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In vitro inhibition of salicylic acid derivatives on human cytosolic carbonic anhydrase isozymes I and II

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ABSTRACT

The inhibition of two human cytosolic carbonic anhydrase (hCA, EC 4.2.1.1) isozymes, hCA I and II, with a series of salicylic acid derivatives was investigated by using the esterase method with 4-nitrophenyl acetate as substrate. IC_{50} values for sulfasalazine, diflunisal, 5-chlorosalicylic acid, dinitrosalicylic acid, 4-aminosalicylic acid, 4-sulfosalicylic acid, 5-sulfosalicylic acid, salicylic acid, acetylsalicylic acid (aspirin) and 3-metylsalicylic acid were of 3.04 μM, 3.38 μM, 4.07 μM, 7.64 μM, 0.13 mM, 0.29 mM, 0.42 mM, 0.56 mM, 2.71 mM and 3.07 mM for hCA I and of 4.49 μM, 2.70 μM, 0.72 μM, 2.80 μM, 0.75 mM, 0.72 mM, 0.29 mM, 0.68 mM, 1.16 mM and 4.70 mM for hCA II, respectively. Lineweaver–Burk plots were also used for the determination of the inhibition mechanism of these substituted phenols, most of which were noncompetitive inhibitors with this substrate. Some salicylic acid derivatives investigated here showed effective hCA I and II inhibitory activity, and might be used as leads for generating enzyme inhibitors eventually targeting other isoforms which have not been assayed yet for their interactions with such agents.

1. Introduction

The carbonic anhydrases (EC. 4.2.1.1) are an expanding family of zinc-containing enzymes, which classically participate in the maintenance of pH homeostasis in human body, catalyzing the reversible hydration of carbon dioxide in a two-step reaction to yield bicarbonate and protons.[1,2] Sixteen isozymes have been described so far, that differ in their subcellular localization, catalytic activity and susceptibility to different classes of inhibitors. Some of these isozymes are cytosolic (CA I, CA II, CA III, CA VII and CA XIII), others are membrane bound (CA IV, CA IX, CA XII and CA XIV), two are mitochondrial (CA VA and CA VB), and one is secreted in saliva (CA VI). It has been reported that CA XV isoform is not expressed in humans or in other primates, but it is abundant in rodents and other higher vertebrates.[3–6] CAs are produced in a variety of tissues where they participate in several important biological processes such as acid–base balance, respiration, carbon dioxide and ion transport, bone resorption, ureagenesis, gluconeogenesis, lipogenesis and body fluid generation.[1,2,4] The two major CA isozymes (CA I and CA II) are present at high concentrations in the cytosol in erythrocytes, and CA II has the highest turnover rate of all the CAs.[3] Many of the CA isozymes involved in these processes are important therapeutic targets with the potential to be inhibited to treat a range of disorders including oedema, glaucoma, obesity, cancer, epilepsy and osteoporosis.[1,5]

Many chemical substances and synthesized drugs affect metabolic processes by changing enzyme activities. Chemicals are generally known to activate or inhibit several enzymes in vivo[7] and affect metabolic pathways. Inhibitory effects of different anions, metal ions, phenols and sulfonamides, which are specific inhibitors have been investigated up to now against many CAs.[5,8–10] CA II inhibitors are used for several aims in particular for the treatment of glucoma, epilepsy, as diuretics or antitumor agents/diagnostic tools.[1–3] For this reason, discovery of novel CA inhibitors targeting various isoenzymes has gained attention nowadays.[1–5]

Salicylic acid derivatives are widely used for treatment of various diseases. For example, acetylsalicylic acid is the most widely used drug in the world, 4-aminosalicylic acid is used for tuberculosis treatment and diflunisal is a strong pain killer and antipyretic.[11] In the present study we have purified carbonic anhydrase I and II from human erythrocytes and examined the in vitro inhibition effects of some salicylic acid derivatives on these important enzymes, by using the esterase activity of CA I and II, with 4-nitrophenyl acetate as substrate.

