# Synthesis of analogues of 5-aminolaevulinic acid and inhibition of 5-aminolaevulinic acid dehydratase[1]

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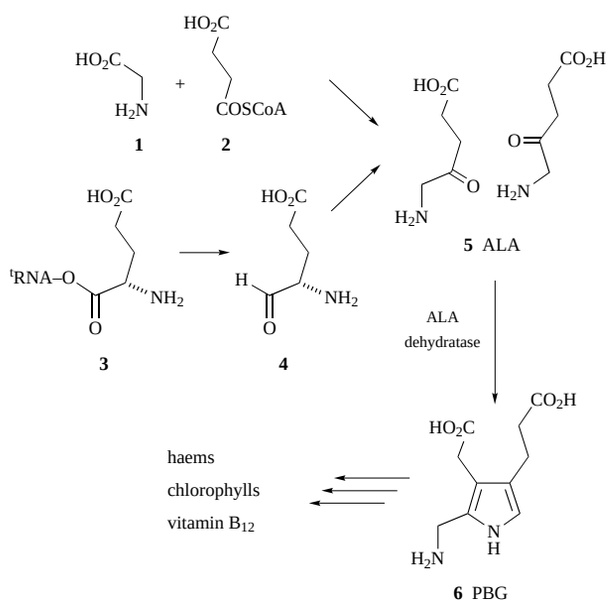
**Syntheses are described of several analogues of 5-aminolaevulinic acid (ALA), which are potential inhibitors of ALA dehydratase (porphobilinogen synthase), an early enzyme of tetrapyrrole biosynthesis. Most of the analogues are relatively weak competitive inhibitors of the enzyme from *Bacillus subtilis* or irreversible inhibitors due to multiple alkylation of the enzyme but the 3-oxa and 3-thia analogues are potent mechanism-based inhibitors which inactivate, by acylation of a nucleophilic residue, probably the active-site lysine residue. The kinetics of the inactivation by 3-thiaALA have implications for the mechanism of the enzymic reaction.**

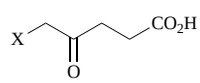
5-Aminolaevulinic acid (ALA) **5** is the first intermediate in the biosynthesis of tetrapyrroles (haems, chlorophylls, vitamin $B_{12}$, *etc.*) which is common to all organisms.[2] In animals, fungi and some bacteria, ALA is made by a pyridoxal phosphate-dependent enzyme, ALA synthase, from glycine **1** and succinyl CoA **2** (Scheme 1). In plants and other bacteria, on the other



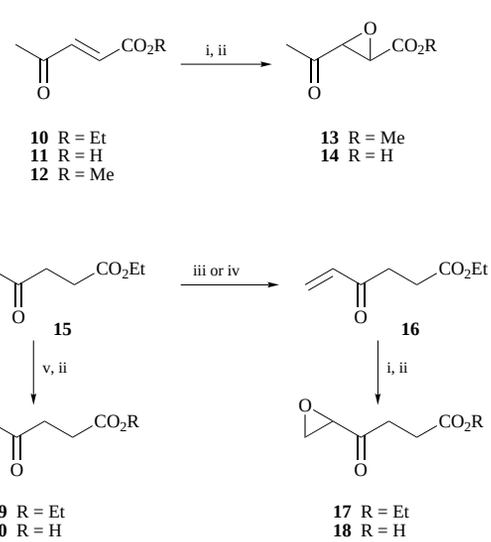

**Scheme 1** Early stages of the biosynthesis of tetrapyrroles

hand, ALA is made by reduction of glutamyl $^t$RNA **3** to the 1-semialdehyde **4**, followed by an intramolecular transamination. In all organisms two molecules of ALA are condensed to give the monopyrrole porphobilinogen (PBG) **6** by ALA dehydratase (PBG synthase, EC 4.2.1.24).

The mechanism of ALA dehydratase is known to involve formation of an intermediate imine between one of the two molecules of ALA and an active-site lysine residue.[3–6] This imine can be reduced with $NaBH_4$, resulting in irreversible inhibition of the enzyme. Among other inhibitors that have been tested with ALA dehydratase (mostly with enzymes from *Rhodopseudomonas spheroides*, *Escherichia coli* and bovine liver) are laevulinic acid **7** (see Scheme 2), which is a competitive inhibitor




**5** X = $NH_2$
**7** X = H
**8** X = Cl
**9** X = F




**Scheme 2** *Reagents and conditions:* i, $H_2O_2$, $K_2CO_3$; ii, NaOH; iii, $CH_2{=}CH_2$, $AlCl_3$, then $Et_3N$; iv, $Bu_3SnCH{=}CH_2$, $Pd^{II}$; v, $CH_2N_2$

with a binding constant similar to the $K_M$ of ALA as substrate and which forms the imine linkage to the enzyme.[3] Also 5-halolaevulinic acids, **8** and **9**, are very good competitive inhibitors[7,8] and the 5-chloro compound **8** inactivates the bovine and *E. coli* enzymes† by alkylation of cysteine residues.[8,10,11]

In view of the fact that tetrapyrrole biosynthesis is essential to all organisms, we were interested in making further analogues of ALA which might inhibit the ALA dehydratase of particular organisms and thus prevent their growth. In addition, by studying the reactions of these analogues with ALA dehydratase we hoped to learn more about the mechanism of

† It is reported that **8** does not readily inactivate *Bradyrhizobium japonicum* ALA dehydratase, however.[9]

this enzyme. In this paper we describe the synthesis of a number of such analogues and their inhibitory properties towards the ALA dehydratase from one organism, the bacterium *Bacillus subtilis*.[1]

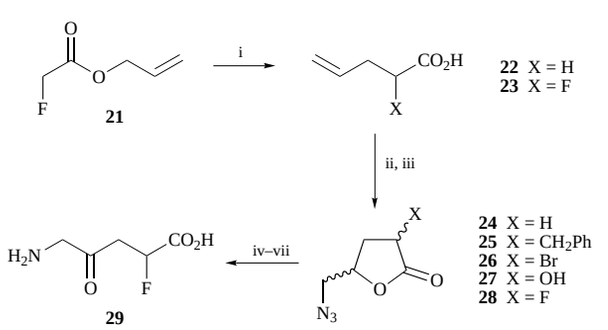
## Results and discussion

### Synthesis of analogues of ALA

Our first targets were dehydrolaevulinic acid **11** and epoxides **14** and **18**, which might alkylate nucleophilic groups in the active site in a similar fashion to that proposed for 5-chlorolaevulinic acid **8**.[8,10,11] The ethyl ester **10** was made by a Wittig reaction between pyruvaldehyde and (ethoxycarbonylmethylene)triphenylphosphorane. Hydrolysis of the ester with a strongly acidic ion exchange resin in water gave the acid **11**. For the synthesis of the corresponding epoxide **14**, the methyl ester **12**, which had recently become commercially available, was used as the starting material. Epoxidation was effected with aqueous potassium carbonate and hydrogen peroxide in 2-methylpropan-2-ol and the resulting ester **13** was rapidly hydrolysed by one equivalent of sodium hydroxide in water to give the carboxylic acid **14** as its sodium salt.

The alternative epoxide **18** was made similarly by epoxidation of enone **16** followed by hydrolysis of the ester group. 2-Methylpropan-2-ol was used as the solvent for the epoxidation reaction because with methanol a major product was the 2-methoxyethyl ketone resulting from conjugate addition of methanol across the C=C double bond. Enone **16** was made by two reported routes from the acid chloride **15**. Reaction of **15** with ethene and $AlCl_3$, to give the 2-chloroethyl ketone, followed by treatment with $Et_3N$ to eliminate HCl[12] was convenient on a large scale but left the product contaminated with diethyl succinate, which was difficult to remove. Alternatively reaction of **15** with tributylvinylstannane with a palladium(II) catalyst[13] gave the enone in high yield and purity but was less suitable on a large-scale due to the cost of the reagents. Reaction of acid chloride **15** with diazomethane gave diazo ketone **19**[14] and hydrolysis of the ester with one equivalent of alkali gave the acid **20** as its sodium salt, which was also tested as an inhibitor of ALA dehydratase (see below).

We have previously reported a synthesis of 2-fluoroALA **29** in 11% overall yield over nine steps starting from diethyl fluoromalonate.[15] Preliminary studies showed that it was a potent inhibitor of ALA dehydratase from *Euglena gracilis*. In order to study this inhibition further, we needed greater quantities of **29** and so we set out to develop a more direct synthetic route employing less expensive starting materials (see Scheme 3).



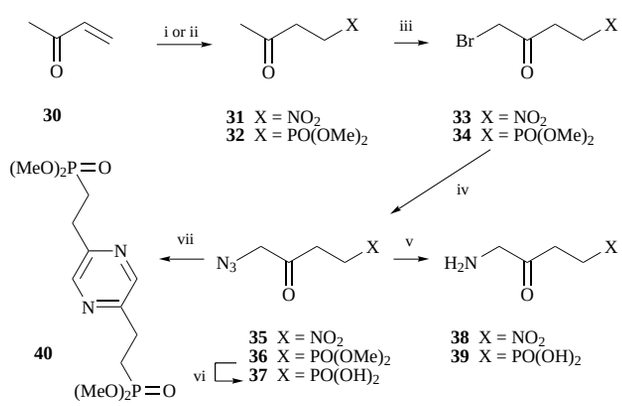

**Scheme 3** *Reagents and conditions:* i, LDA, $Me_3SiCl$; ii, $I_2$, $NaHCO_3$; iii, $NaN_3$, DMF; iv, KOH, MeOH then $CH_2N_2$; v, $CrO_3$, $H_2SO_4$; vi, $H_2$, Pd, HCl; vii, dil. HCl

Initially we investigated fluorination of the known[16,17] azido lactone **24**, made by iodolactonisation of pent-4-enoic acid **22** followed by displacement of the iodide by azide. The enolate derived from **24** by treatment with lithium hexamethyldisilazide reacted with benzyl bromide to give the benzylated lactone **25** (25:1 mixture of diastereoisomers) in 55% yield, and reacted with bromine to give the bromo lactone **26** (10:7 mixture of diastereoisomers) in 90% yield. However, hydroxylation of the enolate using 2-phenylsulfonyl-3-phenyloxaziridine yielded only 21% of the hydroxy lactone **27** and electrophilic fluorination with a variety of different reagents[18–20] and conditions never gave the fluoro lactone **28** in more than 6% yield. Conversion of the bromo lactone **26** into the fluoro lactone **28** was similarly unsuccessful.

In view of the difficulty in fluorinating lactone **24**, a direct synthesis of the fluorinated product **28** from fluoroacetic acid was sought. This synthesis employed the previously reported[21] Ireland–Claisen rearrangement of allyl fluoroacetate **21** to 2-fluoropent-4-enoic acid **23**. The reported synthesis of allyl fluoroacetate **21** involved the preparation and distillation of fluoroacetyl chloride on a large scale (100–200 g). As fluoroacetate derivatives are extremely toxic, we wanted to prepare the allyl ester **21** on a smaller scale and avoid the volatile acid chloride if possible. Therefore **21** was made directly from sodium fluoroacetate by alkylation with allyl bromide in DMF, which produced the desired ester in 85% yield. The Ireland–Claisen rearrangement proceeded essentially as described[21] and then iodolactonisation of the resulting 2-fluoropent-4-enoic acid **23**[21] and reaction with sodium azide in DMF gave the azidomethyl fluoro lactone **28** in 30% overall yield over the four steps from sodium fluoroacetate. This lactone has previously been converted into 2-fluoroALA **29**[15] and the same procedure was followed here with the exception that the final product **29** was purified by cation exchange chromatography on a strongly acidic resin eluting with increasing concentrations of hydrochloric acid.

The overall synthesis of 2-fluoroALA **29** (seven steps, 14% yield from sodium fluoroacetate) is an improvement over our previously reported method[15] and uses a considerably less expensive starting material.

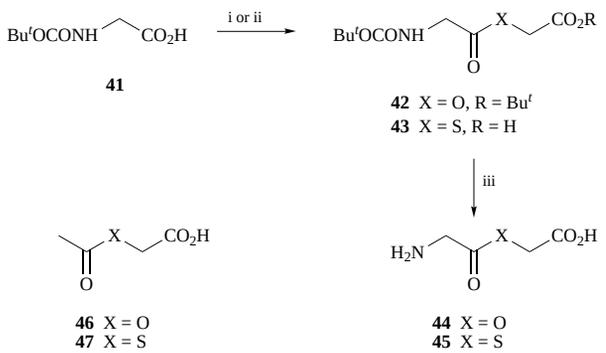
Our next synthetic targets were analogues of ALA having the carboxy group replaced by nitro and phosophono groups, **38** and **39**, Scheme 4. These analogues might be competitive inhibitors and/or substrates for ALA dehydratase. Nitro ketone **31**[22] and keto phosphonate **32**[23] were made by conjugate addition of nitrous acid and dimethyl phosphonic acid respectively to methyl vinyl ketone **30**. Bromination on the methyl group of ketones, **31** and **32**, using bromine in methanol has already been described by some of us in a communication.[24] The resulting bromomethyl ketones, **33** and **34**, were converted into the azidomethyl ketones, **35** and **36**, using sodium azide in DMF. The azido group of the nitro compound **35** was hydrogenated using palladium on carbon in the presence of hydrochloric acid to give the nitro analogue of ALA **38** as its hydrochloride salt (58% over the two steps from **33**). For the phosphonate **36** the hydrogenation was initially performed without the addition of hydrochloric acid. This led to the isolation of the pyrazine **40** resulting from dimerisation of the desired aminomethyl ketone and subsequent aerial oxidation. Therefore, it was decided to




**Scheme 4** *Reagents and conditions:* i, $NaNO_2$, HOAc; ii, $HPO(OMe)_2$; iii, $Br_2$, MeOH; iv, $NaN_3$, DMF; v, $H_2$, Pd, HCl; vi, $Me_3SiBr$; vii, $H_2$, Pd

cleave the phosphonate ester groups first, using $Me_3SiBr$, and then hydrogenate the azide group of the resulting phosphonic acid **37** in the presence of hydrochloric acid. This gives the phosphono analogue of ALA **39** in 79% yield over the three steps from **34**.

