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Tolyporphins L-R: Unusual Tetrapyrroles from a *Brasilonema* sp. of Cyanobacterium

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Abstract

Tolyporphins L-R (**2-8**) have been isolated from a mixed cyanobacterium-microbial culture. The structures of tolyporphins L and M have been revised to four constitutional isomers, isolated as two mixtures of dioxobacteriochlorins (**2/3** and **4/5**). In contrast, tolyporphin P (**6**) is a fully oxidized tetrapyrrole, while tolyporphins Q and R (**7 & 8**) are oxochlorins. X-ray structures are reported for the first time for tolyporphin A (**1**), R (**8**), E (**9**), revealing unexpected stereochemical variation within the series.

Graphical Abstract

INTRODUCTION

Cyanobacteria are abundant producers of bioactive natural products and well recognized for their utility in drug discovery and biotechnology.¹ A plethora of structurally novel bioactive secondary metabolites have been reported from cyanobacteria in the past few decades. Although they are responsible for producing harmful toxins such as microcystins and anatoxins,^{2,3} cyanobacteria are established as an important source of anticancer drugs.⁴ For

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Supporting Information

Copies of the ¹H, ¹³C, and 2D NMR spectroscopic data for all new compounds, and x-ray crystallography data, CIF files, and raw NMR data associated with this article are available free of charge via the Internet at <http://pubs.acs.org>.

example, the FDA approved brentuximab vedotin is an antibody-drug conjugate used to treat Hodgkin's lymphoma and anaplastic large cell lymphoma.⁵ This medication has a derivative of the cyanobacterial natural product dolastatin 10 as a warhead.

The tolyporphins are a distinctive group of cryptically modified porphyrinoid natural products. The strain was collected in Nan Madol, Pohnpei in the Federated States of Micronesia, which is so far the only known producing organism of this family of molecules—further adding to their novelty.^{6–10} The cyanobacterium's original classification of *Tolypothrix nodosa* was based upon morphological taxonomy as it was described as having robust, long, straight trichomes with unilateral false branching and intercalary heterocysts. However, recent 16S rRNA analysis suggests it more closely resembles the relatively new genus *Brasilonema*.^{11,12} The initial bioassay-guided discovery of the tolyporphins was based on their ability to reverse resistance to vinblastine in multi-drug resistant adenocarcinoma cell lines⁶ through efflux pump inhibition,¹³ but subsequent studies have also shown that tolyporphin A (**1**) is an effective agent for photodynamic therapy of cancers.¹⁴ Of particular structural note is the substituent pattern in the tetrapyrrole macrocycle of the tolyporphins, which is significantly different from the tetrapyrroles of life. A majority of the tolyporphins consist of a bacteriochlorin framework with two sites of substitution where either a C-glycoside, -OAc, or -OH resides (C-7 & C-17). Other sites of the macrocycle possess oxo functionalities (C-8 & C-18) or unsubstituted sp² methines (C-2 & C-13) where typically an alkyl propionate unit would reside. As part of a larger study on the chemistry and biology of this cyanobacterium, we report here the isolation and structural elucidation of tolyporphins L-R (**2-8**) along with our efforts to define the absolute configuration for three members of this structural family.

RESULTS AND DISCUSSION

The cyanobacterium was mass cultured in multiple 20 L Pyrex® carboys in pre-sterilized BG-11 media while aerated under continuous illumination of fluorescent light banks. The harvested cells were lyophilized, extracted, and the crude residue was partitioned using a modified Kupchan extraction¹⁵ into three fractions (hexanes, dichloromethane, and aqueous methanol). The dichloromethane and hexanes-soluble extracts were further fractionated over C8 silica gel with the majority of the tolyporphins eluting within the 75% MeOH fraction of the DCM extract as indicated by its deep purple coloration. ¹H-NMR analysis also indicated

tolyporphin-related compounds were in the 90% MeOH fraction of the hexanes partition based on characteristic N-H resonances between -2 to -3 ppm. Distinctive absorption spectral maxima^{16,17} at approximately 400 and 676 nm were also hallmarks of tolyporphin-related compounds and guided their isolation by HPLC. Successive reversed- and normal-phase HPLC on these fractions yielded compounds of sufficient purity for spectroscopic and spectrometric characterization.

