Synthesis of Novel Acceptor Substrates for the Dolichyl Phosphate Mannose Synthase from Yeast

Ines Sprung, Laurence Carmès, Gregory M. Watt, and Sabine L. Flitsch*[a]

Dolichols are polyisoprenoid lipid components of mammalian membranes consisting of an average of 20 head-to-tail linked isoprene units of which the first isoprene is fully saturated. The unusual size of these lipids is intriguing and poses questions about the role of dolichol structure in biological processes. In order to probe structure and function we have synthesised potential dolichyl analogues that retain only the first two isoprene units and carry a second functional group within the terminal lipid chain. Such analogues were evaluated as substrates for a key enzyme in the dolichyl-dependent pathway of glycan biosynthesis, dolichyl phosphate mannose (Dol-P-Man) synthase. It was shown that some functional groups, including labels such as biotin, could be tolerated. When the synthetic analogues were attached to a solid support they were still substrates for the Dol-P-Man system and thus allowed the enzymatic solid-phase synthesis of glycolipids.

KEYWORDS:

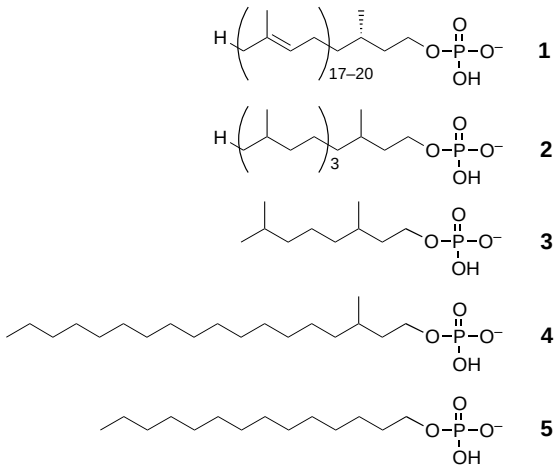
Dol-P-Man synthase · enzymatic synthesis · glycoproteins · lipid phosphates · oligosaccharides

Introduction

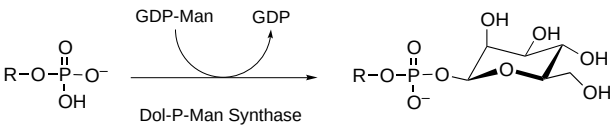
Lipids bearing polyisoprenoid side chains are ubiquitous membrane constituents that play an important role in glycan biosynthesis.[1] Glycosylated phosphoisoprenoids such as dolichyl phosphate mannose (Dol-P-Man) and undecaprenyl diphosphate *N*-acetylglucosamine can act as glycosyl donor substrates in a similar manner to sugar nucleotides. Glycosylated phosphopolyisoprenoid lipids can also serve as carriers of oligosaccharide units in biosynthetic pathways of glycopolymers such as glycoproteins of higher organisms and cell-surface-associated polysaccharides of bacteria.[1] The unusual length of polyisoprenoids such as dolichol is intriguing and has led to proposals that dolichyl-linked intermediates are specifically recognised through dolichyl-binding sites in biosynthetic glycan-processing enzymes.[2]

A difficulty in addressing the specific role of polyisoprenoid structure in biological processes has been the lack of pure lipid for biochemical studies. Polyisoprenoids are not very abundant in natural sources and their chemical synthesis is lengthy. We and others have therefore looked for more accessible lipids than dolichyl phosphate (**1**) that would still be accepted by glycan-processing enzymes and have reported a number of such lipids, in particular phytanyl phosphate (**2**; Scheme 1).

Our choice of enzyme for such studies of substrate analogues has been Dol-P-Man synthase (Scheme 2). Located in endoplasmic reticulum membrane in eucaryotes it catalyses the mannosyl transfer from guanosine diphosphate – mannose (GDP – Man) to the membrane-located lipid dolichyl phosphate (**1**). Dol-P-Man is the essential cofactor in several biosynthetic pathways, including those of glycoproteins (*N*- and *O*-glycan) and glycosyl phosphatidylinositol anchors.[3] Thus, Dol-P-Man synthase catalyses a key



Scheme 1. *Lipid phosphates tested as acceptor substrates for Dol-P-Man synthase.*



Scheme 2. *Mannosyltransferase reaction of Dol-P-Man synthase.*

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reaction in glycan biosynthesis. This is the first reason for choosing this enzyme. Secondly, the enzyme should be fairly sensitive to lipid structure, since the lipid substituent is close to the site of modification in the substrate. One might assume that for reactions later in the pathway, where an outer carbohydrate residue that is quite removed from the lipid is glycosylated by the enzyme, sensitivity towards lipid structure is less pronounced. Thirdly, the lipid substrates for the Dol-P-Man synthase are chemically more accessible than those of more complex glycolipid intermediates, and fourthly, the chemical reaction that is catalysed by this enzyme, the β-mannosylation of a phosphate (Scheme 2), is chemically one of the most challenging linkages to make.[4]

Phytanyl phosphate (**2**) was previously shown to be a reasonable substrate for the Dol-P-Man synthase and could be used with about 60 – 70% efficiency compared to the natural substrate dolichyl phosphate (**1**). Phytanyl phosphate (**2**) analogues were also successfully used as substrates for the β-1,4-mannosyltransferase from yeast[5, 6] and the α-1,3-mannosyltransferase AceA from *Acetobacter xylinum*.[7] In all cases the soluble substrate analogue without the polyisoprenoid was not a substrate for the enzyme, and it appears that there is some minimal requirement for the lipid.

One drawback of using phytanyl derivatives as substrate analogues has been that the saturated hydrocarbon chain is not amenable to further selective functionalisation or modification. We have therefore looked at other lipid analogues bearing functional groups that could be used to probe lipid/protein interactions or could act as attachment sites for labels,[8, 9] to solid supports[10] and in microarrays.[11] So far, fluorescent labels have been introduced into dolichol itself[9] but the low availability of dolichol might limit such an approach. The focus of the present studies was therefore the design and synthesis of lipid analogues that would be easily accessible.[12]

Results and Discussion

Lipid design

In addition to phytanyl phosphate (**2**) we have previously reported the synthesis of dihydrocitronellyl phosphate (**3**), 3-methyloctadecanyl phosphate (**4**) and tetradecanyl phosphate (**5**) and their evaluation against the Dol-P-Man synthase from yeast.[5] Conclusions of these studies were that the 3-methyl group is necessary for substrate recognition (since **5** was not a substrate) and that a minimum chain length was required (since **3** was not a substrate). The stereochemical preference at position 3 had been shown previously to be only small.[13]

Based on these previous studies, the four phosphate lipids **18**, **29**, **31** and **35** (Schemes 3 – 5) were chosen as targets for the present investigation because they retain the diisoprenoid unit close to the phosphate ester, which appeared to be most important for enzyme binding.[5] By first testing **18**, we would establish that a functional group (the imide) was tolerated by the enzyme system. Furthermore, we envisaged **18** to be a starting material for further functionalisation with labels such as biotin (compounds **38** and **41**; Scheme 6) and for final attachement to solid support (**45**; Scheme 7). Compounds **29**, **31** and **35** were designed to test if functionality was tolerated close to the diisoprenyl group. Such compounds would also be more accessible to synthesis.

Chemical synthesis of acceptor analogue 18

The key intermediate for target **18** was protected diol **12**. This was accessible by a published route by selenium dioxide/*tert*-butylperoxide allylic oxidation of **10**, which had been obtained from citronellol (**9**; Scheme 3). The oxidation yielded a mixture of the conjugated aldehyde **11** and the allyl alcohol **12**, in poor yield (12 and 27%). Subsequent reduction of **11** allowed us to obtain **12** in an overall yield of 31%.[14] The conversion of **12** into the bromide **13** was accomplished via the mesylate intermediate with lithium bromide, and subsequent treatment with triphenylphosphine yielded the phosphonium salt **14** in 97% yield.

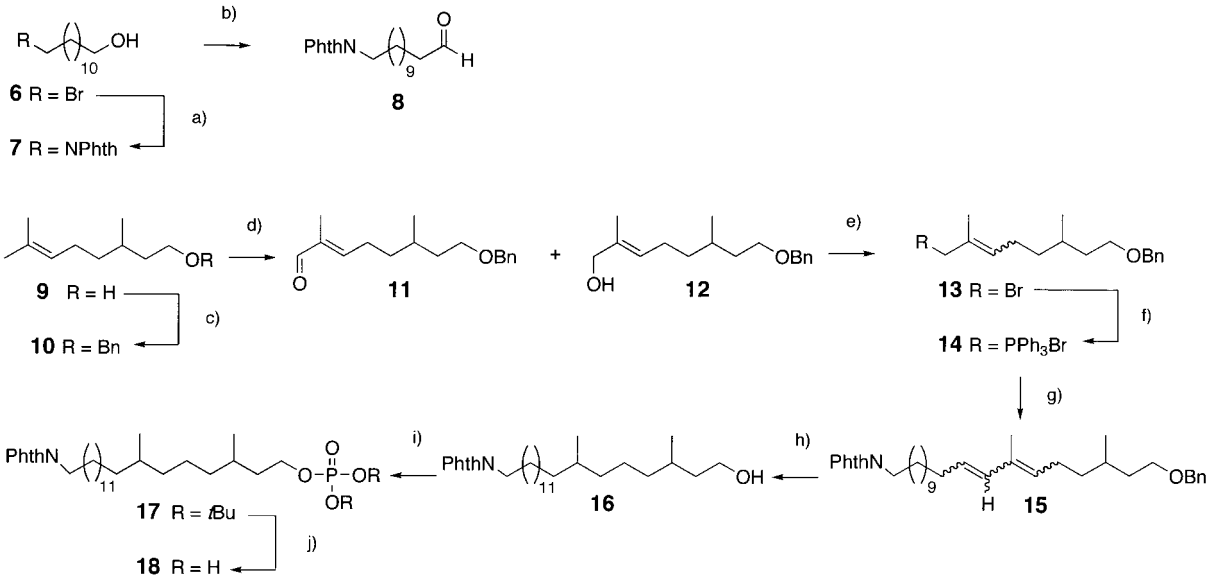
Aldehyde **8** was accessible from commercially available bromododecanol (**6**) by treatment with potassium phthalimide to give the alcohol **7**. The oxidation of the alcohol group with tetrapropylammonium perruthenate and 4-methylmorpholine *N*-oxide yielded the aldehyde **8** in 44% yield. Since the workup is fairly simple and the oxidation gave an acceptable yield it was preferred to the dimethyl sulfoxide oxidation.

The Wittig reaction of aldehyde **8** and phosphonium salt **14** delivered a 1:1 *Z*/*E* isomeric mixture of the unsaturated compound **15** in 52% yield. The subsequent hydrogenation of **15** resulted in saturation of the carbon chain as well as in the deprotection of the alcohol group to give the alcohol **16**. Phosphorylation was achieved with di-*tert*-butyl-diisopropyl-phosphoramidite followed by oxidation with *meta*-chloroperoxybenzoic acid to obtain the phospholipid **17**, and final deprotection of **17** with trifluoroacetic acid delivered the first of our potential substrates **18** for Dol-P-Man synthase.

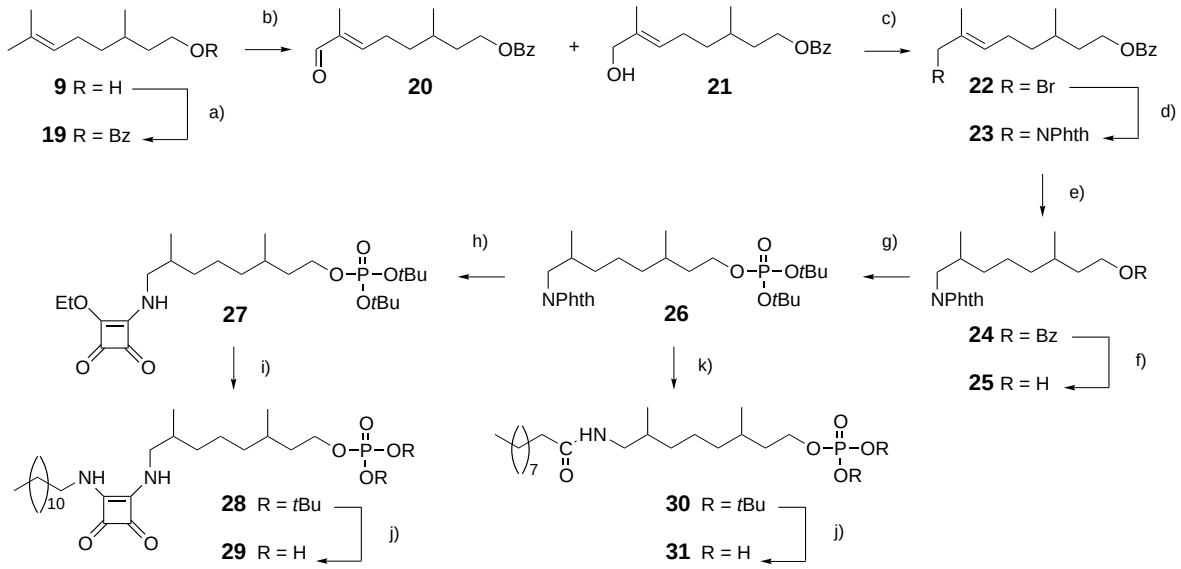
Chemical synthesis of acceptor analogues 29, 31 and 35

Squaric acid diamides were introduced by Tietze and coworkers[15] as very convenient linkers for biomolecules. Two amine moieties can be coupled unsymmetrically with high efficiency in organic or aqueous systems. Thus, the squarate has already been used as a conjugating reagent in neoglycoconjugate synthesis[16] and in enzymatic oligosaccharide synthesis.[17] We therefore chose squarate as a linker and designed target **29** as a potential acceptor analogue to dolichyl and phytanyl phosphates.

The synthesis of the lipid phosphate **29** from citronellol (**9**) is outlined in Scheme 4. Citronellol (**9**) was benzoylated to give **19** in quantitative yield. Treatment of the olefin **19** with selenium dioxide resulted in a mixture of the aldehyde **20** (10%) and the alcohol **21** (9%). In order to increase the amount of desired alcohol **21**, the aldehyde **20** was reduced by $NaBH_4$ to afford **21** in an overall yield of 18%. The allylic bromide **22** was readily prepared from **21** via the mesylate intermediate. Subsequent treatment of **22** with potassium phthalimide resulted in **23** which upon deprotection with hydrazine would yield the primary amine.[18] Reduction of **23** by hydrogenation catalysed by palladium on charcoal afforded **24** in 90% yield.