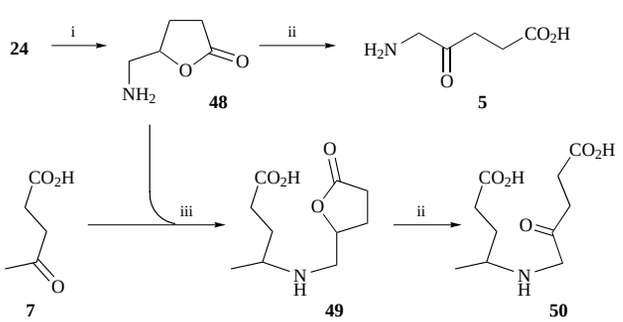
Next we planned to make the 3-oxa and 3-thia derivatives of ALA, **44** and **45**, Scheme 5. It was hoped that these might




**Scheme 5** *Reagents and conditions:* i, $ClCH_2CO_2Bu^t$, $Et_3N$; ii, $ClCO_2Et$, $Et_3N$ then $HSCH_2CO_2H$, $Et_3N$; iii, TFA

inactivate ALA dehydratase by acylating the active-site lysine residue. Alkylation of *N*-Boc-glycine **41** with *tert*-butyl chloroacetate followed by deprotection of the resulting ester **42** using TFA gave 3-oxaALA **44** as its trifluoroacetate salt. Ester **44** has previously been prepared by a similar procedure using benzyl protecting groups in place of *tert*-butyl.[25] For the synthesis of the thioester **45**, *N*-Boc-glycine **41** was activated using EtOCOCl and $Et_3N$ and the mixed anhydride reacted with mercaptoacetic acid. Deprotection of the thioester **43** was again effected with TFA. The substrate analogues lacking the 5-amino group were also studied: acetoxyacetic acid **46** is commercially available and *S*-acetylmercaptoacetic acid **47** was made by acetylation of mercaptoacetic acid using $Ac_2O$ and $Et_3N$.

Our final target was a bisubstrate analogue **50** intended to mimic an intermediate on one of the proposed mechanisms for ALA dehydratase.[26–28] The planned synthesis, shown in Scheme 6, involved reductive amination of laevulinic acid **7**




**Scheme 6** *Reagents and conditions:* i, Raney Ni; ii, $CrO_3$, $H_2SO_4$; iii, $NaBH_3CN$

with amine **48** followed by hydrolysis of the lactone **49** and oxidation of the resulting alcohol to the ketone.

The amine **48** was made in good yield from the corresponding azide **24** by reduction with Raney nickel and isolated as its hydrochloride salt following ion exchange chromatography. Reductive amination of laevulinic acid **7** with amine **48** to give amino acid **49** was effected in 75% yield using $NaBH_3CN$, with NaOAc to help deprotonate the amine hydrochloride. Before attempting the conversion of **49** into **50**, amine **48** was used to test the required procedure. Heating amine **48** in a dilute solution of $CrO_3$ in dil. sulfuric acid resulted in both hydrolysis of the lactone and oxidation of the alcohol to give ALA **5**, isolated in 65% yield after separation from the chromium(III) ions by ion exchange chromatography. The same procedure applied to the amino acid **49** then gave the target bisubstrate analogue **50** in 59% yield.

**Competitive inhibition of ALA dehydratase**

The ALA dehydratase used in this study was the enzyme from *B. subtilis*, whose gene had been cloned and overexpressed in *E. coli*.[29] The standard assay for enzymic activity was used, in which the reaction is stopped after a fixed length of time (10 min in our assays) and the amount of α-free pyrrole formed is determined by reaction with modified Ehrlich's reagent (acidic *p*-dimethylaminobenzaldehyde), giving a pink condensation product ($\lambda_{max}$ 555 nm).[30]

All the analogues of ALA were tested as reversible inhibitors of ALA dehydratase by measuring the rate of the enzymic reaction at various concentrations of ALA in the presence of two different concentrations of the analogue as well as in its absence. Apparent $V_{max}$ and $K_M$ values ($V_{max}'$ and $K_M'$) were obtained from the best fits of curves for the Michaelis–Menten equation to the datapoints. The $K_M$ value for ALA in the absence of any inhibitor, averaged over all the assays, was 0.29 mmol dm$^{-3}$. In all cases the effect of the analogues was primarily to increase the apparent $K_M$ value and little effect was seen on $V_{max}$, which shows that the inhibition is competitive. For some of the analogues a modest decrease in $V_{max}$ was observed but this is attributed to inactivation of the enzyme during the course of the assay (see later for studies of enzymic inactivation). $K_i$ values were calculated using the equation $K_M' = K_M(1 + [I]/K_i)$ and the values obtained for the two different concentrations of inhibitor were averaged. Table 1 summarises the results of these reversible inhibition experiments for all the analogues.

For the ALA dehydratases from bovine liver[8] and *R. spheroides*[3,31] it has been found that the 5-amino group of ALA is relatively unimportant for binding: *i.e.* the $K_i$ value for laevulinic acid **7** is similar to the $K_M$ value for ALA. This is not true for the dehydratase from *B. subtilis*, however, which has a $K_i$ value for laevulinic acid (20 mmol dm$^{-3}$) approximately 70 times greater than the $K_M$ value for ALA. This effect is also seen when comparing the $K_i$ values of **44** and **45** with those of **46** and **47** and in general the inhibition by analogues lacking this amino group (*e.g.* **14**, **18**, **20** and **50**) was disappointing. Some binding is recovered on introduction of the 5-chloro substituent in **8** ($K_i$, 1.6 mmol dm$^{-3}$) but still this compound is not nearly as good an inhibitor of the *B. subtilis* enzyme as of the bovine enzyme[8] ($K_i$, 11 μmol dm$^{-3}$). Considering the effect that the loss of the 5-amino group has on the other compounds, dehydrolaevulinic acid **11** is a surprisingly good inhibitor, which may suggest that this compound can bind in the active site in some alternative fashion.

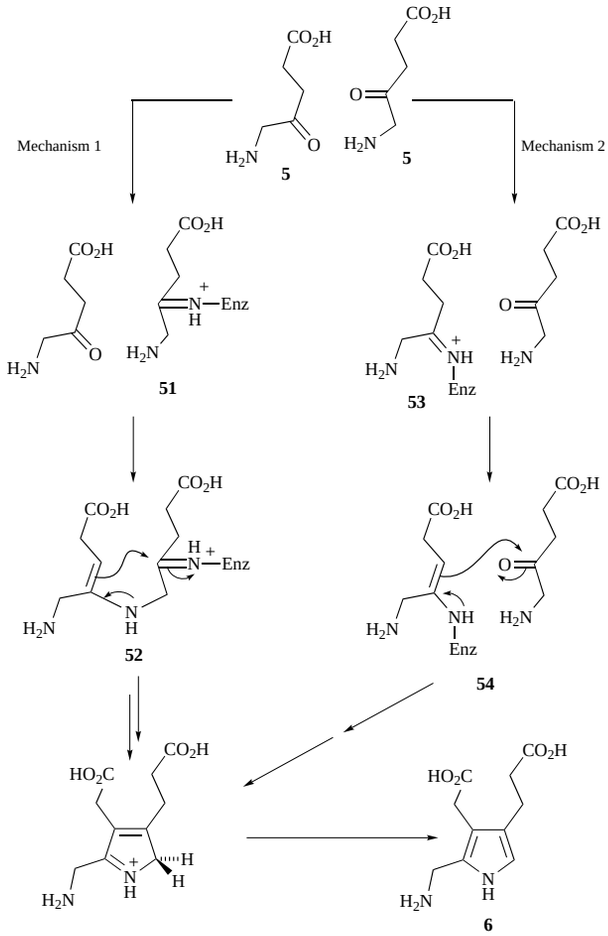
3-ThiaALA **45** ($K_i$, 1.2 mmol dm$^{-3}$) binds slightly worse than ALA but better than 3-oxaALA ($K_i$, 26 mmol dm$^{-3}$). This is reasonable because a sulfur atom is more similar to a methylene group in size and polarity than is an oxygen atom, whereas hydrogen bonding to water is more important for the oxygen atom than the sulfur. The 3-aza derivative of ALA, glycylglycine, has previously been tested as an inhibitor of bovine ALA dehydratase but no inhibition was observed.[7] A similar trend was seen with the corresponding compounds lacking the 5-amino group. Thus the inhibition by the thioester **47** ($K_i$, *ca.* 100 mmol dm$^{-3}$) is approximately five times weaker than that of laevulinic acid and no inhibition at all could be observed with the normal ester **46**.

The phosphonic acid analogue **39** was also tested as a competitive inhibitor but showed only very weak inhibition, $K_i \geqslant 40$ mmol dm$^{-3}$. This may be due to the larger size of the phosphonyl group compared with the carboxy group of ALA or it may be due to the fact that the phosphonic acid will exist as the dianion at pH 9. A similar phosphonic acid lacking the 5-amino group has been tested as an inhibitor of the ALA dehydratase from *R. spheroides* but no inhibition was observed.[31]

No inhibition by the bisubstrate analogue **50** was observed with the ALA dehydratase from *B. subtilis* and so this analogue was also tested as an inhibitor of the bovine liver dehydratase (for which the lack of the amino group at the site equivalent to C-5 of ALA should be less important). Some inhibition was observed in this case ($K_i$, *ca.* 5 mmol dm$^{-3}$) but the compound still binds considerably worse than laevulinic acid ($K_i$, 0.22 mmol dm$^{-3}$, ref. 8).

Two fundamentally different mechanisms can be considered for ALA dehydratase depending on which ALA molecule forms the imine with the active-site lysine residue. In mechanism 1 (Scheme 7),[26–28] the ALA molecule which occupies the P-site (*i.e.* the one that provides the **P**ropionate side-chain of PBG) forms the imine **51** and this imine is the electrophile in the subsequent carbon–carbon bond forming cyclisation of intermediate **52**. In mechanism 2,[3] the ALA molecule which occupies the alternative A-site (provides the **A**cetate side-chain of PBG) is the one that forms the imine **53**; this imine is converted into the enamine **54** which is the nucleophile in the carbon–carbon bond forming step. The bisubstrate analogue **50**, if it formed the imine linkage at the active site, would closely mimic intermediate **52** of mechanism 1 and might, therefore, be expected to inhibit the enzyme strongly. The low level of inhibition observed clearly does not support mechanism 1 but, on the other hand, it might be due to an inability to form the correct imine and so mechanism 1 cannot be ruled out.

After obtaining the above results, the stability of some of the ALA analogues under the conditions of the incubation was checked by $^1$H NMR spectroscopy. The two thioesters **45** and **47** were both sufficiently stable: no decomposition of **45** was seen after 30 min in phosphate-buffered $D_2O$, pH 8.5, at room temperature and only 4% hydrolysis of **47** after 15 min in pH 8.4 buffer at 37 °C. The normal ester **46**, however, showed 20% hydrolysis after 10 min in pH 9.0 buffer at room



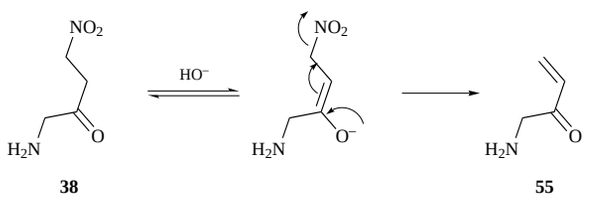

**Scheme 7** Alternative mechanisms for ALA dehydratase

**Table 1** Kinetic parameters for the reversible inhibition of ALA dehydratase from *B. subtilis* by the various analogues of ALA[a]

| Compound | Conc./mmol dm$^{-3}$ | $V_{max}'$ units mg$^{-1}$ | $K_M'$/mmol dm$^{-3}$ | $K_i$/mmol dm$^{-3}$ | Average $K_i$/mmol dm$^{-3}$ |
|---|---|---|---|---|---|
| **7** | 0 | 94.1 ± 1.7 | 0.33 ± 0.03 | | |
| | 10 | 94.3 ± 1.1 | 0.52 ± 0.02 | 17.9 ± 3.1 | |
| | 20 | 93.9 ± 1.46 | 0.62 ± 0.03 | 22.6 ± 3.2 | 20 |
| **8** | 0 | 91.5 ± 0.6 | 0.30 ± 0.01 | | |
| | 5 | 82.0 ± 3.5 | 1.29 ± 0.11 | 1.5 ± 0.2 | |
| | 10 | 72.9 ± 11.9 | 1.96 ± 0.54 | 1.8 ± 0.6 | 1.6 |
| **11** | 0 | 99.0 ± 3.5 | 0.35 ± 0.05 | | |
| | 5 | 92.3 ± 5.0 | 1.70 ± 0.17 | 1.3 ± 0.3 | |
| | 10 | 153.7 ± 29.5 | 6.14 ± 1.48 | 0.6 ± 0.2 | 0.9 |
| **14** | 0 | 152.4 ± 2.0 | 0.28 ± 0.02 | | |
| | 5 | 144.1 ± 2.5 | 0.46 ± 0.03 | 7.3 ± 1.3 | |
| | 9 | 135.4 ± 1.7 | 0.68 ± 0.03 | 6.1 ± 0.6 | 6.5 |
| **18** | 0 | 86.3 ± 0.9 | 0.32 ± 0.02 | | |
| | 1.5 | 82.7 ± 1.4 | 0.64 ± 0.03 | 1.5 ± 0.2 | |
| | 3 | 80.4 ± 2.3 | 1.03 ± 0.06 | 1.3 ± 0.2 | 1.4 |
| **20** | 0 | 94.6 ± 1.4 | 0.24 ± 0.02 | | |
| | 10 | 70.6 ± 1.4 | 0.27 ± 0.03 | 86.1 ± 92.4 | |
| | 15 | 63.7 ± 2.39 | 0.41 ± 0.06 | 21.0 ± 7.1 | 26 |
| **29**[b] | 0 | 99.2 ± 1.7 | 0.26 ± 0.02 | | |
| | 2 | 108.1 ± 3.9 | 0.65 ± 0.07 | 1.3 ± 0.3 | |
| | 3 | 118.5 ± 2.5 | 1.08 ± 0.05 | 0.9 ± 0.1 | 1.0 |
| **38**[b] | 0 | 140.2 ± 1.2 | 0.30 ± 0.01 | | |
| | 0.04 | 138.2 ± 1.9 | 0.34 ± 0.02 | 0.3 ± 0.2 | |
| | 0.1 | 135.2 ± 1.6 | 0.39 ± 0.02 | 0.3 ± 0.1 | 0.3 |
| **44** | 0 | 89.0 ± 1.8 | 0.21 ± 0.03 | | |
| | 30 | 75.9 ± 1.5 | 0.42 ± 0.03 | 29.1 ± 6.0 | |
| | 40 | 68.9 ± 0.8 | 0.54 ± 0.02 | 24.6 ± 3.5 | 26 |
| **45** | 0 | 103.6 ± 0.8 | 0.27 ± 0.01 | | |
| | 0.63 | 107.4 ± 0.8 | 0.42 ± 0.01 | 1.2 ± 0.2 | |
| | 0.95 | 110.0 ± 0.9 | 0.50 ± 0.02 | 1.1 ± 0.1 | 1.15 |
| **47** | 0 | 98.2 ± 1.6 | 0.36 ± 0.02 | | |
| | 162 | 93.2 ± 0.6 | 0.76 ± 0.01 | 141.7 ± 12.5 | |
| | 244 | 114.7 ± 15.7 | 2.06 ± 0.47 | 50.8 ± 14.3 | 100 |