Tolyporphins L-O (**2-5**) were isolated based on HRESIMS data from the 75% MeOH fraction of the DCM partition. Originally, tolyporphins L and M had been partially characterized from this strain using LDI-ToF by Prinsep *et al.*,¹⁸ who proposed two constitutional isomers that each possessed an additional oxygen atom within the C-glycoside compared to tolyporphins B and C. Key evidence for this assignment included MS/MS fragmentations between the tetrapyrrole and the glycoside(s). Specifically, tolyporphin A (**1**) yielded ions at m/z 571.2572 and 399.1832 from successive losses of the two C-glycosides from the core macrocycle, while ions at m/z 173.0808 and 113.0593 were proposed to represent oxonium species of the “whole” C-glycoside and a deacetylated fragment, respectively (Figure 1a). Based on fragments observed at m/z 570 and 544 Prinsep *et al.* concluded one of the C-glycosides contained an additional oxygen atom and was a trihydroxyl sugar (Figure 1b). The exact positioning of this oxygen was not assigned since neither L nor O were isolated in pure form or characterized with additional tools.

As part of a larger project, we recently reisolated several of the known tolyporphins and characterized them by NMR. To our surprise, the initial 1H NMR spectrum of the suspected tolyporphin L and M fraction contained resonances that indicated four distinct compounds. While we were unable to separate the four compounds by RP-HPLC (CH_3CN or $MeOH:H_2O$ on C_{18}), normal-phase HPLC with a hexanes-EtOAc gradient provided sufficient separation between the two sets of isomers. No further separation was possible, so all data was collected on the inseparable mixtures of constitutional isomers (**2-3** and **4-5**). The +ESI-MS/MS spectra of the protonated adduct $[M + H]^+$ in each of the two mixtures (Figure 1b shows a representative spectrum for L/M mixture) resulted in nearly identical ions and closely matched the previously reported MALDI results where the radical cation was selected as the parent ion.¹⁸

The 1H NMR spectrum for the mixture of **2** and **3** indicated a molar ratio of approximately 1.4:1 favoring compound **2**. Analysis of the 1H and ^{13}C NMR data (Table 1) indicated the dioxobacteriochlorin core with two C-glycosides found in **1** was present in both **2** and **3** based on a set of characteristic 1H NMR signals (singlets at δ_H 9.62, 9.57, 9.52, 8.89, and two doublets at δ_H 4.61 and 4.41).⁶ Subsequent analysis of the 2D NMR data confirmed the

core structure and showed the additional oxygen atom required by the molecular formula was attached at C-3' (δ_C 73.2) and C-3'' (δ_C 72.5) in **2** and **3**, respectively, in the form of a hydroxyl group. Specifically, a network of COSY correlations was evident that connect H-1' to H-4' and H-1'' to H-4'' for **2** and **3**, respectively. Due to overlapping signals, 1D-TOCSY data was required to verify the other C-glycoside unit in **2** and **3** was unmodified, and that the oxygen atoms on C-2' and C-2'' in **2** and **3**, respectively, were acetylated. This latter conclusion was based on the chemical shifts of H-2' (δ_H 4.26) and H-2'' (δ_C 4.38), and HMBC correlations to C-7' (δ_C 169.6) and C-7'' (δ_C 169.7), respectively. HMBC correlations from methyl groups H-22 and H-24 to carbonyl carbons (C-8 and C-18) and C-1' or C-1'' established the planar structures by confirming the location of each C-glycoside unit. Finally, analysis of the $^3J_{H-H}$ values obtained from the 1D TOCSY data of **2** revealed the additional hydroxyl group was in an axial orientation based on the observation that H-2' was a doublet of doublets with 10.1 and 3.3 Hz couplings to H-1' and H-3' (Figure 2a). This data also confirmed the rest of the substituents in the C-glycoside units were in the same orientation as in **1** based on the $^3J_{H-H}$ observed. The configuration of the new chiral center in **3** was assigned in a similar fashion using the minor signals in the NMR (Figure 2a). Based on biosynthetic and chemical shift consideration, the relative configuration of C-7 and C-17 is assumed to be the same as in **1**.

The 2:1 mixture of **4** and **5**, respectively, showed a set of resonances in its 1H NMR spectrum (Table 2) characteristic of the core structure of **1**. Similar to **2** and **3**, analysis of the 2D NMR data revealed in **4** and **5** positions C-3' and C-3'' were hydroxylated based on the observation that they resonated at δ_C 76.6 and 76.9 ppm, respectively, but coupling constant analysis of the 1D TOCSY data revealed that **4** and **5** were epimeric to **2** and **3**. The overlapping resonances depicted in Figure 2b at δ_H 3.39 ppm existed as doublets of doublets with one large 9.5 Hz coupling that indicated an axial orientation relative to H-2' in **4** and H-2'' in **5**, and one small 3.2 Hz coupling that implied an equatorial orientation relative to H-4' or H-4'' (Figure 2b). Analysis of the rest of the NMR data confirmed the planar structure, including the location of the acetyl groups on C-2' and C-2'' via HMBC correlations between H-2'/C-7' and H-2''/C-7''. The relative configuration of the C-glycoside units in **4** and **5** was established through detailed analysis of proton-proton coupling constants obtained through 1D TOCSY experiments, while the configuration of C-7 and C-23 are assumed to be conserved within the dioxobacteriochlorin series based on carbon chemical shift similarities at these positions.