2. Results and discussion

2.1. Chemistry

Salicyclic acid **1** and its substituted congeners **2–9** have been investigated as CA inhibitors in this study, together with aspirin

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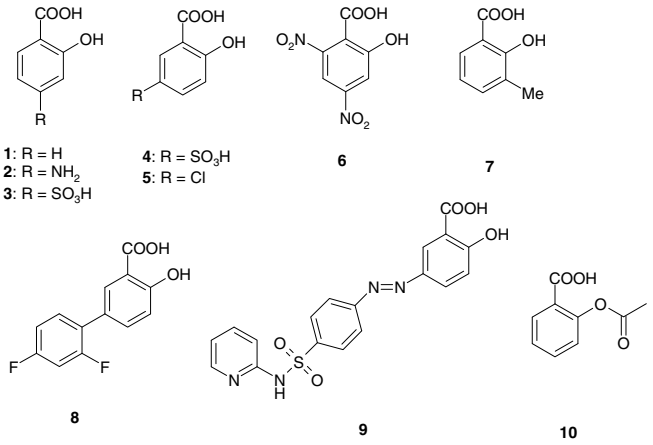
(acetylsalicylic acid) **10**. The rationale of investigating these compounds as CA inhibitors (CAIs) is due to the fact that phenol has been shown to be the only competitive inhibitor with CO_2 as substrate for the main isoform of CA, that is, human CA II (hCA II).[5a] In a very elegant study, Christianson's group reported the X-ray crystal structure for the adduct of hCA II with phenol,[5a] showing this compound to bind to CA by anchoring its OH moiety to the zinc-bound water/hydroxide ion of the enzyme active site through a hydrogen bond as well as to the NH amide of Thr199, an amino acid conserved in all α-CAs and critically important for the catalytic cycle of these enzymes.[1–3] Furthermore, the phenyl moiety of phenol was found to lay in the hydrophobic part of the hCA II active site, where presumably CO_2, the physiologic substrate of the CAs, binds in the precatalytic complex, explaining thus the behavior of phenol as a unique CO_2 competitive inhibitor. Only recently, one of our groups investigated the interactions of phenol and some of its substituted derivatives (as well as bicyclic phenols) with all mammalian isozymes, CA I–XV,[5b–d] evidencing some low micromolar/submicromolar inhibitors as well as the possibility to design isozyme-selective CAIs. Indeed, the inhibition profile of various isozymes with this class of agents is very variable, with inhibition constants ranging from the millimolar to the submicromolar range for many simple phenols.[5] It appeared thus of interest to extend the previous studies,[5] including in this research a phenol with wide clinical applications,[11] salicyclic acid **1**, as well as some of its substituted derivatives incorporating amino, sulfonic acid, chloro, methyl and nitro moieties as substituents at the aromatic ring in different positions. Furthermore, three other derivatives used clinically,[11] that is, diflunisal **8** and sulfasalazine **9**, as well as the acetylated derivative of **1**, acetylsalicylic acid **10** were also included in our study.



2.2. CA purification, assay and inhibition

The purification of the two CA isozymes used here was performed with a simple one step method by a Sepharose-6B-aniline-sulfanilamide affinity column (Table 1).[12–15] Figure 1 shows the SDS–PAGE obtained for determining the purity of the enzymes. Inhibitory effects of salicylic acid derivatives on enzyme activities were tested under in vitro conditions; IC_{50} values were calculated Activity%- [Inhibitor] graphs and are given in Table 2 and K_i values were calculated from Lineweaver–Burk graphs and are given in Table 3.[16,17]

The CA isozymes play important roles in different tissues.[1–4,18,19] It is known that carbonic anhydrase has been purified many times from different organisms and the effects of various chemicals, pesticides and drugs on its activity have been investigated.[20–25] In this study, CA I and II were purified from human erythrocytes by a simple one step procedure using Sephrarose 6B-aniline-sulfanilamide affinity column. The activity of the effluents were determined by the hydratase method, with CO2 as substrate[13] and further kinetic studies were performed using the esterase activity method, with 4-nitrophenyl acetate (NPA) as substrate.[14] hCA I was purified 104.0-fold with specific activity (919.6 EU mg^{-1}) and yield (59.4%). Similarly, hCA II was purified 732.6-fold with specific activity (6469.2 EU mg^{-1}) and yield (53.2%). SDS–PAGE of the enzymes showed a single polypeptide band (Fig. 1).

