Synthesis of a membrane-spanning lipophilic porphyrin with links to two alamethicin fragments on each face

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We have successfully synthesised α,β,α,β-tetrakis(2-amino-5-dodecyloxyphenyl)porphyrin in high yield. Its porphyrin precursor with nitro groups was synthesised without the use of high dilution. The dodecyloxy groups of this precursor increased its solubility. The yield of the desired α,β,α,β-atropisomer of the nitro-precursor could be enriched to 60% by treatment in hot toluene. The reduction of the nitro groups to the amino groups was carried out at room temperature. Thus, α,β,α,β-tetrakis(2-amino-5-dodecyloxyphenyl)porphyrin was synthesized under mild conditions on a multigram scale. This porphyrin possesses two amino groups and two dodecyloxy groups on each face of the porphyrin plane. It was combined with a fragment of alamethicin, a typical membrane peptide, to give a porphyrin–polypeptide hybrid, in which two hydrophobic polypeptides existed on each face of the porphyrin. This conjugate was successfully embedded into the lipid bilayer membrane. The resulting mixed vesicle showed the CD profile of a helical peptide. Thus, a membrane-penetrating columnar structure of α,β,α,β-tetrakis(2-peptidyl-5-dodecyloxyphenyl)porphyrin in the lipid bilayer membrane was suggested.

In nature, the porphyrin framework is part of a variety of biomolecules such as hemes, cytochromes and chlorophylls.[1] Differences in the central metal, degree of ring saturation (chlorophylls and bacteriochlorophylls), ligand coordination to the metal, *etc*., account for the diverse functions and reactivities of porphyrins.[2] Extensive studies have elucidated relationships between the structure and function of porphyrins by the design of sophisticated systems as artificial models, for instance, of O_2 complexation,[3] redox catalysis,[4] energy transport[5] and electron transfer.[6] However, in living systems porphyrins are combined with proteins. These proteins appropriately arrange porphyrins with other coenzymes and often with electron donors or reactants.[7] This ordering of the porphyrins and other functional groups is responsible for their various reactivities. So far, not many model systems have been able to mimic the detailed arrangements of porphyrins and substrates,[8] probably due to synthetic limitations. Besides making efforts to reproduce the natural activity of porphyrins using artificial low molecular weight models, it is still important to carry out structural model studies. In the lipid bilayer membrane the membrane protein is conformationally fixed and the layers are separate from each other. The membrane-penetrating region of the membrane protein consists exclusively of hydrophobic helical peptides.[9] Helical peptides are now routinely synthesised and characterised due to the development of peptide chemistry.[10] By using template-assembled synthetic protein methodology,[11,12] Åkerfeldt *et al.*[13] and ourselves[14] have conjugated porphyrins with helical peptides and successfully incorporated them into the lipid bilayer membrane.

From the pioneering work of Collman's picket fence porphyrin,[15,16] the atropisomers of tetrakis(2-aminophenyl)-porphyrin (TAPP) and their derivatives have often been used as templates on which to tether a variety of functional groups.[17–25] By using the α,α,α,α-atropisomer of TAPP, four helical peptide chains can be combined on one face of the porphyrin plane.[14c] The porphyrin ring occupies the base of the columnar structural conjugate (Fig. 1).[23] We proposed that if we could attach the membrane peptides to both faces of the porphyrin, we could obtain a porphyrin–polypeptide conjugate in which the porphyrin ring lies in the middle. If we could then embed such a conjugate into a lipid bilayer membrane, the porphyrin would be located in the middle of the membrane, isolated from the outer aqueous phase (Fig. 1).[23] This would be interesting because 1) porphyrin prosthetic groups often exist under hydrophobic conditions in proteins or lipids; and 2) in the future, we would be able to connect functional molecules, such as electron carriers, to the peptide as a porphyrin-substrate arranged system.

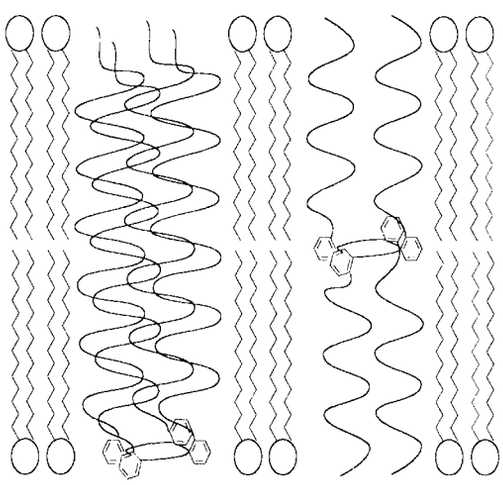

Fig. 1 Illustration of polypeptide–porphyrin conjugates in a lipid bilayer membrane: (left) four helices on one face of the porphyrin; (right) two helices on each face of the porphyrin.

We selected the α,β,α,β-atropisomer of tetrakis(2-aminophenyl)porphyrin (α,β,α,β-TAPP) as a promising candidate onto which to attach the membrane peptides. The α-helix chain is about 10 Å in diameter, so two α-helices would almost cover the porphyrin plane. One disadvantage of α,β,α,β-TAPP as the peptide template is the insoluble nature of its synthetic intermediate, tetrakis(2-nitrophenyl)porphyrin (TNPP), although the reduction of TNPP to TAPP occurs smoothly.[15] We

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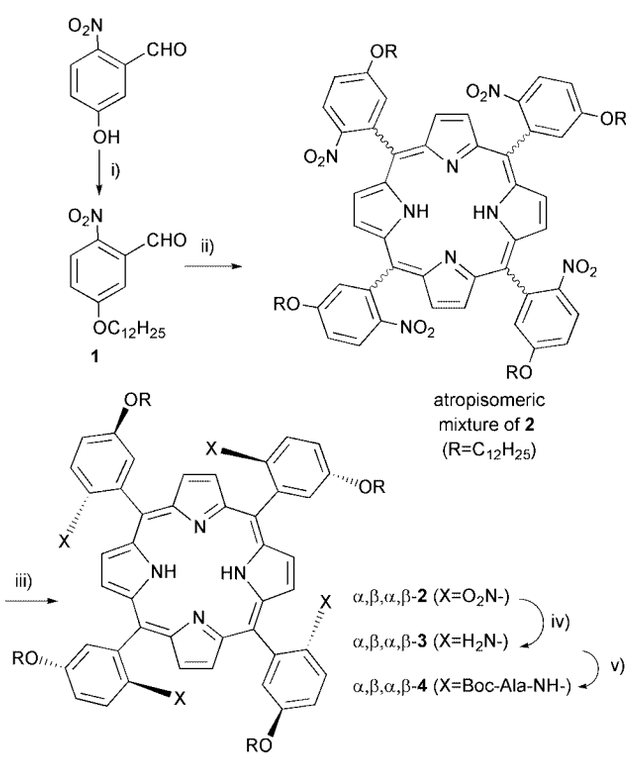
have previously discovered a convenient method to enrich α,β,α,β-TNPP and its derivatives, which involves heating of the atropisomeric mixtures of TNPP in toluene.[26] In later work, Rose *et al*. have refined this thermal isomerisation methodology using hot naphthalene.[27] Thus, α,β,α,β-TNPP derivatives should be available in high yield. However, α,β,α,β-TNPP is highly insoluble and its handling is still tedious. We felt that if we could attach long chain alkoxy groups to TNPP to increase its solubility, and still enrich its α,β,α,β-atropisomer, we could have a better method to obtain the α,β,α,β-TNPP derivative in high yield. Furthermore, the substitutional alkoxy groups might help the porphyrin–polypeptide conjugate penetrate the lipid bilayer membrane. We now wish to report the successful synthesis of α,β,α,β-(alkoxy-linked)TNPP and its conjugation with a typical membrane peptide, alamethicin.

Results and discussion

Porphyrin synthesis

The usual Williamson reaction produced a benzaldehyde derivative with 2-nitro and 5-dodecyloxy groups (2-nitro-5-dodecyloxybenzaldehyde **1**). The modified Lindsey's method with a pyrrole concentration of 0.20 mol dm^{-3} gave a tetraphenylporphyrin derivative with four nitro and four dodecyloxy groups (tetrakis(2-nitro-5-dodecyloxyphenyl)porphyrin **2**) in moderate yield (14% yield after silica gel chromatography) (Scheme 1).[28,29]



Scheme 1 Synthesis of α,β,α,β-**4**. *Reagents and conditions*: i), $C_{12}H_{25}$-Br, K_2CO_3; ii), pyrrole, TFA then DDQ; iii), isomerisation; iv), $SnCl_2$, H^+; v), Boc-Ala-OH, DCC. Compounds **2**–**4**, R = $C_{12}H_{25}$.

