Structural Confirmation of Maitotoxin Based on Complete ^{13}C NMR Assignments and the Three-Dimensional PFG NOESY-HMQC Spectrum

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Maitotoxin (MTX) has attracted considerable attention since it is the most toxic and the largest natural non-biopolymer.[1,2] We recently reported the structure of MTX, including partial stereochemical assignments.[2,3] However, using only NMR data obtained for periodate-degradation products, the entire structure of MTX could not be fully established because the largest fragment (2326 Da) still contained 178 protons, which caused severe overlapping of NMR signals, even in 2D NMR. Therefore, MS/MS experiments with the degradation product played an important role in verifying the structure of the fragment. Since the possible misassignment of NMR signals in these NMR-based studies might have resulted in an incorrect structure, particularly with regard to stereochemistry, we have been seeking further spectroscopic evidence for the present structure (**1**). In the present study, we reexamined the structure using ^{13}C chemical shift data and NOE information derived from the three-dimensional NOESY-HMQC spectrum.

To obtain a ^{13}C-enriched sample, the dinoflagellate *Gambierdiscus toxicus* was grown in media containing 0.01% Na_2CO_3 (99% enriched with ^{13}C). The cells harvested from 2000 L of the media were extracted,[4] and the extract was purified by a modified procedure[5] to give 9 mg of MTX. The abundance of ^{13}C was estimated to be 4%. Pulsed field gradient (PFG) 3D NOESY-HMQC[6] was measured using a JEOL A600 spectrometer (600 MHz) for 6 days to furnish 3D FID matrices (data points: t1(^1H) 128, t2(^{13}C) 32, and t3(^1H) 512), which were then converted to frequency domain data (F1 128, F2 128, and F3 512). The NOESY plane in Figure 1a from 128 2D sheets in the 3D spectrum gave rise to only 30 cross peaks with no overlap, while a conventional 2D NOESY spectrum for the same area showed more than 500 cross peaks, many of which overlapped heavily (see the supporting information for the previous paper[2]). In addition to the separation of signals along the ^{13}C axis, the fact that an NOE interaction gives rise to a single cross peak in a NOESY plane of the 3D spectrum (see Figure 1) reduces the chances of overlap while a pair of cross peaks are observed at symmetrical positions in 2D experiments. PFG neatly eliminated F1 noise due to the peaks of ^{12}C-adjacent protons, which were 50-fold more intense than ^{13}C-satellite signals, and the spectrum was much improved over that measured by the same method without PFG (see the supporting information).