The structure of tolyporphin P (**6**) was similarly deduced through a combination of 1D and 2D NMR techniques. HRESIMS of **6** produced a protonated adduct at m/z 367.1912 [M+H] $^+$ that established the molecular formula to be $C_{24}H_{22}N_4$ (calcd for $C_{24}H_{23}N_4^+$, 367.1917; = -1.4 ppm). The ^{13}C NMR spectrum of **6** (Table 3) included 24 resonances, but curiously lacked the usual carbonyl signals found in this series. Furthermore, the 1H NMR spectrum of **6** contained all the usual aromatic and methyl signals found in tolyporphins A-O, but also contained two additional aromatic singlets at δ_H 9.11. Taken together, the data suggested that **6** contained a macrocyclic ring system with two pyrrole and two pyrrolenine rings, i.e. that **6** was a true porphyrin, unique within the tolyporphins structural family as these are actually chlorins or bacteriochlorins. While this is the first report of **6** as a natural product,

this compound has been used in computational studies examining the electronic effects of substituents in porphinoid systems.¹⁹

The ¹H NMR spectrum of tolyporphin Q (**7**) contained 11 singlets of which seven were methines and four were methyl groups (Table 4) suggestive of an oxochlorin ring system similar to that found in tolyporphin K.⁸ HRMS provided a degree of unsaturation in agreement with the suspected oxochlorin core via a protonated adduct at *m/z* 399.1818 [M + H]⁺ indicative of a molecular formula of C₂₄H₂₂N₄O₂ (calcd for C₂₄H₂₃N₄O₂⁺, 399.1816; = +0.6 ppm). As suspected, the ¹³C NMR spectrum of **7** contained resonances for only one hydroxyl substituent (δ_C 77.6) and one ketone carbonyl (δ_C 208.5 ppm) which could be assigned at C-17 and C-18, respectively. Evidence for these assignments were HMBC correlations from H-15 (δ_H 9.08) to C-17 and HMBC correlations from H₃-24 (δ_H 1.93) to C-16, C-17 and C-18, which established the planar structure as depicted.

HRESIMS provided a protonated adduct at *m/z* 441.1925 [M+H]⁺ for tolyporphin R (**8**) which was consistent with a molecular formula of C₂₆H₂₄N₄O₃ (calcd for C₂₆H₂₅N₄O₃⁺, 441.1921; = +0.9 ppm). The 1D and 2D NMR spectra of **8** (Table 5) were similar to those of **7**, except for the presence of signals indicative of an acetate ester (C-25 and C-26) and a slight downfield shift of C-17 (δ_C 77.6 vs. δ_C 81.1) leading to the proposed structure.

To date, tolyporphin A is the only member of this class of compounds to have its configuration established conclusively. Synthesis of tolyporphin A *O,O*-diacetate and comparison of its ECD spectrum with that of the corresponding derivative of the natural product established a *7R*, *17R*, *1'S*, *2'R*, *3'R*, *4'R*, *5'R*, *1''S*, *2''R*, *3''R*, *4''R*, *5''R* configuration.²⁰ The authors also cautiously speculated that the other analogs shared the same configuration.

After extensive efforts with all members of this structural family, we were able to obtain X-ray quality crystals of three compounds via slow evaporation from an acetonitrile solution. It is important to note that other solvents and mixtures (MeOH, DCM, and DCM/Hexanes) were tested, as was the introduction of silver ions,²¹ and only acetonitrile resulted in X-ray quality crystals. The resolved x-ray structure of **1** confirmed the configurational assignment, made by comparing a derivative, for the first time with the underivatized natural product (Figure 3). Interestingly, the asymmetric unit cell for **1** contained four independent molecules arranged in two offset “sandwiches” in which the sugar moieties were between two of the aromatic cores. On the pyrrole rings the methyl groups and adjacent protons were all at half occupancy reflecting the multiple positions of C-21 and C-24 within the unit cell given the almost symmetrical nature of tolyporphin A (**1**).

