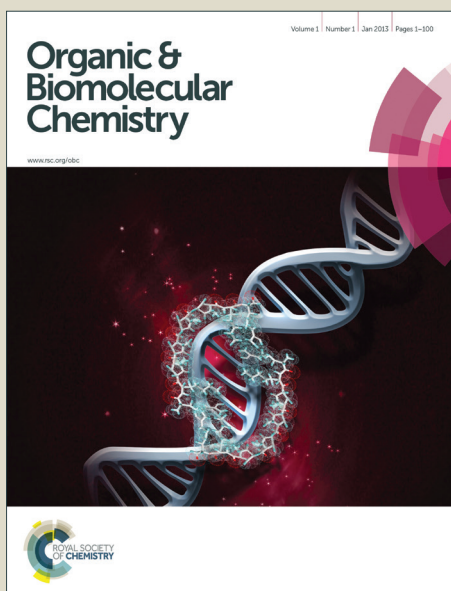


Organic & Biomolecular Chemistry

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Amino acid-linked porphyrin-nitroimidazole antibiotics targeting *Porphyromonas gingivalis*[†]Cite this: *Org. Biomol. Chem.*, 2014, 12, XXXXSimon A. Dingsdag,^{^,a,b} Benjamin C-M. Yap,^{^,a,c} Neil Hunter^{a,b} and Maxwell J. Crossley^{*,c}

The periodontal pathogen *Porphyromonas gingivalis* requires porphyrin supplementation for growth. Previously, in order to inhibit *P. gingivalis* growth, we synthesised very effective 'Trojan horse' ester and amide-linked deuterporphyrin-nitroimidazole (DPIX-Nim) adducts that exploited this requirement to transport metronidazole-derived antibiotics with excellent antimicrobial selectivity and recognition by the HA2 porphyrin binding site. Herein, in the context of developing topical agents to target *P. gingivalis*, L-amino acids are incorporated into adducts as linkers to improve uptake. Ten 13- and 17-propionic amide regioisomers of L-amino acid-linked deuterporphyrin-nitroimidazole adducts were synthesised using a peptide coupling approach. DPIX-Lys regioisomers without attached nitroimidazole were also synthesised as comparison compounds. All the porphyrin adducts bound (K_{d50} 7 to 20 nM) to a recombinant HA2 receptor with similar binding affinity to haem, except the lysine-proline linked DPIX-Lys(Boc)Pro-Nim adducts (K_{d50} 300 nM) and the DPIX-Lys(Nim)-Nim adducts (K_{d50} 200 nM), both of which have large appended groups. DPIX-Lys(Boc)-Nim, DPIX-Lys(OH)-Nim, and DPIX-Pro-Nim adducts were shown to be very effective against *P. gingivalis*. DPIX-Lys(Boc)Pro-Nim adducts and DPIX-Lys(Nim)-Nim adducts showed weak activity. Importantly, DPIX-Lys(Boc)-Nim adducts were selective for *P. gingivalis* and, unlike metronidazole, did not kill a range of other anaerobic bacteria isolated from the human gastrointestinal tract.

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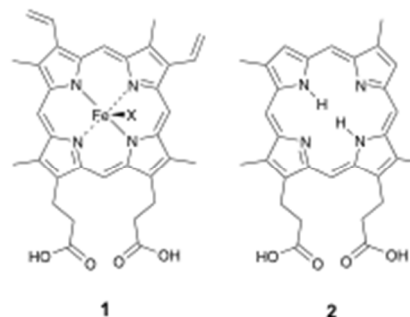
Introduction

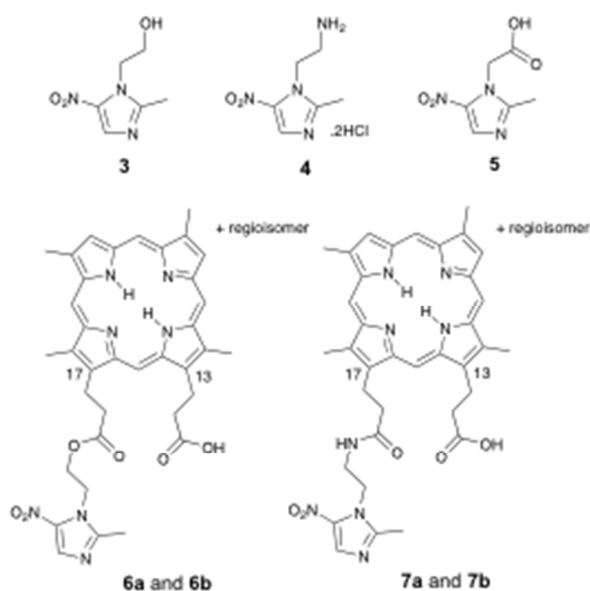
The anaerobic bacterium *Porphyromonas gingivalis*, implicated in periodontal disease, is unable to synthesise porphyrin. The requirement for external porphyrin (for growth) is satisfied *in vitro* by haem **1**. Unlike haem **1**, deuterporphyrin IX (DPIX **2**) does not feature in the bacterial biosynthetic pathway.

DPIX **2** has no central metal iron, and does not contain vinyl groups on the northern face (positions 3 and 8 of the porphyrin macrocycle). Our group previously demonstrated DPIX **2** supports growth of *P. gingivalis*.¹ DPIX **2** is recognised in part by a recombinant preparation of HA2, an outer membrane sub-domain protein, located within the gingipains of *P. gingivalis*.^{2,3}

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[†] Electronic supplementary information (ESI) available: Figs. S1-S6: Growth inhibition of *P. gingivalis* by the substrates 12a/12b, 14a/14b, 17a/17b, 22a/22b, 24a/24b and 29a/29b, respectively, Fig. S7: Enzyme activity studies using lysine-specific gingipain protease, NMR spectra and HRMS of new compounds. See DOI: 10.1039/b000000x/





Broad-spectrum antibiotics, such as metronidazole (Mtz) **3**, are commonly used to treat bacterial infections such as during periodontitis. In this work, we also make use of the derivatives Mtz-NH₂ **4** and Mtz-CO₂H **5**. The nitroimidazole (Nim) antibiotics are pro-drugs, inactive until taken up by anaerobic bacteria and ‘parasites’ (*Entamoeba* and *Giardia*), and activated by nitro group reduction. The human gastrointestinal tract contains extremely complex bacterial populations. Unfortunately, broad-spectrum antibiotics perturb these complex microbial ecosystems within humans and promote drug resistance in bacteria.^{4,5}

The problems associated with the use of broad-spectrum antibiotics prompted our group to synthesise porphyrin-based inhibitors, designed to act as a ‘Trojan-horse’: being recognised and imported as an essential porphyrin nutrient, while also delivering metronidazole derivatives.^{6–8} Facilitated delivery of such nitroimidazole antibiotics was envisaged to overcome indiscriminate suppression of non-target bacteria within the polymicrobial ecosystem of the oral cavity thereby resulting in selectivity for *P. gingivalis*.

In the absence of a structure of the porphyrin binding site on rHA2, a model of the site (Fig. 1) was proposed on the basis of growth and recognition studies.

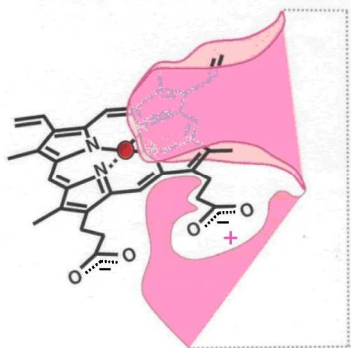


Figure 1 Proposed HA2 binding site.⁶

Based on the model (Fig. 1), we synthesised deuteroporphyrin-nitroimidazole ester adducts **6a** and **6b** and amide adducts **7a** and **7b** whereby the 17-propionate (**a** series) and 13-propionate (**b** series) of the porphyrin is linked directly to the antibiotic. These adducts are recognised by the HA2 receptor of *P. gingivalis*, as strongly as haem **1** to HA2 (Kd₅₀ 10 to 20 nM) and are highly potent towards *P. gingivalis* [Minimum Inhibitory Concentration (MIC) 0.625 to 2 μM].⁶ This activity matches that of metronidazole **3** (MIC 1 μM) and surpasses that of Mtz-NH₂ **4** (MIC 15 μM), suggesting that the pro-drug does not require cleavage from the porphyrin for activity.

Consistent with the binding site model (Fig. 1), the individual deuteroporphyrin-nitroimidazole esters, **6a** and **6b**, were found to bind to rHA2 equally well and they showed the same potency towards *P. gingivalis*. This indicates that either face of the porphyrin ring of deuteroporphyrin can be recognised by rHA2 by binding the 13-propionate or the 17-propionate if the ring is flipped over. Thus both **6a** and **6b** can bind into the rHA2 site by making use of the available propionate thereby leaving the 17-side-chain ester unimpaird in the case of **6a**, and the 13-side-chain ester unimpaird in the case of **6b**. A 1:1 regiosomeric mixture **6a/6b** also showed the same binding and inhibition properties as the individual isomers; thus separation of the regiosomers is not required.

The work herein involves the design, synthesis and evaluation of new porphyrin-based growth inhibitors for *P. gingivalis*. With consideration for topical therapy, amino acids lysine (Lys) and proline (Pro) were incorporated into porphyrin-nitroimidazole adducts as linkers, permitting derivatisation to increase potency and water solubility. Synthesis of amino acid-linked porphyrin-nitroimidazole adducts; DPIX-Lys(Boc)-Nim adducts **12a** and **12b**, DPIX-Lys(H)-Nim adducts **14a** and **14b**, DPIX-Lys(Boc)-Pro-Nim adducts **22a** and **22b**, and DPIX-Pro-Nim adducts **24a** and **24b** is reported herein. DPIX-Lys(Boc)-H adducts **29a** and **29b**, without attached nitroimidazoles function as controls. We also characterise the rate of kill and minimum inhibitory concentrations of **7a** and **7b** (synthesised previously)⁶ and **12a** and **12b** against a range of anaerobic bacteria known to be sensitive to metronidazole (*Fusobacterium nucleatum*, *Bacteroides fragilis*, *Tannerella forsythia* and *Prevotella melaninogenica*). Evaluation of rHA2 binding and growth inhibitory activities against anaerobic bacteria provides a framework for porphyrin-based antibiotics as alternatives to broad-spectrum antibiotics.

