Prymnesin-2: A Potent Ichthyotoxic and Hemolytic Glycoside Isolated from the Red Tide Alga *Prymnesium parvum*

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Prymnesium parvum is one of the most harmful microalgal red tide species worldwide, and it poses a serious threat to fish farming, especially in brackish waters.[2] Over the past 30 years, the organism has been known to produce a potent ichthyotoxin named prymnesin.[3] However, due to extreme difficulty in purification, the toxin has been variously believed to be a saponin,[3] a proteolipid,[4] or a carbohydrate.[5] We previously succeeded in optimizing the culture conditions, achieving 20-fold increase in production of prymnesins.[6] Two prymnesins, prymnesin-1 (PRM1) and prymnesin-2 (PRM2, **1**, Figure 1), were effectively purified and shown to possess potent hemolytic and ichthyotoxic properties.[6] In the present communication, we report structural elucidation of PRM2, the major toxin of the phytoflagellate.

From 400 L of cultures of *P. parvum*, 10 mg of PRM1 and 15 mg of PRM2 were obtained. PRM2 (**1**): pale yellow solid; $[\alpha]^{23}_{D}$ +21.2° (*c* 0.15, dioxane/1% AcOH 7:3); UV λ_{max} (nm, dioxane/1% AcOH 7:3) 244 (ϵ 20 800), 267 (ϵ 24 500), 278 (ϵ 19 800); IR (film) 3300, 2220, 1570, 1280, 1100 cm^{-1}; positive to ninhydrin. The presence of chlorine atoms in the molecule was indicated by energy dispersive X-ray analysis and by absorption at 1280 cm^{-1} in the IR spectrum. To improve the solubility in NMR solvents, PRM2 was converted to its *N*-acetate (NAPRM2, **2**, Figure 1) with Ac_2O in PrOH/H_2O (3:2). Peracetylation (Ac_2O/pyridine) and hydrogenation (Pd/C, H_2) of PRM2 were also carried out in order to locate hydroxyl groups and to confirm the degree of unsaturation in the molecule. To facilitate ^{13}C NMR measurements, ^{13}C-enriched PRM2 was prepared by culturing the organism in the presence of $Na_2{}^{13}CO_3$ (50 mg/L). From 180 L cultures, 1.5 mg of ^{13}C-enriched (5%) NAPRM2 was obtained. The minimum concentration to cause hemolysis of a 1% mouse blood cell suspension and to kill freshwater fish ("white cloud mountain fish", *Tanichthys albonubes*) (pH 8, 2 mM $CaCl_2$) was ~3 nM.

In the positive ion ESI mass spectrum of **1**, two ion clusters having centroids at *m/z* 1969.7 and 1992.3 were observed, corresponding to $[M + H]^+$ and $[M + Na]^+$, respectively. In the spectrum of **2**, the $[M + Na]^+$ ions had a centroid at *m/z* 2034.9. The difference of 42 u indicated the presence of at least one amino group in **1**. In the negative FABMS of **2**, the centroid for $[M - H]^-$ ions was observed at *m/z* 2011, in accordance with ESIMS results. Prominent ions due to elimination of HCl $[M - HCl - H]^-$ appeared at *m/z* 1975 when **2** was kept in pyridine/water (details of its formation will be discussed later). The molecular formula of **2**, including three chlorine atoms, was deduced to be $C_{98}H_{138}Cl_3NO_{36}$ by detailed analysis of the NMR spectra.[7] As cluster ions for both $[M - H]^-$ and $[M - HCl - H]^-$ indicated specific distribution patterns as attributed to natural isotopes ^{35}Cl and ^{37}Cl, simulation of isotope distribution was carried out for the peaks at *m/z* 1975 and 2011. Good agreement between simulated and observed data (see supporting information) verified the molecular formula of **2** deduced from the NMR data and further suggested the molecular formula $C_{96}H_{136}Cl_3NO_{35}$ for **1**.

