Inhibition Studies of Porphobilinogen Synthase from *Escherichia coli* Differentiating between the Two Recognition Sites

Frédéric Stauffer,[b] Eleonora Zizzari,[a] Caroline Engeloch-Jarret,[c] Jean-Philippe Faurite,[a] Janette Bobálová,[d] and Reinhard Neier*[a]

Porphobilinogen synthase condenses two molecules of 5-aminolevulinate in an asymmetric way. This unusual transformation requires a selective recognition and differentiation between the substrates ending up in the A site or in the P site of porphobilinogen synthase. Studies of inhibitors based on the key intermediate first postulated by Jordan allowed differentiation of the two recognition sites. The P site, whose structure is known from X-ray crystallographic studies, tolerates ester functions well. The A site interacts very strongly with nitro groups, but is not very tolerant to ester functions. This differentiation is a central factor in the asymmetric handling of the two identical substrates. Finally, it could be shown that the keto group of the substrate bound at the A site is not only essential for the recognition, but that an increase in electrophilicity of the carbon atom also increases the inhibition potency considerably. This has important consequences for the recognition process at the A site, whose exact structure is not yet known.

KEYWORDS:

biosynthesis · inhibitors · intermediates · lyases · porphobilinogen synthase

Introduction

The biosynthesis of the tetrapyrrolic skeleton of the "pigments of life"[1] has fascinated chemists and biologists since their discovery and their structure determination.[2] Starting from the first dedicated intermediate in the biosynthesis of the tetrapyrrolic dyes, 5-aminolevulinic acid (ALA, **1**), the macrocyclic skeleton is synthesized in only three enzyme-catalyzed steps in a highly convergent way (Scheme 1).[3] Two condensation steps lead first to porphobilinogen (PBG, **2**)[4, 5] and then to the linear compound hydroxybilane, which cyclizes and rearranges to the common precursor of all pigments of life, uroporphyrinogen III (Uro III, **3**).[6] The synthetic efficiency of the biosynthesis and the importance of the tetrapyrrolic cofactors have further enhanced the interest in studies of the biosynthetic pathway.

In this three-step sequence, the first step, formation of PBG (**2**), cannot easily be imitated by chemists (Scheme 2).[7–14] In contrast to this observation, the tetramerization and cyclization have been shown to occur in the presence of acids, yielding the natural regioisomer, Uro III (**3**), as the major product.[15] However, the chemical condensation of ALA (**1**) in the presence of oxygen gives an appreciable amount of the oxidized pyrazine **4**.[16] In the absence of oxygen the symmetric diimine is formed as the major product and a small amount of pseudo-PBG has been observed as a side product.[17, 18] Treating ALA (**1**) with Amberlite TR-45 for a long time has been reported to give some PBG (**2**).[16, 17]

Porphobilinogen synthase (PBGS, E.C. 4.2.1.24), also called 5-aminolevulinate dehydratase (ALAD), the enzyme responsible for the nonsymmetrical condensation of the two molecules of ALA (**1**), has been isolated and studied for a long time.[19] PBGS is widely distributed in bacteria, plants, animals, and humans.[14] All PBGSs studied so far share a considerable degree of homology[20] and most enzymes were shown to be present as octamers.[21, 22] In early studies it could be shown that a Schiff base intermediate between ALA (**1**) and a lysine side chain located in the active site of PBGS can be trapped by reduction with $NaBH_4$.[23] This result was one of the important motivations to draw a close mechanistic analogy between PBGS and the aldolases of

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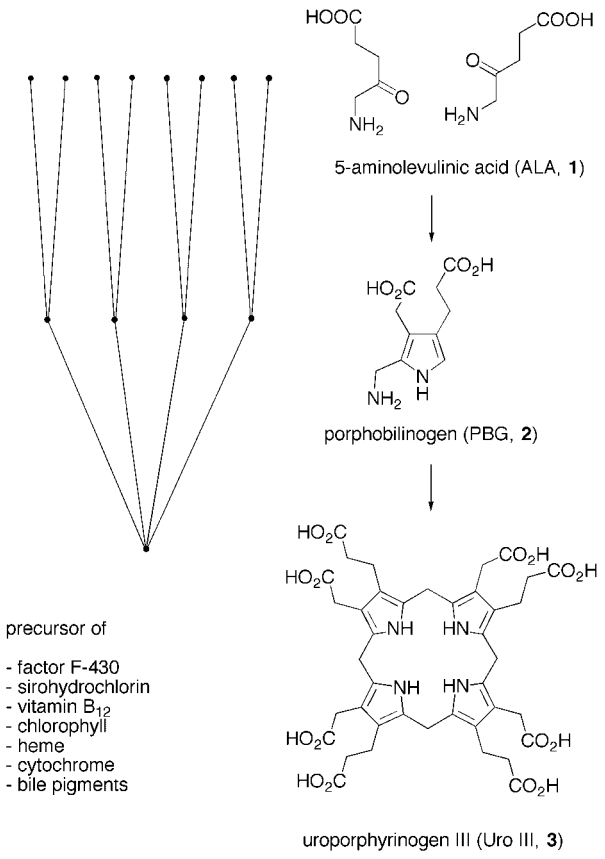
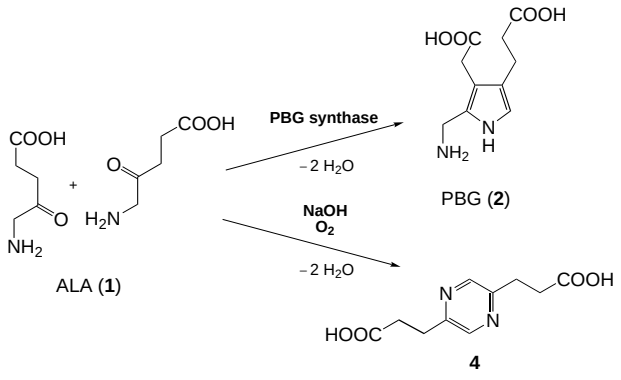


Scheme 1. *The convergent biosynthesis of uroporphyrinogen III (**3**) from eight molecules of 5-aminolevulinic acid (**1**), as determined by the biosynthetic and synthetic studies performed in the groups of Akhtar, Arigoni, Battersby, Eschenmoser, Jordan, Müller, Scott, Shemin, and Thauer (for a review of this pathway see ref. [69]). On the left, the retrosynthetic tree for the transformation of eight molecules of 5-aminolevulinic acid (**1**) into four molecules of porphobilinogen (**2**) and finally into one molecule of uroporphyrinogen III (**3**) is symbolically represented.*



Scheme 2. *The dichotomy between the biosynthetic and the chemical dimerization of 5-aminolevulinic acid (**1**).*

type I.[23] The influence on the catalytic activity of PBGS of bivalent metal ions like Zn^{II} and Mg^{II} was recognized and intensively studied.[24] For the Zn^{II}-containing enzymes, the three cysteine residues binding the metal ion could be identified.[25] For the Mg^{II}-containing enzymes it was assumed that the aspartate-rich region replacing the zinc finger element would form the ligands for the metal ion. One of the most important findings was the determination of the sequence of recognition of the two substrates. Using an elegant pulse-labeling experiment Jordan and Seehra could demonstrate that the first ALA (**1**) recognized by PBGS will form the propionic acid side chain of PBG (**2**).[26] ALA (**1**) forming the propionic acid side chain will first interact with the P site of the enzyme, and the second ALA molecule will interact with the A site.[26] Based on this finding an alternative mechanism for the transformation of ALA (**1**) to PBG (**2**) was proposed.[6, 26, 27] An impressive effort allowed characterization of the complexes between modified PBGS and its natural substrate ALA (**1**) by sophisticated NMR spectroscopic techniques.[28] These spectroscopic measurements identified the Schiff base intermediate formed with the P site of the enzyme and gave indications about the protonation state of both this enzyme – substrate complex and the product – enzyme complex.[28, 29]

Molecular biology has allowed the determination and comparison of the sequences of PBGSs from many sources.[20] More importantly, it became possible to overexpress this enzyme. In recent years the high-resolution X-ray crystallographic structures of PBGSs from three different sources have been published.[25, 30–32] The structures of the enzymes with an empty active site and of the enzymes containing one of the classical inhibitors, levulinic acid, are now available (Brookhaven Protein Data Bank, PDB entries 1aw5, 1ylv, 1b4k). The overall structures of the PBGSs from the three sources *Saccharomyces cerevisiae*, *Escherichia coli*, and *Pseudomonas aeruginosa* are very similar. The three enzymes crystallize as an octamer, which is a tetramer of a dimer. The overall structure of the protein corresponds to a TIM barrel (Figure 1 A).[25, 33] The N-terminal arm is responsible for a large number of contacts between the two units of the homodimer. The Zn^{II} ion present in the active site is coordinated by three cysteines (Cys 133, Cys 135, and Cys 143; numbering for PBGS from *S. cerevisiae*). The X-ray crystallographic structure of PBGS showed not only the lysine residue (Lys 263 for PBGS from *S. cerevisiae*) identified by the reduction with $NaBH_4$, but also the presence of a second lysine residue in the active site (Lys 210, numbering for PBGS from *S. cerevisiae*). The structures containing levulinic acid at the P site clearly indicated the formation of the Schiff base between the carbonyl group and the ε-amino group of Lys 263 (PBGS from *S. cerevisiae*) and the hydrogen bonding of the carboxylate to Tyr 329 and Ser 290 (numbering for PBGS from *S. cerevisiae*).

The structures of PBGS not containing the inhibitor bound to the active site were not well resolved in the region of the amino acids 220 to 233. When the inhibitor was bound to the enzyme this lid covers tightly the active site (see Figures 1 B and C). The structure of PBGS from *P. aeruginosa* could be determined at a high resolution.[30] The overall structure is similar to those observed for the two other PBGSs resolved. Within the same crystal the two active sites of the asymmetric dimer were occupied by a single molecule of levulinic acid, but only one site was completely closed by the lid. Only one Mg^{II} ion is present per dimer. The Mg^{II} ion is not positioned at the active site, but at the interface between the two subunits.[30] A catalytic role has been attributed to the Zn^{II} ions present in the active site. The Mg^{II} ion