Scheme 3. *Synthesis of lipid phosphate* ***18****: a) Potassium phthalimide, DMF, 105 °C, 16 h, 89%; b) TPAP, NMO, molecular sieves, CH_2Cl_2, RT, 16 h, 44%; c) NaH, THF, reflux, 2 h; benzyl bromide, tetrabutylammonium iodide, THF, RT, 18 h, 58%; d) SeO_2, tBuOOH, CH_2Cl_2, H_2O, RT, 16 h, 12% of* ***11****, 27% of* ***12****; $NaBH_4$, H_2O, CH_2Cl_2/EtOH (4:1), RT, 16 h, 33% of* ***12****; e) $MeSO_2Cl$, NEt_3, CH_2Cl_2, −50 °C, 1 h; LiBr, THF, −50 → −30 °C, 2 h, 71%; f) triphenylphosphine, toluene, RT, dark, 6 days, 97%; g) LDA, THF, RT, 45 min,* ***8****, THF, RT, 16 h, 52%; h) H_2, Pd/C, acetone/MeOH (1:1), RT, 16 h, 69%; i) di-tert-butyl diisopropylphosphoramidite, 1H-tetrazole, THF, RT, 2 h; MCPBA, CH_2Cl_2, RT, 16 h, 73%; j) TFA, RT, 10 min. Phth = phthaloyl, Bn = benzyl, DMF =* N,N*-dimethylformamide, TPAP = tetrapropylammonium perruthenate, NMO = 4-methylmorpholine* N*-oxide, THF = tetrahydrofuran, LDA = lithium diisopropylamide, MCPBA =* meta*-chloroperoxybenzoic acid, TFA = trifluoroacetic acid.*



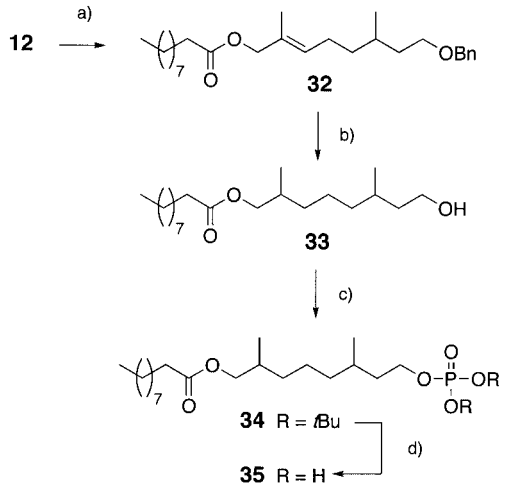
Scheme 4. *Synthesis of lipid phosphates* ***29*** *and* ***31****: a) PhCOCl, pyridine, RT, 18 h, 100%; b) SeO_2, tBuOOH, CH_2Cl_2, H_2O, RT, 18 h, 10% of* ***20****, 9% of* ***21****; $NaBH_4$, H_2O, CH_2Cl_2/EtOH (4:1), RT, 16 h, 100% of* ***21****; c) $MeSO_2Cl$, NEt_3, CH_2Cl_2, −50 °C, 1.5 h; LiBr, THF, −50 → −30 °C, 2.5 h, 75%; d) potassium phthalimide, DMF, 105 °C, 16 h, 80%; e) H_2, Pd/C, MeOH, 18 h, 90%; f) MeONa, MeOH, RT, 20 h, 43%; g) di-tert-butyl diisopropylphosphoramidite, 1H-tetrazole, THF, RT, 1 h; MCPBA, CH_2Cl_2, 0 °C, 1 h, 54%; h) $N_2H_4 \cdot H_2O$, EtOH, RT, 16 h; 3,4-diethoxy-3-cyclobutene-1,2-dione, EtOH, RT, 5 min, 60%; i) $CH_3(CH_2)_{11}NH_2$, EtOH, RT, 5 min, 65%; j) TFA, RT, 10 min, 80% of* ***29****, 88% of* ***31****; k) $N_2H_4 \cdot H_2O$, EtOH, RT, 16 h; decanoyl chloride, Et_3N, THF, RT, 16 h, 61%. Bz = benzoyl.*

The deprotection of the benzoic acid ester **24** with NaOMe proceeded with unexpectedly low yield (43%). Analysis of the main side product revealed that the phthalimide had reacted as well. Perhaps unexpectedly, the acid was formed rather than the ester, which has precedent.[19] Attempts to reclose the phthalimide ring by using different reagents such as 1,1′-carbonyldiimidazole,[20] 2-ethoxy-*N*-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ) or *N*,*N*′-dicyclohexylcarbidiimide (DCC)[21] did not lead to better yields of reaction. Since enough material was available for further synthesis, the reaction sequence was continued at this stage. However, in subsequent work concerned with the synthesis of **35** the benzyl ether was found to be more appropriate and to cause less problems.

The phosphorylation/oxidation step of alcohol **25** following the previously described method led to the phosphate ester **26**.[22] The deprotection of the amine in **26** was achieved by hydrazine hydrate, to give the crude amino compound, which was used without further purification.[23] The amine was then coupled with 3,4-diethoxy-3-cyclobutene-1,2-dione and gave the corresponding squaric acid amide **27**.[15, 24] Under these conditions only the formation of the monoamide **27** was observed and not the synthesis of the symmetrical squaric acid diamide. The reaction of **27** with dodecylamine led to the unsymmetrical squaric acid diamide **28**. Deprotection of the phosphate ester **28** with trifluoroacetic acid (TFA) finally delivered the target substrate **29**.

For comparison with **29** the lipid phosphate **31** containing an amide function was also synthesised. This could easily be achieved by reaction of phthalimide **26** with hydrazine hydrate and by treating the crude product with decanoyl chloride to afford amide **30**, which was deprotected with TFA to give lipid phosphate **31**.

Since amides are known to be much better hydrogen-bond acceptors than esters[25] the ester analogue **35** was also synthesised (Scheme 5). Given the problems discussed before



Scheme 5. *Synthesis of lipid phosphate **35**: a) Decanoyl chloride, pyridine, 0 °C→RT, 18 h, 72%; b) H_2, Pd/C, MeOH, RT, 3 days, 31%; c) di-tert-butyl diisopropylphosphoramidite, 1H-tetrazole, THF, RT, 1 h; MCPBA, CH_2Cl_2, 0 °C, 18 h, 36%; d) TFA, RT, 10 min, 85%.*

for **25**, benzyl ether protection of the terminal alcohol group was chosen. The reaction of alcohol **12** with decanoyl chloride afforded ester **32**. Hydrogenation of **32** led to reduction of the double bond and to the deprotection of the alcohol group in one step to give ester **33**. By phosphorylation/oxidation of **33** and subsequent deprotection of **34** with TFA, **35** was obtained.

Assay of acceptor substrates for Dol-P-Man synthase

Compounds **18**, **29**, **31** and **35** were tested as substrates by incubation with crude microsomal fraction of *Saccharomyces cerevisiae* and radiolabelled ^{14}C-GDP–mannose as reported before.[5] The relative level of transfer of the radiolabelled mannose in the lipid, and thus its suitability as a substrate, was determined by comparison with phytanyl phosphate (**2**), which shows 60–70% of incorporation of the natural substrate, dolichyl phosphate (**1**).

The results, as shown in Table 1, show that compound **18** with 62% of incorporation appears to be a good substrate for Dol-P-Man synthase. The conclusion is that polar groups at the ω-position in **18** can be tolerated.

Table 1. *Incorporation of mannose into acceptor substrates **18**, **29**, **31** and **35** relative to phytanyl phosphate (**2**).*

Substrate	Radioactivity [%][a]
2	100
18	62 ± 16
29	3 ± 1
31	3 ± 1
35	61 ± 10
no substrate	1 ± 0

[a] Relative to phytanyl phosphate (**2**).

When the functional group was introduced closer to the phosphate headgroup (position C-8 rather than C-20) tolerance depended very much on the type of functionality. Compounds **29** and **31** bearing squarate and amide functions at C-8 show no activity as substrates. On the other hand, with an ester at position C-8 (compound **35**) the activity is equivalent to that of **18**.

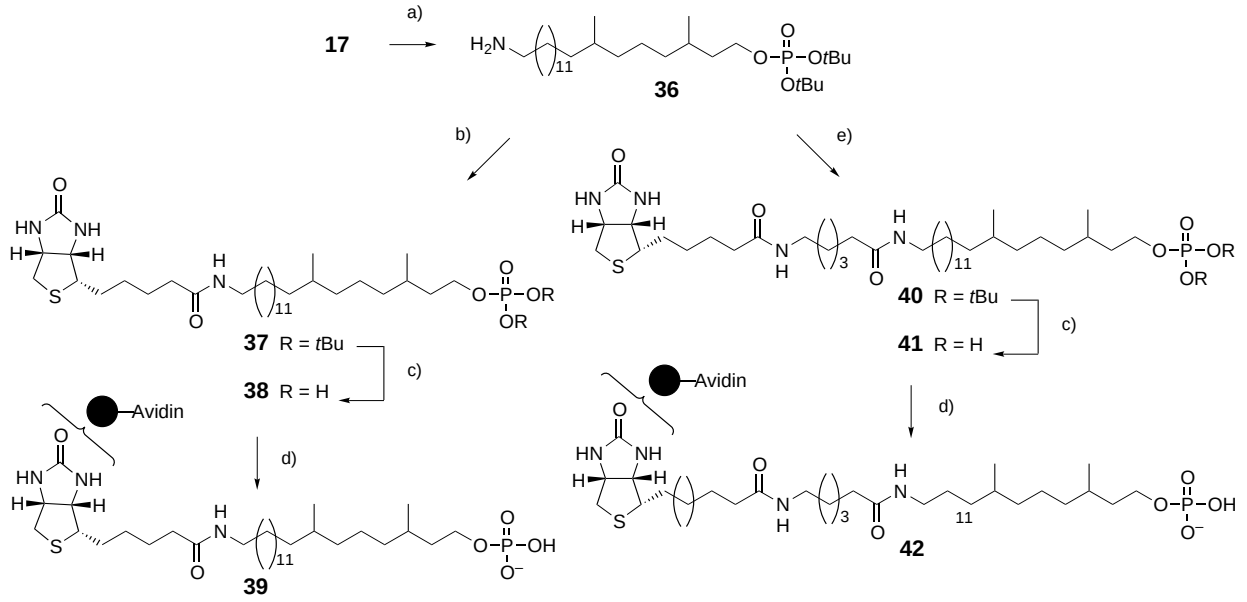
Compounds **29** and **31** were also tested as potential inhibitors by looking at activity of the Dol-P-Man synthase towards phytanyl phosphate (**2**) in the presence and absence of **29** and **31** (data not shown). No inhibitory effect was observed.

Chemical synthesis of biotinylated acceptor analogues 38 and 41

The biological data from phthalimide **18** were very encouraging and suggested that other functional groups, in particular labels for biological work, would be tolerated. Indeed, the phthalimide group had been chosen in the first instance because deprotection would generate an amine that could easily be derivatised.

Thus, **17** was deprotected by treatment with hydrazine hydrate, and the resulting amine **36** was used in further reactions without purification because of its incompatibility with standard chromatographic methods.

Biotin forms a very tight complex with the protein avidin and is therefore commonly used in biological assays as a label.[26] Thus, amine **36** was derivatised by reaction with biotinyl-*N*-hydroxysuccinimide ester to give **37**, which upon deprotection with TFA provided phosphate **38**. Compound **41** with an additional spacer group was made from **40** in a similar way (Scheme 6).



Scheme 6. *Synthesis of biotinyl compounds **38** and **41** and biotin – avidin conjugates **39** and **42**: a) $N_2H_4 \cdot H_2O$, EtOH, RT, 14 h; b)* N*-hydroxysuccinimidobiotin, DMF, RT, 16 h, 63%; c) TFA, RT, 10 min; d) monomeric avidin – agarose, buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, 10 mM mercaptoethanol, 0.5% Triton-X-100 (v/v), pH 7.5); e) biotinyl-*N*-ε-aminocaproyl-*N*-hydroxysuccinimide ester, DMF, RT, 16 h, 52%. Tris = tris(hydroxymethyl)aminomethane.*

Assay of biotinylated substrates 38 and 41 with Dol-P-Man synthase

The results of the enzymatic assay for **38** and **41** are shown in Table 2. Both biotinylated compounds **38** and **41** were only modest substrates for Dol-P-Man synthase with relative incorporations of 12% and 11% respectively compared to phytanyl phosphate (**2**). Nevertheless they were clearly accepted by the enzyme, which prompted us to continue with looking at the enzymatic reactions on solid phase after binding to immobilised avidin. The biotinyl lipids **38** and **41** were linked to monomeric avidin attached to agarose by incubation at 37 °C followed by washing of the resin to remove all unlinked substrate. These immobilised substrate preparations **39** and **42** (Scheme 6) were then subjected to assay conditions as before. Incorporation of radiolabelled mannose from GDP – mannose into the immobilised substrate was determined in two ways: firstly by washing the resin thoroughly and then directly subjecting the resin to scintillation counting (Table 3, first two entries). Activity could clearly be seen for both substrates (**38** and **41**) compared to the control (avidin only, entry 3). Secondly, the product was specifically eluted by treatment with biotin resulting in entries 4 – 6.

Table 2. *Incorporation of mannose into acceptor substrates **38** and **41** relative to phytanyl phosphate (**2**).*

Substrate	Radioactivity [%][a]
2	100
38	12 ± 3
41	11 ± 6
no substrate	2 ± 1

[a] Relative to phytanyl phosphate (**2**).

Table 3. *Incorporation of mannose into acceptor substrates **38** and **41** linked to avidin – agarose.*

	Radioactivity [%][a]
avidin – biotinyl product of **38**	100
avidin – biotinyl product of **41**	83 ± 12
no substrate	18 ± 9
cleaved biotinyl product of **38**	44 ± 12
cleaved biotinyl product of **41**	41 ± 8
no substrate	8 ± 5

[a] Relative to avidin – biotinyl product of **38**.

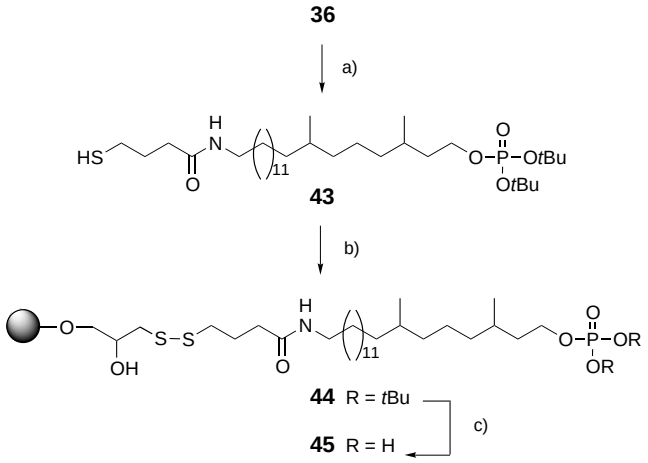
Again, clear incorporation above the control could be seen. However, the yield of eluted product appeared to be only 41 – 44%.