Tetraphenylporphyrin derivatives with *ortho*-substituents exist as atropisomeric mixtures, due to phenyl ring rotation (Scheme 2).[15,16,26,27,30–32] In their case the atropisomers could be separated by HPLC and silica gel chromatography. After porphyrin synthesis of **2** from **1** and pyrrole, the crude reaction mixture was chromatographed over silica gel by eluting with CH_2Cl_2 to remove the non-porphyrin materials. The atropisomeric mixture of **2** thus obtained was analysed by HPLC (Fig. 2, silica gel column, toluene–20% hexane). Fig. 2 shows three signals with retention times of 1.54, 3.12 and 10.60 min (the peak ratio was 24 : 43 : 33), which revealed the α,β,α,β-, α,α,β,β-

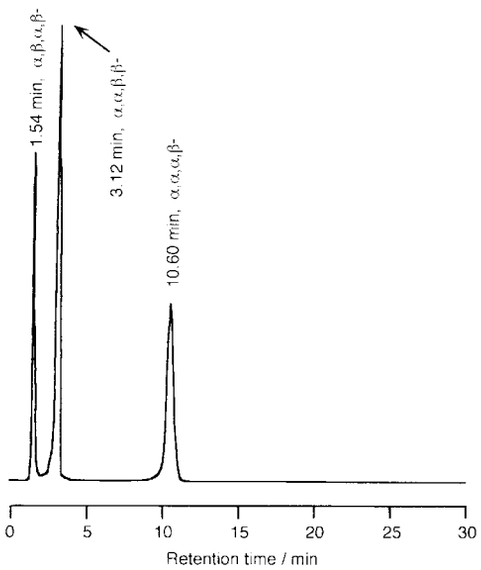
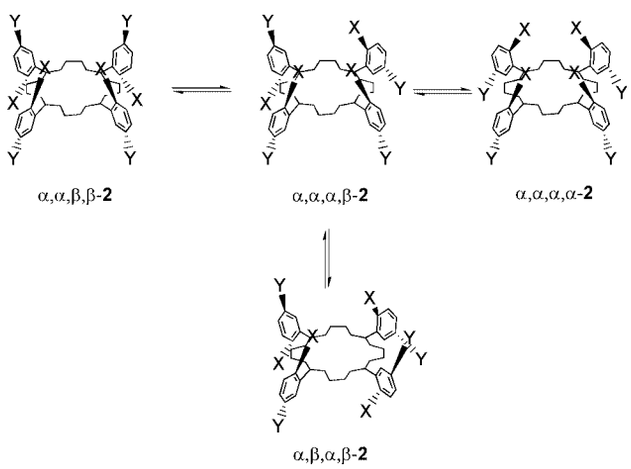


Fig. 2 HPLC profile of **2** after synthesis on a Waters μBondasphere 5μ Si-100 Å column eluting with 1.0 cm^3 min^{-1} of toluene–20% hexane (v/v), detection at 420 nm.



Scheme 2 Atropisomerism of **2**. X = NO_2, Y = $OC_{12}H_{25}$.

and α,α,α,β-atropisomers of **2**, respectively (see below). These three isomers of **2** were successfully separated by silica gel column chromatography.

A ^{1}H NMR evaluation determined the structures of these three isomers. In the α,β,α,β-atropisomer of **2** (α,β,α,β-**2**), eight pyrrole protons were equivalent and also the four phenyl groups were equivalent (Fig. 3). In fact, the isomer that eluted at 1.54 min by HPLC showed one pyrrole signal and one set of phenyl ring signals (Table 1). In α,α,β,β-**2**, two pyrroles (Ha) were situated between Phα and Phβ and the other two pyrroles (Hb) between Phα and Phα (or Phβ and Phβ). In this α,α,β,β-**2**, the four phenyl rings were equivalent, being situated between Ha and Hb. In fact, the isomer that eluted at 3.12 min showed two pyrrole signals and one set of phenyl ring signals. In α,α,α,β-**2**, two pyrroles (Ha) were situated between Phα and Phβ and the other two pyrroles (Hb) between Phα and Phα. In this α,α,α,β-**2**, there were two types of phenyl rings, two that were situated between Ha and Hb, and the other two between Ha and Ha (or Hb and Hb). In fact, the isomer that eluted at 10.60 min showed two pyrrole signals and two sets of phenyl ring signals. Thus, the three isomers were unambiguously determined to be the α,β,α,β-, α,α,β,β- and α,α,α,β-isomers in the order of HPLC elution. The least polar isomer was α,β,α,β-**2**, eluting first by HPLC and having the largest R_f value in the silica gel TLC. We expect the reason for this is that the dipole moments of the nitro groups in α,β,α,β-**2** probably cancelled each other. The basic

nitro groups of α,β,α,β-**2** could hardly interact with the silica gel because of the steric bulk of the dodecyloxy groups. The next isomer was α,α,β,β-**2**, and α,α,α,β-**2** was the most polar of the three.

It should be noted here that α,α,α,α-**2**, the most polar isomer of the possible four, was not detected, either after the synthesis (Fig. 2) or during the thermal isomerisation of any other isomer (see below). The steric hindrance of the alkoxy groups might explain this.

The ratio of the atropisomers of **2**, α,β,α,β-:α,α,β,β-:α,α,α,β-, was 24:43:33 after the synthesis. The presumed statistical abundance for the isomers, α,β,α,β-:α,α,β,β-:α,α,α,β-:α,α,α,α- is 1:2:4:1 (12.5:25:50:12.5, α,α,β,β- is equivalent to α,β,β,α-; α,α,α,β- is equivalent to α,α,β,α-; α,β,α,α- and α,β,β,β-).[15] The atropisomeric mixture of **2** after the synthesis clearly shifted to the less-polar isomers.

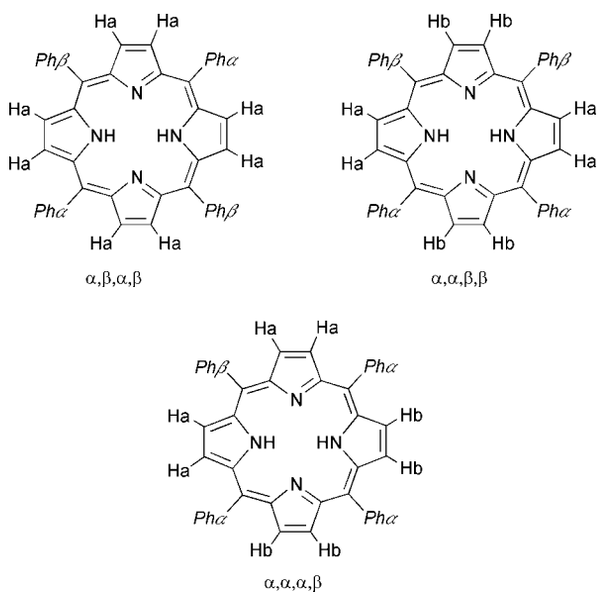


Fig. 3 ^{1}H NMR equivalency for the pyrrole protons of the atropisomeric **2**. *Phα* and *Phβ* denotes the α-Ph and β-Ph rings, respectively. Ha is a pyrrole proton situated between *Phα* and *Phβ*. Hb is a pyrrole proton situated between *Phα* and *Phα* (or *Phβ* and *Phβ*).

The three atropisomers of **2** were isolated by silica gel chromatography, then thermally isomerised. Isomerically pure **2** was injected into toluene pre-heated to 373 K, then a small aliquot was analysed by HPLC at appropriate intervals. Fig. 4 shows the thermal atropisomerisation profiles of the α,β,α,β- (○), α,α,β,β- (□) and α,α,α,β- (△) isomers of **2**. Each isolated isomer reached the equilibrium mixture of α,β,α,β-:α,α,β,β-:α,α,α,β- = 60:16:24 after about 4 h. Fig. 4(b) shows the intermediacy of α,α,α,β-**2** (△) during the isomerisation of α,α,β,β-**2** (□) to α,β,α,β-**2** (○). Fig. 4(c) shows that α,α,α,β-**2** isomerised both to α,α,β,β-**2** and α,β,α,β-**2** during the initial stage, then the produced α,α,β,β-**2** isomerised to α,β,α,β-**2**.

From the initial stage of the isomerisations, the first order rate constants, k_1, and the free energy of activation, $\Delta G^{\ddagger}$, were determined (Table 2). These results indicated the slower isomerisation of α,β,α,β-**2** relative to α,α,β,β-**2** and α,α,α,β-**2**. Isomerisation of α,β,α,β-**2** to α,α,α,β-**2** (the initial product, see Scheme 2) includes rotation of the phenyl group of αβα to ααα. Isomerisation of α,α,β,β-**2** to α,α,α,β-**2** includes the phenyl rotation of αββ to ααβ. The latter rotation should be faster and proceed with a lower $\Delta G^{\ddagger}$ due to steric effects. Isomerisation of α,α,α,β-**2** was indeed slightly faster than that of α,α,β,β-**2**. Isomerisation of α,α,α,β-**2** includes α,α,α,β- to α,β,α,β- (ααα to αβα mode) and α,α,α,β- to α,α,β,β- (ααα to αβα). Therefore, isomerisation of α,α,α,β-**2** was slightly faster than that of α,α,β,β-**2** and showed a similar $\Delta G^{\ddagger}$ value to that of α,α,β,β-**2**.