We report here the first study on the inhibitory effects of salicylic acid derivatives on the CA esterase activity. The previous report by Innocenti et al.[5b] investigated phenols (including salicyclic acid and paracetamol) by using a stopped flow, CO_2 hydration assay for monitoring CA inhibition. Data of Table 2 show the following regarding inhibition of hCA I and II with compounds **1–10:**

(i) Against the slow cytosolic isozyme hCA I, compounds **1–4**, **7** and **10** behave as weak, millimolar inhibitors, with IC_{50}-s in the range of 0.13–3.07 mM. The lead **1** shows an IC_{50} of 0.56 mM, and various substitution patterns such as the introduction of the 4-amino, 4-sulfonic acid-, 5-sulfonic acid or 3-methyl-moieties lead to minor changes in activity, all these compounds behaving as rather weak hCA I inhibitors. The same effect is observed when the inhibition constants are calculated (Table 3) by means of Lineweaver–Burk plots, these compounds showing K_i-s in the range of 0.24–11.42 mM. Acetylsalicylic acid **10** is also a quite weak hCA I inhibitor, but probably this compound is first hydrolyzed (due to the esterase activity of CAs)[14] to salicyclic acid **1** which then inhibits the enzyme in a manner similar to that of all phenols investigated up until now, that is, by hydrogen binding to the zinc-bound water molecule/hydroxide ion within the enzyme cavity.[5] A second group of derivatives, including **5**, **6**, **8** and **9**, show much better inhibitory activity as compared to the previously mentioned salicylates, with IC_{50}-s in the range of 3.04–7.64 μM (corresponding K_i-s of 4.16–14.85 μM, Table 3). These compounds incorporate in their molecules either moieties leading to an acidification of the OH (and COOH) groups of the salicyclic acid scaffold (such as the chlorine atom in 5-position in compound **5** or the two nitro moieties present in **6**), or the bulkier scaffolds present in diflunisal **8** and especially sulfasalazine **9**, which are among the best inhibitors in this series of salicylates. Probably these bulkier moieties present in these derivatives assure better contacts between the enzyme active site and the inhibitor. Work is in progress in our laboratories to resolve the X-ray crystal structures of some of these adducts with isozymes hCA I and/or II, in order to better understand the inhibition mechanism of these derivatives. Data of Table 3 also show that similarly to sulfonamides and inorganic anions,[1,4,8–10] most of the investigated salicyclates act as noncompetitive inhibitors with 4-NPA as substrate, that is, they bind in different regions of the active site cavity as compared to the substrate. However the binding site of 4-NPA itself is unknown, but it is presumed to be in the same region as that of CO_2, the physiological substrate of this enzyme.[5] A competitive inhibitor with 4-NPA has anyhow been detected in this study, which is 5-sulfosalicyclic acid **4**, which may shed new light regarding the interaction of this enzyme with its substrates/inhibitors, provided that a high resolution X-ray structure for the adduct of hCA I with **5** shall be obtained. Compounds **3** and **5** on the other hand showed a behavior of uncompetitive inhibitors with 4-NPA as substrate (Table 3).

(ii) A rather similar activity of these compounds has been observed also for the inhibition of the rapid cytosolic isozyme hCA II (Tables 2 and 3). Thus, the first group of derivatives, including **1–4**, **7** and **10** showed modest hCA II inhibitory activity with

Table 1
Summary of purification procedure for hCA I and hCA II

Purification step	Activity (EU/mL)	Total volume (mL)	Protein (mg/mL)	Total protein (mg)	Total activity	Specific activity (EU/mg)	Yield (%)	Purification factor
Haemolysate	158	40	17.9	716	6320	8.83	100	1
hCA I	469	8	0.51	4.08	3752	919.6	59.4	104
hCA II	841	4	0.13	0.52	3364	6469.2	53.2	732.6

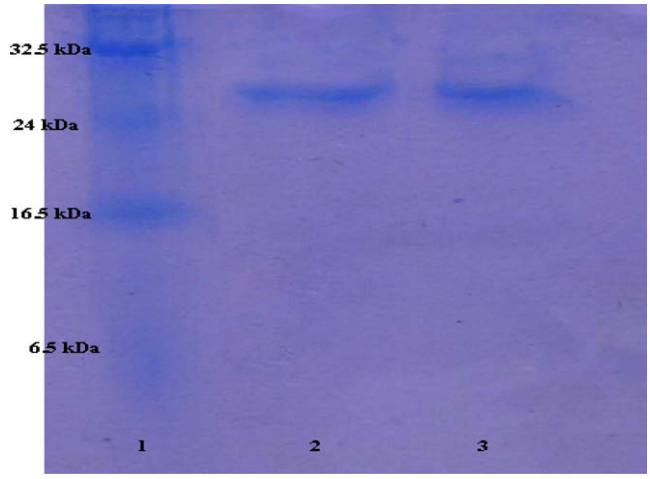


Figure 1. PAGE of the purified CA isozymes. Lane 1: Standards: *E. coli* triosephosphate isomerase (32.5 kDa), soybean trypsin inhibitor (24 kDa), chicken egg white lysozyme (16.5 kDa) and bovine lung aprotinin (6.5 kDa), lane 2: hCA I, lane 3: hCA II.