The poor recovery could be due to the inefficient elution of biotinylated product with biotin. As recommended,[27] further cleavage of biotinyl compounds with a 0.1 M glycine solution at pH 2 was therefore attempted but did not yield any further radiolabelled eluent.

Chemical synthesis of thiol 45

The results with immobilised biotinylated substrates encouraged us to look at covalently (rather than noncovalently) immobilised lipids. It was envisaged that, with a suitable choice of linker, the cleavage of the lipid from solid support could be more efficient. Therefore, in an alternative approach to the biotin – avidin concept, a previously reported disulfide link was chosen to attach the lipid phosphate to the solid support.[17a] The disulfide link not only allows a straightforward attachment but also a mild way of cleavage of the enzymatic product.[28]

The thiol group was conveniently introduced by reacting **36** with γ-thiobutyrolactone (Scheme 7). The reaction of **43** with activated thiopropyl sepharose 6B led to immobilised **44**, which still contained the phosphate protecting groups. The loading of the resin was estimated by determining the concentration of remaining **43** in the filtrate to be about 10 μmol mL^{-1} (33 – 56% of available functional groups on resin, as stated by the manufacturer). Deprotection of the phosphate group in **44** with TFA yielded immobilised phospholipid **45**.



Scheme 7. *Synthesis of thiopropyl sepharose conjugate **45**: a) γ-Thiobutyrolactone, 0.5 M $NaHCO_3$, dithiothreitol, EtOH, N_2, 50 °C, 6 h, 22%; b) thiopropyl sepharose 6B, EtOH/H_2O (2:1), 33 – 56%; c) TFA, RT, 10 min.*

Enzymatic mannosylation of 45 on the solid phase

Compound **45** was incubated as before with crude microsomal Dol-P-Man synthase and a twofold excess of radiolabelled GDP – mannose. Transfer of radiolabels (that is, formation of **46**; Scheme 8) was again measured by counting radioactivity directly on the solid support (Table 4, first entry) and after cleavage of the lipid with thioethanol. The latter should yield a mixture of thiols **47** – **50**. The data clearly showed that mannosyl transfer onto the immobilised lipid has occurred (Table 4, entries 1 and 2). However, the cleavage yield was still only 50%, with the other half remaining on the support after thioethanol treatment (Table 4, entry 3).

Table 4. *Incorporation of mannose into thiopropyl sepharose conjugate **45** and cleavage of products **47** and **48** from resin.*

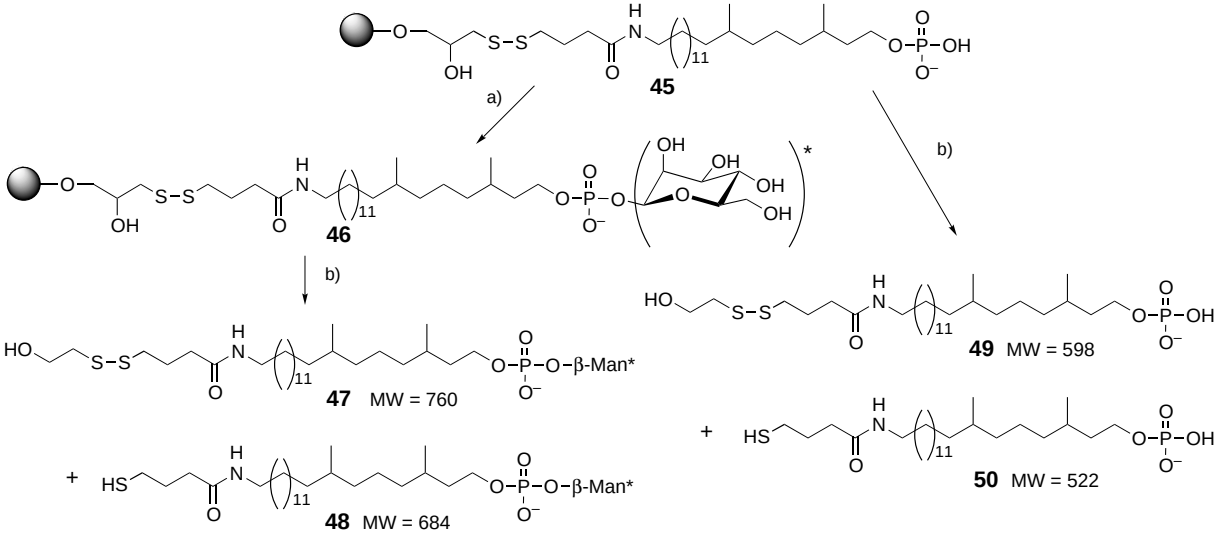
	Radioactivity [%][a]
46	100
cleaved products **47** and **48**	50 ± 5
resin after cleavage	38 ± 20

[a] Relative to **46**.

To obtain further confirmation that mannosyltransfer to the solid-supported lipid substrate had indeed been catalysed by the crude Dol-P-Man synthase, a more detailed analysis was carried out on the incubation products of **45** by liquid chromatography coupled with mass spectrometry (LC – MS). The products were released from the solid support with thioethanol and subjected to LC – MS (Figure 1). The mannosylated products **47** and **48** (molecular weights (MW): 761 and 685) were clearly visible next to lipid precursors **49** and **50** (MW: 599 and 523).

Conclusion

The aim of the present study was to find bifunctional lipid substrates for the Dol-P-Man synthase. A number of novel lipid phosphates (**18**, **29**, **31** and **35**) were synthesised and tested against crude preparation of the enzyme.



Scheme 8. *Enzymatic assay of **45** for Dol-P-Man synthase: a) Dol-P-Man synthase (yeast microsomes), GDP – mannose, GDP – [U-^{14}C]mannose, buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, 0.5% Triton-X-100 (v/v), pH 7.5), 37 °C, 21 h; b) mercaptoethanol (aq), 50 °C, 16 h.*

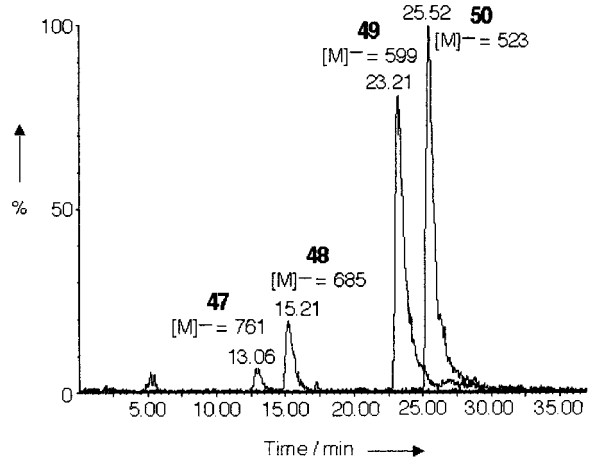


Figure 1. *LC–MS analysis for masses 523, 599, 685 and 761 after enzymatic reaction on* ***45*** *followed by cleavage of products and starting materials from the solid support.*

The tolerance for second functional groups in the lipid substrate was found to be dependent on the nature of the group and the distance from the phosphate headgroup. Thus, in the C-8 position of the lipid an ester was tolerated (as in **35**) but an amide or squarate group was not (**31** and **29**, respectively). One might speculate that this effect is due to the more hydrophobic nature of the ester moiety and to the higher propensity for hydrogen bonding in **31** and **29**. However, when further removed from the phosphate headgroup (as in **18**, **38** and **41**) imide and amide groups were tolerated.

We have also shown that the enzyme can act on a solid support (as in substrate **45**) although the yields need to be improved if this methodology is to be used in solid-phase synthesis.

These results will provide us with useful tools for the study of polyisoprenoid-dependent glycosyltransferases. Labelled substrates such as **38** and **41** can be used in fast enzyme assays that will help us to find and characterise novel glycosyltransferases and develop inhibitors. The results on the solid support indicate that affinity-based assays and purification methods are possible. Finally, these results open up the possibility of solid-phase synthesis of saccharides with either purified or (semi)crude enzyme preparations.

Experimental Section

General: All chemicals were purchased from Sigma–Aldrich or Acros Organics. Solvents and reagents were used as supplied except tetrahydrofuran (THF) and CH_2Cl_2, which were freshly distilled under anhydrous conditions. Radiolabelled GDP-[U-^{14}C]mannose (305 mCi mmol^{-1}) was purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK); Ultima Gold scintillation fluid was from Packard Bioscience (Meriden, USA). ^{1}H and ^{13}C NMR analysis were recorded with a Bruker AC 250, a Varian Gemini 200 or a Bruker WH 360 instrument. Chemical shifts (δ) are reported in parts per million (ppm) downfield from Me_4Si. Coupling constants (J) are reported in Hertz (Hz). IR spectra were recorded as films on sodium chloride plates on a Perkin Elmer Paragon 1000 FT-IR spectrophotometer, only noteworthy absorptions are listed. High-resolution mass spectra (HRMS) were recorded on a Kratos MS50TS instrument and measured by fast atomic bombardment (FAB); the masses are quoted in Daltons. LC–MS was done on a Water Alliance 2790 LC with Micromass Platform II mass spectrometer operated in electrospray + ve/-ve mode.

Flash column chromatography was carried out using either the appropriate sized parallel-sided column filled with silica gel 60 (35–70 μm, Fisher) or by using a FLASH12i chromatography system with prepacked cartridges from Biotage (Hertford, UK). Analytical thin layer chromatography (TLC) was carried out on aluminium-backed plates coated with SiO_2 (silica gel 60 F_{254}, Merck). Components were detected by UV (254 nm) and/or visualised with ammonium molybdate reagent.

The fermentation of yeast was carried out in a 5.0 L Benchtop Fermenter Bioflo 3000 from New Brunswick Scientific (Hatfield, UK). For preparation of microsomes a French press, 'Z Plus' 1.1 KW Benchtop Cell Disrupter, from Constant Systems Ltd. (Northamptonshire, UK) was used. Enzyme assays were carried out in a PierceTherm heating/stirring module from Pierce and Warriner (Chester, UK). The radioactivity was measured by a liquid scintillation analyzer, Tri-Carb 2100TR, from Packard (Pangbourne, UK).

Phytanyl phosphate (**2**) was prepared as described previously.[6d]

12-*N*-Phthalimidododecanol (7): Potassium phthalimide (7.04 g, 38 mmol) was stirred in anhydrous *N*,*N*-dimethylformamide (DMF; 80 mL), and a solution of 12-bromo-1-dodecanol (**6**; 10.00 g, 38 mmol) in anhydrous DMF (80 mL) was added by syringe. The reaction mixture was stirred at 105 °C for 16 h. It was then cooled to room temperature and diluted with water (100 mL) while the colour changed from brown to white. The aqueous layer was extracted with CH_2Cl_2 (3 × 100 mL); the combined organic layers were dried over $MgSO_4$ and concentrated under reduced pressure to obtain a yellow oil which crystallised after some time. The residue was purified by flash column chromatography (petroleum ether/ethyl acetate, 2:1) to obtain **7** as a white solid (11.19 g, 89%). M.p.: 76 °C; ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 1.15$ (m, 19H; CH_2, OH), 1.55 (m, 2H; CH_2CH_2OH), 3.65 (m, 4H; CH_2O, CH_2N), 7.55, 7.85 (m, 4H, Phth) ppm; ^{13}C NMR (63 MHz, $CDCl_3$): $\delta = 25.6$, 26.7, 28.4, 28.9, 29.3, 29.3, 32.6, 37.9 (11 × CH_2), 62.9 (CH_2O), 122.9, 133.7 (4 × CH Phth), 131.9 (2 × C Phth), 168.3 (2 × CON) ppm; IR (Nujol): $\tilde{\nu} = 3550$ (O–H), 1700 (C=O), 1500 (C=C) cm^{-1} ppm; HRMS: calcd. for $C_{20}H_{30}NO_3$ $[M+H]^+$ *m/z*: 332.2226; found: 332.2226.

12-*N*-Phthalimidododecanal (8): Tetrapropylammonium perruthenate (TPAP; 0.60 g, 1.7 mmol) was added to a stirred mixture of **7** (11.19 g, 33.8 mmol), 4-methylmorpholine *N*-oxide (NMO; 6.85 g, 50.6 mmol) and molecular sieves 4 Å (16.80 g) in anhydrous CH_2Cl_2 (110 mL). The reaction mixture was stirred for 16 h and the same amounts of TPAP, NMO and molecular sieves were added again to complete the reaction. The reaction mixture was filtered through celite and washed thoroughly with CH_2Cl_2. The solution containing TPAP was concentrated under reduced pressure to obtain a thick black oil. The crude material was purified by flash column chromatography (petroleum ether/ethyl acetate, 7:1) to obtain **8** as a white solid (4.85 g, 44%). M.p. 72 °C; ^{1}H NMR (200 MHz, $CDCl_3$): $\delta =$ 1.3 (m, 14H; CH_2), 1.65 (m, 4H; CH_2), 2.40 (m, 2H; CH_2CHO), 3.65 (t, ^3J(H,H) = 7.2 Hz, 2H; CH_2N), 7.65, 7.85 (m, 4H; Phth), 9.75 (s, 1H; CHO) ppm; ^{13}C NMR (63 MHz, $CDCl_3$): $\delta = 21.9$, 26.7, 28.5, 29.2, 29.3, 29.6, 37.9, 43.8 (11 × CH_2), 123.0, 133.7, (4 × CH Phth), 132.0 (2 × C Phth), 168.4 (2 × CON), 202.9 (CHO) ppm; IR (Nujol): $\tilde{\nu} = 1720$ (C=O), 1500 (C=C) cm^{-1}; HRMS: calcd. for $C_{20}H_{28}NO_3$ $[M+H]^+$ *m/z*: 330.2069; found: 330.2069.