In order to compare these with our previous results, α,β,α,β-**2** was thermally isomerised at 353 K in toluene. The $\Delta G^{\ddagger}$ value of α,β,α,β-**2** at 353 K was 124.3 kJ mol^{-1} ($k_1 = 2.96 \times 10^{-6}$ s^{-1}), whereas the $\Delta G^{\ddagger}$ of the α,β,α,β-tetrakis(2-nitrophenyl)-porphyrin (α,β,α,β-TNPP) at 353 K was 110 kJ mol^{-1} ($k_1 = 1.19 \times 10^{-4}$ s^{-1}).[26b] The dodecyloxy group at the 5-position slowed the rotation of the phenyl ring. However, the equilibrium ratio of **2** was similar to that of TNPP (α,β,α,β-:α,α,β,β-:α,α,α,β-:α,α,α,α- = 64:14:22:0). TNPP is highly insoluble and hot naphthalene has been proposed as its

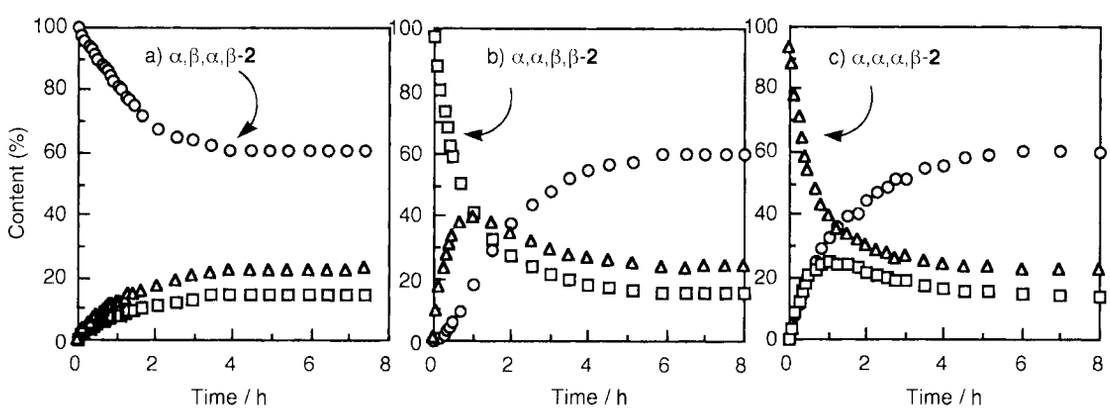


Fig. 4 Thermal isomerisation profiles of (a) α,β,α,β-**2** (O), (b) α,α,β,β-**2** (□) and (c) α,α,α,β-**2** (△) in toluene at 373 K.

Table 1 Experimental data on the atropisomers of **2**

		α,β,α,β-**2**	α,α,β,β-**2**	α,α,α,β-**2**
^{1}H NMR[a]	Pyrrole-β	8.634 (s, 8 H)	8.651 (s, 4 H), 8.607 (s, 4 H)	8.642 (s, 4 H), 8.620 (s, 4 H)
	3-Ph	8.518 (d, 4 H)	8.536 (d, 4 H)	8.570 (d, 2 H), 8.548 (d, 2 H)
	4-Ph	7.405 (d, 4 H)	7.391 (d, 4 H)	7.518 (d, 2 H), 7.492 (d, 2 H)
	6-Ph	7.689 (d, 4 H)	7.593 (d, 4 H)	7.683 (d, 2 H), 7.576 (d, 2 H)
HPLC[b]		1.54	3.12	10.60
TLC[c]		0.90	0.58	0.10
Content[d] (%)		24 (60)	43 (16)	33 (24)

[a] ^{1}H NMR chemical shift in ppm in $CDCl_3$. [b] Retention time/min in silica gel HPLC (toluene–20% hexane). [c] R_f value in TLC (benzene–20% hexane). [d] Content after synthesis (content after thermal isomerisation).

Table 2 Rate constant k_1 and activation free energy $\Delta G^{\ddagger}$ for the atropisomerisation of **2**[a]

Starting isomer	Initial product	$10^4\ k_1/s^{-1}$	$\Delta G^{\ddagger}$/kJ mol^{-1}	Mode of ring rotation[b]
α,β,α,β-**2**	α,α,α,β-**2**	0.25	124.9	αβα to ααα
α,α,β,β-**2**	α,α,α,β-**2**	1.23	120.0	αββ to ααβ
α,α,α,β-**2**	α,β,α,β-**2**/α,α,β,β-**2**	1.32	119.7	ααα to αβα/ααβ

[a] In toluene at 373 K. [b] See text.

isomerisation solvent.[27a] In contrast, **2** is quite soluble in most solvents, so the ease of its purification and handling were compensation for its longer isomerisation time. We have previously synthesized tetrakis(2-nitro-5-methoxycarbonylphenyl)-porphyrin to obtain a quite soluble derivative of TNPP.[26c] Unfortunately, the equilibrium ratio of this porphyrin, α,β,α,β-: α,α,β,β-: α,α,α,β-: α,α,α,α-, was 43 : 21 : 36 : 0 after thermal treatment and the α,β,α,β-content after thermal treatment (43%) was lower than in the case of **2** (64%). The methoxycarbonyl group attached opposite to the nitro group in this porphyrin probably weakened the dipole moment of the nitro groups, therefore, the α,β,α,β-isomer was not satisfactorily enriched. Thus, **2** proved to be the best porphyrin for obtaining the α,β,α,β-isomer bearing the four nitro anchor, which was highly soluble and could be obtained in good yield.

The isomerisation conditions of **2** were briefly examined. In refluxing toluene (383 K), **2** reached equilibrium after 0.5 h, with the same isomer ratio as obtained at 373 K. In CCl_4 (343 K), **2** reached equilibrium with the ratio of α,β,α,β-: α,α,β,β-: α,α,α,β-: α,α,α,α- = 29 : 29 : 42 : 0. In refluxing benzene (353 K), the equilibrium ratio was 37 : 1 : 62 : 0. In *p*-xylene (373 K), the equilibrium ratio was 51 : 22 : 27 : 0. For the examined solvents, toluene gave the best enrichment of the α,β,α,β-isomer.

The preparative synthesis of α,β,α,β-**2** was carried out with 2.5 g of the atropisomeric mixture of **2** in toluene (383 K, 7 h), by which time the α,β,α,β-content was 63%. The chromatographic separation of the equilibrium mixture gave 1.4 g of pure α,β,α,β-**2**, which means that 58% of the atropisomeric mixture was obtained as the α,β,α,β-form. The undesirable atropisomers were recovered (0.50 g), which actually could be converted to the α,β,α,β-rich equilibrium mixture after a second treatment in hot toluene.

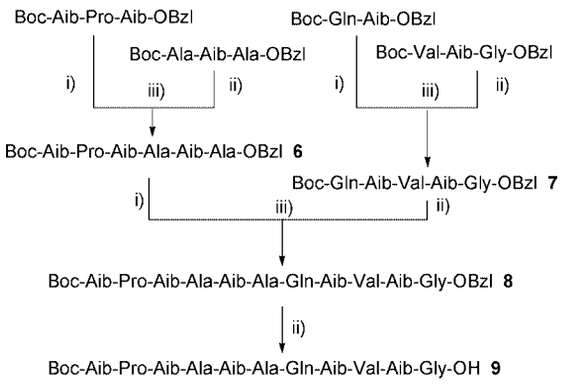
The four nitro groups of **2** were reduced by $SnCl_2{\cdot}2H_2O$ to give tetrakis(2-amino-5-dodecyloxyphenyl)porphyrin. Conventional reduction of TNPP to TAPP includes the use of $SnCl_2{\cdot}2H_2O$ in hot aqueous HCl.[15] We could not apply this method to α,β,α,β-**2**, because 1) **2** is highly lipophilic and insoluble in that solvent and 2) at high temperatures, α,β,α,β-**2** and the product α,β,α,β-**3** might isomerise. Therefore, we tried various milder conditions to reduce **2**. In a highly acidic medium (4 mol dm^{-3} HCl in dioxane) with 1% concentrated aqueous HCl, the quantitative use of $SnCl_2$ (12 equivalents relative to α,β,α,β-**2**) reduced α,β,α,β-**2** to α,β,α,β-**3** at room temperature without noticeable side reactions. The HPLC and ^{1}H NMR analyses indicated that the obtained α,β,α,β-**3** was atropisomerically pure. Addition of a small amount of aqueous HCl was essential, otherwise some side reactions did occur (reduction of the porphyrin ring was detected by UV-Vis spectroscopy). Side reactions also occurred when using an excess amount of $SnCl_2$.