Results and discussion

Design of an inhibitor

A recombinant preparation of the HA2 surface receptor of *P. gingivalis* binds porphyrin independent of the central iron.^{1,2} On this basis, a free-base porphyrin was chosen, designed to render the porphyrin unpalatable to other bacteria with porphyrin scavengers such as the HasA hemophore in *Serratia marcescens* that recognise iron-metallated porphyrin.⁹ Also,

rHA2 recognition is dependent on the structure of the porphyrin macrocycle. At least one free propionic acid side-chain is necessary and certain modifications of the vinyl aspect of these haem analogues are not tolerated.^{1,3}

The synthetic route involves elaboration of an amino acid-linked nitroimidazole derivative, coupled to the carboxylic acid group of DPIIX mono-methyl esters **10** to give the desired mono-amino acid-linked porphyrin-antibiotic adducts. One propionic acid is then free for HA2 recognition. DPIIX mono-methyl esters **10** were chosen as there is no central metal, envisaged thereby to minimize uptake by other bacteria.

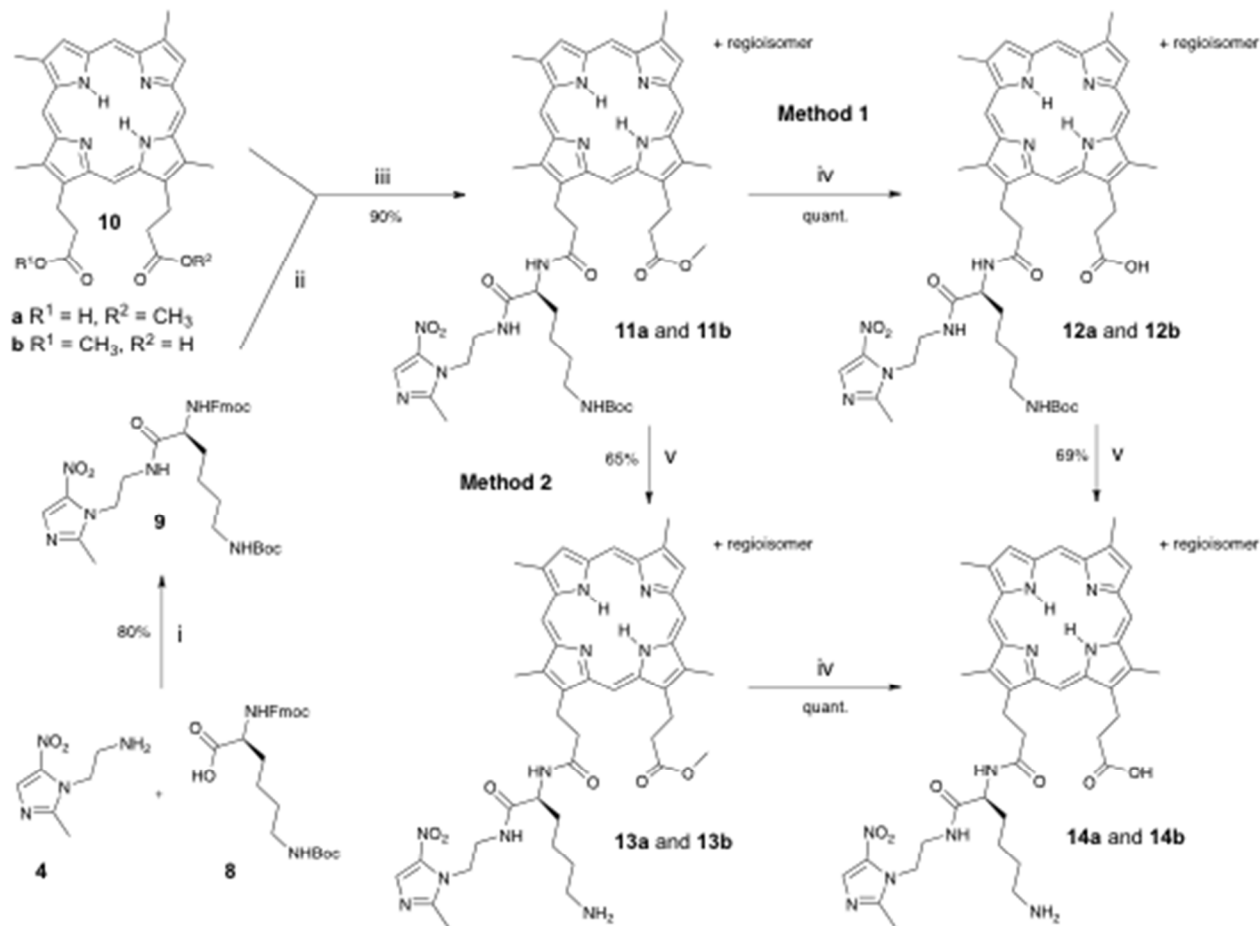
Previous attempts to form a mono-adduct directly between DPIIX **2** and nitroimidazoles resulted in a mixture of products that proved difficult to separate due to the polarity of free carboxylic acid groups.⁶ Further, by-products from the reaction could not be recycled easily. The route using **10** for coupling avoids these issues. Subsequent removal of protecting groups and hydrolysis of the methyl ester should be achieved selectively and in high yield to afford the desired amino acid-linked porphyrin-nitroimidazole adducts.

P. gingivalis Kgp gingipain is lysine-specific.⁶ Using lysine linkers also provides the opportunity to explore gingipain-specific cleavage by *P. gingivalis*.

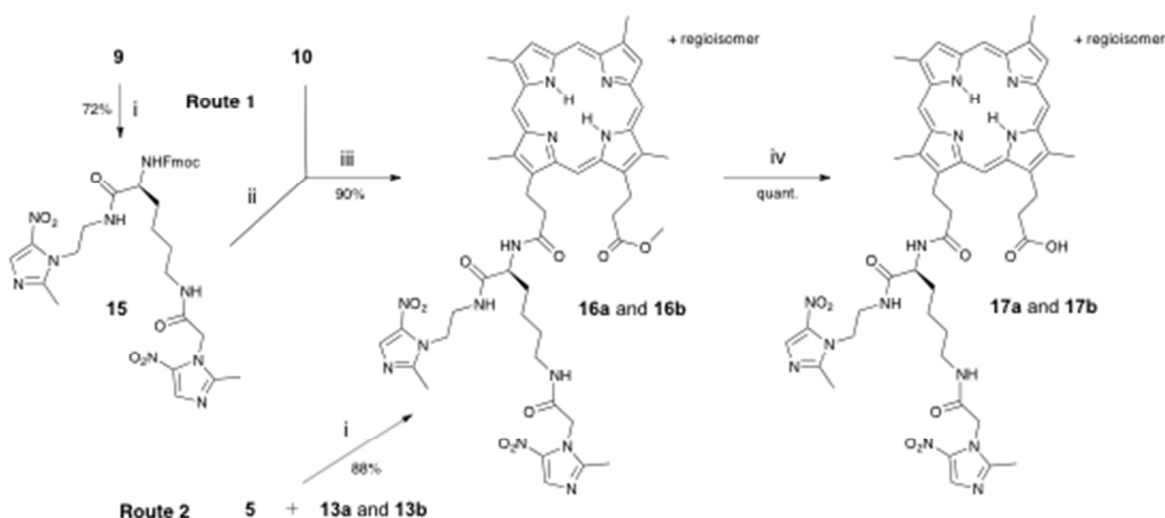
Synthesis

Fmoc-Lys(Boc)-Nim **9** was prepared in 80% yield from Mtz-NH₂ **4** and Fmoc-Lys(Boc)-OH **8** using *o*-(benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HBTU) (Scheme 1). Mtz-NH₂ **4** was prepared in 85% yield from **3** following the literature.⁸ DPIIX mono-methyl esters **10** were prepared in a 49% yield by the controlled hydrolysis of DPIIX dimethyl ester with 4 M hydrochloric acid in refluxing methanol.¹⁰ Fmoc deprotection of **9** by base *in situ* and attachment to the carboxylic acid group of **10** by reaction at its newly liberated amine to give the DPIIX-Lys(Boc)-Nim mono-methyl ester adducts **11a** and **11b**[‡] in 90% yield (Scheme 1). Hydrolysis of adducts **11a** and **11b** by treatment with excess LiOH in a CH₃OH-THF-H₂O mixture overnight at RT gave the free acids **12a** and **12b** in quantitative yield.

The fully deprotected adducts **14a** and **14b** were obtained by two routes. The first route involved Boc de-protection of adducts **12a** and **12b** by treatment with CF₃CO₂H in CH₂Cl₂ overnight to give the desired products **14a** and **14b** in 69% yield. The second route involved reversal of the sequence. Removal of the Boc groups from adducts **11a** and **11b** and subsequent ester hydrolysis of the resultant adducts **13a** and **13b** gave the desired products **14a** and **14b** in 65% yield.



