

Synthesis of Bisubstrate Inhibitors of Porphobilinogen Synthase from *Pseudomonas aeruginosa*

Sabine Gacond^{a)}, Frederic Frère^{b)}, Merle Nentwich^{b)}, Jean-Philippe Faurite^{a)},
Nicole Frankenberg-Dinkel^{b)1)}, and Reinhard Neier^{*a)}

^{a)} Institute of Chemistry, University of Neuchâtel, Rue Emile-Argand 11, case postale 158,
CH-2009 Neuchâtel (phone: +41 32 718 24 28; fax: +41 32 718 25 11; e-mail: reinhard.neier@unine.ch)

^{b)} Institute for Microbiology, Technical University Braunschweig, Spielmannstr. 7, D-38106 Braunschweig

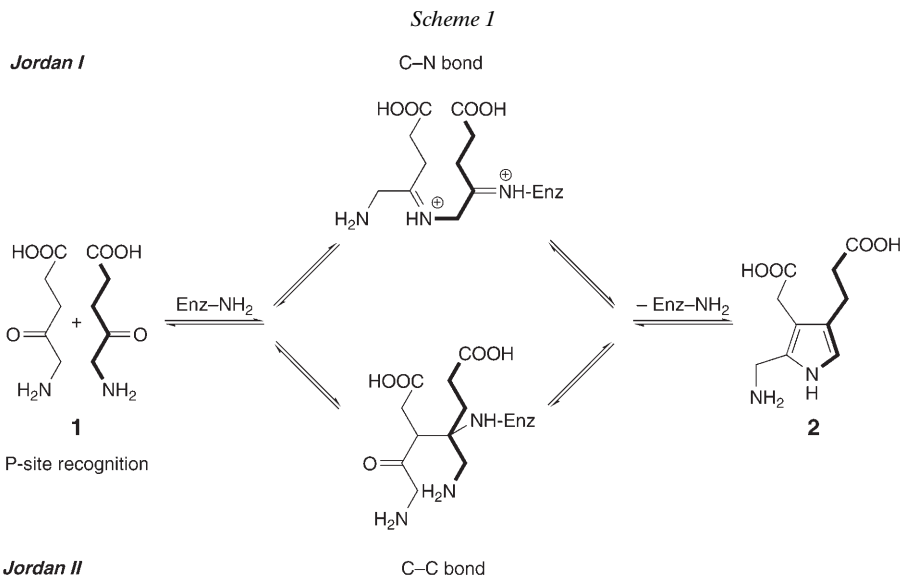
Porphobilinogen synthase (PBGs) synthesizes porphobilinogen **2** (PBG), the common precursor of all natural tetrapyrroles, through an asymmetric condensation of two molecules of 5-aminolevulinic acid **1** (ALA). Symmetrically linked dimers **7–11** derived from levulinic acid **3** (γ -oxovaleric acid) have been synthesized to mimic the assumed bisubstrate bound to the active site of the enzyme. Their inhibition potential was characterized by determination of the IC_{50} and K_i values using PBGS from *Pseudomonas aeruginosa*. The polarity and the size of the functional group linking the two levulinic acid **3** units have a strong influence on the inhibition behavior.

Introduction. – Porphobilinogen synthase (PBGs), also called 5-aminolevulinic acid dehydratase (ALAD; E. C. 4.2.1.24), catalyzes the condensation of two molecules of 5-aminolevulinic acid (ALA, **1**) to produce porphobilinogen (PBG, **2**) [1][2] which is a committed intermediate in the biosynthesis of tetrapyrrolic natural products like porphyrins and chlorophylls.

PBGs has been isolated and studied since 1953 [3]. The order of binding the two substrates to the enzyme has been determined by Jordan and Seehra [4]: binding of ALA (**1**) to the P site of the enzyme, first, and binding of the future acetic acid side ALA (**1**) second. The synthesis of PBG (**2**) requires the sequential formation of both a C–C bond and a C–N bond between the substrate molecules. The C–C bond is formed by an aldol condensation; the C–N bond by a Schiff base formation. Consequently two sequences for the catalytic mechanism are possible (*Scheme 1*). Both possibilities have been discussed [5–8] but a formal proof in favor of one of the mechanisms is still missing.

The active form of PBGS is a homo-octamer, as documented by crystal structures from yeast [9], *Escherichia coli* [6], *Pseudomonas aeruginosa* [10], *Homo sapiens* [11], and *Chlorobium vibrioforme* [12]. The monomers have all a TIM-barrel structure [9][13]. The active site, which is covered by a loop ('active-site flap'), is situated at the C-terminal end of the β_i -strand above the central barrel cavity. The influence of bivalent metal ions (Zn^{2+} and/or Mg^{2+}) on the catalytic activity of PBGS has been

¹⁾ Present address: Physiology of Microorganisms, Ruhr-University-Bochum, Universitätsstrasse 150, D-44780 Bochum.

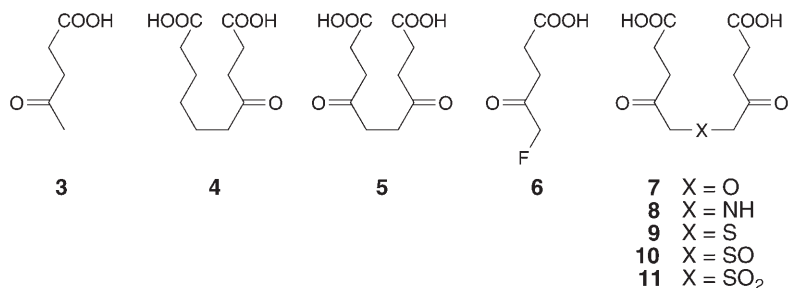


intensively studied [14–16]. The observed metal dependence has been used as basis to tentatively categorize PBGS from different sources.

For several years, our group has been investigating the mechanism of PBGS isolated from *Rhodobacter spheroides* [17] and from *Escherichia coli* [5] by systematic inhibition studies. To test the recognition sites, a series of substrate analogs, product analogs and analogs of postulated intermediates were synthesized, and their inhibition potentials were determined. The results show that two carboxylate groups and two γ -keto groups are essential for a good recognition of the inhibitors in the active site [18][19].

It has been known for many years that the substrate is covalently bound to the P-site lysine [4]. Accordingly, the inhibitor levulinic acid (=4-oxopentanoic acid; **3**) and the substrate 5-aminolevulinic acid (**1**) have been observed crystallographically to form a *Schiff* base to this lysine residue [6][20][21]. The P-site has, therefore, been well characterized. The two irreversible inhibitors, 4-oxosebacic acid (=4-oxodecanedioic acid; **4**) and 4,7-dioxosebacic acid **5**, have been produced to mimic one of the two possible reaction intermediates in which the C–N bond-formation has been observed first [8][22]. The crystal structures of these ligands with Zn-dependent enzymes show that both inhibitors **4** and **5** form a *Schiff* base with P-side lysine [7]. These interactions are similar to all other ligands bound to the P-site exclusively, which have been studied so far. In the case of the 4,7-dioxosebacic acid (**5**), two covalent linkages between the enzyme and the inhibitor have been observed. The crystal structure reveals the presence of a second *Schiff* base in the A-site. Observation of this *Schiff* base indicates the possibility that A-side ALA might form, during the reaction, a bond to the A-site lysine.

Recently, the structure determination of the complex between PBGS and 5-fluorolevulinic acid (**6**) showed that two identical substrate analogs may bind at the



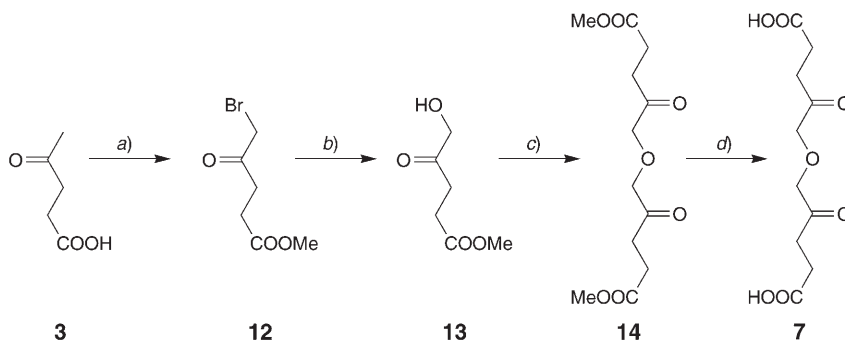
same time to the active site [23]. This X-ray structure provides a detailed model of the native substrate–enzyme complex and fully identifies all residues involved in A-site as well as P-site formation for Zn-independent PBGS. It reveals that an alkaline metal ion may be required in Mg-dependant PBGS to stabilize ALA (**1**) in a defined conformation in the polar A-site and modulate the activity of P-side ALA (**1**) [23].