Analysis of COSY, DQF-COSY, and TOCSY spectra led to elucidation of six sequences of proton spin systems (C1−C2, C5−C6, C9−C70, C72−C82, C83−C86, and C1′−C5′) in **2**. The connectivity from C1 to C10 disrupted by four quaternary carbons (δ C3 78.5, C4 93.6, C7 92.5, C8 82.8) was elucidated by HMBC correlations from H1 to C3, from H5 to C3, C6, and C7, from H6 to C4, C5, and C8, from H9 to C7, and from H10 to C8. The long-range couplings (3 Hz) observed for H2/H5 and H6/H9, the UV maxima (244, 267, 278 nm) for the C7−C12 ynediene, and the agreements of ^{13}C and ^{1}H chemical shifts with those in the literature[8] also supported the partial structure C1−C12. Thus, connectivity from C1 to C70 was established. The *E* geometry of the double bonds was deduced from $^{3}J_{H-H}$ and NOESY data. In the COSY spectrum of NAPRM2, the spin connection from H70 to H72 was not clear due to closeness of their chemical shifts. Those signals were well separated in the spectrum of peracetyl PRM2, allowing us to clarify this part. The disconnection of the spin system between H82 and H83 due to the small coupling was eliminated by HMBC correlation (H83/C82). Both ^{13}C NMR data and cross peaks in the HMBC spectrum (H85/C87, H86/C87, H86/C88, H86/C89) clarified three quaternary carbons (δ C87 76.2, C88 68.4, C89 69.7).[9] The terminal acetylenic proton (H90) was observed at 2.63 ppm (t, $J = 1.2$ Hz) when PRM2 was measured in an acidic solvent (CD_3OD/CD_3COOD 19:1). In CD_3OD/C_5D_5N (1:1), however, a signal (bs, δ 2.98) attributable to H90 and apparent immediately after dissolution gradually disappeared in 12 h (see supporting information), due to exchange of the acidic acetylenic proton with deuterium. The downfield shift of H90 in pyridine solution is compatible with the literature data,[10] as is the chemical shift of C90 (δ 68.8) measured in the acidic solvent.[11] In addition to $J_{H86-H90}$ (1.2 Hz), which was appropriate for $^{6}J_{H-H}$ through two triple bonds, the number of bonds proposed was supported by the absence of additional quaternary carbons between 60 and 100 ppm. Combining these data, a C_{90} carbon chain skeleton was established.

Ether rings from A to N were elucidated by strong negative NOEs between angular protons. The ring system from A to E (6/6/6/7-OH/6) in **1** corresponded to that from A to E of maitotoxin (6/7-OH/6/6/6),[12] with a *trans*-fused ladder-shape structure. Repeating 1,6-dioxadecalin units (F−M) were constructed from the $^{2,3}J_{H-H}$ and NOE data typical for those of *trans*-fused tetrahydropyrans. Linkage of rings M and N was difficult to determine, because all carbons were oxygenated and thus gave rise to methine signals close to each other. However, in the ^{1}H NMR spectra of peracetyl PRM2, acetoxy methine signals were shifted downfield and separated from those of ether methines, thereby allowing precise signal assignments. For

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(7) The spectra were recorded on four NMR spectrometers, 400MHz (JEOL GSX), 500 MHz (Varian Unity plus), 600 MHz (JEOL α-600), and 600 MHz (Bruker AM600), and in three different solvents, CD_3OD/CD_3COOD (19:1) for **1**; dioxane-d_8/D_2O/CD_3COOD (10:10:1) for **2**; and CD_3OD/C_5D_5N (1:1) for **2** and for peracetyl- and perhydro-PRM2.

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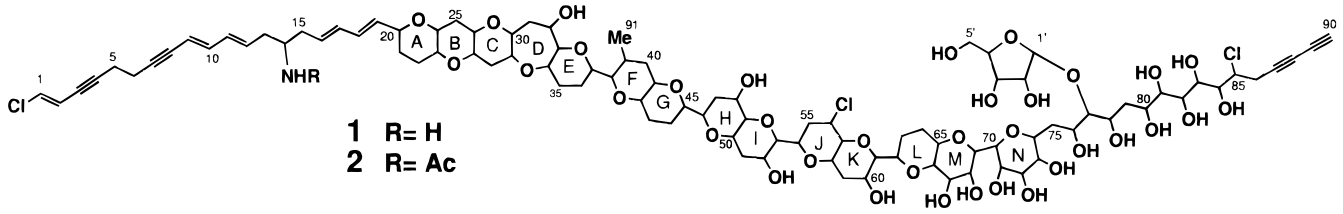

Figure 1. Planar structure of prymnesin-2 (**1**) and *N*-acetylprymnesin-2 (**2**).

verification of the proposed ring combination for L/M/N (6/6−6), NOE data were compared with those in an alternative combination (6/7/7) generated by molecular mechanics calculation (MM2).[13] NOEs observed for H68/H70 and H69/H71 in the spectrum of peracetyl PRM2 agreed with the 6/6−6 model but differed from the calculated interproton distances(>3.77 Å) in the 6/7/7 model. Likewise, unobserved NOEs disagreed with the proximity of protons, H64/H71 (3.27 Å) and H65/H71 (3.34 Å), in the 6/7/7 model (see supporting information).