1-Benzyloxy-3,7-dimethyloct-6-ene (10): [29] A 60% NaH dispersion in mineral oil (3.38 g, 0.141 mol) was washed with hexane (2 × 20 mL)

and anhydrous THF (20 mL). Citronellol (**9**; 20.00 g, 0.128 mol) in anhydrous THF (50 mL) was added to the NaH suspension in anhydrous THF (30 mL). The suspension was heated under reflux for 2 h and then cooled to room temperature. Benzylbromide (21.89 g, 0.128 mol) in anhydrous THF (25 mL) was added to the orange reaction mixture followed by tetrabutylammonium iodide (1.26 g, 3.42 mmol). The reaction mixture was heated under reflux for 18 h while the colour changed to white. After cooling to room temperature the reaction was quenched with a minimum quantity of water and the THF was evaporated under reduced pressure. Afterwards diethyl ether (100 mL) was added, the layers were separated and the aqueous layer was further extracted with diethyl ether (2×100 mL). The combined organic layers were dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by flash column chromatography (petroleum ether/ethyl acetate, 100:1) to obtain **10** as a colourless oil (18.31 g, 58%). ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 0.90$ (d, $^3J(H,H) = 6.6$ Hz, 3H; CH_3), 1.10–2.10 (m, 9H; CH_2), 1.60, 1.69 ($2 \times$ s, 6H; CH_3), 3.42–3.56 (dt, $^3J(H,H) = 7.7$ Hz, $^4J(H,H) = 1.2$ Hz, 2H; CH_2OBn), 4.51 (s, 2H; OCH_2Ph), 5.04–5.16 (m, 1H; CH=C), 7.22–7.38 (m, 5H; Bn) ppm; ^{13}C NMR (63 MHz, $CDCl_3$): $\delta = 17.5$, 19.4 ($2 \times CH_3$), 25.3 (CH_2), 25.6 (CH_3), 29.4 (CH), 36.6, 37.1, 68.6, 72.8 ($4 \times CH_2$), 124.7, 127.3, 127.5, 128.2, 128.7, 128.9 (*C*H=C, $5 \times$ CH Bn), 131.0, 138.5 (CH=*C*, C Bn) ppm; IR (Nujol): $\tilde{\nu} = 1500$ (C=C) cm^{-1}; HRMS: calcd. for $C_{17}H_{27}O$ $[M+H]^+$ *m/z*: 247.2062; found: 247.2066.

8-Benzyloxy-2,6-dimethyl-oct-2-en-1-al (11)/8-Benzyloxy-2,6-dimethyl-oct-2-en-1-ol (12):[30] *t*BuOOH (15.14 g, 168.0 mmol) was added to selenium dioxide (0.25 g, 2.2 mmol) in CH_2Cl_2 (30 mL). The reaction mixture was stirred for 1 min and **10** (18.31 g, 74.4 mmol) in CH_2Cl_2 (20 mL) was added. Water (3 mL) was added and the biphasic mixture was stirred vigorously for 16 h. The reaction mixture was cooled in an ice bath and a saturated solution of $Na_2S_2O_5$ (75 mL) was added until the peroxide test was negative. The organic solvent was evaporated under reduced pressure. The remaining aqueous layer was extracted with diethyl ether (3×75 mL), the combined organic layers were washed with brine and dried over $MgSO_4$. The solution was filtered and concentrated under reduced pressure. The residue was purified by flash column chromatography (petroleum ether/ethyl acetate, gradient from 9:1 to 5:1) to obtain **11** as a yellow oil (2.31 g, 12%) and **12** as a pale yellow oil (5.25 g, 27%).

A solution of $NaBH_4$ (0.39 g, 10.0 mmol) in cold water (10 mL) was added dropwise to the aldehyde **11** (2.31 g, 8.9 mmol) dissolved in EtOH/CH_2Cl_2 (15 mL, 4:1). The reaction mixture was stirred for 16 h. The organic solvents were evaporated under reduced pressure and the resulting residue was dissolved in diethyl ether (10 mL). The organic and aqueous layers were separated. The organic layer was washed carefully with 0.1 M HCl and the aqueous layer was extracted with diethyl ether (2×10 mL). The combined organic layers were washed with water, dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue was purified by flash column chromatography (petroleum ether/ethyl acetate, gradient from 9:1 to 1:1) to obtain **12** as a colourless oil (0.77 g, 33%) in an overall yield of 31%.

1-Benzyloxy-3,7-dimethyl-8-oxooct-6-ene (**11**): ^{1}H NMR (250 MHz, $CDCl_3$): $\delta = 0.93$ (d, $^3J(H,H) = 6.4$ Hz, 3H; CH_3), 1.22–1.72 (m, 5H; CH_2, CH), 1.74 (s, 3H; CH_3), 2.35 (m, 2H; CH_2), 3.50 (m, 2H; OCH_2), 4.50 (s, 2H; OCH_2Ph), 6.46 (td, $^3J(H,H) = 7.3$ Hz, $^4J(H,H) = 1.3$ Hz, 1H; C=CH), 7.26–7.35 (m, 5H; CH Bn), 9.38 (s, 1H; CHO) ppm; ^{13}C NMR (63 MHz, $CDCl_3$): $\delta = 14.0$, 19.1 ($2 \times CH_3$), 26.3 (CH_2), 29.5 (CH), 35.3, 36.3 ($2 \times CH_2$), 68.1, 71.4 ($2 \times OCH_2$), 127.4, 128.1 ($5 \times$ CH Bn), 138.9, 170.9 (*C*=CH, C Bn), 154.9 (C=*C*H), 195.2 (C=O) ppm; IR: $\tilde{\nu} = 1700$ (C=O), 1650 (C=C) cm^{-1}; HRMS: calcd. for $C_{17}H_{24}O_2$, $[M]^+$ *m/z*: 260.1778; found: 260.1798.

8-Benzyloxy-2,6-dimethyloct-2-en-1-ol (**12**): ^{1}H NMR (250 MHz, $CDCl_3$): $\delta = 0.88$ (d, $^3J(H,H) = 6.5$ Hz, 3H; C*H*$_3$), 1.14–1.74 (m, 6H; CH_2, CH, OH), 1.64 (s, 3H; CH_3), 2.05 (m, 2H; CH_2), 3.49 (m, 2H; CH_2O), 3.96 (s, 2H; CH_2O), 4.49 (s, 2H; OCH_2Ph), 5.38 (m, 1H; C=CH), 7.24–7.36 (m, 5H; CH Bn) ppm; ^{13}C NMR (63 MHz, $CDCl_3$): $\delta = 13.5$, 19.3 ($2 \times CH_3$), 24.9 (CH_2), 29.3 (CH), 36.5, 36.6 ($2 \times CH_2$), 68.4, 68.8, 72.7 ($3 \times OCH_2$), 126.3, 127.3, 127.5, 128.2 (C=*C*H, $5 \times$ CH Bn), 134.4, 138.4 (*C*=CH, C Bn) ppm; IR: $\tilde{\nu} = 3390$ (O–H), 1650 (C=C) cm^{-1}; HRMS: calcd. for $C_{17}H_{27}O_2$, $[M+H]^+$ *m/z*: 263.2011; found: 263.2002.

8-Benzyloxy-1-bromo-2,6-dimethyl-2-octene (13): A solution of alcohol **12** (5.57 g, 21 mmol) in anhydrous CH_2Cl_2 (80 mL) was stirred under nitrogen at $-50\,^\circ$C. Triethylamine (4.25 g, 42 mmol) was added to the reaction mixture and after 5 min stirring $MeSO_2Cl$ (4.12 g, 36 mmol) was added, causing an exothermic reaction and a temperature rise to $-20\,^\circ$C. The solution was cooled again to $-50\,^\circ$C and stirred under nitrogen for 1 h while a white solid was formed. A solution of LiBr (6.43 g, 74 mmol) in anhydrous THF (35 mL) was added to the reaction mixture resulting in another temperature rise to $-20\,^\circ$C. The reaction mixture was stirred for 2 h at this temperature whereafter it warmed up gradually to room temperature. The reaction mixture was quenched by pouring it into ice water (150 mL); the aqueous layer was extracted with CH_2Cl_2 (3×100 mL). The combined organic layers were washed with brine and water, dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue was purified by flash column chromatography (petroleum ether/ethyl acetate, 20:1) to obtain **13** as a colourless oil (4.85 g, 71%). ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 0.85$ (m, 3H; CH_3), 1.30 (m, 7H; CH_2), 1.75 (s, 3H; CH_3), 3.42–3.50 (m, 2H; CH_2OBn), 3.90 (s, 2H; CH_2Br), 4.50 (s, 2H; OCH_2Ph), 5.50 (m, 1H; CH=C), 7.22–7.38 (m, 5H; CH Bn) ppm; ^{13}C NMR (63 MHz, $CDCl_3$): $\delta = 14.2$, 20.4 ($2 \times CH_3$), 25.4 (CH_2), 30.1 (CH), 36.6, 37.7, 43.9 ($3 \times CH_2$), 69.2, 73.2 ($2 \times CH_2O$), 127.1, 131.2, 133.6 (*C*H=C, $5 \times$ CH Bn), 127.4, 140.7 (*C*=CH, C Bn) ppm; IR (Nujol): $\tilde{\nu} = 1500$ (C=C) cm^{-1}; HRMS: calcd. for $C_{17}H_{24}BrO$ $[M+H]^+$ *m/z*: 323.1010, 325.0991; found: 323.1010, 325.0908.

8-Benzyloxy-2,6-dimethyl-2-octenyltriphenylphosphonium bromide (14): Triphenylphosphine (9.95 g, 38 mmol) was added to a solution of **13** (6.16 g, 19 mmol) in anhydrous toluene (80 mL), and the reaction mixture was stirred in the dark for 6 days. The milky suspension was filtered and the sticky white solid was washed with toluene (3×40 mL). The residue of the solvent was evaporated under reduced pressure to obtain **14** as a white silvery solid (9.62 g, 97%). M.p. 105 °C; ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 0.80$ (d, $^3J(H,H) = 6.3$ Hz, 3H; CH_3), 0.84–1.71 (m, 7H; CH_2), 1.60 (s, 3H; CH_3), 3.42–3.48 (m, 2H; CH_2OBn), 4.49 (s, 2H; OCH_2Ph), 4.63 (d, $^2J(H,P) = 14.9$ Hz, 2H; CH_2PPh_3Br), 5.29 (m, 1H; CH=C), 7.15–7.87 (m, 20H; Bn) ppm; ^{13}C NMR (63 MHz, $CDCl_3$): $\delta = 18.8$, 19.7 ($2 \times CH_3$), 26.3 (CH_2), 29.9 (CH), 34.6 (d, $^1J(C,P) = 46$ Hz; CH_2PPh_3Br), 36.6, 36.9 ($2 \times CH_2$), 68.9, 73.4 ($2 \times CH_2O$), 118.9 (d, $^1J(C,P) = 85$ Hz; $3 \times$ C–P), 122.2 (*C*=CH), 125.7, 127.9, 128.1, 128.6, 128.8, 129.4, 130.6, 130.7, 134.5, 134.6, 135.3, 135.4, 137.4, 137.3, 139.0 (*C*H=C, $5 \times$ CH Bn, $15 \times$ CH Ph) ppm; ^{31}P NMR (101 MHz, $CDCl_3$): $\delta = 21.0$ (s, CH_2PPh_3Br) ppm; HRMS: calcd. for $C_{35}H_{40}OP$ $[M]^+$ *m/z*: 507.2817; found: 507.2817.

1-Benzyloxy-3,7-dimethyl-20-*N*-phthalimido-6,8-eicosadiene (15): A solution of lithium diisopropylamide (LDA; 12.3 mL, 2 M) was added to a solution of **14** (6.08 g, 11.7 mmol) in anhydrous THF (100 mL). After 45 min stirring at room temperature a solution of **8** (3.84 g, 11.7 mmol) in anhydrous THF (60 mL) was added while the colour changed from dark brown-red to orange. The reaction mixture was stirred for 16 h whereafter it was diluted with diethyl ether (100 mL) and washed with water. The aqueous layer was extracted with

diethyl ether (3×100 mL); the combined organic layers were washed with brine and dried over $MgSO_4$. The solvent was evaporated under reduced pressure. The residue was purified by flash column chromatography (petroleum ether/ethyl acetate, 9:1) to obtain **15** as a *Z*/*E* mixture (1:1) of a pale yellow oil (3.38 g, 52%). ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 0.87$ (m, 3H; CH_3), 1.14 – 2.21 (m, 30H; CH_2), 3.46 – 3.51 (m, 2H; CH_2OBn), 3.66 (t, $^3J(H,H) = 7.3$ Hz, 2H; CH_2N), 4.48 (s, 2H; OCH_2Bn), 5.22 – 5.59 (m, 2H; *CH*=CH), 5.77 (d, $^3J(H,H) =$ 11.8 Hz, 0.5H; CH=*CH*), 6.02 (d, $^3J(H,H) = 15.5$ Hz, 0.5H; CH=*CH*), 7.23 – 7.33 (m, 5H; Bn), 7.65 – 7.84 (m, 4H; Phth) ppm; ^{13}C NMR (63 MHz, $CDCl_3$): $\delta = 12.3$, 16.5 ($2 \times CH_3$), 19.4 ($2 \times CH_3$), 29.4, 29.5 ($2 \times$ CH), 25.4, 26.7, 28.5, 28.6, 29.0, 29.1, 29.4, 29.6, 30.2, 32.8, 36.5, 36.8, 37.9 ($28 \times CH_2$), 68.5, 72.7 ($4 \times CH_2O$), 123.0, 127.3, 127.4, 128.2, 129.5, 130.3, 130.5, 132.6, 133.7, 134.5 ($6 \times$ *C*H=C(H), $18 \times$ CH Bn/Phth), 132.0, 132.4, 133.6, 138.5 (*C*=CH), 168.3 ($4 \times$ CON) ppm; HRMS: calcd. for $C_{37}H_{51}NO_3$ $[M+H]^+$ *m*/*z*: 558.3947; found: 558.3946.

3,7-Dimethyl-20-*N*-phthalimidoeicosanol (16): A solution of **15** (3.38 g, 6.0 mmol) in acetone/MeOH (120 mL, 1:1) was added to palladium on charcoal (10 wt%, 338 mg) in MeOH (60 mL). The reaction mixture was stirred under a slight pressure of hydrogen for 16 h. The reaction mixture was filtered through celite, which was washed thoroughly with acetone. The filtrate was concentrated and the residue dissolved in CH_2Cl_2, washed with water, dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by flash column chromatography (petroleum ether/ethyl acetate, 4:1) to obtain **16** as a white solid (1.97 g, 69%). M.p. 35 °C; ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 0.83$ (d, $^3J(H,H) = 6.5$ Hz, 3H; CH_3), 0.88 (d, $^3J(H,H) = 6.6$ Hz, 3H; CH_3), 1.23 – 1.54 (m, 34H; CH_2, CH), 3.66 (m, 4H; CH_2O, CH_2N), 7.83, 7.70 (m, 4H; Phth) ppm; ^{13}C NMR (63 MHz, $CDCl_3$): $\delta = 19.6$ ($2 \times CH_3$), 24.2, 26.8, 27.0, 28.5, 29.1, 29.4, 29.6, 29.9, 36.9, 37.0, 37.2, 37.3, 38.0, 39.9 ($17 \times CH_2$), 29.4, 32.6 ($2 \times$ CH), 61.1 (CH_2O), 123.0, 133.7 ($4 \times$ CH Phth), 132.0 ($2 \times$ C Phth), 168.4 ($2 \times$ CON) ppm; IR (film): $\tilde{\nu} = 3054$ (O–H), 1716 (C=O) cm^{-1}; HRMS: calcd. for $C_{30}H_{50}NO_3$ $[M+H]^+$ *m*/*z*: 472.3791; found: 472.3793.

