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The Nonenzymatic Cyclic Dimerisation of 5-Aminolevulinic Acid

Anthony R Butler* and Sharon George

Chemistry Department, University of St Andrews,
Fife, Scotland KY16 9ST

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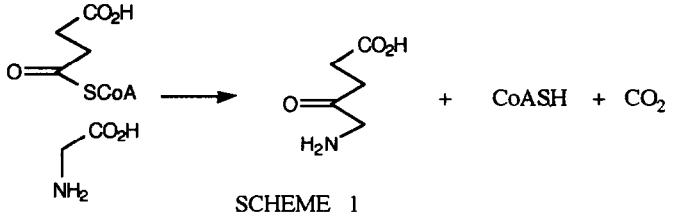
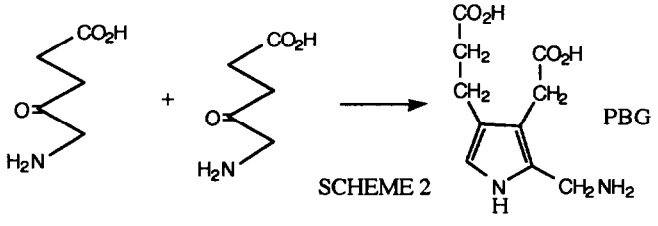
Abstract: The nonenzymatic cyclic dimerisation of 5-aminolevulinic acid (5-ALA) leads to the formation of a pyrazine (**3**) and, under some circumstances, pseudo-porphobilinogen (**1**). On the other hand, the enzyme-catalysed process leads to porphobilinogen (PBG). The products of the former reaction were identified from their NMR spectra and mechanisms for their formation are proposed.

5-Aminolevulinic acid (5-ALA) is the primary building block of porphyrins, chlorophylls, corrins and a wide range of tetrapyrroles. It is synthesised[1] from glycine and succinyl-CoA in a reaction catalysed by the enzyme 5-aminolevulinic acid synthase (EC 2.3.1.37) (Scheme 1). The next step in the biosynthetic pathway[2] is the dimerisation of two molecules of 5-ALA with the elimination of two molecules of water in a Knorr reaction to



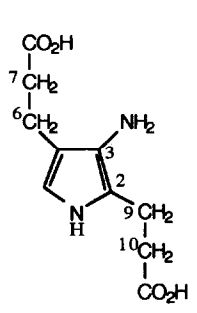
SCHEME 1

give porphobilinogen (PBG) (Scheme 2). The enzyme responsible for this is 5-aminolevulinic acid dehydratase (EC 4.2.1.24), known to exist in almost all living organisms. However, this is not the only way in which two molecules of 5-ALA may condense to give a cyclic product and, in this paper, we give an account of a systematic study of nonenzymatically induced cyclic condensation products of 5-ALA. The products have been identified, in the main, by high field NMR studies. This work complements a study, to be published subsequently, on condensation reactions of 5-ALA which are models for the enzymatically induced formation of PBG.

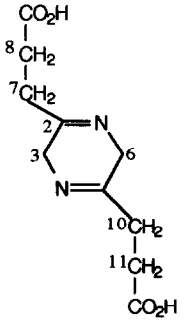


SCHEME 2

Some previous studies of the products obtained by the nonenzymatic condensation of two molecules of 5-ALA gave conflicting results. J. J. Scott[3] reported that anaerobic treatment of 5-ALA with aqueous alkali at 18 °C for several days gave PBG in 3% yield. A. I. Scott *et al.*[4] were unable to substantiate that claim and reported that the only product, obtained in 70% yield, was pseudo-PBG (**1**). PBG and pseudo-PBG differ only in the sense in which condensation occurs, a matter which will be discussed in more detail latter. Scott *et al.*[4] also reported that 5-ALA was converted into PBG in 10% yield in the presence of Amberlite TR-45 resin after 20 days of incubation. Granick and Mauzerall[5] reported that in solution at pH 6.8-8.0 5-ALA dimerises to 2,5-(β-carboxyethyl)dihydropyrazine (**2**) in 10% yield.



