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Facile synthesis of peptide–porphyrin conjugates: Towards artificial catalase

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ABSTRACT

A facile synthetic method for peptide–porphyrin conjugates containing four peptide units on one porphyrin was developed using chemoselective reactions. The key building blocks, 5,10,15,20-tetrakis(3-azidophenyl)porphyrin **1** and 5,10,15,20-tetrakis(5-azido-3-pyridyl)porphyrin **2**, were efficiently synthesized and used as substrates for two well-known chemoselective reactions, traceless Staudinger ligation and copper-catalyzed azide alkyne cycloaddition (so-called click chemistry). Both reactions gave the desired compounds, and click chemistry was superior for our purpose. To confirm the value of the established methodology, nine peptide–porphyrin conjugates were synthesized, and their catalase- and peroxidase-like activity in water was evaluated. Our synthetic strategy is expected to be valuable for the preparation of artificial heme protein models.

1. Introduction

Tetrapyrrolic macrocycles, such as heme, are ubiquitous, and heme binding sites exist within a variety of protein scaffolds, being involved in such diverse tasks as electron transfer, substrate oxidation, ligand sensing, and transport.[1,2] These natural roles of tetrapyrrolic macrocycles stimulated a search for new applications, focusing on new synthetic porphyrins. An important class of these molecules is peptide–porphyrin conjugates, in which minimal peptides are covalently attached to the porphyrins.[1,2] Some elegantly designed examples, with catalytic oxidation,[3–6] ion channel,[7] protein surface recognition,[8–10] and DNA binding[11] functionalities, have been reported.

Traditionally, peptides have been coupled to porphyrin derivatives via amide bonds using standard amide coupling reagents.[3,7–10,12] This approach requires either the presence of protective groups on side-chains, or restriction of the sequence to non-reactive residues, which hampers the systematic investigation of the effect of peptide composition on the overall properties of the molecules. To circumvent these synthetic limitations, several elegant and versatile methods have been developed, which allow rapid preparation of a wide variety of analogues for systematic investigations.

Several peptide–porphyrin conjugates have been obtained directly by solid-phase peptide synthesis. Pavone et al. synthesized porphyrin derivatives with two peptide units by direct incorporation of porphyrin on the solid support, followed by elongation of the peptide moiety.[13] Notably, peptide–porphyrin conjugates with either symmetric (same peptide sequences) or unsymmetric (different peptide sequences) structure were successfully synthesized. An amino acid containing porphyrin moiety on side chain has also been reported, and is compatible with solid-phase peptide synthesis.[14] The methodologies are versatile and elegant, but the synthesis and purification of conjugates containing long peptides could be difficult; this is a general problem of solid-phase peptide synthesis.

As another example of versatile synthesis of peptide–porphyrin conjugates, peptide-sandwiched mesohemes were synthesized in only a few synthetic steps.[15] First, the peptide was synthesized by standard solid-phase methods. The preparation of peptide–porphyrin conjugates was achieved by direct coupling of the unprotected peptides, through the Lys ε-amino group, to the carboxylate in the porphyrin ring. Mono or bis-peptide adducts were obtained by simply changing the number of peptide equivalents relative to the porphyrin. However, the peptide sequences are restricted; for example, the direct incorporation of Lys residues is impossible.

A chemoselective ligation method to generate a thioether bond between thiol (Cys in the peptide) and bromoacetamido porphyrin was also reported.[16] The peptide units can be prepared easily with standard solid-phase methodology. The synthesis is convenient and the thioether bond is fairly stable compared with disulfide[17] or thioester.[18] Many peptide–porphyrin conjugates have been synthesized using this method.[4–6,11,19] The reported examples normally contained multiple peptides, but again the peptide sequence is limited, as Cys residues are difficult to incorporate.

The above reactions enable the convenient preparation of many peptide-based heme protein models. However, alternative methodology, which could overcome the limitations of the above methods, is still needed for the development of more sophisticated heme protein

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models. Such methodology, in combination with already reported technology, should make it possible to achieve multiple substitution with different peptide sequences. Orthogonal ligation reaction would provide convenient access to multi-functionalized peptide–porphyrin conjugates, which could possess more sophisticated functions. We report here an efficient and convenient chemoselective synthesis of peptide–porphyrin conjugates with chemically stable linkages, using chemoselective Staudinger ligation and Huisgen 1,3-dipolar cycloaddition of azides and alkynes (so-called click chemistry). Novel porphyrin building blocks, 5,10,15,20-tetrakis(3-azidophenyl)porphyrin **1** and 5,10, 15,20-tetrakis(5-azido-3-pyridyl)porphyrin **2**, were efficiently synthesized as key intermediates (Fig. 1).

2. Results and discussion

Recently, the unique reactivity of the azide group has attracted much attention in various research areas, including organic chemistry, materials science, bioorganic chemistry, chemical biology, and biology.[20] The azide group shows high selectivity, which enables chemoselective reaction in the presence of other reactive functional groups. Among reactions involving the azide group, Staudinger ligation and click chemistry are the most important from the standpoint of bio-conjugation.

Staudinger ligation has been recognized as a highly chemoselective ligation method for the preparation of bioconjugates,[21,22] exploiting the smooth reaction between an azide and a phosphorane to form a phospha-aza-ylide. This ylide can be trapped by an acyl group with formation of a stable amide bond. Among reported Staudinger ligation procedures, we chose the traceless Staudinger ligation,[23,24] especially the technology introduced by Raines et al., since Raines' method has been proven to be compatible with peptide chemistry and affords simple products (Scheme 1). It is noteworthy that this reaction does not require any catalyst or additive.

Copper-catalyzed azide alkyne cycloaddition, introduced by Sharpless and co-workers[25] and Meldal and co-workers,[26] has also emerged in recent years, and is known as click chemistry. This reaction proceeds selectively and covalently in the presence of Cu(I) as a catalyst, and the chemistry is compatible with a broad array of biological functionalities. We applied this chemistry for the rapid and selective modification of porphyrin (Scheme 2).

As a key porphyrin building block for both Staudinger ligation and copper-catalyzed azide alkyne cycloaddition, 5,10,15,20-tetrakis(3-azidophenyl)porphyrin **1** was initially designed. Tetraphenylporphyrin was selected as the platform porphyrin, since it shows better chemical stability (protection from oxidation at the *meso* positions) and provides a rigid template compared with other generally used porphyrins, such as coproporphyrin. This compound contains four azide groups at the 3-position of the phenyl rings, which are available for both Staudinger ligation and copper-catalyzed azide alkyne cycloaddition. The incorporation of four peptide segments has been shown to be a promising approach to make functional heme protein models;[1,2] a number of interesting functions have been reported, for example, hydroxylase-like,[3]

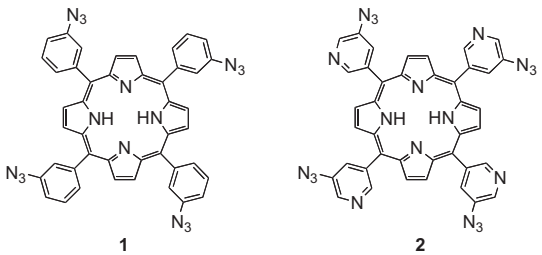
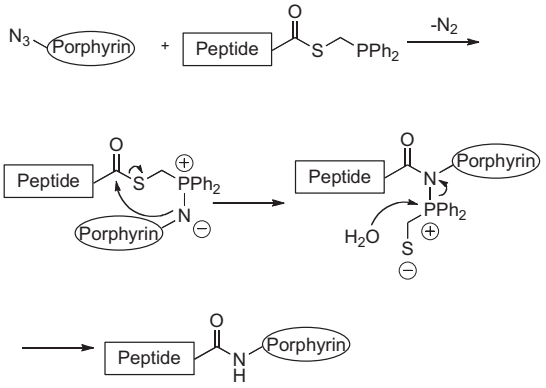
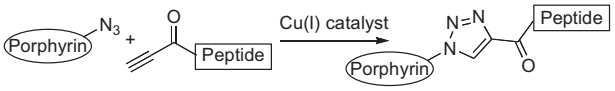


Figure 1. Designed porphyrin building blocks for chemoselective reactions.



Scheme 1. Staudinger ligation for the synthesis of peptide–porphyrin conjugate.

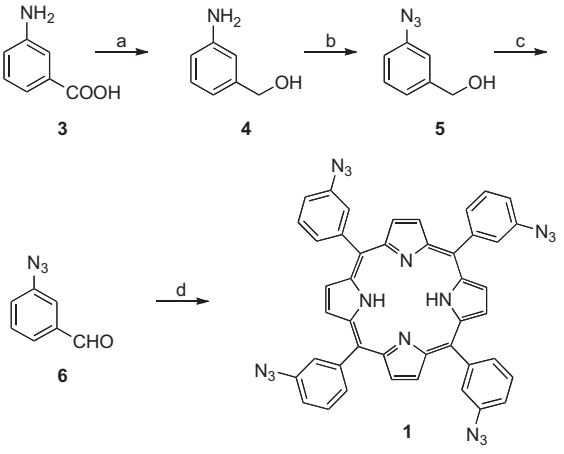


Scheme 2. Click chemistry for the synthesis of the peptide–porphyrin conjugates.

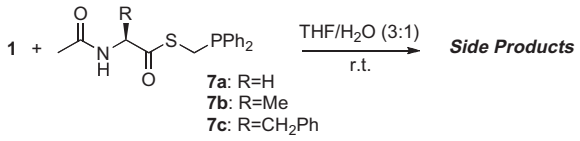
ion-channel,[7] and DNA binding[11] activities. Meta attachment provides favorable interhelical spacing as well as a degree of conformational flexibility about the interannular C–C bond, when each peptide segment folds into helical structure.[7] The desired compound **1** was successfully obtained via the synthetic route shown in Scheme 3. The intermediate 3-azidobenzaldehyde **6** was prepared from 3-aminobenzoic acid **3** in three steps in fairly good yield. Subsequent condensation with equimolar pyrrole gave the desired compound **1**.