[a] Little or no inhibition was observed with compounds **39**, **46** and **50**, so these have not been included in the table. [b] These analogues were not stable under the assay conditions. Elimination of HF from **29** and $HNO_2$ from **38** is thought to occur.

temperature. The nitro analogue **38** was found to be stable enough in aqueous solution as its hydrochloride salt but reacted rapidly in phosphate-buffered $D_2O$ at pH 8. Although the $^1H$ NMR spectrum indicated a mixture of compounds, the appearance of some peaks in the olefinic region (around $\delta$ 6.0) suggested that elimination of $HNO_2$ may have occurred to give the enone **55** (Scheme 8). As a result, it is not possible




38 55

**Scheme 8** Probable mode of decomposition of the nitro analogue **38**

to say what are the compounds which produce the relatively good inhibition observed with this compound. The stability of 2-fluoroALA **29** was not tested using NMR spectroscopy but the yellow coloration that appeared immediately upon adjusting a solution of the hydrochloride to pH 9 suggests decomposition was occurring and the likely initial reaction is elimination of HF to give 2,3-dehydroALA. It may be that this latter compound is responsible for the inhibition observed with 2-fluoroALA **29**.

**Inactivation of ALA dehydratase**

In the absence of thiols, ALA dehydratases are very susceptible to aerial oxidation and we have found this is true of the *B. subtilis* enzyme also. In the cases of the mammalian[32] and bacterial[33] dehydratases, this has been shown to be due to disulfide bond formation between two of the cysteines that are ligands to a $Zn^{2+}$ ion with concomitant loss of the metal ion. However several of the inactivators were found to react with thiols such as dithiothreitol (DTT) and β-mercaptoethanol and so the inactivation experiments were carried out in well degassed thiol-free buffers. Little loss of activity was observed under these conditions and the activities quoted below are in all cases expressed as a percentage of the activity of a parallel control sample which had been treated identically except without inactivator.

Inactivation experiments were carried out by incubating a relatively concentrated solution of thiol-free enzyme with the ALA analogue at 37 °C. At timed intervals, aliquots were taken and added to the assay mixture containing ALA at a final concentration of 2 mmol $dm^{-3}$. The 250-fold dilution at this stage ensured that no effect would be observed due to any reversible inhibition by the analogue. The result was a series of curves for the time-dependent loss of activity [the solid line in Fig. 1(a) is one curve resulting from inactivation by thioester **47**]. For many of the analogues, the enzymic activity followed a pseudo first-order exponential decay and the rate constant was determined by the best fit of the equation $A = A_o e^{-kt}$ to the datapoints.

All the compounds listed in Table 2 were tested for inactivation of ALA dehydratase as described above. The table gives the percentage inactivation observed after 10 min and for those analogues which showed reasonable exponential decay of the enzymic activity after 10 min, the values of $A_o$ and $k$ are also given. It should be noted that the values of $A_o$ are in most cases significantly less than 100%, indicating a rapid loss of a certain amount of activity within the first 10 min before the exponential decay. The explanation of this is not clear. The results shown in Table 2 are discussed below for each analogue in turn.

In addition the reactions of the ALA analogues with the enzyme were also followed by electrospray mass spectrometry (ESMS). In these experiments the enzyme was incubated with various concentrations of inactivators for 15 min at 37 °C. An

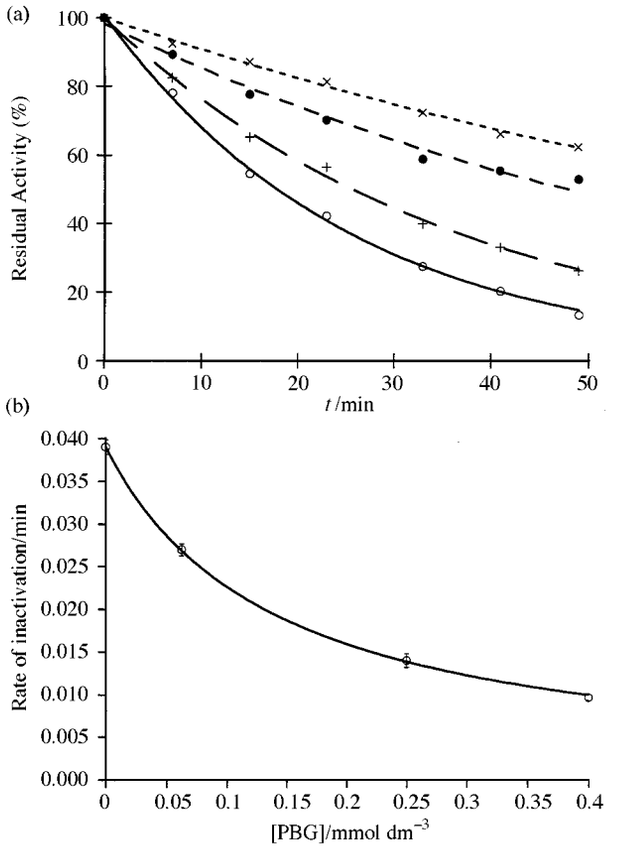



**Fig. 1** Protection of ALA dehydratase by PBG **6** from inactivation by thioester **47** (18 mmol $dm^{-3}$). (a) Loss of enzymic activity plotted against time for four different concentrations of PBG, 0 mmol $dm^{-3}$ (○), 0.0625 mmol $dm^{-3}$ (+), 0.25 mmol $dm^{-3}$ (●) and 0.4 mmol $dm^{-3}$ (×); the lines are the best fit of the equation Activity = $A_o e^{-kt}$ to the datapoints. (b) Rate of inactivation, $k$ derived from the curve-fitting in (a), plotted against [PBG]; the line is the best fit of the equation rate = $a/(b + [PBG])$ to the datapoints, with $a = 0.0054 \pm 0.0001$ and $b = 0.138 \pm 0.003$.

aliquot was then taken for measurement of the enzymic activity while the remainder was extensively dialysed against aqueous ammonium hydrogen carbonate (5 mmol $dm^{-3}$) to remove other ions and small molecules which can interfere with ESMS.

Native ALA dehydratase showed a molecular weight of 36 082 ± 3 Da by ESMS [see Fig. 2(a)] in close agreement with the weight (36 078 Da) calculated from the gene-derived sequence,[34] minus the *N*-terminal methionine. Any metal ions that may have been bound to the enzyme (*e.g.* $Zn^{2+}$ or $Mg^{2+}$) have clearly been largely lost either during preparation of the sample or in the mass spectrometer.‡

Reaction of ALA dehydratase from *B. subtilis* with ALA (0.4 mmol $dm^{-3}$) followed by $NaBH_4$ (1.2 mmol $dm^{-3}$) caused 45% inactivation, showing that this enzyme, like dehydratases from other sources, forms an imine with its substrate. Similar inactivation (50%) was obtained using laevulinic acid **7** (0.094 mmol $dm^{-3}$) followed by $NaBH_4$ (0.19 mmol $dm^{-3}$). ESMS analysis after reaction with ALA and $NaBH_4$ [Fig. 2(b)] showed the appearance of a new major peak corresponding closely to the expected mass increase of 115 Da.

The first ALA analogue to be investigated was 5-chlorolaevulinic acid **8**, a known inactivator of ALA dehydratases from bovine liver[8–10] and *E. coli*.[10] As shown by Table 2, time-

‡ A minor 'shadow' peak, *ca.* 77 mass units higher than the main peak was observed in all samples. This seems to be due to a superimposition of minor peaks due to adducts with buffer components, metal ions *etc.* An unassigned 'shadow' peak, 98 mass units higher than the major peak, was also observed for ALA dehydratase from *E. coli* by Spencer and Jordan.[6]

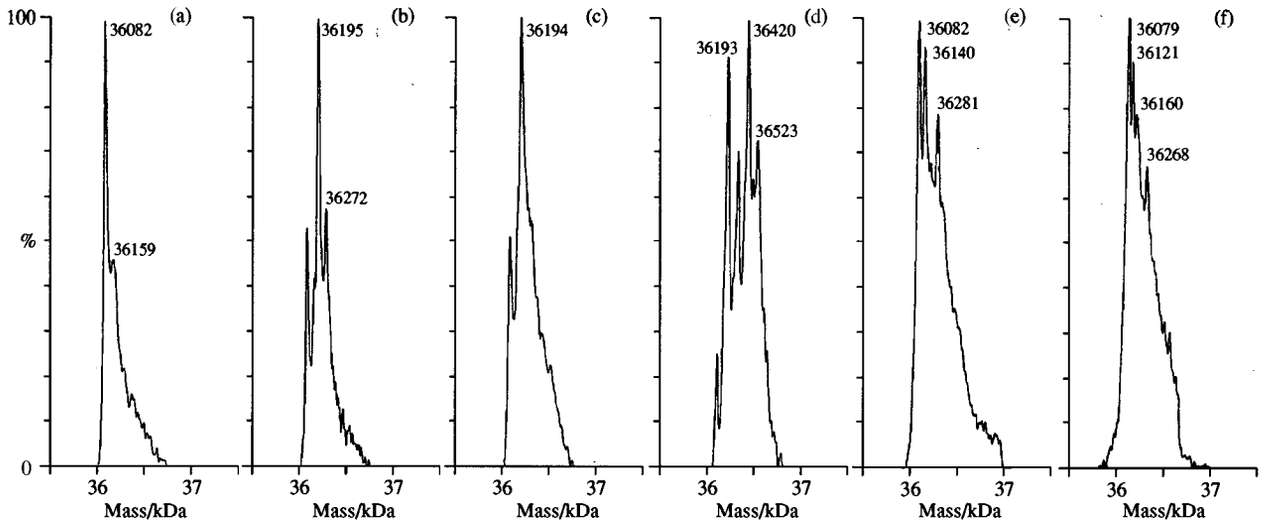



**Fig. 2** Electrospray mass spectra of ALA dehydratase; (a) native enzyme; (b)–(e) after treatment with (b) ALA and $NaBH_4$, (c) 5-chlorolaevulinic acid **8** (2 mmol $dm^{-3}$), (d) **8** (50 mmol $dm^{-3}$), (e) thioester **45** (0.5 mmol $dm^{-3}$) and (f) thioester **47** (12 mmol $dm^{-3}$) for 15 min at 37 °C

**Table 2** Kinetic parameters for the inactivation of ALA dehydratase by the various analogues of ALA

| Compound | Conc./ mmol $dm^{-3}$ | Inactivation (%) in 10 min | $A_o$ (%)[a] | $k$[a]/$10^3$ $min^{-1}$ |
|---|---|---|---|---|
| **8** | 28 | 26 | 84 | 9.6 ± 0.7 |
| | 44 | 32 | 82 | 14.6 ± 1.3 |
| | 59 | 35 | 79 | 21.1 ± 0.6 |
| | 96 | 45 | 73 | 28.9 ± 1.1 |
| **11** | 5 | 17 | 81 | 0.2 ± 0.5 |
| | 10 | 23 | 78 | 1.6 ± 0.4 |
| | 20 | 29 | 71 | 3.5 ± 0.6 |
| | 31 | 41 | 63 | 8.5 ± 0.4 |
| **14** | 2 | 21 | | |
| | 4 | 25 | | |
| | 6 | 41 | | |
| | 8 | 42 | | |
| **20** | 75 | 36 | | |
| | 150 | 60 | 42 | 6.3 ± 0.6 |
| | 275 | 65 | 37 | 9.1 ± 0.9 |
| | 400 | 71 | 32 | 13.2 ± 0.9 |
| **29**[b] | 107 | 2.5 | | |
| | 143 | 17 | | |
| | 178 | 14 | | |
| | 196 | 25 | | |
| | 214 | 30 | | |
| **38**[b] | 16 | 22 | 79 | 5.0 ± 0.7 |
| | 25 | 27 | 77 | 8.0 ± 0.9 |
| | 32 | 30 | 75 | 9.2 ± 0.6 |
| | 45 | 41 | 67 | 18.0 ± 1.3 |
| | 51 | 47 | 65 | 19.8 ± 0.5 |
| **44** | 36 | 29 | | |
| | 54 | 46 | | |
| | 72 | 62 | | |
| | 90 | 70 | | |
| **45** | 0.5 | 21 | 85 | 8.3 ± 1.0 |
| | 1.0 | 44 | 75 | 29.2 ± 1.0 |
| | 1.4 | 59 | 67 | 48.3 ± 1.9 |
| | 1.7 | 72 | 60 | 76.6 ± 2.6 |
| | 2.0 | 79 | 61 | 105.2 ± 15.4 |
| **47** | 5 | 27 | 94 | 25.0 ± 0.8 |
| | 10 | 41 | 97 | 50.2 ± 0.6 |
| | 15 | 59 | 81 | 67.9 ± 2.1 |
| | 20 | 65 | 79 | 81.1 ± 4.6 |
| | 25 | 72 | 65 | 86.2 ± 8.3 |

[a] The values $A_o$ and $k$ are only given for those compounds which gave exponential decay of enzymic activity (activity = $A_o e^{-kt}$) from the first timepoint (usually 10 min) onwards. [b] See footnote b in Table 1.

dependent inactivation was observed but only with concentrations of **8** considerably higher than required for competitive inhibition ($K_i$, 1.6 mmol $dm^{-3}$, Table 1). Furthermore the rate of inactivation increases linearly with the concentration of inactivator with no evidence of saturation kinetics. This suggests that, although 5-chlorolaevulinic acid does bind in the active site (hence the competitive inhibition), the alkylation reaction that causes inactivation does not occur at that site and may not involve prior non-covalent binding of **8** to the enzyme at all.