The crystal structures are compatible with the proposed presence of two *Schiff* bases between the two ALA (**1**) substrates and the lysine residues in the active site, and allows to identify the residues responsible for the recognition of the carboxylic moiety in the A-site. Because of the formation of the two *Schiff* bases with the substrate, the irreversible inhibitor 4,7-dioxosebacic acid (**5**) could also be interpreted as a bisubstrate analog as well as an analog of the postulated intermediate. The aim of this investigation was to contribute to our knowledge about the active site of the enzyme and to elucidate possible mechanisms for the transformation. We have decided to synthesize a new generation of bisubstrate analogs **7–11** in order to understand the bonding of the 4,7-dioxosebacic acid (**5**) to the enzyme. The design features of these inhibitors are: they should contain the two important groups (two carboxylate groups and two γ -keto groups) for a good recognition in the active site, and they should allow to vary the length and the chemical nature of the linker. We report the synthesis of a series of inhibitors where the acid–base character, and the size and polarity of the linking group have been varied. To see if this new family of compounds were good inhibitors, we have studied their inhibition potentials for the Mg-dependant PBGS from *Pseudomonas aeruginosa* by determination of their half maximal inhibition concentrations, the IC_{50} value, their modes of inhibition, and their constants of inhibition, the K_i value.

Results and Discussion. – *Synthesis.* The five specifically designed compounds **7–11** are bisubstrate analogs and contain the two important groups (COOH and γ -oxo functions) for a good recognition in the active site. The difference between the five potential PBGS inhibitors **7–11** is the linker which can be an O-, N-, or S-atom, or bulkier moieties as a sulfinyl or sulfonyl groups. These bisubstrate analogs were synthesized to test the influence of 1) the prolonged linker size compared to 4,7-dioxosebacic acid (**5**) and 2) the volume and polarity of their linker moiety on their potency to inhibit PBGS. The synthesis of the bisubstrate analogs **7–11** is highlighted in the *Schemes 2–4*.

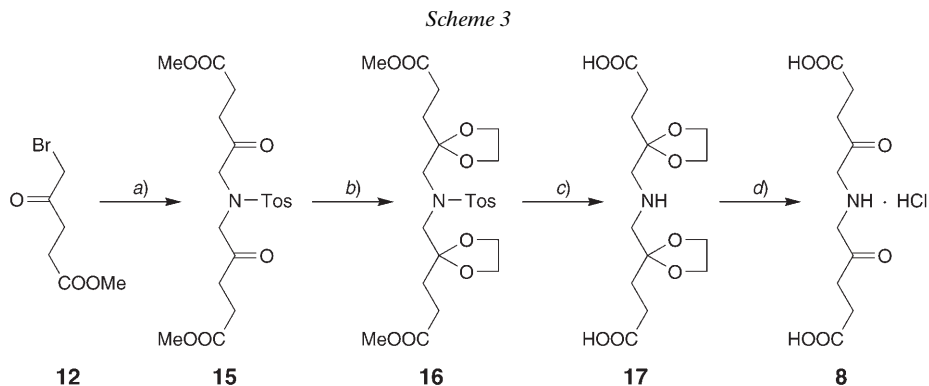
The bromo compound **12** was the precursor for the five targeted inhibitors **7–11** and has already been described in the literature [24]. First, we tried to synthesize the O-linked diacid **7** using classical *Williamson* conditions (*Scheme 2*). The hydroxy ester **13** was synthesized from **12** according to the procedure described in [25], but the *Williamson* reaction between the compounds **12** and **13** gave only traces of the desired O-linked diester **14**. We have decided to change the OH function of **13** to a better leaving group, *e.g.*, a tosyl (Ts) group. For this purpose, we have used the conditions of *Still* and *Daoquan* applied to a α -ceto [26]. The authors obtained a mixture of the protected alcohol and the corresponding ether. The procedure consisted of treating the alcohol **13** with *p*-toluenesulfonyl chloride (TsCl) and Et₃N in CH₂Cl₂. The best conditions, *i.e.*, 1.2 equiv. of TsCl at room temperature for 20 h, gave **14**, without traces of the protected alcohol, in 82% yield (*Scheme 2*). The yield decreased considerably, compared to the optimal value, if the number of equivalents of TsCl was modified. When the reaction was run at lower temperatures, a mixture of **14** and the protected alcohol was obtained. The acidic hydrolysis of **14** gave the desired compound **7**.

Scheme 2



a) Br₂, MeOH, 10° to reflux, 3.5 h; 50%. *b)* Formic acid, 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), r.t., 1 h, Al₂O₃; 47%. *c)* *p*-Toluenesulfonyl chloride (TsCl), Et₃N, CH₂Cl₂, r.t., 20 h; 82%. *d)* 6N HCl, 50°, 2 h; 94%.

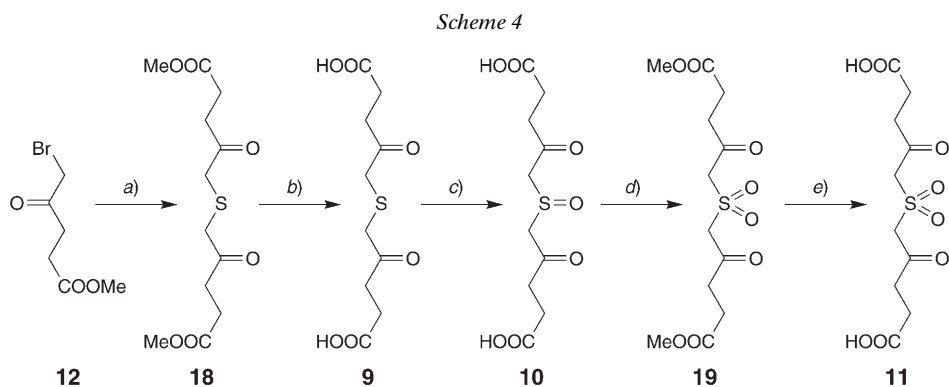
The best way to synthesize the NH-linked diacid **8** was to protect the amino group during the alkylation step followed by the deprotection of the N-atom (*Scheme 3*). The alkylation of *p*-toluenesulfonamide with **12** in the presence of a base formed the desired N-linked diester **15**. The attempts using the same transformation with the (*tert*-butoxy)carbonyl (Boc) and the (benzyloxy)carbonyl protecting groups at the N-atom function were not successful. Classical hydrolysis conditions were tried on **15**, but only the hydrolysis of the two ester functions could be observed. The oxo groups in **15** did not survive the reductive conditions applied for the deprotection of the secondary amine moiety. For this reason, the two oxo groups had to be protected, and, in our hands, the best way was to use acetal groups. The protection of the oxo groups with 1,2-bis(trimethylsilyloxy)ethane afforded **16**. The deprotection of the amine with the sodium naphthalenide [27][28] gave **17**. The final step to obtain the desired compound



a) *p*-Toluenesulfonamide, K_2CO_3 , acetone, reflux, 15 h, Ar; 62%. *b)* 1,2-Bis(trimethylsilyloxy)ethane, trimethylsilyl trifluoromethanesulfonate (TMSOTf), CH_2Cl_2 , -78° to r.t., 5 h, Ar; 82%. *c)* Na, Naphthalene, 1,2-dimethoxyethane (DME), -78° , Ar; 78%. *d)* Conc. HCl, 80° , overnight; 6%.

