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Synthesis of ^{18}O-Labelled Chlorophyll Derivatives at Carbonyl Oxygen Atoms by Acidic Hydrolysis of the Ethylene Ketal and Acetal

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Abstract—The ethylene ketal of pyropheophorbides, chlorophylls possessing the 13-keto carbonyl group and lacking the 13^2-methoxycarbonyl group, reacted with $H_2^{18}O$ (ca. 95% ^{18}O atom) by acidic hydrolysis to give efficiently and regioselectively 13^1-^{18}O-oxo-labelled compounds (ca. 92% ^{18}O). The resulting ^{18}O-labelled chlorin was modified by several chemical reactions to afford some derivatives with little loss of the ^{18}O atom. Following the same procedures, 3^1,13^1-doubly-^{18}O-labelled pyrochlorophyll derivatives were also prepared. All the synthetic ^{18}O-labelled compounds were identified by FAB-mass and vibrational spectra. Especially, in the vibrational spectroscopic results including IR and resonance Raman spectra, an about 30 cm^{-1} wavenumber down-shift of the 3- and/or 13-C=O stretching vibrational bands was observed by exchanging 3^1- or 13^1-oxo-oxygen atom from ^{16}O to ^{18}O.

Introduction

Chlorophylls are naturally occurring magnesium complexes of cyclic tetrapyrroles possessing oxygen atoms at several positions. The oxygen atoms of carbonyl and hydroxy groups in chlorophylls can hydrogen bond and coordinate with any functional groups or atoms including nitrogen, oxygen and metals in the other molecules. In fact, any carbonyl groups of chlorophylls in natural systems form various non-covalently bonding interactions; typically, such interactions make apparatuses of photosynthetical initial stages and control the processes.[1] Therefore, the chlorophyllous oxygen atoms play important roles in photosynthesis. Structural information on photosynthetic apparatus is available not only from X-ray crystallographic[2] and NMR spectroscopic studies,[3] but also from vibrational spectroscopic investigations.[4–6] Vibrational spectra were measured in several samples, for example in a solution and in a solid, giving useful information on the local structure around photosynthetic pigments and/or the peptide scaffolds. Furthermore, specific interaction around the pigments in the ground state as well as short-lived excited states was also observed by resonance Raman (RR) spectroscopy.[7]

Isotope labelling techniques are powerful for assignments of signals on both NMR and vibrational spectra. Many ^2H-, ^{13}C- and ^{15}N-isotope labelling investigations of photosynthetic pigments have been appeared up to date.[8] Some mass spectrometric studies using ^{18}O-labelling of photosynthetic pigments have also been reported[9] but their vibrational spectroscopic reports are unavailable, to our best knowledge, except for one preliminary report in the literature.[10] This is ascribable to the lower availability of ^{18}O-labelled chlorophylls at a high degree of isotopic labelling by simple synthetic procedures. Therefore, we attempted the isotopical conversion of ^{16}O to ^{18}O at the chlorophyllous carbonyl oxygen. In this paper, we report preparation of 13^1-^{18}O-oxo-labelled methyl pyropheophorbide-*a* (**3**) by acidic $H_2^{18}O$ hydrolysis of the ethylene ketal of the unlabelled compound **1** derived from chlorophyll-*a*, the physical properties including vibrational spectra of **3**, and synthetic modification of **3** by several procedures to give a variety of ^{18}O-labelled compounds.

Results and Discussion

In general, ketals and acetals are useful for protection of any carbonyl groups; they tolerate various reactions under neutral and basic conditions, and are easily cleaved by acidic hydrolysis after the reactions. Therefore,

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^{18}O-labelled carbonyl compounds can be afforded after deprotection of the ketal or acetal by action of $H_2^{18}O$ with an acid.

First, we tried to prepare 13^1-^{18}O-oxo-labelled methyl pyropheophorbide-*a* (**3**) possessing a vinyl group at the 3-position (Scheme 1). Methyl pyropheophorbide-*a* (**1**) derived from chlorophyll-*a* was treated with ethylene glycol and trimethylsilyl chloride (TMSCl) to give cyclic ketal **2** at the 13^1-position. Purification of **2** from the reaction mixture must be done by neutral alumina column chromatography because of instability of the ethylene ketal moiety toward acidic conditions, even for silica gel column chromatography. 13^1-Ketal protected chlorin **2** was hydrolyzed by $H_2^{18}O$ (95% ^{18}O atom) with a catalytic amount of trifluoroacetic acid (TFA) to afford a product after neutralization and successive purification by alumina column chromatography and recrystallization. ^{1}H NMR and visible spectra of the product were identical with those of **1**, indicating that the product was methyl pyropheophorbide-*a*. The overall yield of the product from **1** was 67%.

FAB-MS spectrum of the product exhibited the major peaks at $m/z = 550$ or 551 and the values were 2 mass larger than those of M^+ and MH^+ for **1**, respectively, showing that the product contained a singly ^{18}O-labelled compound. IR spectrum of the product in CH_2Cl_2 gave a new band at 1663 cm^{-1}, compared with that of **1** (1692 cm^{-1}, see Fig. 1A). These bands are ascribed to the 13-C=O stretching and about 30 cm^{-1} lower shift in the peaks supported that the 13-C=^{18}O was present in the product.[10] RR spectra of the product and **1** were also measured in the solid state (excited at 457.9 nm). An about 30 cm^{-1} down-shifted 13-C=O band (1688→1660 cm^{-1}) was also observed (see Fig. 1B), indicating the presence of C=^{18}O in the product.

Subsequently, the ^{18}O-labelling degree of the above product was estimated as follows. At first, we attempted to estimate the ^{18}O-labelling degree of the present product from the FAB-MS spectral data: (1) simulation of the pattern of molecular ion peaks and (2) calculation of the total molecular ion intensity, by use of the observed FAB-MS ion peaks of **1** and the ^{18}O-labelled product and the proposed spectral peaks of fully ^{18}O-labelled **3** (plus 2 mass to **1**). The estimation by (1) and (2) led to 92±1 and 94±3% for the ^{18}O-degree, respectively.

For an alternative estimation, the IR spectra in CH_2Cl_2 and tetrahydrofuran (THF) solutions were used. Comparison of the relative deconvolution peak heights in the 13-C=^{16}O stretching bands of unlabelled **1** and the ^{18}O-labelled product (normalized at the unchanged 17^2-C=O peak at 1734 and 1740 cm^{-1} in CH_2Cl_2 and THF solutions, respectively) gave 92±0.2 (in CH_2Cl_2) and 92±1% (in THF) as the ^{18}O-labelling degree. The values estimated from IR spectra were consistent with those from MS spectra. A labelling degree of about 92% is fairly high, considering 95% ^{18}O atom for starting ^{18}O-labelled water and contamination of ^{16}O-water derived from moisture and TFA to the reaction mixture in the deprotected hydrolysis.

The other 13^1-^{18}O-oxo-labelled chlorophyll derivatives possessing a variety of functional groups can be provided by modification of the resulting **3** (including about 8% **1**). We examined several reactions of ^{18}O-labelled **3** and investigated the ^{18}O-labelling degrees of the 13-keto carbonyl oxygen atom in the product (Scheme 2).