Boc-Ala-OH[33] was attached to the NH_2 groups of α,β,α,β-**3** under mild conditions (ice-water temperature, overnight) to form **4** (for clarity, the atropisomeric nomenclature α,β,α,β- is omitted for the porphyrins after **4**). The symmetrical anhydride method, *i.e.*, the pre-treatment of Boc-Ala-OH with DCC then the addition of α,β,α,β-**3**, gave the best results.[34] (Coupling by the activated ester method (mixing Boc-Ala-OH, DCC, HOBt and then α,β,α,β-**3**)[35] failed to form **4**, probably due to poor basicity of the α,β,α,β-**3** NH_2 groups.) After silica gel chromatography, **4** was obtained in 81% yield which was pure in the C4 column HPLC. ^{1}H NMR also confirmed the atropisomeric purity of **4** obtained by this method, though **4** was not soluble in $CDCl_3$, therefore the spectrum was obtained in $(CD_3)_2SO$. In $(CD_3)_2SO$, the pyrrole protons were often observed as two sets of signals, probably due to the slow migration of the porphyrin inner NHs. In fact, the ^{1}H NMR spectrum of **4** in $(CD_3)_2SO$ showed two pyrrole proton signals, however, only one set of phenyl ring signals was present. The 2D (^{1}H–^{1}H) COSY spectrum and 1D variable temperature (VT) spectra were also helpful. From the 2D COSY spectrum (data not shown), the spin–spin couplings between Ala-NH (δ 6.21), Ala-CαH (δ 3.16, overlapped in the solvent peak in 1D but clearly detected in 2D) and Ala-CH_3 (δ −0.14) were observed. The porphyrin ring current caused a high-field shift for the Ala-CH_3 signal. VT NMR was also useful for distinguishing the three singlet peaks around 8.6 ppm. The signal at δ 8.66 (at 373 K) shifted the most with increase in temperature ($\Delta\delta = -4.8 \times 10^{-3}$ ppm K^{-1} *i.e.*, −4.8 ppb K^{-1}), whereas those at 8.61 ($\Delta\delta = 0.6$ ppb K^{-1}) and 8.75 ($\Delta\delta = 0$ ppb K^{-1}) shifted only slightly. The former signal was identified as Ph–NH, which is involved in hydrogen bonding with $(CD_3)_2SO$, and so is most significantly affected by the change in solvent structure caused by increase in temperature.[36] The latter two singlets were identified as the pyrrole protons. Thus, the ^{1}H NMR spectrum of **4** in $(CD_3)_2SO$ was unambiguously assigned, suggesting that **4** was atropisomerically pure and that the aromatic ring had not rotated during the reaction of α,β,α,β-**3** with Boc-Ala-OH. To further ascertain whether **4** was atropisomerically pure, the thermal isomerisation of **4** was attempted in toluene at 373 K. After 6 h, the HPLC analysis showed ~5% of new peak, at the retention time of 30.7 min, in addition to the peak of **4** (25.7 min). The FAB MS spectrum of this thermally treated sample was identical to the spectrum of **4**. These facts suggest that some atropisomerisation of **4** occurred in hot toluene, which supports the fact that **4** was the atropisomerically pure α,β,α,β-form to begin with. Unfortunately, further thermal treatment of this sample caused some degradation, probably Boc group removal (suggested by FAB MS). From these results, we concluded that **4** was the α,β,α,β-form. The CD spectrum of **4** showed a weak induced signal in the porphyrin region.[34]

Synthesis of the porphyrin–polypeptide conjugate

Next, we synthesized a conjugate of the dodecyloxyphenyl-porphyrin **4** with a model membrane peptide. We have already reported the synthesis of the template-assembled alamethicin bundle, which emulates the pore structural ion channel corresponding to the ion potentials.[37] Because alamethicin is rich in 2-aminoisobutyric acid (Aib), this peptide tends to predominantly form a helical structure in the lipid bilayer membrane.[38] Alamethicin involves no amino acid residues with ionic side chains, therefore, this peptide and its fragment are probably monomeric in MeOH. Because the conjugate would bear the peptides on both faces of the porphyrin, we utilized the C-terminal 11 residue fragment (-Aib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-) from the 20 residue alamethicin, considering the thickness of the lipid bilayer. The C-terminus of the fragment was Gly to avoid racemisation during coupling with the porphyrin.

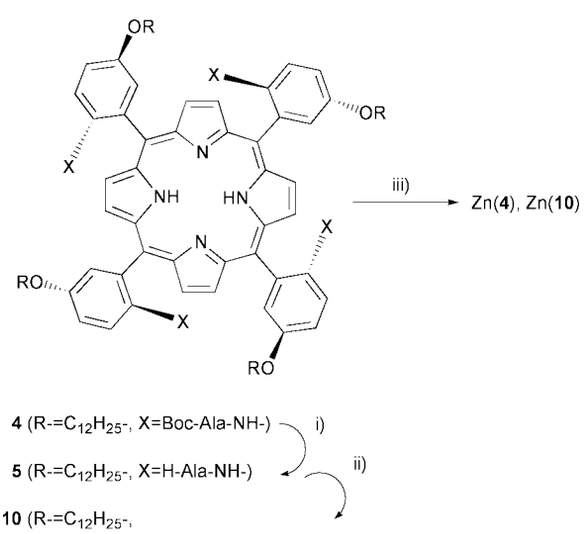
The protected alamethicin fragment **8** was synthesised according to Balaram's strategy *via* solution phase syntheses (Scheme 3).[38a] Where possible, the coupling reactions of



Scheme 3 Synthesis of 11-residue alamethicin fragment **9**. *Reagents and conditions*: i) H_2/Pd; ii) TFA; iii) DCC–HOBt.

the peptide were done with the Pro or Aib positions as the C-terminus to avoid racemisation. Coupling of the N-protected amino acid Boc-Aib-OH and the C-protected amino acid H-Pro-OBzl·HCl with DCC–HOBt in DMF afforded the dipeptide Boc-Aib-Pro-OBzl. The N-terminus Bzl protection was removed with H_2/Pd in MeOH, then further coupling with H-Aib-OBzl·TosOH afforded Boc-Aib-Pro-Aib-OBzl. The N-terminus Bzl protection was again removed and then coupling with the tripeptide H-Ala-Aib-Ala-OBzl·TFA afforded Boc-Aib-Pro-Aib-Ala-Aib-Ala-OBzl **6**. This hexapeptide was chromatographed over silica gel and its purity was confirmed by the C18 HPLC. The pentapeptide Boc-Gln-Aib-Val-Aib-Gly-OBzl **7** was synthesized similarly. Then Boc-Aib-Pro-Aib-Ala-Aib-Ala-OH (generated from **6**) was coupled with H-Gln-Aib-Val-Aib-Gly-OBzl·TFA (generated from **7**) with DCC–HOBt in DMF. Although the reaction was slow (ice-water temperature, 72 h), the reaction gave a clean product which was purified with Sephadex® LH-20 SEC eluting with MeOH and then further purified with C18 HPLC. The obtained 11-peptide **8** was characterised by FAB-MS and ^{1}H NMR (1D and 2D).

The C-terminus Bzl group of **8** was removed with H_2/Pd in AcOH to yield the 11-peptide **9**. This alamethicin fragment **9** (2 × excess was used) was then coupled to **5** (generated by the removal of the Boc groups of **4**) in DMF (Scheme 4). In this reaction, one of the most efficient coupling reagents, HATU,



Scheme 4 Synthesis of porphyrin–polypeptide conjugate **10**. *Reagents and conditions*: i) TFA; ii) **9**, HATU; iii) $Zn(OAc)_2$.

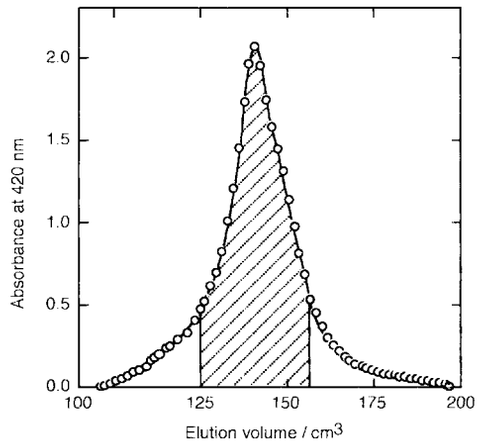


Fig. 5 Elution profile of the porporphyrin–polypeptide conjugate **10** in Sephadex® LH-60 (2.4 × 90 cm) SEC eluting with MeOH. The reaction mixture between **5** and **9** was purified once with Sephadex® LH-60 SEC and the appropriate fractions were collected. This elution profile shows the second SEC. Fractions of 125 to 156 cm^3 (shaded area) were collected.

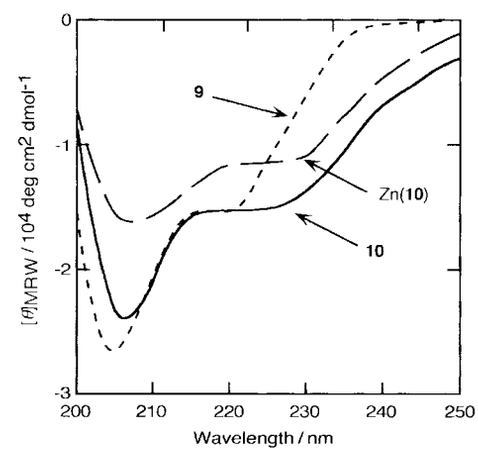


Fig. 6 CD spectra of porphyrin–polypeptide conjugate **10** (——), its Zn complex Zn(**10**) (– – –) and polypeptide **9** (- - -) in MeOH (7.5 μmol dm^{-3} for **10** and Zn(**10**), 30 μmol dm^{-3} for **9**).

was used.[39] The disappearance of **5** was confirmed after 96 h (TLC). The crude reaction product was separated from the free peptide and the reagents by Sephadex® LH-60 SEC eluting with MeOH. A second Sephadex® treatment showed almost exclusively one peak (Fig. 5) and fractions containing only the desired product (checked by analytical SEC) were collected, thus giving a 71% yield of **10**. The Zn complex of **10** (Zn(**10**)) as well as the Zn complex of **4** (Zn(**4**)) was prepared in order to analyse the porphyrin–polypeptide conjugate based on the UV-Vis spectra.