(**1**)



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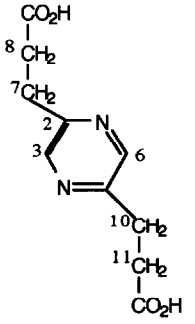
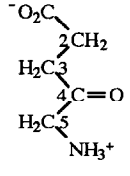


(**3**)

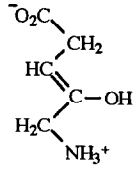
Results and Discussion

The form of 5-ALA in aqueous solution. We used a number of experimental techniques to establish the form of 5-ALA present in aqueous solution. Some possibilities are shown in Scheme 3. Assignments for the ^{13}C NMR spectrum of 5-ALA are shown in Table 1 and are based on the natural abundance spectrum. We then ran the spectrum of 0.25 M [4-^{13}C]-5-ALA (50% enriched) in 0.1 M phosphate buffer (pH 6.8) and obtained 3 more signals at 174.0, 153.4, and 93.4 ppm. The first and second will be discussed later but the third we assign to C-4 of the hydrate **6** on the basis of shifts of known hydrates.[6] There was no evidence, even using the isotopically enriched material, for the presence of the two enol forms **4b** and **5** in detectable amounts.

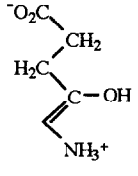
The ^1H NMR spectrum of a 1 M solution of unlabelled ALA in 0.1 M phosphate buffer in D_2O (pD 6.8) contained three major peaks corresponding to the keto form (**4a**) (Table 1). There were also three much smaller peaks with the same splitting pattern. They were shown to be coupled by homonuclear decoupling and we assign



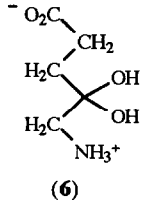
(**4a**)



(**4b**)



(**5**)



(**6**)

SCHEME 3

them to the hydrate (**6**). The methylene protons of **6** are expected to exhibit a set of signals upfield from those of the keto compound. The α-methylene protons of **6** are predicted to be 0.7-0.9 ppm upfield from those of the ketone tautomer and the β-methylene protons are expected to be 0.14-0.20 ppm upfield from the parent signal.[7] The chemical shifts of the signals displayed in Table 1 fit the predicted values very well and they are all signals of

equal intensity, showing that they are from one single tautomer. On the basis of this spectrum 0.4% is the upper limit for the mole fraction of the hydrate form of 5-ALA in aqueous solution at pD 6.8.

Michini and Jaffe[8] obtained supporting evidence for the formation and breakdown of **6** by monitoring oxygen exchange at C-4 using ^{13}C NMR. Oxygen exchange was followed by ^{18}O incorporation from $H_2{}^{18}O$ into [4-^{13}C]-5-ALA (90% enriched). The ^{13}C chemical shift change expected when ^{18}O replaces ^{16}O in an aldehyde is 0.045-0.050 ppm upfield.[9] When [4-^{13}C]-5-ALA was dissolved in 0.1M phosphate buffer (pH 6.8) in buffer containing 50% $H_2{}^{18}O$ the incorporation of ^{18}O onto C-4 was complete in less than 20 min at room temperature, demonstrating the facile formation of the hydrate. The observed isotope shift was -0.046 ppm (3.5 Hz) at 37 °C.

We have further evidence for hydrate formation from ^{17}O NMR studies. The ^{17}O NMR spectra of a large number of liquid organic compounds have been reported by Christ *et al.*[11] Their data provide the basis for the systematisation of ^{17}O chemical shifts. They showed that the ^{17}O resonances of neat aldehydes and ketones fall in the range 530-595 ppm relative to $H_2{}^{17}O$ as an external standard at zero ppm, while the ^{17}O resonances of the hydroxyl groups of alcohols fall near the water line (-37 to +70 ppm) In aqueous solutions of carbonyl compounds known to be hydrated, an ^{17}O resonance due to the gem diol should appear in the range of -37 to +70 ppm. Greenzaid *et al.*[7] were able to detect signals in this range due to hydrated species of a number of carbonyl compounds. These workers also found that the ^{17}O NMR chemical shifts of carbonyl oxygen, and to a lesser extent, those of a gem diol shift to higher field on dilution.