8 was the deprotection of the oxo groups. As acid-catalyzed hydrolysis is the most common method for the deprotection of acetals, **17** was treated with conc. HCl overnight at 80° , and the formation of **8** was observed. During this reaction, a transesterification between the resulting ethane-1,2-diol and **8** was observed. To avoid this problem, we tried an azeotropic distillation with diphenyl ether or heptane in order to remove the water from the reaction medium. However, we did not succeed to isolate **8**. Finally, a change of pH to 2–3 at the end of the reaction with an aqueous solution of NaOH, followed by the evaporation of the solvent and a reprecipitation in a mixture of MeOH/Et₂O, resulted in the isolation of **8**. The isolated product contained small concentrations of NaCl, which did not interfere with the kinetic measurements.

The S-linked diacid **9** was easily obtained by condensation from the corresponding halogen compounds (*Scheme 4*). The S-linked diester **18** was synthesized from **12** in the



a) Na_2S , acetone/ H_2O , r.t., 5.5 h; 79%. *b)* HCl 6N, 50° , 2 h; 89%. *c)* Monoperoxyphthalic acid magnesium salt hexahydrate (MMPP), EtOH 95%, r.t., 20 min, 84%. *d)* *meta*-Chloroperbenzoic acid (*m*-CPBA), CH_2Cl_2 , -40° to r.t., 170 min, 85%. *e)* 6N HCl, r.t., 160 min, 75%.

presence of Na₂S. The acidic hydrolysis of **18** gave the desired compound **9**. Sulfur compounds were easily oxidized to sulfoxide or sulfone compounds depending on the reaction conditions. The oxidation of **18** with 3.5 or 1.2 equiv. of *m*-chloroperbenzoic acid (*m*CPBA) gave the SO₂-linked diester **19** and the corresponding SO-linked diester, respectively. The acidic hydrolysis of **19** led to the desired SO₂-linked diacid **11** (*Scheme 4*). Classical hydrolysis and enzymatic hydrolysis (with pig liver esterase) conditions were tested on the SO-linked diester, but the desired compound **10** was not obtained. At this stage, the oxidation with *m*CPBA of **9** was tried without success. The use of the magnesium monoperoxyphthalate hexahydrate (MMPP) resulted in the oxidation of **9** into a mixture of the desired SO-linked diacid **10** and the SO₂-linked diacid **11** (*Scheme 4*). The use of 1.5 equiv. of MMPP at 40° gave a mixture **10/11** with a ratio of 37:63. The product distribution could be improved to 96:4 when 0.8 equiv. of MMPP at room temperature for 20 min were used. This purity was sufficient for our kinetic studies.

Inhibition Studies. As an initial test, the IC₅₀ values of the bisubstrate analogs **7–11** were determined (*Table*) according to the method described in [29]. When measuring the IC₅₀ values, the time-dependence of inhibition was detected. Specific tests showed time-dependent inhibition for all compounds except **7**. The mode of inhibition for **8–11** was determined to be slow-reversible, and the apparent K_i values of these inhibitors were measured as described in the *Exper. Part* (*Table* and *Fig. 1*). For **7**, the mode of inhibition was determined to be mixed, fast reversible, and K_i and αK_i values were obtained by classical *Michaelis–Menten* kinetics (*Table* and *Fig. 2*). Both methods gave the same picture.

The best inhibitors for PBGS from *Pseudomonas aeruginosa* are compounds **7**, **8**, and **9** (*Table*). SO-Linked diacid **10** and SO₂-linked diacid **11** are weaker inhibitors. The considerably smaller affinity of the compounds **10** and **11** towards the enzyme can be explained by the increased bulkiness of the linker connecting the two identical γ-oxo pentanoic acid parts of the inhibitors. The bulkiness of the inhibitors **10** and **11** correlates with their decreased inhibition potential.

Table. Kinetic Values of Bisubstrate Analog Inhibitors

| Compound | IC ₅₀ [mM] ^{a)} | Time dependence | K _i [μM] | αK _i [μM] |
|-----------|-------------------------------------|-----------------|----------------------------|----------------------|
| 7 | 0.96 ± 0.03 | – | 98 ± 2 ^{b)} | 4200 ± 340 |
| 8 | 0.31 ± 0.01 | + | 200 ± 30 ^{c)} | |
| 9 | 0.34 ± 0.01 | + | 60 ± 20 ^{c)} | |
| 10 | 38 ± 3 | + | 10800 ± 4600 ^{c)} | |
| 11 | 9.9 ± 0.4 | + | 11000 ± 3000 ^{d)} | |

^{a)} Determined with *Eqn. 1*. ^{b)} Determined using *Michaelis–Menten* kinetics. ^{c)} Determined with *Eqn. 3b*. ^{d)} Determined with *Eqn. 4*.

Discussion. – A series of five symmetric di-γ-oxo diacid compounds **7–11** have been synthesized in order to be tested with the enzyme PBGS from *Pseudomonas aeruginosa*. The influence of 1) their increased linker size compared to 4,7-dioxosebacic

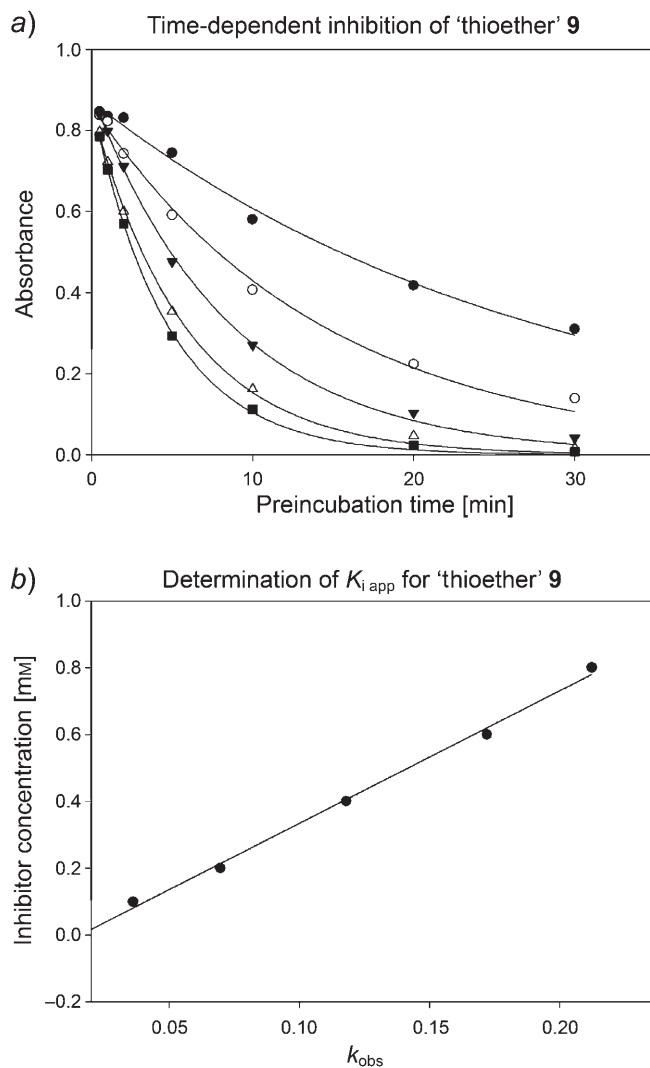


Fig. 1. a) Plots of the absorbance measured at 555 nm, which is proportional to the formation of PBG, vs. preincubation time of the inhibitor **9**. ● = 0.1 mM, ○ = 0.2 mM, ▼ = 0.4 mM, △ = 0.6 mM, ■ = 0.8 mM. The curves were calculated based on Eqn. 2. b) The plot of the inhibitor concentration vs. k_{obs} determined from Fig. 1, a. The straight line is a least-square fit based on Eqn. 3b. The $K_{i,app}$ corresponds to the y-axis intercept.

acid (**5**), and 2) the volume and polarity of their linker moiety on their potency to inhibit PBGS from *Pseudomonas aeruginosa* have been determined.