Table 2
IC_{50} values for the in vitro inhibition of hCA I and hCA II with salicylic acid derivatives **1–10**, by the esterase method with 4-NPA as substrate

Inhibitor	IC_{50} values for hCA I	IC_{50} values for hCA II
Salicylic acid **1**	0.56 mM	0.68 mM
4-Amino salicylic acid **2**	0.13 mM	0.75 mM
4-Sulfosalicylic acid **3**	0.29 mM	0.72 mM
5-Sulfosalicylic acid **4**	0.42 mM	0.29 mM
5-Chlorosalicylic acid **5**	4.07 μM	0.72 μM
4,6-Dinitro salicylic acid **6**	7.64 μM	2.80 μM
3-Metylsalicylic acid **7**	3.07 mM	4.70 mM
Diflunisal **8**	3.38 μM	2.70 μM
Sulfasalazine **9**	3.04 μM	4.49 μM
Acetylsalicylic acid **10**	2.71 mM	1.16 mM

IC_{50}-s in the range of 0.29–4.70 mM (corresponding to K_i-s of 0.74–10.57 mM, Table 3), whereas the remaining four derivatives, that is, the same compounds acting as efficient hCA I inhibitors, showed IC_{50}-s in the range of 0.72–4.49 μM (corresponding K_i-s were in the range of 1.27–9.37 μM, Table 3). Structure–activity relationship (SAR) was thus quite similar in this small group of salicylates both for the inhibition of hCA I and II, although differences of affinity between the two isozymes are evident (e.g., compound **5** is 5.65 times a better hCA II than hCA I inhibitor, considering the IC_{50} values). Again most of these phenols act as noncompetitive inhibitors with 4-NPA as substrate, except for **3** which is a competitive inhibitor and **5** and **7** which act as uncompetitive inhibitors (Table 3). A putative binding mode of salicyclic acid derivatives **1–10** to the CA active site, considering the X-ray crystal structure of the hCA II–phenol adduct reported earlier,[5a] is proposed in Figure 2.

It is probable that the phenolic OH moiety of compounds **1–10** is anchored to the Zn(II) fourth ligand, which is a water molecule/hydroxide ion (depending on the pH), as in the hCA II–phenol adduct investigated by Christianson's group.[5a] A second hydrogen bond involves the phenolic OH and the amide NH moiety of Thr199, an amino acid residue conserved in all CA isozymes. The aromatic ring points toward the hydrophobic pocket of the enzyme where it is presumed that the substrates of CAs bind, which is lined in CA II by residues Val121, Val143, Leu198 and Trp209.[5] It is probable that the COOH moiety present in compounds **1–10** also participates to hydrogen bonds with amino acid residues in its neighborhood (e.g., Thr200), but as no X-ray crystal structures for other phenols than the simple compound showed in Figure 2 are available for the moment, this hypothesis should be checked by resolving the high resolution X-ray crystal structure of some of these derivatives with various CA isozymes. This binding mode proposed by us is in agreement with the noncompetitive inhibition type observed with most of the derivatives investigated here (Table 3).

In a recent study it was reported that thioxolone, a simple compound lacking the sulfonamide, sulfamate, or related functional groups that are typically found in all known CA inhibitors, acts as a CAI, and could represent the starting point for a new class of inhibitors that may have advantages for patients with sulfonamide allergies (thioxolone acts as a prodrug, being hydrolyzed in situ with formation of a mercapto phenol derivative which is the real enzyme inhibitor).[26] However Innocenti et al.[27] showed that compared to sulfonamides thioxolone was inefficient for generating isozyme-selective inhibitors, since except for hCA I which was inhibited in the nanomolar range (K_i of 91 nM), the remaining 12 mammalian CA isoforms (CA II–CA XV) were inhibited with a very flat profile by this compound (K_i-s in the range of only 4.93–9.04 μM). In contrast to thioxolone, 3,5-dichloro-4-hydroxybenzenesulfonamide as well as the clinically used heterocyclic sulfonamide acetazolamide showed K_i-s in the range of 58 nM–78.6 μM and 2.5 nM–200 μM, respectively, against the 13 investigated mammalian CAs. The sulfonamide zinc-binding group is thus superior to the thiol one (from the thioxolone hydrolysis product) for generating CA inhibitors with a varied and sometimes isozyme-selective inhibition profile against the mammalian enzymes. However, it is critically important to explore further classes of potent CAIs in order to detect compounds with a different inhibition profile as compared to the sulfonamides and their bioisosteres and to find novel applications for the inhibitors of these widespread enzymes.