In contrast, the crystal structures of **8** and the known tolyporphin E (**9**) revealed unexpected variations. In **1**, the methyls at C-7 and C-17 were oriented on the same “face” of the macrocyclic ring system. Conversely, **8** had those methyl groups on opposite faces, with the configuration of C-7 inverted. Superimposing the crystal structure of **8** and **9** revealed that the absolute configuration of C-17 was also not conserved even within the oxygenated series (Figure 4). As the biosynthesis of the tolyporphins is largely unknown, these structural details may be useful for evaluating possible biosynthetic proposals. These data also

illustrate that configurational assignments based on a presumed shared biosynthesis must be done with caution, particularly for structures with a high degree of novelty arising from uncharacterized biosynthetic pathways.

Since we were unable to grow X-ray quality crystals of **2-5** and **8**, we compared the ECD spectra of these chiral compounds with that of **1** and **7**. Our ECD spectrum of **1** in MeOH was very similar to that of the diacetate derivative reported previously in CH₂Cl₂,²⁰ with slight bathochromic shifts. We had previously noted this effect on the absorption spectral maxima of **1** in polar solvents, which was attributed to hydrogen bonding, while examining the photochemical properties of the tolporphins.¹⁷ The ECD spectra (Figure 5A) within the dioxobacteriochlorin series **1-5** were in general agreement suggesting the same absolute configuration. Within the oxochlorin series (**7-8**), the ECD spectra (Figure 5B) again match well suggesting these two compounds have the same absolute configuration.

Tetrapyrroles can be delineated into porphyrins, chlorins, or iso/bacteriochlorins based on the number and configuration of π electrons contributing to the aromatic system. Porphinoids are produced by organisms in all kingdoms of life and carry out a multitude of biological functions including photosynthesis, respiration, and electron transport. Along with tolporphin K, the newly isolated P, Q, and R entries are so far the only tolporphin analogs characterized with macrocycles that exist in an oxidation state higher than that of the typically observed bacteriochlorin.

In summary, seven tolporphin derivatives have been characterized by NMR and MS. The general structures of two were previously proposed by analysis of LC-MS data alone, but here shown to be inseparable mixtures of constitutional isomers by NMR, thus highlighting one of the limitations of LC-MS-based structure determinations in the absence of NMR data.

Experimental Section

General Experimental Procedures.—Optical rotations were measured on a Jasco-DIP-700 polarimeter at the sodium line (589 nm). UV spectra were obtained on a Hewlett-Packard 8453 spectrophotometer, and IR spectra were measured as a thin film on a CaF₂ disc using a Perkin Elmer 1600 series FTIR. All 1D and 2D NMR spectra with the exception of ¹³C NMR spectra for compounds **2-8** were acquired on a Varian Unity Inova 500 MHz spectrometer operating at 500 (¹H) or 125 (¹³C) MHz using the residual solvent signals as an internal reference. For the mixtures of **2-3** and **4-5**, ¹³C NMR spectra were acquired on an Agilent 600 MHz spectrometer operating at 150 (¹³C) MHz and referenced similarly. Samples were analyzed in 3 mm Shigemi NMR tubes. High-resolution mass spectrometry data were obtained on an Agilent 6545 LC-MS Q-ToF with ESI ionization in the positive mode. Gradient HPLC separations used a Shimadzu system consisting of LC-20AT Solvent Delivery Modules, an SPD-M20A VP Diode Photodiode Array Detector, and an SCL-20A VP System Controller.

Cultivation of Cyanobacteria.—Cultures were revived from cryostorage and grown in BG-11 media. Phylogenetic characterization and genomic sequencing of this strain has been previously reported.¹⁰ For large-scale harvests, cultures were grown in 20 L Pyrex® carboys and aerated at a flow rate of 5 L/min while under continuous illumination of fluorescent

light banks. Cell material was harvested after 45 days of growth via decantation and filtration. This cell mass was freeze-dried prior to extraction.