3,7-Dimethyl-20-*N*-phthalimidoeicosanylphosphate di-*tert*-butyl ester (17): 1*H*-tetrazole (378 mg, 5.4 mmol) and di-*tert*-butyl diisopropylphosphoramidite (748 mg, 2.7 mmol) were added to a stirred solution of **16** (565 mg, 1.2 mmol) in anhydrous THF (20 mL). The reaction mixture was stirred for 2 h while a white solid was formed. A solution of *meta*-chloroperoxybenzoic acid (MCPBA; 1.305 g of 57% MCPBA, 4.3 mmol) in anhydrous CH_2Cl_2 (20 mL) was added, and the reaction mixture was stirred for 16 h. A 10% Na_2SO_3 solution (70 mL) was added and the biphasic system was stirred for 30 min. The layers were separated by addition of some CH_2Cl_2. The organic layer was washed with saturated $NaHCO_3$ solution, dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by flash column chromatography (petroleum ether/ethyl acetate, 5:1) to obtain **17** as a colourless oil (580 mg, 73%). ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 0.82$ (d, $^3J(H,H) = 6.2$ Hz, 3H; CH_3), 0.89 (d, $^3J(H,H) = 6.2$ Hz, 3H; CH_3), 0.97 – 1.81 (m, 34H; CH_2, CH), 1.45, 1.48 ($2 \times$ s, 18H; CH_3 *t*Bu), 3.67 (t, $^3J(H,H) = 7.3$ Hz, 2H; CH_2N), 3.98 (m, 2H; CH_2O), 7.64 – 7.88 (m, 4H; Phth) ppm; ^{13}C NMR (63 MHz, $CDCl_3$): $\delta = 19.3$, 19.6 ($2 \times CH_3$), 29.7, 29.8 ($6 \times CH_3$ *t*Bu), 24.2, 26.8, 27.0, 28.5, 29.1, 29.4, 29.5, 29.6, 29.9, 36.9, 37.0, 37.1, 37.2, 38.0 ($17 \times CH_2$), 29.1, 32.7 ($2 \times$ CH), 65.1 (d, $^2J(C,P) = 7$ Hz; CH_2OP), 81.8 (d, $^2J(C,P) = 7$ Hz; $2 \times$ COP), 123.0 ($2 \times$ C Phth), 132.1, 133.7 ($4 \times$ CH Phth), 168.4 ($2 \times$ CON) ppm; ^{31}P (101 MHz, $CDCl_3$): $\delta = -8.9$ (s, $CH_2OPO(OtBu)_2$) ppm; IR (film): $\tilde{\nu} =$ 1710 (C=O) cm^{-1}; HRMS: calcd. for $C_{38}H_{67}NO_6P$ $[M+H^+]$ *m*/*z*: 664.4706; found: 664.4704.

20-*N*-Phthalimido-3,7-dimethyleicosanyl dihydrogenphosphate (18): A solution of **17** (4 mg, 6 μmol) in TFA (1 mL) was stirred for 10 min. The TFA was evaporated under reduced pressure to give **18**, which was co-evaporated with toluene (3×3 mL). ^{1}H NMR (250 MHz, $CDCl_3$/MeOD, 1:1): $\delta = 0.90$ (d, $^3J(H,H) = 6.3$ Hz, 3H; CH_3), 0.96 (d, $^3J(H,H) = 6.4$ Hz, 3H; CH_3), 1.15 – 1.76 (m, 36H; CH_2, CH, OH), 3.74 (t, $^3J(H,H) = 7.2$ Hz, 2H; CH_2N), 4.05 (m, 2H; CH_2OP), 7.81 – 7.93 (m, 4H; Phth) ppm; ^{31}P NMR (101 MHz, $CDCl_3$/MeOD, 1:1): $\delta = 0.8$ (s, OPO_3H_2) ppm; HRMS: calcd. for $C_{30}H_{50}NNaO_6P$ $[M+Na]^+$ *m*/*z*: 574.3274; found: 574.3272.

Benzoic acid 3,7-dimethyloct-6-enyl ester (19): Benzoyl chloride (22.3 mL, 0.19 mol) was added to a solution of citronellol (**9**) (25.0 g, 0.16 mol) in pyridine (150 mL) over 30 min at 0 °C whilst stirring. After 10 min a white precipitate of pyridinium hydrogen chloride was observed. The reaction was left to stir at room temperature for 18 h whereafter TLC (hexane/ethyl acetate, 9:1) showed that the reaction was complete. The reaction mixture was diluted with diethyl ether (250 mL) and washed with HCl (1 M, 50 mL), with a saturated solution of $NaHCO_3$ and finally with water. The organic layer was dried over $MgSO_4$, filtered and concentrated under reduced pressure to give **19** as a colourless oil, which was co-evaporated with toluene (3×75 mL) to remove traces of pyridine (41.7 g, 100%). ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 0.97$ (d, $^3J(H,H) = 6.4$ Hz, 3H; CH_3), 1.18 – 1.86 (m, 5H; $2 \times CH_2$, *CH*CH_3), 1.60 (s, 3H; CH_3), 1.67 (s, 3H; CH_3), 2.01 (m, 2H; CH_2), 4.35 (m, 2H; CH_2O), 5.09 (m, 1H; CH=C), 7.38 – 7.65, 8.02 – 8.13 (m, 5H; CH Bz) ppm; ^{13}C NMR (63 MHz, $CDCl_3$): $\delta = 17.5$, 19.3 (CH_3), 25.2 (CH_2), 25.5 (CH_3), 29.3 (CH), 35.3, 36.8 ($2 \times CH_2$), 63.3 (CH_2O), 124.4 (*CH*=C), 128.2, 129.4, 132.6 ($5 \times$ CH Bz), 130.3, 131.2 (*C*=CH, C Bz), 166.4 (COO) ppm; IR: $\tilde{\nu} = 1720$ (C=O) cm^{-1}; HRMS: calcd. for $C_{17}H_{25}O_2$, $[M+H]^+$ *m*/*z*: 261.1855; found: 261.1850.

Benzoic acid 3,7-dimethyl-8-oxooct-6-enyl ester (20)/benzoic acid 8-hydroxy-3,7-dimethyloct-6-enyl ester (21): A mixture of **19** (30.00 g, 0.11 mol) in CH_2Cl_2 (51 mL), *t*BuOOH solution in decane (5 M, 51 mL, 0.25 mol) and water (2.1 mL) was added under argon to a round-bottom flask containing SeO_2 (0.32 g, 2.88 mmol). The biphasic mixture was stirred vigorously at room temperature for 18 h. A saturated solution of $Na_2S_2O_5$ was added under cooling with ice and the mixture was stirred for 16 h. The organic solvent was evaporated under vacuum and the aqueous layer was extracted with diethyl ether (3×100 mL). The combined organic layers were washed with brine, dried over $MgSO_4$ and filtered. After a negative peroxide test, the organic solvent was evaporated under reduced pressure. Purification by flash column chromatography (heptane/ethyl acetate, gradient from 9:1 to 4:1) afforded **20** (3.10 g, 10%) and **21** (2.73 g, 9%).

Further reduction of **20** was achieved by dropwise addition of $NaBH_4$ (0.47 g, 12.42 mmol) in cold water to a solution of **20** (3.10 g, 11.29 mmol) in EtOH/CH_2Cl_2 (4:1, 25 mL) and stirring for 16 h. The solution was concentrated under reduced pressure and the residue was diluted in CH_2Cl_2 (50 mL) and HCl (0.1 M) until the compound was completely dissolved. The aqueous layer was removed and the organic layer was washed with water, dried over $MgSO_4$, filtered and concentrated under reduced pressure to obtain **21** (3.12 g, 100%) as a colourless oil in an overall yield of 5.90 g (18%).

Benzoic acid 3,7-dimethyl-8-oxooct-6-enyl ester (**20**): ^{1}H NMR (250 MHz, $CDCl_3$): $\delta = 1.02$ (d, $^3J(H,H) = 6.4$ Hz, 3H; CH_3), 1.17 – 1.88 (m, 5H; CH_2, CH), 1.75 (s, 3H; CH_3), 2.39 (m, 2H; CH_2), 4.37 (m, 2H; CH_2O), 6.47 (tq, $^3J(H,H) = 7.4$ Hz, $^4J(H,H) = 1.4$ Hz, 1H; C=CH), 7.39 – 7.60, 8.00 – 8.05 (m, 5H; CH Bz), 9.37 (s, 1H; CHO) ppm; ^{13}C NMR (63 MHz, $CDCl_3$): $\delta = 9.0$, 19.1 (CH_3), 26.3 (CH_2), 29.5 (CH), 35.2 ($2 \times CH_2$), 62.9 (CH_2O), 128.2, 129.3, 132.8 ($5 \times$ CH Bz), 130.1, 139.2 (*C*=CH, C Bz), 154.5 (*CH*=C), 166.5 (COO), 195.2 (CHO) ppm; IR: $\tilde{\nu} = 1720$ (C=O) cm^{-1}; HRMS: calcd. for $C_{17}H_{23}O_3$, $[M+H]^+$ *m*/*z*: 275.1647; found: 275.1648.

Benzoic acid 8-hydroxy-3,7-dimethyloct-6-enyl ester (**21**): ^{1}H NMR (250 MHz, $CDCl_3$): $\delta = 0.96$ (d, $^3J(H,H) = 6.4$ Hz, 3H; CH_3), 1.20 – 1.83

(m, 5H; 2 × CH_2, CH), 1.64 (s, 3H; CH_3), 2.04 (m, 2H; CH_2), 3.51 (br s, 1H; OH), 3.96 (s, 2H; CH_2OH), 4.34 (m, 2H; CH_2O), 5.38 (m, 1H; CH=C), 7.38 – 7.57, 8.00 – 8.09 (m, 5H; CH Bz) ppm; ^{13}C NMR (63 MHz, $CDCl_3$): δ = 13.5, 19.3 (CH_3), 24.8 (CH_2), 29.4 (CH), 35.3, 36.4 (2 × CH_2), 63.3 (CH_2O), 68.7 (CH_2OH), 126.0 (*C*H=C), 128.2, 129.3, 132.7 (5 × CH Bz), 130.2, 134.5 (C=H, C Bz), 166.5 (COO) ppm; IR: $\tilde{\nu}$ = 3420 (O–H), 1720 (C=O) cm^{-1}; HRMS: calcd. for $C_{17}H_{25}O_3$, $[M+H]^+$ *m/z*: 277.1804; found: 277.1804.

Benzoic acid 8-bromo-3,7-dimethyloct-6-enyl ester (22): Triethylamine (5.95 mL, 42.70 mmol) was added to a cooled (−50 °C) and stirred solution of **21** (5.90 g, 21.35 mmol) in CH_2Cl_2 (87 mL). After 5 min $MeSO_2Cl$ (2.80 mL, 36.30 mmol) was added and a white precipitate was observed. The reaction mixture was stirred for 1.5 h at this temperature whereafter a solution of LiBr (6.49 g, 74.72 mmol) in THF (25 mL) was added. The temperature was allowed to rise to −20 °C and the reaction was stirred for 2.5 h. The reaction mixture was poured into ice water (140 mL). The aqueous layer was extracted with CH_2Cl_2 (3 × 75 mL); the combined organic layers were dried over $MgSO_4$, filtered and concentrated under reduced pressure. Purification of the residue by flash column chromatography (petroleum ether/ethyl acetate, 99:1) afforded **22** as a colourless oil (5.40 g, 75%). ^{1}H NMR (250 MHz, $CDCl_3$): δ = 0.96 (d, ^3J(H,H) = 6.4 Hz, 3H; CH_3), 1.26 – 1.83 (m, 5H; CH_2, CH), 1.74 (s, 3H; CH_3), 2.05 (m, 2H; CH_2), 3.93 (s, 2H; CH_2Br), 4.35 (m, 2H; CH_2O), 5.58 (t, ^3J(H,H) = 7.0 Hz, 1H; CH=C), 7.39 – 7.57, 8.01 – 8.05 (m, 5H; CH Bz) ppm; ^{13}C NMR (63 MHz, $CDCl_3$): δ = 14.4, 19.2 (CH_3), 25.5 (CH_2), 29.3 (CH), 35.2, 35.9, 41.6 (3 × CH_2), 63.1 (CH_2O), 128.1, 129.3, 132.6 (5 × CH Bz), 131.2 (C=*C*H), 130.2, 132.8 (*C*=CH, C Bz), 166.4 (C=O) ppm; IR: $\tilde{\nu}$ = 1718 (C=O) cm^{-1}; HRMS: calcd. for $C_{17}H_{24}O_2Br$, $[M+H]^+$ *m/z*: 339.0960, 341.0939; found: 339.0957, 341.0940.

Benzoic acid 8-phthalimido-3,7-dimethyloct-6-enyl ester (23): A solution of **22** (1.50 g, 4.42 mmol) in DMF (5 mL) was added under nitrogen to potassium phthalimide (0.80 g, 4.34 mmol). The mixture was heated for 16 h at 105 °C. The solution was diluted with water and the product extracted with CH_2Cl_2 (3 × 15 mL). The organic layers were dried over $MgSO_4$, filtered and concentrated under reduced pressure. Purification of the residue by flash column chromatography (petroleum ether/ethyl acetate, gradient from 19:1 to 9:1) afforded **23** as a colourless oil (1.42 g, 80%). ^{1}H NMR (250 MHz, $CDCl_3$): δ = 0.92 (d, ^3J(H,H) = 6.3 Hz, 3H; CH_3), 1.14 – 1.81 (m, 5H; CH_2, CH), 1.61 (s, 3H; CH_3), 2.01 (m, 2H; CH_2), 4.15 (s, 2H; CH_2N), 4.30 (m, 2H; CH_2O), 5.34 (tq, ^3J(H,H) = 7.1 Hz, ^4J(H,H) = 1.3 Hz, 1H; CH=C), 7.36 – 7.54, 7.63 – 7.84, 7.97 – 8.03 (m, 9H; Phth, Bz) ppm; ^{13}C NMR (63 MHz, $CDCl_3$): δ = 14.4, 19.2 (2 × CH_3), 25.0 (CH_2), 29.5 (CH), 35.2, 36.2, 44.8 (3 × CH_2), 63.2 (CH_2O), 123.0, 133.7 (4 × CH Phth), 128.1, 129.3, 132.6 (5 × CH Bz), 127.5 (C=*C*H), 128.9 (*C*=CH), 130.2, 131.8 (2 × C Phth, C Bz), 166.4 (COO), 168.0 (CON) ppm; IR: $\tilde{\nu}$ = 1770, 1720 (C=O) cm^{-1}; HRMS: calcd. for $C_{25}H_{28}NO_4$, $[M+H]^+$ *m/z*: 406.2018; found: 406.2018.

