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Complete NMR signal assignment of palytoxin and *N*-acetylpalytoxin

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Abstract—Palytoxin is the second largest-sized natural product that does not contain repeating units such as amino acids or monosaccharides. The molecular formula of palytoxin is $C_{129}H_{223}N_3O_{54}$ and the molecule contains 64 chiral centers. We assigned all hydrogen and carbon NMR signals of palytoxin and *N*-acetylpalytoxin using multidimensional Fourier transform techniques. Although the complete assignment was difficult due to the spectral complexity, advances in NMR spectrometry and techniques such as gradient enhancement and 3D Fourier transform have enabled us to completely assign the ^1H and ^{13}C signals of palytoxin and also the ^{15}N signals of *N*-acetylpalytoxin. This NMR data will contribute to further studies of the structure and biosynthetic pathways of the palytoxin family compounds, including conformational and dynamic properties of the molecule. © 2001 Elsevier Science Ltd. All rights reserved.

We report here the complete chemical shift assignment of the ^1H, ^{13}C and ^{15}N NMR signals of palytoxin and *N*-acetylpalytoxin (Tables 1 and 2). This study represents the feasibility of applying modern 2D and 3D NMR techniques for a large-molecule natural product without enrichment of ^{13}C and ^{15}N for the first time. An important first step in studying the molecular conformation of palytoxin bound to putative membrane proteins, which is thought to have a key function of the toxity, is the NMR assignments.

Palytoxin is an extremely poisonous and complex natural product in marine environments.[1] The LD_{50} value of palytoxin reaches a maximal level of 25 ng/kg in rabbits.[2] Palytoxin induces a contractile response and also produces membrane depolarization on various muscle sites.[3] Recently, the biological interaction on Na/K–ATPase was investigated by several groups.[4] Palytoxin is known to bind to the Na/K-pump and inhibit its ATPase activity. The toxin induces a nonselective cation conductance in membranes, and this effect is blocked by ouabain. The molecular formula of palytoxin is $C_{129}H_{223}N_3O_{54}$ and the molecule contains 64 chiral centers. The primary structure of palytoxin was identified by chemical degradation and fragment analysis using X-ray diffraction and ^1H NMR, and the absolute stereochemistry was determined using a synthetic method.[5]

In the present report, all NMR spectra were measured with palytoxin (4 mg) and *N*-acetylpalytoxin (34 mg) in CD_3OD or CD_3OH, which was obtained from *Palythoa tuberculosa*. No correlation was obtained between ^{15}N and ^1H by HMBC using 4 mg of palytoxin. The choice of solvent was critical at the initial stage of structure elucidation using NMR. Water would be an ideal solvent, in that it provides a similar environment as the biological assay system. However, the ^1H NMR spectrum of palytoxin in neutral water was broad, and difficult to analyze. We found that methanol was suitable for NMR analysis of palytoxin, because it gave sharp signals. It is difficult to account for the line broadening of the ^1H NMR spectrum. Possibly, palytoxin has slow conformational tumbling or an equilibrium is reached between multiple molecule clusters in water solution. When the solvent was switched from water to methanol, the ^1H NMR spectrum of the molecule showed characteristic sharp signals due to a fast averaged conformation or a monomer. The different dynamic properties in the different solvents might

Keywords: natural products; toxin; palytoxin; *N*-acetylpalytoxin; Na/K–ATPase; ^1H NMR; ^{13}C NMR; ^{15}N NMR; 2D HSQC–TOCSY; 3D TOCSY–HSQC.

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Table 1. ^{1}H, ^{13}C NMR chemical shift data of *N*-acetylpalytoxin and palytoxin