Characterisation of 10 in MeOH

The UV-Vis spectrum of **10** in MeOH was somewhat red-shifted relative to **4** (Table 3), both in the Soret and Q regions. This fact might suggest that **10** was in a somewhat assembled structure in MeOH, where some interaction between the porphyrin rings might result in a red- or blue-shifted spectrum.[40] Another possible reason for the red-shifted absorption of **10** was that the near-by peptide moiety afforded a polar environment for **10**.[41] The Zn complex of **10** (Zn(**10**)) also showed a somewhat red-shifted spectrum compared to the corresponding porphyrin Zn complex (Zn(**4**)).

The CD spectra of **10** in MeOH (7.5 μmol dm^{-3} for **10**) showed a typical Cotton effect for a helical peptide (Fig. 6), with the double minima at 207 nm and 225 nm (Table 3).[38a,42] $[\theta]_{MRW}$ values ($[\theta]_M$ per amino acid residue) at around 222 nm

Table 3 UV-Vis and CD spectra of porphyrin **4**, polypeptide **9** and the conjugate **10** in MeOH and in vesicle

Compound	Solvent	λ_{max}/nm [a]					λ_{min}/nm ($[\theta]_{MRW}$/deg cm^2 dmol^{-1}) [b]	
4	MeOH	419	514	546	590	652		
Zn(**4**)	MeOH	425		556	594			
10	MeOH	421	516	550	590	648	207 (−23600)	225 (−15400)
Zn(**10**)	MeOH	427		558	596		207 (−16700)	225 (−11500)
9	MeOH						205 (−26600)	219 (−15500)
10 [c]	Vesicle	431	523	555	592	645	205	225

[a] UV-Vis spectra. [b] CD spectra. [c] The mixed vesicle obtained from **10** and L-α-phosphatidylcholine (1 : 200) in 20 mmol dm^{-3} TRIS buffer.

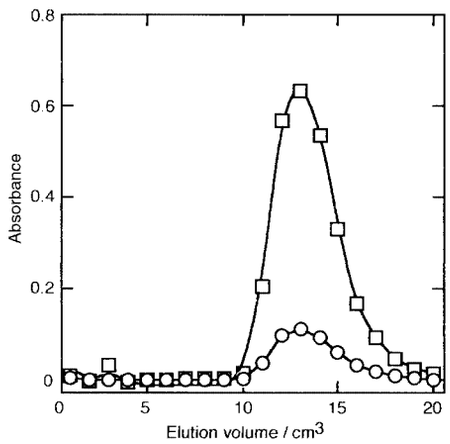


Fig. 7 Elution profile of the mixed vesicle obtained from porphyrin–polypeptide conjugate **10**/L-α-phosphatidylcholine (1 : 200) in Sephadex® G-75 (1.5 × 20 cm) SEC eluting with 20 mmol dm^{-3} TRIS buffer (pH 7.4) detected at 210 (□) and 431 nm (○).

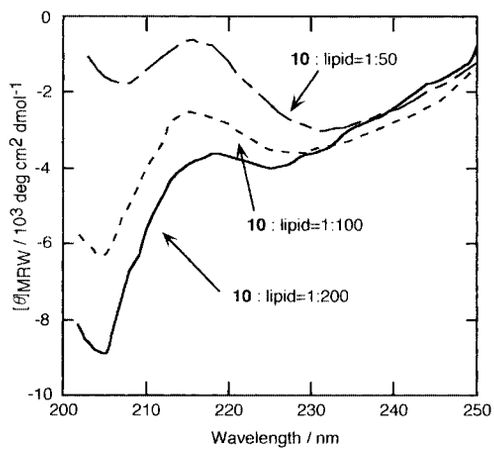


Fig. 8 CD spectra of the mixed vesicle obtained from the porphyrin–polypeptide conjugate **10**/L-α-phosphatidylcholine **10** in the molar ratio of (1 : 200 ——), (1 : 100 – –) and (1 : 50 –·–) in 20 mmol dm^{-3} TRIS buffer (pH 7.4).

are often employed to evaluate the helicity of a peptide.[42,43] For **10**, the $[\theta]_{MRW}$ at 225 nm was −15400 deg cm^2 dmol^{-1}, which was similar to that of peptide **9** ($[\theta]_{MRW}$ at 219 nm was −15500, 30 μmol dm^{-3} for **9**). One would therefore expect the helicity of the peptide moiety of **10** to be similar to that of **9**. However, the $[\theta]_{min}$ for **10** was red-shifted to 225 nm (relative to **9**, 219 nm) and was observed as a large peak compared to **9**. Such a red-shift in the CD spectra was also observed in our former template-assembled alamethicin bundle.[37] If the porphyrin template structure of **10**, bound to two peptide chains on each face, is correct the two peptide chains have a chance to interact with each other. Because alamethicin and its fragments reportedly take a 3_{10} helix structure in solution,[38b] the large Cotton effect of **10** at 225 nm might support a somewhat assembled helix structure in **10**. The UV-Vis spectrum of **10**, which suggested the interaction of porphyrins, also supported the assembled structure of **10** in MeOH. These facts suggested that **10** may well have the columnar structure (at least as a monomer unit) depicted in Fig. 1. Fig. 6 also showed that Zn(**10**) was less helical than **10** or **9** suggesting that the polar Zn-porphyrin group may distort the helical structure.

Incorporation of 10 into the lipid bilayer membrane

The alamethicin-tethered porphyrin **10** was successfully incorporated into the unilamellar vesicles of egg yolk L-α-phosphatidylcholine. The mixed vesicle was prepared by admixing **10** and egg yolk L-α-phosphatidylcholine in pH 7.4 buffer solution with sonication to obtain a clean solution of the mixed vesicle.[36,44] Fig. 7 shows the SEC profile (Sephadex® G-75, pH 7.4 TRIS buffer) of the vesicle obtained in this way (molar ratio of **10** : L-α-phosphatidylcholine was 1 : 200). The porphyrin (431 nm) and the lipid (210 nm) eluted at the same elution volume indicating the successful incorporation of **10** into the lipid bilayer membrane. Its Aib-rich peptide chain and dodecyloxy groups may cause **10** to be stably embedded in the lipid. The red-shifted λ_{max} (around 10 nm) in the UV-Vis spectra of **10** in the vesicle and the decreased absorption (ε at 431 nm was *ca.* 220000) clearly indicate that the porphyrin rings are in a hydrophilic environment, though an exact ε was not obtained due to the turbidity of the sample. So, the porphyrin ring in the vesicle might well exist in an environment more hydrophilic than MeOH. Fig. 8 shows the CD spectra of the porphyrin–polypeptide conjugate **10** in the vesicle in the same buffer. Some helix-like CD profiles were observed with the $[\theta]_{min}$ at 225 and 205 nm. This indicates that the peptide moiety of **10** takes a helical structure in the vesicle, probably penetrating the membrane. When the molar ratio of **10** : L-α-phosphatidylcholine was increased from 1 : 200 to 1 : 100 and 1 : 50, the retention times of the obtained mixed vesicles were the same, indicating that the size of the mixed vesicle was independent of the amount of **10**. However, the CD spectra of the mixed vesicle were different (Fig. 8). This is probably because the mixed vesicle became unstable as the content of **10** increased, therefore, some light scattering might occur. It is interesting that Zn(**10**) did not form a stable mixed vesicle with L-α-phosphatidylcholine under the same conditions. The SEC analysis of the attempted preparation of the mixed vesicle of Zn(**10**) and L-α-phosphatidylcholine showed that only the lipid formed the vesicle and Zn(**10**) lay on top of the SEC column. This suggests that for embedding Zn(**10**) into the lipid bilayer membrane was unfavourable. The peptide moiety of Zn(**10**) shown by CD spectroscopy to be less helical, which might suggest some aggregation through the peptide moiety may occur.

Conclusions

We have synthesized α,β,α,β-tetrakis(2-amino-5-dodecyloxyphenyl)porphyrin α,β,α,β-**3** by a straightforward procedure in high yield. The tetranitroporphyrin precursor **2** was synthesised using the modified Lindsey's method without high dilution. Its dodecyloxy groups increased its solubility and their steric and electronic effects allowed the easy enrichment of the desired α,β,α,β-atropisomer in hot toluene. Quantitative reduction of

the nitro groups to the amino groups was performed under mild conditions. Thus, α,β,α,β-**3** was synthesized under mild conditions on a multigram scale. Because α,β,α,β-**3** possessed two amino groups on each face of the porphyrin plane, it could tether two functional groups to each face to form a columnar structure with the porphyrin at its middle. Four chains of alamethicin fragments, a hydrophobic membrane peptide rich in Aib, were combined to give the porphyrin–polypeptide hybrid **10**, in which two polypeptides (and two dodecyloxy groups) existed on both faces of the porphyrin. This conjugate was successfully incorporated into the L-α-phosphatidylcholine vesicle. The mixed vesicle showed a CD profile for the helical peptide, thus a membrane penetrating columnar structure of **10** in the lipid bilayer membrane was suggested. In order to fix the porphyrin into the lipid membrane or the micelle, various groups have studied lipophilic tailored porphyrins.[45] Our system includes the hydrophobic polypeptides to which some functional group could be connected. The construction of a further sophisticated system combined with more rigorous analyses is under way.