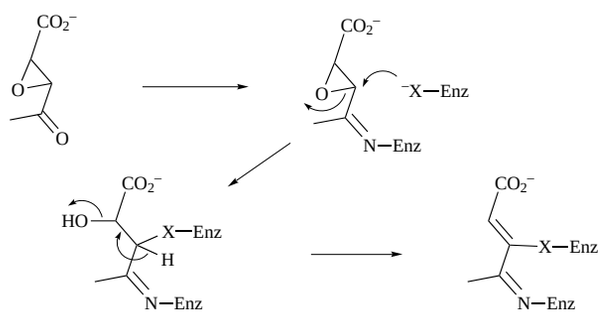
ESMS analysis was performed after reaction of the enzyme with six different concentrations of 5-chlorolaevulinic acid **8**. At the lowest concentration (2 mmol $dm^{-3}$) relatively clean monoalkylation was observed [Fig. 2(c)] but virtually no inactivation. With **8** at 50 mmol $dm^{-3}$, a series of peaks corresponding to mono- to tetra-alkylation was observed [Fig. 2(d)] but the degree of inactivation was modest (45%). At the highest concentration (100 mmol $dm^{-3}$) the major species were tri- and tetra-alkylated and yet the level of inactivation was still only 59%. Comparison of the peak heights with the residual activity indicates that even the trialkylated enzyme must retain substantial activity.

The conclusion from these experiments is that 5-CLA is not an active site-directed inhibitor of ALA dehydratase from *B. subtilis* but simply a non-specific alkylating agent, most likely to alkylate cysteine residues. There are only four cysteines encoded by the gene sequence[34] and this matches the maximum number of alkylations observed per enzyme subunit. The similarity between this ALA dehydratase and that from *E. coli* (48% identity[34]) suggests that the three cysteine residues which are conserved are likely to be involved[35] in binding $Zn^{2+}$. At least two of these three cysteines will be alkylated in the trialkylated enzyme and, as it is unlikely that $Zn^{2+}$ would still bind after alkylation of at least two of its ligands, it would seem that this $Zn^{2+}$ ion (if present) is not essential for catalysis. It has been proposed, however, that the ALA dehydratase from *E. coli* contains two zinc binding sites, one 'catalytic' and one 'non-catalytic', and that the cysteine residues are ligands to the 'non-catalytic' zinc.[35]

The lesson to be learnt from this example is that it is dangerous to propose that a compound is a specific active-site directed inhibitor unless (a) it is effective at concentrations comparable to its $K_i$ value and (b) it has been demonstrated that a single modification event leads to inactivation of the enzyme. For the inactivation of bovine liver ALA dehydratase by **8**, criterion (b) has been satisfied[8] but the concentrations required for inactivation (>1 mmol $dm^{-3}$) are well in excess of the $K_i$ value (11 μmol $dm^{-3}$) and saturation kinetics were not observed. For inactivation of *E. coli* dehydratase[11] neither (a) nor (b) has been demonstrated.

The results with 4-oxopent-2-enoic acid (2,3-dehydrolaevulinic acid) **11** indicate that it also is a non-specific alkylating agent similar to 5-chlorolaevulinic acid. Thus **11** only inactivated slowly, even at concentrations (31 mmol $dm^{-3}$) much higher than its $K_i$ value (0.9 mmol $dm^{-3}$). ESMS confirmed that multiple alkylation had occurred: this time, peaks for enzyme which had been alkylated up to six times were visible even though the level of inactivation was only 43%.

2,3-Epoxylaevulinic acid **14** showed some inactivation of ALA dehydratase at moderate concentrations (2–8 mmol $dm^{-3}$) but the enzymic activity did not decay towards 0% with time but instead levelled off after about 30 min with 40% or more of the activity remaining. It is possible that **14** is unstable under the incubation conditions and had completely decomposed after 30 min. ESMS of dehydratase after reaction with **14** (6 mmol $dm^{-3}$; 37% inactivation) showed the major species as unmodified enzyme (except that oxidation to produce a disulfide linkage may have occurred, as a change in mass of only 2 Da could not be detected) but a minor peak appeared 95 Da higher (36 172 Da), which increased in size with increasing concentration of inactivator. It is difficult to be sure that this small peak arises from modification of the enzyme by **14** but, if it does, it would correspond most closely to [enzyme + **14** − $2H_2O$] (expected mass increase 94 Da). Loss of one molecule of water could result from an elimination reaction following nucleophilic opening of the epoxide, and loss of the second molecule of water could result from imine formation between the ketone and an amino group on the enzyme. Scheme 9 shows a tentative




**Scheme 9** Possible reaction between epoxide **14** and ALA dehydratase

proposal of how such a modification could occur if there is a nucleophilic group $X^-$ in the active site in an appropriate position to attack C-3 of the ALA analogue.

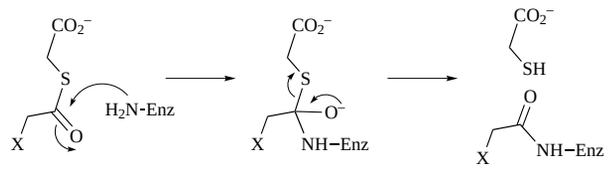
In contrast to the 2,3-epoxide **14**, the 5,6-epoxide **18** showed no detectable inactivation of ALA dehydratase, even at concentrations as high as 160 mmol $dm^{-3}$, despite the fact that it is a relatively good competitive inhibitor.

5-Diazolaevulinic acid **20** required high concentrations to achieve any significant inactivation. After a large loss of activity within the first 10 min, only slow further inactivation was observed. In accord with this, ESMS showed little change from the native enzyme after reaction with **20** at 50 or 75 mmol $dm^{-3}$.

As noted above, 1-amino-4-nitrobutanone **38** is unstable under the incubation conditions and probably eliminates $HNO_2$ to give enone **55** (among other products), which would be expected to alkylate the enzyme in a similar way to dehydrolaevulinic acid **11**. Time-dependent inactivation of dehydratase by **38** was indeed observed. The ESMS spectrum after reaction of the enzyme with **38** at 20 mmol $dm^{-3}$ showed a large number of peaks but the highest peak (and the first one above that for native enzyme) is at 36 159 Da, consistent with the monoadduct of the enzyme with enone **55** (calculated mass, 36 163 Da).

It was also suspected that 2-fluoroALA **29** is unstable at pH 9 and so for this compound inactivation experiments were performed at pH 5.4. However, little inactivation was observed and concentrations of **29** between 107 and 214 mmol $dm^{-3}$ were needed to obtain any significant loss of activity. After an initial drop, the enzymic activity remained fairly constant for 20–30 min and then began to fall. This suggests that the 2-fluoroALA does not itself inactivate the enzyme but decomposes slowly to a product which does inactivate.

The esters **44** and **46** and the thioesters **45** and **47** were designed to inactivate ALA dehydratase by acylation of the active-site lysine residue by the mechanism shown in Scheme 10. When 2-glycyloxyacetic acid **44** was tested at concentrations




**45** X = $NH_2$
**47** X = H

**56** X = $NH_2$
**57** X = H

**Scheme 10** Proposed reaction between thioesters **45** and **47** and ALA dehydratase

between 36 and 90 mmol $dm^{-3}$, considerable inactivation was observed within the first 10 min but thereafter there was little further decrease in activity. With acetoxyacetic acid **46** no inactivation was observed, which was not unexpected in view of the fact that it showed no binding to dehydratase in the competitive inhibition experiments.

The thioesters **45** and **47** are much better competitive inhibitors than esters **44** and **46** and they also proved to be much better at inactivating the enzyme. 2-Glycylthioacetic acid **45** was tested at concentrations between 0.5 and 2.0 mmol $dm^{-3}$ and 2-acetylthioacetic acid **47** was tested at concentrations between 5 and 25 mmol $dm^{-3}$. In both cases higher concentrations were not used because the inactivation would have been too rapid to follow using our standard procedure, which involved taking aliquots every 10 min. It is clear that both thioesters show substantial rates of inactivation at concentrations equal to or less than their $K_i$ values (1.15 and *ca.* 100 mmol $dm^{-3}$ respectively). ESMS spectra of ALA dehydratase after reaction with **45** at 0.5 mmol $dm^{-3}$ (47% inactivation) and with **47** at 12 mmol $dm^{-3}$ (44% inactivation) are given in Fig. 2(e) and 2(f). In both spectra higher mass peaks appear [*e.g.* at 36 281 Da in Fig. 2(e)] which are thought to be due to covalent adducts of native or modified enzyme with DTT. The major feature of the spectra, however, is the appearance of peaks at 36 140 in Fig. 2(e) and at 36 121 in Fig. 2(f), which are attributable to the monoglycyl- **56** and monoacetyl-enzyme **57** respectively. At the higher concentrations peaks appear which are probably due to diacylated enzyme [*e.g.* at 36 160 in Fig. 2(*f*)]. It is clear from the degree of inactivation that the monoacylated enzyme must be largely (and probably entirely) inactive in both cases. These two thioesters, therefore, meet the criteria laid down above for true active-site directed inactivators. It is most probable that they do indeed react with ALA dehydratase as shown in Scheme 10 but it does still need to be proved that it is the active-site lysine residue which is acylated as proposed. After the completion of this work, a communication was published describing the inactivation of *E. coli* ALA dehydratase by thioester **45**.[36] In that work covalent attachment of the glycyl moiety of **45** to the enzyme was demonstrated by $^{13}C$ NMR spectroscopy rather than ESMS as here. The normal ester **44** was also tested but no inactivation was observed.[36]

For most of the inhibitors listed in Table 2 the rate of inactivation is approximately proportional to the concentration of inhibitor, as would be expected for a simple bimolecular reaction with no formation of a non-covalent complex prior to the irreversible inactivation step. (Alternatively, if an initial non-covalent complex is involved, its dissociation constant should be higher than the concentrations of inhibitor studied, so that saturation kinetics are not observed.) For 2-glycylthioacetic acid **45**, however, the graph of rate of inactivation *vs.* concentration unexpectedly curves upwards (Fig. 3).

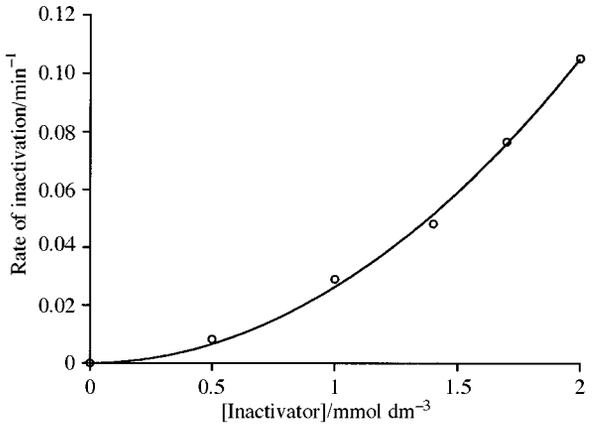


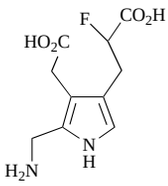

**Fig. 3** Rate of inactivation of ALA dehydratase by thioester **45** plotted against concentration of **45**. The curve is the best fit of the equation rate = $k$[Inactivator]$^2$ to the datapoints, with $k = 0.0263 \pm 0.0004$.

In fact the rate is approximately proportional to the concentration of inhibitor squared. This implies that *two* molecules of **45** have to bind before attack of the active-site amine on the thioester occurs. This is not unreasonable as ALA dehydratase obviously has binding sites for two ALA molecules per active site.

These results with thioester **45** suggest that for ALA, which is very similar in structure, it may also be the case that two molecules bind non-covalently before attack of the active-site amine on the ketone occurs to form the imine. Previously it has been assumed that the first molecule of ALA to bind to the enzyme forms the imine and then the second molecule binds subsequently[26,27] and the results of an experiment, in which the initial enzyme–ALA complex was reacted with $NaBH_4$, lent support to this assumption.[28] If it is correct that two molecules of ALA have to bind in the active site before imine formation occurs, however, then the conclusion[25–27] that imine formation with the active site lysine residue occurs in the P-site needs to be re-evaluated. Until further information becomes available, both mechanisms shown in Scheme 7 should continue to be considered as possibilities.

Finally, the finding that thioesters **45** and **47** are active-site directed inhibitors has allowed the affinity of the product, PBG **6**, for the active site to be measured. It was not possible to measure a $K_i$ value for PBG as a competitive inhibitor because the excess PBG present caused too large a background reading in the assay of enzymic activity. However, a binding constant could be calculated from the protection that PBG provides from inactivation by thioester **47** because the incubation mixture for the inactivation reaction is diluted 250-fold before the assay for residual enzymic activity. Fig. 1(a) shows the data for the inactivation of ALA dehydratase by **47** in the presence of PBG at 0, 62.5, 250 and 400 μmol dm$^{-3}$. The rates of inactivation were calculated by line-fitting (as described above) and are plotted against the concentration of PBG in Fig. 1(b). Saturation kinetics were not observed for the inactivation of ALA dehydratase by **47** and so the inactivation can be treated as a simple second order reaction between the enzyme and **47**. In these circumstances, the rate of inactivation in the presence of a protecting agent P is given by the equation $V = V_{max}K_P/(K_P + [P])$ where $K_P$ is the dissociation constant for P. The value of $K_P$ for PBG was calculated to be 138 ± 4 μmol dm$^{-3}$ by fitting a curve of equation $y = a/(b + x)$ to the datapoints in Fig. 1(b).