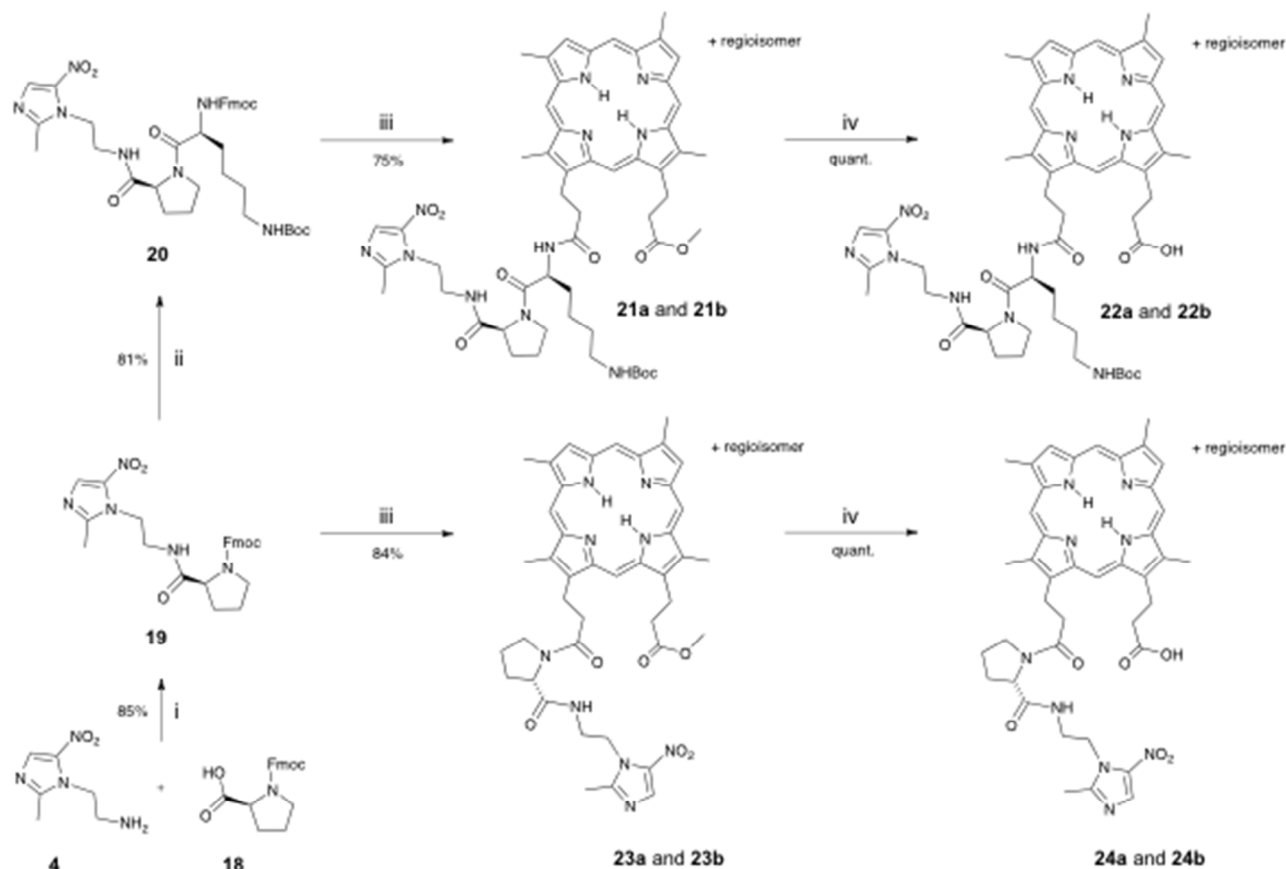
Scheme 1 Reagents and conditions: i HBTU, DIPEA, stir under N₂, 3 h, then HOBt; ii DBU, CH₂Cl₂, stir under N₂, 30 min; iii HOBt, HBTU, DIPEA, stir under N₂, overnight; iv LiOH in CH₃OH:THF:H₂O (3:4:1) stir, overnight; v TFA, CH₂Cl₂, stir under N₂, 3 h.



Scheme 2 Reagents and conditions: i v TFA, CH_2Cl_2 , stir under N_2 , 3 h, then Mtz-CO₂H **5**, HBTU, DIPEA, stir under N_2 , overnight; ii ii DBU, CH_2Cl_2 , stir under N_2 , 30 min then HOBt, HBTU, DIPEA, stir under N_2 , overnight; iv LiOH in $\text{CH}_3\text{OH}:\text{THF}:\text{H}_2\text{O}$ (3:4:1) stir, overnight.

Adducts **17a** and **17b**, with two nitroimidazoles attached, were synthesised in a bid to improve potency and this was also achieved by two routes (Scheme 2). The first route involved prior preparation of the di-Nim-linked lysine **15**. Mtz-CO₂H **5** was prepared from Mtz **3** in 32% yield following the

literature.¹¹ The di-Nim-linked lysine **15** was prepared by initial Boc de-protection of **9** and subsequent coupling to **5** with HBTU (Scheme 2). Fmoc de-protection of **15** with DBU and coupling to the DPIX mono-methyl esters **10** and **16b** in a 90% yield. Hydrolysis to the corresponding free



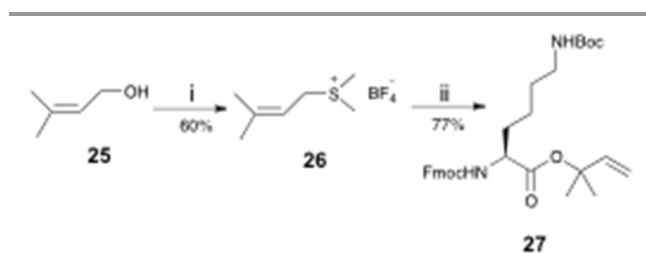
Scheme 3 i HBTU, DIPEA, stir under N_2 , overnight; ii DBU, CH_2Cl_2 , stir under N_2 , 30 min; then HOBt, Fmoc-Lys(Boc)-OH **8**, HBTU, DIPEA, stir under N_2 , overnight; iii DBU, CH_2Cl_2 , stir under N_2 , 30 min; then DPIX mono-methyl esters **10**, HOBt, HBTU, DIPEA, stir under N_2 , overnight; iv LiOH in THF, CH_3OH and water mixture, stir, overnight.

acids **17a** and **17b** occurred quantitatively. The second route involved initial Boc de-protection of conjugates **13a** and **13b**, coupling of **5** with HBTU, followed by quantitative hydrolysis.

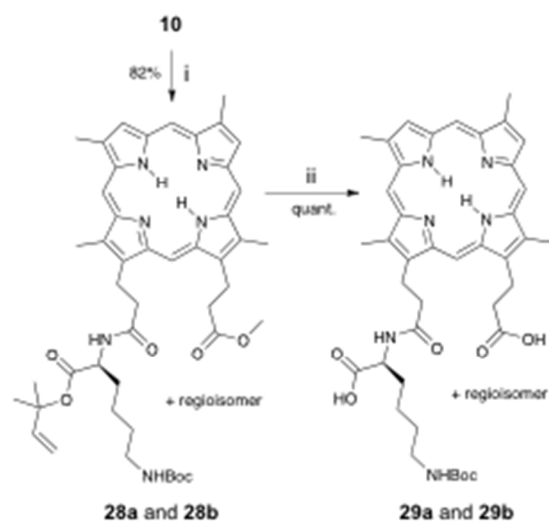
DPIX-Lys(Boc)-Pro-Nim adducts **22a** and **22b**, whereby a proline-lysine linker was used to bridge the nitroimidazole and the porphyrin, were synthesised in good overall yield (Scheme 3). First, the Fmoc-Pro-Nim **19** was prepared in 85% yield from Mtz-NH₂ **4** and Fmoc-Pro-OH **18** with the use of peptide coupling reagent HBTU. Fmoc-Lys(Boc)-Pro-Nim **20** was then prepared in 81% yield by HBTU-induced coupling of Fmoc-Lys(Boc)-OH **8** and the amine derived by *in situ* base deprotection of the Fmoc-Pro-Nim derivative **19**. In turn the Fmoc-Lys(Boc)-Pro-Nim **20** was Fmoc-deprotected and coupled *in situ* to the DPIX mono-methyl esters **10** which gave the methyl ester adducts **21a** and **21b** in a 81% yield. Hydrolysis of the methyl esters gave the DPIX-Lys(Boc)-Pro-Nim adducts **22a** and **22b** in quantitative yield.

DPIX-Pro-Nim adducts **24a** and **24b**, whereby a proline linker was used to bridge the nitroimidazole and the porphyrin, were synthesised as a control for the enzyme activity experiment. Fmoc-Pro-Nim derivative **19** was Fmoc deprotected by base *in situ* and attached at its newly liberated amine to the carboxylic acid group of **10** to give the DPIX-Pro-Nim mono-methyl ester adducts **23a** and **23b** (Scheme 3) in 84% yield. Subsequent hydrolysis with LiOH dissolved in a CH₃OH-THF-H₂O mixture at RT overnight gave the corresponding free acids **24a** and **24b** in quantitative yield.

Controls **29a** and **29b**, essentially **12a** and **12b** without nitroimidazole attached were synthesised (Schemes 4 and 5). First, preparation of the triply-protected lysine **27** involved reacting prenyldimethylsulfonium tetrafluoroborate **26** with Fmoc-Lys(Boc)-OH **8** with catalytic CuBr. The sulfonium salt **26** was prepared from 3-methyl-2-buten-1-ol **25** in 60% yield following the literature (Scheme 4).¹² Fmoc-Lys(Boc)-dimethylallyl ester **27** was Fmoc deprotected using DBU and then attached to the DPIX mono-methyl esters **10** by an amide linkage through one of the propionic acid side-chains of the porphyrin macrocycle to give diesters **28a** and **28b** in 82% yield (Scheme 5). Subsequent hydrolysis of the methyl and dimethylallyl esters to give adducts **29a** and **29b** occurred quantitatively on treatment over night under standard conditions.



Scheme 4 Reagents and conditions: i (CH₃)₂S, HBF₄ (54 wt % in diethyl ether), CH₂Cl₂, stir under N₂ at 0 °C, 6 h, then at room temperature, 14 h; ii Fmoc-Lys(Boc)-OH **8**, K₂CO₃, CuBr, CH₂Cl₂, stir under N₂ at 25 °C, overnight.



Scheme 5 Reagents and conditions: i Fmoc-Lys(Boc)-dimethylallyl ester **27**, DBU, CH₂Cl₂, stir under N₂, 30 min; then HOBt, HBTU, DIPEA, stir under N₂, overnight; ii LiOH in CH₃OH:THF:H₂O (3:4:1) stir, overnight.

rHA2 Binding

DPIX-Lys(Boc)-Nim adducts **12a** and **12b**, DPIX-Lys(H)-Nim adducts **14a** and **14b**, DPIX-Pro-Nim adducts **24a** and **24b**, and DPIX-Lys(Boc)-H adducts **29a** and **29b** bind rHA2 as measured by solid-phase ELISA (where binding affinity to rHA2 was defined as the K_{d50}) with similar binding affinities (K_{d50} 7 to 20 nM) to that of haem **1** (Fig. 2).