Experimental

Materials and methods

HPLC analysis was carried out using a Hitachi L-7100 intelligent pump equipped with a Hitachi L-7420 UV-Vis detector. The analyses were performed on 1) a Waters μBondasphere® 5 μ Si-100 Å (4.6 × 250 mm) column eluting with toluene–20% (v/v) hexane (1.0 cm^3 min^{-1}) and detection at 420 nm; 2) same as 1) but eluting with toluene–1% propan-2-ol; 3) a Wakogel® C4 4.6 × 150 mm column eluting with a linear gradient of H_2O–30% CH_3CN–0.1% TFA to CH_3CN–0.1% TFA over 15 min, then CH_3CN–0.1% TFA (1.0 cm^3 min^{-1}) and detection at 420 nm; 4) a MS-GEL® C18PAC DF-5-120 Å 4.6 × 150 mm column eluting with a linear gradient of H_2O–10% CH_3CN–0.1% TFA to CH_3CN–0.1% TFA over 30 min (1.0 cm^3 min^{-1}) and detection at 220 nm. Semi-preparative HPLC was carried out on a Wakopak® WS-II 5C18 100 × 250 mm column eluting with H_2O–50% CH_3CN–0.1% TFA (3.0 cm^3 min^{-1}) and detection at 220 nm. Analytical size exclusion chromatography was performed on a TSKgel® G3000H_{XL} (7.8 × 300 mm) eluting with DMF (0.8 cm^3 min^{-1}) and detection at 420 nm. TLC analyses were performed on Merck 5715 plates. Typical eluents for the TLC were 1) benzene–20% hexane, 2) benzene–10% CH_3CN, 3) $CHCl_3$–10% MeOH, 4) $CHCl_3$–10% MeOH–2% AcOH and 5) $CHCl_3$–20% MeOH–4% AcOH. FAB MS spectra were obtained with a JEOL SX-102 mass spectrometer. High-resolution MS spectra (HIMS) were calibrated with CsI. ^1H NMR spectra were measured with a JEOL JNM α-500 spectrometer operating at 500.00 MHz and the chemical shifts were determined with respect to TMS. The spectra was obtained with 32768 data points over 20 ppm spectrum range, resulting in the 0.31 Hz (6.1 × 10^{-4} ppm) resolution. VT-NMR was performed at 298, 303, 308, 313 and 318 K. UV-Vis spectra were recorded on a Hitachi U-2010 spectrophotometer. CD spectra were recorded on a JASCO J-720 spectropolarimeter using a quartz cell of 1 mm pathlength at 298 K.

The amino acid derivatives and the reagents for the peptide synthesis were from Watanabe Chemical Industries, Ltd. (Hiroshima, Japan). L-α-Phosphatidylcholine (type XVI-E: from fresh egg yolk) was from Sigma. Other reagents and solvents were from Wako Pure Industries, Ltd.

Synthesis of porphyrin

2-Nitro-5-dodecyloxybenzaldehyde (1). In 20 cm^3 of DMF, 2-nitro-5-hydroxybenzaldehyde (2.0 g, 12 mmol), 1-bromododecane (8.6 cm^3, 36 mmol) and K_2CO_3 (5.0 g, 36 mmol) were mixed and refluxed for 8 h. After filtration and evaporation, the residue was dissolved in hot ethyl acetate, washed with aqueous K_2CO_3 and again evaporated. The resultant solid was recrystallised from ethyl ether, giving 3.8 g (11 mmol, 95%) of **1**. TLC, R_f ($CHCl_3$) 0.82 (Found: C, 68.3; H, 8.6; N, 4.3. Calc. for $C_{19}H_{29}NO_4$: C, 68.0; H, 8.7; N, 4.2%); δ_H ($CDCl_3$), 0.88 (3 H, t, CH_3), 1.27 (20 H, m, CH_2), 4.10 (2 H, t, OCH_2), 7.18 (2 H, m, ArH), 8.15 (1 H, d, ArH), 10.47 (1 H, s, CHO).

5,10,15,20-Tetrakis(2-nitro-5-dodecyloxyphenyl)porphyrin (2).[28,29] In 100 cm^3 of CH_2Cl_2 (distilled from K_2CO_3), **1** (6.7 g, 20 mmol), pyrrole (1.4 cm^3, 20 mmol) and TFA (0.77 cm^3, 10 mmol) were mixed. The mixture was stirred overnight and then 2,3-dichloro-5,6-dicyanobenzoquinone (3.4 g, 15 mmol) was added. After 2 h, triethylamine (1.4 cm^3, 10 mmol) was added and the mixture was evaporated. The solid was chromatographed over silica gel (6.0 × 60 cm) eluting with CH_2Cl_2. The fast-eluting red band was then collected, leaving the non-porphyrin black materials in the column. The eluent was evaporated to obtain 1.1 g of an atropisomeric mixture of **2** (0.70 mmol, 14% yield), R_{fl} 0.90, 0.58 and 0.10. The atropisomeric mixture of **2** thus obtained was chromatographed over silica gel (6.0 × 60 cm) eluting with benzene–20% hexane to separate the atropisomers.

α,β,α,β-**2**; 0.26 g (24% of atropisomeric mixture of **2**), TLC, R_{fl} 0.90; HPLC(1), 1.54 min; δ_H ($CDCl_3$), −2.49 (2 H, br s, NH), 0.86 (12 H, quintet, CH_3), 1.85 (80 H, m, CH_2), 4.12 (8 H, t, OCH_2), 7.41 (4 H, d, 4-Ph), 7.69 (4 H, d, 6-Ph), 8.52 (4 H, d, 3-Ph), 8.63 (8 H, s, py); FAB MS (3-nitrophenyl octyl ether), *m*/*z* 1532 (M^+); HIMS, Found: 1530.91856. Calc. for $C_{92}H_{122}N_8O_{12}$: 1530.91816; λ_{max} (CH_2Cl_2), 422 (relative absorption 100), 518 (6.67), 552 (2.64), 595 (2.31), 653 (1.15).

α,α,β,β-**2**; 0.46 g (43%), TLC, R_{fl} 0.58; HPLC(1), 3.12 min; δ_H ($CDCl_3$), −2.47 (2 H, br s, NH), 0.84 (12 H, t, CH_3), 1.83 (80 H, m, CH_2), 4.12 (8 H, m, OCH_2), 7.39 (4 H, d, 4-Ph), 7.59 (4 H, d, 6-Ph), 8.54 (4 H, d, 3-Ph), 8.61 (4 H, s, py), 8.65 (4 H, s, py). FAB MS and UV-Vis spectra were the same as α,β,α,β-**2**.

α,α,α,β-**2**; 0.35 g (33%), TLC, R_{fl} 0.10; HPLC, 10.60 min; δ_H ($CDCl_3$), −2.47 (2 H, br s, NH), 0.84 (12 H, t, CH_3), 1.82 (80 H, m, CH_2), 4.11 (8 H, m, OCH_2), 7.49 (2 H, d, 4-Ph), 7.52 (2 H, d, 4-Ph), 7.58 (2 H, d, 6-Ph), 7.68 (2 H, d, 6-Ph), 8.55 (2 H, d, 3-Ph), 8.57 (2 H, d, 3-Ph), 8.62 (4 H, s, py), 8.64 (4 H, s, py). FAB MS and UV-Vis spectra were the same as α,β,α,β-**2**.

Thermal isomerisation of atropisomers of 2 (analytical).[26] In a septum-capped test tube, 3.0 cm^3 of toluene was pre-heated to 383 K. A solution of isomerically pure **2** (1.0 mg) in 100 μl of toluene was added and then 20 μl of the mixture was periodically withdrawn to analyse the atropisomeric ratio by HPLC(1). At least three independent experiments were averaged to obtain the time course shown in Fig. 4. The initial stage of the reaction was analysed by the first-order rate equation; $k_1t = \ln([A_0]/[A])$, where A_0 and A denote the ratio of the specific atropisomer. The activation free energy, $\Delta G^{\ddagger}$, was determined from the equation; $\Delta G^{\ddagger} = -RT \ln (k_1h/kT)$, where h and k denote Planck's constant and Boltzmann's constant, respectively.

Preparative thermal isomerisation of atropisomer mixture of 2 to obtain α,β,α,β-2. In 500 cm^3 of toluene, 2.5 g of the atropisomeric mixture of **2** was refluxed for 7 h, after which time HPLC analysis indicated that the mixture had reached equilibrium. After evaporation, the mixture was dissolved in benzene and chromatographed over silica gel (6.0 × 70 cm) eluting with benzene–20% hexane to isolate α,β,α,β-**2** (1.5 g, 58% based on the starting atropisomeric mixture). TLC and HPLC analyses indicated that the obtained α,β,α,β-**2** was pure. After the elution of α,β,α,β-**2**, the undesirable atropisomers were eluted with CH_2Cl_2–10% MeOH to recover 0.50 g of α,α,β,β-**2** and α,α,α,β-**2**, which could be further subjected to thermal isomerisation.