Extraction and Isolation of Tolyporphins from UH strain HT-58-2.—The freeze-dried alga (79 g) was extracted with 1:1 CH₂Cl₂:2-propanol four times overnight. The combined extract was filtered and the solvent removed *in vacuo*. The crude residue (4.7 g) was partitioned via a modified Kupchan partition into hexanes, DCM, and aqueous methanol soluble fractions. The residue from the hexanes and DCM partitions was separately dry-loaded on to two C8 silica gel columns and eluted with a H₂O-MeOH step gradient modified with 0.1% formic acid. Five fractions of increasing MeOH content (25%, 50%, 75%, 90%, and 100%) were collected. The 75% MeOH fraction of the DCM partition and the 90% MeOH fraction of the hexanes partition were then subjected to further purification via HPLC. Initial HPLC chromatographic separation was performed using a linear gradient of H₂O-ACN modified with 0.1% formic acid (70-100% ACN in water over 25 min and then 100% ACN over 15 min on a Gemini 5 μ , C₁₈, 250 x 21.2 mm, flowrate 15 mL/min, PDA detection) with the following retention times: **2-4** t_R 23.7 min, **5** t_R 29.7 min, **6** t_R 21.5 min, **7** t_R 24.2 min. Final purification of these compounds was achieved by normal phase chromatography (a linear gradient of hexanes-EtOAc from 10-80% over 30 min and then 100% EtOAc over 15 min; Luna Si (2), 10 μ , 100 Å, 250 x 10 mm, flowrate 3.5 mL/min, detection at 400 nm). This afforded mixtures of **2** and **3** (0.9 mg, 0.002% yield, 96.6% purity, t_R 31.2 min) and **4** and **5** (2.3 mg, 0.005% yield, 99.3% purity t_R 33.6 min) and **6** (1.0 mg, 0.02% yield, 96.2% purity, t_R 9.5 min), **7** (3.5 mg, 0.074% yield, 94.2% purity, t_R 15.8 min), and **8** (1.0 mg, 0.02% yield, 96.4% purity, t_R 12.3 min). Final purity was determined by integration of the TIC from LC-MS analysis of the purified samples (0.75 min at 10% ACN in H₂O with 0.1% formic acid then a linear gradient to 100% ACN with 0.1% formic acid over 9 minutes then hold at that solvent composition for 3 more min; Eclipse Plus C₁₈, 1.8 μ m, 2.1 x 50 mm, flowrate 0.5 mL/min, PDA detection). Retention times of the compounds reported in this run are: **2/3** t_R 7.66 min, **4/5** t_R 7.66 min, **6** t_R 8.72 min, **7** t_R 7.43 min, and **8** t_R 8.12 min.

Tolyporphin L & M (2 & 3): amorphous dark purple solid; $[\alpha]_D^{22}$ -21 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ϵ) 677 (4.7), 644 (3.5), 613 (3.5), 545 (3.5), 506 (3.6), 401 (5.1), 298 (4.0), 277 (4.0) nm; ECD (MeOH), nm (ϵ): 400 (+0.85), 382 (+0.80), 319 (-0.30); IR (CaF₂) ν_{max} 3447, 2928, 1734, 1716, 1705, 1699, 1683 cm⁻¹; see Table 1 for tabulated NMR spectroscopic data; HRMS (ESI-TOF) m/z [M+H]⁺ Calcd for C₃₈H₄₅N₄O₁₀⁺ 717.3130; Found: 717.3138.

Tolyporphin N & O (4 & 5): amorphous dark purple solid; $[\alpha]_D^{22}$ -54 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ϵ) 677 (4.7), 644 (3.5), 613 (3.5), 545 (3.5), 504 (3.6), 401 (5.1), 298 (3.9), 277 (4.0) nm; ECD (MeOH), nm (ϵ): 395 (+0.45), 323 (-0.19); IR (CaF₂) ν_{max} 3450, 2929, 1737, 1714, 1705, 1699, 1682 cm⁻¹; see Table 2 for tabulated NMR spectroscopic data; HRMS (ESI-TOF) m/z [M+H]⁺ Calcd for C₃₈H₄₅N₄O₁₀⁺ 717.3130; Found: 717.3117.

Tolyporphin P (6): amorphous red-purple solid; $[\alpha]_D^{22} -30$ (c 1.5, CHCl_3); UV (CHCl_3) λ_{max} (log ϵ) 619 (3.1), 563 (3.5), 527 (3.5), 494 (3.7), 398 (4.9) nm; IR (CaF_2) ν_{max} 3426, 1714 cm^{-1} ; see Table 3 for tabulated NMR spectroscopic data; HRMS (ESI-TOF) m/z $[\text{M} + \text{H}]^+$ Calcd for $\text{C}_{24}\text{H}_{23}\text{N}_4^+$ 367.1917; Found: 367.1912.