The amino group was located at C14 because of both the downfield shift of H14 by 0.7 ppm after acetylation and the 51 ppm shift of its carbon signal. Location of hydroxyl groups was established by comparing the proton chemical shifts of **2** with those of peracetyl PRM2. ^{1}H signals of acetoxy methines, except for H56 and H85, were shifted downfield by 0.45−1.55 ppm, thus clearly separated from those due to ether methines. The position of one chlorine at C1 was suggested by both $^1J_{Cl-H1}$ (200 Hz) and its chemical shift (δ 6.54, 131.8). The other two chlorine atoms were located at C56 (δ 60.3) and C85 (δ 64.1), based on the chemical shifts of the carbons and on unobserved downfield shifts of methine protons after acetylation. Lowered electron density at C85 and C86 due to adjacent tandem triple bonds led to liberation of C85 Cl and H86 in a basic solvent (pyridine-d_5/CD_3OD 1:1). The resultant enediyne structure gave rise to coupled proton signals (δ H85 6.97, H86 6.05) in the ^{1}H NMR spectra. The occurrence of ions corresponding to dehydrochlorinated NAPRM2 in the FABMS was thus explained. The chemical shifts of C1′−C5′ [14] and cross peaks in the HMBC spectrum (H1′/C4′, H1′/C77) permitted us to assign both the sequence to be a pentofuranose and the position of a glycosidic bond at C77. Chiral GC analysis of the hydrolysis product of **1** confirmed that the sugar is L-xylose.

In the positive ESIMS spectrum of perhydro-PRM2, molecular-related ions were observed at about *m/z* 1963, which was 32 u smaller than the expected value.[15] Extensive analysis of both ^{1}H−^{1}H COSY and NOE difference spectra revealed that the chlorine atom at C1 had been substituted by a hydrogen atom during hydrogenation. The nature of unsaturation including five double and four triple bonds in **1** was thus verified.

The above spectral information led to the planar structure of PRM2 (**1**), with a molecular formula of $C_{96}H_{136}Cl_3NO_{35}$. The molecule has these unique structural features: contiguous ether rings, repetitive 1,6-dioxadecalin units, conjugated double and triple bonds, chlorine and nitrogen atoms, and an uncommon L-xylose. Furthermore, the molecule is constructed of a C_{90} unbranched carbon chain except for a single methyl at C39. All 16 hydroxyls except for one at C32 are concentrated on carbons C48−C84. This feature, together with a xylose moiety at C77, imparts the molecule its amphoteric nature, thus leading to interaction with biomembranes. Elucidation of the structure of PRM1 and of the stereochemistry of **1** are in progress.

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Supporting Information Available: Table of ^{13}C and ^{1}H NMR assignments of **2**; ^{1}H−^{1}H COSY spectrum of **2** with the assignments of cross peaks; ESI mass spectra of **1** and **2**; FABMS spectrum of **2** and theoretical ion distribution for compositions of both $C_{98}H_{138}Cl_3NO_{36}$ and $C_{98}H_{137}Cl_2NO_{36}$; partial 1D ^{1}H spectra (2.4−3.1 ppm) of **2**, indicating the time course of disappearance of the signal (δ 2.98) in CD_3OD/C_5D_5N (1:1); figures for both terminal parts indicating chemical shifts; HMBC correlations and coupling constants; partial NOESY spectrum of NAPRM2, showing correlations from oxymethine protons; and NOE data for the selection of linkage combination of rings L−N (8 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

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(13) The stereochemical data for these two structures calculated by MM2 were very similar, except for the relative positions of H69 and H70 (see supporting information).

(14) ^{1}H and ^{13}C NMR data were as follows: C1′ (δ 5.38, 106.2); C2′ (δ 4.20, 74.6); C3′ (δ 4.18, 72.8); C4′ (δ 4.41, 88.2); C5′a,b (δ 3.68, 3.73, 64.5).

(15) We believe that a mechanical problem inherent to a quadrupole mass spectrometer in measuring a chlorinated large molecule resulted in the smaller difference (32 instead of 34 u) than it should have been.