Analysis of the IC_{50} values of these compounds showed that they are all potent inhibitors for the PBGS from *Pseudomonas aeruginosa*. Three compounds, **7**, **8** and **9**, seem to be very good inhibitors, as their IC_{50} values are better than for the 4,7-

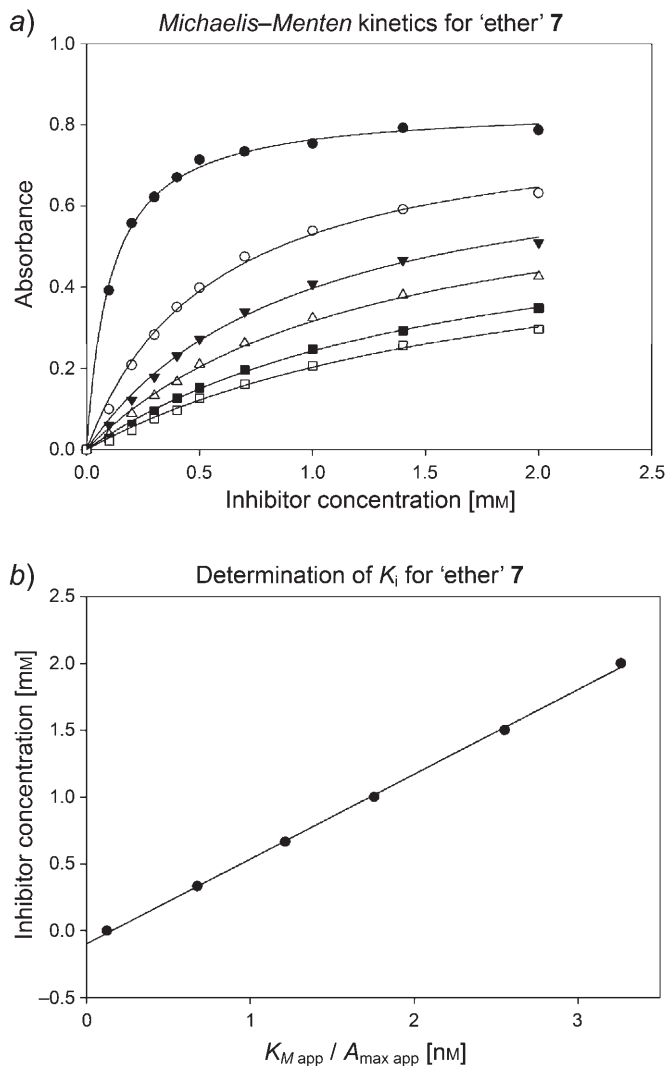


Fig. 2. a) Plots of absorbance at 555 nm, which is proportional to the formation of PBG, vs. substrate concentration for different concentrations of the inhibitor **7**. ●=0 mM, ○=0.33 mM, ▼=0.66 mM, △=1.0 mM, ■=1.5 mM, □=2.0 mM. The curves are least-square fits to the *Michaelis–Menten* equation $A = \frac{A_{\max \text{ app}} \cdot [S]}{K_{M \text{ app}} + [S]}$. b) The straight line is a least square fit to the equation $[I] = \frac{A_{\max}}{K_M} \cdot \frac{K_{M \text{ app}}}{A_{\max \text{ app}}} \cdot K_i - K_i$, which can be deduced for mixed inhibitors. The K_i value corresponds to the y-axis intercept. c) The straight line is a least-square fit to the equation $[I] = \frac{A_{\max}}{A_{\max \text{ app}}} \cdot \alpha K_i - \alpha K_i$. The αK_i value corresponds to the y-axis intercept. The following symbols and abbreviations were used: [I]: inhibitor concentration, K_M : *Michaelis* coefficient, $K_{M \text{ app}}$: apparent *Michaelis* coefficient in presence of inhibitor ($K_{M \text{ app}} = K_M$ in absence of inhibitor), A_{\max} : maximal absorption (linear to maximal velocity for a given enzyme concentration), $A_{\max \text{ app}}$: apparent maximal absorption in presence of inhibitor ($A_{\max \text{ app}} = A_{\max}$ in absence of inhibitor), K_i : inhibition coefficient for interaction between free enzyme and inhibitor, αK_i : inhibition coefficient for interaction between enzyme–substrate complex and inhibitor.

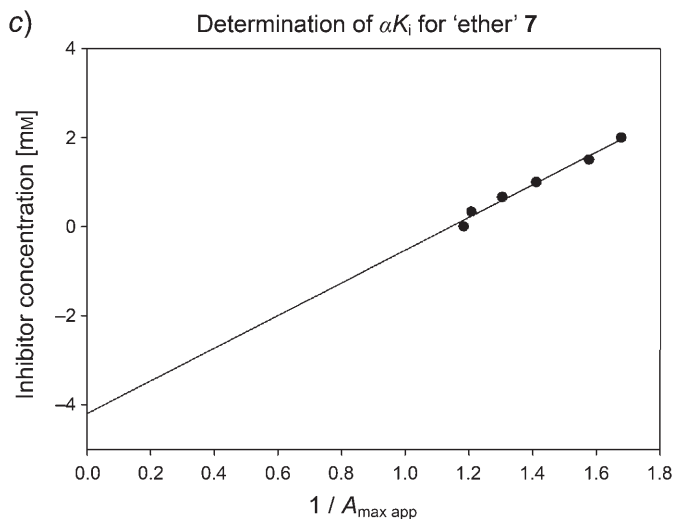


Fig. 2 (cont.)

dioxosebasic acid (**5**; $IC_{50}=2.1$ mM) [8]. The IC_{50} values of these three inhibitors are also by roughly a factor of ten smaller than the IC_{50} value of hydroxy-levulinic acid ($IC_{50}=4.05$ mM; data not shown), which is known to be a good substrate analogue. Within the set of inhibitors, NH-linked diacid **8**, which is an H-bond donor in its protonated form, and S-linked diacid **9** which is a weak H-bond acceptor, are the most potent inhibitors with respect to their IC_{50} values. Both have relatively small linker moieties. The other inhibitor with a small linker moiety, O-linked diacid **7**, is an H-bond acceptor and exhibits an IC_{50} value which is by one order of magnitude higher than the values for compounds **8** and **9**. Interestingly, **7** differs from the other inhibitors in showing fast reversible binding. Nevertheless the K_i values of the **7–9** indicate a comparable inhibition potency.

The weakest inhibitors in the set, indicated by either IC_{50} or K_i values, are SO-linked diacid **10** and SO_2 -linked diacid **11** which carry bulky H-bond-accepting linker groups. These results can be tentatively interpreted as an indication that an optimal size for dioxo-diacid inhibitors is reached with compounds **7**, **8**, and **9**. Also H-bond-accepting moieties (*i.e.*, **10** and **11**) in this position seem to be obstructive. Based on these promising findings, we have planned further kinetic studies and co-crystallization experiments of the new compounds **7–11** with PBGS from *Pseudomonas aeruginosa*. These results have already been published since the submission of the manuscript [29].

This research was supported by a grant from the *Swiss National Science Foundation* (Grant 2000-104007) to R. N., as well as grants from the *Deutsche Forschungsgemeinschaft* and the *Fonds der Chemischen Industrie* to N. F.-D.

Experimental Part

General. THF (*Fluka, purum*) was distilled under N_2 over K and benzophenone; 1,2-dimethoxyethane (DME) was distilled under N_2 over Na and benzophenone; other solvents (*Fluka, puriss.*) were used without further treatment. TLC: silica gel 60 F_{254} plates (aluminium, 0.2 mm; *Merck*). Flash column

chromatography (FC): silica gel 60 (0.04–0.063 mm, *Fluka*). M.p.: *Büchi 510* apparatus, uncorrected. IR Spectra: *Perkin-Elmer 1720X* FT-IR spectrophotometer; in cm^{-1} . NMR Spectra: *Bruker AMX-400* and *Varian Gemini XL-200*; Me_4Si as external reference ($\delta=0$); temp.: 298 K; all coupling constants J in Hz. MS: EI (70 eV): *PolarisQ (Agilent)*; ESI and APCI: *ThermoFinnigan LCQ* (San José, California); HR-MS: *Bruker BioAPEX II Daltonics*. Elemental analyses were performed by 'l'Ecole d'ingénieur' of Fribourg.