Benzoic acid 8-phthalimido-3,7-dimethyloctyl ester (24): A solution of **23** (1.42 g, 3.50 mmol) in MeOH (38 mL) was added to palladium on charcoal (10 wt%, 0.14 g). The reaction mixture was stirred under a slight pressure of hydrogen at room temperature for 18 h. The suspension was filtered through celite, which was washed thoroughly with MeOH. The solution was concentrated under reduced pressure to give **24** in a diastereomeric mixture (1:1), as a colourless oil (1.28 g, 90%). ^{1}H NMR (250 MHz, $CDCl_3$): δ = 0.87 (d, ^3J(H,H) = 6.7 Hz, 3H; CH_3), 0.92 (dd, ^3J(H,H) = 6.4 Hz, ^4J(H,H) = 1.9 Hz, 3H; CH_3), 0.98 – 2.32 (m, 10H; CH_2, CH), 3.49 (m, 2H; CH_2N), 4.31 (m, 2H; CH_2O), 7.39 – 7.55, 7.68 – 7.86, 8.01 – 8.05 (m, 9H; CH Phth, CH Bz) ppm; ^{13}C NMR (63 MHz, $CDCl_3$): δ = 17.2, 17.3 (2 × CH_3), 19.3, 19.4 (2 × CH_3), 23.8, 23.9 (2 × CH_2), 29.7 (2 × CH), 32.3 (2 × CH), 34.4 (2 × CH_2), 35.2, 35.3 (2 × CH_2), 36.8 (2 × CH_2), 44.0 (2 × CH_2), 63.3 (2 × CH_2O), 122.9, 133.6 (8 × CH Phth), 128.1, 129.3, 132.6 (10 × CH Bz), 130.2, 131.8 (4 × C Phth, 2 × C Bz), 166.4 (2 × COO), 168.5 (2 × CON) ppm; IR: $\tilde{\nu}$ = 1770, 1720 (C=O) cm^{-1}; HRMS: calcd. for $C_{25}H_{30}NO_4$, $[M+H]^+$ *m/z*: 408.2174; found: 408.2175.

8-Phthalimido-3,7-dimethyloctan-1-ol (25): A solution of NaOMe in MeOH (0.5 M, 1.44 mL, 0.70 mmol) was added to **24** (290 mg, 0.70 mmol). The solution was stirred for 20 h where after HCl (0.5 M, 10 mL) was added. The product was extracted with ethyl acetate (3 × 5 mL). The combined organic layers were dried over $MgSO_4$, filtered and concentrated under reduced pressure. Flash column chromatography (petroleum ether/ethyl acetate, 7:3) of the residue afforded **25** in a diastereomeric mixture (1:1), as a colourless oil (94 mg, 43%). ^{1}H NMR (250 MHz, $CDCl_3$): δ = 0.83 (m, 6H; 2 × CH_3), 1.07 – 2.00 (m, 11H; CH_2, CH, OH), 3.45 (m, 2H; CH_2N), 3.61 (m, 2H; CH_2O), 7.65 – 7.82 (m, 4H; CH Phth) ppm; ^{13}C NMR (63 MHz, $CDCl_3$): δ = 17.3 (2 × CH_3), 19.4, 19.5 (2 × CH_3), 23.8, 23.9 (2 × CH_2), 29.1, 29.2 (2 × CH), 32.3, 32.4 (2 × CH), 34.3, 34.4 (2 × CH_2), 36.8, 37.0 (2 × CH_2), 39.6, 39.8 (2 × CH_2), 44.0 (2 × CH_2), 60.8, 60.9 (2 × CH_2O), 123.0, 133.7 (8 × CH Phth), 131.8 (4 × C Phth), 168.6 (2 × CON) ppm; IR: $\tilde{\nu}$ = 3460 (O–H), 1770, 1710 (C=O) cm^{-1}; HRMS: calcd. for $C_{18}H_{26}NO_3$, $[M+H]^+$ *m/z*: 304.1913; found: 304.1913.

Phosphoric acid di-*tert*-butyl 8-phthalimido-3,7-dimethyloctyl ester (26): Di-*tert*-butyl diisopropylphosphoramidite (69 μL, 0.22 mmol) was added to a solution of **25** (44 mg, 0.14 mmol) and 1*H*-tetrazole (30 mg, 0.44 mmol) in THF (0.9 mL) under argon. After 1 h stirring the reaction mixture was cooled with ice and a solution of MCPBA (106 mg of 57% MCPBA, 0.35 mmol) in CH_2Cl_2 (0.9 mL) was added. It was stirred for a further hour. An aqueous solution of Na_2SO_3 (10%, 5.0 mL) was added and the mixture was stirred for 5 min. The layers were separated; the organic layer was washed with a saturated solution of $NaHCO_3$, dried over $MgSO_4$, filtered and concentrated under reduced pressure. Purification of the residue by flash column chromatography (petroleum ether/ethyl acetate, 7:3) afforded **26** in a diastereomeric mixture (1:1), as a colourless oil (39 mg, 54%). ^{1}H NMR (250 MHz, $CDCl_3$): δ = 0.87 (m, 6H; 2 × CH_3), 1.16 – 1.95 (m, 10H; CH_2, CH), 1.46 (s, 18H; CH_3 *t*Bu), 3.54 (m, 2H; CH_2N), 3.91 – 4.00 (m, 2H; CH_2O), 7.67 – 7.74, 7.79 – 7.86 (m, 4H; CH Phth) ppm; ^{13}C NMR (63 MHz, $CDCl_3$): δ = 17.2 (2 × CH_3), 19.1, 19.2 (2 × CH_3), 23.9 (2 × CH_2), 29.1 (2 × CH), 29.6, 29.7 (12 × CH_3 *t*Bu), 32.4 (2 × CH), 34.4 (2 × CH_2), 36.8 (2 × CH_2), 37.0 (2 × CH_2), 44.0 (2 × CH_2), 64.9 (d, ^2J(C,P) = 7 Hz; 2 × CH_2OP), 81.7 (d, ^2J(C,P) = 7 Hz; 4 × POC(CH_3)$_3$), 123.0, 133.7 (8 × CH Phth), 131.8 (4 × C Phth), 168.5 (2 × CON) ppm; ^{31}P NMR (101 MHz, $CDCl_3$): δ = −9.0 (s, CH_2OPO(O*t*-Bu)$_2$) ppm; IR: $\tilde{\nu}$ = 1770, 1710 (C=O) cm^{-1}; HRMS: calcd. for $C_{26}H_{43}NO_6P$, $[M+H]^+$ *m/z*: 496.2828; found: 496.2827.

Phosphoric acid di-*tert*-butyl (8-(2-ethoxy-3,4-dioxocyclobut-1-enylamino)-3,7-dimethyloctyl) ester (27): Hydrazine hydrate (65 μL, 1.33 mmol) was added to a solution of **26** (66 mg, 0.13 mmol) in EtOH (135 μL). The reaction mixture was stirred for 16 h while the formation of a white precipitate was observed. The reaction mixture was concentrated under reduced pressure and THF was added for further precipitation. The mixture was filtered and the filtrate was concentrated. The residue was diluted with EtOH (820 μL) and 3,4-diethoxy-3-cyclobutene-1,2-dione (20 μL, 0.13 mmol) was added. After 5 min TLC (petroleum ether/ethyl acetate, 9:11) showed no remaining starting material and the formation of a new product. The solvent was evaporated under reduced pressure and purification of the residue by flash column chromatography (petroleum ether/ethyl acetate, 9:11) afforded **27** in a diastereomeric mixture (1:1), as a colourless oil (39 mg, 60%). ^{1}H NMR (250 MHz, $CDCl_3$): δ = 0.89 (m, 6H; 2 × CH_3), 1.06 – 1.71 (m, 10H; CH_2, CH), 1.44 (t, ^3J(H,H) = 7.0 Hz, 3H; CH_3CH$_2$O), 1.46 (s, 18H; CH_3 *t*Bu), 3.33 (m, 2H; CH_2N), 3.96 (m, 2H; CH_2OP), 4.76 (q, ^3J(H,H) = 7.0 Hz, 2H; OCH_2), 6.69 (m, 1H; NH) ppm;

^{13}C NMR (63 MHz, $CDCl_3$): $\delta = 15.7$ ($2 \times OCH_2\mathit{C}H_3$), 16.9, 17.0 ($2 \times CH_3$), 19.3, 19.5 ($2 \times CH_3$), 23.8 ($2 \times CH_2$), 29.0 ($2 \times CH$), 29.7 ($12 \times \mathit{C}H_3$ *t*Bu), 33.6, 33.8 ($2 \times CH_2$), 34.1, 34.2 ($2 \times CH$), 36.3, 36.6 ($2 \times CH_2$), 36.9 ($2 \times CH_2$), 50.6, 50.7 ($2 \times CH_2$), 64.9 (d, ^2J(C,P) = 6 Hz; $2 \times CH_2OP$), 69.4 ($2 \times O\mathit{C}H_2CH_3$), 82.0 (d, ^2J(C,P) = 7 Hz; $2 \times PO\mathit{C}(CH_3)_3$), 172.7, 176.8 ($4 \times$ C=C), 182.8 ($2 \times$ C=O), 189.1 ($2 \times$ C=O) ppm; ^{31}P NMR (101 MHz, $CDCl_3$): $\delta = -9.0$ (s; $CH_2OPO(O\mathit{t}Bu)_2$) ppm; HRMS: calcd. for $C_{24}H_{45}NO_7P$, $[M+H]^+$ *m/z*: 490.2933; found: 490.2932.

Phosphoric acid di-*tert*-butyl (8-(2-dodecylamino-3,4-dioxocyclobut-1-enylamino)-3,7-dimethyloctyl) ester (28): A solution of **27** (39 mg, 82 μmol) diluted in EtOH (500 μL) was added slowly to a solution of dodecylamine (20 μL, 88 μmol) in EtOH (500 μL). After 5 min, TLC (petroleum ether/ethyl acetate, 1:4) showed no remaining starting material and the formation of a new product. The solvent was evaporated under reduced pressure. Purification of the residue by flash column chromatography (petroleum ether/ethyl acetate, 1:4) afforded **28** in a diastereomeric mixture (1:1), as a colourless oil (33 mg, 65%). ^{1}H NMR (250 MHz, $CDCl_3$): $\delta = 0.86$ (m, 9H; $3 \times CH_3$), 1.22 – 1.96 (m, 30H; CH_2, CH), 1.46 (s, 18H; CH_3 *t*Bu), 3.50 – 3.64 (m, 4H; $2 \times CH_2N$), 3.96 (m, 2H; CH_2OP), 7.10 (br s, 1H; NH), 7.30 (br s, 1H; NH) ppm; ^{13}C NMR (63 MHz, $CDCl_3$): $\delta = 14.0$, 17.2 ($4 \times CH_3$), 20.1, 20.2 ($2 \times CH_3$), 22.5 ($4 \times CH_2$), 22.6 ($2 \times CH_2$), 26.4 ($2 \times CH_2$), 29.0 ($2 \times CH$), 29.2 ($2 \times CH_2$), 29.3 ($2 \times CH_2$), 29.4 ($2 \times CH_2$), 29.5 ($4 \times CH_2$), 29.7, 29.8 ($12 \times \mathit{C}H_3$ *t*Bu), 31.4 ($2 \times CH_2$), 31.8 ($2 \times CH_2$), 32.4 ($2 \times CH_2$), 34.0 ($2 \times CH$), 34.9 ($2 \times CH_2$), 35.8 ($2 \times CH_2$), 44.3 ($2 \times CH_2N$), 49.4 ($2 \times CH_2N$), 65.8 (d, ^2J(C,P) = 9 Hz; $2 \times CH_2OP$), 82.9 ($4 \times PO\mathit{C}(CH_3)_3$), 182.6 ($2 \times$ C=O), 182.8 ($2 \times$ C=O) ppm; ^{31}P NMR (101 MHz, $CDCl_3$): $\delta = -10.5$ (s, $CH_2OPO(O\mathit{t}Bu)_2$) ppm; HRMS: calcd. for $C_{34}H_{65}N_2O_6P$, $[M]^+$ *m/z*: 628.4580; found: 628.4580.

Phosphoric acid mono-(8-(2-dodecylamino-3,4-dioxo-cyclobut-1-enylamino)-3,7-dimethyl-octyl) ester (29): TFA (300 μL) was added to **28** (6 mg, 9.5 μmol). The solution was stirred for 10 min and then concentrated under reduced pressure to give **29**, which was co-evaporated with toluene (3×1 mL) under reduced pressure to remove traces of TFA. Final yield of **29** was 5 mg (80%). ^{1}H NMR (250 MHz, $CDCl_3$): $\delta = 0.89$ (m, 9H; $3 \times CH_3$), 1.10 – 1.76 (m, 34H; CH_2, CH, OH), 3.50 (m, 4H; $2 \times CH_2N$), 3.99 (m, 2H; CH_2OP) ppm; ^{31}P NMR (101 MHz, dimethyl sulfoxide): $\delta = -0.1$ (s, $CH_2OPO(OH)_2$) ppm; HRMS: calcd. for $C_{26}H_{50}N_2O_6P$, $[M+H]^+$ *m/z*: 517.3406; found: 517.3407.