Tolyporphin Q (7): amorphous purple solid; $[\alpha]_D^{22} -27$ (c 1.0, MeOH); UV (MeOH) λ_{max} (log ϵ) 666 (3.7), 637 (4.0), 585 (3.3), 542 (3.5), 504 (3.5), 394 (4.8), 326 (4.0), 218 (3.8) nm; ECD (MeOH), nm (ϵ): 420 (+1.00), 387 (−2.0); IR (CaF_2) ν_{max} 3401, 2929, 1717, 1587 cm^{-1} ; see Tables 4 for tabulated NMR spectroscopic data; HRMS (ESI-TOF) m/z $[\text{M} + \text{H}]^+$ Calcd for $\text{C}_{24}\text{H}_{23}\text{N}_4\text{O}_2^+$ 399.1816; Found: 399.1818.

Tolyporphin R (8): an amorphous red-purple solid; $[\alpha]_D^{22} -60$ (c 0.1, MeOH); UV (MeOH) λ_{max} (log ϵ) 636 (4.1), 585 (3.3), 542 (3.4), 395 (4.7), 326 (3.9), 219 (3.9) nm; ECD (MeOH), nm (ϵ): 410 (+0.1), 396 (−0.05); IR (CaF_2) ν_{max} 3426, 1714 cm^{-1} ; see Tables 5 for tabulated NMR spectroscopic data; HRMS (ESI-TOF) m/z $[\text{M} + \text{H}]^+$ Calcd for $\text{C}_{26}\text{H}_{25}\text{N}_4\text{O}_3^+$ 441.1921; Found: 441.1925.

X-ray crystallographic analysis.—All data were collected at 100 K using a Bruker MicroStar system that incorporates a rotating-anode Cu $K\alpha$ source and microfocus optics. Standard procedures for data collection and processing were used that included APEX3, SAINT and OLEX2 libraries of programs. For tolyporphin A, crystal quality was an issue, but despite a high value for Z' and high thermal activity, utility-quality data were obtained. Also for tolyporphin A, it was impossible to produce satisfactory models for a large quantity of disordered solvent. This was treated using the program SQUEEZE; found: 875e/unit cell. Deposition CCDC Number 1940600, 1940601 and 1940602 contain the supplementary crystallographic data for this paper. These data are provided free of charge by the joint Cambridge Crystallographic Data Centre and Fachinformationszentrum Karlsruhe Access Structures service www.ccdc.cam.ac.uk/structures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
Extracted +ESI-MS/MS spectra of a) tolporphins A (1) (b) L/M (2 and 3) All spectra were collected at a collision energy of 25 eV.

Figure 2.
Key regions of the ^1H NMR spectra of **2-3** (a.) and **4-5** (b.) used to assign the configuration of the new chiral center.

Figure 3.
Crystal structure of **1**. Structure on the right has C-glycosides removed for clarity.

Figure 4.
Structures of 7 and 9.

Figure 5.
ECD spectra of (a.): **1** (red), **2/3** (blue), **4/5** (orange), and (b.): **7** (red) and **8** (blue).

Table 1.NMR Spectroscopic Data (500 MHz in CD₃OD) for Tolyporphin L (**2**) and M (**3**).

Position	Tolyporphin L (2)		Tolyporphin M (3)	
	δ_{H} (J in Hz)	δ_{C} , type	δ_{H} (J in Hz)	δ_{C} , type
1	-	137.9, C	-	136.4, C
2	8.89, s	126.4, CH	^a 8.91, s	126.1, CH
3	-	^a 138.3, C	-	138.0, C
4	-	^a 138.5, C	-	138.0, C
5	9.67, s	98.8, CH	9.78, s	99.5, CH
6	-	162.0, C	-	163.0, C
7	-	57.3, C	-	57.0, C
8	-	208.4, C	-	208.72, C
9	-	^b 149.0, C	-	^a 147.9, C
10	9.62, s	95.0, CH	9.64, s	95.2, CH
11	-	^c 135.9, C	-	^b 137.1, C
12	-	^c 137.5, C	-	^b 137.5, C
13	8.89, s	126.4, CH	^a 8.89, s	126.8, CH
14	-	137.9, C	-	138.0, CH
15	9.57, s	101.6, CH	9.50, s	100.9, CH
16	-	163.5, C	-	162.4, C
17	-	56.8, C	-	57.1, C
18	-	208.67, C	-	208.5 C
19	-	^b 145.6, C	-	^a 146.7, C
20	9.52, s	96.8, CH	9.53, s	96.7, CH
21	3.48, s	^d 13.1, CH ₃	3.51, s	^d 13.1, CH ₃
22	1.97, s	21.3, CH ₃	2.00, s	21.7, CH ₃
23	3.51, s	^d 13.2, CH ₃	3.49, s	^d 13.2, CH ₃
24	1.99, s	21.7, CH ₃	1.96, s	21.3, CH ₃
1'	4.61, d (10.1)	81.6, CH	4.41, d (9.9)	85.2, CH
2'	3.21, dd (10.1, 3.3)	69.6, CH	4.38, ddd (10.0, 10.0, 4.7)	69.5, CH
3'	3.42, dd (3.4, 3.3)	73.2, CH	(eq.) 1.91, ddd (13.1, 4.4, 3.2) (ax.) 1.54, m	38.4, CH ₂
4'	3.57, brs	73.9, CH	3.74, brs	69.7, CH
5'	4.26, m	73.0, CH	3.94, m	79.1, CH
6'	1.59, d (6.5)	17.6, CH ₃	1.59, d (6.6)	17.1, CH ₃
7'	-	-	-	169.6, C
8'	-	-	0.36, s	19.6, CH ₃
1''	4.41, d (9.9)	84.5, CH	4.59, d (10.1)	81.0, CH