DPIX-Lys-diNim adducts **17a** and **17b** (K_{d50} 200 nM) and DPIX-Lys(Boc)-Pro-Nim adducts **22a** and **22b** (K_{d50} 300 nM), with larger appended groups had a significantly reduced affinity for rHA2, probably because of some size restrictions around the second propionate group. This might also have resulted from π - π interaction between the two nitroimidazoles or the nitroimidazole and the porphyrin ring leading to a greater three-dimensional bulk, thereby interfering with HA2 recognition. NMR spectroscopy rules this out, however, as the chemical shifts of the nitroimidazole protons

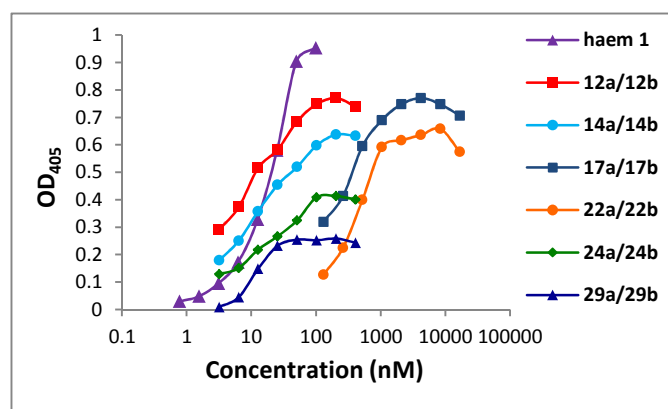


Figure 2: Binding curves of haem **1** and porphyrin-nitroimidazole adducts **12a/12b**, **14a/14b**, **22a/22b**, **24a/24b**, porphyrin-bis(nitroimidazole) adducts **17a/17b** and the porphyrin-lysine controls **29a/29b** to rHA2.

are essentially the same across the range of adducts prepared in this study, even when such a π - π interaction cannot occur.

Bacteriology

Growth inhibition profiles in CDC media of *P. gingivalis* and a range of other anaerobic bacteria isolated from the human

gastrointestinal tract, were determined in order to understand the behaviour of porphyrin nitroimidazole adducts (Fig. 5 and Table 1). To accommodate growth requirements of all nominated bacteria it was necessary to use enriched media (eBHI). On eBHI media, rate of kill comparisons were made to assess effectiveness of uptake and processing.

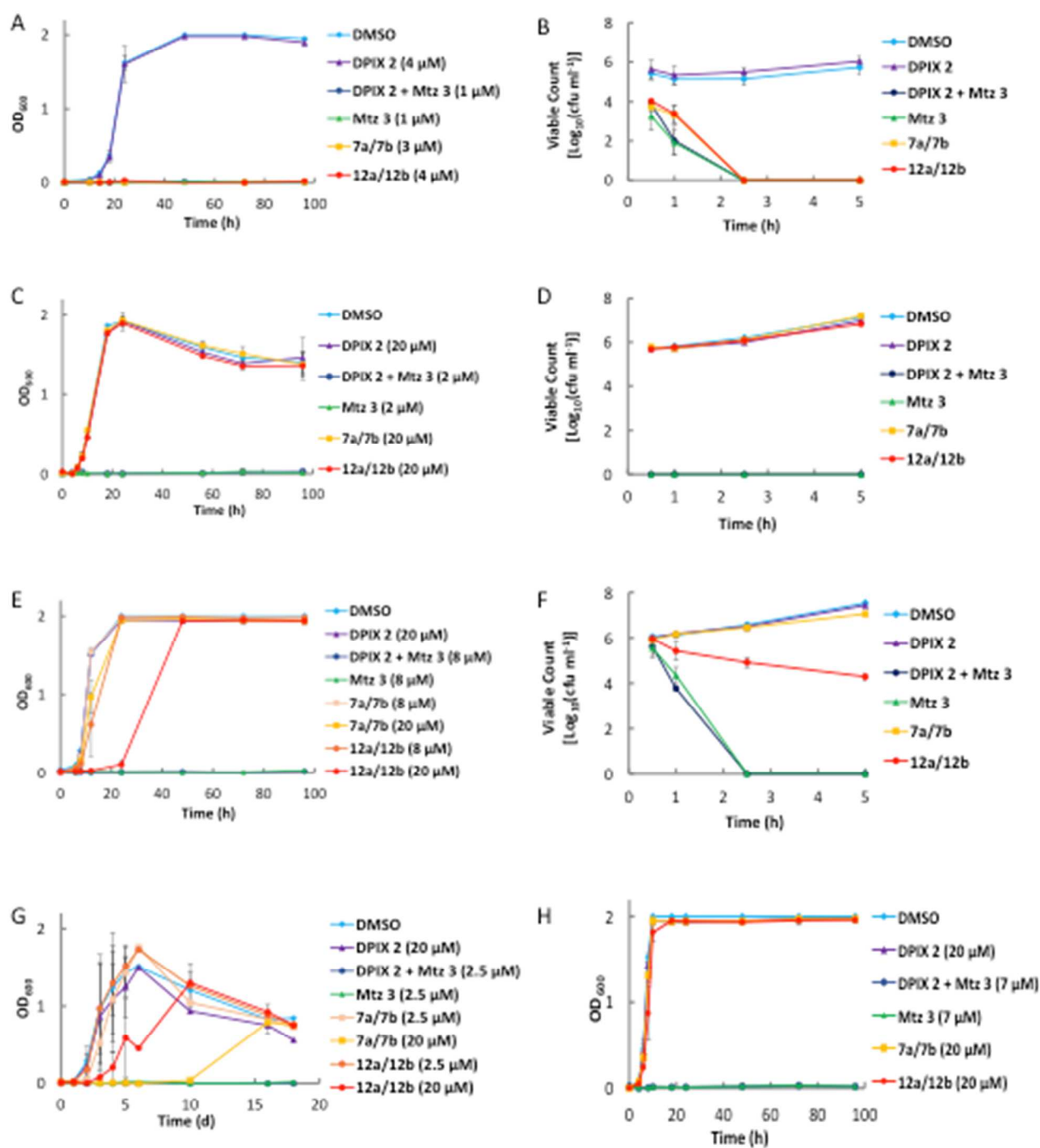


Figure 5: Growth curves or rate of kill assays for bacterial strains. A) growth of *P. gingivalis* in eBHI media; B) viable count of *P. gingivalis* on solid eBHI media with 5 μM treatments; C) *F. nucleatum* in eBHI media; D) viable count of *F. nucleatum* with 20 μM treatments; E) growth of *P. melaninogenica* in eBHI media; F) viable count of *P. melaninogenica* on solid eBHI media with 20 μM treatments; G) growth of *T. forsythia* in eBHI and H) growth of *B. fragilis* in eBHI media.

Table 1: Summary of adduct activity for *P. gingivalis* grown in CDC broth.^a

Adduct	MIC (μM)
Mtz 3	1-2
Mtz-NH ₂ 4	10-20
12a and 12b	1-2
14a and 14b	10-20
17a and 17b	Not killed (at 20 μM)
22a and 22b	Not killed (at 20 μM)
24a and 24b	1
29a and 29b	Growth supported

^a See Fig. S1-S6 for growth curves.

DPIX-Lys(Boc)-Nim adducts **12a** and **12b**, whereby Mtz-NH₂ **4** is attached to DPIX **2** via a Boc-protected lysine, have an MIC of <4 μM for *P. gingivalis* (in eBHI, Fig. 5A). In eBHI Mtz **3** has a lower MIC (< 1 μM , Fig. 5A). It was found that 5 μM Mtz **3** killed *P. gingivalis* within 2.5 hours, as did equimolar **12a** and **12b** and **7a** and **7b** (in eBHI, Fig. 5B). The MIC for **12a** and **12b** in CDC was 1 μM (Table 1, Fig. S1). In previous experiments (using CDC media), no growth of *P. gingivalis* was observed in Mtz **3** at <2 μM and in DPIX-Nim **7a** and **7b** at <1.25 μM .⁶

In eBHI 20 μM Mtz **3** rapidly killed *F. nucleatum* (<30 minutes), whilst **12a** and **12b** and **7a** and **7b** did not affect *F. nucleatum* (Fig. 5C). The MIC of Mtz **3** for *F. nucleatum* was 2 μM (Fig. 5D). Like *F. nucleatum*, *B. fragilis* was unaffected by adducts in eBHI media, but no growth was detected in the presence of 7 μM Mtz **3** (Fig. 5H).

It was found that 20 μM Mtz **3** killed *P. melaninogenica*, within 2.5 hours (Fig. 5F) and the MIC was 8 μM (Fig. 5E). DPIX-Lys(Boc)-Nim adducts **12a** and **12b** caused a dose-dependent initial reduction in *P. melaninogenica* (Fig. 5E and Fig. 5F) with recovery between 24-48 hours similar to the biomass of untreated controls (Fig. 5F). Adducts **7a** and **7b** (20 μM) affected the growth of *P. melaninogenica* slightly (Fig. 5E and Fig. 5F). The growth rate of *T. forsythia* was slowed by **12a** and **12b** as well as **7a** and **7b** in a dose-dependent manner (Fig. 5G). Mtz **3** at 2.5 μM prevented growth of *T. forsythia*. Indeed, *P. melaninogenica* and *T. forsythia* grew in 50 μM **7a** and **7b** and **12a** and **12b**, respectively.

DPIX-Lys(H)-Nim adducts **14a** and **14b**, essentially adducts **12a** and **12b** without a Boc group, are active against *P. gingivalis* at 20 μM (Table 1, Fig. S2).

In an attempt to increase the potency of adducts against *P. gingivalis*, the DPIX-Lys(Nim)-Nim adducts **17a** and **17b**, with two nitroimidazoles attached, were synthesised. However, adducts **17a** and **17b** are inactive against *P. gingivalis* at 20 μM (Table 1, Fig. S3).

DPIX-Lys(Boc)-Pro-Nim adducts **22a** and **22b**, with a proline-lysine linker bridging Mtz **3** to DPIX **2**, retarded growth, but did not kill *P. gingivalis* at 20 μM . This could be due to weaker binding affinity for rHA2 (Fig. S4).

DPIX-Pro-Nim adducts **24a** and **24b**, whereby Mtz-NH₂ **4** is attached to DPIX **2** via a proline, are as active as the DPIX-

Lys(Boc)-Min adducts **12a** and **12b**, with an MIC less than 1 μM (Table 1, Fig S5).

DPIX-Lys(Boc)-OH adducts **29a** and **29b**, essentially **12a** and **12b** without nitroimidazole attached, were synthesised as a control. Controls **29a** and **29b** supported growth of *P. gingivalis* at 20 μM (Table 1, Fig. S6). This was expected as no antibiotic is attached, so these controls behave like DPIX **2** (support growth). Controls **29a** and **29b** were shown to bind rHA2 with a similar binding affinity to that of haem **1** (Fig. 2).