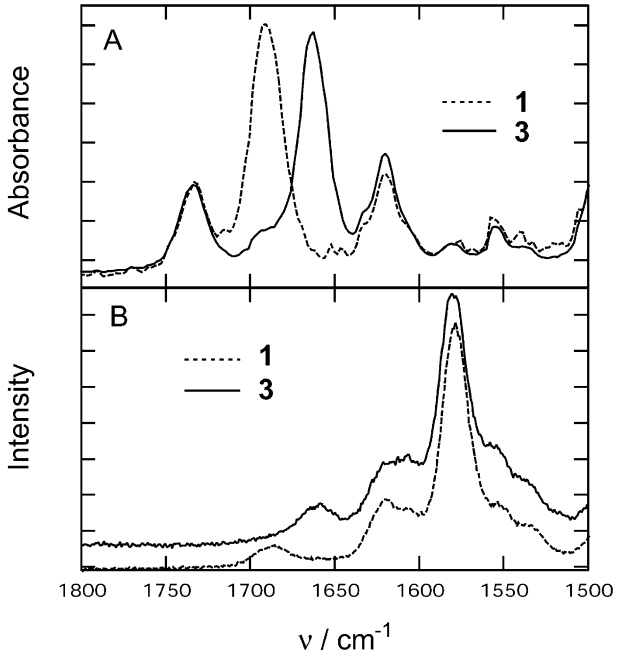
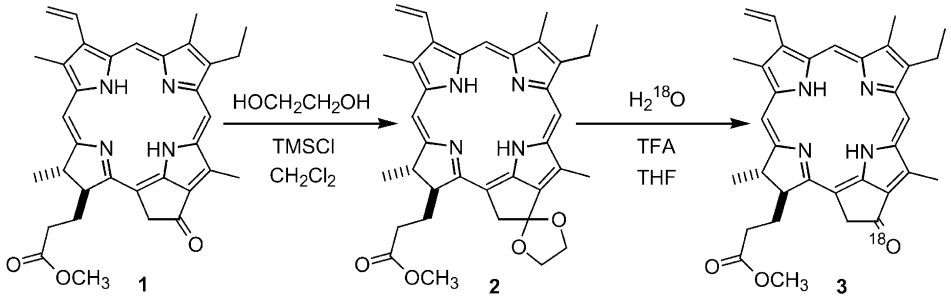
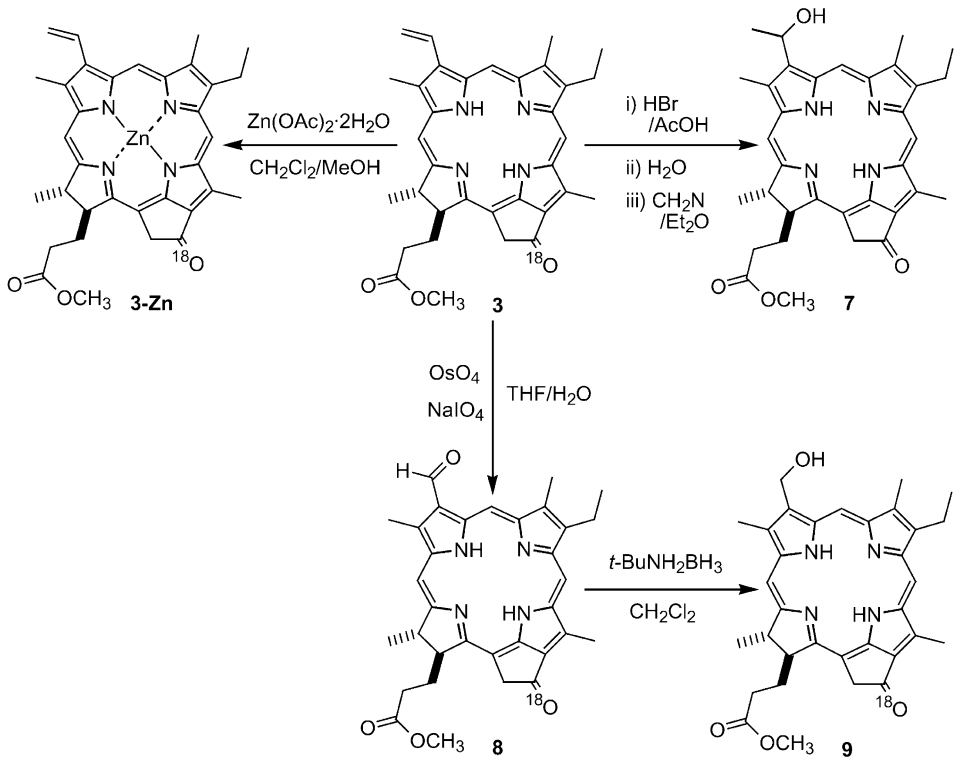


Figure 1. IR spectra of **1** (solid line) and **3** (broken line) in CH_2Cl_2 solution (A) and their RR spectra in the solid state excited at 457.9 nm (B).



Scheme 1. Synthesis of 13^1-^{18}O-oxo-labelled methyl pyropheophorbide-*a* (**3**).



Scheme 2. Chemical modification of **3** possessing the 13^1-^{18}O atom.

The 3-vinyl group of **3** (ca. 92% ^{18}O atom) was oxidized by OsO_4 and $NaIO_4$ to afford the 3-formyl group as in **8**. Regioselective reduction of the 3-formyl group of **8** by t-$BuNH_2{\cdot}BH_3$ gave 3-hydroxymethyl-chlorin **9**. From their FAB-MS and IR spectra, the products **8** and **9** possessed the ^{18}O-oxo-group at the 13^1-position in 90 ± 3 and $90\pm1\%$, respectively. During the above oxidation and reduction, little undesired delabelling was observed.

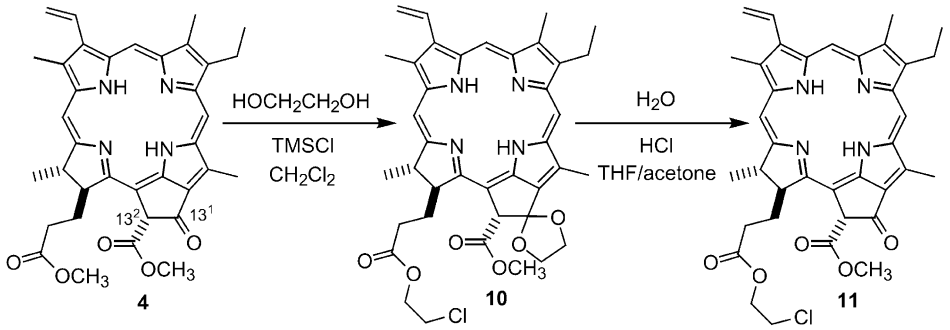
The vinyl group of **3** was hydrated by treatment with HBr/AcOH($=CH_3COOH$), followed by H_2O to give methyl bacteriopheophorbide-*d* possessing the 3-(1-hydroxyethyl) group. The FAB-MS and IR spectra showed that the product was ^{18}O-unlabelled compound **7** because of 13^1-oxygen exchange from ^{18}O to ^{16}O by the action of acidic water. In other words, the 13-keto carbonyl oxygen atom of chlorophyll derivatives was exchangeable in acidic water.[10,11] Treatment with acidic water must be avoided in any synthetic procedures for 13^1-^{18}O-oxo-labelled compounds.