The same experiment was repeated using 9-fluoroPBG[37] **58** and laevulinic acid **7** as the protecting agents. Considerably more fluoroPBG than PBG was required to provide the same level of protection. As a result there was a greater background reading in the enzymic assay due to the added pyrrole and, consequently, a greater error in the readings. The value




**58**

obtained for the dissociation constant of fluoroPBG **58** was 1.3 ± 0.2 mmol dm$^{-3}$. For laevulinic acid **7** a dissociation constant of 3.6 ± 0.5 mmol dm$^{-3}$ was obtained. Somewhat surprisingly this value is significantly lower than the $K_i$ value for laevulinic acid as a competitive inhibitor (20 mmol dm$^{-3}$). The explanation presumably lies in the fact that laevulinic acid could bind on either of the two sides of the active site (A-site or P-site) and will have different dissociation constants at the two sites.

## Conclusion

The enzymic experiments described here have shown that 5-chlorolaevulinic acid **8** and 2,3-dehydrolaevulinic acid **11**, despite their similarity to ALA **5**, inactivate *B. subtilis* ALA dehydratase by non-specific alkylation reactions and are not active-site directed inhibitors. Electrospray mass spectrometry (ESMS) proved particularly useful in demonstrating this fact but it could also be deduced from the kinetics of the inactivation process.

In contrast the thioesters **45** and **47** (3-thiaALA and 3-thialaevulinic acid) proved to be potent inactivators of ALA dehydratase. ESMS showed that the enzyme becomes acylated and that a single acylation reaction, presumably on the active-site lysine residue, is sufficient to cause inactivation. The strategy of replacing a ketone group of the substrate by a thioester could well be a successful approach in developing mechanism-based inhibitors of other Schiff's base-forming enzymes such as aldolases, decarboxylases, dehydratases and transaminases.

The kinetics of inhibition of ALA dehydratase by thioester **45** suggested that two molecules have to bind before attack by the active-site lysine occurs. If this is also true for the natural substrate, ALA, it has implications concerning the correct mechanism for the enzyme and means that both mechanisms shown in Scheme 7 should continue to be considered as possibilities.

## Experimental

### General directions

Melting points were recorded using Buchi 510 and Reichert hotstage melting point apparatus and are uncorrected. Infrared spectra were recorded using Perkin-Elmer 1310 and 1710 Fourier Transform spectrometers as a thin film, as a solution in 0.5 mm NaCl cells or as a KBr disc, as indicated. NMR spectra were recorded using Bruker WM-200, WM-250, WH-400 and AC-250 spectrometers; coupling constants $J$ are quoted in Hz. In the $^{13}C$ NMR spectra, the number of hydrogen atoms attached to each carbon atom was deduced, where indicated, using the APT $J$-modulated spin-echo pulse sequence. Mass spectra were recorded on Kratos MS30 and MS50 mass spectrometers in electron impact mode unless otherwise stated. FAB (+ve and −ve ion mode) spectra were recorded on a Kratos MS80 spectrometer. Reagents were purchased from Aldrich Chemical Company or Sigma Chemical Company unless otherwise stated. Solvents were distilled and, where indicated, dried by standard techniques. Analytical thin layer chromatography was carried out on Kieselgel 60 silica plates (0.25 mm). Flash column chromatography was carried out using 230–400 mesh Kieselgel 60 silica.

The cloning and overexpression of the *hemB* gene from *B. subtilis* and the purification of the resulting ALA dehydratase will be described elsewhere.[29] The stock solutions of purified enzyme contained protein (4.26, 0.64 and 0.42 mg $cm^{-3}$) dissolved in Tris·HCl buffer (20 mM; pH 7.5) containing NaCl (500 mM), zinc sulfate (0.1 mM) and β-mercaptoethanol (20 mM). These were mixed with 5 or 10% glycerol and stored at −70 °C. Electrospray mass spectra were recorded using a VG BioQ quadrupole mass spectrometer. Ultraviolet–visible spectra were recorded on a Kontron Instruments Uvikon 860 (room temperature) and a Varian Carey 1E UV–Visible spectrophotometer (temperature controlled with a Grant LTD6 thermostatted water bath), using 1 cm polystyrene or 1 cm or 1 mm quartz cells.

**Ethyl 5-chloro-4-oxopentanoate**

Ethyl succinyl chloride **15** (5 $cm^3$, 35.1 mmol) was reacted with diazomethane followed by hydrogen chloride as described.[14] Bulb-to-bulb distillation of the product gave the chloro ketone (5.47 g, 87%) as a liquid, bp 92–102 °C (0.5 mmHg) [lit.,[38] bp 56 °C (0.005 mmHg)] (Found: $MH^+$, 179.0475. $C_7H_{12}O_3{}^{35}Cl$ requires *MH*, 179.0475); $\nu_{max}$(thin film)/$cm^{-1}$ 1731br (C=O); $\delta_H$(200 MHz, $CDCl_3$) 1.22 (3 H, t, *J* 7, $CH_2CH_3$), 2.57 and 2.85 (each 2 H, t, *J* 7, $CH_2CH_2$), 4.09 (2 H, q, *J* 7, $CH_2CH_3$) and 4.12 (2 H, s, $CH_2Cl$); $\delta_C$(100 MHz, APT, $CDCl_3$) 14.1 ($CH_2CH_3$), 28.1 and 34.3 ($CH_2CH_2$), 48.2 ($CH_2Cl$), 60.8 ($CH_2CH_3$), 172.3 ($CO_2$) and 201.3 (C=O); *m*/*z* (CI) 198 and 196 ($M + NH_4^+$, 31 and 100%), 181 and 179 ($MH^+$, 25 and 69), 129 ($MH - CH_2Cl$), 100 ($MH - CH_2Cl - Et$) and 55 (25).

**5-Chloro-4-oxopentanoic acid 8**

Ethyl 5-chloro-4-oxopentanoate (4 g, 22.4 mmol) was hydrolysed in hydrochloric acid as described[14] to give the acid **8** (3.04 g, 90%), mp 69–71 °C (lit.,[14] 68–70 °C) (Found: $MH^+$, 151.0162. $C_5H_8O_3{}^{35}Cl$ requires *MH*, 151.0162); $\delta_H$(200 MHz, $CD_3OD$) 2.59 and 2.82 (each 2 H, t, *J* 6, $CH_2CH_2$) and 4.33 (2 H, s, $CH_2Cl$); $\delta_C$(100 MHz, APT, $CD_3OD$) 28.8 and 35.3 ($CH_2CH_2$), 48.4–49.7 ($CH_2Cl$ plus $CD_3OD$), 176.1 ($CO_2H$) and 203.2 (C=O); *m*/*z* (FAB +ve) 151 ($MH^+$, 40%), 150 ($M^+$, 47), 149 (M − H, 50), 123 (89), 121 (90), 115 (M − Cl, 65) and 105 (100).

**Ethyl 4-oxopent-2-enoate 10**

A solution of (ethoxycarbonylmethylene)triphenylphosphorane[39] (21.3 g, 61 mmol) in a small volume of dichloromethane was added slowly to aqueous pyruvaldehyde (40 wt%; 46.7 $cm^3$, 305 mmol) at room temperature. The mixture was diluted with *N*,*N*-dimethylformamide (30 $cm^3$), stirred for 20 h until TLC showed the reaction was complete, then added to water and extracted with dichloromethane. The organic extract was washed successively with water and brine, dried ($MgSO_4$) and evaporated under reduced pressure to give the ester **10** (8.3 g, 96%) as an oil, shown by $^1H$ NMR spectroscopy to consist of a mixture of *E* and *Z* isomers (9 : 1). The isomers were separated by flash column chromatography, eluting with hexane–ethyl acetate (3 : 1). *E* isomer: $R_f$ 0.80 (EtOAc–hexane, 1 : 1); $\delta_H$($CD_3COCD_3$, 60 MHz) 1.4 (3 H, t, *J* 7, $CH_2CH_3$), 2.3 (3 H, s, $COCH_3$), 4.15 (2 H, q, *J* 7, $CH_2CH_3$) and 6.6 and 6.9 (each 1 H, d, *J* 15, CH=CH); *Z* isomer: $R_f$ 0.74 (EtOAc–hexane, 1 : 1); $\delta_H$($CD_3COCD_3$, 60 MHz) 1.3 (3 H, t, *J* 7, $CH_2CH_3$), 2.3 (3 H, s, $COCH_3$), 4.15 (2 H, q, *J* 7, $CH_2CH_3$) and 5.9 and 6.4 (each 1 H, d, *J* 12, CH=CH).

**(*E*)-4-Oxopent-2-enoic acid 11**

Ethyl (*E*)-4-oxopent-2-enoate **10** (800 mg, 5.63 mmol) was stirred with Dowex 50X8-100 ($H^+$) ion exchange resin (5 g) in water (15 $cm^3$) for 2 h at room temperature. The mixture was filtered and the residue washed with hydrochloric acid (1 mol $dm^{-3}$; 2 × 15 $cm^3$). The filtrate and washings were extracted with diethyl ether (3 × 50 $cm^3$). The combined extracts were dried ($MgSO_4$) and evaporated under reduced pressure to give the acid **11** (540 mg, 84%), mp 120–122 °C (from acetone; lit.,[40] 120–121 °C); $\delta_H$(200 MHz, $D_2O$) 2.20 (3 H, s, $CH_3$), 6.42 and 6.65 (each 1 H, d, *J* 16, HC=CH).

**Methyl *trans*-2,3-epoxy-4-oxopentanoate 13**

Saturated aqueous potassium carbonate (110 $mm^3$) was added dropwise to a stirred solution of methyl (*E*)-4-oxopent-2-enoate **12** (200 mg, 1.56 mmol), 30% hydrogen peroxide (312 $mm^3$) and water (1 $cm^3$) in 2-methylpropan-2-ol (2 $cm^3$). The mixture was stirred overnight at room temperature, then diluted with water (10 $cm^3$) and extracted with diethyl ether (2 × 20 $cm^3$). The organic extracts were dried ($MgSO_4$) and evaporated under reduced pressure. The residue was purified by flash chromatography, eluting with light petroleum–EtOAc (1 : 1), to give recovered enone **12** (26 mg, 13%), $R_f$ 0.71, and *epoxide* **13** (169 mg, 75%), $R_f$ 0.60, as an oil (Found: $M + NH_4^+$, 162.0766. $C_6H_8O_4$ requires $M + NH_4$, 162.0766); $\nu_{max}$(thin film)/$cm^{-1}$ 3012 (H-C), 1755 (ester) and 1716 (ketone); $\delta_H$(200 MHz, $CDCl_3$) 2.08 (3 H, s, $CH_3CO$), 3.57 and 3.59 (each 1 H, d, *J* 2, CH-CH) and 3.76 (3 H, s, $OCH_3$); $\delta_C$(100 MHz, APT, $CDCl_3$) 24.6 ($CH_3CO$), 51.6 and 57.7 (2 × CH), 52.9 ($OCH_3$), 167.2 ($CO_2$) and 202.4 (C=O); *m*/*z* (CI) 162 ($M + NH_4^+$, 28%), 145 ($MH^+$, 4) and 44 ($CO_2^+$, 100).

**Ammonium *trans*-2,3-epoxy-4-oxopentanoate 14**

Aqueous sodium hydroxide (1.95 mol $dm^{-3}$; 308 $mm^3$, 0.6 mmol) was added dropwise to a stirred solution of epoxy ester **13** (78.6 mg, 0.549 mmol) in water (1 $cm^3$). The solution was stirred at 0 °C for 10 min and then treated with Dowex X8-400 ($NH_4^+$) ion exchange resin until the pH dropped to 8–9. The ion exchange resin was filtered off and the filtrate evaporated under reduced pressure to give the ammonium salt of the *epoxy acid* **14** as a solid (80 mg, 96%); $\nu_{max}$(KBr)/$cm^{-1}$ 3250–3000 ($NH_4^+$ and C-H), 1617 and 1400 (C=O and $CO_2^-$); $\delta_H$(400 MHz, $D_2O$) 2.20 (3 H, s, $CH_3$) and 3.42 and 3.66 (each 1 H, d, *J* 2, CH-CH); $\delta_C$(100 MHz, APT, $D_2O$) 25.0 ($CH_3$), 54.1 and 56.8 (2 × CH), 173.1 ($CO_2$) and 207.9 (C=O).

**Ethyl 5,6-epoxy-4-oxohexanoate 17**

Saturated aqueous potassium carbonate (59 $mm^3$) was added slowly to a solution of the enone **16** (107 mg, 0.69 mmol) in 2-methylpropan-2-ol (1.15 $cm^3$), 30% $H_2O_2$ (170 $mm^3$) and water (572 $mm^3$) at 0 °C. The solution was stirred at 0 °C for 1 h and at room temperature for a further 2 h, then diluted with water (10 $cm^3$) and extracted with diethyl ether (3 × 10 $cm^3$). The combined extracts were dried ($MgSO_4$) and evaporated under reduced pressure to give the *epoxide* **17** as an oil (98 mg, 83%) which was homogeneous by TLC (Found: $M + NH_4^+$, 190.1079. $C_8H_{12}O_4$ requires $M + NH_4$, 190.1079), $R_f$ 0.32 (light petroleum–EtOAc, 1 : 1); $\nu_{max}$(thin film)/$cm^{-1}$ 1728 (ester) and 1717 (ketone); $\delta_H$(200 MHz, $CDCl_3$) 1.25 (3 H, t, *J* 7, $CH_2CH_3$), 2.49–2.73 (4 H, m, $CH_2CH_2$), 2.99 (1 H, dd, *J* 6 and 3) and 3.03 (1 H, dd, *J* 6 and 4, $CH_2O$), 3.47 (1 H, dd, *J* 4 and 3, CH-O) and 4.12 (2 H, q, *J* 7, $CH_2CH_3$); $\delta_C$(100 MHz, APT, 100 MHz) 14.6 ($CH_2CH_3$), 27.3 and 31.1 ($CH_2CH_2$), 46.1 ($CH_2$-O), 53.5 (CH-O), 60.8 ($CH_2CH_3$), 172.4 ($CO_2Et$) and 206.1 (C=O); *m*/*z* (CI) 190 ($M + NH_4^+$, 100%), 173 ($MH^+$, 50).