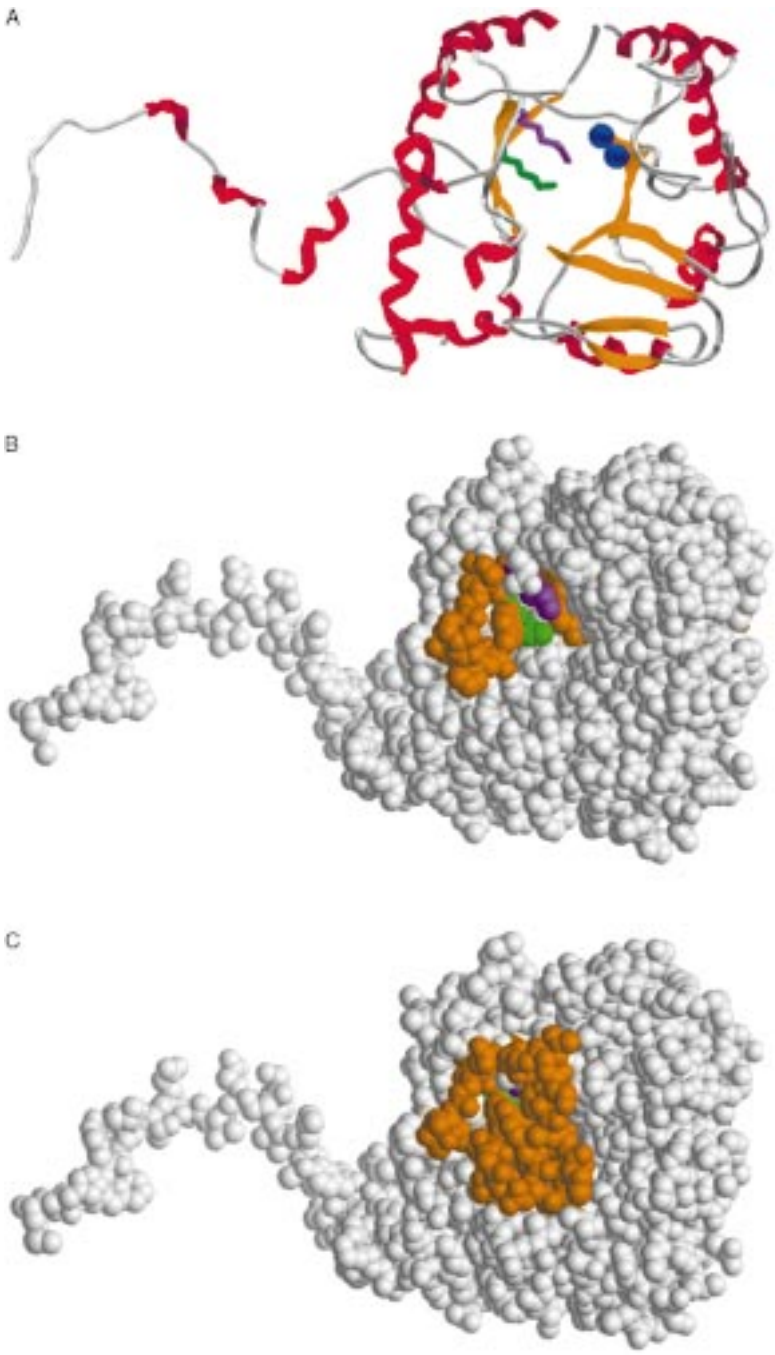


Figure 1. *A: Structure of PBGS from* S. cerevisiae. *The TIM barrel fold and the N-terminal arm are clearly visible in the schematic presentation of the secondary structure elements. The two lysines of the active site are colored in green (Lys 263) and purple (Lys 210); the two Zn^{II} ions are colored in blue. B: Space-filling model of PBGS without an inhibitor bound in the active site. The lid part of the enzyme is colored in orange. The active site is accessible from the outside. C: The structure of PBGS bound to levulinate. The lid is much more structured and covers the active site almost completely . The two lysine residues of the active site are not accessible from the outside. All structures were drawn with the program SYBYL 6.6 (TRIPOS Inc., St. Louis) by using the coordinates deposited in the Brookhaven Protein Databank (PDB entries 1aw5 and 1ylv, which were determined at 2.3 and 2.15 Å resolution, respectively).*

must play another role and an allosteric interaction of the metal ion has been proposed that induces the complete closure of the lid.[30]

Right from the beginning of the studies of PBGS, inhibition experiments were undertaken to explore the active site of the enzyme.[34–36] Our group has studied the inhibition behavior of PBGS isolated from *Rhodobacter sphaeroides* and from *E. coli*.[14, 37] The goal of our systematic studies is to characterize the recognition sites of PBGS sufficiently, so that adequate analogues of the postulated intermediates can be synthesized and their inhibition behavior can be tested. The long-term objective is to obtain information relevant for the detailed understanding of the mechanism and at the same time to have a collection of adequate inhibitors available suited for cocrystallization with the enzyme.

Several groups have made significant contributions to our knowledge of the inhibition behavior of substrate analogues.[14, 38, 39] These systematic studies have been facilitated by the availability of large quantities of recombinant PBGS from organisms like *E. coli*, *S. cerevisiae*, and *Pisum sativum*.[38] Only recently we could show that the kinetics of PBGS depend highly on the concentration of the natural substrate used during the test.[41] Using substrate concentrations between 4 and 80 μM a parabolic curve is observed if an inverse plot (1/[S] against 1/*v*) is used to analyze the experimental results. This parabolic curve is compatible with a mechanism in which two substrate molecules are sequentially recognized at the active site of the enzyme (Figure 2). The Michaelis – Menten constants for the two sites can be calculated to be $K_{M,1} = 4.6$ μM and $K_{M,2} =$ 66 μM. If, however, substrate concentrations between 80 and 400 μM are used, simple Michaelis – Menten kinetics are observed. This behavior has been interpreted as the consequence of saturating the first recognition site of PBGS and thereby observing only the competition between binding of our inhibitors and the recognition of the second substrate by the enzyme saturated at the P site with the natural substrate.

This peculiarity of PBGS allows one to draw conclusions not only from the size of the inhibition constants, but also from the change in inhibition behavior. We interpret pure competitive inhibition as a consequence of competition between the natural substrate and the inhibitor for the A site of the enzyme. Observation of mixed or uncompetitive inhibition is a clear sign for a more complex interaction; probably the inhibitor competes with the natural substrate also for the P site. In the absence of functional groups capable of forming irreversible covalent bonds with the enzyme, slow binding or even irreversible inhibition is an indication that several functional groups interact synergistically with the amino acids of the active site. It is probable that interactions at both sites of the enzyme, the A and the P site, are responsible for this behavior.

PBGS will recognize two δ-amino acids **1** first, which will be transformed into an aromatic amino dicarboxylic acid **2**. What-

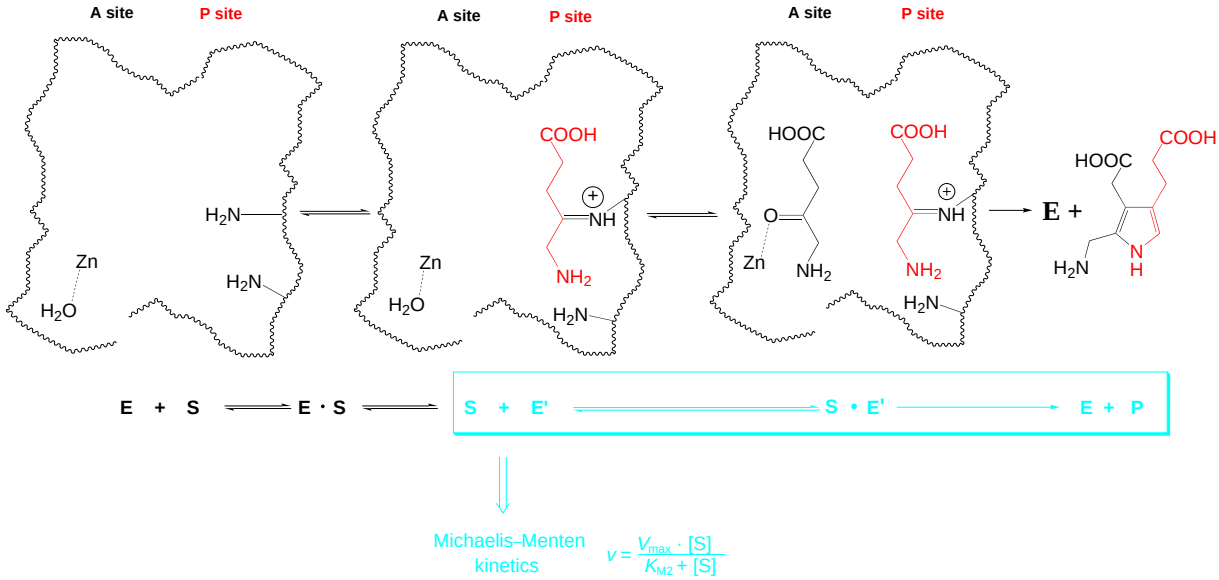
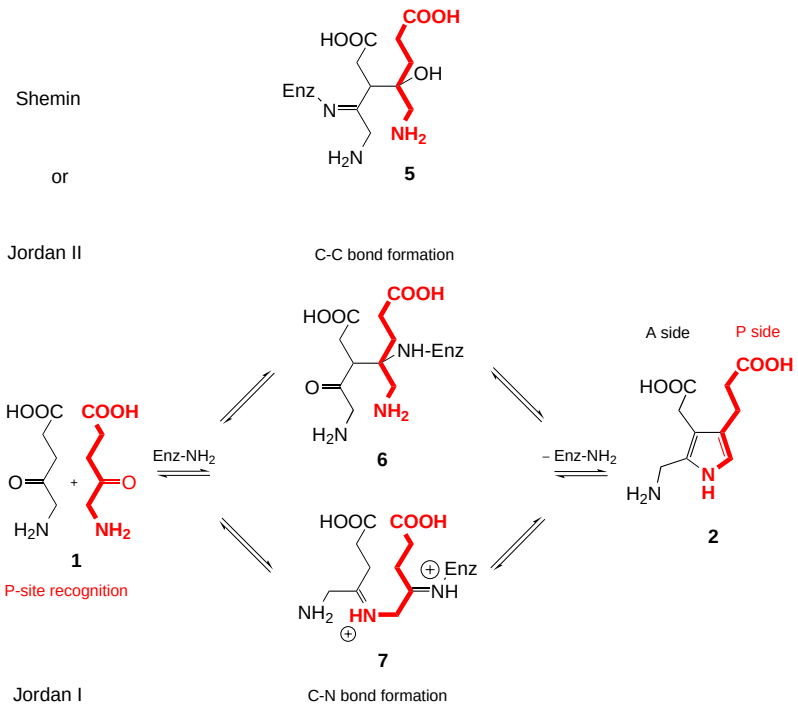


Figure 2. *Mechanistic and kinetic interpretation of the fact that a Michaelis – Menten kinetics is observed for the inhibition of PBGS.*

ever is the mechanism of this transformation, at one point an aliphatic dicarboxylic acid must be present as intermediate. In a recent study we have shown that aliphatic γ-ketodicarboxylates are recognized by PBGS.[41] The chain length is an important factor determining the inhibition behavior as well as the size of the inhibition constant. 4-Oxopimelic acid is a moderate competitive inhibitor, whereas 4-oxosebacic acid is an irreversible inhibitor. We have interpreted this significant change in the inhibition behavior as a function of the chain length as a hint in favor of the mechanism first proposed by Jordan (Scheme 3, mechanism Jordan I).[41]