Position	Tolyporphin L (2)		Tolyporphin M (3)	
	δ_H (J in Hz)	δ_C , type	δ_H (J in Hz)	δ_C , type
2''	4.26, m	69.8, CH	3.11, dd (10.1, 3.3)	69.4, CH
3''	(eq.) 1.86, ddd (12.9, 4.7, 3.1) (ax.) 1.55, m	37.9, CH ₂	3.39, dd (3.4, 3.3)	72.5, CH
4''	3.71, brm	69.4, CH	3.59, brm	73.6, CH
5''	3.92, m	78.5, CH	4.28, m	72.8, CH
6''	1.60, d (6.6)	16.9, CH ₃	1.62, d (6.6)	17.5, CH ₃
7''	-	169.7, C	-	-
8''	0.61, s	19.8, CH ₃	-	-

^{a,b,c,d} Values within a column with the same superscript may be interchanged. Superior peak shapes and dispersion in the glycoside region of the isomer mixtures was observed in CD₃OD over CDCl₃.

Table 2.NMR Spectroscopic Data (500 MHz) for Tolyporphin N (**4**) and O (**5**).

Position	Tolyporphin N (4)		Tolyporphin O (5)	
	δ_{H} (J in Hz)	δ_{C} , type	δ_{H} (J in Hz)	δ_{C} , type
1	-	136.4, C	-	137.5, C
2	8.88, brs	126.1, CH	8.88, brs	126.4, CH
3	-	138.0, C	-	^a 138.5, C
4	-	138.0, C	-	^a 138.3, C
5	9.77, s	99.3, CH	9.67, s	98.8, CH
6	-	162.5, C	-	162.1, C
7	-	57.0, C	-	57.3, C
8	-	208.29, C	-	208.4, C
9	-	^a 147.8, C	-	^b 148.9, C
10	9.65, s	95.3, CH	9.62, s	95.0, CH
11	-	^b 137.9, C	-	^c 137.5, C
12	-	^b 137.1, C	-	^c 135.8, C
13	8.91, brs	126.8, CH	8.91, brs	126.4, CH
14	-	138.0, C	-	137.9, C
15	9.50, s	100.9, CH	9.55, s	101.4, CH
16	-	162.7, C	-	163.2, C
17	-	57.1, C	-	56.8, C
18	-	208.42, C	-	208.3, C
19	-	^a 146.8, C	-	^b 145.6, C
20	9.53, s	96.7, CH	9.53, s	96.8, CH
21	3.48, s	13.1, CH ₃	3.50, s	^d 13.1, CH ₃
22	1.99, s	21.3, CH ₃	1.99, s	21.8, CH ₃
23	3.48, s	13.1, CH ₃	3.46, s	^d 13.2, CH ₃
24	1.96, s	21.8, CH ₃	1.98, s	21.3, CH ₃
1'	4.20, d (9.5)	84.9, CH	4.42, d (9.8)	84.5, CH
2'	2.88, dd (9.5, 9.5)	71.6, CH	4.25, ddd (11.1, 10.1, 4.7)	69.6, CH
3'	3.39, dd (9.5, 3.2)	76.6, CH	(eq.) 1.87, ddd (12.9, 4.8, 3.1) (ax.) 1.60, m	37.9, CH ₂
4'	3.66, dd (3.2, 1.0)	73.3, CH	3.72, brm	69.4, CH
5'	3.94, m (obscured)	76.6, CH	3.94, m (obscured)	78.5, CH
6'	1.66, d, (6.2)	17.6, CH ₃	1.60, d (6.3)	17.5, CH ₃
7'	-	-	-	169.6, C
8'	-	-	0.60, s	19.8, CH ₃
1''	4.42, d (9.8)	85.2, CH	4.18, d (9.5)	85.6, CH
2''	4.38, ddd (10.0, 9.8, 4.5)	69.7, CH	2.96, dd (9.5, 9.5)	71.7, CH