We first conducted the Staudinger ligation with amino acid phosphinothioesters (Scheme 4). The amino acid derivatives (**7a–c**) were synthesized according to the literature.[27] The ligation of **1** with **7a–c** was carried out in THF/H_2O (3:1) at room temperature, since this condition has been used for Staudinger ligation of peptides.[24,27] After 30 h, the reaction mixtures were examined by HPLC and HRMS.

Although the small amount of the desired compounds were obtained, three side products were produced almost exclusively. These products were identified as the amino acid–porphyrin conjugates with substitution of 3, 2, and 1 amino acids, in which unreacted azide groups were reduced to amines. This result suggested



Scheme 3. Reagents and conditions: (a) $LiAlH_4$, THF, reflux, 15 h (91%); (b) (1) $NaNO_2$, HCl, 0 °C, 20 min, (2) NaN_3, H_2O, 0 °C, 30 min (96%); (c) $(C_5H_6N)_2{\cdot}Cr_2O_7$, CH_2Cl_2, rt, 14 h (83%); (d) (1) pyrrole, $BF_3{\cdot}Et_2O$, $CHCl_3$, rt, 19 h, (2) DDQ, rt, 3 h (31%).



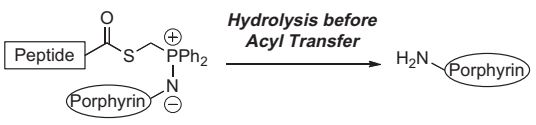
Scheme 4. Initial attempt to obtain amino acid–porphyrin conjugates.

that the *S*→*N* acyl transfer step is slow, probably due to steric hindrance, so that hydrolysis occurred before the acyl transfer step (Scheme 5).[23]

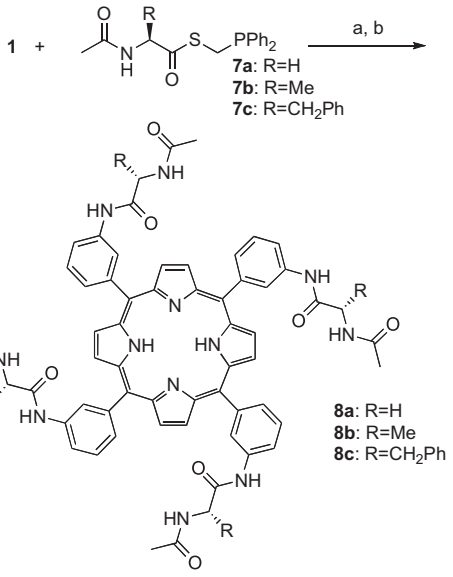
To avoid the early-stage hydrolysis pathway, absolute solvent was used for completion of the acyl transfer. Following the addition of a small amount of H_2O, hydrolysis proceeded to give the amide-linked conjugate. Among the conditions we examined, *N*-methyl-2-pyrrolidone (NMP) and DMF/THF (3:1) with molecular sieves 4A gave good results. Also, the reaction temperature needed to be raised to 50 °C, and 2 equiv (to the azide group) of amino acid derivative was necessary to complete the ligation. Under these conditions, tetra-substituted amino acid–porphyrin conjugates were obtained in good yields (Scheme 6).

Traceless Staudinger ligation with porphyrin **1** worked well, affording the desired conjugates, but three drawbacks were found, which might hamper versatile synthesis of peptide–porphyrin conjugates. First, the phosphinothioester is highly prone to oxidation. The requisite phosphine is susceptible to air oxidation,[28,29] and synthesis and purification need to be performed immediately prior to use. The C-terminal modified peptide phosphinothioester could be synthesized by solid-phase peptide synthesis with safety-catch resin according to the literature.[30] The C-terminal modification and the cleavage from the resin were performed simultaneously. The peptide phosphinothioester, unlike amino acid phosphinothioester, is difficult to synthesize on a large scale, and work-up, such as purification by HPLC and lyophilization of purified fractions, normally takes several hours. Special care would be necessary to prevent air oxidation. Second, optimization of reaction conditions is difficult. Solvents in which porphyrin **1** is soluble are not good solvents for peptide phosphinothioester, especially for polar peptides. We used NMP as a solvent, but the solubility of porphyrin **1** is still poor, and the solvent is difficult to remove due to its high boiling point. Case-by-case optimization would be required, depending on the peptide sequence. Also, complete formation of the iminophosphorane intermediate required a fairly long reaction time (12–20 h). Third, diphenylphosphinothiol, which is necessary for phosphinothioester synthesis, has a strong odor.

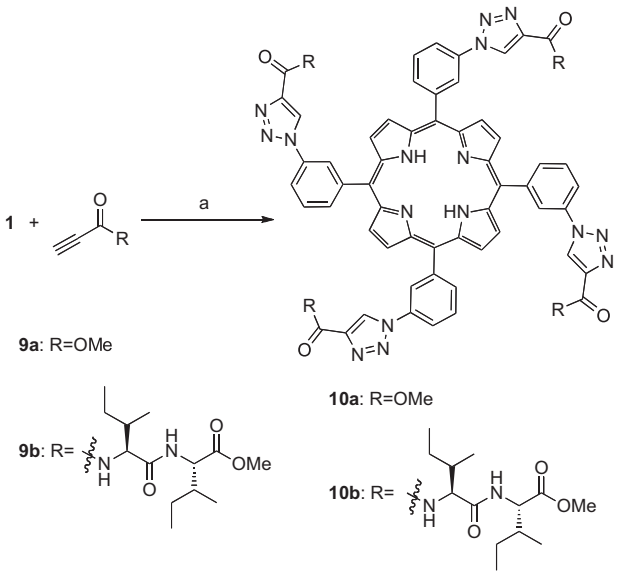
Next, we examined the synthesis of porphyrin conjugate via copper-catalyzed azide alkyne cycloaddition (Schemes 2 and 7). First, propiolic acid methyl ester was used as a substrate. When only $[Cu(CH_3CN)_4]PF_6$ was used as the catalyst, simple insertion reaction of copper into 5,10,15,20-tetrakis(3-azidophenyl)porphyrin occurred almost exclusively, and no click product was obtained. TBTA [tris(benzyltriazolylmethyl)amine] is a multidentate ligand, which forms a complex with Cu(I) and enhances the reactivity.[31] We envisioned that the use of Cu(I)–TBTA as a catalyst would suppress the metal insertion reaction into the porphyrin ring and effectively catalyze the azide alkyne cycloaddition reaction. As expected, the reaction proceeded efficiently within 2 h and gave the desired compound in high yield, with Cu(I)–TBTA complex as a



Scheme 5. Plausible side-reaction of traceless Staudinger ligation.



Scheme 6. Reagents and conditions: (a) molecular sieves 4A, DMF/THF (3:1), 50 °C, 12 h (**7a**); NMP, 50 °C, 20 h (**7b** and **7c**); (b) H_2O, rt, 3 h, (84%, **8a**; 80%, **8b**; 90%, **8c**). Yields were determined by HPLC.



Scheme 7. Reagents and conditions: (a) $[Cu(CH_3CN)_4]PF_6$, TBTA, THF, rt, 2 h (94%, **10a**; 99%, **10b**).

catalyst. The ligation reaction with peptide was then performed, and proceeded efficiently with Cu(I)–TBTA catalyst.

Recently, several groups have reported functionalization of a porphyrin ring with peptides via click chemistry. Ikawa et al. synthesized N-fused porphyrin modified with a single oligoarginine segment by direct incorporation of porphyrin into the peptide on a solid support; the side-chains were fully protected.[32] This strategy is easy and convenient, but attachment of multiple peptide segments to a single porphyrin would be difficult.

Click chemistry with Cu(I)–TBTA was found to be efficient for the preparation of peptide–porphyrin conjugates. The reaction proceeds smoothly. Both propiolic acid-modified peptide and porphyrin are stable under usual conditions and can be stored without special care. Also no odoriferous compounds are required. Thus, we decided to use click chemistry for further investigation.

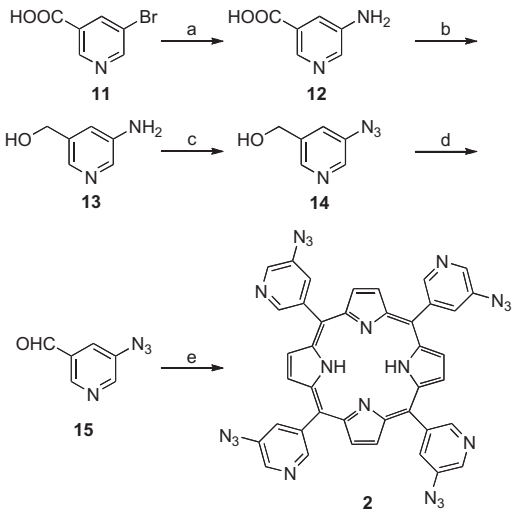
However, as mentioned above, a solubility problem with porphyrin again emerged: good solvents for peptides are poor solvents for porphyrin.

In order to circumvent the solubility problem, we designed and synthesized another porphyrin, 5,10,15,20-tetrakis(5-azido-3-pyridyl)porphyrin **2**. Tetrapyridylporphyrin is generally more soluble in polar solvents than tetraphenylporphyrin. Also, tetrapyridylporphyrin could be easily derivatized by simple alkylation of the pyridine rings to *N*-alkylpyridiniumylporphyrin, which is water-soluble. Porphyrin **2** was synthesized according to Scheme 8. Starting from 5-bromonicotinic acid **11**, amination with ammonia followed by reduction gave 3-amino-5-hydroxymethylpyridine **13**. The amino group was converted to azide to afford 3-azido-5-hydroxymethylpyridine **14** via Sandmeyer reaction. The alcohol **14** was oxidized to give 5-azido-3-pyridinecarbaldehyde **15**, and the condensation of **15** with an equimolar amount of pyrrole gave the desired porphyrin **2**.