**Sodium 5,6-epoxy-4-oxohexanoate 18**

A solution of sodium hydroxide (5.1 mg, 0.128 mmol) in water (70 $mm^3$) was stirred with epoxide **17** (22 mg, 0.13 mmol) for 10 min at room temperature. The solvent was evaporated under reduced pressure at room temperature to give the sodium salt of *epoxy acid* **18**, as a solid (21 mg, 98%); $\nu_{max}$(KBr)/$cm^{-1}$ 1719 (C=O), 1600 and 1441 ($CO_2^-$); $\delta_H$(200 MHz, $D_2O$) 2.44 and 2.81 (each 2 H, t, *J* 7, $CH_2CH_2$), 2.91 (1 H, dd, *J* 6 and 3) and 3.10 (1 H, dd, *J* 6 and 5, $CH_2O$) and 3.79 (1 H, dd, *J* 5 and 3, CH-O); $\delta_C$(100 MHz, APT, $D_2O$) 29.9 and 34.5 ($CH_2CH_2$), 47.0 ($CH_2O$), 52.6 (CH-O), 180.5 ($CO_2$) and 205.3 (C=O).

**Ethyl 5-diazo-4-oxopentanoate 19**

Ethyl succinyl chloride **15** (400 mm$^3$, 2.81 mmol) was added dropwise to a solution of triethylamine (391 mm$^3$, 2.81 mmol) in excess ethereal diazomethane under argon in a 'clearfit' apparatus. The mixture was stirred for 1 h at room temperature, then water (50 cm$^3$) was added and the organic layer was washed with saturated aqueous sodium hydrogen carbonate (3 × 75 cm$^3$), dried ($MgSO_4$) and evaporated under reduced pressure to give the diazoketone[14] **19** (395 mg, 83%) as a yellow oil; $R_f$ 0.56 (light petroleum–EtOAc, 1:2), $\nu_{max}$(thin film)/cm$^{-1}$ 2105 (C=N=N), 1732 (ester) and 1644 (ketone); $\delta_H$(200 MHz, $CDCl_3$) 1.26 (3 H, t, *J* 7, $CH_2CH_3$), 2.56 (4 H, s, $CH_2CH_2$), 4.14 (2 H, q, *J* 7, $CH_2CH_3$) and 5.29 (1 H, br s, *H*C=N); $\delta_C$(100 MHz, APT, $CDCl_3$) 14.2 ($CH_3$), 28.1 and 35.0 (br, $CH_2CH_2$), 54.6 (br, $CHN_2$), 60.7 ($CH_3CH_3$) and 172.6 ($CO_2Et$) (signal for ketone obscured by background noise).

**Sodium 5-diazo-4-oxopentanoate 20**

A mixture of aqueous sodium hydroxide (0.5 mol dm$^{-3}$; 1.17 cm$^3$, 0.585 mmol) and diazo ester **19** (100 mg, 0.588 mmol) was stirred at room temperature for 10 min and then extracted with diethyl ether (2 × 2 cm$^3$). The aqueous layer was evaporated under reduced pressure to give the sodium salt of *acid* **20** as a solid; $R_f$ 0.17 (Bu$^n$OH–$H_2O$–$CH_3CO_2H$, 12:5:3); $\nu_{max}$(KBr)/cm$^{-1}$ 2110 (C=N=N), 1568br (C=O and $CO_2^-$) and 1380 ($CO_2^-$); $\lambda_{max}$($H_2O$)/nm 245 ($\varepsilon$/dm$^3$ mol$^{-1}$ cm$^{-1}$ 370) and 275 (540); $\delta_H$(200 MHz, $D_2O$) 2.50 and 2.65 (each 2 H, t, *J* 6, $CH_2CH_2$) and 5.93 (exchanges in $D_2O$, s, HC=N); $\delta_C$(100 MHz, APT, $D_2O$) 32.0 and 35.9 ($CH_2CH_2$), 56.6 (t, *J* 30, DC=N), 180.5 ($CO_2^-$) and 199.2 (C=O).

**5-Azidomethyl-3-benzyltetrahydrofuran-2-one 25**

A solution of lithium hexamethyldisilazide (0.92 mmol) in THF (2.92 cm$^3$) was stirred at −78 °C and a solution of the azidomethyl lactone **24**[16] (100 mg, 0.71 mmol) in THF was added, followed, after 5 min, by benzyl bromide (85 mm$^3$, 0.92 mmol). The reaction mixture was allowed to warm up to room temperature, diluted with water (3 cm$^3$) and extracted with dichloromethane (2 × 3 cm$^3$). The combined extracts were dried ($MgSO_4$) and evaporated under reduced pressure. Flash chromatography, eluting with EtOAc–light petroleum (1:2), gave the *lactone* **25** (91 mg, 55%) as an oil, shown to be a mixture of diastereoisomers (ratio 25:1) by NMR spectroscopy (Found: M + $NH_3^+$, 248.1273. $C_{12}H_{13}N_3O_2$ requires *M* + $NH_3$, 248.1273); $R_f$ 0.43 (EtOAc–light petroleum 1:1); $\nu_{max}$(thin film)/cm$^{-1}$ 2040 ($N_3$) and 1745 (C=O); $\delta_H$(250 MHz, $CDCl_3$) (major diastereoisomer) 2.10 (2 H, dd, *J* 9 and 6, $CHCH_2CH$), 2.78 (1 H, dd, *J* 13.5 and 6.5, C*H*HPh), 3.01–3.13 (1 H, m, $CHCH_2Ph$), 3.19 (1 H, dd, *J* 13.5 and 4.5, CH*H*Ph), 3.39 (1 H, dd, *J* 13 and 4.5) and 3.53 (1 H, dd, *J* 13 and 4, $CH_2N_3$), 4.41 (1 H, tt, *J* 6 and 4, CH-O) and 7.15–7.40 (5 H, m, Ph); (minor diastereoisomer, distinguishable signals) 1.70–1.90 (1 H, m) and 2.10–2.30 (1 H, m, $CHCH_2CH$); $\delta_C$(100 MHz, APT, $CDCl_3$) (major diastereoisomer) 29.7 ($CH_2Ph$), 36.7 ($CHCH_2CH$), 40.8 ($CHCH_2Ph$), 54.3 ($CH_2N_3$), 75.9 (CH-O), 126.95 (*p*-CH), 128.9 and 128.85 (*o*- and *m*-CH), 137.85 (phenyl-C) and 178.0 (C=O); (minor diastereoisomer) 30.7 ($CH_2Ph$), 36.7 ($CHCH_2CH$), 42.3 ($CHCH_2Ph$), 53.3 ($CH_2N_3$), 76.4 (CH-O), 126.85 (*p*-CH), 128.8 (*o*- and *p*-CH), 138.7 (phenyl-C) and 177.1 (C=O); *m*/*z* (CI) 248 (M + $NH_3^+$, 100%), 231 ($M^+$, 4), 223 (52) and 206 (M + $NH_3$ − $N_3$, 93).

**5-Azidomethyl-3-bromotetrahydrofuran-2-one 26**

A solution of lithium hexamethyldisilazide (0.90 mmol) in THF (2.90 cm$^3$) was stirred at −78 °C and a solution of azidomethyl lactone **24**[16] (100 mg, 0.71 mmol) in THF (2 cm$^3$) was added. This solution was then added dropwise by means of a cannula to a solution of bromine (44 mm$^3$, 0.85 mmol) in THF (4 cm$^3$) maintained at −78 °C.[41] After 15 min, pH 7 buffer (1 cm$^3$) in THF (2 cm$^3$) was added. The solution was warmed to room temperature, washed with aqueous sodium metabisulfite (2 × 5 cm$^3$), dried ($MgSO_4$), filtered and evaporated under reduced pressure. The residue was purified by flash chromatography, eluting with EtOAc–light petroleum (1:4), to give the *bromo lactone* **26** (140 mg, 90%) as an oil, shown to be two diastereoisomers (ratio 10:7) by TLC and NMR spectral analysis; $R_f$ 0.49 and 0.41 (EtOAc–light petroleum 1:1); $\nu_{max}$(thin film)/cm$^{-1}$ 2040 ($N_3$) and 1780 (C=O); $\delta_H$(250 MHz, $CDCl_3$) (major diastereoisomer) 2.51 (1 H, ddd, *J* 15, 6 and 3) and 2.68 (1 H, ddd, *J* 15, 8 and 7, $CH_2CHBr$), 3.50 (1 H, dd, *J* 14 and 4) and 3.77 (1 H, dd, *J* 14 and 3.5, $CH_2N_3$), 4.51 (1 H, dd, *J* 7 and 3, CHBr) and 4.86 (1 H, ddt, *J* 8, 6 and 4, CH-O); (minor diastereoisomer) 2.45 (1 H, ddd, *J* 14, 8 and 7.5) and 2.97 (1 H, ddd, *J* 14, 9 and 7, $CH_2CHBr$), 3.63 (2 H, d, *J* 5, $CH_2N_3$), 4.56 (1 H, t, *J* 9, CHBr) and 4.66 (1 H, tt, *J* 7.5 and 5, CH-O); $\delta_C$(100 MHz, APT, $CDCl_3$) (major diastereoisomer) 36.0 ($CH_2CHBr$), 37.6 (CHBr), 53.6 ($CH_2N_3$), *ca.* 77.6 (obscured by solvent, CH-O) and 171.6 (C=O); (minor diastereoisomer) 35.6 ($CH_2CHBr$), 36.4 (CHBr), 53.1 ($CH_2N_3$), *ca.* 76.8 (obscured by solvent, CH-O) and 171.6 (C=O); *m*/*z* (EI) 163 and 165 (M − $CH_2N_3$) and 135 and 137 (M − $COCH_2N_3$).

**Allyl fluoroacetate 21**

[**CAUTION:** Sodium fluoroacetate and fluoroacetate esters are extremely toxic.] A solution of sodium fluoroacetate (5 g, 50 mmol) in *N*,*N*-dimethylformamide (300 cm$^3$) and allyl bromide (130 cm$^3$, 1.5 mol) was heated at 70 °C for 48 h. The solution was cooled, mixed with diethyl ether (200 cm$^3$), washed with water (4 × 400 cm$^3$), dried ($MgSO_4$) and evaporated under reduced pressure to give the ester **21** as an oil (5.02 g, 85%) which was homogeneous by TLC and had the same NMR spectral data as described;[21] $R_f$ 0.57 (light petroleum–EtOAc, 1:1).

**5-Azidomethyl-3-fluorotetrahydrofuran-2-one 28**

A solution of 5-iodomethyl-3-fluorotetrahydrofuran-2-one[21] (160 mg, 0.656 mmol) in *N*,*N*-dimethylformamide (2 cm$^3$) was stirred with sodium azide (156 mg, 2.40 mmol) under argon at room temperature for 48 h. Dichloromethane (25 cm$^3$) was added and the mixture was washed with water (3 × 25 cm$^3$), dried ($MgSO_4$) and evaporated under reduced pressure to give the azidomethyl lactone **28** as an oil (83 mg, 80%), consisting of two diastereoisomers (ratio 3:1 by NMR spectroscopy) which could be separated by PLC for NMR analysis, but were not separated for the further reactions to give 2-fluoroALA **29** (Found: $M^+$, 159.0448. $C_5H_6FN_3O_2$ requires *M*, 159.0443); $R_f$ 0.56 and 0.46 (light petroleum–EtOAc, 1:1). Spectroscopic data were the same as previously described.[15]

**4-Nitrobutan-2-one 31**

A suspension of sodium nitrite (12.3 g, 178 mmol) in a mixture of methyl vinyl ketone (12.5 g, 178 mmol), THF (100 cm$^3$) and acetic acid (30 cm$^3$) was stirred vigorously for 2 h, then mixed with water (100 cm$^3$) and extracted with ethyl acetate (3 × 100 cm$^3$). The combined organic layers were washed successively with hydrochloric acid (2 mmol dm$^{-3}$; twice) and saturated aqueous sodium hydrogen carbonate (twice), dried ($MgSO_4$) and evaporated under reduced pressure. The residue was distilled (bp 66–68 °C/5 mmHg) to give the nitro ketone **31**[22] (12.5 g, 60%); $R_f$ 0.55 (EtOAc–hexane, 1:1); $\delta_H$(60 MHz, $CDCl_3$) 2.2 (3 H, s, Me), 3.1 (2 H, t, *J* 6, $CH_2CO$) and 4.6 (2 H, t, *J* 6, $CH_2NO_2$).

**1-Bromo-4-nitrobutan-2-one 33**

Bromo ketone **33** was prepared as described previously[24] (Found: C, 24.2; H, 2.9; N, 7.1. $C_4H_6BrNO_3$ requires C, 24.5; H, 3.1; N, 7.15%); $R_f$ 0.60 (EtOAc–hexane, 3:1); $\delta_H$(60 MHz, $CDCl_3$) 3.2 (2 H, t, *J* 6, $CH_2CO$), 4.0 (2 H, s, $CH_2Br$) and 4.6 (2 H, t, *J* 6, $CH_2NO_2$).