Methyl 5-Bromo-4-oxopentanoate (12) [24]. Br_2 (44 ml, 860 mmol) was added dropwise to levulinic acid (**3**; 100 g, 860 mmol) in MeOH (800 ml) at 0° for 2 h. After the addition was completed, the mixture was stirred at reflux for 90 min. After removal of the solvent, the residue was dissolved in CH_2Cl_2 (800 ml) and washed with NaHCO_3 (1×200 ml) and with H_2O (3×200 ml). The org. layer was dried (MgSO_4) and concentrated, and the residue was purified by distillation (*Widmer* column, 50 cm, $7 \cdot 10^{-2}$ mm Hg) to afford **12** (91.6 g, 50%). Colorless liquid. B.p. 60° at $7 \cdot 10^{-2}$ mm Hg. R_f (hexane/AcOEt 1:2) 0.5. IR (KBr, film): 2999m, 2954s, 2849w, 1733s, 1439s, 1411s, 1360s, 1322s, 1208s, 1176s, 1123m, 1079s, 1024s, 988s, 862m, 844m, 694m. $^1\text{H-NMR}$ (400 MHz, CDCl_3): 3.95 (s, $\text{CH}_2(5)$); 3.67 (s, Me); 2.95 (t, $J=6.5$, $\text{CH}_2(3)$); 2.65 (t, $J=6.5$, $\text{CH}_2(2)$). $^{13}\text{C-NMR}$ (100 MHz, CDCl_3): 201.1 (C(4)); 173.2 (C(1)); 52.4 (Me); 34.8 (C(3)); 34.5 (C(5)); 28.5 (C(2)).

Methyl 5-Hydroxy-4-oxopentanoate (13) [25]. 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU; 11.7 g, 77.0 mmol) was added dropwise to a mixture of **12** (12.6 g, 60.2 mmol) and of HCOOH (3.44 g, 74.7 mmol) in benzene (85 ml) at 10° . The soln. was stirred at r.t. for 1 h, and then the reaction was quenched with H_2O (100 ml). The mixture was extracted with CH_2Cl_2 (3×100 ml), and the combined org. extracts were washed successively with 0.1N HCl (250 ml) and H_2O (100 ml). The org. layer was dried (Na_2SO_4) and concentrated, and the residue was hydrolyzed on a column of aluminium oxide (neutral; $\text{CH}_2\text{Cl}_2/\text{MeOH}$ 1:1). The crude product was purified by micro-distillation ($P 6.5 \cdot 10^{-2}$ mm Hg) to give **13** (4.10 g, 47%). Colorless liquid. B.p. $55-56^\circ$ at $6.5 \cdot 10^{-2}$ mm Hg. R_f (hexane/AcOEt 2:1) 0.2. IR (KBr, film): 3450s (br.), 3001m, 2956m, 2918m, 2852m, 1729s (br.), 1439s, 1413s, 1364s, 1320s, 1256s, 1211s, 1175s, 1125m, 1079s, 1016s, 980m. $^1\text{H-NMR}$ (400 MHz, CDCl_3): 4.30 (s, $\text{CH}_2(5)$); 3.67 (s, Me); 2.68 (s, $\text{CH}_2(2)$, $\text{CH}_2(3)$). $^{13}\text{C-NMR}$ (100 MHz, CDCl_3): 208.5 (C(4)); 173.1 (C(1)); 68.5 (C(5)); 52.3 (Me); 33.1 (C(3)); 27.8 (C(2)).

Methyl 5-[4-(Methoxycarbonyl)-2-oxobutoxy]-4-oxopentanoate (14). TsCl (3.14 g, 16.4 mmol) was added to **13** (2.03 g, 13.9 mmol) and Et_3N (1.66 g, 16.5 mmol) in CH_2Cl_2 (20 ml), and the mixture was stirred at r.t. for 20 h. The mixture was concentrated, and the residue was purified by FC (1% AcOEt in CH_2Cl_2) to afford **14** (1.57 g, 82%). Pale yellow liquid. R_f (1% AcOEt in CH_2Cl_2) 0.4. IR (KBr, film): 2997m, 2954s, 1733s, 1632m, 1438s, 1403s, 1359s, 1328s, 1200s (br.), 1088s, 1025s, 994s, 967m, 872m, 844m, 818m, 775s, 727m. $^1\text{H-NMR}$ (400 MHz, CDCl_3): 4.15 (s, $\text{CH}_2(5)$); 3.68 (s, Me); 2.90 (t, $J=6.4$, $\text{CH}_2(3)$); 2.67 (t, $J=6.4$, $\text{CH}_2(2)$). $^{13}\text{C-NMR}$ (100 MHz, CDCl_3): 201.4 (C(4)); 172.9 (C(1)); 52.1 (Me); 48.3 (C(5)); 34.4 (C(3)); 27.9 (C(2)). EI-MS: 179, 167, 165 (100), 135, 133, 115, 97, 87, 69, 55.

5-(4-Carboxy-2-oxobutoxy)-4-oxopentanoic Acid (7). A soln. of **14** (0.64 g, 2.33 mmol) and HCl 6N (75 ml) was stirred at 50° for 2 h. The solvent was evaporated, and the residue was purified by FC (MeOH) and then by recrystallization in hexane/AcOEt to provide **7** (0.45 g, 78%). White solid. M.p. 86° . R_f ($\text{CH}_2\text{Cl}_2/\text{AcOEt}$ 10:3) 0.35. IR (KBr): 3445–2316 (br.), 1736s, 1690s, 1433m, 1410s, 1366m, 1324m, 1296s, 1212m, 1096m, 1038m, 951m, 863w, 770m, 732w, 621m. $^1\text{H-NMR}$ (400 MHz, CDCl_3): 4.14 (s, $\text{CH}_2(5)$); 2.90 (t, $J=6.4$, $\text{CH}_2(3)$); 2.71 (t, $J=6.4$, $\text{CH}_2(2)$). $^{13}\text{C-NMR}$ (100 MHz, CDCl_3): 201.3 (C(4)); 178.3 (C(1)); 48.2 (C(5)); 34.2 (C(3)); 27.9 (C(2)). APCI-MS: 229 ($[\text{M} - \text{H}_2\text{O} + \text{H}]^+$). EI-MS: 133, 101, 73, 55 (100).

Methyl 5-[4-(Methoxycarbonyl)-2-oxobutyl](4-methylphenylsulfonyl)amino]-4-oxopentanoate (15). *p*-Toluenesulfonamide (4.00 g, 23.4 mmol) and K_2CO_3 (6.60 g, 47.6 mmol) in acetone (150 ml) were stirred at reflux for 50 min under Ar. Compound **12** (9.99 g, 47.8 mmol) was added dropwise with a syringe, and the heterogenous mixture was stirred at reflux for 3 h. The solid was filtered, and the soln. was concentrated. The residue was separated by FC (hexane/AcOEt (1:2)) and purified by recrystallization in *i*-PrOH to afford **15** (6.24 g, 62%). White solid. M.p. 109° . R_f (hexane/AcOEt 1:2) 0.32. IR (KBr): 3075w, 3063w, 3025w, 3012w, 2955m, 2940m, 2921m, 1739s, 1722s, 1599m, 1496w, 1451m, 1440s, 1414s, 1358s, 1329s, 1305s, 1200s, 1183s, 1174s, 1155s, 1119m, 1093s, 1019m, 998m, 983s, 975s, 887m, 881m, 816m, 804m, 781s, 716m, 662s, 555s, 540s. $^1\text{H-NMR}$ (400 MHz, CDCl_3): 7.68 (d, $J=8.4$, H–C(2,6)

of tosyl); 7.29 (*d*, $J=8.4$, H-C(3,5) of tosyl); 4.18 (*s*, CH₂(5)); 3.65 (*s*, COOMe); 2.73–2.69 (*m*, CH₂(3)); 2.63–2.59 (*m*, CH₂(2)); 2.42 (*s*, Me-C(4) of tosyl). ¹³C-NMR (100 MHz, CDCl₃): 204.2 (C(4)); 173.1 (C(1)); 144.0 (C(4) of tosyl); 136.0 (C(1) of tosyl); 129.8 (C(3,5) of tosyl); 127.6 (C(2,6) of tosyl); 56.6 (C(5)); 52.0 (COOMe); 34.1 (C(3)); 27.9 (C(2)); 21.7 (Me-C(4) of tosyl). ESI-MS: 450 ([*M*+Na]⁺). Anal. calc. for C₁₉H₂₅NO₈S (427.48): C 53.39, H 5.89, N 3.28; found C 53.33, H 5.92, N 3.18.