3. Conclusions

Salicylic acid **1** and its derivatives **2–10** used in this study affect the activity of CA isozymes due to the presence of the different functional groups (OH and COOH) moieties present in their aromatic scaffold. Our findings here indicate thus another class of possible CAIs of interest, in addition to the well-known sulfonamides/sulfamates/sulfamides, the phenols bearing *ortho* carboxylic acid moieties. Indeed, some salicylic acid derivatives investigated here showed effective CA I and II inhibitory activity, in the low micromolar range, by the esterase method which usually gives K_i-s an order of magnitude higher as compared to the CO_2 hydrase assay.[28] These findings point out that substituted salicylic acids may be

Table 3
K_i values for the inhibition of hCA I and hCA II with the salicylic acid derivatives **1**–**10**

Inhibitor	K_i values for hCA I	Inhibition type	K_i values for hCA II	Inhibition type
Salicylic acid **1**	0.62 mM	Noncompetitive	0.74 mM	Noncompetitive
4-Aminosalicylic acid **2**	0.24 mM	Noncompetitive	1.23 mM	Noncompetitive
4-Sulfosalicylic acid **3**	0.48 mM	Uncompetitive	1.06 mM	Competitive
5-Sulfosalicylic acid **4**	0.77 mM	Competitive	1.92 mM	Noncompetitive
5-Chlorosalicylic acid **5**	4.16 μM	Uncompetiti ve	1.27 μM	Uncompetitive
4,6-Dinitrosalicylic acid **6**	14.85 μM	Noncompetitive	5.45 μM	Noncompetitive
3-Metylsalicylic acid **7**	11.42 mM	Noncompetitive	10.57 mM	Uncompetitive
Diflunisal **8**	8.45 μM	Noncompetitive	9.37 μM	Noncompetitive
Sulfasalazine **9**	6.87 μM	Noncompetitive	8.72 μM	Noncompetitive
Acetylsalicylic acid **10**	7.53 mM	Noncompetitive	3.66 mM	Noncompetitive

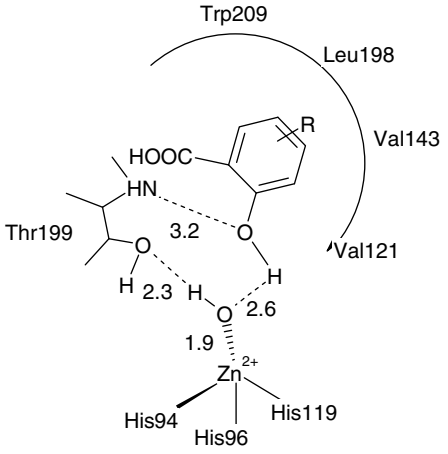


Figure 2. Putative binding mode of salicyclic acid derivatives **1**–**10** to the CA active site, considering the X-ray crystal structure of the hCA II–phenol adduct reported earlier.[5a] Figures represent distances (in Å) and correspond to the hCA II–phenol adduct reported by Christianson's group.[5a]

used as leads for generating potent CAIs eventually targeting other isoforms which have not been assayed yet for their interactions with such agents.

4. Experimental

4.1. Chemicals

Sepharose-6B, protein assay reagents, 4-nitrophenylacetate and chemicals for electrophoresis were purchased from Sigma–Aldrich Co. (Sigma–Aldrich Chemie GmbH Export Department Eschenstrasse 5, 82024 Taufkirchen, Germany). All other chemicals were analytical grade and obtained from Merck (Merck KGaA Frankfurter strasse 250, D 64293 Darmstadt, Germany).