Gingipain activity studies

The *P. gingivalis* Kgp gingipain is lysine-specific and is associated with porphyrin accumulation at the surface of the bacterium. Preliminary work demonstrated that a peptide-biotin compound with a lysine-proline sequence was preferentially cleaved by *P. gingivalis* Kgp whereas this linkage is inefficiently cleaved by trypsin.^{6, 13}

In vitro studies demonstrated efficient cleavage of DPIX-Lys(Boc)-Pro-Nim adducts **22a** and **22b** by purified Kgp (see Figs S7a-S7c). A *kgp*⁻ mutant of *P. gingivalis* 33277 (KDP 129) and wild type were grown in the presence of adducts **22a** and **22b** with similar growth inhibitory activities observed for both adducts. In contrast the proline-linked DPIX-Pro-Nim adducts **24a** and **24b** were not cleaved by Kgp but were as active as metronidazole **3** for *P. gingivalis* (see Table 1). These results suggest that Kgp catalytic activity is not required for uptake and processing by *P. gingivalis* (see Figs S7a-S7c).¹⁴

Confocal studies

H413 are a human oral epithelial cell line. H413 readily take up **12a** and **12b** (Fig. 6). Concentrated depots of **12a** and **12b** were observed in the cytoplasm of the epithelial cells. Amide-linked adducts **6a** and **6b** and **7a** and **7b** were not taken up by epithelial cells (results not shown). The lysine-linked adducts **12a** and **12b** may therefore be effective against intracellular *P. gingivalis*.

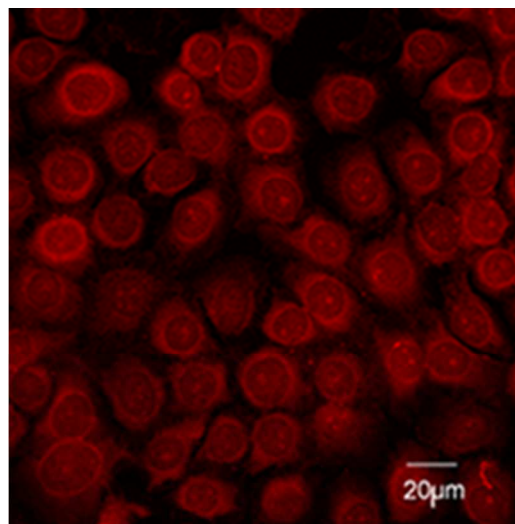


Figure 6: Confocal microscopy showing uptake of fluorescent **12a** and **12b** by oral epithelial cells.

The surface receptor CD24 is highly expressed on diseased epithelium next to affected teeth.^{15,16} CD24-specific antibody attached to a peptide linker on the lysine side chain of **12a** and **12b** could localize the adducts to the tissue interface of disease sites. Studies are ongoing in our laboratories to further develop antibody CD24-mediated targeting.

Conclusions

A successful methodology for the synthesis of amino acid-linked porphyrin-nitroimidazole antibiotics targeting *P. gingivalis* has been developed. It involves a standard peptide-like coupling between the carboxylic acid of a free-base porphyrin DPIX **2** and an amino group of an antibiotic-appended amino acid or dipeptide and it affords the target compounds in good overall yields. DPIX-Lys(Boc)-Nim adducts **12a** and **12b** have been shown to have potential for selective action against *P. gingivalis*. The inactivity of adducts **17a** and **17b** suggests that certain modifications are not allowed with respect to HA2 recognition and highlights the need to refine the previously proposed HA2 binding site (Fig. 1) by indicating restriction in the space around the modified propionate. A new HA2 binding site model is proposed and is depicted in Fig. 6.

It has been shown that some adducts, that bind rHA2 as well as haem **1**, are ineffective in inhibiting the growth of *P. gingivalis*. This result highlights that not only is capture by HA2 necessary but for a drug to be active it needs to be successfully transported into the cell and/or utilised by the organism. The amino acid-linked porphyrin-nitroimidazole adducts with growth supporting or growth inhibitory activities are taken up by *P. gingivalis*. Findings from this study have also shown that Kgp is not critical for the activity of certain adducts as these adducts have the same albeit reduced growth inhibitory activities against both the *Kgp* mutant and wild type of *P. gingivalis*.

Mtz **3** killed all anaerobic bacteria tested. DPIX-Lys(Boc)-Nim adducts **12a** and **12b** (also **7a** and **7b**) kill *P. gingivalis* with a similar potency to that of Mtz **3**. In contrast, all

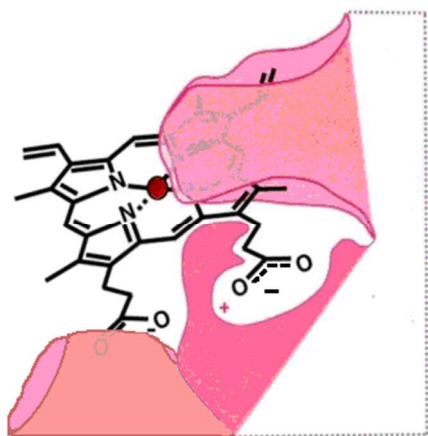


Fig. 6 Newly proposed HA2 binding site showing space restrictions around the propionate region.

anaerobic bacteria tested grew in the presence of **12a** and **12b** in concentrations approximately ten fold higher than the lethal Mtz **3** concentration. Indeed, *P. melaninogenica* and *T. forsythia* grew in 50 μ M **7a** and **7b** and **12a** and **12b**, respectively.

To facilitate comparisons between fastidious *T. forsythia* and other anaerobes, we elected to use nutrient rich eBHI media (which is more nutritious than CDC media). In eBHI, *F. nucleatum* was unaffected by **7a** and **7b**, whereas we previously demonstrated retarded growth in the presence of **7a** and **7b** in CDC media.⁶ Indeed, **12a** and **12b** were more effective for *P. gingivalis* in CDC than in eBHI media. We also note the rate of growth and final biomass was higher for all organisms tested in eBHI medium. We cannot discount increased fitness of the organisms in eBHI medium, although these results indicate competition for uptake of adducts with other porphyrin sources, via porphyrin uptake pathways. We have not explicitly proved this. Thus, the effectiveness of porphyrin adducts will be impacted by local concentration of nutrients (including porphyrin).

Data herein inform practical considerations for the selective control of bacteria using porphyrin-based antibiotics.

Experimental

General procedures. Melting points were recorded on a Gallenkamp melting point apparatus and are uncorrected. ¹H Nuclear Magnetic Resonance (¹H NMR) spectra were recorded on a Bruker Avance DPX 300 spectrometer at a frequency of 300.13 MHz at 300 K. *J* values are given in Hz. MALDI-TOF mass spectra were recorded on a Micromass Tof Spec 2E spectrometer. ElectroSpray Ionisation (ESI) mass spectra were recorded on a ThermoQuest Finnigan LCQ Deca ion trap mass spectrometer. Infrared absorption spectra were recorded on a Shimadzu Model 8400 FTIR spectrophotometer and electronic absorption spectra were carried out on a Cary 5E UV-Vis spectrophotometer. Analytical thin layer chromatography (TLC) was performed using Merck Kieselgel silica gel 60 F-254 precoated sheets (0.2 mm) and preparative column chromatography was carried out routinely on Merck Kieselgel 60 silica gel (SiO₂, 0.040-0.063 mm). Analytical and preparative reverse phase HPLC was carried out on a Waters 600E solvent delivery system with a Rheodyne 7125 injector and a Waters 486 UV detector using the solvent system stated. Amino acids are all of the naturally occurring L-configuration and are referred to using their standard three-letter codes as monomers.

Preparation of Precursor Protected L-Amino Acids and Dipeptides

Fmoc-Lys(Boc)-Nim 9. Fmoc-Lys(Boc)-OH **8** (900 mg, 1.92 mmol) was dissolved in *N,N'*-dimethylformamide (DMF, 3 cm³), followed by CH₂Cl₂ (17 cm³). To this was added Mtz-NH₂ **4** (480 mg, 1.98 mmol), *N,N'*-diisopropylethylamine (DIPEA, 1.032 cm³, 5.92 mmol) and HBTU (745 mg, 1.98

mmol) and the mixture was stirred at room temperature under N₂ for 3 h. CHCl₃ (50 cm³) was added followed by H₂O (50 cm³). The aqueous layer was extracted with CHCl₃ (3 × 50 cm³) and the combined organic extracts were washed with H₂O (3 × 50 cm³) and brine solution (3 × 50 cm³), dried over anhydrous sodium sulfate, filtered and the solvent removed under reduced pressure. The residue was purified by silica gel chromatography (1:1 Hexane:EtOAc ramping to neat EtOAc). The fractions that contained the first band were discarded. The fractions containing the second band were evaporated to dryness to yield Fmoc-Lys(Boc)-Nim **9** (953 mg, 80%) as an off-white solid. ¹H NMR (300 MHz; CDCl₃) δ_H 1.42 (9H, s, *t*Bu-H), 2.48 (3H, s, N=C-CH₃), 3.08-3.10 (2H, overlapping t, -CH₂), 3.57-3.60 (2H, overlapping t, -CH₂), 4.07-4.23 (4H, overlapping t, -CH₂), 4.19 (1H, t, *J* 3.1, -CH), 4.26 (1H, m, αH), 4.41-4.44 (6H, overlapping t, -CH₂), 4.72 (1H, br t, NH), 5.47 (1H, br d, NH), 6.85 (1H, br t, NH), 7.27-7.78 (8H, m, Ar-H), 7.92 (1H, s, NO₂C=CH); *m/z* (ESI-MS) 621.1 [(M + H)⁺ requires 621.3]; *m/z* (FTICR-MS) 621.3034 [(M + H)⁺, calcd for [C₃₂H₄₀N₆O₇ + H⁺]: 621.3042].