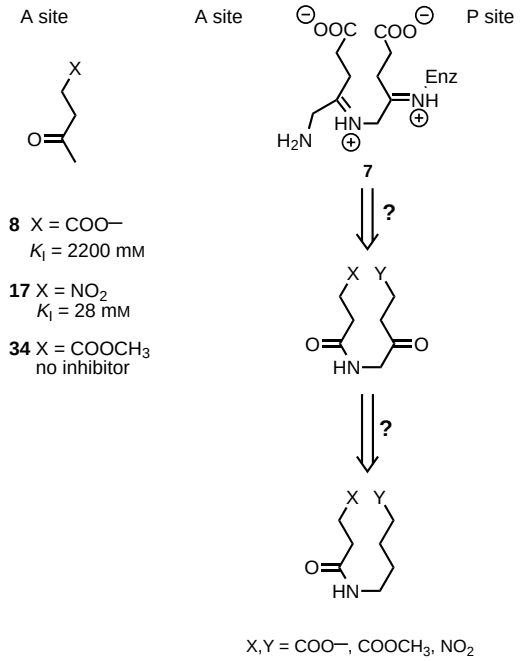
Concentrating the mechanistic analysis on the key step, which will connect for the first time the two identical substrates, thereby creating the differentiation between the two ALA molecules (**1**), three proposals have been discussed. The first proposal made by Shemin over 30 years ago used a close analogy with the aldolases of type I as a guideline. The central point of this mechanism is the formation of a Schiff base intermediate between the enzyme and the A-site substrate. This Schiff base is then transformed into its corresponding enamine, which has the correct reactivity to form the crucial C–C bond leading to the intermediate **5**. Despite its attractiveness from the mechanistic point of view,[23] this proposal has been largely dismissed due to the fact that Shemin proposed the recognition of the A-site substrate first. This is clearly not compatible with the results of Jordan's pulse-labeling experiments. The formation of the same C–C bond, leading to the intermediate **6** as the key compound joining the two substrate molecules for the first time, has been proposed by Jordan later (Jordan II mechanism).[6] His proposal incorporates the sequence of recognition events. The mechanism postulated by Jordan first (Jordan I mechanism) proposes the formation of the key intermediate **7**, which involves the Schiff base between the two reacting starting materials. This mechanism has the advantage of following the mechanism proposed for the Knorr pyrrole synthesis.[40] However, it has been recognized that stereoelectronically this ring closure reaction is not favorable.[42] It is worth mentioning that it has not been possible to trap this second Schiff base despite considerable efforts.[43]



Scheme 3. *The three key intermediates postulated by Shemin and Jordan for the biosynthesis of PBG (**2**). Enz = enzyme = PBGS.*

Results and Discussion

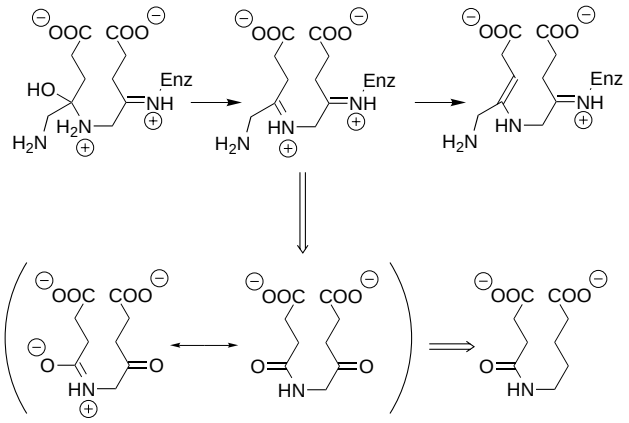
Studies with substrate analogues showing competitive inhibition behavior had shown that for a good recognition the γ-keto function was necessary.[44, 45] Replacing the amino group of the substrate had no significant effect. This result is compatible with our kinetic interpretation postulating the interaction of these inhibitors at the A site. The amino group at this position does not undergo any change during the reaction. Finally, the carboxylate is important for a good recognition. Replacing the carboxylate by an ester group reduces the affinity considerably, usually K_I increases by at least a factor of 10 – 20. The carboxylate function can be substituted by a nitro group and a considerable increase in the affinity can be observed: K_I decreases by a factor of about 100. Synthesizing and testing dicarboxylates and analogues thereof, which contain the three functional groups mentioned above, should allow analysis not only of the recognition selectivity at the A site, but also at the P site (Scheme 4).



Scheme 4. *A comparison of the Jordan I intermediate with the simplified structures was used to construct families of inhibitors. The inhibition potency of the substrate analogues* ***8****,* ***17****, and* ***34*** *has been determined for comparison with the inhibition constants of the more complex analogues of intermediates.*[37, 45, 68]

To work out this analysis, the inhibitors have to be recognized at both sites and we have to be able to predict the orientation of the inhibitor in the active site. The analysis of all the reported inhibition studies of PBGS indicates that replacing the keto function of an inhibitor reduces substantially its affinity for the A site. We hoped, however, that the A site would be more tolerant to the introduction of an amide function than the P site, where a keto function is necessary to form the Schiff base.

Introducing an amide function at this position could have additional advantages (Scheme 5). The key intermediate of the Jordan I mechanism possesses an iminium ion connecting the C4



Scheme 5. *Comparison of intermediates postulated in the Jordan I mechanism (top row) with the analogues containing an amide function as the central part (bottom row).*

atom of the A-site ALA with the amino group of the P-site ALA. Furthermore, in the next step in the formation of the enamine, the C4 atom stays sp^2-hybridized. This double-bond character was not completely imitated by using the dioxosebacic acid as inhibitor. An amide function should at least partially imitate this double-bond character. At the same time the amide group mimics also the charge distribution found in the iminium ion and to some extent also the polarity that is found when the amine group of the P-site substrate is attacking the carbonyl function of the A-site substrate. The major problem in using an amide function resides in the fact that there is a restricted rotation around this partial C-N double bond. It is known that the s-*trans* conformer is preferred in solution for secondary amides, whereas we have to suppose that the diacids are bound in a conformation that is close to the relative position of the two carboxylate groups as they are found in the product.

In view of these arguments we decided to study a series of substrate analogues first to obtain data which should allow to evaluate the importance of the replacement of the γ-keto function by an amide group (Tables 1 and 2). The comparison of the inhibition potency of the nitro compounds (see Table 2) with the corresponding carboxylates (see Table 1) shows that in three out of five cases the nitro compound is better recognized than the carboxylate. The difference between the nitro compound and the carboxylates is usually considerable. In the case of compounds **17** and **8**, the nitro compound is almost 80 times better recognized than the carboxylate. The difference for the α-fluoroketones **18** and **9** is, however, rather small: Recognition of the nitro compound is only better by a factor of four. The introduction of an α-fluoro substituent increases the electrophilicity of the carbon atom of the ketone and should reduce the nucleophilicity of its oxygen atom. The inhibition potency seems to be considerably increased by this change in electron density. This comes as a surprise, because most hypotheses published so far have assumed that the keto function acts as a ligand for a Lewis acid.[46–49] It has been speculated that Zn^{II} would function as the Lewis acid, which activates the ketone and facilitates thereby the transformation into the enol or the enolate ion. The

Table 1. *Inhibition of PBGS by substrate analogues in which the keto function is modified.*

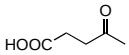
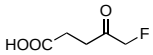
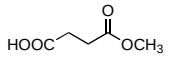
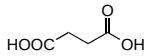
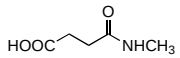
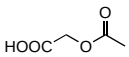
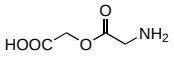
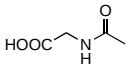
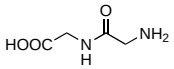
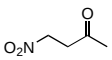
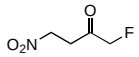
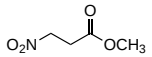
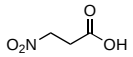
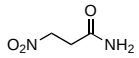
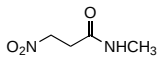
Compound		K_I [μM]	Inhibition type/remarks
	**8**	2200	competitive
	**9**	85	competitive
	**10**	38400	competitive
	**11**	12500	competitive
	**12**	36400	competitive
	**13**	24500	competitive
	**14**	18200	competitive
	**15**	18800	competitive
	**16**	> 150000	not detectable

Table 2. *Inhibition of PBGS by substrate analogues containing a nitro function instead of the carboxy group.*

Compound		K_I [μM]	Inhibition type/remarks
	**17**	28	competitive
	**18**	21	competitive
	**19**	1880	competitive
	**20**	44900	competitive
	**21**	–	interference with the Ehrlich reaction
	**22**	222800	competitive

observation of this strong increase in inhibition potency is not in accordance with these postulated interactions with the enzyme active site. The combination of two factors that individually increase the inhibition potency seems not to be additive. The nitro fluoro compound **18** shows only a slight decrease in K_I compared to the nitroketone **17**, whereas the introduction of the fluoro substituent into levulinic acid decreases the K_I considerably: A change of K_I from 2200 μM for **8** to 85 μM for compound **9** is observed. Changing the keto function for a carboxylate function or amide function thereof reduces the inhibition potency considerably. The K_I value of compounds **20** and **22**, in which the keto function has been replaced by a deactivated carbonyl group, is 1600- and 8000-fold bigger, respectively, than the K_I value for the standard compound **17**. The amide cannot be considered to be an inhibitor at all because the K_I is so much bigger than the K_M of the natural substrate. The inhibition constant for the primary amide compound **21** could not be determined because this inhibitor interfered with the Ehrlich's reaction, which is used to determine the amount of PBG formed.[50] The comparison of the series of compounds in which the modification of the keto function into an amide or into an ester function has been made at the end of the chain or within the chain is quite interesting. Introducing an amide group at the end of the chain could mimic the imine or the iminium ion **7** formed between the two substrate molecules in the mechanism Jordan I (see Scheme 4). The amide, and to some extent also the ester in the chain, could be considered as a mimic for the enamine or the enolate necessary for the ring closure reaction. Comparing the inhibitors **10** and **12** with the compounds **13** and **15** shows that there is at most a slight difference between those two arrangements of the functional groups. All of these compounds are weak or even very weak inhibitors judged by their K_I values. A very interesting observation is the fact that introducing the amino function at the end of the chain, with the hope of adding another recognition site and thereby increasing the affinity to the active site, does not influence the K_I value in the expected direction. The values for compounds **13** and **14** are almost identical. In the case of the amides **15** and **16** the introduction of the additional amino function transforms a weak inhibitor into a compound that is no longer recognized by the enzyme, an observation already reported by Leeper.[51] This shows that we cannot expect simple additivity in our series of inhibitors. If the interaction at one site is optimal, the trials to improve the inhibition potency by adding additional functional groups (in the hope of increasing the number of contacts) are often not successful. The other clear conclusion is that for inhibitors which interact at the A site of the enzyme the replacement of the keto function by an amide group reduces the inhibition potency substantially. As amides should be good ligands for Lewis acids, this observation is surprising, but the conclusion is similar as the one drawn from the introduction of the fluoro substituent in the 5-position.