Zinc metallation of **3** was achieved by standard procedures ($Zn(OAc)_2{\cdot}2H_2O$ in $MeOH–CH_2Cl_2$, stirring at room temperature). Slight loss of 13^1-^{18}O oxygen atom was observed during the reaction to give **3-Zn** ($89\pm1\%$ ^{18}O atom). We conclude that the ^{18}O-oxo-label was fairly resistant under the above functionalization conditions except for severe acidic conditions.

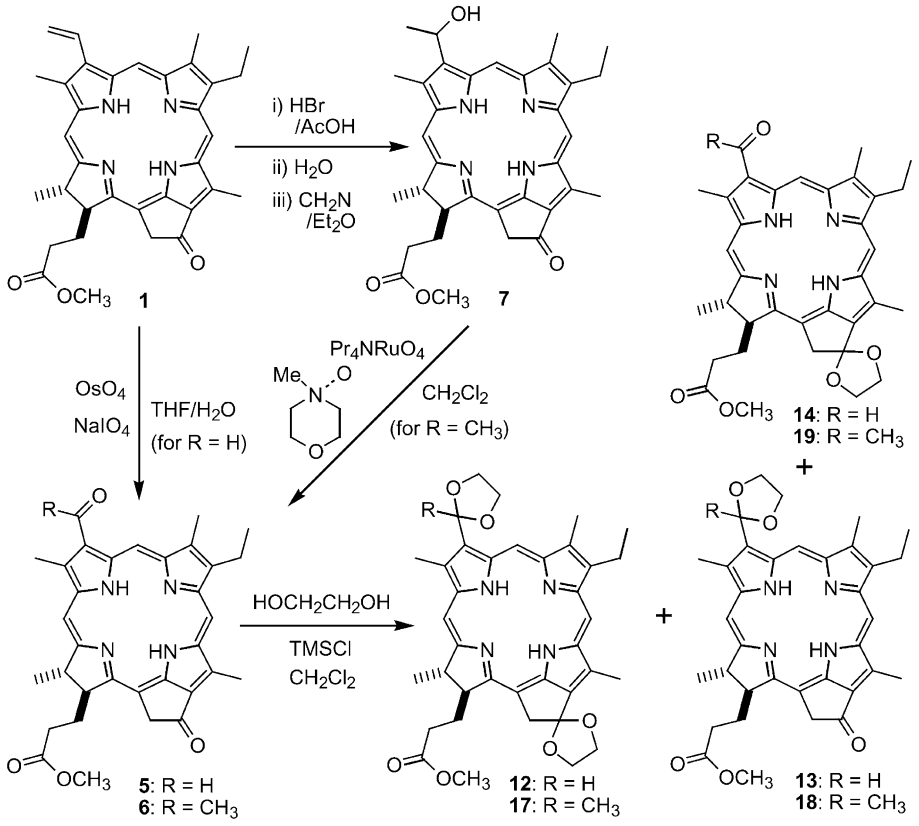
The ^{18}O-labelling method described above for **1**→**3** through ketal protection-deprotection was applied to the other chlorophyll derivatives such as methyl pheophorbide-*a* (**4**), methyl pyropheophorbide-*d* (**5**), methyl 3-devinyl-3-acetyl-pyropheophoebide-*a* (**6**) and methyl bacteriopheophorbide-*d* (**7**).

Cyclic ketal protection of **4** (see Scheme 3) with ethylene glycol was not achieved by the above conditions for **1**→**2** due to the steric hindrance of the neighboring 13^2-methoxycarbonyl group. Then, the protection of **4** was examined using 10-fold the amount of reagents and for a longer reaction time (2 days). After purification of the reaction mixture, overreacted compound **10** possessing 13^1-ketal and 2-chloroethyl ester was isolated in 54% yield. The resulting 13^1-ketal **10** could not be hydrolyzed using the above acidic conditions for **2**→**3**. A 10-fold concentration of an acid as well as 10 times the volume of water were necessary for the deprotection of **10** and the total yield of **4**→**11** was at most 19%, which is inadequate for efficient preparation of the ^{18}O-labelled compound. Thus, ^{18}O-labelling of **4** through ketal protection-deprotection was not performed here.

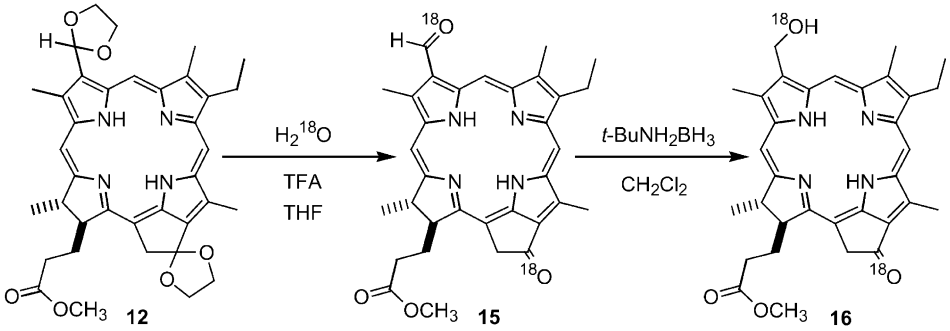
3-Formyl-13^1-oxo-chlorin **5** (see Scheme 4) reacted with ethylene glycol in the presence of TMSCl to give doubly protected **12** at the 3-formyl and 13-keto groups as a main product and two mono-protected minor products, 3-formyl- and 13-keto-protected chlorins **13** and **14**. Only 3^1-acetal-13^1-ketal **12** could be separated (50%) from the reaction mixture by alumina column chromatography due to the least polarity in the reaction mixture of **5** and **12**–**14**. The other two products **13** and **14** have similar polarities as **5** and these three chlorins could not be separated from each other on an alumina column. Hydrolysis of **12** with acidic $H_2^{18}O$ gave cleanly 3^1,13^1-doubly-^{18}O-labelled methyl pyropheophorbide-*d* (**15**, see Scheme 5), which was confirmed by the FAB-MS



Scheme 3. Ketalation of methyl pheophorbide-*a* (**4**) and deprotection of the ketal.



Scheme 4. Ketalation of 3-formyl- and 3-acetyl-13^1-oxo-chlorins **5** and **6**.



Scheme 5. Synthesis of 3^1,13^1-doubly-^{18}O-labelled chlorophyll derivatives **15** and **16**.

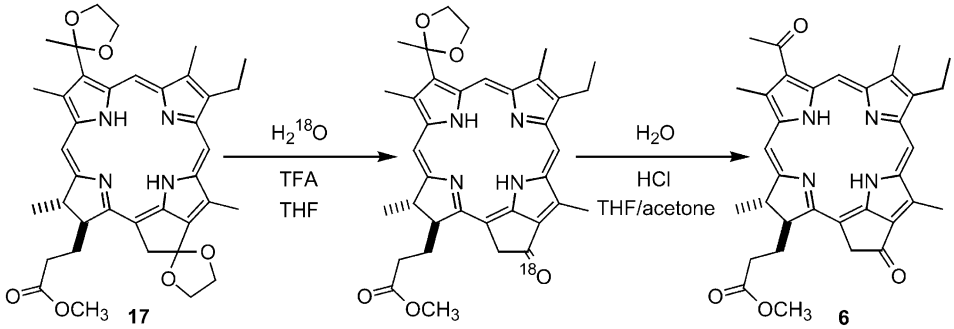
and IR spectra. Especially, IR data of **15** in CH_2Cl_2 indicate that both 3- and 13-C=O stretching vibrational bands of methyl pyropheophorbide-*d* were down-shifted to about 30 cm^{-1} by exchanging the carbonyl oxygen atom from ^{16}O to ^{18}O; 1678→1649 and 1697→1669 cm^{-1}, respectively, for **5**→**15**.