The ^{17}O NMR spectra of 1 M 5-ALA·HCl in ordinary water and in $H_2{}^{17}O$ (4% atom) were acquired after equilibrating the respective solutions in a thermostatic bath at 37 °C for 10 min. The former revealed only the natural abundance $H_2{}^{17}O$ resonance at zero ppm. The latter, however, showed, in addition to the $H_2{}^{17}O$ resonance at zero, the ^{17}O carbonyl resonances of 5-ALA at 521.8 and 252.1 ppm. The latter is within the range expected for a carboxyl group and was not observed immediately but increased in intensity with time. The former is correct for the carbonyl group of 5-ALA and incorporation of ^{17}O into the carbonyl group is most likely to occur *via* formation of the gem diol. There was, however, no signal due to the gem diol itself, probably because of its low intensity and proximity to the strong water line. This was the case even with $H_2{}^{17}O$ (12% atom)

Table 1. NMR Spectra of 5-ALA in aqueous buffer

Compound	Carbon	Shift/ppm	Proton	Shift/ppm	J/Hz
4a	1	177.7	2-CH_2	2.70(t)	6.38
	2	28.2	3-CH_2	2.90(t)	6.38
	3	34.8	5-CH_2	4.14(s)	
	4	204.2			
	5	47.5			
6	4	93.4	2-CH_2	2.55(t)	7.74
			3-CH_2	2.06(t)	7.74
			5-CH_2	3.10(s)	

and water suppression with 0.1 M manganese triflate. In conclusion, there can be no doubt that 5-ALA does

form a hydrate and this form must be taken into account when we consider the reaction mechanism of dimerisation.

Hydrogen exchange on 5-ALA. It is assumed that, in acid solution, hydrogen exchange on the methylene groups of 5-ALA occurs through the enol form and thus exchange can be used as proof of enol formation. That exchange at C-5 does occur has been demonstrated by Jaffe and Markham[11] but we were particularly interested in comparing C-5 with C-3 under our experimental conditions. 5-ALA·HCl (1μmole) was dissolved in phosphate buffer in D_2O (0.5 cm^3). The time of 5-ALA dissolution was taken as zero and the sample was immediately placed in a 5 mm NMR tube and inserted into the NMR probe at 37 °C. Spectra were acquired at appropriate time intervals in order to monitor the exchange of protons at C-3 and C-5 and the spectra were processed by resolution enhancement. The spectra show that the C-5 methylene is a singlet at 4.30 ppm while the proton of CHD is a three line spectrum (1:1:1) at 4.28 ppm, J_{HD} = 2.65 Hz. The C-3 methylene is a regular triplet at 2.96 ppm J_{HH} = 5.56 Hz; the C-3 CHD is a 1:2:1 triplet of 1:1:1: triplets, which is difficult to decipher. It is probably easier to monitor exchange at C-3 by observing changes in the signals of the C-2 methylene group. Initially this was a 1:2:1 triplet 2.69 ppm J_{HH} = 6.60 Hz. When adjacent to CHD the C-2 methylene becomes an isotope shifted doublet at 2.685 ppm, J_{HH} = 6.32 Hz and the signal is slightly broadened by unresolved coupling to deuterium. When adjacent to CD_2 the signals become a singlet at 2.68 ppm substantially broadened by unresolved coupling to the deuteriums. From the spectra in it is clear that enolisation occurs much more readily at C-5 than at C-3 by a factor of about 6. This result is fully in accord with the results of Jaffe and Rajagopalan[12] who undertook a much fuller study of this matter. They detected that there was rapid exchange of protons on C-3 and C-5 at pH 6.8 and when the pH was lowered exchange was much slower. Equally exchange on levulinic acid is much slower than on 5-ALA, presumably because of the electron withdrawing properties of the $-NH_3^+$ group. The mechanism of enolisation has been widely discussed and will not be considered further. The significant fact to note is that the case of 5-ALA enolisation at C-5 occurs more readily than to C-3.