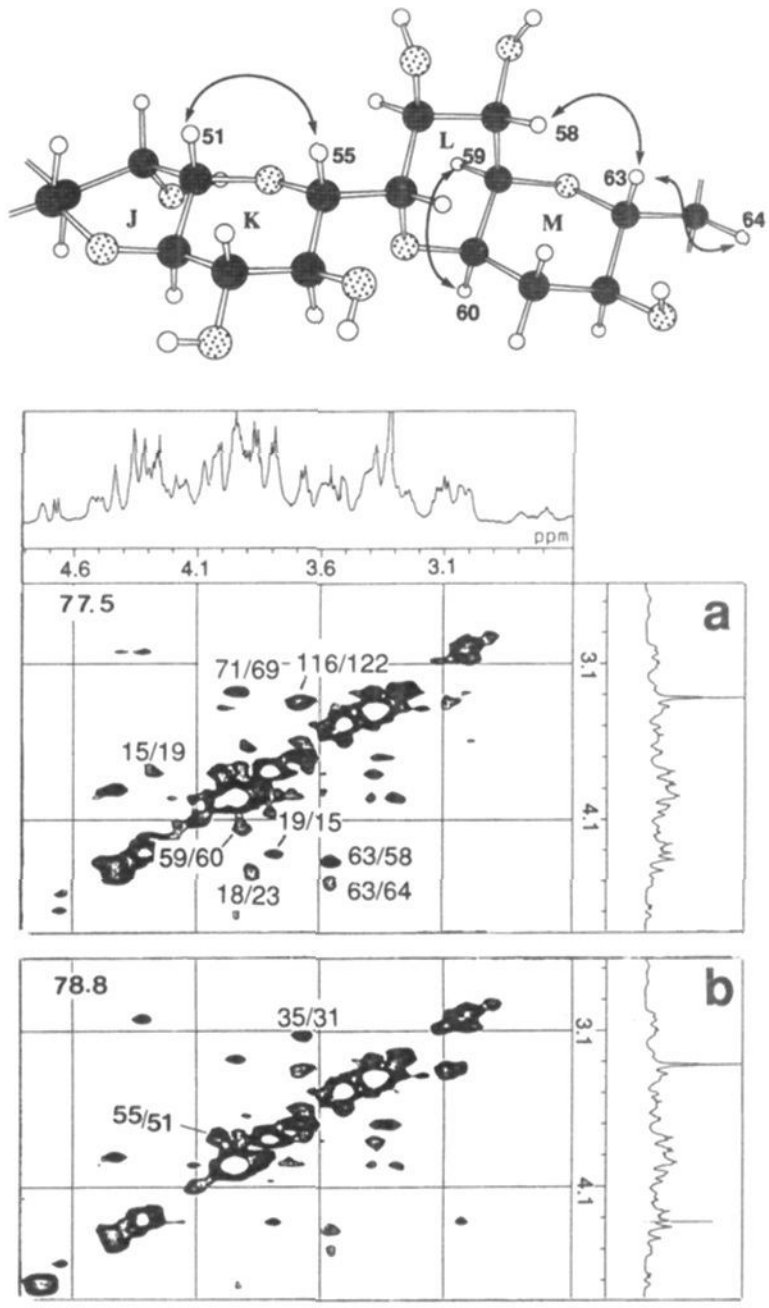

Figure 1. Two NOE planes of 3D PFG NOESY-HMQC. The spectrum was measured with the use of 9 mg of maitotoxin in 0.4 mL of C_5D_5N/CD_3OD (1:1). An NOE interaction in the charts is represented as a single cross peak at the chemical shift (on the horizontal axis of F3) of the proton whose adjacent carbon possesses a chemical shift at about $\delta_{^{13}C}$ 77−79. Since a and b correspond to $\delta_{^{13}C}$ 77.5 and 78.8, respectively, some cross peaks, such as H63/H58 and H116/H122, are shown in both planes whereas those of H15/H19, H18/H23, and H35/H31 only appear in either a or b. In conventional 2D NOESY, NOEs from H35 and H55 could not be precisely assigned due to overlap of ^1H signals; H35 at $\delta_{^1H}$ 3.65 and H55 at $\delta_{^1H}$ 4.03 overlap H16/H116 and H50/H62, respectively. However, their ^{13}C chemical shifts are separated enough to be distinguishable in the 3D experiment (see Figure 2). The arrows in the top drawing indicate NOEs observed in the NOE planes.

Angular protons and methyls, except for those with very close ^1H chemical shifts (e.g., H42/H46, Me155/Me156, Me158/Me159, and Me161/Me162), give rise to prominent NOEs in the 3D spectrum (Figure 2). Trans-cisoid fusion in clusters of ethercycles (rings A−F, P−V, and W−F′), which had been deduced from MS/MS and NMR data for the degradation products,[1−3] were confirmed without ambiguity.

Assignments of the ^{13}C NMR signals should provide further evidence regarding stereochemistry, because ^{13}C chemical shifts are significantly affected by the orientation of ring substituents. Since 3D experiments are not suitable for the precise determination of ^{13}C chemical shifts due to the small number of data

(1) Murata, M.; Iwashita, T.; Yokoyama, A.; Sasaki, M.; Yasumoto, T. *J. Am. Chem. Soc.* **1992**, *114*, 6594−6596.
(2) Murata, M.; Naoki, H.; Iwashita, T.; Matsunaga, S.; Sasaki, M.; Yokoyama, A.; Yasumoto, T. *J. Am. Chem. Soc.* **1993**, *115*, 2060−2062.
(3) Murata, M.; Naoki, H., Matsunaga, S.; Satake, M.; Yasumoto, T. *J. Am. Chem. Soc.* **1994**, *116*, 7098−7107.
(4) Yokoyama, A.; Murata, M.; Oshima, Y.; Iwashita, T.; Yasumoto, T. *J. Biochem.* **1988**, *104*, 184−187.
(5) The purification procedure was modified as follows. Extraction and partition were the same as previously reported.[4] The butanol fraction was purified by successive chromatography on a poly(divinylbenzene) column (Amberchrome CG-161, TOSOH HAAS) by stepwise elution with MeOH/H_2O, a gel permeation column (ToyoPearl HW40) with CD_3CN/H_2O (4:6), and an HPLC column (Develosil TMS-5) with 10 mM potassium phosphate buffer (pH 6.8) in CH_3CN/H_2O (40:60).
(6) Geertem, W. V.; Boelens, R.; Kaptein, R.; Burgering, M.; Van Zijil, P. C. M. *J. Biomol. NMR* **1992**, *2*, 301−305. The pulse sequence is available in the supporting information. 3D measurement was carried out with a spectral width of 4000 Hz (6.67 ppm) for F1/F3 (^1H) and of 12 600 Hz (84 ppm) for F2 (^{13}C).