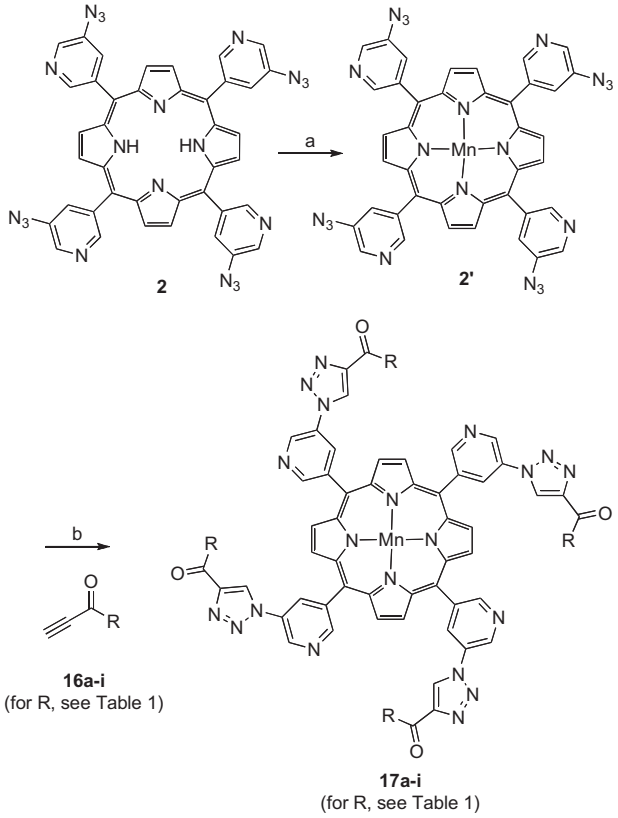
Using the newly synthesized porphyrin, we synthesized nine different peptide–manganese porphyrin conjugates and examined their catalase-like activity. Catalase catalyzes the decomposition of H_2O_2, a reactive oxygen species, to generate H_2O and O_2.

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

Mn-porphyrins are known to exhibit catalase-like activity.[33,34] Mn-porphyrins have been shown to be effective in ameliorating oxidative stress, inflammation and injury in a large number of in vitro and animal models of human disease. Recently, orally available Mn-porphyrins were reported as potential oral therapeutic agents to treat cellular damage caused by oxidative stress.[35,36] We speculated that modification of Mn-porphyrins with peptides could affect their function, since we have found that the modification of Mn–salen complex with various functionalities altered the catalase activity.[37] Manganese was inserted into porphyrin **2** and N-terminally modified peptide segments were conjugated via click chemistry (Scheme 9). The peptide segments were synthesized by Fmoc solid-phase peptide synthesis and the coupling of propiolic acid was performed with EEDQ.[26] The peptides were purified by reverse-phase HPLC. The azide alkyne cycloaddition reaction proceeded efficiently with Cu(I)–TBTA catalyst in DMSO and the conjugation products were purified by HPLC.



Scheme 8. Reagents and conditions: (a) NH_3 aq, $CuSO_4 \cdot 5H_2O$, 170–180 °C, 19 h (93%); (b) (1) $SOCl_2$, MeOH, reflux, 2 d (2) $LiAlH_4$, THF, 0 °C, 0.5 h, then rt, 2 d (69%); (c) (1) $NaNO_2$, HCl, 0 °C, 0.5 h, (2) NaN_3, 0 °C, 1 h, then rt, 2 h (86%); (d) IBX, DMSO, rt, 1 h (96%); (e) pyrrole, AcOH, 85 °C, 2 h (12%).



Scheme 9. Reagents and conditions: (a) $Mn(OAc)_2 \cdot 4H_2O$, AcOH, rt to 100 °C, 0.5 h (86%); (b) $[Cu(CH_3CN)_4]PF_6$, TBTA, DMSO, rt (for yields, see Table 1).

The synthesized peptide–porphyrin conjugates are summarized in Table 1. Citrulline (Cit), which contains a ureido group, was incorporated to the peptide segments with the aim of enhancing the catalase-like activity. We have shown that a ureido group-containing Mn–salen complex exhibited efficient catalase-like activity.[37] The yields of click chemistry ranged from 43% to 79%.

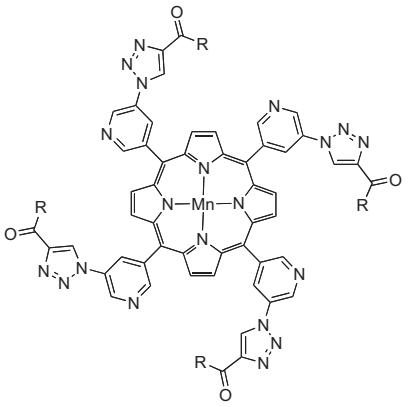
As control compounds, manganese 5,10,15,20-tetrakis(1-methyl-5-azidopyridinium-3-yl)porphyrin **18**, manganese(III) 5,10,15,20-tetrakis(3-pyridyl)porphyrin **20′** and manganese(III) 5,10,15,20-tetrakis(5-amino-3-pyridyl)porphyrin **21′** were prepared (Scheme 10–12, respectively). Also, Mn–salen complex, EUK-113,[38] was used as a control (see Supplementary data for the structure).

Catalase-like activities of the prepared Mn-porphyrins were determined in aqueous phosphate buffer solution (pH 7.4) by real-time monitoring of molecular oxygen concentration using a polarographic oxygen electrode at 25 °C (Table 2). All compounds, including control porphyrins, were soluble in neutral aqueous buffer at the concentration of 10 μM. All the Mn-porphyrins showed catalase-like activity and some conjugates, for example **17a**, showed improved activity.

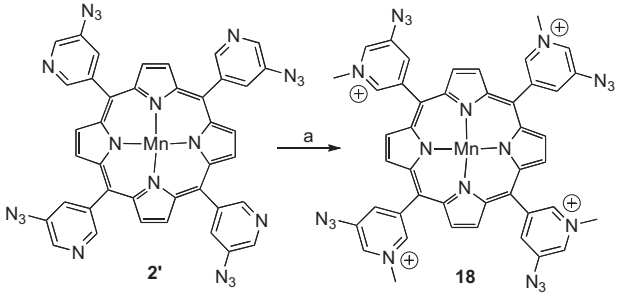
However, the enhancement of activity was not very significant and the origin of the difference is unclear, though it might be due to the flexibility of the peptide backbones. Careful design of the peptide sequence to display appropriate functional groups at precisely controlled positions might improve the activity.

Peroxidase-like activities of the prepared Mn-porphyrins were determined in potassium phosphate solution (pH 7.4, containing 0.1% DMSO) by monitoring the production of ABTS radical over time (Table 3).[38] Various metal complexes which display catalase-like activity, such as Mn–salens[36,38] and Mn-porphyrins,[36] also exhibit peroxidase-like activity. All the Mn-porphyrins showed weak peroxidase-like activities. Among the conjugates, compounds **17g**, **17h**

Table 1
Peptide–Mn-porphyrin conjugates synthesized via click chemistry



Compound	R	Yield (%)
17a	Lys-Gly-Cit-NH_2	43
17b	Lys-Gly-Gly-Cit-NH_2	48
17c	Gly-Lys-NH_2	57
17d	Gly-Gly-Lys-NH_2	53
17e	Gly-Lys-Cit-NH_2	62
17f	Gly-Cit-Lys-NH_2	60
17g	Glu-Gly-Cit-NH_2	78
17h	Gly-Gly-Glu-NH_2	79
17i	Gly-His-Gly-NH_2	56

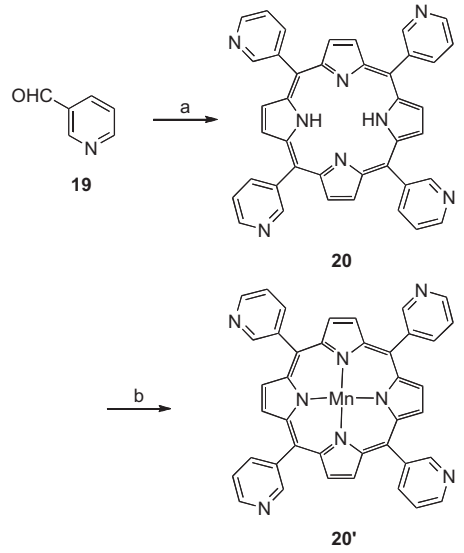


Scheme 10. Reagents and conditions: (a) TsOMe, DMF, 100 °C, 4 h (92%).

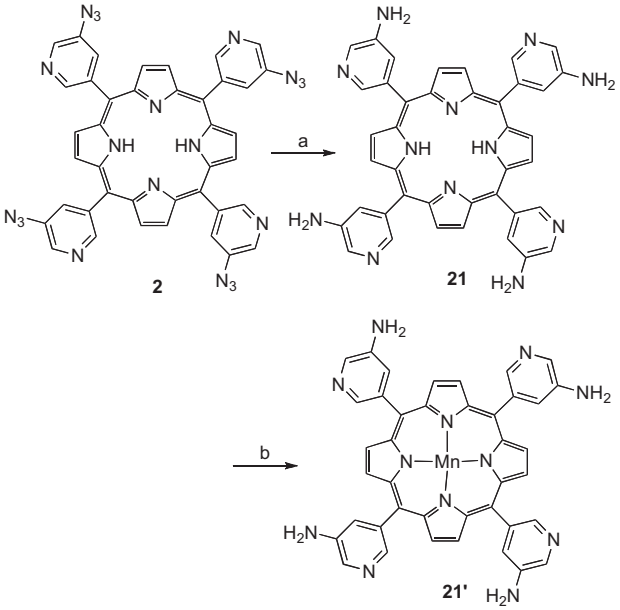
(containing Glu) and **17i** (containing His) showed higher activities than the others, even though these conjugates showed weaker catalase-like activity. This result suggests that peptide sequence could be a determinant of the peroxidase-like activity.