α,β,α,β-5,10,15,20-Tetrakis(2-amino-5-dodecyloxyphenyl)-porphyrin (3).[15] To a solution of α,β,α,β-**2** (1.0 g, 0.65 mmol) in 100 cm^3 of 4.0 mol dm^{-3} HCl–dioxane (Watanabe Chemicals), $SnCl_2 \cdot 2H_2O$ (1.8 g, 7.8 mmol, 12 equivalents relative to α,β,α,β-**2**) and 1.0 cm^3 of concentrated aqueous HCl were added. The green reaction mixture was monitored by TLC after neutralising the TLC spots with NH_3 vapour. After 2 h, the solvent was evaporated and aqueous NH_3 (7 mol dm^{-3}, 20 cm^3) was added. This alkaline mixture was filtered and the black filtrate was then washed with CH_2Cl_2 (300 cm^3) to extract the porphyrins. The CH_2Cl_2 extract was washed with brine, dried ($MgSO_4$) and evaporated to give almost pure **3** (0.90 mg, 0.64 mmol, 98%), which could be recrystallised from CH_2Cl_2–MeOH. TLC, R_{f2} 0.78; HPLC(2), 1.30 min; δ_H ($CDCl_3$), −2.73 (2 H, br s, NH), 0.83 (12 H, t, CH_3), 1.23 (64 H, m, CH_2), 1.44 (8 H, quintet, CH_2), 1.80 (8 H, quintet, CH_2), 3.40 (8 H, br s, NH_2), 4.01 (8 H, t, OCH_2), 7.04 (4 H, d, 4-Ph), 7.21 (4 H, dd, 3-Ph), 7.50 (4 H, d, 6-Ph), 8.92 (8 H, s, py); FAB MS (3-nitrophenyl octyl ether), *m*/*z* 1412 (M^+); HIMS, Found: 1411.0254. Calc. for $C_{92}H_{130}N_8O_4$: 1411.0214.

α,β,α,β-5,10,15,20-Tetrakis(2-(Boc-Ala-NH)-5-dodecyloxyphenyl)porphyrin (4). To a solution of Boc-Ala-OH (0.67 g, 3.5 mmol) in 16 cm^3 of CH_2Cl_2, DCC (0.36 g, 1.8 mmol) was added at ice-water temperature. After 30 min, 0.31 g (0.22 mmol) of α,β,α,β-**3** was added and the mixture was stirred overnight. After evaporation, the residue was taken up in 30 cm^3 of ethyl acetate, washed successively with aqueous citric acid and aqueous $NaHCO_3$ and again evaporated. The obtained crude product was purified by silica gel chromatography (toluene–10% CH_3CN), yielding 0.38 g (0.18 mmol, 81%) of pure **4**. TLC, R_{f2} 0.53; HPLC(3), 25.66 min (Found: C, 70.4; H, 8.7; N, 7.8. Calc. for $C_{124}H_{182}N_{12}O_{16} \cdot 1H_2O$: C, 70.4; H, 8.8; N, 8.0%); δ_H ($(CD_3)_2SO$), −2.82 (2 H, br s, NH), −0.14 (12 H, s, Ala-CH_3), 0.83 (12 H, t, CH_3), 0.92 (36 H, s, Boc), 1.19 (56 H, m, CH_2), 1.32 (8 H, quintet, CH_2), 1.41 (8 H, quintet, CH_2), 1.75 (8 H, quintet, CH_2), 3.16 (overlapped with solvent, Ala-αH), 4.06 (8 H, t, OCH_2), 6.21 (4 H, s, Ala-NH, $\Delta\delta = -7.2$ ppb K^{-1}), 7.35 (4 H, s, 6-Ph), 7.41 (4 H, d, 3-Ph), 7.92 (4 H, d, 6-Ph), 8.61 (4 H, s, py, $\Delta\delta = -0.6$ ppb K^{-1}), 8.66 (4 H, s, Ph-NH, $\Delta\delta = -4.8$ ppb K^{-1}), 8.75 (4 H, s, py, $\Delta\delta = 0$ ppb K^{-1}); FAB MS (3-nitrobenzyl alcohol), *m*/*z* 2097 (M^+); λ_{max} (MeOH), 419 (ε/dm^3 mol^{-1} cm^{-1} 319000), 514 (19100), 546 (5110), 590 (5740), 652 (3300); CD (5.5×10^{-5} mol cm^{-3} in MeOH), $[\theta]_M$ at 419 nm, -5.20×10^4 deg cm^2 $dmol^{-1}$.

Preparation of the Zn complex of 4, Zn(4). A CH_2Cl_2 (0.50 cm^3) solution of **4** (10 mg, 4.8 μmol) and a MeOH (0.30 cm^3) solution of $Zn(OAc)_2 \cdot 2H_2O$ (3.5 mg, 16 μmol) were mixed and stirred vigorously for 6 h. After evaporation, the residue was taken up in CH_2Cl_2, washed thoroughly with H_2O, dried with Na_2SO_4, filtered and again evaporated. The yield of Zn(**4**) was quantitative. FAB MS (3-nitrobenzyl alcohol), *m*/*z* 2162 (M^+); λ_{max} (MeOH), 425 (ε/dm^3 mol^{-1} cm^{-1} 420000), 556 (17000), 594 (3600).

α,β,α,β-5,10,15,20-Tetrakis(2-(H-Ala-NH)-5-dodecyloxyphenyl)porphyrin (5). At ice-water temperature, 19 mg (9.1 μmol) of **4** was added to 1.0 cm^3 of TFA and the mixture was stirred for 30 min. After evaporation, the residue was washed twice with diethyl ether–petroleum ether and then dried in a NaOH desiccator. The crude **5** thus obtained (15 mg, 7.0 μmol as the **5**·TFA salt) was sufficiently pure for further synthesis. HPLC (3), 18.74 min; δ_H ($(CD_3)_2SO$), −2.85 (2 H, br s, NH), −0.39 (12 H, d, Ala-CH_3), 0.84 (12 H, t, CH_3), 1.23 (56 H, m, CH_2), 1.34 (8 H, quintet, CH_2), 1.43 (8 H, quintet, CH_2), 1.79 (8 H, quintet, CH_2), 3.06 (4H, m, Ala-αH), 4.12 (8 H, t, OCH_2), 7.41 (4 H, s, Ph), 7.50 (4 H, d, Ph), 7.71 (4H, s, H_2N-Ala), 7.74 (4H, s, H_2N-Ala), 7.84 (4 H, d, Ph), 8.59 (4 H, s, py), 8.87 (4 H, s, Ph-NH), 9.22 (4 H, s, py).

Synthesis of the alamethicin fragment[38]

Synthesis of Boc-Aib-Pro-Aib-Ala-Aib-Ala-OBzl (6) from Boc-Aib-Pro-Aib-OH and TFA·H-Ala-Aib-Ala-OBzl. In 40 cm^3 of DMF, Boc-Aib-OH (4.1 g, 20 mmol), HCl·H-Pro-OBzl (4.8 g, 20 mmol), $HOBt \cdot H_2O$ (3.1 g, 20 mmol), NEt_3 (3.4 ml, 24 mmol) and DCC (5.0 g, 24 mmol) were mixed at ice-water temperature and stirred overnight.[35] The mixture was filtered, evaporated, taken up in ethyl acetate and then washed with aqueous citric acid and aqueous $NaHCO_3$. After evaporation, the residue was solidified by adding diethyl ether–petroleum ether and then recrystallised from ethyl acetate–diethyl ether–petroleum ether to obtain sufficiently pure Boc-Aib-Pro-OBzl (5.6 g, 14 mmol, 72%). TLC, R_{f3} 0.73. To this sample of Boc-Aib-Pro-OBzl (3.8 mg), dissolved in 19 cm^3 of MeOH, 10% Pd/C (0.10 g) was added and hydrogenated with H_2 at atmospheric pressure for 5 h. After filtration and evaporation, the residue was solidified by adding diethyl ether–petroleum ether to obtain quantitatively sufficiently pure Boc-Aib-Pro-OH. TLC, R_{f4} 0.50. Boc-Aib-Pro-OH (2.9 g, 9.5 mmol) was then coupled with TosOH·H-Aib-OBzl in a similar manner to that above, yielding Boc-Aib-Pro-Aib-OBzl (4.3 g, 9.0 mmol, 95%). TLC, R_{f3} 0.49. Boc-Ala-Aib-Ala-OBzl was synthesized in a similar manner through Boc-Ala-Aib-OBzl (TLC, R_{f3} 0.67), Boc-Ala-Aib-OH (TLC, R_{f4} 0.52) and Boc-Ala-Aib-Ala-OBzl (TLC, R_{f3} 0.55).