Cyclic condensation products from 5-ALA. The self condensation products of 5-ALA in acetate buffer (pH 4.6), carbonate buffer (pH 9.7) and phosphate buffer (pH 11.6) were investigated. In each case 5-ALA (0.25 mmole) was refluxed in 1 cm^3 of the buffer for 6 h. The solvent was removed by evaporation, D_2O was added to redissolve the material and the solution analysed by NMR spectroscopy. The only condensation product formed in any of the buffers was 2,5-(β-carboxyethyl)pyrazine (**3**), as suggested by Jaffe and Rajagopalan.[12]

The pyrazine was characterised by ^{13}C and ^1H NMR spectroscopy (Table 2) and the designation is unambiguous. Further proof comes from our observation that when [4-^{13}C]-5-ALA is dissolved in buffer, a signal at 153.4 ppm was observed and this clearly corresponds to the formationof some **3** even under the very mild conditions of that experiment and it is C-4 of 5-ALA which would end up as C-2, C-5 of **3**. In that early experiment there was also an additional signal at 174.0 ppm and this we assign to C-2/C-5 of the dihydropyrazine (**2**). It is possible that **3** is formed from **2** by aerial oxidation and we carried out the condensation under anaerobic conditions to confirm this point.

Anaerobic condensation of 5-ALA. In this experiment we used different conditions for the formation of the dihydropyrazine (**2**). A deaerated solution of 5 M NaOH (0.5 cm^3) was added to a deaerated sample of 5-ALA·HCl (0.25 mmole) under a stream of argon using a vacuum line. The reaction mixture was well stirred at 70 °C in a water bath for 4 h and then transferred to an evacuated 5 mm NMR tube under argon.

Table 2. ^{13}C and ^{1}H NMR assignments of 2, 5-(β-carboxyethyl)pyrazine(**3**)

	^{13}C chemical shifts/ppm				^{1}H chemical shifts/ppm		
Atom	acetate buffer	carbonate buffer	phosphate buffer	Atom	acetate buffer	carbonate buffer	phosphate buffer
C2/C5	156.0	155.7	155.7	3/6-CH	8.47(s)	8.69(s)	8.65(s)
C3/C6	146.0	145.3	145.4	7/10-CH_2	3.10(t) J=7.29Hz	3.23(t) J=6.83Hz	3.21(t) J=6.83Hz
C7/C10	31.9	31.0	31.1	8/11-CH_2	2.79(t) J=7.29Hz	2.91(t) J=6.83Hz	2.92(t) J=6.83Hz
C8/C11	36.1	34.9	35.0				
C9/C12	180.5	179.2	179.3				

This treatment of 5-ALA·HCl lead to a mixture of two condensation products 2, 5-(β-carboxyethyl)dihydropyrazine (**2**) and pseudo-PBG (**1**) in the ratio 2:1, identified by their ^{13}C and ^{2}H NMR spectra (Table 3). The latter clearly differentiates between PBG and pseudo-PBG

Frank and Stratmann,[13] in a similar study, suggest that the minor product was, in fact, PBG rather than pseudo-PBG but the NMR spectrum clearly indicates that this is not the case. They reached their conclusion on the basis of the Ehrlich test, the reaction between 4-dimethylaminobenzaldehyde and a pyrrole with a free

Table 3. NMR spectra of dihydropyrazine (**2**) and pseudo-porphobilinogen (**1**)

Compound	Carbon	Shift/ppm	Proton	Shift/ppm	J/Hz
2	C-2/C-5	175.2	2-CH_2	2.70(t)	6.38
	C-3/C-6	53.0	3-CH_2	2.90(t)	6.38
	C-7/C-10	35.77	5-CH_2	4.14(s)	
	C-8/C-11	35.71			
	C-9/C-11	183.7			
1	C-2/C-4	120.4,117.3	5-CH	6.40(s)	
	C-3/C-5	125.1,114.3	6-CH_2	2.60(t)	6.83
	C-6/C-9	23.1,23.8	7-CH_2	2.40(t)	6.83
	C-7/C-10	39.8,40.3	9-CH_2	2.75(t)	6.83
	C-8/C-11	184.6,185.2	10-CH_2	2.43(t)	6.83

α-position. This reaction has been studied in some detail by Alexander and Butler.[14] Both PBG and pseudo-PBG have free α-positions and so the test will not distinguish between them and, in any event, Treibs and Herrmann[15] have shown that a free α-position is not necessary for formation of a colour.