3. Conclusions

We have developed a convenient and versatile strategy for the preparation of peptide–porphyrin conjugates. Novel porphyrin building blocks with azide functionality, **1** and **2**, were designed and synthesized. Various other porphyrins with azide functionality should be accessible using compounds **6** and **15**. Both Staudinger ligation and click chemistry worked, though click chemistry gave better results for our purpose. Using click chemistry, we successfully synthesized nine peptide–Mn-porphyrin conjugates and then evaluated their catalase-like and peroxidase-like activities. The activities were not very striking, but appeared to be dependent upon the peptide moieties. Here, we attached the peptide segment at the N-terminus, but by using amino acids with an alkyne side chain, such as propargylglycine, modification at an arbitrary position would become possible.[26] Further study is in progress in our laboratory.



Scheme 11. Reagents and conditions: (a) pyrrole, AcOH, reflux, 2 h (16%); (b) $Mn(OAc)_2{\cdot}4H_2O$, AcOH, rt to 100 °C, 1 h (34%).



Scheme 12. Reagents and conditions: (a) PPh_3, THF/H_2O, reflux, 5 d (77%); (b) $Mn(OAc)_2{\cdot}4H_2O$, AcOH, rt to 100 °C, 20 min (52%).

4. Experimental

4.1. Syntheses

4.1.1. General

Proton nuclear magnetic resonance (^1H NMR) spectra were recorded on a JEOL JNM GSX-400 (400 MHz) pulse Fourier-transform NMR spectrometer. Chemical shifts are expressed in parts per million (ppm) downfield from internal tetramethylsilane ($\delta = 0$) and coupling constants are reported in hertz (Hz). Splitting patterns are indicated as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet. Column chromatography was carried out using BW-200 or BW-300 (Fuji Silysia, Japan; these silica gels are stable in high MeOH ratio eluent) unless otherwise noted, and thin layer chromatography (TLC) was performed on Merck precoated plates (silica gel 60 F_{254}, 0.25 mm). FAB mass spectra were recorded on

Table 2
Catalase-like activity of peptide–Mn-porphyrin conjugates

Compounds	Initial rate (μM O_2/min)	Endpoint (maximal μM O_2)
18	74	68
20′[a]	33	63
21′	42	90
EUK-113	59	37
17a	70	99
17b	58	87
17c	60	101
17d	58	103
17e	49	102
17f	60	97
17g	36	85
17h	36	92
17i[a,b]	43	51

Catalase-like activity was examined by monitoring the conversion of H_2O_2 to O_2 using a Clark-type polarographic oxygen electrode. To a solution of Mn-porphyrin (10 μM) in 50 mM sodium phosphate buffer (pH 7.4), 10 mM (final concentration) H_2O_2 was added at 25 °C under Ar.
[a] DMSO (1%, final concentration) was used.
[b] Some precipitate was appeared during the assay.

Table 3
Peroxidase-like activity of peptide–Mn-porphyrin conjugates

Compounds	Initial rate (ABTS μM/min)
18	2.77
20′	0.40
21′	0.19
EUK-113	18.52
17a	0.57
17b	0.71
17c	0.38
17d	0.44
17e	0.22
17f	0.25
17g	1.80
17h	2.07
17i	3.16

Peroxidase-like activity was examined by monitoring the conversion of ABTS to ABTS radical cation. Assay solution consisted of potassium phosphate buffer (50 mM, pH 7.4) containing 0.1% DMSO, ABTS (0.5 mM), H_2O_2 (0.2 mM) and Mn-porphyrin (10 μM).

a JEOL JMS-LCmate spectrometer. MALDI-TOF-MS spectra were recorded on a Shimadzu AXIMA-CFR, and ESI-Mass spectra were recorded on a Bruker FT-MS APEX II. Toluene, DMF, and CH_2Cl_2 were distilled over CaH_2. Cyclohexane, THF, and ether were distilled from Na. Methanol was distilled over Mg/I_2. TMEDA was distilled from *n*-butyric anhydride.

4.1.2. 3-Aminobenzyl alcohol (4)

To $LiAlH_4$ (2.10 g, 55.3 mol) was slowly added THF (20 mL) at 0 °C under an atmosphere of argon. To the mixture was slowly added 3-aminobenzoic acid **3** (5.00 g, 36.5 mmol) in THF (55 mL). The reaction mixture was stirred at 0 °C under an atmosphere of argon for 0.5 h, then refluxed in an oil bath for 15 h. The resulting mixture was cooled to 0 °C. Water (2.1 mL), 15% NaOH aq (2.1 mL) and water (6.3 mL) were added successively at 0 °C, and the mixture was stirred at room temperature. The solid in the mixture was removed by filtration through Celite. The solvent was removed under reduced pressure, and the residue was purified by column chromatography [silica, MeOH/$CHCl_3$ (3:97)] to give a yellow solid (4.09 g, 91%). Mp 90–91 °C; IR (KBr) ν 3365, 3298, 3197 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 1.25 (s, 1H), 3.71 (s, 2H), 4.61 (s, 2H), 6.49 (dd, J = 7.9, 2.3 Hz, 1H), 6.71 (s, 1H), 6.74 (dd, J = 7.6, 0.7 Hz, 1H), 7.14 (t, J = 7.6 Hz, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 65.4, 113.5, 114.4, 117.1, 117.1, 129.5, 146.7; EI-MS m/z 123 (M^+); EI-HRMS m/z M^+ calcd for C_7H_9ON, 123.06842; found, 123.06927.

4.1.3. 3-Azidobenzyl alcohol (5)

3-Aminobenzyl alcohol **4** (3.00 g, 24.4 mmol) was dissolved in 6.0 M HCl (25 mL) at 0 °C. To the mixture was added dropwise 1.2 M $NaNO_2$ aq (30 mL, 36.5 mmol) over 20 min. The mixture was stirred at 0 °C for 20 min, followed by the addition of 1.6 M NaN_3 aq (60 mL, 97.4 mmol). The reaction mixture was stirred at 0 °C for 30 min. The solution was extracted with ether (80 mL × 3). The organic layer was washed with brine, saturated $NaHCO_3$ aq and brine. The solution was dried over $MgSO_4$, filtrated and concentrated. The residue was purified by column chromatography [silica, AcOEt/*n*-hexane (1:5)] to give a yellow oil (3.49 g, 96%). IR (neat) ν 3323, 2113, 1607, 1589, 1485 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 2.34 (s, 1H), 4.64 (s, 2H), 6.93 (dd, J = 7.9, 2.0 Hz, 1H), 7.01 (s, 1H), 7.09 (d, J = 7.6 Hz, 1H), 7.31 (t, J = 7.9 Hz, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 64.63, 117.3, 123.2, 129.9, 140.3, 142.9; EI-MS m/z 149 (M^+); EI-HRMS m/z M^+ calcd for $C_7H_7ON_3$, 149.05892; found, 149.05977.

4.1.4. 3-Azidobenzaldehyde (6)

To a solution of 3-azidobenzyl alcohol **5** (2.00 g, 13.4 mmol) in dry CH_2Cl_2 (45 mL) were added Celite (7.57 g) and PDC (7.57 g, 20.1 mmol) at room temperature under an atmosphere of argon. The mixture was stirred at room temperature under an atmosphere of argon for 14 h. The solid in the mixture was removed by filtration. The solvent was removed under reduced pressure, and the residue was purified by column chromatography [silica, AcOEt/*n*-hexane (1:9)] to give a yellow oil (1.64 g, 83%). IR (neat) ν 2839–2734, 2118, 1699, 1586, 1480, 1452, 1297 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.27 (ddd, J = 7.9, 2.3, 1.3 Hz, 1H), 7.53 (t, J = 7.6 Hz, 1H), 7.55 (dd, J = 2.3, 1.3 Hz, 1H), 7.65 (td, J = 7.6, 1.3 Hz, 1H), 10.00 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 119.0, 124.9, 126.7, 130.5, 137.8, 141.5, 191.3; EI-MS m/z 147 (M^+); EI-HRMS m/z M^+ calcd for $C_7H_5ON_3$, 147.04327; found, 147.04230.

4.1.5. 5,10,15,20-Tetrakis(3-azidophenyl)porphyrin (1)

To a solution of 3-azidobenzaldehyde **6** (500 mg, 3.40 mmol) in $CHCl_3$ (332 mL) was added molecular sieves 4A (1.0 g). The mixture was bubbled with argon to remove oxygen. To the mixture were added pyrrole (181 mL, 3.40 mmol) and $BF_3{\cdot}Et_2O$ (144 mL, 1.13 mmol) at room temperature under an atmosphere of argon. The reaction flask was shielded from light and the mixture was stirred for 19 h, then DDQ (515 mg, 2.27 mmol) was added and stirring was continued for 3 h. To the resulting mixture was added Et_3N (164 mL, 1.13 mmol) and stirring was continued for 5 min. The reaction mixture was filtered through a pad of alumina (4 × 15 cm) and eluted with $CHCl_3$. The resulting mixture was evaporated and the residue was dissolved in $CHCl_3$. The solution was filtered through a pad of silica gel (4 × 15 cm), eluting with $CHCl_3$. The eluate was evaporated and the residue was purified by column chromatography [silica, CH_2Cl_2/*n*-hexane (1:1)] to give a purple solid (207 mg, 31%). Mp >300 °C; IR (KBr) ν 3453, 2122 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ −2.85 (s, 2H), 7.48 (dd, J = 7.9, 2.4 Hz, 4H), 7.74 (t, J = 7.6 Hz, 4H), 7.89 (s, 4H), 8.00 (d, J = 7.3 Hz, 4H), 8.85 (s, 8H); ^{13}C NMR (125 MHz, CD_2Cl_2) δ 119.0, 119.5, 125.5, 128.5, 131.7, 139.3, 144.0; FAB-MS m/z 780 (M+H); Anal. Calcd for $C_{44}H_{26}N_{16}{\cdot}0.5MeOH$: C, 67.25; H, 3.55; N, 28.20. Found: C, 67.12; H, 3.60; N, 28.10.