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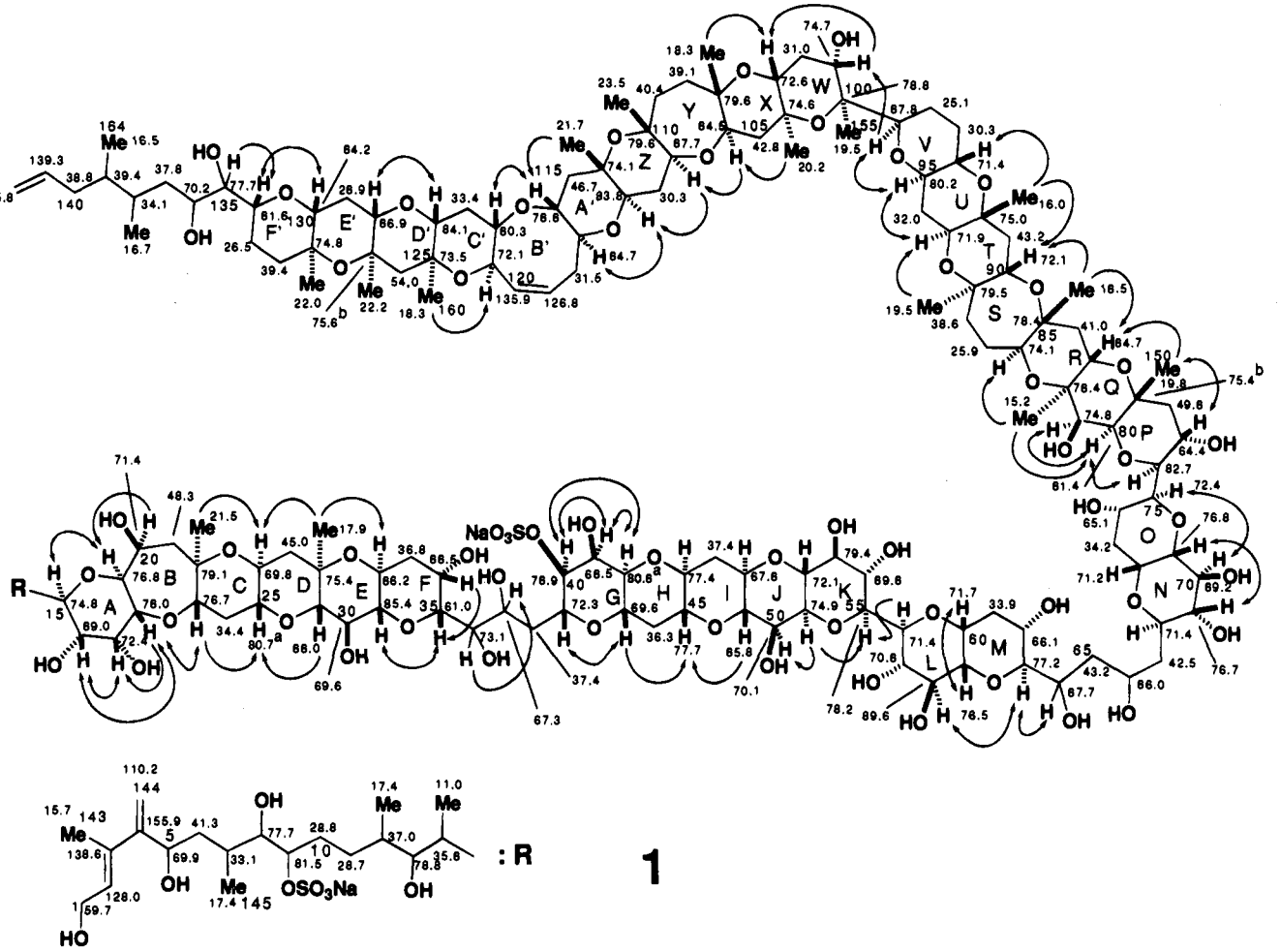