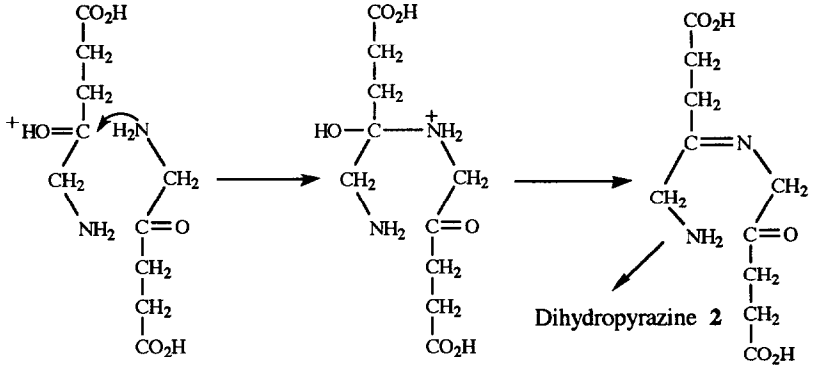
When the reaction mixture was exposed to air, there was a slow and irreversible conversion of **2** into **3**. The ^{13}C and ^{1}H chemical shifts of **3** are listed in Table 2. Thus, our suggestion on this point was confirmed.

The ^{13}C assignments of **1**, **2**, and **3** were confirmed by repeating the condensation using [4-^{13}C]-5-ALA

in 5 M NaOH and then exposing the product mixture to air. In the case of the dihydropyrazine, the enhanced signal for C-2/C-5 appeared at 175.2 ppm since these carbons are 20% enriched. The C-3/C-6 and C-7/C-10 methylene resonances at 53.00 and 35.77 ppm respectively each had satellite peaks on either side of the parent and one eighth the intensity of the latter ($^1J_{C2,C3}$ = 36.6 Hz, $^1J_{C2C7}$ = 47.4 Hz). The resonances at 35.71 and 183.7 ppm were assigned to the C-8/C-11 and C-9/C-12 carbons respectively, their intensities being greater than the remaining methylene carbon resonances in the spectrum.

In the case of pseudo-PBG, two resonances at 117.3 and 120.4 ppm appear largely enhanced suggesting that these are resonances from C-2 and C-4 which originate from the enriched C-4 of 5-ALA. However, these resonances cannot be unambiguously assigned. The resonances at 114.3 and 125.1 ppm were assigned to C-5 and C-3, respectively ($^1J_{C4C5}$ = 15.9Hz; $^1J_{C2C3}$ = 18.3Hz). The resonances at 23.1 ppm and 23.8 ppm each have satellite peaks on both sides of the parent signal and about one eighth the intensity of the latter. These, therefore, correspond to the C-6 and C-9 resonances, although they cannot be distinguished. The resonance at 23.1 ppm had satellite peaks which were 47.0 Hz apart and the resonance at 23.8 ppm had satellite peaks which were 41.8 Hz apart. Resonances at 39.8 and 40.3 ppm correspond to the methylene carbons C-7 and C-10, or *vice versa*, and resonances at 184.6 and 185.2 ppm correspond to C-8 and C-11, or *vice versa*.

In the case of the pyrazine the enhanced signals, corresponding to C-2/C-5, appeared at 156.3 ppm. The resonances at 145.5 and 183.8 ppm were unambiguously assigned to C-3/C-6 and C-9/C-12, respectively. Satellite peaks were observed on both sides of the parent signal at 33.2 ppm, showing this to be the C-7/C-10 resonance ($^1J_{C2C7}$ = 34.4 Hz). The C-8/C-11 methylene carbons have a chemical shift at 39.0 ppm.



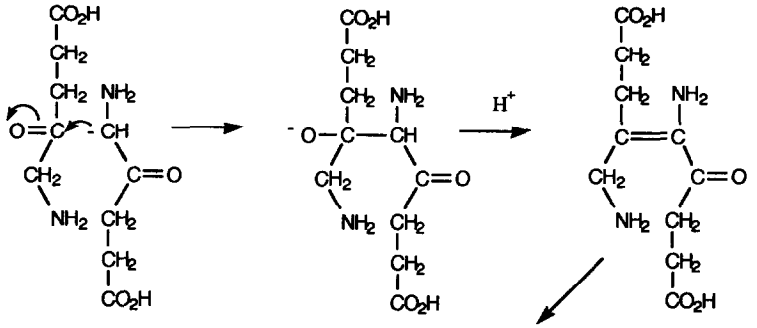
SCHEME 4

The ^1H resonances of the dihydropyrazine, pseudo-PBG and pyrazine were assigned after performing a ^{13}C-^1H correlation on the partially oxidised reaction mixture.