4.1.6. General procedure for the synthesis of *N*-acetyl amino acid thioester (7a–c)

These compounds were synthesized according to the reported procedure with slight modifications. To a stirred solution of

N-acetyl amino acid and (diphenylphosphino)methanethiol, which was synthesized according to the literature, (1.5 equiv) in dry DMF under an atmosphere of argon were added DCC (1.5 equiv) and DMAP (0.2 equiv). The reaction mixture was stirred for 12 h at room temperature. The solid was removed by filtration and the filtrate was concentrated under reduced pressure. The crude product was purified by silica gel chromatography (1:1 EtOAc/*n*-hexane) to give a colorless solid.

4.1.7. AcGlySCH$_2$PPh$_2$ (7a)

Yield: 67%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.44–7.40 (m, 4H), 7.36–7.35 (m, 6H), 5.94 (m, 1H), 4.18 (d, J = 5.6 Hz, 2H), 3.54 (d, J = 3.9 Hz, 2H), 2.03 (s, 3H); FAB-MS m/z 332 (M+H).

4.1.8. AcAlaSCH$_2$PPh$_2$ (7b)

Yield: 53%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.45–7.40 (m, 4H), 7.37–7.34 (m, 6H), 5.84 (d, J = 7.6 Hz, 1H), 4.69 (quintet, J = 7.6 Hz, 1H), 4.11 (m, 2H), 2.00 (s, 3H), 1.30 (d, J = 7.1 Hz, 3H); FAB-MS m/z 346 (M+H).

4.1.9. AcPheSCH$_2$PPh$_2$ (7c)

Yield: 48%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.57–7.36 (m, 10H), 7.26–7.21 (m, 5H), 5.99 (d, J = 8.3 Hz, 1H), 4.95–4.93 (m, 1H), 3.60–3.45 (m, 2H), 3.07 (dd, J = 5.6 Hz, 14.1 Hz, 1H), 2.96 (dd, J = 7.1 Hz, 14.1 Hz, 1H); FAB-MS m/z 422 (M+H).

4.1.10. General procedure for the synthesis of *N*-acetyl amino acid–porphyrin conjugate (8a–c)

Method A. Azidoporphyrin **1** (3.9 mg, 5.0 μmol) and *N*-acetyl amino acid phosphinothioester (20 μmol for **7a**, 40 μmol for **7b** and **7c**) were dissolved in dry NMP and the reaction mixture was stirred for 20 h at 50 °C. After cooling to room temperature, H_2O (0.1 mL) was added and the whole was stirred at room temperature for an additional 3 h, then concentrated under reduced pressure. The residue was applied to an alumina column (eluted with 1:1 EtOAc/*n*-hexane to EtOAc) to remove NMP. The colored fractions were collected and concentrated. The resulting purple solid was further purified by RP-HPLC.

4.1.11. Compound 8a

Yield: 71% (determined by HPLC). ^{1}H NMR (600 MHz, CD_3OD/$CDCl_3$ = 1:10) δ 8.88 (broad, 8H), 8.05 (d, J = 7.8 Hz, 4H), 7.95 (d, J = 7.8 Hz, 4H), 7.71 (t, J = 7.8 Hz, 4H), 4.07 (s, 8H), 2.05 (s, 12H); ESI-HRMS m/z M+H calcd for $C_{60}H_{54}N_{12}O_8$, 1071.4266; found, 1071.4335.

4.1.12. Compound 8b

Yield: 80% (determined by HPLC). ^{1}H NMR (600 MHz, CD_3OD/$CDCl_3$ = 1:10) δ 8.89 (broad, 8H), 8.40 (s, 4H), 8.06 (d, J = 7.2 Hz, 4H), 7.95 (d, J = 7.2 Hz, 4H), 7.71 (t, J = 7.2 Hz, 4H), 4.62 (q, J = 4.2 Hz, 4H), 2.05 (s, 12H), 1.47 (d, J = 4.2 Hz, 12H); ESI-HRMS m/z M+H calcd for $C_{64}H_{62}N_{12}O_8$, 1127.4892; found, 1127.5003

4.1.13. Compound 8c

Yield: 90% (determined by HPLC). ^{1}H NMR (600 MHz, CD_3OD/$CDCl_3$ = 1:10) δ 8.88 (broad, 8H), 8.23 (s, 4H), 7.97–7.21 (m), 4.81 (m, 4H), 3.19 (m, 4H), 3.07 (m, 4H), 1.98 (m, 12H); ESI-HRMS m/z M+H calcd for $C_{88}H_{78}N_{12}O_8$, 1431.6143; found, 1431.6471.

Method B. Thioester **7a** (13 mg, 40 μmol) and molecular sieves 4A were charged in a round-bottomed flask and dried in vacuo, followed by addition of dry DMF (0.6 mL) under an atmosphere of argon with stirring. To a stirred solution was added azidoporphyrin **1** (3.9 mg, 5 μmol) in freshly distilled THF (0.2 mL) under an atmosphere of argon. Stirring was continued at 50 °C for 12 h, then H_2O (80 μL) was added and stirring was continued at room temperature for 3 h. The reaction mixture was then concentrated under reduced pressure to afford a crude purple solid, which was purified by RP-HPLC to give **8a**. Yield: 84%.

4.1.14. 5,10,15,20-Tetrakis{3-[(4-methoxycarbonyl)-1*H*-1,2,3-triazol-1-yl]phenyl}porphyrin (10a)

To a mixture of TBTA (34.1 mg, 64.2 mmol) and [Cu(CH_3CN)$_4$]-PF_6 (23.9 mg, 64.2 mmol) was added deaerated THF (15 mL) at room temperature under an atmosphere of argon, and the mixture was stirred for 30 min. Then methyl propiolate (21.4 mL, 257 mmol) was added, and stirring was continued at room temperature under an atmosphere of argon for 30 min. To the mixture was added azidoporphyrin **1** (50.0 mg, 64.2 mmol) in THF (20 mL), and the mixture was stirred at room temperature under an atmosphere of argon for 2 h. The solvent was removed under reduced pressure, and the residue was purified by column chromatography [silica, MeOH/$CHCl_3$ (1:49)]. The product was recrystallized from CH_2Cl_2/*n*-hexane to give a purple solid (67.5 mg, 94%). Mp >300 °C; IR (KBr) ν 3448, 1733 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6, 70 °C) δ −2.78 (s, 2H), 3.87 (s, 12H), 8.06 (t, J = 7.9 Hz, 4H), 8.38 (d, J = 7.3 Hz, 4H), 8.48 (dd, J = 8.5, 1.8 Hz, 4H), 8.87 (s, 4H), 8.98 (s, 8H), 9.64 (s, 4H); ^{13}C NMR (125 MHz, DMSO-d_6, 70 °C) δ 51.5, 118.4, 120.0, 125.5, 127.3, 128.3, 131.4, 134.5, 134.8, 139.5, 142.5, 160.2; FAB-MS m/z 1116 (M+H); Anal. Calcd for $C_{60}H_{42}N_{16}O_8{\cdot}H_2O$: C, 63.60; H, 3.91; N, 19.78. Found: C, 63.88; H, 4.19; N, 19.62.

4.1.15. H$_2$N-Ile-OMe

To MeOH (10 mL) was slowly added thionyl chloride (2.6 mL) at 0 °C, and the mixture was stirred for 10 min. Then H_2N-L-Ile-OH (1.31 g, 10.0 mmol) was added, and stirring was continued at room temperature for 7 h. The solvent was removed under reduced pressure. To the residue was added satd $NaHCO_3$ aq and the mixture was extracted with CH_2Cl_2. The organic layer was dried over $MgSO_4$, filtered and concentrated to give a colorless oil (772 mg, 53%) without purification. ^{1}H NMR (400 MHz, CD_3OD) δ 0.85 (t, J = 7.4 Hz, 3H), 0.85 (t, J = 6.9 Hz, 3H), 1.20–1.09 (m, 1H), 1.44–1.34 (m, 1H), 1.70–1.60 (m, 1H), 3.29 (d, J = 4.9 Hz, 1H), 3.63 (s, 3H); FAB-MS m/z 154 (M+H).

4.1.16. BocHN-Ile-OH

A solution of H_2N-L-Ile-OH (1.31 g, 10.0 mmol) in 1,4-dioxane (20 mL), water (10 mL), and 1 M NaOH aq (10 mL) was stirred at 0 °C. Then Boc_2O (2.40 g, 11.0 mmol) was added, and the mixture was stirred at room temperature for 9 h. Further Boc_2O (0.24 g, 1.0 mmol) was added and stirring was continued for 15 h. Then the solution was concentrated to about 15–20 mL, cooled in an ice-water bath, covered with a layer of AcOEt (about 30 mL), and acidified to pH 2–3 with a dilute solution of $KHSO_4$. The aqueous phase was extracted with AcOEt. The organic layer was washed with water, dried over Na_2SO_4, filtered and concentrated to give a colorless solid (2.43 g, quant.) without purification. ^{1}H NMR (400 MHz, CD_3OD) δ 0.91 (t, J = 7.4 Hz, 3H), 0.94 (d, J = 6.9 Hz, 3H), 1.28–1.15 (m, 1H), 1.23 (t, J = 7.3 Hz, 1H), 1.55–1.36 (m, 1H), 1.44 (s, 9H), 1.88–1.79 (m, 1H); FAB-MS m/z 232 (M+H).