Fmoc-Lys(Nim)-Nim 15. Fmoc-Lys(Boc)-Nim **9** (150 mg, 0.242 mmol) was dissolved in CF₃CO₂H:CH₂Cl₂ (3:1, 20 cm³) and the mixture was stirred at room temperature under N₂ for 3 h. The solvent was removed under reduced pressure and DMF (1 cm³) was added, followed by CH₂Cl₂ (9 cm³). To this was added Mtz-CO₂H **5** (46 mg, 0.248 mmol), followed by HBTU (96 mg, 0.253 mmol) and DIPEA (0.18 cm³, 1.033 mmol) and the mixture was stirred at room temperature under N₂ overnight. The solvents were removed and the residue was purified by silica gel chromatography eluting with CH₃OH:CH₂Cl₂ (1:4) to yield Fmoc-Lys(Nim)-Nim **15** (120 mg, 72%) as an off-white solid. ¹H NMR (300 MHz; CDCl₃) δ_H 2.46/2.48 (6H, s, N=C-CH₃), 3.22-3.26 (2H, overlapping t, -CH₂), 3.58-3.62 (2H, overlapping t, -CH₂), 4.07-4.12 (2H, overlapping t, -CH₂), 4.15-4.21 (2H, overlapping t, -CH₂), 4.24 (1H, m, αH), 4.39-4.44 (6H, m, -CH₂), 4.84 (2H, overlapping t, -CH₂), 5.63 (1H, br s, NH), 6.77 (1H, br t, NH), 7.01 (1H, br t, NH), 7.24-7.76 (8H, m, Ar-H), 7.90/7.93 (2H, s, NO₂C=CH); *m/z* (ESI-MS) 688.5 [(M + H)⁺ requires 688.7].

Fmoc-Pro-Nim 19. Fmoc-Pro-OH **18** (500 mg, 1.48 mmol) was dissolved in *N,N'*-dimethylformamide (DMF, 3 cm³), followed by CH₂Cl₂ (17 cm³). To this was added Mtz-NH₂ **4** (360 mg, 1.49 mmol), *N,N'*-diisopropylethylamine (DIPEA, 0.780 cm³, 4.48 mmol) and HBTU (560 mg, 1.49 mmol) and the mixture was stirred at room temperature under N₂ for 3 h. CHCl₃ (50 cm³) was added followed by H₂O (50 cm³). The aqueous layer was extracted with CHCl₃ (3 × 50 cm³) and the combined organic extracts were washed with H₂O (3 × 50 cm³) and brine solution (3 × 50 cm³), dried over anhydrous sodium sulfate, filtered and the solvent removed under reduced pressure. The residue was purified by silica gel chromatography (1:1 Hexane:EtOAc ramping to neat EtOAc). The fractions that contained the first band were discarded. The fractions containing the second band were evaporated to dryness to yield

Fmoc-Pro-Nim **19** (620 mg, 85%) as an off-white solid. ¹H NMR (300 MHz; CDCl₃) δ_H 2.12-2.38 (4H, m, -CH₂), 2.45 (3H, s, N=C-CH₃), 3.30-3.45 (2H, m, -CH₂), 3.55-3.60 (2H, overlapping t, -CH₂), 4.20 (1H, t, *J* 3.1, -CH), 4.23 (1H, m, αH), 4.37-4.41 (4H, overlapping t, -CH₂), 6.55 (1H, br t, NH), 7.24-7.75 (8H, m, Ar-H), 7.88 (1H, s, NO₂C=CH); *m/z* (ESI-MS) 490.4 [(M + H)⁺ requires 490.2]; *m/z* (FTICR-MS) 490.2001 [(M + H)⁺, calcd for [C₂₆H₂₇N₅O₅ + H⁺]: 490.2012].

Fmoc-Lys(Boc)-Pro-Nim 20. Fmoc-Pro-Nim **19** (100 mg, 0.203 mmol) was dissolved in DMF (1 cm³) followed by CH₂Cl₂ (9 cm³). To this was added DBU (0.039 cm³, 0.252 mmol) and the mixture was stirred at room temperature under N₂ for 30 min. The mixture was quenched with HOBt (33 mg, 0.246 mmol) and to this was added Fmoc-Lys(Boc)-OH **8** (95 mg, 0.203 mmol), *N,N'*-diisopropylethylamine (DIPEA, 0.174 cm³, 0.999 mmol) and HBTU (155 mg, 0.411 mmol) and the mixture was stirred at room temperature under N₂ for 3 h. CHCl₃ (50 cm³) was added followed by H₂O (50 cm³). The aqueous layer was extracted with CHCl₃ (3 × 50 cm³) and the combined organic extracts were washed with H₂O (3 × 50 cm³) and brine solution (3 × 50 cm³), dried over anhydrous sodium sulfate, filtered and the solvent removed under reduced pressure. The residue was purified by silica gel chromatography (1:1 Hexane:EtOAc ramping to neat EtOAc). The fractions which contained the first band were discarded. The fractions containing the second band were evaporated to dryness to yield Fmoc-Lys(Boc)-Pro-Nim **20** (117 mg, 81%) as an off-white solid. ¹H NMR (300 MHz; CDCl₃) δ_H 1.42 (9H, s, *t*Bu-H), 2.11-2.36 (4H, m, -CH₂), 2.47 (3H, s, N=C-CH₃), 3.08-3.10 (2H, overlapping t, -CH₂), 3.30-3.46 (2H, m, -CH₂), 3.55-3.61 (2H, overlapping t, -CH₂), 4.08-4.22 (4H, overlapping t, -CH₂), 4.20 (1H, t, *J* 3.1, -CH), 4.24 (1H, m, αH) 4.27 (1H, m, αH), 4.40-4.43 (6H, overlapping t, -CH₂), 4.61 (1H, br t, NH), 5.45 (1H, br d, NH), 6.75 (1H, br t, NH), 7.25-7.77 (8H, m, Ar-H), 7.90 (1H, s, NO₂C=CH); *m/z* (ESI-MS) 718.2 [(M + H)⁺ requires 718.3]; *m/z* (FTICR-MS) 718.3461 [(M + H)⁺, calcd for [C₃₇H₄₇N₇O₈ + H⁺]: 718.3486].

Synthesis of Deuteroporphyrin-metronidazole adducts

DPIX-Lys(Boc)-Nim adducts 12a and 12b. Fmoc-Lys(Boc)-Nim **9** (43 mg, 0.069 mmol) was dissolved in CH₂Cl₂ (10 cm³). To this was added DBU (0.013 cm³, 0.084 mmol) and the mixture was stirred at room temperature under N₂ for 30 min. The mixture was quenched with HOBt (11 mg, 0.082 mmol) and to this was added DPIX mono-methyl esters **10** (30 mg, 0.057 mmol), DIPEA (44 mm³, 0.257 mmol) and HBTU (43 mg, 0.114 mmol). The mixture was stirred at room temperature under N₂ overnight. The mixture was filtered and the solvent removed. The residue was purified by silica gel chromatography (3:97 CH₃OH:CH₂Cl₂) to yield DPIX-Lys(Boc)-Nim methyl esters **11a** and **11b** (47 mg, 90%) as a reddish-black solid. *m/z* (ESI-MS) 905.5 [(M + H)⁺ requires 905.5]; *m/z* (FTICR-MS) 905.4671 [(M + H)⁺, calcd for [C₄₈H₆₀N₁₀O₈ + H⁺]: 905.4669]. Hydrolysis of **11a** and **11b**

(100 mg, 0.110 mmol) occurred with LiOH (222 mg, 9.30 mmol) in CH₃OH:THF:H₂O (3:4:1, 20 cm³) with stirring overnight under N₂ at room temperature. The solvent was removed to give DPIIX-Lys(Boc)-Nim adducts **12a** and **12b** (99 mg, quant.) as a black solid. λ_{\max} (CH₃OH)/nm 399 (log ϵ dm³ mol⁻¹ cm⁻¹ 5.18), 497 (4.04), 530 (3.87), 565 (3.78), 619 (3.44); ν_{\max} (KBr)/cm⁻¹ 3453w (NH), 3040m (OH), 2990m (aromatic CH), 2860m (aliphatic CH), 1711s (C=O), 1651/1463m (C=C), 1513/1367w (N=O), 1166m (C–O); ¹H NMR (300 MHz; DMSO-D₆) δ_{H} -4.00 (2H, s, inner NH), 1.36 (9H, s, *t*Bu-H), 1.85/1.99 (3H, s, N=C–CH₃), 2.52–2.55 (4H, overlapping t, –CH₂), 3.05–3.17 (4H, overlapping t, –CH₂), 3.25–3.30 (2H, overlapping t, –CH₂), 3.67 (3H, s, –CH₃), 3.71 (3H, s, –CH₃), 3.76/3.78 (3H, s, –CH₃), 3.76–3.81 (2H, overlapping t, –CH₂), 3.80/3.81 (3H, s, –CH₃), 4.15–4.20 (2H, overlapping t, –CH₂), 4.22 (1H, m, α H), 4.40–4.45 (6H, overlapping t, –CH₂), 6.40 (1H, br s, O=C–NH), 7.89/7.92 (1H, s, NO₂C=CH), 7.99 (1H, br s, –NH), 9.34/9.37 (2H, s, β -pyrrolic H), 10.30–10.38 (4H, overlapping s, *meso* H), 12.45 (1H, br s, O=C–OH); *m/z* (ESI-MS) 891.4 [(M + H)⁺ requires 891.5]; *m/z* (FTICR-MS) 891.4532 [(M + H)⁺, calcd for [C₄₇H₅₈N₁₀O₈ + H⁺]: 891.4512].