Phosphoric acid di-*tert*-butyl (8-decanoylamino-3,7-dimethyl-octyl) ester (30): Hydrazine hydrate (38 μL, 790 μmol) was added to a solution of **26** (39 mg, 79 μmol) in EtOH (80 μL). The reaction mixture was stirred for 16 h while the formation of a white precipitate was observed. The reaction mixture was then concentrated under reduced pressure and THF was added for further precipitation. The mixture was filtered and the solution was concentrated. The residue was diluted in THF (0.5 mL) and was added slowly to a solution of decanoyl chloride (16 μL, 79 μmol) and Et_3N (11 μL, 79 μmol) in THF (500 μL). The solution was stirred for 16 h. Water (5 mL) was added and the product was extracted with ethyl acetate (3×10 mL). The combined organic layers were dried over $MgSO_4$, filtered and concentrated under reduced pressure. Purification of the residue by flash column chromatography (petroleum ether/ethyl acetate, 1:1) afforded **30** in a diastereomeric mixture (1:1), as a colourless oil (25 mg, 61%). ^{1}H NMR (250 MHz, $CDCl_3$): $\delta = 0.85$ (m, 9H; $3 \times CH_3$), 1.08 – 1.71 (m, 24H; CH_2, CH), 1.45 (s, 18H; CH_3 *t*Bu), 2.15 (t, ^3J(H,H) = 7.6 Hz, 2H; CH_2CONH), 3.00 – 3.18 (m, 2H; CH_2N), 3.96 (m, 2H; CH_2OP), 5.72 (m, 1H; NH) ppm; ^{13}C NMR (63 MHz, $CDCl_3$): $\delta = 14.0$ ($2 \times CH_3$), 17.4, 17.5 ($2 \times CH_3$), 19.2, 19.3 ($2 \times CH_3$), 22.5 ($2 \times CH_2$), 23.8 ($2 \times CH_2$), 25.8 ($2 \times CH_2$), 28.8, 29.0 ($2 \times CH$), 29.1, 29.2 ($2 \times CH_2$), 29.3 ($2 \times CH_2$), 29.7, 29.8 ($12 \times CH_3$ *t*Bu), 31.7 ($2 \times CH_2$), 33.0, 33.1 (CH), 34.2 ($2 \times CH_2$), 34.4 ($2 \times CH_2$), 36.5 ($2 \times CH_2$), 36.8 ($2 \times CH_2$), 37.0 ($2 \times CH_2$), 37.1 ($2 \times CH_2$), 45.1, 45.2 ($2 \times CH_2N$), 65.0 (d, ^2J(C,P) = 7 Hz; CH_2OP), 81.8 (d, ^2J(C,P) = 7 Hz; $PO\mathit{C}(CH_3)_3$), 172.2 ($2 \times$ C=O) ppm; ^{31}P NMR (101 MHz, $CDCl_3$): $\delta = -8.9$ (d, ^2J(C,P) = 7 Hz; $CH_2OPO(O\mathit{t}Bu)_2$) ppm; HRMS: calcd. for $C_{28}H_{59}NO_5P$, $[M+H]^+$ *m/z*: 520.4131; found: 520.4130.

Phosphoric acid mono-(8-decanoylamino-3,7-dimethyl-octyl) ester (31): TFA (490 μL) was added to **30** (8 mg, 15 μmol). The solution was stirred for 10 min and then concentrated under reduced pressure to give **31**, which was co-evaporated from toluene (3×1 mL) under reduced pressure to remove traces of TFA. Final yield of **31** was 5.5 mg (88%). ^{1}H NMR (250 MHz, $CDCl_3$): $\delta = 0.86$ (m, 9H; $3 \times CH_3$), 1.09 – 1.75 (m, 26H; CH_2, CH, OH), 2.27 (m, 2H; CH_2CO_2), 3.11 (m, 2H; CH_2N), 4.05 (m, 2H; CH_2OP), 6.50 (m, 1H; NH) ppm; ^{31}P NMR (101 MHz, $CDCl_3$): $\delta = 2.1$ (s; $CH_2OPO(O\mathit{t}Bu)_2$) ppm; HRMS: calcd. for $C_{20}H_{43}NO_5P$, $[M+H]^+$ *m/z*: 408.2879; found: 408.2879.

Decanoic acid (8-benzyloxy-2,6-dimethyloct-2-enyl) ester (32): Decanoyl chloride (0.36 mL, 2.28 mmol) was added to a solution of **12** (0.50 g, 1.90 mmol) in pyridine (1.80 mL) under stirring at 0 °C over 5 min whereafter a white precipitate was observed. The reaction was left to stir at room temperature for 18 h. The reaction mixture was diluted with diethyl ether (25 mL) and was washed with HCl (1 M) then with a saturated solution of $NaHCO_3$ and with water. The organic layer was dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue was co-evaporated with toluene (3×10 mL) under reduced pressure to remove traces of pyridine. Purification of the residue by flash column chromatography (petroleum ether/ethyl acetate, 49:1) afforded **32** as a colourless oil (0.58 g, 72%). ^{1}H NMR (250 MHz, $CDCl_3$): $\delta = 0.87$ (m, 6H; $2 \times CH_3$), 1.17 – 1.71 (m, 19H; CH_2, CH), 1.63 (s, 3H; CH_3), 2.04 (m, 2H; CH_2), 2.31 (t, ^3J(H,H) = 7.5 Hz, 2H; CH_2CO_2), 3.49 (m, 2H; CH_2O), 4.44 (s, 2H; CH_2O), 4.49 (s, 2H; OCH_2Ph), 5.43 (m, 1H; C=CH), 7.24 – 7.35 (m, 5H; CH Bn) ppm; ^{13}C NMR (63 MHz, $CDCl_3$): $\delta = 13.7$, 14.0, 19.3 ($3 \times CH_3$), 22.5, 24.9, 25.0, 29.0, 29.1, 29.3 ($6 \times CH_2$), 29.5 (CH), 31.7, 34.2, 36.4, 36.5 ($4 \times CH_2$), 68.4, 69.9, 72.8 ($3 \times OCH_2$), 127.3, 127.4, 128.2, 129.7 (C=CH, $5 \times$ CH Bn), 129.7, 138.5 (C=CH, C Bn), 173.6 (COO) ppm; IR: $\tilde{\nu} = 1740$ (C=O) cm^{-1}; HRMS: calcd. for $C_{27}H_{45}O_3$, $[M+H]^+$ *m/z*: 417.3369; found: 417.3368.

Decanoic acid (8-hydroxy-2,6-dimethyloctyl) ester (33): A solution of **32** (460 mg, 1.11 mmol) in MeOH (12 mL) was added to palladium on charcoal (10 wt%, 46 mg). The reaction mixture was stirred under a slight pressure of hydrogen for 3 days. The suspension was then filtered through celite, which was washed thoroughly with MeOH. The solution was concentrated under reduced pressure. Purification of the residue by flash column chromatography (petroleum ether/ethyl acetate, 9:1) afforded **33** in a diastereomeric mixture (1:1), as a colourless oil (112 mg, 31%). ^{1}H NMR (250 MHz, $CDCl_3$): $\delta = 0.85$ (m, 9H; CH_3), 1.02 – 1.49 (m, 19H; CH_2, CH), 1.55 (m, 4H; CH_2), 1.69 (m, 1H; CH), 2.26 (t, ^3J(H,H) = 7.5 Hz, 2H; CH_2CO_2), 2.89 (br s, 1H; OH), 3.60 (m, 2H; CH_2O), 3.68 – 3.94 (m, 2H; CH_2O) ppm; ^{13}C NMR (63 MHz, $CDCl_3$): $\delta = 13.9$ ($2 \times CH_3$), 16.7 ($2 \times CH_3$), 19.3, 19.4 ($2 \times CH_3$), 22.5 ($2 \times CH_2$), 23.9 ($2 \times CH_2$), 24.8 ($2 \times CH_2$), 29.0 ($2 \times CH_2$), 29.1 ($2 \times CH_2$), 29.2 ($2 \times CH$), 29.2 ($2 \times CH_2$), 31.7 ($2 \times CH_2$), 32.3 ($2 \times CH$), 33.3, 33.4 ($2 \times CH_2$), 34.2 ($2 \times CH_2$), 37.0, 37.1 ($2 \times CH_2$), 39.6, 39.7 ($2 \times CH_2$), 60.8 ($2 \times CH_2O$), 69.0 ($2 \times CH_2O$), 174.0 ($2 \times$ COO) ppm; IR: $\tilde{\nu} = 3446$ (O–H), 1740 (C=O) cm^{-1}; HRMS: calcd. for $C_{20}H_{41}O_3$, $[M+H]^+$ *m/z*: 329.3056; found: 329.3055.

Phosphoric acid di-*tert*-butyl (8-decanoyloxo-3,7-dimethyloctyl) ester (34): Di-*tert*-butyl diisopropylphosphoramidite (180 μL, 0.57 mmol) was added to a solution of **33** (125 mg, 0.38 mmol) and 1*H*-tetrazole (80 mg, 1.14 mmol) in THF (2.4 mL) under argon. After stirring for 1 h the reaction mixture was cooled with ice and a solution of MCPBA (279 mg of 57% MCPBA, 0.92 mmol) in CH_2Cl_2

(2.4 mL) was added and stirred for 18 h. A olution of Na_2SO_3 (10%, 10.0 mL) was added and the mixture was stirred for 5 min. The layers were separated; the organic layer was washed with a saturated solution of $NaHCO_3$, dried over $MgSO_4$, filtered and concentrated under reduced pressure. Purification of the residue by flash column chromatography (petroleum ether/ethyl acetate, 17:3) afforded **34** in a diastereomeric mixture (1:1), as a colourless oil (72 mg, 36%). ^{1}H NMR (250 MHz, $CDCl_3$): $\delta = 0.84$ (m, 9H; CH_3), 1.09 – 1.69 (m, 24H; CH_2, CH), 1.44 (s, 18H; CH_3 *t*Bu), 2.26 (t, ^3J(H,H) = 7.5 Hz, 2H; CH_2CO_2), 3.76 – 3.98 (m, 4H; CH_2OP, CH_2OCO) ppm; ^{13}C NMR (63 MHz, $CDCl_3$): $\delta = 13.9$ (2 × CH_3), 16.6, 16.7 (2 × CH_3), 19.1, 19.2 (2 × CH_3), 22.5 (2 × CH_2), 23.9 (2 × CH_2), 24.9 (2 × CH_2), 29.0 (2 × CH), 29.0 (2 × CH_2), 29.1 (2 × CH_2), 29.3 (2 × CH_2), 29.6, 29.7 (12 × CH_3), 31.7 (2 × CH_2), 32.4 (2 × CH), 33.4 (2 × CH_2), 34.2 (2 × CH_2), 36.9, 37.0 (2 × CH_2), 37.1, 37.2 (2 × CH_2), 64.9 (d, ^2J(H,H) = 6 Hz; 2 × CH_2OP), 68.9, 69.0 (2 × OCH_2), 81.7 (d, ^2J(H,H) = 7 Hz; 2 × $OC(CH_3)_3$), 174.0 (2 × C=O) ppm; ^{31}P NMR (101 MHz, $CDCl_3$): $\delta = -8.8$ (s, $CH_2OPO(OtBu)_2$) ppm; IR: $\tilde{\nu} = 1740$ (C=O) cm^{-1}; HRMS: calcd. for $C_{28}H_{58}O_6P$, $[M+H]^+$ *m/z*: 521.3971; found: 521.3972.

Phosphoric acid mono-(8-decanoyloxo-3,7-dimethyloctyl) ester (35): TFA (1.1 mL) was added to **34** (18 mg, 34 μmol). The solution was stirred for 10 min and then concentrated under reduced pressure to give **35**, which was co-evaporated with toluene (3 × 1 mL) to remove traces of TFA. Final yield of **35** was 12 mg (85%). ^{1}H NMR (250 MHz, $CDCl_3$): $\delta = 0.88$ (m, 9H; 3 × CH_3), 1.12 – 1.74 (m, 26H; CH_2, CH, OH), 2.30 (t, ^3J(H,H) = 7.4 Hz, 2H; CH_2CO_2), 3.74 – 4.06 (m, 4H; CH_2O, CH_2OP) ppm; ^{31}P NMR (101 MHz, $CDCl_3$): $\delta = 2.2$ (s, $CH_2OPO(OH)_2$) ppm; HRMS: calcd. for $C_{20}H_{42}O_6P$, $[M+H]^+$ *m/z*: 409.2719; found: 409.2719.

20-Amino-3,7-dimethyleicosanylphosphate di-*tert*-butyl ester (36): Hydrazine hydrate (136 mg, 2.71 mmol) was added to **17** (180 mg, 0.271 mmol) in EtOH (8.5 mL) and the solution was stirred for 14 h while the formation of a white precipitate was observed. The solvent was evaporated. CH_2Cl_2 (5 mL) was added to the residue and the mixture was filtered, dried over $MgSO_4$ and filtered again. After evaporation of the solvent under reduced pressure, **36** was recovered as a pale yellow oil (150 mg), which was used in the next step without purification. ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 0.83$ (d, ^3J(H,H) = 6.2 Hz, 3H; CH_3), 0.89 (d, ^3J(H,H) = 6.4 Hz, 3H; CH_3), 1.00 – 1.81 (m, 36H; CH_2, CH, NH_2), 1.51, 1.48 (2 × s, 18H; CH_3 *t*Bu), 2.68 (t, ^3J(H,H) = 6.8 Hz, 2H; CH_2N), 3.98 (m, 2H; CH_2OP) ppm; ^{13}C NMR (63 MHz, $CDCl_3$): $\delta = 19.2$, 19.3 (2 × CH_3), 29.7 (6 × CH_3 *t*Bu), 24.1, 26.7, 26.9, 27.0, 29.0, 29.3, 29.5, 29.6, 29.7, 29.9, 30.1, 30.2, 33.3, 36.9, 37.0, 37.2, 41.9 (17 × CH_2), 29.5, 32.6 (2 × CH), 65.1 (d, ^2J(C,P) = 7 Hz; CH_2OP), 81.7 (d, ^2J(C,P) = 8 Hz, 2 × COP) ppm; ^{31}P NMR (101 MHz, $CDCl_3$): $\delta = -8.8$ (s, $CH_2OPO(OtBu)_2$) ppm; HRMS: calcd. for $C_{30}H_{65}NO_4P$ $[M+H]^+$ *m/z*: 534.4651; found: 534.4653.