Position	Tolyporphin N (4)		Tolyporphin O (5)	
	δ_H (J in Hz)	δ_C , type	δ_H (J in Hz)	δ_C , type
3''	(eq.) 1.92, ddd (13.0, 4.5, 3.0) (ax.) 1.62, m	38.4, CH ₂	3.39, dd (9.5, 3.2)	76.9, CH
4''	3.75, brm	69.7, CH	3.68, dd (3.2, 1.1)	73.3, CH
5''	3.94, m (obscured)	79.1, CH	3.94, m (obscured)	77.1, CH
6''	1.60, d (6.4)	17.5, CH ₃	1.65, d (6.1)	17.6, CH ₃
7''	-	169.5, C	-	-
8''	0.36, s	19.6, CH ₃	-	-

^{a,b,c,d} Values within a column with the same superscript may be interchanged. Superior peak shapes and dispersion in the glycoside region of the isomer mixtures was observed in CD₃OD over CDCl₃.

Table 3.NMR Spectroscopic Data (500 MHz, CDCl₃) for Tolyporphin P (**6**).

Position	δ_{H} (J in Hz)	δ_{C} , type
1	-	140.6, C
2	9.09, s	129.0, CH
3	-	140.8, C
4	-	140.0, C
5	9.98, s	102.3, CH
6	-	156.1, C
7	-	140.6, C
8	9.11, s	129.0, CH
9	-	152.1, C
10	10.07, s	99.6, CH
11	-	140.2, C
12	-	140.8, C
13	9.09, s	128.9, CH
14	-	140.9, C
15	10.09, s	100.1, CH
16	-	152.6, C
17	-	140.0, C
18	9.11, s	128.8, CH
19	-	156.0, C
20	10.16, s	97.3, CH
21	3.76, s	13.7, CH ₃
22	3.76, s	13.7, CH ₃
23	3.74, s	13.7, CH ₃
24	3.75, s	13.7, CH ₃

Table 4.NMR Spectroscopic Data (600 MHz, CDCl₃) for Tolyporphin Q (**7**).

Position	δ_{H} (J in Hz)	δ_{C} , type
1	-	134.8, C
2	8.24, s	123.7, CH
3	-	^a 136.3, C
4	-	^a 133.8, C
5	9.34, s	100.5, CH
6	-	151.9, C
7	-	143.6, C
8	8.61, s	130.9, CH
9	-	152.6, C
10	9.49, s	101.5, CH
11	-	^b 136.3, C
12	-	^b 138.4, C
13	8.75, s	124.8, CH
14	-	138.7, C
15	9.08, s	93.5, CH
16	-	162.4, C
17	-	77.6, C
18	-	208.5, C
19	-	142.4, C
20	8.98, s	94.8, CH
21	3.34, s	13.1, CH ₃
22	3.48, s	13.5, CH ₃
23	3.60, s	13.4, CH ₃
24	1.93, s	24.5, CH ₃
NH 1	^a -4.18, br	-
NH 2	^a -4.30, br	-

^{a,b} Values within a column with the same superscript may be interchanged.

Table 5.NMR Spectroscopic Data (500 MHz, CDCl₃) for Tolyporphin R (**8**).

Position	δ_{H} (J in Hz)	δ_{C} , type
1	-	136.5, C
2	8.99, s	125.0, CH
3	-	^a 135.1, C
4	-	^a 137.5, C
5	10.00, s	101.3, CH
6	-	152.7, C
7	-	143.0, C
8	8.79, s	131.4, CH
9	-	153.0, C
10	9.79, s	102.3, CH
11	-	^b 138.6, C
12	-	^b 136.5, C
13	8.89, s	124.7, CH
14	-	138.8, C
15	9.13, s	92.5, CH
16	-	*160.4, C
17	-	81.1, C
18	-	*202.7, C
19	-	144.2, C
20	9.79, s	95.8, CH
21	3.70, s	13.5, CH ₃
22	3.59, s	13.6, CH ₃
23	3.67, s	13.5, CH ₃
24	2.15, s	22.0, CH ₃
25	-	^a 169.7, C
26	2.25, s	20.3, CH ₃

^{a,b} Values with the same superscript may be interchanged.* Chemical shift for ¹³C resonance reported based on experiment at 150 MHz with a relaxation delay of 1.2 s.