4.1.17. Boc-HN-Ile-Ile-OMe

To a solution of H_2N-Ile-OMe (200 mg, 1.28 mmol) and BocHN-Ile-OH (350 mg, 1.51 mmol) in CH_2Cl_2 (20 mL) were added WSCD·HCl (290 mg, 1.51 mmol) and HOBt·H_2O (232 mg, 1.51 mmol), and the mixture was stirred for 16 h. The solvent was removed under reduced pressure and the residue was dissolved in $NaHCO_3$ aq. The aqueous phase was extracted with CH_2Cl_2. The organic solvent was removed under reduced pressure and the residue was dissolved in 2 M HCl. The aqueous phase was extracted with CH_2Cl_2. The organic layer was washed with water and dried over Na_2SO_4, filtered and concentrated to give a

white solid (400 mg, 81%) without purification. ^{1}H NMR (400 MHz, CD_3OD) δ 0.89–0.84 (m, 12H), 1.27–1.06 (m, 2H), 1.55–1.36 (m, 2H), 1.39 (m, 9H), 1.71 (br d, J = 6.3 Hz, 1H), 1.89–1.80 (m, 1H), 3.65 (s, 3H), 3.88 (d, J = 7.9 Hz, 1H), 4.34 (d, J = 5.9 Hz, 1H); FAB-MS m/z 359 (M+H).

4.1.18. H_2N-Ile-Ile-OMe

To Boc-HN-Ile-Ile-OMe (400 mg, 1.12 mmol) was added 4 M HCl/1,4-dioxane (6 mL), and the mixture was stirred for 1 h. The solvent was removed under reduced pressure. The residue was dissolved in CH_2Cl_2. The organic phase was extracted with 2 M HCl. The aqueous layer was basified with Na_2CO_3 to pH 9–10. The aqueous phase was extracted with CH_2Cl_2. The organic layer was dried over Na_2SO_4, filtered and concentrated to give a colorless oil (163 mg, 57%). ^{1}H NMR (400 MHz, CD_3OD) δ 0.91–0.85 (m, 12H), 1.26–1.02 (m, 2H), 1.52–1.41 (m, 2H), 1.71–1.63 (m, 1H), 1.89–1.81 (m, 1H), 3.12 (d, J = 5.6 Hz, 1H), 3.66 (s, 3H), 4.35 (d, J = 5.9 Hz, 1H); FAB-MS m/z 259 (M+H).

4.1.19. *N*-Propiolyl-Ile-Ile-OMe (9b)

To a solution of DCC (71 mg, 279 μmol) in CH_2Cl_2 (2.3 mL) was added propiolic acid (16 μL, 260 μmol) at 0 °C. After 10 min, to the mixture was added a solution of H_2N-Ile-Ile-OMe (60 mg, 232 μmol) in CH_2Cl_2 (1 mL), and the mixture was stirred at 0 °C for 1 h. After this time, the mixture was allowed to warm to room temperature and stirred for 5 h. After filtration, the solvent was removed under reduced pressure. The residue was purified by column chromatography [silica, $CHCl_3$/MeOH (99:1)] to give a pale yellow solid (90.3 mg, mixture). ^{1}H NMR (400 MHz, CD_3OD) δ 0.85–0.79 (m, 12H), 1.31–0.99 (m, 2H), 1.52–1.34 (m, 2H), 1.82–1.68 (m, 2H), 3.51 (s, 1H), 3.60 (s, 3H), 4.22 (d, J = 8.9 Hz, 1H), 4.27 (d, J = 5.9 Hz, 1H); ^{13}C NMR (125 MHz, CD_3OD) δ 11.1, 11.7, 15.6, 15.9, 26.0, 26.3, 26.7, 34.7, 37.7, 38.2, 52.3, 154.5, 173.3, 173.3; FAB-MS m/z 311 (M+H).

4.1.20. Compound 10b

To a mixture of TBTA (39 mg, 73.5 μmol) and $[Cu(CH_3CN)_4]PF_6$ (27 mg, 72.5 μmol) was added deaerated THF (5 mL) under an atmosphere of argon, and the mixture was stirred for 30 min. Then a solution of compound **9b** (90 mg) in deaerated THF (2.5 mL) was added, and the mixture was stirred for 30 min. Next, compound **1** (11 mg, 14.1 μmol) in deaerated THF (3 mL) was added, and stirring was continued for 1 h. A solution of compound **9b** (22 mg) in deaerated THF (2.5 mL) was added to the reaction mixture and stirring was continued for 2 h. The solvent was removed under reduced pressure, and the residue was purified by column chromatography [silica, MeOH/$CHCl_3$ (1:49 to 1:14)]. The product was recrystallized from CH_2Cl_2/*n*-hexane to give a purple solid (28.9 mg, 99%). Mp 200–201 °C; IR (KBr) ν 3399, 1655 cm^{-1}; ^{1}H NMR (400 MHz, CD_2Cl_2) δ −2.91 (s, 2H), 1.33–0.80 (m, 60H), 1.92–1.63 (m, 12H), 3.49 (s, 12H), 4.56 (s, 4H), 4.75 (s, 4H), 8.20–7.23 (m, 20H), 8.85–8.58 (m, 16H); ^{13}C NMR (125 MHz, CD_2Cl_2) δ 11.5, 11.6, 15.6, 25.5, 30.1, 38.1, 52.2, 56.8, 57.9, 119.0, 120.3, 124.5, 126.5, 128.5, 135.6, 143.9, 160.0, 171.0, 172.7; MALDI-TOF-MS m/z 2021 (M+H), 2043 (M+Na), 2059 (M+K); Anal. Calcd for $C_{108}H_{130}N_{24}O_{16}\cdot 2H_2O$: C, 63.08; H, 6.57; N, 16.35. Found: C, 63.15; H, 6.48; N, 16.17.

4.1.21. 5-Aminonicotinic acid (12)

This compound was synthesized according to the reported procedure.[39] To 5-bromonicotinic acid **11** (5.16 g, 25.5 mmol) and $CuSO_4\cdot 5H_2O$ (1.28 g, 5.1 mmol) was added NH_3 aq (10 mL), and the mixture was stirred at 170–180 °C for 19 h in a glass-lined autoclave. The dark-colored solution was transferred after cooling, treated with Na_2S aq to remove copper ions, and then filtered. The filtrate was adjusted to pH 4–5 and concentrated. The precipitate was collected by filtration to give a brown solid (3.27 g, 93%), which was used for the next step without purification. IR (KBr) ν 3341, 3197, 2477, 2143, 1658, 1591, 1469 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6) δ 5.59 (s, 2H), 7.41 (dd, J = 2.9, 2.0 Hz, 1H), 8.10 (d, J = 2.4 Hz, 1H), 8.24 (d, J = 1.5 Hz, 1H); EI-MS m/z 138 (M^+); HREI-MS m/z M^+ calcd for $C_6H_6O_2N_2$, 138.04293; found, 138.04319.

4.1.22. 3-Amino-5-hydroxymethylpyridine (13)

This compound was synthesized according to the reported procedures with some modification.[40,41] To compound **12** (4.96 g, 35.9 mmol) in MeOH (220 mL) was slowly added $SOCl_2$ (50 mL, 682.3 mmol) at 0 °C and the mixture was stirred at reflux for 2 d. The solvent was removed under reduced pressure. The residue was dissolved in a minimum volume of H_2O at 0 °C, and to the solution was added satd Na_2CO_3 aq until the product precipitated. The insoluble material was collected by filtration to give the methyl ester (4.30 g, 28.3 mmol, 79%) without purification.

To $LiAlH_4$ (4.29 g, 113.0 mmol) was slowly added THF (25 mL) at 0 °C under an atmosphere of argon. To the mixture was slowly added the methyl ester (4.30 g, 28.3 mmol) in THF (63 mL). The mixture was stirred at 0 °C for 0.5 h, and then at room temperature for 2 d. The resulting mixture was cooled to 0 °C, then carefully acidified (2 M HCl aq) to pH 3 and thereafter basified (solid Na_2CO_3) to pH 8. The mixture was filtered through Celite® and the solvent was removed under reduced pressure. The residue was dissolved in satd Na_2CO_3 aq and extracted with AcOEt. The organic layer was dried over Na_2SO_4, filtered and concentrated. The residue was purified by column chromatography [silica, MeOH/$CHCl_3$ (1:9)] to give a yellow oil (3.04 g, 87%). ^{1}H NMR (400 MHz, CD_3OD) δ 4.53 (s, 2H), 7.16 (s, 1H), 7.76 (s, 1H), 7.83 (d, J = 2.2 Hz, 1H); FAB-MS m/z 125 (M+H).

4.1.23. 3-Azido-5-hydroxymethylpyridine (14)

To compound **13** (3.28 g, 26.4 mmol) in 10% HCl (40 mL) was slowly added $NaNO_2$ (2.12 g, 31.7 mmol) in H_2O (40 mL) at 0 °C. The reaction mixture was stirred for 0.5 h, then NaN_3 (2.06 g, 31.7 mmol) was slowly added at 0 °C. Stirring was continued at this temperature for 1 h, then at room temperature for 2 h. The mixture was basified with satd Na_2CO_3 aq, and extracted with AcOEt and CH_2Cl_2. The organic layer was dried over Na_2SO_4, filtered and concentrated at reduced pressure. The residue was purified by column chromatography [silica, $CHCl_3$ to MeOH/$CHCl_3$ (1:9)] to give a yellow oil (3.41 g, 86%). IR (neat) ν 3298, 2357, 2116 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.32 (s, 1H), 4.72 (s, 2H), 7.43–7.42 (m, 1H), 8.16 (d, J = 2.7 Hz, 1H), 8.25 (d, J = 1.5 Hz, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 61.6, 124.6, 137.3, 138.0, 139.7, 144.0; EI-MS m/z 150 (M^+); HREI-MS m/z M^+ calcd for $C_6H_6ON_4$, 150.05416; found, 150.05454.