Figure 2. ^{13}C NMR assignments of maitotoxin and NOEs observed in the 3D NOESY-HMQC experiment. Chemical shifts are observed in C_5D_5N/CD_3OD (1:1). The center peak of $^{13}CD_3OD$ was taken as the standard signal at 48.94 ppm. Arrows (single-headed) indicate NOEs in the NOESY plane corresponding to $\delta_{^{13}C}$ of the carbon adjacent to a proton (a tail of an arrow). Double-headed arrows denote that NOEs were observed in both directions. NOE interactions are mainly depicted for angular protons and methyls. Other interactions, such as those due to methylenes, are not shown for clarity. Assignments designated "a" are interchangeable. Assignments designated "b" are interchangeable.

points, 2D HSQC and ^{13}C-observed H−C COSY spectra were measured to assign protonated carbons. Quaternary carbons were determined by HMBC experiments. These NMR measurements led to revision of the previous ^1H NMR assignments[3] for H_2−88, H_2−98 and H_2−132.[7]

The chemical shift data shown in Figure 2 further supported the present structure, particularly around methyl-bearing quaternary carbons. For example, ether carbons showing high-field shifts, such as C26, C32, C83, C90, C93, C96, C103, and C121, are neighbored by an angular methyl across an ether bond; and methylene groups with high-field shifts, such as C102, C112, and C129, are located at the C-4 position of a 2,6-dimethyltetrahydropyran system. Using all of these data, the proposed structure and configuration for MTX[2,3] have been verified with much better accuracy. Stereochemical assignments for the remaining portions (C5−C14, C36−C37, C64−C66, and C135−C139) are currently being performed by synthetic and spectroscopic methods.[8]

Despite the frequent use of heteronuclear 3D NMR spectroscopy for analyses of proteins or polynucleotides, there are a very limited number of applications suitable for determining the structure of natural non-biopolymers. Among other reasons, natural non-biopolymers, particularly secondary metabolites, usually possess molecular masses of less than 1500 Da, and their structures can be determined by 2D NMR experiments. In addition, unlike proteins, which can feasibly be ^{13}C-labeled by recombinant DNA techniques, secondary metabolites are hardly enriched with ^{13}C at the concentrations necessary for 3D experiments. As far as we know, this is the first report of heteronuclear 3D NMR spectroscopy being used as a realistic method for elucidating the structure of natural non-biopolymers. Recently, natural products with large molecular weights have been found in marine organisms (e.g., zooxanthellatoxins[9] and prymnesins[10]). Since these natural products are mostly noncrystalline, 3D NMR can possibly serve as a powerful tool for structural elucidation.

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Supporting Information Available: A pulse sequence of PFG 3D HMQC-NOESY used in this study, ^{13}C-observed 2D H−C COSY, selected 3D HMQC-NOESY charts, a 3D chart without PFG, and 1D ^1H NMR and 1D ^{13}C NMR spectra (22 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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(7) $\delta_{^1H}$ in 1:1 C_5D_5N/CD_3OD: H_2-88, 1.73/1.81; H_2-98, 1.56/1.82; and H_2-132, 1.50/1.74.

(8) Sasaki, M.; Nonomura, T.; Murata, M.; Tachibana, K. *Tetrahedron Lett.* **1994**, *35*, 5023−5026.

(9) Nakamura, H.; Asari, T.; Murai, A.; Kan, Y.; Kondo, T.; Yoshida, K.; Ohizumi, Y. *J. Am. Chem. Soc.* **1995**, *117*, 550−551.

(10) Igarashi, T.; Satake, M.; Yasumoto, T. *Symposium paper of 36th symposium on the chemistry of natural products*; Organizing Committee of the Symposium: Hiroshima, 1994; p 89.