**1-Azido-4-nitrobutan-2-one 35**

1-Bromo-4-nitrobutan-2-one **33**[24] was stirred with a solution of sodium azide (0.87 g, 13.4 mmol) in DMF (30 $cm^3$) under nitrogen at −24 °C for 2.5 h. The mixture was poured into brine and extracted with ethyl acetate (3 × 50 $cm^3$). The combined extracts were washed once with water and twice with brine, then dried ($MgSO_4$) and evaporated under reduced pressure. The residue was purified by column chromatography, eluting with ethyl acetate–hexane (1 : 1) to give the *azide* **35** (0.55 g, 78%) as an oil (Found: C, 30.2; H, 3.8; N, 35.6. $C_4H_6N_4O_3$ requires C, 30.4; H, 3.8; N, 35.4%); $R_f$ 0.60 (EtOAc–hexane, 1 : 1); $\nu_{max}$(thin film)/$cm^{-1}$ 2950 and 2900 (CH), 2106 ($N_3$), 1725 (C=O) and 1545 and 1370 ($NO_2$); $\delta_H$(60 MHz, $CDCl_3$) 3.5 (2 H, t, *J* 6, $CH_2CO$), 4.5 (2 H, s, $CH_2N_3$) and 5.0 (2 H, t, *J* 6, $CH_2NO_2$); $\delta_C$(50 MHz, $CDCl_3$) 33.5 (C-3), 57.2 (C-1), 69.3 (C-4) and 207.2 (C-2).

**1-Amino-4-nitrobutan-2-one hydrochloride 38**

A solution of azide **35** (280 mg, 1.78 mmol) in methanol was stirred with palladium on carbon (10%; 400 mg) and conc. hydrochloric acid (1 $cm^3$) under an atmosphere of hydrogen at room temperature for 4 h, then filtered through Celite and evaporated under reduced pressure. The residue was recrystallised from methanol–ethyl acetate to give the *amine hydrochloride* **38** (220 mg, 74%) as a powder, mp 117–120 °C (Found: C, 28.3; H, 5.33; N, 16.8. $C_4H_9ClN_2O_3$ requires C, 28.5; H, 5.38; N, 16.6%); $\nu_{max}$(KBr)/$cm^{-1}$ 3400 (NH), 2950 and 2885 (CH), 1720 (C=O) and 1545 and 1370 ($NO_2$); $\delta_H$(200 MHz, $D_2O$) 3.32 (2 H, t, *J* 6, $CH_2CO$), 4.21 (2 H, s, $CH_2NH_2$) and 4.86 (2 H, t, *J* 6, $CH_2NO_2$); $\delta_C$(50 MHz, $CDCl_3$) 38.6 (C-3), 49.8 (C-1), 71.5 (C-4) and 204.2 (C-2).

**Dimethyl 3-oxobutylphosphonate 32**

A solution of dimethyl phosphite (6.71 g, 61 mmol) and methyl vinyl ketone (3.88 g, 55.4 mmol) in methanol (50 $cm^3$) was stirred at 65 °C. After 30 min a solution of sodium methoxide (1.25 g, 23.1 mmol) in methanol (10 $cm^3$) was added. After 20 h the solution was evaporated under reduced pressure and the residue was distilled (101–103 °C, 4 mmHg) to give the phosphonate ester[23] **32** (4.18 g, 42%) as an oil; $R_f$ 0.31 (EtOAc–hexane, 3 : 1); $\nu_{max}$(thin film)/$cm^{-1}$ 2875 (CH), 1710 (C=O) and 1240 and 1040 (P-O); $\delta_H$(200 MHz, $CDCl_3$) 2.03 (2 H, dt, *J* 18 and 7, $CH_2P$), 2.19 (3 H, s, MeC=O), 2.74 (2 H, dt, *J* 12 and 7, $CH_2C$=O) and 3.74 (6 H, d, *J* 11, OMe); $\delta_C$(50 MHz, $CDCl_3$) 18.3 (d, *J* 145, C-1), 29.6 (s, *Me*C=O), 36.2 (d, *J* 4, C-2), 52.5 (d, *J* 6.5, OMe) and 205.6 (d, *J* 14, C-3).

**Dimethyl 4-bromo-3-oxobutylphosphonate 34**

Bromo ketone **34** was prepared as described previously[24] (Found: C, 27.5; H, 4.45. $C_6H_{12}BrO_4P$ requires C, 27.8; H, 4.67%); $R_f$ 0.17 (EtOAc–hexane, 3 : 1); $\delta_H$(200 MHz, $CDCl_3$) 1.98 (2 H, dt, *J* 18 and 6, $CH_2P$), 2.88 (2 H, dt, *J* 12 and 6, $CH_2CO$), 3.65 (6 H, d, *J* 11, OMe) and 3.90 (2 H, s, $CH_2Br$); $\delta_C$(50 MHz, $CDCl_3$) 17.6 (d, *J* 145, C-1), 31.8 (d, *J* 3.5, C-4), 33.2 (d, *J* 14.5, C-2), 51.6 (d, *J* 6.5, OMe) and 198.8 (d, *J* 14, C-3).

**Dimethyl 4-azido-3-oxobutylphosphonate 36**

Bromo ketone **34** (0.70 g, 2.7 mmol) was stirred with a solution of sodium azide (0.53 g, 8.1 mmol) in DMF (30 $cm^3$) at −24 °C under nitrogen for 1 h. The mixture was poured into saturated brine and extracted with ethyl acetate (3 × 50 $cm^3$). The combined extracts were washed once with water and twice with brine, then dried ($MgSO_4$) and evaporated under reduced pressure to give the *azide* **36** (0.5 g, 94%) as an oil (Found: C, 32.5; H, 5.22; N, 19.1. $C_6H_{12}N_3O_4P$ requires C, 32.6; H, 5.47; N, 19.0%); $R_f$ 0.16 (EtOAc–hexane, 3 : 1); $\nu_{max}$(thin film)/$cm^{-1}$ 2959 (CH), 2106 ($N_3$), 1731 (C=O) and 1248 and 1036 (P-O); $\delta_H$(200 MHz, $CDCl_3$) 1.79 (2 H, dt, *J* 18 and 6, $CH_2P$), 2.45 (2 H, dt, *J* 14 and 6, $CH_2CO$), 3.43 (6 H, d, *J* 11, OMe) and 3.74 (2 H, s, $CH_2N_3$); $\delta_C$(50 MHz, $CDCl_3$) 17.9 (d, *J* 144.5, C-1), 32.6 (d, *J* 3.5, C-2), 52.4 (d, *J* 7.5, OMe), 57.1 (s, C-4) and 202.4 (d, *J* 13.5, C-3).

**2,5-Bis(2-dimethoxyphosphorylethyl)pyrazine 40**

A solution of azide **36** (270 mg, 0.94 mmol) in methanol was stirred with palladium on carbon (200 mg) under an atmosphere of hydrogen at room temperature for 1 h, then filtered through Celite and evaporated under reduced pressure. The residue was purified by column chromatography to give the *pyrazine* **40** (144 mg, 67%) as an oil (Found: $M^+$, 352.0961. $C_{12}H_{22}N_2O_6P_2$ requires *M*, 352.0953); $\delta_H$(200 MHz, $CDCl_3$) 2.17–2.34 (4 H, m, $CH_2P$), 3.02–3.10 (4 H, m, $CH_2Ar$), 3.74 (12 H, d, *J* 8.5, OMe) and 8.43 (2 H, s, ArH); $\delta_C$(50 MHz, $CDCl_3$) 24.6 (d, *J* 142, $CH_2P$), 27.7 (d, *J* 4, *C*$H_2$Ar), 52.7 (d, *J* 6.5, OMe), 143.5 (s, C-3 and -6) and 153.3 (d, *J* 15.5, C-2 and -5).

**4-Amino-3-oxobutylphosphonic acid hydrochloride 39**

A solution of azide **36** (160 mg, 0.72 mmol) and bromotrimethylsilane (440 mg, 2.87 mmol) in dichloromethane was stirred under nitrogen for 1 h, then mixed with water (5 $cm^3$) and stirred for a further 10 min. The aqueous layer was separated and evaporated under reduced pressure to give the crystalline phosphonic acid **37** (140 mg), mp 58–60 °C, which was used in the next reaction without further purification; $\delta_H$(200 MHz, $CDCl_3$) 1.85–2.08 (2 H, m, $CH_2P$), 2.45–2.71 (2 H, m, $CH_2CO$) and 3.90 (2 H, s, $CH_2N_3$); $\delta_C$(50 MHz, $CDCl_3$) 23.0 (d, *J* 138.5, C-1), 36.1 (d, *J* 3.5, C-2), 49.2 (s, C-4) and 205.8 (d, *J* 13.5, C-3).

A solution of the above phosphonic acid **37** (140 mg) in methanol was stirred with palladium on carbon (400 mg) and conc. hydrochloric acid (5 $cm^3$) under an atmosphere of hydrogen at room temperature for 1 h, then filtered through Celite and evaporated under reduced pressure. The residue was dissolved in a small amount of ethanol and twice the volume of propylene oxide was added. The solution was kept at 4 °C and the precipitate was then collected to give the *amino phosphonic acid hydrochloride* **39** (124 mg, 84%) as a powder, mp 61–62 °C (Found: C, 23.3; H, 5.8; N, 6.9. $C_4H_{11}ClNO_4P$ requires C, 23.6; H, 5.45; N, 6.9%); $\delta_H$(200 MHz, $D_2O$) 1.87–2.03 (2 H, m, $CH_2P$), 2.75–2.91 (2 H, m, $CH_2CO$), 4.16 (2 H, br s, $CH_2N$); $\delta_P$(121.5 MHz, $D_2O$) 26.1.

***tert*-Butoxycarbonylmethyl *tert*-butoxycarbonylaminoacetate 42**

Triethylamine (92 $mm^3$, 0.661 mmol) was added dropwise to a stirred solution of *N*-(*tert*-butoxycarbonyl)glycine **41** (100 mg, 0.571 mmol) and *tert*-butyl chloroacetate (100 mg, 0.664 mmol) in ethyl acetate (1 $cm^3$) and the mixture heated at reflux under argon overnight. Hydrochloric acid (0.5 mol $dm^{-3}$; 20 $cm^3$) was added and the mixture was extracted with ethyl acetate (2 × 20 $cm^3$). The organic extracts were washed with saturated aqueous sodium hydrogen carbonate (2 × 20 $cm^3$), dried ($MgSO_4$) and evaporated under reduced pressure. The residue was purified by flash chromatography, eluting with ethyl acetate, to give the *ester* **42** (92 mg, 58%) as an oil (Found: $MH^+$, 290.1625. $C_{13}H_{23}NO_6$ requires *MH*, 290.1604); $R_f$ 0.1 (dichloromethane–MeOH, 9 : 1); $\nu_{max}$(thin film)/$cm^{-1}$ 3510–3350 (NH), 2970–2891 (CH), 1751, 1720 and 1715 (ester) and 1510 (urethane); $\delta_H$(200 MHz, $CDCl_3$) 1.45 and 1.47 (each 9 H, s, Bu$^t$), 4.02 (2 H, br d, *J* 5.5, $CH_2N$), 4.56 (2 H, s, $CH_2O$) and 5.05 (1 H, br s, NH); $\delta_C$(100 MHz, APT, $CDCl_3$) 28.0 and 28.3 (2 × *Me*$_3$C), 42.2 ($CH_2N$), 61.6 ($CH_2O$), 80.1 and 82.8 (2 × Me$_3$*C*) and 155.7, 166.4 and 169.9 (3 × C=O); *m*/*z* (CI) 307 ($M + NH_4^+$, 60%), 290 ($MH^+$, 4), 251 ($M + NH_4 - C_4H_8$, 82), 234 ($MH - C_4H_8$, 62), 195 (98), 178 ($M + NH_4 - CH_2NCO_2Bu^t$, 79), 160 ($MH - CH_2NHCO_2Bu^t$, 39), 134 (81) and 57 ($C_4H_9$, 10).

**2-Glycyloxyacetic acid 44**

A solution of the protected ester **42** (124 mg, 0.402 mmol) in trifluoroacetic acid (1 $cm^3$, 13 mmol) was stirred at room tem-

perature for 2 h and then evaporated under reduced pressure to give the ester **44**[25] as the solid trifluoroacetate salt (100 mg, 95%); $\delta_H$(200 MHz, $D_2O$) 3.92 (2 H, s, $NCH_2$) and 4.72 (2 H, s, $OCH_2$).

**2-Glycylthioacetic acid 45**

A solution of *N*-(*tert*-butoxycarbonyl)glycine **41** (100 mg, 0.57 mmol) in ethyl acetate (1 $cm^3$) was stirred with triethylamine (90 $mm^3$, 0.645 mmol) and ethyl chloroformate (60 $mm^3$, 0.63 mmol) under argon at room temperature for 15 min. A mixture of mercaptoacetic acid (40 $mm^3$, 0.576 mmol) and triethylamine (90 $mm^3$, 0.645 mmol) was added and the mixture was stirred for 1 h and then filtered, washing the residue with ethyl acetate (5 $cm^3$). The filtrate and washings were dried ($MgSO_4$) and evaporated under reduced pressure. The residue was purified by flash chromatography, eluting with light petroleum–dichloromethane–acetic acid (6:3:1), to give the thioester **43** (88 mg, 62%) as an oil, $R_f$ 0.32 (dichloromethane–acetic acid, 9:1), as well as the intermediate mixed anhydride (11 mg, 8%) ($R_f$ 0.57) and starting material **41** (21 mg, 21%) ($R_f$ 0.75). The $^1H$ NMR spectrum of **43** showed a mixture of two rotamers about the N–CO bond; $\delta_H$(200 MHz, $CDCl_3$) 1.46 (9 H, s, Bu$^t$), 3.73 (2 H, s, $CH_2S$), 3.98 (1 H, br s) and 4.09 (1 H, br d, *J* 6, $CH_2N$), 5.24 and 6.36 (each 0.5 H, br s, NH) and 7.76 (1 H, br s, $CO_2H$).