4.1.24. 5-Azido-3-pyridinecarbaldehyde (15)

IBX[42] (7.68 g, 27.4 mmol) was dissolved in DMSO (62 mL), and the mixture was stirred for 10 min. To the solution was added compound **14** (3.43 g, 22.8 mmol) in DMSO (30 mL). Stirring was continued for 1 h, then H_2O was added at 0 °C to give a white precipitate. The mixture was filtered and the solution was extracted with 50% AcOEt/*n*-hexane. The organic layer was dried over Na_2SO_4, filtered and the solvent was removed under reduced pressure. The residue was purified by column chromatography [silica, AcOEt/*n*-hexane (1:4), gradient] to give a white solid (3.26 g, 96%). IR (neat) ν 2124, 1701 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.83 (dd, J = 2.7, 1.7 Hz, 1H), 8.57 (d, J = 2.7 Hz, 1H), 8.85 (d, J = 1.7 Hz, 1H), 0.13 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 124.0, 131.7, 138.1, 146.2, 148.0, 189.6; EI-MS m/z 148 (M^+); HREI-MS m/z M^+ calcd for $C_6H_4ON_4$, 148.03851; found, 148.03728.

4.1.25. 5,10,15,20-Tetrakis(5-azido-3-pyridyl)porphyrin (2)

AcOH (450 mL) was heated at 85 °C for 10 min. To the solvent was added compound **15** (3.66 g, 24.7 mmol) in AcOH (44 mL), followed by pyrrole (1.31 mL, 24.7 mmol). The reaction mixture was stirred at 85 °C for 2 h and cooled to room temperature. The solvent was removed under reduced pressure. The residue was dissolved in satd $NaHCO_3$ aq and extracted CH_2Cl_2. The organic layer was dried over Na_2SO_4, then filtered and the solvent was removed under reduced pressure. The residue was purified by column chromatography [silica, MeOH/$CHCl_3$ (1:49), gradient] and gel permeation chromatography to give a purple solid (582 mg, 12%). IR (disc) ν 3432, 2109 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ −2.86 (s, 2H), 8.19 (s, 4H), 8.83 (d, J = 2.4 Hz, 4H), 8.89 (s, 8H), 9.23 (s, 4H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 29.7, 52.7, 115.7, 130.8, 136.1, 138.2, 140.9, 149.8; FAB-MS m/z 783 (M+H).

4.1.26. Manganese(III) 5,10,15,20-tetrakis(5-azido-3-pyridyl)porphyrin acetate (2′)

A mixture of compound **2** (20 mg, 25.6 μmol) and $Mn(OAc)_2 \cdot 4H_2O$ (50 mg, 204.4 μmol) in AcOH (2 mL) was gradually heated from room temperature to 100 °C for 30 min. The solution was cooled to room temperature and the solvent was removed under reduced pressure. The residue was dissolved in 28% NH_3 aq and the solvent was removed under reduced pressure. The residue was dissolved in MeOH and the solvent was removed under reduced pressure twice. The residue was taken up in $CHCl_3$, and the solution was filtered through Celite. The filtrate was evaporated under reduced pressure, then the residue was dissolved in AcOH and the solvent was removed under reduced pressure. The residue was recrystallized from $CHCl_3$/*n*-hexane to afford a green solid (20 mg, 86%). FAB-MS m/z 835 (M−OAc).

4.1.27. General procedure for the synthesis of *N*-propiolyl peptide (16a–i)

These compounds were synthesized according to the standard procedures for Fmoc solid-phase peptide synthesis. Fmoc-NH-SAL-resin was treated with a solution of 20% piperidine/DMF for 15 min. The resin was washed with DMF, then treated with a solution of Fmoc amino acid (3 equiv), HBTU (3 equiv), $HOBt \cdot H_2O$ (3 equiv) and DIPEA (6 equiv) in DMF for 30 min. The Fmoc group was removed with 20% piperidine/DMF. Propiolic acid (3 equiv) and EEDQ (3 equiv) were mixed in DMF and transferred to the resin (for reaction overnight). The resin was cleaved, and the product was purified by RP-HPLC.

4.1.28. *N*-Propiolyl-Lys-Gly-Cit-NH_2 (16a)

Yield: 88%. ^1H NMR (400 MHz, CD_3OD) δ 1.54–1.39 (m, 4H), 1.75–1.61 (m, 4H), 1.85–1.78 (m, 2H), 2.89 (t, J = 7.3 Hz, 2H), 3.12–3.05 (m, 2H), 3.61 (s, 1H), 3.85 (s, 2H), 4.32–4.26 (m, 2H); ^{13}C NMR (125 MHz, CD_3OD) δ 23.6, 27.5, 28.0, 30.2, 31.8, 40.4, 40.5, 43.7, 54.3, 55.4, 77.0, 77.8, 154.9, 162.3, 171.5, 174.1, 176.9; FAB-MS m/z 412 (M+H).

4.1.29. *N*-Propiolyl-Lys-Gly-Gly-Cit-NH_2 (16b)

Yield: 83%. ^1H NMR (400 MHz, CD_3OD) δ 1.57–1.39 (m, 4H), 1.75–1.61 (m, 4H), 1.88–1.79 (m, 2H), 2.89 (t, J = 7.6 Hz, 2H), 3.11–3.07 (m, 2H), 3.62 (s, 1H), 3.85 (d, J = 2.0 Hz, 2H), 3.86 (s, 2H), 4.33–4.27 (m, 2H); ^{13}C NMR (125 MHz, CD_3OD) δ 23.6, 27.6, 28.0, 30.1, 31.9, 40.4, 40.5, 43.6, 43.9, 54.4, 55.2, 77.1, 77.8, 154.9, 162.3, 171.7, 172.3, 174.2, 176.9; FAB-MS m/z 469 (M+H).

4.1.30. *N*-Propiolyl-Gly-Lys-NH_2 (16c)

Yield: quant. ^1H NMR (400 MHz, CD_3OD) δ 1.53–1.36 (m, 2H), 1.73–1.58 (m, 3H), 1.94–1.85 (m, 1H), 2.91 (t, J = 7.4 Hz, 2H), 3.65 (s, 1H), 3.87 (d, J = 16.4 Hz, 1H), 3.94 (d, J = 16.4 Hz, 1H), 4.37 (dd, J = 9.5, 4.7 Hz, 1H); ^{13}C NMR (125 MHz, CD_3OD) δ 23.7, 27.9, 32.4, 40.5, 43.8, 53.9, 76.7, 77.8, 155.3, 170.9, 176.7; FAB-MS m/z 255 (M+H).

4.1.31. *N*-Propiolyl-Gly-Gly-Lys-NH_2 (16d)

Yield: 96%. ^1H NMR (400 MHz, CD_3OD) δ 1.52–1.37 (m, 2H), 1.78–1.62 (m, 3H), 1.94–1.85 (m, 1H), 2.92 (t, J = 7.1 Hz, 2H), 3.65 (s, 1H), 3.88 (d, J = 3.8 Hz, 2H), 3.91 (d, J = 1.9 Hz, 2H), 4.34 (dd, J = 9.7, 4.7 Hz, 1H); ^{13}C NMR (125 MHz, CD_3OD) δ 23.7, 27.9, 32.2, 40.5, 43.8, 43.9, 54.1, 76.8, 77.8, 155.4, 171.7, 171.8, 176.8; FAB-MS m/z 312 (M+H).

4.1.32. *N*-Propiolyl-Gly-Lys-Cit-NH_2 (16e)

Yield: quant. ^1H NMR (400 MHz, CD_3OD) δ 1.50–1.41 (m, 1H), 1.60–1.52 (m, 1H), 1.74–1.63 (m, 2H), 1.89–1.81 (m, 2H), 2.92 (t, J = 7.4 Hz, 2H), 3.13 (t, J = 6.8 Hz, 2H), 3.64 (s, 1H), 3.91 (d, J = 0.9 Hz, 1H), 3.96 (s, 1H), 4.38–4.32 (m, 1H); ^{13}C NMR (125 MHz, CD_3OD) δ 23.5, 27.7, 27.9, 30.2, 32.2, 40.5, 40.5, 43.7, 54.2, 54.5, 76.7, 77.8, 155.3, 171.1, 173.9, 176.8; FAB-MS m/z 412 (M+H).

4.1.33. *N*-Propiolyl-Gly-Cit-Lys-NH_2 (16f)

Yield: quant. ^1H NMR (400 MHz, CD_3OD) δ 1.51–1.41 (m, 2H), 1.61–1.52 (m, 2H), 1.76–1.62 (m, 4H), 1.89–1.81 (m, 2H), 2.93 (t, J = 7.3 Hz, 2H), 3.18–3.10 (m, 2H), 3.63 (s, 1H), 3.88 (d, J = 16.5 Hz, 1H), 3.95 (d, J = 16.5 Hz, 1H), 4.34–4.29 (m, 2H); ^{13}C NMR (125 MHz, CD_3OD) δ 23.7, 27.7, 27.9, 29.7, 32.3, 40.6, 40.6, 43.6, 54.1, 55.0, 76.7, 77.8, 155.3, 162.3, 171.3, 174.3, 176.7; FAB-MS m/z 412 (M+H).

4.1.34. *N*-Propiolyl-Glu-Gly-Cit-NH_2 (16g)

Yield: quant. ^1H NMR (400 MHz, D_2O) δ 1.39–1.35 (m, 2H), 1.59–1.47 (m, 1H), 1.71–1.62 (m, 1H), 1.87–1.78 (m, 1H), 2.00–1.91 (m, 1H), 2.32 (t, J = 7.2 Hz, 2H), 2.92 (t, J = 6.9 Hz, 2H), 3.36 (s, 1H), 3.75 (s, 2H), 4.10 (dd, J = 9.5, 4.8 Hz, 1H), 4.21 (dd, J = 8.6, 5.9 Hz, 1H); ^{13}C NMR (125 MHz, D_2O) δ 27.0, 27.4, 29.9, 31.4, 41.0, 4.2, 54.9, 55.2, 77.0, 79.2, 156.2, 163.0, 172.9, 175.0, 178.2, 178.3; FAB-MS m/z 413 (M+H).