A final point has to be discussed in this context. The nitro function seems to imitate the carboxylate very well. The nitro analogues are often better recognized than the carboxylates themselves. Those cases in which the introduction of a nitro group has considerably enhanced the recognition have been shown to be due to the interaction of the nitronate anion with the active site of the enzyme. Careful titration of compounds **17** and **22** allowed us to determine the pK_a values. It was necessary to wait five minutes after each addition of base to establish the equilibrium. The pK_a values of **17** and **22** are 8.05 and 8.68, respectively. This means that a considerable percentage of our inhibitors is present in their deprotonated form. Without spectroscopic information on the enzyme – inhibitor complex we cannot be sure which form is present in the active site. However, it seems reasonable to assume that the negatively charged nitronate could be an excellent substitute for

the carboxylate. This would be an additional reason why the nitro compounds are generally such excellent inhibitors of PBGS.[37]

The studies of a series of linear dicarboxylic acids containing one or two keto functions had shown that PBGS is very sensitive to the chain length. The sebacic acid derivatives, close analogues of the postulated key intermediate of the Jordan I mechanism, generally showed the highest inhibition efficiency.[41] It was also shown that a three-point interaction was in general sufficient for an excellent recognition of the inhibitor at the active site. Based on these results we synthesized a series of analogues in which we kept the keto function and the relative arrangements of the functional groups constant (Table 3). Instead of the second keto function, which should be recognized at the A site, we introduced an amide group. Keeping this basic skeleton constant, we varied the groups at the end of the chain from carboxylate to an ester or nitro group. We synthesized most of the variations of these end groups in order to analyze the recognition selectivity at the two sites of the enzyme. Compound **23**, which contains two carboxylates, was our standard for this series of tests. This compound was an irreversible inhibitor, like the corresponding diketone or 4-oxosebacic acid (**30**). Despite the substantial reduction of recognition of an amide at the A site, which we had established before, compound **23** interacts strongly with the active site of PBGS. Exchanging the carboxylate at the A site for a methyl ester function (→**24**) reduced the interaction considerably and an uncompetitive inhibitor with a K_i value of 243 μM was obtained. The uncompetitive inhibition behavior is a good indication that our inhibitor still interacts not only in the A site, but probably at both sites at the same time. However, the potency of this inhibitor is strongly reduced, as was expected from the studies of the substrate analogues. Simply changing the position of the ester group from the A site to the P site (→**25**) again resulted in an irreversible inhibitor. This observation is compatible with a greater tolerance for an ester function at the P site than at the A site. The amino acids responsible for the interaction with the P-site carboxylate are known from the X-ray crystallographic studies of PBGS containing levulinate bound to the active site.[36, 38, 40] The carboxy group of the levulinic acid forms three hydrogen bonds: one with Tyr 329 and two with Ser 290. It can be envisaged that with a methyl ester function only a partial disruption of the hydrogen bonds occurs. This would explain the reduction in recognition, rather than a total loss. The fact that the A-site recognition seems to be less tolerant to esters is in favor of an on – off mechanism for the recognition. The interaction could be due to the fact that there are hydrogen bonds to one amino acid only. This interpretation will have to be tested as soon as the A site of PBGS can be identified by an X-ray crystallographic analysis. Introducing a nitro function at the A site as in **26** and **27** still leads to irreversible inhibition. This result is expected and in complete accordance with the results obtained with the substrate analogues. Introduction of the nitro group at the P site (→**28** and **29**) gives competitive/mixed inhibitors. These inhibitors are slightly better than the inhibitor containing the ester function at the A site (**24**), but their inhibition behavior and their K_i values of 20 and 30 μM, respectively, indicate that the nitro group is by far not such a good group at the P site than it is at the A site. It seems clear from these results that PBGS has two distinct recognition mechanisms for the two carboxylates. The P-site carboxylate can be replaced by an ester function with only a slight loss in activity. Replacing the P-site carboxylate by a nitro group is much less favorable. However, at the A site the nitro group is by far the best group for obtaining a high affinity. The ester function is not well tolerated at this part of the active site. The tolerance of the P site to the ester function is compatible with data available from the X-ray crystallographic structures. For a rationalization of the inversed behavior of the A site, we have to wait for results of X-ray crystallographic analyses that should allow us to identify the groups responsible for the recognition mechanism.

Finally, a minimal version of the analogues of the Jordan I intermediate was tested (Table 4). 4-Oxosebacic acid (**30**) is an irreversible inhibitor.[41] The nitro analogue **31** is a competitive inhibitor with a K_i value of 4900 μM. Probably this compound is no longer recognized as an analogue of the intermediate, but it is a substrate analogue with a long chain. This result is in agreement with the observation reported above. When the keto function is replaced by an amide function, very weak competitive inhibitors like **32** and **33** are obtained. It is clear that the absence of the keto function is not compensated for by the correct chain length and the presence of two carboxylate functions. The K_i values are in the order of 20 mM, which means a very weak inhibition.

Table 3. *Inhibition of PBGS by analogues of the Jordan I key intermediate.*

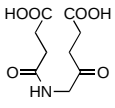
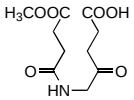
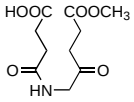
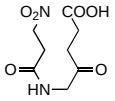
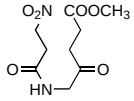
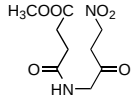
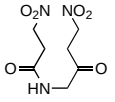
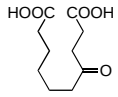
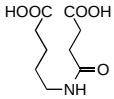
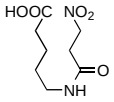
Compound		K_i [μM]	Inhibition type/remarks
	**23**	–	irreversible
	**24**	243	uncompetitive
	**25**	–	irreversible
	**26**	–	irreversible
	**27**	–	irreversible
	**28**	20	competitive/mixed
	**29**	30	competitive/mixed

*Table 4. Inhibition of PBGS by analogues of 4-oxosebacic acid (**30**).*

Compound		K_I [μM]	Inhibition type/remarks
	**30**	–	irreversible
	**31**	4900	competitive
	**32**	22900	competitive
	**33**	18200	competitive/activator

Conclusion

We could show that substrate analogues in which the keto function had been replaced is not well tolerated at the A site of PBGS. However, an increase in the electrophilicity of the carbon atom of the keto function increases the inhibition potency considerably. This is compatible with an important interaction with a nucleophile, but not really in accordance with an interaction with a Lewis acid such as a Zn^{II} ion. The amide function, however, is well tolerated at the A site if it is incorporated into an analogue of the Jordan I key intermediate. This observation allowed us to distinguish the recognition selectivities at both sites of the enzyme. The P site of the enzyme tolerates the substitution of the carboxylate by an ester group. The nitro function is accepted, but does not lead to an increase in inhibition potential. This result can be interpreted on the basis of the known structure of PBGS. On the other hand, the A site accepts the replacement of the carboxylate by a nitro function very well; in most cases a considerable increase in the interaction can be observed. Esters at the A site, however, are not tolerated.

This study clearly shows the importance of systematic studies of specifically designed inhibitors to increase our knowledge about the active site of enzymes. It is clear that definitive proof of the observed results will have to wait until adequate X-ray crystallographic structures become available. Finally, it is important to realize that we have interpreted our results on the basis of the postulated key intermediates. However, at this stage it is difficult—if not impossible—to distinguish between a mechanism based on the binding of intermediates and a mechanism based on the recognition of a bisubstrate compound.[52]

Experimental Section

Biochemical studies

Materials: *E. coli* (CR 261) was a gift from C. Roessner (Texas A&M University). Cultivation of *E. coli* and purification of PBGS were performed as described previously.[53]

PBGS assay and determination of kinetic constants: The PBGS assay is a colorimetric assay based on the reaction between PBG and 4-dimethylaminobenzaldehyde[50] (Ehrlich's reagent). The assay for *E. coli* PBGS contained 4–6.4 μg PBGS and the inhibitor in 1.5 mL of 0.1 M sodium phosphate buffer (NaH_2PO_4/Na_2HPO_4 mixture, pH 8.1, 12.3 mM mercaptoethanol, 10 mM $MgCl_2 \cdot 6H_2O$, 10 μM $ZnCl_2$). The preincubation took place at 37 °C for 30–45 min. The substrate was added in varying concentrations and the solution was incubated for 14 min, after which the PBGS-catalyzed reaction was stopped by adding 1 mL of the stop reagent (20% (w/v) trichloroacetic acid, 10 mM $HgCl_2$) at 0 °C. After centrifugation (4 min, 3600 *g*) 1 mL of the supernatant was treated with 1 mL of Ehrlich's reagent (1.1 g 4-dimethylaminobenzaldehyde in 20 mL perchloric acid and 35 mL acetic acid). This solution was centrifuged (4 min, 3600 *g*) again. The quantity of product formed was determined by measuring the absorbance at 554 nm ($\varepsilon = 62000\ mol^{-1}\ cm^{-1}$).

The type of inhibition was determined by using Eadie–Hofstee, Lineweaver–Burk, and Hanes plots. The K_I values shown in the different tables are the average of at least three independent assays. K_I values were calculated from the apparent K_M values deduced from the hyperbolic plot, which is typical of Michaelis–Menten kinetics.

Estimation of the specific activity: Protein concentration was estimated with the Bio-Rad Protein Assay using the color change of Coomassie Brilliant Blue G-250. This color change is followed by measuring the absorbance at 596 nm.[54] The concentration of PBGS is deduced by comparison with a standard curve. Using this value together with the rate of PBG formation allows the specific activity to be calculated.

Dialysis assay: 4–6.4 μg PBGS were dissolved in 10 mL of 0.1 M sodium phosphate buffer (see above). This solution (4 mL) was placed in two different 5-mL tubes, one containing 2.5–10 mg of the inhibitor, the second (without inhibitor) was used as a reference. After 24 h at RT, the specific activities were determined. 1 mL of each solution was placed in a dialysis tube and separately dialyzed against 1.5 L of 0.1 M sodium phosphate buffer (see above). After dialysis for 66 h at 4 °C, the specific activities were determined again.

Chemical syntheses

General: THF (purum, Fluka) was distilled over K and benzophenone under N_2; the other solvents (puriss., Fluka) were used without further treatment. TLC: silica gel 60 F_{254} plates (aluminium, 0.2 mm; Merck). Flash column chromatography: silica gel 60 (0.04–0.063 mm, Fluka) at ca. 0.2 bar. Determination of melting points: Büchi 510 apparatus, uncorrected. IR spectra: Perkin–Elmer 1720 X FT-IR spectrophotometer; absorption bands given in cm^{-1}. NMR spectra: Bruker AMX-400 and Bruker Avance 400 (^1H NMR: 400 MHz, ^{13}C NMR: 100 MHz); Varian Gemini XL-200 and Varian Gemini 2000 (^1H NMR: 200 MHz, ^{13}C NMR: 50 MHz); Me_4Si as external reference ($\delta = 0$); temperature: 298 K; all coupling constants *J* given in Hz. Mass spectrometry: EI (70 eV) and CI (NH_3 as ionisation gas): Nermag RC 30–10; ESI-MS and APCI-MS: Finnigan LCQ. HR-MS: Bruker FTMS 4.7T BioAPEX II (ESI-MS, positive and negative mode). GC: Perkin–Elmer SIGMA 3B, Dual FID Chromatograph, SE 54 column (Macherey&Nagel, $l = 25$ m, ID = 0.32 mm, $d_f = 0.25$ μm), P (program: 5 min at 70 °C, 70 → 150 °C at 8 °C min^{-1}, 10 min at 150 °C). Elemental analyses were performed by the Microanalytical Laboratory of Ciba-Specialty SA, Marly, and by "l'Ecole d'Ingénieur" of Fribourg.