Methyl 3-(2-((2-[2-(Methoxycarbonyl)ethyl][1,3]dioxolan-2-yl)methyl)(4-methylphenylsulfonyl)-amino)methyl[1,3]dioxolan-2-yl)propanoate (16). Trimethylsilyl trifluoromethanesulfonate (TMSOTf; 0.04 ml, 0.22 mmol) and 1,2-bis(trimethylsiloxy)ethane (1.29 g, 6.26 mmol) were added successively to **15** (1.02 g, 2.38 mmol) in CH₂Cl₂ (5 ml) at –40° and under Ar. The mixture was stirred at –40° for 1 h and then at r.t. for 5 h. The org. layer was washed with NaHCO₃ (2 × 75 ml), dried (Na₂SO₄), and concentrated. The residue was purified by FC (hexane/AcOEt 1:1) to afford **16** (1.0 g, 82%). Slimy liquid. *R_f* (hexane/AcOEt 1:1) 0.4. IR (KBr, film): 2953*s*, 2893*s*, 1732*s*, 1598*s*, 1495*m*, 1435*s*, 1335*s*, 1155*s*, 1091*s*, 1034*s*, 951*s*, 931*s*, 895*s*, 865*m*, 816*s*, 805*s*, 756*s*, 716*m*, 659*s*, 546*s*. ¹H-NMR (400 MHz, CDCl₃)²⁾: 7.70 (*d*, $J=8.2$, H-C(2,6) of tosyl); 7.25 (*d*, $J=8.2$, H-C(3,5) of tosyl); 3.81–3.71 (*m*, H_a-C(4',5')); 3.69–3.63 (*m*, H_b-C(4',5')); 3.66 (*s*, CH₂(5)); 3.65 (*s*, COOMe); 2.40 (*s*, Me-C(4) of tosyl); 2.32 (*t*, $J=7.5$, CH₂(2)); 1.91 (*t*, $J=7.5$, CH₂(3)). ¹³C-NMR (100 MHz, CDCl₃): 174.9 (C(1)); 142.9 (C(4) of tosyl); 138.9 (C(1) of tosyl); 129.8 (C(3,5) of tosyl); 127.8 (C(2,6) of tosyl); 110.5 (C(4)); 64.9 (C(4',5')); 51.7 (COOMe); 49.6 (C(5)); 31.1 (C(3)); 28.4 (C(2)); 21.6 (Me-C(4) of tosyl). ESI-MS: 538 ([*M*+Na]⁺). HR-MS: 538.1717 ([*M*+Na]⁺; calc. 538.1723).

3-(2-(((2-(2-Carboxyethyl)[1,3]dioxolan-2-yl)methyl)amino)methyl[1,3]dioxolan-2-yl)propanoic Acid (17). A soln. of sodium naphthalene in freshly distilled 1,2-dimethoxyethane (DME) was prepared by adding Na (0.21 g, 9.24 mmol) to a mixture of naphthalene (1.39 g, 10.9 mmol) in DME (15 ml) and stirring the resulting mixture at r.t. for 2 h under Ar. A soln. of **16** (0.68 g, 1.33 mmol) in DME (10 ml) was cooled at –78° under Ar. The sodium naphthalene soln. was added dropwise to the well-stirred soln. of **16**, until a light green color persisted. The reaction was quenched with H₂O (0.3 ml), leading to complete decoloration. The soln. was concentrated, and the residue was purified by FC (AcOEt/MeOH 2:1 to 1:2) to afford **17** (0.35 g, 78%). Pale yellow solid. M.p. 27°. *R_f* (AcOEt/MeOH 1:2) 0.19. IR (KBr): 3430*s* (br.), 2969*m*, 2899*m*, 1630*s*, 1567*s*, 1491*m*, 1407*s*, 1372*m*, 1303*m*, 1213*m*, 1138*m*, 1048*m*, 952*w*, 853*w*, 646*w* (br.). ¹H-NMR (400 MHz, CD₃OD)²⁾: 4.08–4.04 (*m*, H_a-C(4',5')); 4.03–4.00 (*m*, H_b-C(4',5')); 3.10 (*s*, CH₂(5)); 2.27 (*t*, $J=7.3$, CH₂(2)); 1.98 (*t*, $J=7.3$, CH₂(3)). ¹³C-NMR (100 MHz, CD₃OD): 181.2 (C(1)); 109.7 (C(4)); 66.2 (C(4',5')); 52.4 (C(5)); 32.8 (C(2)); 32.6 (C(3)). ESI-MS: 332 ([*M*-H]⁻). HR-MS: 332.1346 ([*M*-H]⁻; calc. 332.1345).

5-(4-Carboxy-2-oxobutyl)amino]-4-oxopentanoic Acid 8. Compound **17** (1.14 g, 3.43 mmol) and conc. HCl (10 ml) were stirred at 80° overnight. The pH was adjusted to 2–3 with an aq. soln. of NaOH, and the soln. was concentrated. The residue was dissolved in MeOH, and the insoluble solid was filtered. The solvent was removed, and the crude product was purified by recrystallization in MeOH/Et₂O. The beige solid **8** (0.06 g, 6%) was recovered by centrifugation. The yield was not optimized. M.p. 207°. IR (KBr): 3459*w*, 2978*m*, 2950*m*, 2918*m*, 2759*m*, 2631*m*, 2544*m*, 1738*s*, 1487*m*, 1426*m*, 1399*s*, 1384*s*, 1324*m*, 1293*m*, 1221*m*, 1192*m*, 1133*m*, 1099*s*, 1066*m*, 1011*s*, 969*s*, 914*s*, 839*m*, 824*m*, 719*m*, 667*m*, 620*m*, 547*m*. ¹H-NMR (200 MHz, D₂O): 4.08 (*s*, CH₂(5)); 2.74 (*t*, $J=6.5$, CH₂(3)); 2.46 (*t*, $J=6.5$, CH₂(2)). ¹³C-NMR (100 MHz, D₂O): 204.0 (*m*³⁾, C(4)); 179.1 (C(1)); 53.9 (*m*³⁾, C(5)); 35.5 (C(3)); 29.3 (C(2)). ESI-MS: 246 ([*M*+H]⁺), 268 ([*M*+Na]⁺), 244 ([*M*-H]⁻). HR-MS: 246.0976 ([*M*+H]⁺; calc. 246.0978), 268.0796 ([*M*+Na]⁺; calc. 268.0797).

Methyl 5-([4-(methoxycarbonyl)-2-oxobutyl]sulfonyl)-4-oxopentanoate 18. Na₂S (3.79 g, 48.6 mmol) in H₂O (25 ml) was added to **12** (10.0 g, 47.9 mmol) in acetone (120 ml). The yellow soln. was stirred at r.t. for 8 h before removal of the solvents. The residue was separated by FC (hexane/AcOEt 1:2) and purified by cold reprecipitation in MeOH/AcOEt to afford **18** (5.48 g, 79%). White solid. M.p. 32°. *R_f* (hexane/AcOEt 1:2) 0.4. IR (KBr): 2950*w*, 2883*w*, 1735*s*, 1699*s*, 1436*m*, 1412*s*, 1382*m*, 1330*s*, 1292*m*, 1252*m*, 1205*s*, 1103*m*, 1020*m*, 991*m*, 864*m*, 627*w*. ¹H-NMR (400 MHz, CDCl₃): 3.67 (*s*, COOMe); 3.36

2) Assignments according to the C-atom numbering as for **15**. Primed numbering is used for dioxolane C-atoms.

3) The *multiplet* is explained by H/D exchange due to the NMR solvent.

(s, CH₂(5)); 2.88 (*t*, *J* = 6.5, CH₂(3)); 2.61 (*t*, *J* = 6.5, CH₂(2)). ¹³C-NMR (100 MHz, CDCl₃): 203.8 (C(4)); 173.3 (C(1)); 52.2 (COOMe); 41.0 (C(5)); 35.9 (C(3)); 28.2 (C(2)). ESI-MS: 313 ([*M*+Na]⁺). Anal. calc. for C₁₂H₁₈O₆S (290.34): C 49.64, H 6.25; found C 49.80, H 6.27.

