# Mechanistic Studies on the Phytylation Step in Bacteriochlorophyll *a* Biosynthesis: an Application of the $^{18}O$ Induced Isotope Effect in $^{13}C$ N.M.R. Spectroscopy

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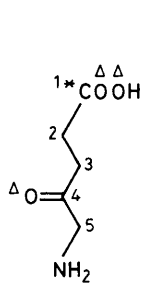
It is shown that bacteriochlorophyll *a* (**3b**) biosynthesised from [1-$^{13}C$; 1,1,4-$^{18}O_3$]-5-aminolaevulinic acid (**1**, C* = $^{13}C$; $O^{\Delta}$ = $^{18}O$) in growing cultures of *Rhodopseudomonas sphaeroides* contains oxygen-18 in both the bridge ($^{16}O{=}^{13}C{-}^{18}O{-}$) and non-bridge ($^{18}O{=}^{13}C{-}^{16}O{-}$) oxygen of the phytyl ester linkage [–C(:O)–O–phytyl].

All naturally occurring chlorophylls are esterified[1] at the ring D propionate carboxy group with a long-chain alcohol moiety. The nature of this esterifying alcohol varies considerably and has been shown to be phytol for plant chlorophylls[2] and bacteriochlorophyll *a* from *Rhodopseudomonas sphaeroides*,[3] geranylgeraniol for bacteriochlorophyll *a* from *Rhodospirillum rubrum*,[4] and farnesol for *Chlorobium* chlorophylls.[5] Until recently the esterification process was assumed to be merely a reversal of the hydrolytic reaction catalysed by chlorophyllase;[6] however, two independent reports have suggested that a novel enzyme (chlorophyll synthetase), unique to the biosynthetic pathway, is responsible for the transformation. First,[7] in the incorporation of [1,1,4-$^{18}O_3$]-5-aminolaevulinic acid (**1**) (ALA; $O^{\Delta}$ = $^{18}O$) into bacteriochlorophyll *a* (**3b**), it was found that the bridge oxygen of the latter contained $^{18}O$. Secondly, Rüdiger and co-workers[8] have shown the incorporation of geranylgeraniol pyrophosphate into a chlorophyll fraction using a cell-free system from maize seedlings. The results outlined above are in accord with a mechanism[7] in which the ester bond formation occurs by the nucleophilic attack of the C-17[3] carboxylate anion of bacteriochlorophyllide *a* on the isoprenyl pyrophosphate, *viz.* Scheme 1.

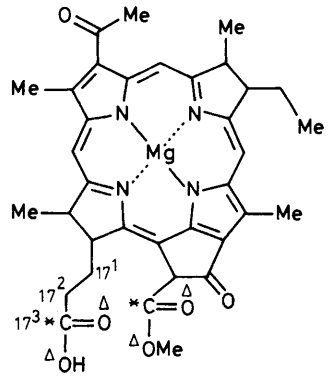
A mandatory requirement of this mechanism is that both C-17[3] carboxy-oxygen atoms of bacteriochlorophyllide *a* are retained in bacteriochlorophyll *a*. However, the experimental demonstration of this mechanistic facet relying solely upon an $^{18}O$ labelling approach is hampered by difficulties in the mass spectral analysis of bacteriochlorophyll *a* containing multiply labelled sites with low isotopic enrichments. Hence, an approach based on the $^{18}O$ induced isotope effect on $^{13}C$ n.m.r. was utilised. Since its theoretical inception in 1977 by Jameson,[9] and subsequent experimental observation by Risley and Van Etten,[10] the aforementioned technique has been increasingly utilised particularly in the delineation of secondary metabolite biosynthesis.[11] We envisaged that incorporation of ALA labelled strategically at the C-1 position with $^{13}C$ and $^{18}O$ (**1**; C* = $^{13}C$, $O^{\Delta}$ = $^{18}O$) into bacteriochlorophyllide *a* (**2**; C* = $^{13}C$, $O^{\Delta}$ = $^{18}O$) and thence into bacteriochlorophyll *a* (**3b**; C* = $^{13}C$, $O^{\Delta}$ = $^{18}O$) *via R. sphaeroides* would enable us to analyse the isotopic status of both bridge and non-bridge oxygens at C-17[3] in bacteriochlorophyll *a* by utilising the distinctive upfield shift $^{18}O$ exerts relative to its $^{16}O$ counterpart. Such an approach would allow a critical scrutiny of the proposed mechanism.