Starting materials: (4-Nitro-2-oxo-1-butyl)ammonium chloride (**35**),[55] monobenzyl succinate (**36**),[56] and (4-carboxy-2-oxo-1-butyl)-ammonium chloride (**1**)[57] were synthesized according to the literature. (4-Methoxycarbonyl-2-oxo-1-butyl)ammonium chloride (**37**): A commercial sample (>98%, Sigma) as well as a specifically

synthesized sample were used. 3-Methoxycarbonylpropionyl chloride (**38**) (purum, Fluka) as well as all the other starting materials were commercially available (Fluka or Aldrich).

Levulinic acid (**8**), succinic acid (**11**), acetoxyacetic acid (**13**), *N*-acetylglycine (**15**) and glycylglycine (**16**), 3-nitropropionic acid (**20**), and monomethylsuccinate (**10**) were commercially available (Fluka or Aldrich). 5-Fluorolevulinic acid (**9**),[58, 59] aminoacetic acid carboxymethylester (**14**),[60] and 4-nitrobutan-2-one (**17**)[61, 62] were synthesized according to the literature.

The synthesis and characterization of the following starting materials or intermediates is available as Supporting Information: 3-nitropropionyl chloride (**39**), 1-diazo-4-nitrobutanone (**40**), [(4-benzyloxycarbonyl)-1-butyl]ammonium *p*-toluenesulfonate (**41**), [4-(methoxycarbonyl)-1-butyl]ammonium *p*-toluenesulfonate (**42**), [4-(benzyloxycarbonyl)-2-oxo-1-butyl]ammonium *p*-toluenesulfonate (**43**), methyl 5-[(3-nitropropionyl)amino]pentanoate (**44**), benzyl 5-{[3-(benzyloxycarbonyl)propionyl]amino}pentanoate (**45**), benzyl 5-{[3-(benzyloxycarbonyl)propionyl]amino}-4-oxopentanoate (**46**), methyl 5-{[3-(benzyloxycarbonyl)propionyl]amino}-4-oxopentanoate (**47**), benzyl 5-{[3-(methoxycarbonyl)propionyl]amino}-4-oxopentanoate (**48**), methyl 4-oxo-4-(2-oxocyclohexyl)butyrate (**49**), ethyl 9-bromo-4-oxononanoate (**50**), intermediate **51**, ethyl 9-nitro-4-oxononanoate (**52**).

***N*-Methylsuccinamic acid (12):** An 8 M solution of methylamine in ethanol (5.5 mL, 44 mmol) was added to an ice-cooled solution of succinic anhydride (2.0 g, 20 mmol) in ethanol (10 mL) and the reaction mixture was stirred at 0 °C for 90 min and then for 1 h at RT. Evaporation of the solvent, addition of 1 M HCl (40 mL) and ethyl acetate (50 mL), saturation with NaCl, extraction with ethyl acetate (8×50 mL), drying over $MgSO_4$, filtration, and evaporation of the solvent gave the crude product (1.65 g, 63 %). Subsequent purification by recrystallization in THF/diethyl ether yielded **12** (1.0 g, 38 %) as a white solid. M.p. 108 °C (ref. [64]: 111 °C). $R_f = 0.12$ (CH_2Cl_2/methanol, 9:1); IR (KBr plates, film): $\tilde{\nu} = 3600 - 2400$ (m), 3356 (vs), 2981 (m), 2929 (m), 2732 (m), 2642 (m), 2546 (m), 1715 (s), 1635 (s), 1569 (s), 1455 (m), 1422 (s), 1413 (s), 1370 (m), 1337 (m), 1277 (m), 1239 (s), 1179 (s), 1163 (s), 1085 (m), 1028 (m), 986 (m), 960 (m), 939 (m), 839 (m), 703 (m), 556 (m); ^{1}H NMR (200 MHz, [D$_6$]DMSO): $\delta = 2.28$ ("t", $^3J_{3\text{-}2} = 6.2$, 2 H, $H_2C(3)$), 2.41 ("t", $^3J_{2\text{-}3} = 6.4$, 2 H, $H_2C(2)$), 2.55 (d, $^3J_{5\text{-}HN} = 4.8$, 3 H, $H_3C(5)$), 7.76 (m, 1 H, HN) 12.06 (s, 1 H, HO); ^{13}C NMR (50 MHz, [D$_6$]DMSO): $\delta = 25.5$ (C(5)); 29.2, 30.0 (C(2), C(3)); 171.4, 174.0 (C(1), C(4); MS (ESI, negative mode): *m/z*: 130 $[M-1]^-$, 112 $[M-1-H_2O]^-$, 86 $[M-1-CO_2]^-$.

1-Fluoro-4-nitro-butan-2-one (18):[63] Pyridine hydrofluoride (Fluka, contains ca. 70 % HF; 10 mL) was added over 30 min to a solution of 1-diazo-4-nitrobutan-2-one (**40**) (1.43 g, 10 mmol) in diethyl ether (15 mL) at 15 °C in a high-density polyethylene (HDPE) container containing HF. The reaction mixture was stirred for 2 h at RT. Work-up with water (20 mL) followed by extraction of the aqueous layer with chloroform (6×50 mL), washing of the organic layers with anhydrous KF, filtration, and evaporation of the solvent gave the crude product (610 mg, 45 %) as an orange liquid. Subsequent purification by flash chromatography (silica gel; *n*-hexane/CH_2Cl_2, 2:1) gave the final product **18** (260 mg, 19 %) as a pure liquid (> 99 % (GC), $t_r = 9.6$). $R_f = 0.48$ (ethyl acetate/*n*-hexane, 2:1); IR (KBr plates, film): $\tilde{\nu} =$ 3028 (w), 2973 (w), 2933 (w), 1745 (s), 1558 (vs), 1427 (m), 1398 (m), 1378 (s), 1082 (m), 1045 (m), 998 (m); ^{1}H NMR (200 MHz, $CDCl_3$): $\delta =$ 3.25 (td, $^3J_{3\text{-}4} = 5.9$, $^4J_{3\text{-}F} = 2.1$, 2 H, $H_2C(3)$), 4.72 (t, $^3J_{4\text{-}3} = 6.0$, 2 H, $H_2C(4)$), 4.92 (d, $^2J_{1\text{-}F} = 47.4$, 2 H, $H_2C(1)$); ^{13}C NMR (50 MHz, $CDCl_3$): $\delta =$ 34.4 (C(3)), 67.9 (C(4)), 84.7 (d, $^1J_{1\text{-}F} = 184$, 1 C, C(1)), 202.7 (d, $^2J_{2\text{-}F} = 21$, 1 C, C(2)); ^{19}F NMR (188 MHz, $CDCl_3$): $\delta = 8.16$ (t, $^2J_{F\text{-}H5} = 47.7$, 1 F, FC(5)); MS (ESI, positive mode): 136 $[M+1]^+$, 89 $[M+1-HNO_2]^+$; HR-MS: *m/z*: calcd for $C_4H_6FNO_3$ 158.02239 $[M+Na]^+$, found 158.02211.

Methyl 3-nitropropionate (19): Oxalyl chloride (3.54 g, 21 mmol) and DMF (1 drop) were added under N_2 to an ice-cooled solution of 3-nitropropionic acid (2.51 g, 19.8 mmol) in CH_2Cl_2 (30 mL). The reaction mixture was stirred for 15 min at 0 °C and then for 2 h 45 min at RT. Half of the solvent was evaporated, the reaction mixture was ice-cooled and methanol (5 mL) was added dropwise; the mixture was stirred for 10 min at 0 °C and 20 min at RT. Evaporation of the solvent gave a crude product as a yellowish liquid; purification by bulb-to-bulb distillation ($p = 0.5 - 0.07$ mm Hg, $T = 60$ °C) yielded **19** (2.04 g, 78 %) as a colorless solid. $R_f = 0.47$ (ethyl acetate/*n*-hexane, 1:1); IR (KBr plates, film): $\tilde{\nu} = 3009$ (w), 2960 (w), 1739 (s), 1558 (vs), 1441 (s), 1407 (m), 1380 (s), 1342 (m), 1258 (m), 1218 (s), 1201 (s), 1181 (s), 872 (m), 850 (m); ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 4.64$ (t, $^3J_{3\text{-}2} = 6.2$, 2 H, $H_2C(3)$), 3.72 (s, 3 H, $H_3C(1^1)$), 2.97 (t, $^3J_{3\text{-}2} = 6.0$, 2 H, $H_2C(2)$); ^{13}C NMR (50 MHz, $CDCl_3$): $\delta = 169.9$ (C(1)), 69.6 (C(3)); 52.3 ($C(1^1)$), 30.7 (C(2)).

3-Nitropropionamide (21):[65] 3-Nitropropionyl chloride (**39**) (1.38 g, 10 mmol) was added to a 12 % ice-cooled aqueous solution of NH_3 (10 mL). After 10 min, the reaction mixture was acidified to pH 1 with 32 % HCl saturated with NaCl. Extraction with ethyl acetate ($8 \times$ 40 mL), drying over $MgSO_4$, filtration, and evaporation of the solvent gave the crude solid (1.02 g, 86 %). Subsequent purification by preabsorbtion onto silica and purification by flash chromatography (silica gel; ethyl acetate) yielded **21** (401 mg, 77 %) as a white solid. $R_f = 0.14$ (ethyl acetate). IR (KBr plates, film): $\tilde{\nu} = 3394$ (s), 3203 (m), 2973 (w), 2927 (w), 2855 (m), 2799 (m), 1710 (s), 1663 (s), 1630 (s), 1555 (vs), 1429 (s), 1402 (m), 1382 (m), 1310 (m), 1248 (m), 595 (m); ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 2.72$ (t, $^3J_{2\text{-}3} = 6.0$, 2 H, $H_2C(2)$), 4.66 (t, $^3J_{3\text{-}2} = 6.0$, 2 H, $H_2C(3)$), 7.03 (s, 1 H, HN), 7.51 (s, 1 H, HN); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 16.4$ (C(6)), 28.2 (C(5)), 36.5 (C(2)), 42.5 (C(3)), 178.2 (C(1)), 210.7 (C(4)).