5-[(4-Carboxy-2-oxobutyl)sulfanyl]-4-oxopentanoic Acid (**9**). A soln. of **18** (1.54 g, 5.32 mmol) and 6*N* HCl (75 ml) was stirred at 50° for 2 h. The solvent was evaporated, and the residue was purified by recrystallization in *i*-PrOH to give **9** (1.24 g, 89%). White solid. M.p. 156°. *R*_f (MeOH) 0.7. IR (KBr): 3384–2537 (br.), 1694s, 1445*m*, 1426*m*, 1408s, 1383s, 1364*m*, 1329s, 1285*m*, 1257*m*, 1228s, 1193*m*, 1143*w*, 1115*w*, 1096*m*, 1075*m*, 1040*m*, 907*m*, 888*m*, 865*m*, 618*m*, 525*w*, 505*w*. ¹H-NMR (400 MHz, DMSO): 12.14 (s, COOH); 3.46 (s, CH₂(5)); 2.76 (*t*, *J* = 6.5, CH₂(3)); 2.41 (*t*, *J* = 6.5, CH₂(2)). ¹³C-NMR (100 MHz, DMSO): 204.5 (C(4)); 173.6 (C(1)); 40.6 (C(5)); 35.4 (C(3)); 27.7 (C(2)). ESI-MS: 285 ([*M*+Na]⁺), 280 ([*M*+H₂O]⁺), 263 ([*M*+H]⁺). Anal. calc. for C₁₀H₁₄O₆S (262.28): C 45.79, H 5.38; found C 45.63, H 5.25.

5-[(4-Carboxy-2-oxobutyl)sulfonyl]-4-oxopentanoic Acid (**11**). *m*-Chloroperbenzoic acid (*m*-CPBA) (2.06 g, 11.9 mmol) was added dropwise to a soln. of **18** (1.00 g, 3.45 mmol) in CH₂Cl₂ (80 ml) at –40°. After 20 min, the cooling bath was removed, and the mixture was stirred at r.t. for 2.5 h. NaHSO₃ (25 ml) was added to the soln., and the org. phase was washed with Na₂CO₃ (1 × 50 ml) and brine (2 × 50 ml), dried (MgSO₄), and concentrated. The residue was separated by FC (hexane/AcOEt 1:2) and purified by cold reprecipitation in MeOH/*i*-PrOH to provide **19** (0.94 g, 85%). White solid. M.p. 40°. *R*_f (hexane/AcOEt 1:2) 0.3. IR (KBr): 3001*m*, 2974*m*, 2957*m*, 2940*m*, 2921*m*, 1746s, 1717s, 1438*m*, 1416*m*, 1398*m*, 1383*m*, 1373*m*, 1364*m*, 1333*m*, 1314s, 1277*m*, 1257*m*, 1216s, 1196s, 1176s, 1142s, 1099*m*, 1081*m*, 1044*w*, 988*m*, 970*m*, 852*m*, 790*m*, 748*m*, 676*w*, 551*m*, 512*m*. ¹H-NMR (400 MHz, CDCl₃): 4.33 (s, CH₂(5)); 3.69 (s, COOMe); 2.95 (*t*, *J* = 6.3, CH₂(3)); 2.65 (*t*, *J* = 6.3, CH₂(2)). ¹³C-NMR (100 MHz, CDCl₃): 197.9 (C(4)); 172.6 (C(1)); 62.9 (C(5)); 52.2 (COOMe); 39.2 (C(3)); 27.6 (C(2)). ESI-MS: 345 ([*M*+Na]⁺). Anal. calc. for C₁₂H₁₈O₈S (322.34): C 44.72, H 5.63; found C 44.84, H 5.62.

A soln. of **19** (4.4 g, 13.7 mmol) and 6*N* HCl (400 ml) was stirred at 50° for 160 min. The solvent was evaporated, and the residue was purified by recrystallization in THF to give **11** (3.02 g, 75%). White solid. M.p. 156°. IR (KBr): 3422–2565 (br.), 2967s, 2921s, 1723s, 1697s, 1429s, 1400s, 1372*m*, 1361*m*, 1327s, 1300*m*, 1256s, 1237s, 1206*m*, 1186*m*, 1153*m*, 1119s, 1079s, 1036*m*, 1018*m*, 942*m*, 866*m*, 855*m*, 648*w*, 522*w*, 486*m*. ¹H-NMR (400 MHz, DMSO): 12.22 (s, COOH); 4.58 (s, CH₂(5)); 2.84 (*t*, *J* = 6.5, CH₂(3)); 2.43 (*t*, *J* = 6.5, CH₂(2)). ¹³C-NMR (100 MHz, DMSO): 198.7 (C(4)); 173.3 (C(1)); 63.3 (C(5)); 38.7 (C(3)); 27.4 (C(2)). ESI-MS: 293 ([*M*–H][–]). Anal. calc. for C₁₀H₁₄O₈S (294.28): C 40.82, H 4.80; found C 40.78, H 4.76.

5-[(4-Carboxy-2-oxobutyl)sulfinyl]-4-oxopentanoic Acid (**10**). Monoperoxyphthalic acid magnesium salt hexahydrate (MMPP; 0.77 g, 1.55 mmol) in H₂O (10 ml) was added to **9** (0.51 g, 1.95 mmol) in EtOH (10 ml). The heterogeneous mixture was heated until solubilization and then stirred at r.t. for 20 min. The obtained solid was filtered and yielded **10**. The soln. was concentrated, and the residue was treated in MeOH. The insoluble solid was again filtered. The white solid **10** (0.45 g, 84%) was obtained but was contaminated with 4% of **11**. M.p. 172°. *R*_f (AcOEt/MeOH 1:1) 0.12. IR (KBr): 3275–2886 (br.), 1751*m*, 1700s, 1717s, 1446*m*, 1406*m*, 1357*m*, 1325*m*, 1301*m*, 1255*w*, 1230*m*, 1214*m*, 1166*m*, 1095*m*, 1031s, 1015*m*, 993*m*, 898*w*, 689*w*, 618*w*. ¹H-NMR (400 MHz, DMSO): 12.18 (s, COOH); 4.13 (*d*, *J* = 14.7, CH₂(5)); 4.03 (*d*, *J* = 14.7, CH₂(5)); 2.80 (*dt*, *J* = 18.3, *J* = 6.2, CH₂(3)); 2.76 (*dt*, *J* = 18.3, *J* = 6.2, CH₂(3)); 2.44 (*t*, *J* = 6.2, CH₂(2)). ¹³C-NMR (100 MHz, DMSO): 202.3 (C(4)); 173.5 (C(1)); 61.9 (C(5)); 38.7 (C(3)); 27.4 (C(2)). ESI-MS: 277 ([*M*–H][–]).

Biochemical Studies. PBGS Purification. The procedure for the PBGS isolation and purification has been published in [29].

PBGS Assay and Determination of IC₅₀ Values. The PBGS is a colorimetric assay based on the reaction between PBG (**2**) and 4-(dimethylamino)benzaldehyde (*Ehrlich's* reagent) [30]. The assay conditions were kinetic buffer (Bis-Tris propane, pH 8.5 (100 mM), MgCl₂ (10 mM)), inhibitors (between 125 mM and 125 · 10^{–3} μM), PBGS (4 μg ml^{–1}), and ALA (**1**; 2 mM). PBGS and inhibitor were preincubated for 30 min at 37° prior to addition of the ALA (**1**) soln., preheated to 37°. Reaction was allowed to proceed for 15 min and stopped by adding the mixture (350 μl) to the stop reagent (350 μl; 25% Cl₃CCOOH). After centrifugation (3 min, 13500 rpm), the supernatant (450 μl) was treated with the *Ehrlich's* reagent (450 μl; 4-(dimethylamino)benzaldehyde (0.4 g) in AcOH (10 ml) and HClO₄

(10 ml)). After 10–15 min, the quantity of product formed was determined by measuring the absorbance at 555 nm ($\epsilon = 60200 \text{ M}^{-1} \text{ cm}^{-1}$). The experimental data were fit against the *Eqn. 1*, assuming a linear proportionality between the absorbance and the product concentration. The definitions of the parameters are: v is the velocity, $[I]$ is the inhibitor concentration (in mM), IC_{50} is the inhibitor concentration giving 50% inhibition and h is the *Hill* coefficient.

$$v_i/v_o = 1/(1 + ([I]/IC_{50}))^h \quad (1)$$

The values for IC_{50} to give the best fit to *Eqn. 1* were determined by using the regression wizard of sigmaplot 9.0.