The ^{18}O-labelling degree of the product was estimated using IR and FAB-MS spectral data. The decrease of the vibrational 13-C=^{16}O peak at 1697 cm^{-1} indicated 93±1% for the 13^1-^{18}O-labelling degree. The other decrease of the 3-C=^{16}O peak at 1678 cm^{-1} could not be confirmed because the IR lights of the region were absorbed by the produced 13-C=^{18}O (1669 cm^{-1}). Simulation of the pattern of molecular ion peaks by using the observed FAB-MS of **5** and the product led to the ratio of 84±3:15±3:1 for di-^{18}O-, mono-^{18}O and unlabelled compounds in the product. A combination of ^{18}O-degrees estimated from the above IR and FAB-MS data showed that the product was a 84:6:9:1 mixture of 3^1,13^1-di-^{18}O (**15**), 3^1-mono-^{18}O, 13^1-mono-^{18}O (**8**) and unlabelled methyl pyropheophorbide-*d* (**5**). As a result, both the ^{18}O-labelling degrees of the 3-formyl and 13-keto carbonyl groups for **5**→**15** were estimated to be about 90 and 93%, respectively, which were almost identical to the value for **1**→**3**.

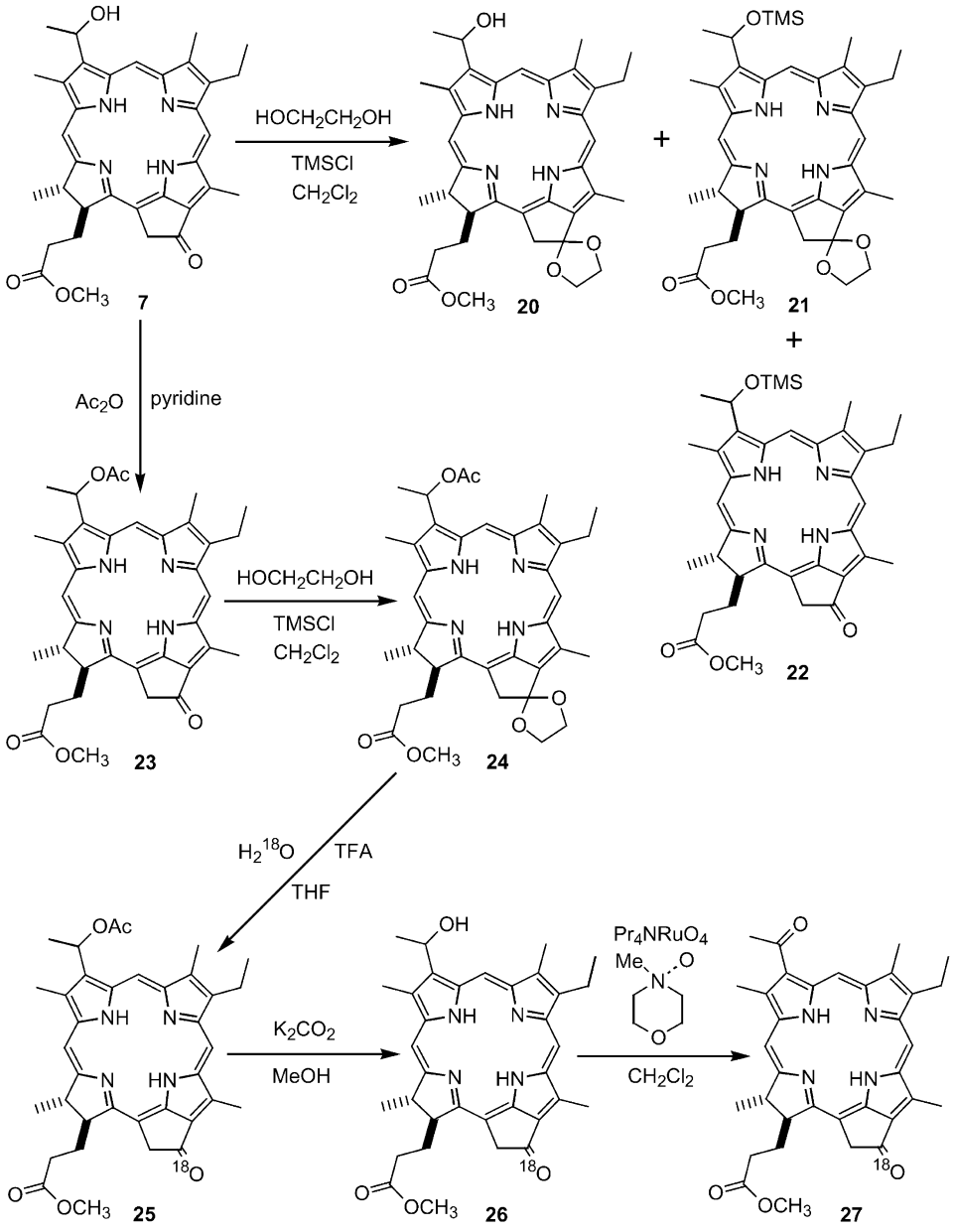
The 3-formyl group of **15** was selectively reduced by *t*-butylamine borane complex to give the 3-hydroxymethyl group. The FAB-MS spectra clearly showed the presence of double ^{18}O atoms, that is 3^1-^{18}OH and 13-C=^{18}O groups (ca. 84%), in the reduced product. The IR spectra in THF indicated that both the 13-C=^{18}O peaks (ca. 93%) of **9** (mono-^{18}O) and **16** (di-^{18}O) were situated at 1670 cm^{-1} and were shifted 30 cm^{-1} lower than the corresponding unlabelled 13-C=^{16}O (1700 cm^{-1}). The 3^1-OH stretching vibrational peak was at around 3430 cm^{-1} as a broad band and gave no clear isotopic down-shift. Considering the reported data that frequency of OH stretching vibration moved at most 10 cm^{-1} lower by ^{16}O→^{18}O,[12] the 3^1-O-H bands were so broad that an apparent shift could not be detected in the present compounds. Similar estimation from the IR and FAB-MS data gave no loss of ^{18}O-label during the above reduction.