20-*N*-Biotinylamino-3,7-dimethyleicosanylphosphate di-*tert*-butyl ester (37): A solution of **36** in anhydrous DMF (5 mL) was added to biotin-*N*-hydroxysuccinimide ester (120 mg, 0.35 mmol) in anhydrous DMF (5 mL) and the reaction mixture was stirred for 16 h. The solvent was evaporated under reduced pressure and the residue was purified by flash column chromatography (CH_2Cl_2/EtOH, 9:1) to obtain **37** as a white amorphous solid (168 mg, 63%). M.p. 157 °C; ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 0.81$ (d, ^3J(H,H) = 6.3 Hz, 3H; CH_3), 0.87 (d, ^3J(H,H) = 6.4 Hz, 3H; CH_3), 1.06 – 1.78 (m, 58H; CH_2, CH), 1.46 (s, 18H; *t*Bu), 2.18 (t, ^3J(H,H) = 7.4 Hz, 2H; CH_2CONH), 2.73 (d, ^2J(H,H) = 12.8 Hz, 1H; CH_2S), 2.90 (dd, ^2J(H,H) = 12.8 Hz, ^3J(H,H) = 4.8 Hz, 1H; CH_2S), 3.17 (m, 3H; CH_2NH, CHS), 3.96 (m, 2H; CH_2OP), 4.30, 4.49 (2 × m, 2H; C*H*NH), 5.50, 6.34 (2 × s, 2H; NHCONH), 6.00 (t, ^3J(H,H) = 5.4 Hz, 1H; NHCO) ppm; ^{13}C NMR (63 MHz, $CDCl_3$): $\delta = 19.2$, 19.5 (2 × CH_3), 29.7 (6 × CH_3 *t*Bu), 24.2, 25.6, 26.9, 27.9, 28.0, 29.2, 29.5, 29.6, 29.8, 29.9, 35.9, 36.9, 37.1, 39.4, 40.4 (22 × CH_2), 29.1, 32.6 (2 × CH), 55.4 (CHS), 60.1, 61.6 (2 × CHNH), 65.1 (d, ^2J(C,P) = 6 Hz, CH_2OP), 81.8 (d, ^2J(C,P) = 7 Hz, 2 × COP), 163.7 (NHCONH), 173.0 (CONH) ppm; ^{31}P NMR (101 MHz, $CDCl_3$): $\delta = -9.7$ (s, $CH_2OPO(OtBu)_2$) ppm; HRMS: calcd. for $C_{40}H_{79}N_3O_6PS$ $[M+H]^+$ *m/z*: 760.5427; found: 760.5427.

20-*N*-Biotinylamino-3,7-dimethyleicosanyl dihydrogenphosphate (38): TFA (1 mL) was added to **37** (5 mg, 6.6 μmol) and the reaction mixture was stirred for 10 min. The TFA was evaporated under reduced pressure to give **38**, which was co-evaporated with toluene (3 × 3 mL). ^{31}P NMR (101 MHz, $CDCl_3$): $\delta = -10.0$ (s, $CH_2OPO(OH)_2$); HRMS: calcd. for $C_{32}H_{63}N_3O_6SP$ $[M+H]^+$ *m/z*: 648.4175; found: 648.4177.

20-*N*-(Biotinyl-*N*-ε-aminocaproyl)-amino-3,7-dimethyleicosanyl-phosphate di-*tert*-butyl ester (40): A solution of **36** (35 mg, 0.066 mmol) in anhydrous DMF (1 mL) was added to a stirred solution of biotinyl-*N*-ε-aminocaproyl-*N*-hydroxysuccinimide ester (30 mg, 0.066 mmol) in anhydrous DMF (1 mL) and stirred for 16 h. The solvent was evaporated under reduced pressure and the residue was purified by flash column chromatography (CH_2Cl_2/EtOH, 9:2) to obtain **40** as a white solid (30 mg, 52%). M.p. 165 °C; ^{1}H NMR (250 MHz, $CDCl_3$): $\delta = 0.86$ (d, ^3J(H,H) = 6.4 Hz, 3H; CH_3), 0.92 (d, ^3J(H,H) = 6.5 Hz, 3H; CH_3), 1.23 – 1.79 (m, 46H; CH_2, CH), 1.50 (s, 18H; CH_3 *t*Bu), 2.24 (m, 4H; CH_2CON), 2.77 (d, ^2J(H,H) = 12.7 Hz, 1H; CH_2S), 2.96 (dd, ^2J(H,H) = 12.7 Hz, ^3J(H,H) = 4.9 Hz, 1H; CH_2S), 3.25 (m, 5H; CH_2N, CHS), 4.01 (m, 2H; CH_2OP), 4.35, 4.55 (2 × m; 2H, 1H; CHN), 5.50, 6.31 (2 × s, 2H; NHCONH), 5.94, 6.31 (2 × t, ^3J(H,H) = 5.2 Hz, 2H; NHCO) ppm; ^{13}C NMR (63 MHz, $CDCl_3$): $\delta = 19.8$, 20.1 (CH_3), 30.2 (6 × CH_3 *t*Bu), 24.7, 25.6, 25.9, 26.7, 27.4, 28.3, 29.5, 29.8, 30.1, 36.1, 36.8, 37.7, 39.5, 40.0, 40.9 (27 × CH_2), 29.7, 33.2 (2 × CH), 55.9 (CHS), 60.7, 62.2 (2 × CHNH), 65.7 (d, ^2J(C,P) = 6 Hz; CH_2OP), 82.4 (d, ^2J(C,P) = 7 Hz; 2 × $OC(CH_3)_3$), 162.5 (NHCONH), 173.1, 173.2 (CONH) ppm; ^{31}P NMR (101 MHz, $CDCl_3$): $\delta = -8.5$ (s, $CH_2OPO(OtBu)_2$) ppm; HRMS: calcd. for $C_{46}H_{90}N_4O_7SP$ $[M+H]^+$ *m/z*: 873.6268; found: 873.6261.

20-*N*-(Biotinyl-*N*-ε-aminocaproyl)-amino-3,7-dimethyleicosanyl dihydrogenphosphate (41): TFA (1 mL) was added to **40** (5 mg, 5.7 μmol) and the reaction mixture was stirred for 10 min. The TFA was evaporated under reduced pressure to give **41**, which was co-evaporated with toluene (3 × 3 mL). HRMS: calcd. for $C_{38}H_{74}N_4O_7SP$ $[M+H]^+$ *m/z*: 761.5016; found: 761.5025.

20-(3′-Mercaptopropionylamido)-3,7-dimethyleicosanylphosphate di-*tert*-butyl ester (43): γ-Thiobutyrolactone (92 μL, 1.05 mmol) and dithiothreitol (68 mg, 0.44 mmol) were added to a solution of **36** (92 mg, 0.17 mmol) in degassed aqueous sodium bicarbonate (0.5 M, 1 mL) and EtOH (1 mL). The reaction mixture was stirred at 50 °C under a nitrogen atmosphere for 6 h. The reaction mixture was then acidified with 1 M HCl to pH 7 and CH_2Cl_2 (5 mL) was added. The organic layer was separated and the aqueous layer was extracted with CH_2Cl_2 (2 × 5 mL). The combined organic layers were washed with water, dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue was purified by flash column chromatography (petrol ether/ethyl acetate, gradient from 5:1 to 1:1) to obtain **43** as a sticky white solid (24 mg, 22%). ^{1}H NMR (250 MHz, $CDCl_3$): $\delta = 0.81$ (d, ^3J(H,H) = 6.5 Hz, 3H; CH_3), 0.87 (d, ^3J(H,H) = 6.5 Hz, 3H; CH_3), 1.12 – 1.35 (m, 35H; CH_2, CH, SH), 1.46 (s, 18H; *t*Bu), 1.93 (quintet, ^3J(H,H) = 7.0 Hz, 2H; $HSCH_2CH_2CH_2CO$), 2.29, 2.55, 2.58 (3 × t, ^3J(H,H) = 7.0 Hz, 4H; CH_2CON, $HSCH_2$), 3.20, 3.22 (2 × t, ^3J(H,H) = 7.0 Hz, 2H; CH_2N), 3.96 (m, 2H; CH_2OP), 5.60 (br s, 1H; NH) ppm; ^{13}C NMR (63 MHz, $CDCl_3$): $\delta = 19.3$, 19.6 (2 × CH_3), 29.8 (6 × CH_3 *t*Bu), 24.0, 24.2, 26.8, 27.0, 29.4, 29.5, 29.7, 29.9, 34.6, 36.9, 37.0, 37.2, 39.4, (20 × CH_2), 29.2, 32.6 (2 × CH), 65.1 (d, ^2J(C,P) = 6 Hz, CH_2OP), 81.8, (d, ^2J(C,P) = 7 Hz, 2 × $OC(CH_3)_3$), 171.8 (CONH) ppm; ^{31}P NMR (101 MHz, $CDCl_3$): $\delta = -9.0$ (s, $CH_2OPO(OtBu)_2$) ppm; HRMS: calcd. for $C_{34}H_{71}NO_5PS$ $[M+H]^+$ *m/z*: 636.4791; found: 636.4769.

Thiopropyl sepharose conjugate 44: Activated thiopropyl sepharose 6B gel (700 mg, degree of functionalization: 18 – 31 μmol 2-pyridyl disulfide per mL of drained gel) was re-swollen in water (6 mL) for 15 min at room temperature whereafter the additives were washed away with water (150 mL) added in small portions over 15 min. A solution of **43** (15.7 mg, 24.7 μmol) in EtOH (5 mL) was added to the gel resuspended in water (2.5 mL) and the reaction mixture was rotated for 16 h. The resin was filtered and washed with EtOH (25 mL) and water (25 mL) in small portions. The solvent of the filtrate was evaporated and the residue was dissolved in CH_2Cl_2 (30 mL).

For recording of a calibration curve of **43** by LC – MS, the following solutions of **43** in acetonitrile/water (9:1) were prepared: 0.030 mM, 0.020 mM, 0.015 mM and 0.010 mM. By means of integration of the chromatogram of the molecular peak the number of moles in the filtrate (30 mL) could be established. It contained 6.36 μmol of **43**, which left a total yield for coupling on solid phase of 18.3 μmol (28 – 48%) with 2.1 mL of gel containing 37.8 – 65.1 μmol active groups.

Thiopropyl sepharose conjugate, deprotected (45): TFA (6 mL) was added to the thiopropyl sepharose conjugate **44** (18.3 μmol) and the reaction mixture was shaken for 10 min. The resin was filtered, washed with water (60 mL), with EtOH (60 mL) and again with water (60 mL) in small portions. The resin **45** was stored as a suspension in water (4.5 mL) at 4 °C.

Fermentation of *Saccharomyces cerevisiae*:[31] A saturated culture (10 mL) of *Saccharomyces cerevisiae* (Baker's yeast, derived from a single colony) in buffered mineral dextrose (BMD) medium was used as a 2 v/v% inoculum for BMD medium (500 mL). The culture was grown at 30 °C and 250 rpm for 16 h. The cells were harvested by centrifugation (3000 rpm, 25 °C, 10 min) and were resuspended in BMD medium (200 mL). The cell suspension was then added to the fermenter with sterilized Basal Salts medium (2 L) containing 2 w/v% glucose. The media was adjusted to pH 5.0 with NH_4OH. Sterilized PTM_1 trace salts solution (8.7 mL) and a sterilized vitamin solution (20 mL), consisting of calcium pantothenate (120 mg mL^{-1}), inositol (600 mg mL^{-1}), pyridoxine HCl (120 mg mL^{-1}) and thiamine HCl (120 mg mL^{-1}), were then added to the medium. The cells were grown at 30 °C with aeration and agitation and were continuously supplemented with a sterile glucose solution (25 w/v%) containing PTM_1 trace salts solution (6 mL) and vitamin solution (1 mL) over 48 h. Cells were harvested by centrifugation at 4000 rpm for 20 min at 4 °C to yield wet cells (490 g). The cell pellet was divided into 20-g portions and stored at − 80 °C.

Preparation of yeast microsomes:[5] A 20-g batch of cells was thawed and resuspended in tris(hydroxymethyl)aminomethane (Tris)/Mg^{2+} buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, 10 mM mercaptoethanol, pH 7.5), to a total volume of 40 mL. The cells were disrupted in a French press at 40 000 psi. The cellular debris was removed by centrifugation at 4000 rpm for 20 min at 4 °C. Centrifugation of the supernatant at 18 500 rpm for 45 min at 4 °C yielded pellets containing microsomes. Aliquots were stored at − 80 °C prior to assay for Dol-P-Man synthase. Protein content was measured by Bradford assay.[32]

Enzymatic assay of 2, 18, 29, 31, 35, 38 and 41 for Dol-P-Man synthase:[5] Acceptor lipids (100 μM) were each incubated with crude microsomes (1 – 2 mg), GDP – mannose (20 μM), GDP – [U-^{14}C]mannose (0.1 μCi mL^{-1}) and buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, 10 mM mercaptoethanol, 0.5% Triton-X-100 (v/v), pH 7.5, 1 mL) at 37 °C for 1 h. Aliquots (100 μL) were removed into an equal volume of $CHCl_3$/MeOH (1:1, v/v). The aqueous phase was removed after centrifugation. The organic phase was washed twice with water and incorporation of radioactivity into organic soluble material was measured by scintillation counting.

Enzymatic assay of biotinyl compounds 38 and 41 on monomeric avidin – agarose for Dol-P-Man synthase and cleavage of products: Substrates **38** and **41** (100 μM) were linked to monomeric avidin – agarose by incubation in buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, 10 mM mercaptoethanol, 0.5% Triton-X-100 (v/v), pH 7.5, 1 mL) at 37 °C for 1 h. Resins were washed ten times with buffer and then incubated with crude microsomes (1 – 2 mg), GDP – mannose (20 μM), GDP – [U-^{14}C]mannose (0.042 μCi mL^{-1}) and buffer (1 mL) at 37 °C for 1.5 h. Suspensions were centrifuged and resins were washed ten times with water. Cleavage was achieved by washing of the resins with buffer containing biotin (1 mg mL^{-1}) fifteen times. The combined buffer fractions were subjected to scintillation counting.

Enzymatic assay with thiopropyl sepharose conjugate 45 for Dol-P-Man synthase and cleavage of products from the solid support: Thiopropyl sepharose conjugate **45** (500 μM) was incubated with microsomes (3 – 4 mg), GDP – mannose (1 mM), GDP – [U-^{14}C]mannose (0.0625 μCi mL^{-1}) and buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, 0.5% Triton-X-100 (v/v), pH 7.5, 2 mL) at 37 °C for 16 h. Incubation was continued for 5 h after a further addition of microsomes (3 – 4 mg). Resin was washed eight times with water and divided into two equal parts. Incorporation of radioactivity into one resin portion was measured by scintillation counting. Cleavage was achieved by heating of the remaining resin with aqueous mercaptoethanol (770 mL, 10 v/v%) at 50 °C for 16 h. After centrifugation, resin was washed four times with EtOH and the combined liquid fractions were subjected to scintillation counting.

For LC – MS analysis the assay was carried out simultaneously without radiolabelled GDP – mannose. Combined EtOH washings were used for measurements undiluted.

LC – MS analysis: For all measurements a C18 reversed-phase column (150 × 2.00 mm, 3μm) was used. Samples (injection volume 50 μL) were run with a water/acetonitrile gradient from 50:50 to 5:95 at a flow rate of 0.2 mL min^{-1}. Samples for a calibration curve of **43** were prepared as described before and scanned for positive ionisation from mass 50 to 800 (cone voltage 35, source temperature 140 °C). Assay samples were scanned for negative ionisation (cone voltage 45) under previous conditions.

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