***N*-Methyl-3-nitropropionamide (22):** A solution of 3-nitropropionyl chloride (**39**) (543 mg, 3.95 mmol) in CH_2Cl_2 (4 mL) was added to an 8 M ice-cooled solution of methylamine in ethanol (1.1 mL, 8.7 mmol) over 20 min. After 90 min the cooling bath was removed and the reaction mixture was stirred for 2 h at RT. Addition of an 1 M aqueous solution of HF (20 mL) and CH_2Cl_2 (30 mL), extraction with ethyl acetate (3×30 mL), drying over $MgSO_4$, filtration, and evaporation of the solvent gave the crude product (1.46 g, > 100 %) as a brownish oil. Subsequent purification by flash chromatography (silica gel; ethyl acetate/*n*-hexane, 2:1) yielded **22** (401 mg, 77 %) as a white solid. $R_f = 0.32$ (CH_2Cl_2/ethyl acetate/methanol, 10:1:1); IR (KBr plates, film): $\tilde{\nu} = 3337$ (s), 3305 (s), 3114 (w), 3022 (w), 2945 (m), 2924 (m), 2851 (w), 1674 (s), 1645 (vs), 1551 (s), 1416 (s), 1377 (s), 1340 (m), 1278 (s), 1248 (m), 1204 (m), 1165 (m), 1090 (m), 1030 (m), 952 (m), 874 (m), 738 (m), 581 (m), 492 (m); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 2.78$ (d, $^3J_{4\text{-}HN} = 4.8$, 3 H, $H_3C(4)$), 2.81 (t, $^3J_{2\text{-}3} = 6.2$, 2 H, $H_2C(2)$), 4.67 (t, $^3J_{3\text{-}2} = 6.1$, 2 H, $H_2C(3)$), 6.34 (s, 1 H, HN); ^{13}C NMR (100 MHz, $CDCl_3$, DEPT): $\delta = 26.3$ (C(4)), 32.2 (C(2)), 70.2 (C(3)), 169.0 (C(1)); MS (ESI, positive mode): 155 $[M+Na]^+$, 133 $[M+1]^+$; elemental analysis (%): calcd for $C_4H_8N_2O_3$ (132.12): C 36.36, H 6.10, N 20.20; found: C 36.10, H 6.15, N 21.76.

5-Amino-*N*-(3-carboxypropionyl)-4-oxopentanoic acid (23): A solution of the ester **46** (300 mg, 0.73 mmol) and of 10 % Pd/C (30 mg) in THF (40 mL) was shaken under H_2 (4 bar) for 20 h. The reaction mixture was filtered through celite and the catalyst washed with THF (40 mL). The filtrate was evaporated to give the crude product (160 mg) as a white solid. Subsequent recrystallization from THF/diethyl ether yielded **23** (85 mg, 61 %) as a white solid. M.p. 160 – 161 °C (ref. [66]: 197 – 199 °C); $R_f = 0.51$ (ethyl acetate/methanol/

acetic acid, 8:1:1); IR (KBr): $\tilde{\nu}$ = 3650 – 2300 (m) (3039), 3386 (m), 3341 (m), 2942 (m), 1724 (s), 1691 (vs), 1634 (s), 1530 (m), 1434 (m), 1410 (m), 1386 (m), 1305 (m), 1221 (m), 1195 (m), 630 (m); ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 2.36 – 2.47 (m, 6H, H$_2$C(2,7,8)), 2.63 (t, $^3J_{3\text{-}2}$ = 6.3, 2H, H$_2$C(3)), 3.91 (d, $^3J_{5\text{-}HN}$ = 5.4, 2H, H$_2$C(5)), 8.19 (t, $^3J_{HN\text{-}5}$ = 5.5, 1H, HN), 10.75 – 11.45 (s, 2H, HO); ^{13}C NMR (100 MHz, [D$_6$]DMSO, DEPT, HETCOR): δ = 27.6 (C(2)); 29.2, 29.8 (C(7), C(8)); 34.1 (C(3)); 48.5 (C(5)); 171.5 (C(6)); 173.8, 173.9 (C(1), C(9)); 205.9 (C(4)); MS (ESI, negative mode): *m/z*: ms 230 [$M-1$]$^-$, ms-ms (230) 212 [$M-1-$H$_2$O]$^-$, ms-ms-ms (230, 212) 194 [$M-1-2$H$_2$O]$^-$, ms-ms-ms-ms (230, 212, 194) 150 [$M-1-2$H$_2$O$-$CO$_2$]$^-$, ms-ms-ms-ms-ms (230, 212, 194, 150) 122 [$M-1-2$H$_2$O$-$CO$_2-$C$_2$H$_4$]$^-$; elemental analysis (%): calcd for C$_9$H$_{13}$NO$_6$ (231.20): C 46.75, H 5.67, N 6.06; found: C 46.79, H 5.72, N 5.67.

5-{[3-(Methyloxycarbonyl)propionyl]amino}-4-oxopentanoic acid (24): A solution of the ester **48** (335 mg, 1 mmol) and of 10% Pd/C (35 mg) in CH$_2$Cl$_2$ (40 mL) was shaken under H$_2$ (4 bar) for 16 h. The reaction mixture was filtered through celite and the catalyst washed with THF (20 mL). The filtrate was evaporated to give the crude product (260 mg) as a white solid. The recrystallization from THF/*n*-hexane yielded **24** (166 mg, 68%) as a white solid. M.p. 103 – 104 °C; R_f = 0.22 (CH$_2$Cl$_2$/methanol, 9:1); IR (KBr): $\tilde{\nu}$ = 3500 – 2500 (w) (3027), 3344 (m), 2958 (w), 2938 (w), 1733 (s), 1695 (s), 1641 (vs), 1521 (m), 1437 (m), 1411 (m), 1376 (m), 1354 (m), 1310 (m), 1196 (m), 1173 (m), 1140 (m), 631 (m), 493 (m); ^{1}H NMR (400 MHz, [D$_6$]DMSO): 2.40 (t, $^3J_{2\text{-}3}$ = 6.4, 2H, H$_2$C(2)), 2.43 ("t", $^3J_{7\text{-}8\,or\,8\text{-}7}$ = 6.1, 2H, H$_2$C(7 or 8)), 2.50 ("t", $^3J_{8\text{-}7\,or\,7\text{-}8}$ = 6.6, 2H, H$_2$C(8 or 7)), 2.62 (t, $^3J_{3\text{-}2}$ = 6.4, 2H, H$_2$C(3)), 3.57 (s, 3H, H$_3$C(10), 3.92 (d, $^3J_{5\text{-}HN}$ = 5.6, 2H, H$_2$C(5)), 8.22 (t, $^3J_{HN\text{-}5}$ = 5.5, 1H, HN) 11.2 – 13.0 (s, 1H, HO); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 27.6 (C(2)); 28.8, 29.7 (C(7), C(8)); 34.7 (C(3)); 48.5 (C(5)); 51.5 (C(10)); 171.3 (C(6)); 172.9 (C(9)), 173.8 (C(1)); 205.8 (C(4)); MS (ESI, negative mode): *m/z*: ms 244 [$M-1$]$^-$, 212 [$M-1-$CH$_4$O]$^-$, ms-ms (244) 212 [$M-1-$CH$_3$OH]$^-$, ms-ms-ms (244, 212) 194 [$M-1-$CH$_3$OH$-$H$_2$O]$^-$, ms-ms-ms-ms (244, 212, ms-ms-ms (244, 212) 194 [$M-1-$CH$_4$O$-$H$_2$O]$^-$, ms-ms-ms-ms (244, 212, 194) 150 [$M-1-$CH$_4$O$-$H$_2$O$-$CO$_2$]$^-$, ms-ms-ms-ms-ms (244, 212, 194, 150) 122 [$M-1-$CH$_4$O$-$H$_2$O$-$CO$_2-$(CO or C$_2$H$_4$)]$^-$; elemental analysis (%): calcd for C$_{10}$H$_{15}$NO$_6$ (245.23): C 48.98, H 6.16, N 5.71; found: C 48.78, H 6.27, N 5.82; HR-MS: *m/z*: calcd for C$_{10}$H$_{16}$NO$_6$ 246.09721, found: 246.09720.

3-{[4-(Methoxycarbonyl)-2-oxobutyl]carbamoyl}propionic acid (25): A solution of the ester **47** (600 mg, 1.79 mmol) and of 10% Pd/C (60 mg) in CH$_2$Cl$_2$ (75 mL) was shaken under H$_2$ (4 bar) for 20 h. The reaction mixture was filtered through celite and the catalyst washed with THF (100 mL). The filtrate was evaporated to give the crude product (650 mg) as a white solid. The recrystallization from THF/diethyl ether yielded **25** (350 mg, 80%) as a white solid. M.p. 107 °C; R_f = 0.22 (CH$_2$Cl$_2$/methanol, 9:1); IR (KBr): $\tilde{\nu}$ = 3650 – 2500 (m), 3348 (s), 3063 (m), 3027 (m), 2958 (m), 2927 (m), 2858 (m), 1730 (s), 1718 (vs), 1697 (s), 1636 (vs), 1525 (s), 1441 (m), 1423 (m), 1394 (m), 1384 (m), 1357 (s), 1311 (m), 1286 (m), 1202 (s), 1184 (m), 1140 (m), 1107 (m), 978 (m), 635 (m), 537 (m), 492 (m); ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 2.38 – 2.44 (m, 4H, H$_2$C(7,8)), 2.49 (t, $^3J_{2\text{-}3}$ = 6.6, 2H, H$_2$C(2)), 2.70 (t, $^3J_{3\text{-}2}$ = 6.5, 2H, H$_2$C(3)), 3.58 (s, 3H, H$_3$C(10)), 3.93 (d, $^3J_{5\text{-}HN}$ = 5.6, 2H, H$_2$C(5)), 8.19 (t, $^3J_{HN\text{-}5}$ = 5.4, 1H, HN); ^{13}C NMR (100 MHz, [F$_6$]DMSO): δ = 27.2 (C(2)); 29.2, 29.9 (C(7), C(8)); 33.9 (C(3)); 48.5 (C(5)); 51.5 (C(10)); 171.5 (C(4)); 172.7 (C(9)); 173.9 (C(1)); 205.7 (C(6)); MS: *m/z* (%): 246 (6) [M+1]$^+$, 228 (4) [$M-$OH]$^+$, 214 (8) [$M-$CH$_3$O]$^+$, 196 (10) [$M-$CH$_5$O$_2$]$^+$, 168 (12) [$M-$C$_2$H$_5$O$_3$]$^+$, 140 (4) [$M-$C$_3$H$_5$O$_4$]$^+$, 131 (12) [C$_5$H$_9$NO$_3$]$^{+\bullet}$, 115 (100) [C$_5$H$_7$O$_3$]$^+$, 114 (16), 113 (40) [C$_5$H$_7$NO$_2$]$^{+\bullet}$, 101 (27) [C$_4$H$_5$O$_3$]$^+$, 87 (19) [C$_4$H$_7$O$_2$]$^+$, 73 (12) [C$_3$H$_5$O$_2$]$^+$, 55 (27); elemental analysis (%): calcd for C$_{10}$H$_{15}$NO$_6$ (245.23): C 48.98, H 6.16, N 5.71; found: C 49.03, H 6.17, N 5.73.