We examined ^{18}O-labelling at the 3- and/or 13-carbonyl group(s) of methyl 3-devinyl-3-acetyl-pyropheophorbide-*a* (**6**). Similar to the protection of **5**, the reaction of **6** with ethylene glycol and TMSCl gave major 3^1,13^1-double ketal **17** as well as the other two minor 3^1-katal **18** and 13^1-ketal **19** (see Scheme 4). Only **17** could be separated and purified from the reaction mixture (vide supra) to give an analytical pure sample in 63% yield. Under the same deprotection conditions for **2**→**3**, $H_2^{18}O$-hydrolysis of **17** led to mono-cleavage at the 13^1-ketal moiety to give 3^1-ketal-13^1-^{18}O-oxo-chlorin exclusively (see Scheme 6). The selective deprotection is ascribed to the difference in the stability of both the ketal moieties. The 13^1-ketal is less stable than the 3^1-ketal because the former spiro type has more structural strain in a moiety. A greater volume of aqueous acidic solution containing a larger quantity of acid was required for deprotection of the 3^1-ketal, similar to the deprotection of **10**→**11**. Such procedures are not useful for ^{18}O-labelling because of the waste of expensive $H_2^{18}O$, so the ^{18}O-acetyl-chlorin corresponding to **6** was not prepared here.

As described above, the 13^1-^{18}O-oxo form of **7** as in **26** could not be prepared from acidic hydration of **3** (see Scheme 2). Deprotection of **19** (Scheme 4) followed by selective reduction of the 3-acetyl group should give the desired **26** but the isolation of **19** could not be achieved (vide supra). Then, cyclic ketal protection of **7** was examined (Scheme 7). Reaction of **7** with ethylene glycol and TMSCl gave the desired **20** and also the other two products **21** and **22** due to reaction of the 3^1-hydroxy group with TMSCl. On an alumina column, **20** could not be separated from starting **7**, and the isolation of **20** was extremely difficult. The 3^1-hydroxy group of **7** was thus protected by acetic anhydride in pyridine to give acetate **23**. The resulting **23** reacted with ethylene glycol and TMSCl to give 13^1-ketal **24**, which was easily purified by alumina column chromatography and recrystallization. Hydrolysis of 13^1-ketal **24** with acidic $H_2^{18}O$ provided 13^1-^{18}O-oxo-labelled **25** (93±1% ^{18}O-atom) without undesired hydrolysis of the 3^1-acetate. When ^{18}O-labelled 3^1-acetate **25** was deprotected by methanolysis under basic conditions, **26** was efficiently prepared with little loss of 13^1-^{18}O atom (92±2% ^{18}O-atom). Furthermore, the 3^1-OH of **26** was oxidized by *N*-methylmorphorine-*N*-oxide in the presence of perruthenate to give 3-acetyl-13^1-^{18}O-oxo-labelled chlorin **27** (88±2% ^{18}O-atom), which was not synthesized from 3-acetyl-13^1-oxo-chlorin **6** by the above direct ^{18}O-labelling methods because of inaccessibility of intermediate **19**.



Scheme 6. Deprotection of 3^1,13^1-doubly-ketalated chlorin **17**.



Scheme 7. Synthesis of 13^1-^{18}O-oxo-labelled methyl bacteriopheophorbide-*d* (**26**) and 3-devinyl-3-acetyl-pyropheophorbide-*a* (**27**).

Conclusion

Various regioselectively ^{18}O-labelled chlorophyll derivatives at the 3-formyl, 3-hydroxymethyl and 13-keto groups were efficiently prepared by ^{18}O-labelling methods using acidic $H_2^{18}O$-hydrolysis of the ethylene ketal and/or acetal protected chlorophylls. The ^{18}O-labelling degree was about 92% from ca. 95% ^{18}O-atom of water and the ^{18}O-label was fairly resistant to several reactions except for acidic conditions. The procedures could not be applied to 13^1-^{18}O-labelling of **4** possessing the 13^2-COOMe due to the inefficient (de)protection. The present ^{18}O-labelled chlorophyll derivatives indicate an about 30-cm^{-1} down-shift of the carbonyl stretching bands in vibrational spectroscopies including IR and RR, compared with the corresponding unlabelled chlorophylls. Such ^{18}O-labelled chlorophylls should be useful for structural elucidation of complex natural systems involving chlorophylls as in photosynthetic apparatus, through reconstruction of chlorophyll–protein systems and the preparation of model systems.

Experimental

General

Fourier-transfer IR spectra were recorded at room temperature on a Shimadzu FTIR-8600 spectrophotometer; CH_2Cl_2 and THF solutions were measured in a 0.1-mm KBr cell. RR spectra of chlorins **1** and **3** were obtained with a 3.5 cm^{-1} resolution using a JASCO-NR-1800 system. The samples were irradiated with the 457.9 nm line of an Ar^+ ion laser (Spectra-Physics Stabilite®−2017, ~20 mW). Proton NMR measurement was performed on a Bruker AC-300

FT-NMR spectrometer (300 MHz). FAB-MS were recorded on a JEOL GCmate II spectrophotometer; FAB-mass samples were dissolved in CH_2Cl_2 and *m*-nitrobenzyl alcohol was used as the matrix. CH_2Cl_2 and THF for IR measurements and ^{18}O-labelling reaction and ketalation solvents were freshly distilled over CaH_2 before use. Flash column chromatography (FCC) was carried out on silica gel (Merck Kieselgel 60, 9358). Neutral alumina (Merck; Brockmann Grade III, i.e., deactivated with 6% water) was used for gravity column chromatography.

Compounds and synthetic procedures

Unlabelled chlorophyll derivatives, such as methyl pyropheophorbide-*a* (**1**),[13] the ethylene ketal form **2**[14,15] of **1**, methyl pheophorbide-*a* (**4**),[16] methyl pyropheophorbide-*d* (**5**),[17] methyl 3-devinyl-3-hydroxymethyl-pyropheophorbide-*a*,[17] methyl 3-devinyl-3-acetyl-pyropheophorbide-*a* (**6**)[18] and methyl bacteriopheophorbide-*d* (**7**)[13] were prepared according to the literature. Protection of carbonyl group with ethylene glycol and TMSCl in dry CH_2Cl_2,[15] oxidation of the 3-vinyl to 3-formyl by $NaIO_4$ and OsO_4 in aqueous THF,[17] selective reduction of the 3-formyl to 3-hydroxymethyl group by *t*-$BuNH_3BH_3$ in dry CH_2Cl_2,[17] zinc-metallation of a free base chlorin by $Zn(OAc)_2{\cdot}2H_2O$ in MeOH and CH_2Cl_2[18] and oxidation of the 3-(1-hydroxyethyl) to 3-acetyl group by *N*-methylmorpholine-*N*-oxide and tetra-propylammonium perruthenate in dry CH_2Cl_2[18] were done according to the procedures reported by Tamiaki et al.

Determination of ^{18}O-labelling degree

^{18}O-Labelling degrees of 13^1-^{18}O-labelled products were determined by an average of four individual values estimated by using the following four different methods: (1) simulation of the pattern of molecular ion peaks from FAB-MS spectra, (2) calculation of the total molecular ion intensity from FAB-MS spectral data, (3) deconvolution peak height of 13-C=^{16}O stretching band from IR spectra in CH_2Cl_2 and (4) in THF.