Boc-Aib-Pro-Aib-OH was generated from Boc-Aib-Pro-Aib-OBzl (93%, TLC, R_{f3} 0.36). TFA·H-Ala-Aib-Ala-OBzl was generated by TFA treatment (ice-water temperature, 30 min) of Boc-Ala-Aib-Ala-OBzl (89%, TLC, R_{f3} 0.25). The coupling of Boc-Aib-Pro-Aib-OH and TFA·H-Ala-Aib-Ala-OBzl was similarly carried out as described above, yielding 3.6 g of crude **6**. This was chromatographed over silica gel ($CHCl_3$–2% MeOH) yielding 2.4 g of pure **6** (58% based on Boc-Aib-Pro-Aib-OH and TFA·H-Ala-Aib-Ala-OBzl). TLC, R_{f3} 0.40; HPLC(4), 23.96 min; FAB MS (glycerol), *m*/*z* 703 $(M + H)^+$.

Synthesis of Boc-Gln-Aib-Val-Aib-Gly-OBzl (7) from Boc-Gln-Aib-OH and TFA·H-Val-Aib-Gly-OBzl. Boc-Gln-Aib-OBzl was synthesized as above (75%, TLC ($CHCl_3$–5% MeOH), R_f 0.23) and then deprotected to give Boc-Gln-Aib-OH (quantitative, TLC, R_{f4} 0.19). Boc-Val-Aib-OBzl was synthesized (94%, TLC, R_{f3} 0.86) and then deprotected to give Boc-Val-Aib-OH which was solidified by adding H_2O (60%, TLC, R_{f4} 0.71). Boc-Val-Aib-Gly-OBzl was synthesized (80%, TLC, R_{f3} 0.65) and TFA·H-Val-Aib-Gly-OBzl was generated (70%, TLC, R_{f4} 0.21) in a similar way.

The coupling of Boc-Gln-Aib-OH and TFA·H-Val-Aib-Gly-OBzl was carried out in a similar manner, yielding 2.7 g of crude **7**. This was chromatographed over silica gel ($CHCl_3$–3% MeOH) yielding 2.3 g of pure **7** (69% based on Boc-Gln-Aib-OH and TFA·H-Val-Aib-Gly-OBzl). TLC, R_{f3} 0.20; HPLC(4), 18.84 min; FAB MS (glycerol), *m*/*z* 664 $(M + H)^+$.

Boc-Aib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-OBzl (8). Boc-Aib-Pro-Aib-Ala-Aib-Ala-OH was quantitatively generated from **6** (TLC, R_{f4} 0.16; HPLC(4), 18.49 min). TFA·H-Gln-Aib-Val-Aib-Gly-OBzl was quantitatively generated from **7** (TLC, R_{f4} 0; HPLC(4), 14.20 min). The coupling of Boc-Aib-Pro-Aib-Ala-Aib-Ala-OH and TFA·H-Gln-Aib-Val-Aib-Gly-OBzl was carried out at ice-water temperature for 72 h, yielding 1.3 g of crude **8**. This was first chromatographed over silica gel ($CHCl_3$–3% MeOH), then further chromatographed over Sephadex® LH-20 (2.0 × 80 cm, MeOH) SEC. Collecting the appropriate fractions gave 0.91 g of almost pure **8**. This was further purified by semi-preparative HPLC to yield pure **8** (0.26 g, 16% based on Boc-Aib-Pro-Aib-Ala-Aib-Ala-OH and TFA·H-Gln-Aib-Val-Aib-Gly-OBzl). TLC, R_{f4} 0.22, R_{f5} 0.99; HPLC(4), 24.52 min; FAB MS (glycerol), *m*/*z* (relative

intensity) 1157 (10%) $(M + H)^+$, 992 (100) $(M - NHCH_2COOBzl)^+$, 907 (25) $(M - NHC(CH_3)_2CONHCH_2COOBzl)^+$; δ_H ($(CD_3)_2SO$), 0.80 (3 H, d, Val-γH), 0.85 (3 H, d, Val-γH), 1.4 (45 H, m, Boc, Aib-βH, Ala-βH), 1.63 (1 H, m, Pro-γH), 1.86 (1 H, m, Pro-γH), 1.97 (2 H, m, Pro-βH, Gln-βH), 2.11 (1 H, m, Gln-βH), 2.26 (4 H, m, Pro-βH, Gln-γH × 2, Val-βH), 3.40 (1 H, m, Pro-δH), 3.68 (1 H, m, Gly-αH), 3.79 (1 H, m, Gln-αH), 3.90 (1 H, m, Pro-δH), 3.94 (2 H, m, Ala-αH, Gly-αH), 4.00 (1 H, m, Ala-αH), 4.10 (1 H, m, Val-αH), 4.14 (1 H, t, Pro-αH), 5.07 (2 H, s, CH_2Ph), 6.70 (1 H, br s, Gln-NH_2), 6.85 (1 H, t, Val-NH), 7.14 (1 H, br s, Gln-NH_2), 7.32 (5 H, m, CH_2*Ph*), 7.45 (2 H, m, Aib-NH, Ala-NH), 7.48 (1 H, s, Aib-NH), 7.56 (1 H, d, Ala-NH), 7.59 (1 H, s, Aib-NH), 7.76 (1 H, s, Aib-NH), 7.77 (1 H, m, Gln-NH), 7.86 (1 H, t, Gly-NH), 7.88 (1 H, s, Aib-NH).

Boc-Aib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-OH (9). In 10 cm^3 of AcOH, **8** (0.20 g, 0.17 mmol) and 10% Pd/C (0.20 g) were mixed and hydrogenated with H_2 at atmospheric pressure for 5 h. After filtration and evaporation, the residue was triturated by adding diethyl ether to obtain sufficiently pure **9** (0.16 g, 0.15 mmol, 91%). TLC, R_{f4} 0.06, R_{f5} 0.14; HPLC(4), 20.33 min; FAB MS (glycerol), *m*/*z* (relative intensity) 1068 (45%) $(M + H)^+$, 992 (100) $(M - NHCH_2COOH)^+$, 907 (54) $(M - NHC(CH_3)_2CONHCH_2COOH)^+$.

Synthesis of porphyrin–polypeptide conjugate

α,β,α,β-5,10,15,20-Tetrakis(2-(Boc-Aib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Ala-NH)-5-dodecyloxyphenyl)-porphyrin (10). To 0.30 cm^3 of DMF were added **9** (40 mg, 37 μmol), **5** (10 mg, 4.7 μmol), HATU (14 mg, 37 μmol) and diisopropylethylamine (13 mm^3, 74 μmol) at ice-water temperature.[39] The mixture was stirred at that temperature for 24 h when further HATU (14 mg, 37 μmol) and diisopropylethylamine (9.6 mm^3, 56 μmol) were added. The mixture was stirred for another 72 h then evaporated. The residue was dissolved in 0.50 cm^3 of MeOH and chromatographed over Sephadex® LH-60 (2.4 × 90 cm, MeOH) SEC. The appropriate fractions were collected, evaporated and then further chromatographed over Sephadex® LH-60. The fractions were analysed by analytical SEC and those containing only **10** were collected. The yield of **10** was 20 mg (3.4 μmol, 71%). TLC, R_{f5} 0.78; HPLC (SEC), 10.92 min; FAB MS, 5897 (M^+); λ_{max} (MeOH), 421 (ε/dm^3 mol^{-1} cm^{-1} 400000), 516 (22300), 550 (7600), 590 (7600), 648 (3200).

Preparation of the Zn complex of 10, Zn(10). A CH_2Cl_2 (0.50 cm^3) solution of **10** (2.0 mg, 0.37 μmol) and a MeOH (0.30 cm^3) solution of $Zn(OAc)_2 \cdot 2H_2O$ (0.73 mg, 3.3 μmol) were mixed and stirred vigorously for 6 h. A further solution of $Zn(OAc)_2 \cdot 2H_2O$ (0.73 mg, 3.3 μmol) in MeOH (0.30 cm^3) was added and the mixture stirred for another 6 h. After evaporation, H_2O and CH_2Cl_2 were added to the residue. The mixture was vigorously stirred and the organic layer was washed thoroughly with H_2O, dried with Na_2SO_4, filtered and then evaporated. The crude product was chromatographed over Sephadex® LH-60 (MeOH) SEC. The appropriate fractions were collected and evaporated to yield 3.5 mg (0.59 μmol, 78%) of Zn(**10**). FAB MS, 5961 (M^+); λ_{max} (MeOH), 427 (ε/dm^3 mol^{-1} cm^{-1} 310000), 558 (14100), 596 (2550).

Preparation of the vesicles incorporating the porphyrin–peptide conjugate[36,44]

In a 10 cm^3 pear-shaped flask, 0.50 cm^3 of a 10 mmol dm^{-3} solution of L-α-phosphatidylcholine in MeOH–$CHCl_3$ (1:1, v/v) and 0.50 cm^3 of a 50 μmol dm^{-3} solution of **10** in MeOH were mixed (**10**:L-α-phosphatidylcholine was 1:200). The solvent was evaporated by a stream of N_2. The dried lipid with the porphyrin–peptide conjugate was hydrated in 5.0 cm^3 of TRIS buffer (pH 7.4, 20 mmol dm^{-3}) using a bath-type sonicator (5 min). The obtained suspension was twice sonicated at 298 K for 10 min under an N_2 atmosphere using a Branson Sonifier model 250 at a 20 W intensity with 5 min intervals. The vesicle solution was kept standing for 1 h, then chromatographed over Sephadex® G-75 (1.5 × 20 cm) SEC with the same buffer.

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