Autoclaving a solution of [1-$^{13}C$]ALA (50 mg; 90% enriched in $^{13}C$) in [$^{18}O$]water (75 μl, containing 98 atom % excess of $^{18}O$) for 1.5 h in the presence of a trace of HCl led to the formation of [1-$^{13}C$; 1,1,4-$^{18}O_3$]ALA (**1**; C* = $^{13}C$, $O^{\Delta}$ = $^{18}O$). The resulting material which contained $^{18}O$ at C-4 as well as C-1 was either analysed directly by $^{13}C$ n.m.r. spectroscopy to yield the distribution of $^{18}O$ at C-1 or incubated at pH 6.9 for 24 h to exchange the relatively labile $^{18}O$ at C-4 and then oxidised with $NaIO_4$ to give succinic acid. The later, after conversion into its bis(trimethylsilyl) ester derivative was analysed by g.c.–mass spectrometry. Both analyses showed the ALA to contain 69.1 atom % excess of $^{18}O$ at each of the two C-1 oxygen atoms (specifically: 19.7% $^{16}O_2$, 32.5% $^{18}O^{16}O$, 47.8% $^{18}O_2$).

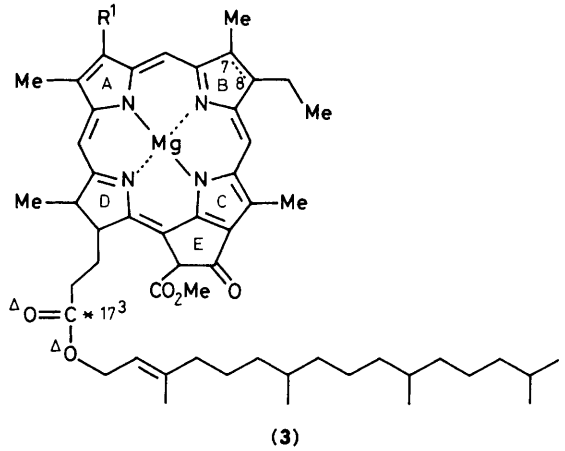
Conditions for the manipulation of *R. sphaeroides* preferentially to incorporate exogenously added ALA have previously been established in our laboratory.[7] Hence a culture medium (360 ml) containing a freshly grown inoculum of *R. sphaeroides* (40 ml) was supplemented with [4-$^{14}C$; 1-$^{13}C$; 1,1,4-$^{18}O_3$]ALA (30 μmol; $^{14}C$ specific activity 7.3 × $10^4$ d.p.m./μmol; $^{18}O$ distribution as above) and the growth allowed to proceed for 24 h at 27 °C under illumination from a 60 W tungsten lamp. The cells were harvested and processed to give, after purification, bacteriochlorophyll *a* (3.4 μmol) containing 0.44 × $10^6$ d.p.m. of $^{14}C$, thus showing that 22% of the biosynthetic pigment had originated from exogenously added ALA. In order to aid facile n.m.r. analysis, eight parallel 400 ml incubations were performed, each containing



(1) ALA



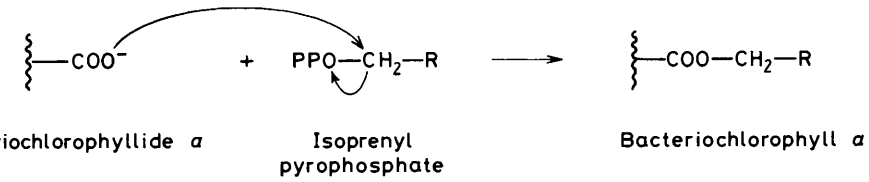
(2)



(3)