Synthesis of 13^1-^{18}O-oxo-labelled methyl pyropheophorbide-*a* (3). A dry 0.08 M THF solution (1.3 mL) of TFA and $H_2^{18}O$ (0.1 mL, ca. 95% ^{18}O atom, Rotem Industries, Ltd.) was added to a dry THF solution (2.5 mL) of 13^1-ketal **2** (19.5 mg). After stirring at 35 °C under N_2 overnight, CH_2Cl_2 and an aqueous 4% $NaHCO_3$ solution were added to the reaction mixture. The organic phase was washed with distilled water (twice), dried over anhydrous Na_2SO_4, filtered and evaporated to dryness. The residue was purified by alumina column with CH_2Cl_2. The main black fraction was recrystallized from CH_2Cl_2 and MeOH to give a 8 ± 1:92 ± 1 mixture of **1** and **3** as a black solid (16.6 mg, 92% yield); ^1H NMR ($CDCl_3$) δ 9.42, 9.32, 8.54 (each 1H, s, 5-, 10-, 20-H), 7.96 (1H, dd, $J=12$, 18 Hz, 3-CH), 6.29 (1H, dd, $J=1$, 18 Hz, 3^1-CH-*trans* to 3-CH), 6.15 (1H, dd, $J=1$, 12 Hz, 3^1-CH-*cis* to 3-CH), 5.26, 5.10 (each 1H, d, $J=20$ Hz, 13^1-CH_2), 4.48 (1H, dq, $J=2$, 7 Hz, 18-H), 4.29 (1H, m, 17-H), 3.63 (2H, q, $J=8$ Hz, 8-CH_2), 3.64, 3.62, 3.40, 3.19 (each 3H, s, 2-, 7-,12-CH_3, CO_2CH_3), 2.76–2.47, 2.39–2.20 (each 2H, m, 17-CH_2CH_2), 1.82 (3H, d, $J=7$ Hz, 18-CH_3), 1.67 (3H, t, $J=8$ Hz, 8^1-CH_3), 0.41, −1.73 (each 1H, s, NH); VIS (CH_2Cl_2) λ_{max} 667 (relative intensity, 0.44), 610 (0.07), 539 (0.09), 509 (0.10), 414 (1.00), 322 nm (0.18); IR (CH_2Cl_2) 1734 (17^2-C=O), 1692 (13-C=^{16}O), 1663 (13-C=^{18}O), 1620 cm^{-1} (C=C); MS (FAB) found: *m*/*z* 550, calcd for $C_{34}H_{36}N_4O_2^{18}O$: M^+, 550.

Synthesis of 13^1-^{18}O-labelled methyl pyropheophorbide-*d* (8). According to the reported procedure,[17] oxidation of **3** gave 3-formyl-chlorin **8** (84% yield, $90\pm3\%$-^{18}O); ^1H NMR and VIS, see data of unlabelled **5** in ref 17; IR (CH_2Cl_2) 1734 (17^2-C=O), 1697 (13-C=^{16}O), 1669 (13-C=^{18}O, 3-C=^{16}O), 1616 cm^{-1} (C=C); MS (FAB) found: *m*/*z* 552, calcd for $C_{33}H_{34}N_4O_3^{18}O$: M^+, 552.

Synthesis of 13^1-^{18}O-labelled methyl 3-devinyl-3-hydroxymethyl-pyropheophorbide-*a* (9). Reduction[17] of **8** gave 3-hydroxymethyl-chlorin **9** (93% yield, $90\pm1\%$-^{18}O); ^1H NMR and VIS, see data of the corresponding unlabelled chlorin in ref 17; IR (THF) 3431 (3^1-O-H), 1741 (17^2-C=O), 1701 (13-C=^{16}O), 1670 (13-C=^{18}O), 1620 cm^{-1} (C=C); MS (FAB) found: *m*/*z* 554, calcd for $C_{33}H_{36}N_4O_3^{18}O$: M^+, 554.

Synthesis of 13^1-^{18}O-labelled zinc methyl pyropheophorbide-*a* (3-Zn). Zinc metallation[18] of **3** gave **3-Zn** (82% yield, $89\pm1\%$-^{18}O); ^1H NMR and VIS, see data of unlabelled **1-Zn** in ref 15; IR (CH_2Cl_2) 1734 (17^2-C=O), 1683 (13-C=^{16}O), 1655 (13-C=^{18}O), 1618 cm^{-1} (C=C); MS (FAB) found: *m*/*z* 612, calcd for $C_{34}H_{34}N_4O_2^{18}O^{64}Zn$: M^+, 612.

Ketalation and esterification of methyl pheophorbide-*a* (4) to 10. 13^2-Methoxycarbonyl-chlorin **4** (*a*/*a*′ = 9/1, 32.0 mg) was dissolved in dry CH_2Cl_2 (10 mL). Ethylene glycol (1.5 mL) and TMSCl (1.5 mL) were added, and the reaction mixture was stirred at room temperature for 2 days. After pouring into an aqueous 1 N NH_4OH solution (100 mL) at 0 °C, the reaction mixture was extracted with CH_2Cl_2. The combined extracts were washed by water (twice), dried over anhydrous Na_2SO_4, filtered and evaporated to dryness. The residue was purified by alumina column chromatography (CH_2Cl_2) and the main pale green fraction was recrystallized from CH_2Cl_2 and MeOH to give **10** (*a*/*a*′ = 7/1, 19.7 mg, 54% yield) as a green solid; ^1H NMR ($CDCl_3$) δ (*a*/*a*′) 9.84/80, 9.70/68, 8.89/82 (each 1H, s, 5-, 10-, 20-H), 8.21/19 (1H, dd, $J=12$, 18 Hz, 3-CH), 6.35 (1H, d, $J=18$ Hz, 3^1-CH-*trans* to 3-CH), 6.19 (1H, d, $J=12$ Hz, 3^1-CH-*cis* to 3-CH), 6.18/04 (1H, s, 13^1-CH), 4.80–4.52 (6H, m, OCH_2CH_2O, 17-, 18-H), 4.30–4.10 (2H, m, 17^2-CO_2CH_2), 3.83 (2H, q, $J=7$ Hz, 8-CH_2), 3.76, 3.61, 3.55/54, 3.39/38 (each 3H, s, 2-, 7-, 12-CH_3, 13^2-CO_2CH_3), 3.51–3.44 (2H, m, CH_2Cl), 2.76–2.13 (4H, m, 17-CH_2CH_2), 1.82 (3H, d, $J=7$ Hz, 18-CH_3), 1.75 (3H, t, $J=7$ Hz, 8^1-CH_3), −1.23/12, −3.06/−2.93 (each 1H, s, NH); VIS (CH_2Cl_2) λ_{max} 654 (rel. 0.28), 598 (0.03), 499 (0.10), 399 nm (1.00); MS (FAB) found: *m*/*z* 699, calcd for $C_{39}H_{43}N_4O_6^{35}Cl$: MH^+, 699.