DPIIX-Lys(H)-Nim adducts 14a and 14b. Method 1. Hydrolysis of DPIIX-Lys(Boc)-Nim methyl esters **11a** and **11b** (30 mg, 0.033 mmol) occurred quantitatively with LiOH (22 mg, 0.921 mmol) in CH₃OH:THF:H₂O (3:4:1, 20 cm³) with stirring overnight under N₂ at room temperature. The solvent was removed to give DPIIX-Lys(Boc)-Nim adducts **12a** and **12b** as a black solid. To this was added CH₂Cl₂ (10 cm³) and CF₃CO₂H (2 cm³) and the mixture was stirred for 3 h at room temperature under N₂. The solvent was removed and sodium hydrogen carbonate was added until the pH of the solution was approximately 6–7. The solvent was removed and the residue was passed through a reverse phase Zorbax Rx-SIL column eluting with CH₃OH:H₂O:CH₃CO₂H (80:20:0.5) over a gradient to CH₃OH:CH₃CO₂H (100:0.5) to yield DPIIX-Lys(H)-Nim adducts **14a** and **14b** (17 mg, 69%) as a black solid. λ_{\max} (CHCl₃)/nm 395 (log ϵ dm³ mol⁻¹ cm⁻¹ 4.40), 495 (3.69), 528 (3.45), 565 (3.35), 618 (3.14); ν_{\max} (KBr)/cm⁻¹ 3492w (NH), 3065m (OH), 2980m (aromatic CH), 2864m (aliphatic CH), 1710s (C=O), 1680/1466m (C=C), 1573/1365w (N=O), 1190m (C–O); ¹H NMR (300 MHz; DMSO-D₆) δ_{H} -4.01 (2H, s, inner NH), 1.78/1.91 (3H, s, N=C–CH₃), 2.39–2.45 (4H, overlapping t, –CH₂), 3.00–3.13 (4H, overlapping t, –CH₂), 3.21–3.26 (2H, overlapping t, –CH₂), 3.63 (3H, s, –CH₃), 3.67 (3H, s, –CH₃), 3.72/3.73 (3H, overlapping s, –CH₃), 3.72–3.77 (2H, overlapping t, –CH₂), 3.75/3.77 (3H, s, –CH₃), 4.05–4.11 (2H, overlapping t, –CH₂), 4.15 (1H, m, α H), 4.35–4.40 (6H, overlapping t, –CH₂), 6.32 (1H, br s, O=C–NH), 7.32–7.44 (2H, br s, –NH), 7.86/7.87 (1H, s, NO₂C=CH), 9.32/9.34 (2H, s, β -pyrrolic H), 10.26–10.32 (4H, overlapping s, *meso* H), 12.45 (1H, br s, O=C–OH); *m/z* (ESI-MS) 791.5 [(M + H)⁺ requires 791.9]; *m/z* (FTICR-MS) 791.3988 [(M + H)⁺, calcd for [C₄₂H₅₀N₁₀O₆ + H⁺]: 791.4004]. Retention time: 9.2 min; ZorbaxRx-SIL975.9; 1mL/min.

Method 2. DPIIX-Lys(Boc)-Nim methyl esters **11a** and **11b** (30 mg, 0.033 mmol) was dissolved in CH₂Cl₂ (10 cm³). To this was added CF₃CO₂H (2 cm³) and the mixture was stirred for 3 h at room temperature under N₂. The solvent was removed to yield DPIIX-Lys(H)-Nim methyl esters **13a** and **13b** as a black solid *m/z* (ESI-MS) 805.47, [(M + H)⁺ requires 805.42]. To the residue was added LiOH (222 mg, 9.30 mmol) in CH₃OH:THF:H₂O (3:4:1, 20 cm³) and the mixture was stirred at room temperature under N₂ overnight. The solvent was removed to yield a black solid. The residue was passed through a reverse phase Zorbax Rx-SIL column eluting with CH₃OH:H₂O:CH₃CO₂H (80:20:0.5) over a gradient to CH₃OH:CH₃CO₂H (100:0.5) to yield DPIIX-Lys(H)-Nim adducts **14a** and **14b** (17 mg, 65%) as a black solid with identical characterisation to the product from Method 1.

DPIIX-Lys(Nim)-Nim 17a and 17b. Fmoc-Lys(Nim)-Nim **15** (80 mg, 0.116 mmol) was dissolved in DMF (1 cm³) followed by CH₂Cl₂ (9 cm³). To this was added DBU (0.270 cm³, 0.174 mmol) and the mixture was stirred for 30 min at room temperature under N₂. The mixture was quenched with HOBT (25 mg, 0.186 mmol) and to this was added DPIIX mono-methyl esters **10** (30 mg, 0.057 mmol), DIPEA (88 mm³, 0.505 mmol) and HBTU (44 mg, 0.114 mmol). The mixture was stirred overnight at room temperature under N₂. The mixture was filtered and the solvent removed. The residue was purified by silica gel chromatography (5:95 CH₃OH:CH₂Cl₂) to yield DPIIX-Lys(Nim)-Nim methyl esters **16a** and **16b** (50 mg, 90%) as a black solid. *m/z* (ESI-MS) 972.9 [(M + H)⁺ requires 972.4]; *m/z* (FTICR-MS) 972.4454 [(M + H)⁺, calcd for [C₄₉H₅₇N₁₃O₉ + H⁺]: 972.4475]. Hydrolysis of **16a** and **16b** (40 mg, 0.042 mmol) occurred with LiOH (222 mg, 9.30 mmol) in CH₃OH:THF:H₂O (3:4:1, 10 cm³) with stirring overnight at room temperature. The solvent was removed to give adducts **17a** and **17b** (39 mg, quant.) as a black residue. The residue was passed through a reverse phase Zorbax Rx-SIL column eluting with CH₃OH:H₂O:CH₃CO₂H (80:20:0.5) over a gradient to CH₃OH:CH₃CO₂H (100:0.5) to yield DPIIX-Lys(Nim)-Nim adducts **17a** and **17b** as a black solid. λ_{\max} (CH₃OH)/nm 398 (log ϵ dm³ mol⁻¹ cm⁻¹ 5.19), 497 (4.03), 528 (3.89), 564 (3.79), 618 (3.45); ν_{\max} (KBr)/cm⁻¹ 3455w (NH), 3048m (OH), 2992m (aromatic CH), 2863m (aliphatic CH), 1715s (C=O), 1650/1462m (C=C), 1512/1369w (N=O), 1164m (C–O); ¹H NMR (300 MHz; DMSO-D₆) δ_{H} -4.04 (2H, s, inner NH), 2.27/2.30 (6H, s, N=C–CH₃), 2.84–2.90 (2H, overlapping t, –CH₂), 3.06–3.13 (4H, overlapping t, –CH₂), 3.15–3.18 (2H, overlapping t, –CH₂), 3.30–3.55 (4H, overlapping t, –CH₂), 3.60 (3H, s, –CH₃), 3.64 (3H, s, –CH₃), 3.72 (3H, s, –CH₃), 3.75 (3H, s, –CH₃), 3.80–3.89 (2H, overlapping t, –CH₂), 3.97–4.01 (2H, overlapping t, –CH₂), 4.15–4.19 (1H, m, α H), 4.30–4.39 (6H, overlapping t, –CH₂), 7.86/7.88 (2H, s, NO₂C=CH), 8.32–8.36 (2H, m, –NH), 9.12–9.18 (1H, br s, –NH), 9.30/9.32 (2H, s, β -pyrrolic H), 10.24–10.28 (4H, overlapping s, *meso* H), 10.68 (1H, broad s, O=C–OH); *m/z* (ESI-MS) 958.5 [(M + H)⁺ requires 958.4]; *m/z* (FTICR-MS) 958.4500 [(M + H)⁺, calcd for [C₄₈H₅₅N₁₃O₉ + H⁺]: 958.4530].

Synthesis of DPIX-Lys(Boc)-Pro-Nim adducts 22a and 22b.

Fmoc-Lys(Boc)-Pro-Nim **20** (50 mg, 0.070 mmol) was dissolved in DMF (2 cm³) followed by CH₂Cl₂ (8 cm³). To this was added DBU (0.014 cm³, 0.090 mmol) and the mixture was stirred at room temperature under N₂ for 30 min. The mixture was quenched with HOBt (12 mg, 0.089 mmol) and to this was added DPIX mono-methyl esters **10** (30 mg, 0.057 mmol), DIPEA (44 mm³, 0.257 mmol) and HBTU (43 mg, 0.114 mmol). The mixture was stirred at room temperature under N₂ overnight. The mixture was filtered and the solvent removed. The residue was purified by silica gel chromatography (3:97 CH₃OH:CH₂Cl₂) to yield DPIX-Lys(Boc)-Pro-Nim mono-methyl esters **21a** and **21b** (43 mg, 75%) as a reddish-black solid. *m/z* (ESI-MS) 1002.4 [(M + H)⁺ requires 1002.5]; *m/z* (FTICR-MS) 1002.5120 [(M + H)⁺, calcd for [C₅₃H₆₇N₁₁O₉ + H⁺]: 1002.5123]. Hydrolysis of methyl esters **21a** and **21b** (20 mg, 0.0200 mmol) occurred with LiOH (100 mg, 4.19 mmol) in CH₃OH:THF:H₂O (3:4:1, 20 cm³) with stirring overnight under N₂ at room temperature. The solvent was removed to give DPIX-Lys(Boc)-Pro-Nim **22a** and **22b** (19 mg, quant.) as a black solid. λ_{\max} (CH₃OH)/nm 398 (log ϵ dm³ mol⁻¹ cm⁻¹ 5.15), 495 (4.05), 530 (3.83), 563 (3.75), 617 (3.43); ν_{\max} (KBr)/cm⁻¹ 3454w (NH), 3042m (OH), 2992m (aromatic CH), 2863m (aliphatic CH), 1714s (C=O), 1650/1465m (C=C), 1513/1365w (N=O), 1170m (C-O); ¹H NMR (300 MHz; DMSO-D₆) δ_{H} -3.98 (2H, s, inner NH), 1.34 (9H, s, *t*Bu-H), 2.10-2.33 (4H, m, -CH₂), 2.46 (3H, s, N=C-CH₃), 2.72-2.77 (2H, overlapping t, -CH₂), 3.09-3.18 (4H, overlapping t, -CH₂), 3.21-3.28 (2H, overlapping t, -CH₂), 3.33-3.49 (2H, m, -CH₂), 3.55-3.62 (2H, overlapping t, -CH₂), 3.66 (3H, s, -CH₃), 3.69 (3H, s, -CH₃), 3.72/3.75 (3H, s, -CH₃), 3.78/3.81 (3H, s, -CH₃), 4.09-4.21 (4H, overlapping t, -CH₂), 4.22 (1H, m, α H) 4.26 (1H, m, α H), 4.41-4.45 (6H, overlapping t, -CH₂), 4.64 (1H, br t, NH), 5.43 (1H, br d, NH), 6.60 (1H, br t, NH), 7.92 (1H, s, NO₂C=CH), 9.22/9.26 (2H, s, β -pyrrolic H), 10.03-10.10 (4H, overlapping s, *meso* H), 12.32 (1H, br s, O=C-OH); *m/z* (ESI-MS) 988.7 [(M + H)⁺ requires 988.5]; *m/z* (FTICR-MS) 988.4966 [(M + H)⁺, calcd for [C₅₂H₆₅N₁₁O₉ + H⁺]: 988.4967].

