. Docking poses of compounds **4a**, **4b**, **4d**, **8g**, **8c** and **8i** present convincing binding patterns, exemplified by the docked conformations of structures **8g** and **8i** in Figure 2. It is worth noting that the docking pose of **8g** shows almost perfect overlay with fosmidomycin at both the metal binding site and at the phosphonic acid binding site, with an almost fully retained hydrogen bond network.

All 14 compounds were screened for inhibitory activity against *Mt*DXR at 100 μM (Table 1). Next, the IC_{50} values were determined for inhibitors with more than 35% inhibition at 100 μM (**4b**, **8g**, **8i**, and **8j**).[24,25] Only one of the seven analogs with phosphonic acid replacements, the isoxazole carboxylic acid **8g**, showed *Mt*DXR inhibition (IC_{50} = 151 μM). Interestingly, it has been indicated (preliminary data) that monoester **8h** has some activity against TB.[10]

Synthesis and biological evaluation of the molecules with a modified hydroxamic part (**8b–8f**, **8i–8j**) led to two new active fosmidomycin analogs (**8i** and **8j**). Heterocyclic structure **8i** inhibited *Mt*DXR at 53 μM. Catechol-containing phosphonic acid **8j** has been reported to inhibit *Escherichia coli* DXR with an IC_{50} of 4.5 μM.[7] In the case of *Mt*DXR, **8j** inhibited it with an IC_{50} of 41 μM. Although active in the enzyme assay, **8g**, **8i** and **8j** did not provide MIC value below 512 μg/mL. Compound **8e** was previously shown to have inhibitory activity in a whole-cell assay of

Table 1
Evaluation of FR900098/fosmidomycin analogs

Modification of the phosphonic acid part			Modification of the hydroxamic acid part		
	%Inh at 100 μm	IC_{50} (μm)		%Inh at 100 μm	IC_{50} (μm)
Fosmidomycin, R = H **FR900098**, R = CH_3		0.08 ± 0.02 0.16 ± 0.03	**8b**	8 ± 6	
4a	0		**8c**	0	
4b	36 ± 14	>100	**8d**	7 ± 4	
4c	29 ± 12				
4d	0		**8e**	11 ± 6	
8a	13 ± 4		**8f**	11 ± 7	
8g	41 ± 1	151 ± 22	**8i**	61 ± 5	53 ± 13
8h	23 ± 7		**8j**	78 ± 3	41 ± 10

IC_{50} values[22] were determined for inhibitors with more than 35% inhibition at 100 μM. Only **8e** showed an MIC value below 512 μg/mL (256 μg/mL).

Catharanthus roseus ajmalicine and the activity of the compound was proposed to be due to DXR inhibition.[13] The fact that this compound did not show activity against *Mt*DXR, but still exhibited an MIC of 256 μg/mL, might indicate that **8e** acts on another target.

In summary, 14 FR900098/fosmidomycin-derived inhibitors substituted with either acidic or metal coordinating moieties were prepared and evaluated against *Mt*DXR. The results showed that it is possible to replace both the phosphonic acid group and the hydroxamic acid group in FR900098 and to achieve active inhibitors. Further development of these inhibitors may be worthwhile in an attempt to improve both *Mt*DXR binding and *Mt* whole-cell activity.

Acknowledgments

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Preparation of compound **8j**: To a solution of dibenzyl methylphosphonate **17** (138 mg, 0.5 mmol) in THF (2 mL) was added *n*-BuLi (0.25 mL, 0.62 mmol, 2.5 M in hexanes) at −78 °C under N_2 atmosphere and stirred at −78 °C for 1 h. Then added a solution of 3,4-dibenzyloxybenzaldehyde (159 mg, 0.5 mmol) in THF (2 mL) at −78 °C and the reaction mixture was brought to room temperature over 4 h. The reaction was cooled to −78 °C and a solution of NaO^tBu (58 mg, 0.6 mmol) in THF (2 mL) was added and stirred for 10 min. The reaction was quenched with saturated aq NH_4Cl and extracted with EtOAc to obtain the crude compound **18**. Compound **18** (107 mg) was taken in methanol (5 mL) and added 10% Pd/C (40 mg) and glacial acetic acid (0.05 mL). The contents were stirred under the atmosphere of hydrogen for 16 h at room temperature. The reaction mixture was filtered on a pad of celite and concentrated. The crude product was purified by preparative LC/MS (water/MeOH, gradient, 30–60% MeOH, 20 min). The isolated yield was 0.018 g, 17% (overall yield). LC/MS: *m*/*z* 219 (M+1). HRMS for $C_8H_{12}O_5P$ calcd 219.0422, found 219.0424. ^1H NMR (400 MHz, D_2O): δ 1.81–1.93 (m, 2H), 2.58–2.68 (m, 2H), 6.57–6.62 (m, 1H), 6.68–6.75 (m, 2H). ^{13}C NMR (100 MHz, D_2O): δ 27.6 (J = 3.9 Hz), 28.8 (J = 131.9 Hz), 115.8, 116.2, 120.2, 134.5 (J = 17.5 Hz), 142.0, 143.8.

Inhibition of *Mt*DXR-activity was measured in a spectrophotometric assay[3,24,25] by monitoring the NADPH-dependent rearrangement and reduction of DXP to form MEP using the absorption of NADPH at 340 nm wavelength. Assay reactions had a final volume of 50 μL and contained 50 mM HEPES-NaOH pH 7.5, 100 mM NaCl, 1.5 mM $MnCl_2$, 0.2 mM NADPH, 0.19 μM *Mt*DXR, 0.2 mM DXP and inhibitory compound at various concentrations. Initial screening for inhibition was performed with an inhibitor concentration of 100 μM. IC_{50}-measurements were performed using six reactions with inhibitor concentrations ranging between 0.01 and 1000 μM. Reactions were initiated by adding DXP and followed simultaneously in a 96-well plate (UV-Star, Greiner) at 22 °C with a spectrophotometer (Envision 2140 Multilabel Reader, PerkinElmer). Absorbance at 340 nm was measured every 5 s during a 500 s period. The slope of the linear phase of each reaction was used to calculate the initial velocity. This was compared to the velocity of the uninhibited reaction and used to calculate enzyme activity. Enzyme activities were plotted against the corresponding inhibitor concentration and data points were fitted to (equation 1), $Y = \mathrm{Lo} + \frac{\mathrm{Hi}-\mathrm{Lo}}{1+\frac{X}{IC_{50}}}$ where Hi is the estimated highest enzyme activity at zero inhibitor concentration, Lo is the estimated lowest enzyme activity at infinite inhibitor concentration, X is the concentration of inhibitor and Y is the measured enzyme activity. IC_{50}-values presented are the average value of three independent experiments.