5-(3-Nitropropionylamino)-4-oxopentanoic acid (26): A solution of pig liver esterase (4 mg, 130 U mg^{-1}) in (NH$_4$)$_2$SO$_4$ buffer (3 M, pH 7.2; 0.4 mL) was added to a solution of the ester **27** (565 mg, 2.3 mmol) in water (40 mL). The reaction mixture was maintained for 9 h at pH 7.2 by adding 0.3 M NaOH then acidified to pH 1.5 with 32% HCl, saturated with NaCl, and extracted with ethyl acetate (8 × 50 mL). The organic layers were dried over MgSO$_4$ and filtered, and the solvent was evaporated to give the crude product (400 mg) as a brown oil. Subsequent recrystallization from THF yielded **26** (154 mg, 29%) as a white solid. M.p. 148 °C; R_f = 0.37 (CH$_2$Cl$_2$/methanol, 7:3); IR (KBr): $\tilde{\nu}$ = 3650 – 2400 (w) (3069), 3317 (s), 2967 (w), 2922 (w), 1723 (s), 1699 (s), 1650 (vs), 1550 (s), 1433 (m), 1422 (m), 1407 (m), 1398 (m), 1389 (m), 1270 (m), 1234 (m); ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 2.41 (t, $^3J_{2\text{-}3}$ = 6.5, 2H, H$_2$C(2)), 2.63 (t, $^3J_{3\text{-}2}$ = 6.5, 2H, H$_2$C(3)), 2.84 (t, $^3J_{7\text{-}8}$ = 5.9, 2H, H$_2$C(7)), 3.97 (d, $^3J_{5\text{-}HN}$ = 5.5, 2H, H$_2$C(5)), 4.69 (t, $^3J_{8\text{-}7}$ = 6.2, 2H, H$_2$C(8)), 8.40 (t, $^3J_{HN\text{-}5}$ = 5.2, 1H, HN); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 27.6 (C(2)), 31.2 (C(7)), 34.1 (C(3)), 48.5 (C(5)), 70.6 C(8), 169.0 C(6), 173.7 C(1), 205.4 C(4); MS (ESI, negative mode): *m/z*: ms 231 [$M-1$]$^-$, 184 [$M-1-$HNO$_2$]$^-$, ms-ms (231) 184 [$M-1-$HNO$_2$]$^-$, ms-ms-ms (231, 184) 166 [$M-1-$HNO$_2-$H$_2$O]$^-$, 140 [$M-1-$HNO$_2-$CO$_2$]$^-$, 112 [$M-1-$HNO$_2-$CO$_2-$C$_2$H$_4$]$^-$; HR-MS: *m/z*: calcd for C$_8$H$_{12}$N$_2$NaO$_6$ 255.058750, found: 255.058743.

Methyl 5-[(3-nitropropionyl)amino)]-4-oxopentanoate (27): The acyl chloride **39** (808 mg, 6 mmol) was added dropwise to a solution of **37** (1.1 g, 6 mmol) in CH$_2$Cl$_2$ (50 mL) under N$_2$ at 0 °C, followed by the addition of triethylamine (1.23 g, 12 mmol) over 30 min. The reaction mixture was stirred for 1 h at 0 °C and 1 h at RT. Work-up with 1 M HCl (30 mL) and CH$_2$Cl$_2$ (30 mL) followed by extraction of the aqueous layer with CH$_2$Cl$_2$ (3 × 50 mL), drying of the organic layers over MgSO$_4$, filtration, and evaporation of the solvent gave the crude product (990 mg) as a yellow solid. Subsequent recrystallization from ethyl acetate yielded **27** (657 mg, 44%) as a white solid. M.p. 96 °C; R_f = 0.51 (CH$_2$Cl$_2$/methanol, 9:1); IR (KBr): $\tilde{\nu}$ = 3650 – 2800 (w), 3300 (s), 3104 (w), 3027 (w), 2984 (w), 2955 (w), 2921 (w), 2854 (w), 1744 (s), 1727 (s), 1650 (s), 1573 (m), 1552 (vs), 1442 (m), 1411 (m), 1403 (m), 1377 (m), 1366 (m), 1348 (m), 1282 (m), 1213 (m), 1181 (m), 1097 (m), 1031 (m); ^{1}H NMR (400 MHz, [D$_6$]DMSO): 2.63 – 2.67 (m, 2H, H$_2$C(2)), 2.72 – 2.76 (m, 2H, H$_2$C(3)), 2.90 (t, $^3J_{8\text{-}9}$ = 6.2, 2H, H$_2$C(8)), 3.66 (s, 3H, H$_3$C(6)), 4.21 (d, $^3J_{5\text{-}HN}$ = 4.7, 2H, H$_2$C(5)), 4.69 (t, $^3J_{9\text{-}8}$ = 6.2, 2H, H$_2$C(8)), 6.58 (s, 1H, HN); ^{13}C NMR (100 MHz, CDCl$_3$): δ = 27.5 (C(2)), 32.1 (C(8)), 34.5 (C(3)), 49.3 (C(5)), 51.9 (C(6)), 69.9 (C(9)), 168.3 (C(7)), 172.8 (C(1)), 203.7 (C(4)); MS (EI): *m/z* (%): 246 (3) [M]$^{+\bullet}$, 215 (2) [$M-$CH$_3$O]$^+$, 115 (29) [C$_5$H$_7$O$_3$]$^+$, 87 (11), 85 (14), 84 (37) [C$_4$H$_4$O$_2$]$^{+\bullet}$, 59 (22), 57 (14), 56 (13), 55 (100); elemental analysis (%): calcd for C$_9$H$_{14}$N$_2$O$_6$ (246.22): C 43.90, H 5.73, N 11.38; found: C 43.84, H 5.54, N 11.25.

Methyl [(4-nitro-2-oxobutyl)carbamoyl]propionate (28): The acyl chloride **38** (808 mg, 6 mmol) was added dropwise to a solution of **35** (460 mg, 2.74 mmol) in CH$_2$Cl$_2$ (30 mL) under N$_2$ at 0 °C, followed by the addition of triethylamine (554 mg, 5.48 mmol) over 30 min. The reaction mixture was stirred for 1 h at 0 °C and for 2 h at RT. Work-up with 1 M HCl (40 mL) and CH$_2$Cl$_2$ (10 mL) followed by extraction of the aqueous layer with CH$_2$Cl$_2$ (4 × 40 mL), drying of the organic layers over MgSO$_4$, filtration, and evaporation of the solvent gave the crude product (640 mg) as a yellow solid. Subsequent purification by flash chromatography (silica gel; CH$_2$Cl$_2$/methanol, 9:1) and recrystallization from ethyl acetate yielded **28** (280 mg, 37%) as a white solid. M.p. 101 – 102 °C; R_f = 0.52 (CH$_2$Cl$_2$/methanol, 9:1); IR (KBr): $\tilde{\nu}$ = 3700 – 2800 (w), 3284 (s), 3107 (m), 2951 (w), 2925 (m), 1737 (vs), 1641 (s), 1573 (s), 1551 (s), 1433 (s), 1419 (s), 1390 (s), 1356 (m), 1341 (m), 1277 (m), 1252 (m), 1193 (s), 1176 (s), 1104 (m), 1066 (m), 1035 (m), 1016 (m), 981 (m); ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 2.43 – 2.47 (m, 2H, H$_2$C(2)), 2.50 – 2.54 (m, 2H, H$_2$C(3)), 3.11 (t, $^3J_{8\text{-}9}$ = 5.8, 2H, H$_2$C(8)), 3.58 (s, 3H, H$_3$C(5)), 3.99 (d, $^3J_{6\text{-}HN}$ = 5.7, 2H, H$_2$C(6)), 4.70 (t,

$^3J_{9\text{-}8}=5.8$, 2H, $H_2C(8)$), 8.27 (t, $^3J_{6\text{-}HN}=5.4$, 1H, HN); ^{13}C NMR (100 MHz, [D$_6$]DMSO, HETCOR): $\delta=28.8$ (C(3)), 29.6 (C(2)), 35.6 (C(8)), 48.4 (C(6)), 51.4 (C(5)), 69.4 (C(9)), 171.4 (C(4)), 172.9 (C(1)), 204.0 C(7); MS (CI): *m/z* (%): 247 (10) $[M+1]^+$, 232 (5) $[M+1-CH_3]^{+\bullet}$, 215 (2) $[M-CH_3O]^+$, 201 (12), 200 (100) $[M-NO_2]^+$, 168 (13) $[M-CH_4O_3N]^+$, 132 (11) $[C_5H_{10}NO_3]^+$, 115 (8) $[C_5H_7O_3]^+$, 100 (5) $[C_4H_4O_3]^+$, 55 (8); elemental analysis (%): calcd for $C_9H_{14}N_2O_6$ (246.22): C 43.90, H 5.73, N 11.38; found: C 44.14, H 5.79, N 11.30.

3-Nitro-*N*-(4-nitro-2-oxobutyl)propionamide (29): The acyl chloride **39** (79 mg, 0.59 mmol) was added to a suspension of **35** (100 mg, 0.59 mmol) in THF (30 mL) under N_2 at 0 °C, followed by the addition of triethylamine (1 g, 10 mmol) over 30 min. The reaction mixture was stirred for 1 h at 0 °C and for 1 h at RT. Work-up with 1 M HCl (30 mL) and diethyl ether (50 mL) followed by saturation with NaCl and extraction of the aqueous layer with ethyl acetate (6 × 30 mL), drying of the organic layers, filtration, and evaporation of the solvent gave the crude product (130 mg) as a yellow solid. Subsequent purification by flash chromatography (silica gel; *n*-hexane/ethyl acetate, 4:1) yielded **29** (41 mg, 30%) as a white solid. M.p. 129–130 °C; $R_f=0.56$ (CH_2Cl_2/methanol, 9:1); IR (KBr): $\tilde{\nu}=3700-2800$ (m), 3293 (s), 3103 (w), 2973 (w), 2917 (w), 1732 (s), 1683 (m), 1651 (s), 1561 (s), 1541 (vs), 1426 (s), 1409 (m), 1398 (m), 1376 (s), 1357 (m), 1278 (m); ^{1}H NMR (400 MHz, [D$_6$]DMSO): $\delta=2.85$ (t, $^3J_{2\text{-}3}=5.9$, 2H, $H_2C(2)$), 3.12 (t, $^3J_{6\text{-}7}=5.7$, 2H, $H_2C(6)$), 4.04 (d, $^3J_{4\text{-}HN}=5.6$, 2H, $H_2C(4)$), 4.70 (t, $^3J_{3\text{-}2\,or\,7\text{-}6}=5.9$, 2H, H_2C(3 or 7)), 4.71 (t, $^3J_{7\text{-}6\,or\,3\text{-}2}=5.8$, 2H, H_2C(7 or 3)), 8.45 (t, $^3J_{HN\text{-}4}=5.4$, 1H, HN); ^{13}C NMR (100 MHz, [D$_6$]DMSO): $\tilde{\nu}=$ 31.2, 35.6 C(2), C(6); 48.4 (C(4)); 69.4, 70.6 (C(3), C(7)); 169.2 (C(1)); 203.6 (C(3)); MS (ESI, positive mode): *m/z*: 256 $[M+Na]^+$, 251 $[M+NH_4]^+$, 234 $[M+1]^+$, ms-ms (234) 187 $[M+1-HNO_2]^+$, ms-ms-ms (234, 187) 140 $[M+1-2HNO_2]^+$, ms-ms-ms-ms (234, 187, 140) 122 $[M+1-2HNO_2-H_2O]^+$; elemental analysis (%): calcd for $C_8H_{12}N_2O_6$ (232.19): C 36.06, H 4.75, N 18.02; found: C 36.09, H 4.86, N 17.70.