Deprotection of 13^1-ketal 10 to 11. A mixture of aqueous 1 M HCl solution (2 mL) and acetone (1.5 mL) was added to a THF solution (4 mL) of **10** (12.9 mg). After stirring at 35 °C under N_2 for 3 h, the reaction mixture was worked-up similarly with synthesis of **2**→**3**, to give 2-chloroethyl pheophorbide-*a* (**11**) as a black solid (*a*/*a*′ = 6/1, 4.2 mg, 35% yield); ^{1}H NMR ($CDCl_3$) δ (*a*/*a*′) 9.53/49, 9.39/35, 8.56/50 (each 1H, s, 5-, 10-, 20-H), 8.00/7.98 (1H, dd, *J* = 12, 8 Hz, 3-CH), 6.28 (1H, dd, *J* = 1, 18 Hz, 3^1-CH-*trans* to 3-CH), 6.25/15 (1H, s, 13^1-CH), 6.19 (1H, dd, *J* = 1, 12 Hz, 3^1-CH-*cis* to 3-CH), 4.47 (1H, q, *J* = 7 Hz, 18-H), 4.32–4.11 (3H, m, 17-H, 17^2-CO_2CH_2), 3.67 (2H, q, *J* = 8 Hz, 8-CH_2), 3.87/82, 3.73, 3.41/39, 3.24/22 (each 3H, s, 2-, 7-, 12-CH_3, 13^2-CO_2CH_3), 3.50 (2H, t, *J* = 6 Hz, CH_2Cl), 2.72–2.51, 2.43–2.16 (each 2H, m, 17-CH_2CH_2), 1.82 (3H, d, *J* = 7 Hz, 18-CH_3), 1.70 (3H, t, *J* = 8 Hz, 8^1-CH_3), 0.56/67, −1.61/43 (each 1H, s, NH); VIS (CH_2Cl_2) λ_{max} 667 (rel. 0.43), 610 (0.08), 538 (0.10), 508 (0.10), 475 (0.04), 413 (1.00), 327 nm (0.22); MS (FAB) found: *m*/*z* 654, calcd for $C_{37}H_{39}N_4O_5^{35}Cl$: M^+, 654.

Synthesis of 3^1-ethylene acetal and 13^1-ethylene ketal 12 of methyl pyropheophorbide-*d* (5). Ketalation[15] of **5** gave **12** (50% yield) as the first fraction on an alumina column (CH_2Cl_2) and a mixture of undesired 3^1-acetal **13**, 13^1-ketal **14** and starting material **5** as the second yellow brown fraction. **12**; green solid (from CH_2Cl_2 and hexane); ^{1}H NMR ($CDCl_3$) δ 10.04, 9.68, 8.91 (each 1H, s, 5-, 10-, 20-H), 7.44 (1H, s, 3-CH), 5.20, 5.05 (each 1H, d, *J* = 16 Hz, 13^1-CH_2), 4.73–4.48 (9H, m, 3^1-, 13^1-OCH_2CH_2O, 18-H), 4.44–4.41 (1H, m, 17-H), 3.84 (2H, q, *J* = 7 Hz, 8-CH_2), 3.64, 3.61, 3.59, 3.40 (each 3H, s, 2-, 7-, 12-CH_3, CO_2CH_3), 2.85–2.53, 2.39–2.21 (each 2H, m, 17-CH_2CH_2), 1.83 (3H, d, *J* = 7 Hz, 18-CH_3), 1.75 (3H, t, *J* = 7 Hz, 8^1-CH_3), −1.40, −3.17 (each 1H, s, NH); VIS (CH_2Cl_2) λ_{max} 649 (rel. 0.34), 595 (0.03), 525 (0.02), 497 (0.09), 396 nm (1.00); MS (FAB) found: *m*/*z* 638, calcd for $C_{37}H_{42}N_4O_6$: M^+, 638.

Synthesis of 3^1-13^1-^{18}O-oxo-labelled methyl pyropheophorbide-*d* (15). Similarly with synthesis of **2**→**3**, deprotection of **12** (9.2 mg) gave **15** (6.0 mg, 75% yield) purified by FCC with 5–7 (v/v)% Et_2O–CH_2Cl_2 as the main red brown fraction; 3^1,13^1-^{18}O-**15** : 3^1-^{18}O/13^1-^{16}O: 3^1-^{16}O/13^1-^{18}O-**8**: 3^1,13^1-^{16}O-**5** = 84:6:9:1; black solid (from CH_2Cl_2 and hexane); ^{1}H NMR and VIS, see data of **5** in ref 17; IR (CH_2Cl_2) 1734 (17^2-C=O), 1697 (13-C=^{16}O), 1669 (13-C=^{18}O), 1649 (3-C=^{18}O), 1616 cm^{-1} (C=C); MS (FAB) found: *m*/*z* 554, calcd for $C_{33}H_{34}N_4O_2^{18}O_2$: M^+, 554.

Synthesis of 3^1,13^1-^{18}O-labelled methyl 3-devinyl-3-hydroxymethyl-pyropheophorbide-*a* (16). Reduction[17] of **15** gave **16** (90% yield); 3^1,13^1-^{18}O-**16**: 3^1-^{18}O/13^1-^{16}O: 3^1-^{16}O/13^1-^{18}O-**9**: 3^1,13^1-^{16}O = 84:6:9:1; ^{1}H NMR and VIS, see data of the corresponding unlabelled chlorin in ref 17; IR (THF) 3427 (3^1-^{18}O-H), 1741 (17^2-C=O), 1701 (13-C=^{16}O), 1670 (13-C=^{18}O), 1620 cm^{-1} (C=C); MS (FAB) found: *m*/*z* 556, calcd for $C_{33}H_{36}N_4O_2^{18}O_2$: M^+, 556.

Synthesis of 3^1,13^1-ethylene ketal 17 of methyl 3-devinyl-3-acetyl-pyropheophorbide-*a* (6). Ketalation[15] of **6** gave **17** (63% yield) as the first green fraction on alumina column chromatography (CH_2Cl_2) and a mixture of undesired 3^1-ketal **18**, 13^1-ketal **19** and starting material **6** as the second yellow brown fraction. **17**; green solid (from CH_2Cl_2 and hexane); ^{1}H NMR ($CDCl_3$) δ 10.58, 9.69, 8.88 (each 1H, s, 5-, 10-, 20-H), 5.18, 5.04 (each 1H, d, *J* = 16 Hz, 13^1-CH_2), 4.74–4.51 (5H, m, 13^1-OCH_2CH_2O, 18-H), 4.43–4.37, (3H, m, 3^1-OCH_2, 17-H), 4.13–3.98, (2H, m, 3^1-OCH_2), 3.85 (2H, q, *J* = 7 Hz, 8-CH_2), 3.67, 3.68, 3.58, 3.43 (each 3H, s, 2-, 7-, 12-CH_3, CO_2CH_3), 2.81–2.47, 2.42–2.13 (each 2H, m, 17-CH_2CH_2), 2.39 (3H, s, 3^1-CH_3), 1.82 (3H, d, *J* = 7 Hz, 18-CH_3), 1.76 (3H, t, *J* = 7 Hz, 8^1-CH_3), −1.36, −3.19 (each 1H, s, NH); VIS (CH_2Cl_2) λ_{max} 647 (rel. 0.32), 591 (0.03), 524 (0.03), 496 (0.09), 396 nm (1.00); MS (FAB) found: *m*/*z* 652, calcd for $C_{39}H_{44}N_4O_6$: M^+, 652.