4.1.35. *N*-Propiolyl-Gly-Gly-Glu-NH_2 (16h)

Yield: 94%. ^1H NMR (400 MHz, D_2O) δ 1.85–1.75 (m, 1H), 2.03–1.95 (m, 1H), 2.30 (dt, J = 7.3, 1.7 Hz, 2H), 3.36 (s, 1H), 3.79 (s, 1H), 3.85 (s, 1H), 4.18 (dd, J = 9.6, 4.9 Hz, 1H); ^{13}C NMR (125 MHz, D_2O) δ 27.4, 31.4, 43.8, 44.0, 54.1, 76.8, 78.9, 156.6, 172.9, 172.9, 177.3, 178.4; FAB-MS m/z 313 (M+H).

4.1.36. *N*-Propiolyl-Gly-His-Gly-NH_2 (16i)

Yield: 56%. ^1H NMR (400 MHz, CD_3OD) δ 3.14–3.12 (m, 1H), 3.49–3.47 (m, 1H), 3.69 (s, 1H), 3.93–3.85 (m, 4H), 4.74 (dd, J = 7.0, 5.4 Hz, 1H), 7.37 (d, J = 1.0 Hz, 1H), 8.77 (s, 1H); ^{13}C NMR (125 MHz, CD_3OD) δ 27.9, 42.8, 43.8, 53.4, 76.7, 77.8, 119.0, 130.6, 135.0, 155.3, 170.9, 172.2, 174.3; FAB-MS m/z 321 (M+H).

4.1.37. General procedure for the synthesis of peptide–Mn-porphyrin conjugates (17a–i)

To a mixture of TBTA (5 equiv) and $[Cu(CH_3CN)_4]PF_6$ (5 equiv) was added DMSO under an atmosphere of argon, and the mixture was stirred for 30 min. To the mixture was added a solution of *N*-propiolyl peptide (>5 equiv) in DMSO. The mixture was stirred for 30 min, then a solution of compound **2′** in DMSO (final concentration: 6.5 mM compound **2′** in DMSO) was added, and stirring was continued. To the resulting mixture was added $CHCl_3$ and the precipitate was filtered. The precipitate was purified by RP-HPLC to give a dark red solid.

4.1.38. Compound 17a

Yield: 43%. MALDI-TOF-MS m/z 2481 (M−CF_3COO+H).

4.1.39. Compound 17b

Yield: 48%. MALDI-TOF-MS *m*/*z* 2709 (M−CF_3COO+H).

4.1.40. Compound 17c

Yield: 57%. MALDI-TOF-MS *m*/*z* 1853 (M−CF_3COO+H).

4.1.41. Compound 17d

Yield: 53%. MALDI-TOF-MS *m*/*z* 2081 (M−CF_3COO+H).

4.1.42. Compound 17e

Yield: 62%. MALDI-TOF-MS *m*/*z* 2481 (M−CF_3COO+H).

4.1.43. Compound 17f

Yield: 60%. MALDI-TOF-MS *m*/*z* 2481 (M−CF_3COO+H).

4.1.44. Compound 17g

Yield: 78%. MALDI-TOF-MS *m*/*z* 2507 (M−OAc+Na).

4.1.45. Compound 17h

Yield: 79%. MALDI-TOF-MS *m*/*z* 2106 (M−OAc+Na).

4.1.46. Compound 17i

Yield: 56%. MALDI-TOF-MS *m*/*z* 2117 (M−CF_3COO+H).

4.1.47. Manganese(III) 5,10,15,20-tetrakis(5-azido-1-methylpyridinium-3-yl)porphyrin pentachloride (18)

To a solution of compound **2′** (20 mg, 22.4 μmol) in DMF (4 mL) was added TsOMe (440 μL, 2.91 mmol). The mixture was stirred at 100 °C for 4 h, then the solvent was removed under reduced pressure and the residue was dissolved in $CHCl_3$. The solution was extracted with H_2O and the aqueous layer was removed under reduced pressure. To the residue was added NH_4PF_6 aq and the precipitate was collected by filtration. To the precipitate was added *tetra-n*-butylammonium chloride in acetone and the precipitate was filtered to give a purple solid (22 mg, 92%). ESI-MS *m*/*z* 298 (M/3), 261 (M−N_2/3), 224 (M/4), 196 (M−N_2/4).

4.1.48. 5,10,15,20-Tetrakis(3-pyridyl)porphyrin (20)

To a mixture of 3-pyridinecarbaldehyde **19** (0.439 mL, 4.67 mmol) and pyrrole (0.249 mL, 4.67 mmol) was added EtCOOH (20 mL). The reaction mixture was stirred at reflux in air for 2 h, then evaporated under reduced pressure. The residue was purified by column chromatography [silica, MeOH/$CHCl_3$ (1:20)] to give a purple solid (0.164 mg, 23%). Mp >300 °C; IR (KBr) ν 3434 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ −2.83 (s, 2H), 7.78 (dd, *J* = 7.3, 5.5 Hz, 4H), 8.54 (d, *J* = 6.1 Hz, 4H), 8.87 (s, 8H), 9.07 (dd, *J* = 4.9, 1.2 Hz, 4H), 9.47 (s, 4H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 116.7, 122.1, 122.1, 137.6, 141.0, 141.0, 149.3, 153.6; FAB-MS *m*/*z* 620 (M+H).

4.1.49. Manganese(III) 5,10,15,20-tetrakis(3-pyridyl)porphyrin acetate (20′)

The mixture of compound **20** (60 mg, 97 μmol) and $Mn(OAc)_2\cdot 4H_2O$ (190 mg, 756 μmol) in AcOH (8 mL) was gradually heated from room temperature to 100 °C for 1 h. The solution was cooled to room temperature and the solvent was removed under reduced pressure. The residue was dissolved in 28% NH_3 aq and the solvent was removed under reduced pressure. The residue was dissolved in MeOH and the solvent was removed under reduced pressure twice. The residue was taken up in $CHCl_3$, and the solution was filtered through Celite. The filtrate was evaporated under reduced pressure, and the residue was dissolved in AcOH. The solvent was removed under reduced pressure, and the residue was purified by PTLC [silica, MeOH/$CHCl_3$ (3:17)]. The product was recrystallized from $CHCl_3$/*n*-hexane to afford a green solid (24 mg, 34%). FAB-MS *m*/*z* 671 (M−OAc).

4.1.50. 5,10,15,20-Tetrakis(5-amino-3-pyridyl)porphyrin (21)

To a solution of compound **2** (30 mg, 38 μmol) in THF/H_2O (1:1) (3 mL) was added PPh_3 (50 mg, 192 μmol) and the mixture was refluxed for 5 d. The solvent was removed under reduced pressure and the residue was dissolved in 2 M HCl. PPh_3 and Ph_3P = O were extracted with $CHCl_3$. The aqueous layer was basified with 2 M NaOH and extracted with $CHCl_3$. The organic layer was dried over Na_2SO_4, filtered and evaporated under reduced pressure. The residue was purified by column chromatography [silica, MeOH/$CHCl_3$ (3:17 to 1:4), gradient] to give a red-purple solid (20 mg, 77%). ^{1}H NMR (400 MHz, CD_3OD) δ 7.80–7.76 (m, 4H), 8.28 (d, *J* = 2.5 Hz, 4H), 8.41–8.40 (s, 4H), 8.81 (br s, 8H); ^{13}C NMR (100 MHz, CD_3OD) δ 118.0, 128.2, 128.3, 136.5, 136.7, 139.6, 142.8, 145.2; FAB-MS *m*/*z* 679 (M+H).

4.1.51. Manganese(III) 5,10,15,20-tetrakis(5-amino-3-pyridyl) porphyrin trifluoroacetate (21′)

A mixture of compound **21** (7 mg, 10.3 μmol) and $Mn(OAc)_2\cdot 4H_2O$ (20 mg, 82.5 μmol) in AcOH (1 mL) was gradually heated from room temperature to 100 °C for 20 min. The solution was cooled to room temperature and the solvent was removed under reduced pressure. The residue was purified by reversed-phase HPLC (Inertsil ODS-3 using a gradient of 0.1% TFA/CH_3CN in 0.1% TFA/H_2O, 10–12% over 20 min at 10 mL/min) to give a dark red solid (7 mg, 52%). FAB-MS *m*/*z* 731 (M−OAc+H).

4.2. Catalase-like activity

The ability of Mn-porphyrins and EUK-113 to catalyze the conversion of hydrogen peroxide to oxygen was measured according to the method described by Doctrow et al.[38] with some modification. Briefly, the activity was determined by monitoring the concentration of oxygen using a Clark-type polarographic oxygen electrode (UC-12-SOL, Central Kagaku Corp.). To a solution of Mn-Porphyrin or EUK-113 (10 μM) in 50 mM sodium phosphate buffer (pH 7.4), hydrogen peroxide (10 mM, final concentration) was added. The reaction mixture was stirred at 25 ± 0.2 °C under an atmosphere of argon. Initial rates were calculated by linear regression using the data from 0–5 s of the reaction.

4.3. Peroxidase-like activity

Peroxidase-like activity was examined according to the method described by Doctrow et al.[38] with some modification. Briefly, the activity was assayed by spectrophotometric monitoring of ABTS oxidation. To the assay mixture, consisting of 50 mM potassium phosphate (pH 7.4), 0.5 mM ABTS, and 10 μM Mn-porphyrin or EUK-113, hydrogen peroxide (0.2 mM, final concentration) was added and the spectral change at 740 nm was monitored. Assays were conducted at 25 ± 0.2 °C. ABTS oxidation was estimated using a $\Delta\varepsilon_{740}$ value of 20,300 $M^{-1}cm^{-1}$.

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Supplementary data

Supplementary data (chemical structure of EUK-113) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.07.018.

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