In summary, we see that the cyclic dimerisation of 5-ALA at moderate pH gives exclusively a dihydropyrazine in a reaction favoured by acid. The mechanism for this reaction is shown in Scheme 4. There appears to be no role for the hydrate, although we know that form does exist in aqueous solution. Although the -NH_2 group will be substantially protonated the pK_a is such (8.9) that there will always be some of the free base. Nevertheless, the reaction is favoured by acid because of the need to protonate the carbonyl group.

The reaction in 5 M NaOH to give pseudo-PBG probably involves the carbanion and it is resonable to assume that the ease of carbanion formation (C-3 or C-5) will parallel the readiness with which the enol is formed (ie. C-5 is more acidic than C-3). The mechanism for production of pseudo-PBG is shown in Scheme 5. In

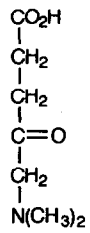
view of what has been proposed in Scheme 4 for formation of the dihydropyrazine, it is difficult to see why some of this product is obtained under strongly alkaline conditions.



pseudo-PBG (1)

SCHEME 5

Anaerobic condensation of ALA with N,N-dimethylaminolevulinic acid. In order to provide some evidence for the above we examined the condensation of 5-ALA with *N,N*-dimethyl-5-amino-levulinic acid (**7**). With **7**, no pyrazine or pyrrole can form but **7** can react with 5-ALA to give pyrrole (**8**), but not a dihydropyrazine. 5-ALA was added dropwise to an alkaline solution of **7** until equimolar amounts had been mixed. The products of reaction were determined without isolation. Examination of the ^{13}C and ^{1}H NMR spectra (Table 4) showed three condensation products in approximately equal amounts. Two were immediately identified as **1** and **3** formed, as expected, by the cyclisation of two 5-ALA molecules. Compound **7** remained largely unreacted but some of **8** had been formed from the condensation of one molecule of 5-ALA with one molecule of **7**. This observation is entirely consistent with the mechanism proposed for the formation of pseudo-PBG from 5-ALA.



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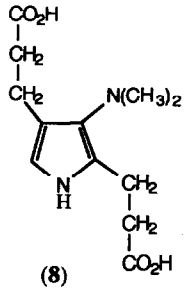

(8)

Table 4. NMR spectra of compound (**8**)

Carbon	Shift/ppm	Proton	Shift/ppm
C-2/C-4	124.4, 118.3	5-CH	6.60(s)
C-3/C-5	127.3, 116.7	6-CH_2	2.60(t)
C-6/C-9	24.0, 24.2	7-CH_2	2.22(t)
C-7/C-10	42.5, 42.9	9-CH_2	2.54(t)
C-8/C-11	185.2, 185.6	10-CH_2	2.27(t)

The information acquired in this study will be used in a study of Knorr-type pyrrole products obtained by the condensation of 5-ALA with some diketones. These reactions are models for the formation of PBG from 5-ALA. What is clear for the enzyme catalysed process is that any spontaneous reaction is more likely to lead to pseudo-PBG rather than PBG. Any enzyme mechanism must address this aspect.

Experimental

Materials. 5-ALA·HCl was purchased from the Sigma Chemical Company. All other chemicals were AnalaR grade whenever possible. Water containing oxygen-17 was obtained from Yeda Isotopes Ltd, Israel. Acetate, carbonate and phosphate buffers were made in the normal way. Carbon-13 labelled 5-ALA at the 4-position was made by minor modifications of the methods of Pichat and Herbert[16] and of Tschudy and Collins.[17] *Techniques.* Proton and carbon-13 NMR spetra were obtained using a Bruker AM300 spectrometer. The former were referenced with respect to external dioxan set at 3.7 ppm and the latter set at 67.4 ppm. Oxygen-17 NMR spectra were obtained at 67.798 MHz on a Bruker MSL 500 spectrometer using 16 K data points. They were acquired unlocked in a 10 mm high resolution probe using a pulse width of 26 μs (27 μs for a 90° flip angle), a recycle time of 0.41 s and a spectral width of 100 KHz. They were referenced with respect to water as an internal standard, set at zero, and were proton decoupled. All spectra were acquired at a probe temperature of 37 °C. A Griffin digital pH meter was used.

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