4.2. Purification of carbonic anhydrase isozymes from human erythrocytes by affinity chromatography

Erythrocytes were purified from fresh human blood obtained from the Blood Center of the Research Hospital at Atatürk University. The blood samples were centrifuged at 1500 rpm for 15 min and the plasma and buffy coat were removed. The red cells were isolated and washed twice with 0.9% NaCl, and hemolysed with 1.5 volumes of ice-cold water. The ghost and intact cells were removed by centrifugation at 20,000 rpm for 30 min at 4 °C. The pH of the hemolysate was adjusted to 8.7 with solid Tris.[9] Firstly, Sepharose-6B oxidized by $KMnO_4$ and subsequently activated by $SOCl_2$. After that, aniline attached to the activated gel as an spacer arm and finally diazotized sulfanilamide clamped to the *para* position of aniline molecule as ligand. The hemolysate was applied to the prepared Sepharose-6B-aniline-sulfanylamide affinity column equilibrated with 25 mM Tris–HCl/0.1 M Na_2SO_4 (pH 8.7). The affinity gel was washed with 25 mM Tris–HCl/22 mM Na_2SO_4 (pH 8.7). The human carbonic anhydrase (hCA I and hCA II) isozymes were eluted with 1 M NaCl/25 mM Na_2HPO_4 (pH 6.3) and 0.1 M CH_3COONa/0.5 M $NaClO_4$ (pH 5.6), respectively. All procedures were performed at 4 °C.[12]

4.3. Hydratase activity assay

Carbonic anhydrase activity was assayed by following the hydration of CO_2 according to the method described by Wilbur and Anderson.[13] CO_2-hydratase activity as an enzyme unit (EU) was calculated by using the equation $(t_0 - t_c/t_c)$ where t_0 and t_c are the times for pH change of the nonenzymatic and the enzymatic reactions, respectively.

4.4. Esterase activity assay

Carbonic anhydrase activity was assayed by following the change in absorbance at 348 nm of 4-nitrophenylacetate (NPA) to 4-nitrophenylate ion over a period of 3 min at 25 °C using a spectrophotometer (CHEBIOS UV–VIS) according to the method described by Verpoorte et al.[14] The enzymatic reaction, in a total volume of 3.0 mL, contained 1.4 mL of 0.05 M Tris–SO_4 buffer (pH 7.4), 1 mL of 3 mM 4-nitrophenylacetate, 0.5 mL H_2O and 0.1 mL enzyme solution. A reference measurement was obtained by preparing the same cuvette without enzyme solution. The inhibitory effects of sulfasalazine, diflunisal, 5-chlorosalicylic acid, dinitrosalicylic acid, 4-aminosalicylic acid, 4-sulfosalicylic acid, 5-sulfosalicylic acid, salicylic acid, acetyl salicylic acid and 3-metylsalicylic acid were examined. All compounds were tested in triplicate at each concentration used. Different inhibitor concentrations were used. hCA I enzyme activities were measured for sulfasalazine (1.5–4 μM), diflunisal (1.15–6.9 μM), 5-chlorosalicylic acid (0.94–7.48 μM), dinitrosalicylic acid (2.3–22.5 μM), 4-aminosalicylic acid (0.062–0.39 mM), 4-sulfosalicylic acid (0.15–1.02 mM), 5-sulfosalicylic acid (0.16–0.81 mM), salicylic acid (0.19–1.41 mM), acetyl salicylic acid (1.43–3.58 mM) and 3-metylsalicylic acid (0.5–3.18 mM) at cuvette concentrations and hCA II enzyme activities were measured for sulfasalazine (0.875–8.75 μM), diflunisal (1.15–6.9 μM), 5-chlorosalicylic acid (0.1–1.9 μM), dinitrosalicylic acid (0.9–4.5 μM), 4-aminosalicylic acid (0.078–0.78 mM), 4-sulfosalicylic acid (0.2–1.02 mM), 5-sulfosalicylic acid (0.16–1.35 mM), salicylic acid (0.09–0.94 mM), acetyl salicylic acid (0.36–2.86 mM) and 3-metylsalicylic acid (1.02–8.2 mM) at cuvette concentrations. Control cuvette activity in the absence of inhibitor was taken as 100%. For each inhibitor an Activity%- [Inhibitor] graph was drawn. To determine K_i values, three different inhibitor concentrations were tested; in these experiments, 4-nitrophenylace-

tate was used as substrate at five different concentrations (0.15–0.75 mM). The Lineweaver–Burk curves were drawn.[17]

4.5. Protein determination

Protein during the purification steps was determined spectrophotometrically at 595 nm according to the Bradford method, using bovine serum albumin as the standard.[15]

4.6. SDS–polyacrylamide gel electrophoresis

SDS–polyacrylamide gel electrophoresis was performed after purification of the enzymes. It was carried out in 10% and 3% acrylamide for the running and the stacking gel, respectively, containing 0.1% SDS according to Laemmli. A 20 mg sample was applied to the electrophoresis medium. Gels were stained for 1.5 h in 0.1% Coomassie Brilliant Blue R-250 in 50% methanol and 10% acetic acid, then destained with several changes of the same solvent without the dye.[16] The electrophoretic pattern was photographed (see Fig. 1).

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