(3a) Plant chlorophyll *a*, R = vinyl; $\Delta^7$

(3b) Bacteriochlorophyll *a* from *R. sphaeroides*, R = Ac; no $\Delta^7$




**Scheme 1**

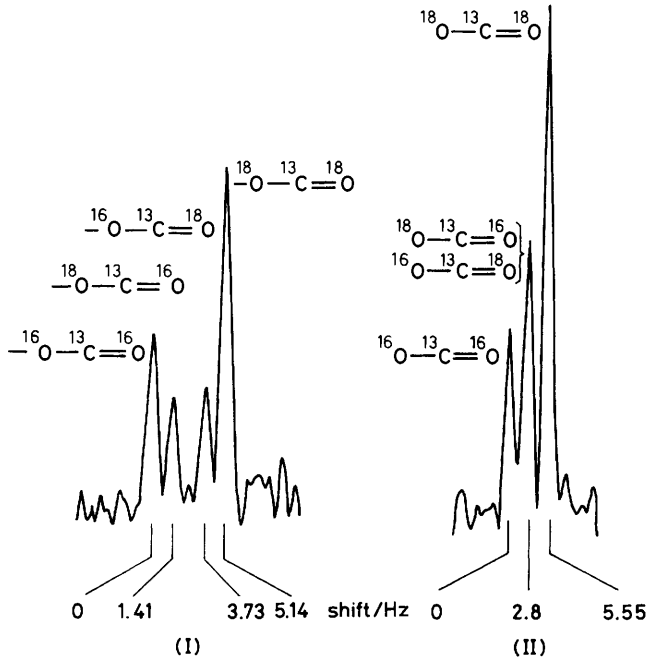



**Figure 1.** Partial $^{13}C$ n.m.r. spectrum of (I) bacteriochlorophyll *a* and (II) ALA illustrating the isotopic distribution at C-17[3] of bacteriochlorophyll *a* (δ 173.860) and at C-1 of ALA (δ 176.273). Isotope shifts shown are relative to the ($^{16}O$–$^{13}C$=$^{16}O$) species; the percentage of each isotopomer is quoted in the text. Spectroscopic parameters were as follows: (I) bacteriochlorophyll *a* (25 mg) was dissolved in [$^2H_6$]acetone–[$^2H_4$]methanol (4:1 v/v; 0.6 ml) and the spectrum accumulated at 297 K in a 5 mm bore tube using a sweep width of 2000 Hz with 64 K data block, 2883 scans, pulse angle 48°, and an acquisition time of 4.096 s. For resolution enhancement a line broadening factor of −0.7 was applied together with a Gaussian multiplier of 0.2; 0.061 Hz/data point, and the free induction decay was zero filled to 16K prior to Fourier transformation. (II) [1-$^{13}C$; 1,1,4-$^{18}O_3$]ALA (1 mg) was dissolved in [$^2H_4$]methanol (0.5 ml) and the spectrum accumulated at 273 K in a 5 mm bore tube using a sweep width of 3125 Hz, 32K data block, 372 scans, pulse angle 72°, and an acquisition time of 5.243 s. Spectra for both samples were obtained using a Bruker WH400 spectrometer operating at 100.63 MHz with quadrature detection and broadband decoupling.

30 μmol of ALA and yielding 27.4 μmol of bacteriochlorophyll *a* in total.

The high-resolution 100 MHz $^{13}C$ n.m.r. spectrum of the biosynthetic sample of bacteriochlorophyll *a* showed the C-17[3] resonance at δ 173.86 to consist of four components (Figure 1) corresponding to: ($^{16}O$–$^{13}C$=$^{16}O$), ($^{18}O$–$^{13}C$=$^{16}O$) ($^{16}O$–$^{13}C$=$^{18}O$), and ($^{18}O$–$^{13}C$=$^{18}O$) species respectively. The upfield isotope shifts for the last three species (+1.41, +3.73, and +5.14 Hz) are consistent with values reported for model compounds.[12] A comparison of the isotopic ratios determined from the intensities of the $^{13}C$ resonances for C-17[3] [23.3%($^{16}O$–$^{13}C$=$^{16}O$); 15%($^{18}O$–$^{13}C$=$^{16}O$); 16.6% ($^{16}O$–$^{13}C$=$^{18}O$), and 45.1%($^{18}O$–$^{13}C$=$^{18}O$)] with those for C-1 of the starting ALA demonstrates that retention of $^{18}O$ at *both* oxygen atoms was in excess of 95%. As expected, the intensities of ($^{18}O$–$^{13}C$=$^{16}O$) and ($^{16}O$–$^{13}C$=$^{18}O$) at C-17[3] of bacteriochlorophyll *a* are equal and their sum (31.6%) is equivalent to the intensity of singly labelled (–C$^{18}O^{16}$OH) oxygen species in the starting ALA (32.5%).

The above results are in accord with the proposed mechanism (*vide supra*) whereby phytylation occurs by nucleophilic attack of the C-17[3] carboxy group of (**2**) on the isoprenyl pyrophosphate moiety to yield bacteriochlorophyll *a* (**3b**) with concomitant retention of both C-17[3] oxygen atoms. To our knowledge this is the first application of the $^{18}O$ induced isotope effect on $^{13}C$ n.m.r. spectra to show unambiguously the presence of bridge, non-bridge, and dual labelled oxygen species within an ester group of a biosynthetic natural product. Observation of the latter and the fact that $^{18}O$ retention at both oxygens was in excess of 95% verifies the proposed mechanism and implies the biosynthetic flux through the pathway operating via this mechanism under *in vivo* conditions must be at least 90%† for *R. sphaeroides*.

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† Mean of three independent experiments.