A solution of protected thioester **43** (100 mg, 0.402 mmol) in trifluoroacetic acid (0.5 $cm^3$, 6.5 mmol) was stirred at room temperature for 1 h and then evaporated under reduced pressure to give the thioester **45**[42] as the solid trifluoroacetate salt (100 mg, 95%); $\delta_H$(200 MHz, $D_2O$) 3.88 (2 H, s, $CH_2CO_2$) and 4.21 (2 H, s, $CH_2N$).

**5-Aminomethyltetrahydrofuran-2-one hydrochloride 48**

A solution of azido lactone **24**[16] (428 mg, 3.03 mmol) in diethyl ether (90 $cm^3$) and water (80 $cm^3$) was stirred with Raney nickel (50% slurry in water; 15 $cm^3$) at room temperature for 20 min and then filtered through Celite, washing with acetonitrile (150 $cm^3$). The filtrate and washings were acidified with conc. hydrochloric acid and evaporated under reduced pressure. The residue was purified on a column of Dowex-50 X8-400 ($H^+$) ion exchange resin (3 cm × 7 cm) eluted with dilute hydrochloric acid (10 $cm^3$ each of 0, 50, 100, 150 and 200 mmol $dm^{-3}$). The aminomethyl lactone hydrochloride eluted at 150 mmol $dm^{-3}$ and the hydrolysed lactone at 200 mmol $dm^{-3}$. Evaporation of the appropriate fractions under reduced pressure gave the aminomethyl lactone hydrochloride **48**[17] as a solid (391 mg, 85%) and the opened lactone as an oil (10 mg, 3%); both gave ninhydrin-positive spots on TLC; $\nu_{max}$(KBr)/$cm^{-1}$ 3000 ($NH_3^+$) and 1762 (C=O); $\delta_H$(200 MHz, $D_2O$) 2.01 (1 H, dtd, *J* 13, 9 and 8) and 2.38–2.55 (1 H, m, $CH_2$CH-O), 2.67 and 2.70 (each 1 H, m, $CH_2$C=O), 3.22 (1 H, dd, *J* 14 and 9) and 3.38 (1 H, dd, *J* 14 and 3, $CH_2N$) and 4.90 (1 H, dtd, *J* 9, 7 and 3, CH-O); $\delta_C$(100 MHz, APT, $D_2O$) 23.8 and 27.4 ($CH_2CH_2$), 42.2 ($CH_2N$), 77.2 (CH-O) and 180.1 (C=O).

**5-(4-Carboxybutan-2-ylaminomethyl)tetrahydrofuran-2-one 49**

A solution of aminomethyl lactone hydrochloride **48** (323 mg, 2.13 mmol) and sodium acetate (175 mg, 2.13 mmol) in methanol (6 $cm^3$) was stirred with laevulinic acid **7** (218 $mm^3$, 2.13 mmol) and sodium cyanoborohydride (134 mg, 2.13 mmol) at room temperature for 3 days and then evaporated under reduced pressure. The residue was purified on a column of Dowex-50 X8-400 ($H^+$) ion exchange resin as for lactone **48** above. Evaporation of the appropriate fractions under reduced pressure gave the *lactone amino acid hydrochloride* **49** (344 mg, 75%) as a solid (Found: $MH^+$, 216.1236. $C_{10}H_{18}NO_4$ requires *MH*, 216.1236); $R_f$ 0.5 (Bu$^n$OH–$H_2O$–$CH_3CO_2H$, 12:5:3); $\nu_{max}$(thin film)/$cm^{-1}$ 3500–3300 (N-H), 3100–2900 ($CO_2H$), 1767 (lactone) and 1710 ($CO_2H$). NMR spectroscopy indicated a mixture of two diastereoisomers (*ca.* 1:1); $\delta_H$(200 MHz, $D_2O$) 1.33 (3 H, br d, *J* 7, $CH_3$), 1.80–2.21 (2 H, m, $CH_2$CHN), 2.40–2.61 (4 H, m, $CH_2CO_2H$ and $CH_2$CH-O), 2.62–2.84 (2 H, m, $CH_2$C=O), 3.28–3.53 (3 H, m, $CH_2$NHC*H*) and 4.80–5.03 (1 H, m, CH-O); $\delta_C$(100 MHz, APT, $D_2O$) 14.3 and 14.5 ($CH_3$), 24.0, 24.1, 26.4, 26.8, 27.4 (2 C) and 29.0 (2 C) (2 × $CH_2CH_2$), 47.4 and 47.5 ($CH_2N$), 53.9 ($CHCH_3$), 76.4 and 76.5 (CH-O), 176.2 and 176.3 ($CO_2H$) and 180.0 (C=O); *m*/*z* (CI) 216 ($MH^+$, 100%), 215 ($M^+$, 32), 198 (MH − $H_2O$, 80), 142 (M − $CH_2$-$CH_2CO_2H$, 2), 130 ($HO_2CCH_2CH_2CHMeHN^+$=$CH_2$, 8) and 112 (9).

**5-Aminolaevulinic acid hydrochloride 5**

A solution of aminomethyl lactone hydrochloride **48** (24 mg, 0.158 mmol) and chromium trioxide (24 mg, 0.240 mmol) in 5% aqueous sulfuric acid (2 $cm^3$, 1.99 mmol) was heated at reflux for 4 h then stirred at room temperature overnight. Methanol (0.05 $cm^3$) was added and the mixture was stirred at room temperature for 10 min. The methanol was then evaporated under reduced pressure and barium(II) chloride (414 mg, 1.99 mmol) was added. The barium(II) sulfate precipitate was filtered off through Celite and the filtrate evaporated under reduced pressure. The residue was purified on a column of Dowex-50 X8-400 ($H^+$) ion exchange resin (1 × 5 cm) using dil. hydrochloric acid (20 $cm^3$ each of 0, 50, 100, 150 and 200 mmol $dm^{-3}$). The chromium salt eluted between 0–50 mmol $dm^{-3}$ and 5-aminolaevulinic acid (ALA) eluted at 200 mmol $dm^{-3}$. Evaporation of the appropriate fractions under reduced pressure gave ALA hydrochloride **5** as a solid (17 mg, 65%), identical to authentic ALA hydrochloride by TLC ($R_f$ 0.30, Bu$^n$OH–$H_2O$–$CH_3CO_2H$, 12:5:3) and NMR spectroscopy; $\delta_H$(200 MHz, $D_2O$) 2.73 and 2.92 (each 2 H, t, $CH_2CH_2$) and 4.15 (2 H, s, $CH_2N$); $\delta_C$(100 MHz, APT, $D_2O$) 26.8 and 33.8 ($CH_2CH_2$), 46.5 ($CH_2N$), 178.2 ($CO_2H$) and 203.6 (C=O).

**5-(4-Carboxybutan-2-ylamino)laevulinic acid 50**

A solution of lactone amino acid hydrochloride **49** (57 mg, 0.265 mmol) and chromium trioxide (40 mg, 0.40 mmol) in 5% aqueous sulfuric acid (2 $cm^3$, 1.99 mmol) was heated at reflux for 1 h. Methanol (0.1 $cm^3$) was added and the mixture was stirred at room temperature for 10 min. The methanol was then evaporated under reduced pressure and barium(II) chloride (414 mg, 1.99 mmol) was added. The barium sulfate precipitate was filtered off through Celite and the filtrate was evaporated under reduced pressure. The residue was purified on a column of Dowex-50 X8-400 ($H^+$) ion exchange resin (2 × 5 cm), eluting with dil. hydrochloric acid (30 $cm^3$ each of 0, 50, 100, 150 and 200 mmol $dm^{-3}$), to give (eluting at 150 mmol $dm^{-3}$) the *amino diacid* **50** (36 mg, 59%) as a solid (Found: M + $NH_4^+$ − $H_2O$, 231.1345. $C_{10}H_{19}N_2O_4$ requires *M* + $NH_4$ − $H_2O$, 231.1345); $R_f$ 0.41 (Bu$^n$OH–$H_2O$–$CH_3CO_2H$, 12:5:3); $\nu_{max}$(thin film)/$cm^{-1}$ 3500–3300 (N-H), 3100–2900 ($CO_2H$) and 1726 and 1720 (C=O); $\delta_H$(200 MHz, $D_2O$) 1.35 (3 H, d, *J* 7, $CH_3$), 1.89 (1 H, m) and 2.14 (1 H, m, $CH_2$CH), 2.55 (2 H, t, *J* 7, $CH_2CH_2$CH), 2.74 (2 H, t, *J* 7) and 2.93 (2 H, t, *J* 7, $CH_2CH_2CO$), 3.34–3.51 (1 H, m, $CHCH_3$) and 4.30 (2 H, s, $CH_2N$); $\delta_C$(100 MHz, APT, $D_2O$) 14.9 ($CH_3$), 26.9 and 27.0 (2 × $CH_2CO_2H$), 29.3 and 34.2 ($CH_2CO$ and $CH_2$CH), 51.5 ($CH_2N$), 53.7 (CH), 176.2 and 176.4 (2 × $CO_2H$) and 203.4 (C=O); *m*/*z* (CI) 231 (M + $NH_4^+$ − $H_2O$, 10%), 214 (MH − $H_2O$, 100), 198 (28), 196 (MH − $2H_2O$, 26) and 100 (25).

Also obtained (eluting at 200 mmol $dm^{-3}$) was 4-aminopentanoic acid (3 mg, 11%) (Found: $MH^+$, 118.0868. $C_5H_{11}NO_2$ requires *MH*, 118.0868); $R_f$ 0.51 (Bu$^n$OH–$H_2O$–$CH_3CO_2H$, 12:5:3); $\delta_H$(200 MHz, $D_2O$) 1.33 (3 H, d, *J* 7, $CH_3CH$), 1.81–2.21 (2 H, m, $CH_2$CH), 2.55 (2 H, t, *J* 7, $CH_2CO$) and 3.45 (1 H, br sextet, *J* 7, $CHCH_3$); *m*/*z* (CI) 118 ($MH^+$, 100%) and 100 (47).

**Assay of ALA dehydratase**[30]

ALA dehydratase from *B. subtilis* (1.5–2.0 μg) was preincubated for 10 min at 37 °C in bis-tris propane buffer (12 mmol $dm^{-3}$; pH 9; 40 $mm^3$) containing DTT (5.55 mmol $dm^{-3}$), sodium chloride (140 mmol $dm^{-3}$), zinc sulfate (50 μmol $dm^{-3}$) and magnesium sulfate (500 μmol $dm^{-3}$). This solution was then added to bis-tris propane buffer (109 mmol $dm^{-3}$; pH 9; 460 $mm^3$), containing various concentrations of ALA (obtained by neutralisation of ALA·HCl and used immediately) and a known concentration of inhibitor, which had been preincubated for 5 min at 37 °C. After 10 min the reaction was stopped by the addition of a solution of mercury(II) chloride (100 mmol $dm^{-3}$) in 10% aqueous trichloroacetic acid (0.5 $cm^3$) followed by modified Ehrlich's reagent[30] (1 $cm^3$). The absorbance at 555 nm was measured 20–23 min after the addition of the Ehrlich's reagent. All assays were carried out in duplicate or triplicate. The molar absorption coefficient of the product of the reaction between PBG and Ehrlich's reagent is $6.2 \times 10^4$ $dm^3$ $mol^{-1}$ $cm^{-1}$ at 555 nm. The activity is expressed in units $mg^{-1}$ where a unit is defined as 1 μmol of PBG produced $h^{-1}$ at 37 °C.

For ALA dehydratase from bovine liver (Sigma), the enzyme (13 μg) was preincubated for 10 min at 37 °C in sodium phosphate buffer (100 mmol $dm^{-3}$; pH 6.8; 187.5 $mm^3$) containing DTT (20 mmol $dm^{-3}$) and zinc sulfate (100 μmol $dm^{-3}$). This solution was then added to sodium phosphate buffer (75 mmol $dm^{-3}$; pH 6.8; 312.5 $mm^3$), containing various concentrations of ALA, which had been preincubated for 5 min at 37 °C. The remainder of the assay followed the procedure described above.

**Inactivation of ALA dehydratase**

Stock solution of enzyme (4.26 mg $cm^{-3}$; 60 $mm^3$; 256 μg) was incubated in bis-tris propane buffer (24 mmol $dm^{-3}$; pH 9; 120 $mm^3$) containing DTT (11.1 mmol $dm^{-3}$), sodium chloride (280 mmol $dm^{-3}$), zinc sulfate (50 μmol $dm^{-3}$) and magnesium sulfate (500 μmol $dm^{-3}$) for 10 min at 37 °C. The solution was then dialysed under anaerobic conditions against the same degassed buffer (180 $cm^3$) lacking the DTT for 6 h at 4 °C. After the dialysed enzyme solution had been removed from the dialysis bag, the bag was washed with more of this buffer (240 $mm^3$). The enzyme solution and washings were incubated with an aqueous solution of inactivator (0.25 vol) of known concentration at 37 °C. At timed intervals, aliquots (2 $mm^3$) were removed and diluted with ice-cold buffer (59 $mm^3$) containing DTT. These aliquots were kept on ice until the assay was commenced by the addition of bis-tris propane buffer (114 mmol $dm^{-3}$; pH 9; 0.44 $cm^3$) containing ALA (final ALA concentration, 2 mmol $dm^{-3}$). The remainder of the assay was as described above except that the solutions were clarified by centrifugation after addition of Ehrlich's reagent.

**Preparation of samples of ALA dehydratase for electrospray mass spectrometry**

Stock solutions of enzyme (0.64 mg $cm^{-3}$) were incubated with an equal volume of buffer containing DTT and then dialysed against buffer free of DTT essentially as described above. The dialysed enzyme solution and washings were incubated with various concentrations of inactivator for 15 min at 37 °C. An aliquot (2 $mm^3$) was removed for assay of activity as above and the remaining enzyme solution was dialysed against aqueous ammonium hydrogen carbonate (5 mmol $dm^{-3}$; 10 000 vol) for 16 h at 4 °C. 10% Aqueous acetic acid (0.1 vol) was added immediately before injection of an aliquot (20 $mm^3$, 100–200 pmol of enzyme) into the mass spectrometer.

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