Hydrolysis of 3^1,13^1-ketal 17. Similarly with synthesis of **2**→**3**, deprotection of **17** gave 3^1-ketal of 13^1-^{18}O-oxo-labelled methyl 3-acetyl-pyropheophorbide-*a* (84% yield, 88 ± 1%-^{18}O) as the main brown fraction of alumina column; brown solid (from CH_2Cl_2 and hexane); ^{1}H NMR ($CDCl_3$) δ 10.19, 9.54, 8.59 (each 1H, s, 5-, 10-, 20-H), 5.28, 5.12 (each 1H, d, *J* = 16 Hz, 13^1-CH_2), 4.49 (1H, q, *J* = 7 Hz, 18-H), 4.31 (1H, d, *J* = 9 Hz, 17-H), 4.45–4.35, 4.12–4.02 (each 2H, m, 3^1-OCH_2CH_2O), 3.72 (2H, q, *J* = 8 Hz, 8-CH_2), 3.69, 3.60, 3.52, 3.30 (each 3H, s, 2-, 7-, 12-CH_3, CO_2CH_3), 2.77–2.50, 2.40–2.18 (each 2H, m, 17-CH_2CH_2), 2.33 (3H, s, 3^1-CH_3), 1.81 (3H, d, *J* = 7 Hz, 18-CH_3), 1.71 (3H, t, *J* = 8 Hz, 8^1-CH_3), 0.33, −1.83 (each 1H, s, NH); VIS (CH_2Cl_2) λ_{max} 662 (rel. 0.50), 606 (0.08), 538 (0.10), 506 (0.09), 474 (0.04), 410 (1.00), 319 nm (0.20); IR (CH_2Cl_2) 1734 (17^2-C=O), 1696 (13-C=^{16}O), 1663 (13-C=^{18}O), 1622 cm^{-1} (C=C); MS (FAB) found: *m*/*z* 610, calcd for $C_{36}H_{40}N_4O_4^{18}O$: M^+, 610.

Similarly with synthesis of **10**→**11**, deprotection of the above 3^1-ketal afforded ^{18}O-unlabelled ketone **6** (46% yield).

Ketal-protection of methyl bacteriopheophorbide-*d* (7) to 20. According to the reported procedure,[15] reaction of **7** (9.6 mg) with ethylene glycol and TMSCl gave expected 13^1-ketal **20** and also undesired trimethyl silyl ether **22** and its ketal **21**. The reaction mixture was purified with an alumina column. 13^1-Ketal (un)protected chlorin **21** and **22** possessing the 3^1-TMSO group were eluted with CH_2Cl_2 as the first and second fractions, respectively. As the third fraction, a mixture of **7** and **20** was eluted (CH_2Cl_2) together and desired **20** could not be isolated because of their similar polarities.

Synthesis of 13^1-^{18}O-labelled methyl bacteriopheophorbide-*d* (26). 3-(1-Hydroxyethyl)-chlorin **7** (50.6 mg) was dissolved in pyridine (3 mL). Acetic anhydride (0.2 mL) was added into the pyridine solution. After stirring for 24 h at room temperature, the reaction mixture was poured into water and successively extracted with

CH_2Cl_2. The combined extracts were washed with aq 2% HCl and water (twice), dried over anhydrous Na_2SO_4, filtered and evaporated to dryness. Purification by FCC with 5–7 (v/v)% Et_2O–CH_2Cl_2 as the main black fraction and recrystallization from CH_2Cl_2 and hexane gave **23** (36.6 mg, 67% yield); black solid; ^{1}H NMR, VIS and FAB-MS spectra in ref 19; IR (CH_2Cl_2) 1736 (3^2-, 17^2-C=O), 1691 (13-C=O), 1622 cm^{-1} (C=C).

According to the reported procedure,[15] ketalation of **23** gave 3^1-acetate ester and 13^1-ethylene ketal **24** of methyl bacteriopheophorbide-*d* (65% yield) by purification with alumina column chromatography (CH_2Cl_2); green solid (from CH_2Cl_2 and hexane); ^{1}H NMR ($CDCl_3$) δ 10.03, 9.70, 8.86 (each 1H, s, 5-, 10-, 20-H), 7.46 (1H, m, 3^1-H), 5.19, 5.04 (each 1H, d, J=16 Hz, 13^1-CH_2), 4.77–4.51 (5H, m, OCH_2CH_2O, 18-H), 4.42 (1H, d, J=8 Hz, 17-H), 3.86 (2H, q, J=7 Hz, 8-CH_2), 3.65, 3.59, 3.58, 3.44 (each 3H, s, 2-, 7-, 12-CH_3, CO_2CH_3), 2.84–2.52, 2.41–2.15 (each 2H, m, 17-CH_2CH_2), 2.28 (3H, d, J=5 Hz, 3^1-CH_3), 2.27 (3H, s, 3^1-$OCOCH_3$), 1.83 (3H, d, J=7 Hz, 18-CH_3), 1.77 (3H, t, J=7 Hz, 8^1-CH_3), −1.36, −3.19 (each 1H, s, NH); VIS (CH_2Cl_2) λ_{max} 647 (rel. 0.31), 590 (0.04), 523 (0.03), 497 (0.09), 395 nm (1.00); MS (FAB) found: m/z 652, calcd for $C_{38}H_{44}N_4O_6$: M^+, 652.

Similarly with synthesis of **2**→**3**, deprotection of **24** (7.4 mg) gave **25** (6.1 mg, 88% yield, 93±1%-^{18}O); black solid (from CH_2Cl_2 and hexane); ^{1}H NMR and VIS, see data of unlabelled **23** in ref 19; IR (CH_2Cl_2) 1735 (3^2,17^2-C=O), 1695 (13-C=^{16}O), 1664 (13-C=^{18}O), 1618 cm^{-1} (C=C); MS (FAB) found: m/z 610, calcd for $C_{36}H_{40}N_4O_4^{18}O$: M^+, 610.

Potassium carbonate (71 mg) was dissolved in a methanol solution (10 mL) of **25** (9.9 mg) at 0 °C under N_2. After stirring at room temperature for 2 h, the reaction mixture was poured into water and extracted with several portions of $CHCl_3$. The combined organic layers were washed with water (twice), dried over anhydrous Na_2SO_4, filtered and evaporated to dryness. Purification by FCC with 7–12 (v/v)% Et_2O–CH_2Cl_2 and recrystallization from CH_2Cl_2 and hexane gave **26** (7.6 mg, 82% yield, 91±1%-^{18}O) as an epimeric mixture of 3^1-*R*/*S*=1/1; black solid; ^{1}H NMR and VIS, see data of unlabelled **7** in ref 13; IR (THF) 3417 (3^1-OH), 1734 (17^2-C=O), 1697 (13-C=^{16}O), 1663 (13-C=^{18}O), 1622 cm^{-1} (C=C); MS (FAB) found: m/z 568, calcd for $C_{34}H_{38}N_4O_3^{18}O$: M^+, 568.

Synthesis of 13^1-^{18}O-labelled methyl 3-devinyl-3-acetylpyropheophorbide-*a* (27). Oxidation[18] of **26** gave **27** (95% yield, 88±2%-^{18}O); ^{1}H NMR and VIS, see data of unlabelled **6** in ref 18; IR (CH_2Cl_2) 1734 (17^2-C=O), 1697 (13-C=^{16}O), 1666 (13-C=^{18}O, 3-C=^{16}O), 1618 cm^{-1} (C=C); MS (FAB) found: m/z 566, calcd for $C_{34}H_{36}N_4O_3^{18}O$: M^+, 566.

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