Synthesis of DPIX-Pro-Nim adducts 24a and 24b. Fmoc-Pro-Nim **19** (33 mg, 0.067 mmol) was dissolved in DMF (1 cm³) followed by CH₂Cl₂ (9 cm³). To this was added DBU (0.013 cm³, 0.084 mmol) and the mixture was stirred at room temperature under N₂ for 30 min. The mixture was quenched with HOBt (11 mg, 0.082 mmol) and to this was added DPIX mono-methyl esters **10** (30 mg, 0.057 mmol), DIPEA (44 mm³, 0.257 mmol) and HBTU (43 mg, 0.114 mmol). The mixture was stirred at room temperature under N₂ overnight. The mixture was filtered and the solvent removed. The residue was purified by silica gel chromatography (3:97 CH₃OH:CH₂Cl₂) to yield DPIX-Pro-Nim methyl esters **23a** and **23b** (37 mg, 84%) as a reddish-black solid. *m/z* (ESI-MS) 774.5 [(M + H)⁺ requires 774.4]; *m/z* (FTICR-MS) 774.3647 [(M + H)⁺, calcd for [C₄₂H₄₇N₉O₆ + H⁺]: 774.3649]. Hydrolysis of methyl esters **23a** and **23b** (20 mg, 0.026 mmol) occurred with LiOH (100

mg, 4.19 mmol) in CH₃OH:THF:H₂O (3:4:1, 20 cm³) with stirring overnight under N₂ at room temperature. The solvent was removed to give DPIX-Pro-Nim adducts **24a** and **24b** (19 mg, quant.) as a black solid. λ_{\max} (CH₃OH)/nm 398 (log ϵ dm³ mol⁻¹ cm⁻¹ 5.15), 495 (4.02), 531 (3.88), 565 (3.76), 620 (3.43); ν_{\max} (KBr)/cm⁻¹ 3456w (NH), 3041m (OH), 2994m (aromatic CH), 2862m (aliphatic CH), 1712s (C=O), 1648/1460m (C=C), 1512/1364w (N=O), 1164m (C-O); ¹H NMR (300 MHz; DMSO-D₆) δ_{H} -3.98 (2H, s, inner NH), 2.11-2.36 (4H, m, -CH₂), 2.43 (3H, s, N=C-CH₃), 2.58-2.61 (2H, overlapping t, -CH₂), 3.01-3.09 (2H, overlapping t, -CH₂), 3.21-3.26 (2H, overlapping t, -CH₂), 3.30-3.44 (2H, m, -CH₂), 3.53-3.55 (2H, m, -CH₂), 3.62 (3H, s, -CH₃), 3.65 (3H, s, -CH₃), 3.70/3.72 (3H, s, -CH₃), 3.78/3.81 (3H, s, -CH₃), 4.11-4.15 (2H, overlapping t, -CH₂), 4.22 (1H, m, α H), 4.35-4.40 (2H, overlapping t, -CH₂), 6.60 (1H, br t, NH), 7.75 (1H, s, NO₂C=CH), 9.22/9.26 (2H, s, β -pyrrolic H), 10.02-10.10 (4H, overlapping s, *meso* H), 12.28 (1H, br s, O=C-OH); *m/z* (ESI-MS) 760.5 [(M + H)⁺ requires 760.3]; *m/z* (FTICR-MS) 760.3487 [(M + H)⁺, calcd for [C₄₁H₄₅N₉O₆ + H⁺]: 760.3493].

DPIX-Lys(Boc)OH 29a and 29b. Dimethyl (3-methyl-2-butenyl)sulfonium tetrafluoroborate **26** was prepared from 3-methyl-2-buten-1-ol **25** following the literature in a 60% yield.¹⁶ A solution of **26** (350 mg, 2.67 mmol) in CH₂Cl₂ (10 cm³) was added to a stirred suspension of Fmoc-Lys(Boc)-OH **8** (1.26 g, 2.70 mmol), K₂CO₃ (373 mg, 2.70 mmol) and CuBr (4 mg, 0.029 mmol) in CH₂Cl₂ (10 cm³) at room temperature overnight. The mixture was filtered and the filtrate evaporated under reduced pressure. The crude product was purified by silica gel chromatography (1:99 CH₃OH:CH₂Cl₂) and the solvent was removed to yield Fmoc-Lys(Boc)-DMA **27** (1.12 g, 77%) as an off-white solid. *m/z* (ESI-MS) 559.1 [(M + Na)⁺ requires 559.3]. Fmoc-Lys(Boc)-DMA **27** (160 mg, 0.298 mmol) was dissolved in CH₂Cl₂ (20 cm³). To this was added DBU (0.50 cm³, 0.322 mmol) and the mixture was stirred at room temperature under N₂ for 1 h. HOBt (45 mg, 0.335 mmol) was added followed by DPIX mono-methyl esters **10** (120 mg, 0.229 mmol), HBTU (173 mg, 0.458 mmol) and DIPEA (0.157 cm³, 0.901 mmol). The mixture was stirred at room temperature under N₂ overnight. Solvent was removed and the residue was purified by silica gel chromatography using CH₃OH:CH₂Cl₂ (2:98). The solvent was removed to give the desired compounds **28a** and **28b** (154 mg, 82%) as a black solid. *m/z* (ESI-MS) 821.3 [(M + H)⁺ requires 821.0]; *m/z* (FTICR-MS) 821.4596 [(M + H)⁺, calcd for [C₄₇H₆₀N₆O₇ + H⁺]: 821.4602]. Hydrolysis of **28a** and **28b** (50 mg, 0.061 mmol) occurred with LiOH (222 mg, 9.30 mmol) in CH₃OH:THF:H₂O (3:4:1, 20 cm³) with stirring overnight at room temperature. The solvent was removed to give DPIX-Lys(Boc)OH regioisomers **29a** and **29b** (49 mg, quant.) as a black residue. The residue was passed through a reverse phase Zorbax Rx-SIL column eluting with CH₃OH:H₂O:CH₃CO₂H (80:20:0.5) over a gradient to CH₃OH:CH₃CO₂H (100:0.5) to yield **29a** and **29b** as a black solid. λ_{\max} (CH₃OH)/nm 399 (log ϵ dm³ mol⁻¹ cm⁻¹ 5.17), 496 (4.04), 529 (3.86), 564 (3.78), 618 (3.44); ν_{\max} (KBr)/cm⁻¹

3455w (NH), 3041m (OH), 2988m (aromatic CH), 2858m (aliphatic CH), 1712s (C=O), 1649/1460m (C=C), 1510/1364w (N=O), 1165m (C–O); ^1H NMR (300 MHz; DMSO- D_6) δ_{H} –4.19 (2H, s, inner NH), 1.27 (9H, s, *t*Bu-H), 3.08-3.22 (4H, overlapping t, –CH₂), 3.29-3.33 (2H, overlapping t, –CH₂), 3.49/3.50 (3H, s, –CH₃), 3.54/3.58 (3H, s, –CH₃), 3.54-3.58 (2H, overlapping t, –CH₂), 3.70 (3H, s, –CH₃), 3.71 (3H, s, –CH₃), 3.92-3.96 (1H, m, α H), 4.23-4.30 (2H, overlapping, –CH₂), 4.32-4.38 (4H, overlapping t, –CH₂), 4.42-4.50 (2H, overlapping t, –CH₂), 5.11-5.22 (1H, overlapping d, –NH), 6.21-6.23 (1H, br t, –NH), 9.03 (2H, s, β -pyrrolic H), 9.90-10.02 (4H, overlapping s, *meso* H); *m/z* (ESI-MS) 738.9 [(M + H)⁺ requires 738.9]; *m/z* (FTICR-MS) 738.9243 [(M + H)⁺, calcd for [C₄₁H₅₀N₆O₇ + H⁺]: 738.9250].

General procedures for biological testing.

Recombinant HA2 (rHA2) preparation, purification and ELISA were performed as described previously.^{1,2,6}

For growth curves, *P. gingivalis* was cultured in CDC broth supplemented with menadione and haemin (at 37 °C in an atmosphere containing CO₂ (5%), H₂ (10%) and N₂ (85%))². A *kgp*[–] mutant of *P. gingivalis* (KDP 129) was cultured in BHI broth containing brain heart infusion (37 g L^{–1}, Oxoid), yeast extract (5g L^{–1}, Oxoid), L-cysteine (0.5 mg L^{–1}, Fluka) supplemented with menadione (1 mg L^{–1}) and haemin (5 mg L^{–1}) **1**. *Fusobacterium nucleatum* ATCC 25586, *Tannerella forsythia* ATCC 43037, *Prevotella melaninogenica* ATCC 25845 and *Bacteroides fragilis* ATCC 25285 were grown in ‘enriched BHI’ (eBHI), which was BHI with additional fetal bovine serum (5% v/v) and N-acetylmuramic acid (10 mg L^{–1}, Sigma) supplementation.

All rate of kill experiments were performed on solid eBHI with sheep blood (4% v/v) and agar (15 g L^{–1}). For experimental evaluation log phase cultures were passaged as a 1% (v/v) inoculum (at 5 × 10⁵ cfu ml^{–1}) into media supplemented with menadione only. Under this condition the bacteria had sufficient haem **1** reserves to grow through a complete cycle as reported previously.^{2,6}

Bacterial growth was monitored at 600 nm (with correction against reagent blanks). Data show mean values and standard error of the mean. Porphyrins, adducts and metronidazole derivatives were prepared as 5-10 mM stock solutions in DMSO (Sigma) for dilution into growth media. To control the possible inhibition of bacterial growth by DMSO, all cultures were adjusted to contain levels of this solvent equivalent to the highest concentration used in the experiment (1% v/v). All biological testing results were representative of at least two experiments.

Kgp (1 μL of a 1 μM solution) was incubated with porphyrin adducts (200 μM in 100 μL) in the presence of cysteine (1 μM) for 1 h at 37 °C and the products of the reaction were monitored by HPLC-MS using a solvent system of CH₃CN:H₂O:CF₃CO₂H (10:90:0.5) over a gradient to CH₃CN:H₂O:CF₃CO₂H (60:40:0.5). In a control experiment, the *Kgp* was first inactivated with TLCK (4 mM) for 30 min before incubation

with porphyrin adducts (200 μM) in the presence of cysteine (1 μM) for 1 h at 37 °C (see supplementary information).

For confocal studies, monolayer cultures of H413 oral epithelial cells were incubated for 15 min with 20 μM adduct, washed and examined by confocal microscopy.

Extensive details of H413 cell culture and confocal microscopy were recently described by us.^{15,16}

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