4-Oxodecandioic acid (30): A solution of 87% potassium hydroxide (2.3 g, 41.5 mmol) in water (2 mL) was heated to 100 °C and the diketone **49** (2.2 g, 10.4 mmol) was added. After 3 min the solution was cooled with an ice bath, and water (5 mL) and 32% HCl (5 mL) were added. A white solid precipitated from the acidified solution which was filtered to give the crude product (1.85 g) as a white solid. Subsequent recrystallization from THF/*n*-hexane yielded **30** (1.71 g, 76%) as a white solid. M.p. 107 °C (ref. [67]: 109–110 °C); $R_f=0.52$ (ethyl acetate/methanol/acetic acid, 7:2:1); IR (KBr): $\tilde{\nu}=3650-2300$ (m), 2938 (m), 1698 (vs), 1413 (m), 1295 (m), 1246 (m), 1216 (m), 1106 (m), 938 (m); ^{1}H NMR (400 MHz, [D$_6$]DMSO, COSY): 1.18–1.26 (m, 2H, $H_2C(7)$), 1.45 (qi, $^3J_{6\text{-}5}=^3J_{6\text{-}7}=7.8$, 2H, $H_2C(6)$), 1.47 (qi, $^3J_{8\text{-}7}=^3J_{8\text{-}9}=7.6$, 2H, $H_2C(8)$), 2.18 (t, $^3J_{9\text{-}8}=7.4$, 2H, $H_2C(9)$), 2.38 (t, $^3J_{2\text{-}3}=6.4$, 2H, $H_2C(2)$), 2.41 (t, $^3J_{5\text{-}6}=7.3$, 2H, $H_2C(5)$), 2.62 (t, $^3J_{3\text{-}2}=6.5$, 2H, $H_2C(3)$), 12.02 (s, 2H, HO); ^{13}C NMR (100 MHz, [D$_6$]DMSO, HETCOR): $\delta=23.0$ (C(6)); 24.4 (C(8)); 27.8 (C(2)); 28.2 (C(7)); 33.6 (C(9)); 36.7 (C(3)); 41.7 (C(5)); 173.9, 174.6 (C(1), C(10)); 209.2 (C(4)); MS (EI): *m/z* (%): 217 (2) $[M+1]^+$, 199 (7) $[M-OH]^+$, 181 (6) $[M+1-2H_2O]^+$, 153 (7), 143 (8) $[M-C_3H_5O_2]^+$, 125 (29) $[M-C_3H_7O_3]^+$, 116 (47) $[C_5H_8O_3]^{+\bullet}$, 101 (41) $[C_5H_9O_2]^+$, 98 (95) $[C_5H_6O_2]^{+\bullet}$, 97 (30), 73 (77) $[C_3H_5O_2]^+$, 69 (100), 55 (55); calcd for $C_{10}H_{16}O_5$ 216.23.

9-Nitro-4-oxononanoate (31): A solution of pig liver esterase (3 mg, 130 U mg^{-1}) in $(NH_4)_2SO_4$ buffer (3 M, pH 7.2; 0.3 mL) was added to a solution of the ester **52** (360 mg, 1.47 mmol) in water (40 mL). The reaction mixture was maintained for 5 h at pH 7.2 by adding 0.2 M NaOH, then acidified to pH 1 with 32% HCl, saturated with NaCl and extracted with ethyl acetate (6 × 50 mL). The organic layers were dried over $MgSO_4$, filtered, and the solvent was evaporated to give the crude product (320 mg) as a liquid. Subsequent purification by flash chromatography (silica gel; CH_2Cl_2/methanol, 96:4) yielded **31** (186 mg, 58%) as a white solid. M.p. 62–63 °C; $R_f=0.26$ (CH_2Cl_2/methanol, 9:1); IR (KBr): $\tilde{\nu}=3600-2400$ (m) (3043), 2938 (m), 2893 (m), 2869 (m), 1722 (s), 1704 (s), 1551 (vs), 1474 (m), 1438 (m), 1411 (s), 1399 (s), 1389 (s), 1360 (m), 1336 (m), 1254 (m), 1233 (m), 1224 (vs), 1191 (m), 1152 (m), 1108 (m), 1075 (m), 911 (m), 633 (m); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta=1.36$ ("qi", $^3J_{7\text{-}6}=^3J_{7\text{-}8}$ 7.8, 2H, $H_2C(7)$), 1.62 (tt, $^3J_{6\text{-}7}=7.8$, $^3J_{6\text{-}5}=7.2$, 2H, $H_2C(6)$, 1.99 (tt, $^3J_{8\text{-}7}=7.7$, $^3J_{8\text{-}9}=7.0$, 2H, $H_2C(8)$), 2.47 (t, $^3J_{5\text{-}6}=7.2$, 2H, $H_2C(5)$), 2.59–2.62 (m, 2H, $H_2C(2)$), 2.67–2.70 (m, 2H, $H_2C(3)$), 4.36 (t, $^3J_{9\text{-}8}=7.0$, 2H, $H_2C(9)$), 9.7–11.0 (s, 1H, HO); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta=22.7$ (C(6)); 25.6 (C(7)); 27.0 (C(8)); 27.7 (C(2)); 36.7 (C(3)); 41.9 (C(5)); 75.3 (C(9)); 178.7 (C(1)); 208.3 (C(4)); MS (ESI, negative mode): *m/z*: ms 216 $[M-1]^-$, ms-ms (216) 169 $[M-1-HNO_2]^-$, ms-ms-ms (216, 169) 151 $[M-1-HNO_2-H_2O]^-$, 125 $[M-1-HNO_2-CO_2]^-$, 107 $[M-1-HNO_2-H_2O-CO_2]^-$; HR-MS: *m/z*: calcd for $C_9H_{15}NNaO_5$ 240.0842, found: 240.0844.

5-[(3-Carboxypropionyl)amino]pentanoic acid (32): A solution of the ester **45** (3.05 g, 7.7 mmol) and of 10% Pd/C (300 mg) in CH_2Cl_2 (120 mL) was shaken under H_2 (4 bar) for 20 h. The reaction mixture was filtered through celite and the catalyst washed with THF (40 mL). The filtrate was evaporated to give the crude product (1.77 g) as a white solid. The recrystallization from THF/diethyl ether yielded **32** (1.29 g, 77%) as a white solid. M.p. 141 °C; $R_f=0.52$ (CH_2Cl_2/methanol/acetic acid, 8:1:1); IR (KBr): $\tilde{\nu}=3650-2300$ (m), 3315 (s), 3070 (s), 2964 (s), 2882 (m), 1693 (vs), 1629 (s), 1552 (s), 1473 (m), 1463 (m), 1431 (s), 1321 (s), 1271 (m), 1203 (s), 927 (m), 680 (m); ^{1}H NMR (400 MHz, [D$_6$]DMSO): $\delta=1.33-1.41$ (m, 2H, $H_2C(4)$), 1.43–1.51 (m, 2H, $H_2C(3)$), 2.18 (t, $^3J_{2\text{-}3}=7.3$, 2H, $H_2C(2)$), 2.27 (t, $^3J_{7\text{-}8}=7.1$, 2H, $H_2C(7)$), 2.38 ("t", $^3J_{8\text{-}7}$ 6.9, 2H, $H_2C(8)$), 3.01 (td, $^3J_{5\text{-}4}=6.9$, $^3J_{5\text{-}HN}=$ 5.5, 2H, $H_2C(5)$), 7.83 (t, $^3J_{HN\text{-}5}=5.5$, 1H, HN), 10.8–11.9 (s, 2H, HO); ^{13}C NMR (100 MHz, [D$_6$]DMSO, DEPT, HETCOR): $\delta=22.1$ (C(3)); 28.8 (C(4)); 29.6 (C(8)); 30.3 (C(7)); 33.6 (C(2)); 38.3 (C(5)); 171.0 (C(6)); 174.1, 174.6 (C(1), C(9)); MS (CI): *m/z* (%): 218 (100) $[M+1]^+$, 200 (50) $[M-OH]^+$, 182 (23) $[M+1-2H_2O]^+$,174 (36) $[M+1-CO_2]^+$), 118 (31) $[C_4H_8NO_3]^+$, 100 (82) $[C_4H_6NO_2]^+$; elemental analysis (%): calcd for $C_9H_{15}NO_5$ (217.22): C 49.76, H 6.96, N 6.45; found: C 50.13, H 7.02, N 6.06; HR-MS: *m/z*: calcd for $C_9H_{15}NNaO_5$ 240.08424, found: 240.08422.

5-[(3-Nitropropionyl)amino]pentanoic acid (33): A solution of pig liver esterase (3 mg, 130 U mg^{-1}) in $(NH_4)_2SO_4$ buffer (3 M, pH 7.2; 0.3 mL) was added to a solution of the ester **44** (1.2 g, 5.17 mmol) in water (50 mL). The reaction mixture was maintained for 4 h at pH 7.3 by adding 0.5 M NaOH, then acidified at pH 1.5 with 32% HCl, saturated with NaCl and extracted with ethyl acetate (4 × 50 mL). The organic layers were dried over $MgSO_4$ and filtered, and the solvent was evaporated to give the crude product (1.01 g) as an orange solid. Subsequent recrystallization from THF/diethyl ether yielded **33** (810 mg, 72%) as a white solid. M.p. 81 °C; $R_f=0.52$ (ethyl acetate/methanol, 2:1); IR (KBr): $\tilde{\nu}=3600-2400$ (m), 3298 (s), 3071 (m), 2948 (m), 2878 (m), 1692 (s), 1638 (s), 1551 (vs), 1477 (m), 1464 (m), 1416 (m), 1377 (m), 1334 (m), 1280 (m), 1218 (m), 1192 (m), 924 (m), 872 (m), 681 (m); ^{1}H NMR (400 MHz, [D$_6$]DMSO): $\delta=1.35-1.42$ (m, 2H, $H_2C(4)$), 1.45–1.52 (m, 2H, $H_2C(3)$), 2.20 (t, $^3J_{2\text{-}3}=7.3$, 2H, $H_2C(2)$), 2.73 (t, $^3J_{7\text{-}8}=6.0$, 2H, $H_2C(7)$), 3.04 (td, $^3J_{5\text{-}4}=7.0$, $^3J_{5\text{-}HN}=5.3$, 2H, $H_2C(5)$), 4.68 (t, $^3J_{8\text{-}7}=6.0$, 2H, $H_2C(8)$), 8.04 (t, $^3J_{HN\text{-}5}=5.3$, 1H, HN), 12.00 (s, 1H, HO); ^{13}C NMR (100 MHz, [D$_6$]DMSO, HETCOR): $\delta=21.9$ (C(3)), 28.5 (C(4)), 31.4 (C(7)), 33.2 (C(2)), 38.2 (C(5)), 70.7 (C(8)), 168.3 (C(6)), 174.4 (C(1)); MS (CI): *m/z* (%): 236 (50) $[M+18]^+$, 219 (100) $[M+1]^+$, 201 (10) $[M-OH]^+$, 187 (15), 172 (21) $[M-NO_2]^+$, 101 (15); elemental analysis (%): calcd for $C_8H_{14}N_2O_5$ (218.21): C 44.03, H 6.47, N 12.84; found: C 44.24, H 6.53, N 12.67.

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