Determination of Time-Dependent or -Independent Inhibition, Mode of Inhibition and K_i Values. Time dependence of inhibition was determined by pre-incubating the inhibitor with a fixed concentration of PBGS ($4 \mu\text{g ml}^{-1}$) and ALA (**1**; 2 mM) in kinetic buffer (Bis-Tris propane, pH 8.5 (100 mM), MgCl_2 (10 mM)) between 30 s and 30 min. The PBGS activity was determined as described above. In case time-independent inhibition was observed, the K_i values were established using *Michaelis–Menten* kinetics. The apparent *Michaelis* constant, $K_{M\text{app}}$, and the maximal absorbance A_{max} (correlated linear to v_{max}) were determined for each inhibitor concentration. For mixed inhibition behavior, plots of inhibitor concentration vs. $K_{M\text{app}}/A_{\text{maxapp}}$ and inhibitor concentration vs. $1/A_{\text{maxapp}}$ yield K_i and αK_i , resp. For inhibitors that show time-dependent behavior, mode of inhibition and potency were determined as described by *Copeland* [31]. The dependance of product formation (measured at absorbance of 555 nm) on the preincubation time of the inhibitor with the enzyme was determined. The obtained data were used to determine k_{obs} applying *Eqn. 2*:

$$A/A_0 = e^{-k_{\text{obs}}t} \quad (2)$$

The dependence of k_{obs} on inhibitor concentration directly yields the apparent inhibition constant $K_{i\text{app}}$ through the application of *Eqn. 3*, with k_4 representing the dissociation rate constant for a simple reversible slow binding mode of time-dependent inhibition ($\text{E} + \text{I} \xrightleftharpoons[k_4]{k_3} \text{E-I}$):

$$k_{\text{obs}} = k_4 \cdot (1 + [I]/K_i) \quad (3a)$$

or

$$[I] = K_i \cdot (k_{\text{obs}}/k_4) - K_i \quad (3b)$$

So a plot of $[I]$ vs. k_{obs} yields K_i as y-axis intercept. In the case of **11**, the apparent K_i value was only determinable if a *Hill* coefficient h was introduced into *Eqn. 3a* to give *Eqn. 4*.

$$k_{\text{obs}} = k_4 \cdot \left(1 + ([I]/K_i)^h\right) \quad (4)$$

In this case K_i was determined *via* non-linear regression using sigmaplot 9.0.

REFERENCES

- [1] D. Shemin, *Method. Enzymol.* **1970**, *XVII Part A*, 205.
- [2] A. M. Cheh, J. B. Neilands, *Top. Curr. Chem.* **1976**, *29*, 123.
- [3] J. E. Falk, E. I. B. Dresel, E. Rimington, *Nature* **1953**, *172*, 292.
- [4] P. M. Jordan, J. S. Seehra, *J. Chem. Soc., Chem. Commun.* **1980**, 240.
- [5] R. Neier, in ‘Advances in Nitrogen Heterocycles’, Ed. C. J. Moody, J. A. I. Greenwich, 1996, Vol. 2, p. 35.
- [6] P. T. Erskine, E. Norton, J. B. Cooper, R. Lambert, A. Coker, G. Lewis, P. Spencer, M. Sarwar, S. P. Wood, M. J. Warren, P. M. Schooling-Jordan, *Biochemistry* **1999**, *38*, 4266.
- [7] P. T. Erskine, L. Coates, R. Newbold, A. A. Brindley, F. Stauffer, S. P. Wood, M. J. Warren, J. B. Cooper, P. M. Schooling-Jordan, R. Neier, *FEBS Lett.* **2001**, *503*, 196.

- [8] J. Kervinen, E. K. Jaffe, F. Stauffer, R. Neier, A. Wlodawer, A. Zdanov, *Biochemistry* **2001**, *40*, 8227.
- [9] P. T. Erskine, N. Senior, S. Awan, R. Lambert, G. Lewis, I. J. Tickle, M. Sarwar, P. Spencer, P. Thomas, M. J. Warren, P. M. Shoolingin-Jordan, *Nature Struct. Biol.* **1997**, *4*, 1025.
- [10] N. Frankenberg, P. R. Erskine, J. Cooper, P. M. Shoolingin-Jordan, D. Jahn, D. Heinz, *J. Mol. Biol.* **1999**, *289*, 591.
- [11] S. Breinig, J. Kervinen, L. Stith, A. S. Wasson, R. Fairmann, A. Wlodawer, A. Zdanov, E. K. Jaffe, *Nature Struct. Biol.* **2003**, *10*, 757.
- [12] L. Coates, G. Beaven, P. T. Erskine, S. I. Beale, Y. J. Avissar, R. Gill, F. Mohammed, S. P. Wood, P. Shoolingin-Jordan, J. B. Cooper, *J. Mol. Biol.* **2004**, *342*, 563.
- [13] D. W. Banner, A. C. Bloomer, G. A. Petsko, D. C. Philipps, C. I. Pogson, I. A. Wilson, *Nature* **1975**, *255*, 609.
- [14] E. K. Jaffe, *Acta Crystallogr., Sect. D* **2000**, *56 (Pt 2)*, 115.
- [15] E. K. Jaffe, *J. Biol. Inorg. Chem.* **2003**, *8*, 176.
- [16] E. K. Jaffe, *Chem. Biol.* **2003**, *10*, 25.
- [17] R. M. Lüönd, J. Walker, R. Neier, *J. Org. Chem.* **1992**, *57*, 5005.
- [18] C. Jarret, F. Stauffer, M. E. Henz, M. Marty, R. M. Lüönd, J. Bobalova, P. Schurmann, R. Neier, *Chem. Biol.* **2000**, *7*, 185.
- [19] F. Stauffer, E. Zizzari, C. Soldermann-Pissot, J.-P. Faurite, R. Neier, *Chem. Biol.* **2001**, *55*, 314.
- [20] P. T. Erskine, R. Newbold, J. Roper, A. Coker, M. J. Warren, P. M. Shoolingin-Jordan, S. P. Wood, J. B. Cooper, *Protein Sci.* **1999**, *8*, 1250.
- [21] P. T. Erskine, R. Newbold, A. A. Brindley, S. P. Wood, P. M. Shoolingin-Jordan, M. J. Warren, J. B. Cooper, *J. Mol. Biol.* **2001**, *312*, 133.
- [22] E. K. Jaffe, J. Kervinen, J. Martins, F. Stauffer, R. Neier, A. Wlodawer, A. Zdanov, *J. Biol. Chem.* **2002**, *277*, 19792.
- [23] F. Frere, W. D. Schubert, F. Stauffer, N. Frankenberg, R. Neier, D. Jahn, D. W. Heinz, *J. Mol. Biol.* **2002**, *320*, 237.
- [24] S. F. MacDonald, *Can. J. Chem.* **1974**, *52*, 3257.
- [25] R. M. Lüönd, J. Walker, R. Neier, *J. Org. Chem.* **1992**, *57*, 5005.
- [26] I. W. Still, W. Daoquan, *Phosphorus, Sulfur Silicon Relat. Elem.* **1991**, *62*, 83.
- [27] W. D. Closson, S. Ji, S. Schulenberg, *J. Am. Chem. Soc.* **1970**, *92*, 650.
- [28] W. D. Closson, P. A. Wriede, S. Bank, *J. Am. Chem. Soc.* **1966**, *88*, 1581.
- [29] F. Frere, M. Nentwich, S. Gacond, D. M. Heinz, R. Neier, N. Frankenberg-Dinkel, *Biochemistry* **2006**, *45*, 8243.
- [30] D. Mauzerall, S. Granick, *J. Biol. Chem.* **1956**, *219*, 435.
- [31] R. A. Copeland, in 'Enzymes, a practical introduction to structure, mechanism and data analysis', Wiley-VCH, 2000, pp. 237.