No.	*N*-Acetylpalytoxin				Palytoxin			
	^{13}C (δ)	Mult.	^{1}H (δ)	^{1}H (δ)	^{13}C (δ)	Mult.	^{1}H (δ)	^{1}H (δ)
1	175.88	s	–		175.92	s	–	
2	75.66	d	4.12		75.70	d	4.09	
3	34.59	d	2.19		34.73	d	2.17	
3-Me	13.90	q	0.88		13.99	q	0.88	
4	41.59	t	1.77	1.41	41.73	t	1.77	1.40
5	66.57	d	4.52		66.62	d	4.50	
6	131.82	d	5.50		131.85	d	5.49	
7	138.16	s	–		138.28	s	–	
7-Me	13.12	q	1.72		13.17	q	1.72	
8	80.83	d	3.94		80.91	d	3.92	
9	72.18	d	3.83		72.34	d	3.81	
10	29.21	t	2.13	1.73	29.23	t	2.12	1.73
11	76.04	d	4.19		76.19	d	4.18	
12	73.76	d	3.65		73.88	d	3.64	
13	75.01	d	3.58		75.17	d	3.54	
14	71.56	d	3.61		71.68	d	3.60	
15	72.76	d	3.65		72.91	d	3.62	
16	71.09[*1]	d	4.05		71.28[*2]	d	4.03	
17	71.56[*1]	d	4.05		71.68[*2]	d	4.04	
18	73.18	d	3.56		73.27	d	3.54	
19	71.17	d	3.79		71.35	d	3.79	
20	71.01	d	3.88		71.11	d	3.87	
21	27.26	t	1.49	1.38	27.38	t	1.48	1.39
22	26.81	t	1.48	1.35	26.93	t	1.47	1.35
23	34.90	t	1.65	1.55	35.03	t	1.64	1.55
24	28.31	t	1.36		28.44	t	1.36	
25	39.61	t	1.26		39.72	t	1.26	
26	29.61	d	1.67		29.70	d	1.67	
26-Me	19.29	q	0.92		19.30	q	0.92	
27	40.66	t	1.47	0.93	40.78	t	1.47	0.91
28	80.11	d	3.98		80.17	d	3.97	
29	82.34	s	–		82.31	s	–	
29-Me	20.99	q	1.19		21.01	q	1.18	
30	45.61	t	1.71	1.16	45.74	t	1.70	1.14
31	25.42	d	2.05		25.55	d	2.04	
31-Me	21.85	q	0.92		21.89	q	0.91	
32	43.60	t	1.69	1.10	43.74	t	1.67	1.09
33	109.22	s	–		109.23	s	–	
34	38.50	t	1.61		38.64	t	1.60	
35	23.86	t	1.42		23.98	t	1.41	
36	30.85[*3]	t	1.33		30.98[*4]	t	1.31	
37	30.79[*3]	t	1.33		30.93[*4]	t	1.31	
38	30.68[*3]	t	1.33		30.81[*4]	t	1.31	
39	31.19	t	1.35		31.29	t	1.36	
40	39.04	t	1.49		39.20	t	1.48	
41	69.19	d	3.79		69.26	d	3.80	
42	39.18	t	1.87	1.45	39.37	t	1.86	1.44
43	64.76	d	4.40		64.86	d	4.39	
44	73.71	d	3.65		73.88	d	3.65	
45	73.96	d	3.97		74.28	d	3.95	
46	68.11	d	3.69		68.25	d	3.67	
47	101.05	s	–		101.24	s	–	
48	41.45	d(t)	1.83		41.95	d(t)	1.83	
49	72.28	d	3.95		72.40	d	3.94	
50	43.95	d	2.28		44.07	d	2.26	
50-Me	16.54	q	1.04		16.58	q	1.03	
51	134.44	d	5.62		134.46	d	5.62	
52	134.72	d	5.52		134.74	d	5.51	
53	73.97	d	4.07		74.06	d	4.05	
54	34.77	t	1.78	1.62	34.93	t	1.77	1.61
55	27.61	t	1.69	1.47	27.79	t	1.69	1.46
56	72.94	d	3.76		73.11	d	3.74	
57	72.76	d	3.86		72.81	d	3.85	

Table 1. (Continued)

No.	*N*-Acetylpalytoxin				Palytoxin			
	^{13}C (δ)	Mult.	^{1}H (δ)	^{1}H (δ)	^{13}C (δ)	Mult.	^{1}H (δ)	^{1}H (δ)
58	73.93	d	3.90		74.19	d	3.87	
59	32.87	t	2.28	1.68	33.05	t	2.27	1.66
60	70.04	d	3.87		70.18	d	3.85	
61	76.43	d	3.17		76.57	d	3.15	
62	73.09	d	3.75		73.11	d	3.74	
63	36.65	t	1.99	1.70	36.77	t	1.96	1.70
64	71.78	d	3.67		71.77	d	3.68	
65	72.07	d	3.77		72.20	d	3.76	
66	36.84	t	2.04	1.55	37.01	t	2.04	1.53
67	77.07	d	3.46		77.22	d	3.44	
68	75.87	d	3.15		76.04	d	3.12	
69	79.54	d	3.39		79.74	d	3.36	
70	75.69	d	3.12		75.85	d	3.09	
71	77.02	d	3.45		77.08	d	3.44	
72	41.28	t	2.06	1.45	41.51	t	2.04	1.43
73	64.93	d	4.85		64.99	d	4.84	
74	133.29	d	5.39		133.47	d	5.37	
75	130.05	d	6.01		130.04	d	6.00	
76	128.80	d	6.46		128.87	d	6.46	
77	133.83	d	5.79		133.88	d	5.78	
78	38.50	t	2.41		38.64	t	2.42	
79	71.12	d	3.98		71.20	d	3.93	
80	76.20	d	3.28		76.29	d	3.27	
81	73.02	d	3.71		73.04	d	3.63	
82	34.18	t	2.75	2.40	34.35	t	2.75	2.39
83	130.05	d	5.69		130.18	d	5.69	
84	132.62	d	5.96		132.64	d	5.95	
85	146.54	s	–		146.73	s	–	
85′	114.89	t	5.08	4.94	114.86	t	5.07	4.94
86	34.18	t	2.33	2.25	34.30	t	2.34	2.25
87	32.95	t	1.72	1.60	33.13	t	1.72	1.59
88	74.05	d	3.72		74.19	d	3.71	
89	73.93	d	3.51		74.02	d	3.50	
90	77.70	d	3.37		77.82	d	3.35	
91	32.99	d	1.89		33.00	d	1.89	
91-Me	15.60	q	0.92		15.65	q	0.91	
92	27.74	t	2.22	1.31	27.86	t	2.21	1.30
93	74.72	d	4.06		74.83	d	4.03	
94	72.88	d	3.68		73.04	d	3.65	
95	74.55	d	3.63		74.73	d	3.61	
96	75.87	d	3.19		76.01	d	3.15	
97	69.62	d	4.33		69.71	d	4.32	
98	132.32	d	5.57		132.43	d	5.55	
99	135.26	d	5.73		135.28	d	5.71	
100	71.89	d	4.36		71.90	d	4.36	
101	71.63	d	3.69		71.77	d	3.68	
102	40.11	t	1.59		40.21	t	1.58	
103	68.41	d	4.22		68.39	d	4.22	
104	40.35	t	1.76	1.40	40.53	t	1.74	1.38
105	76.04	d	4.53		76.14	d	4.51	
106	36.73	t	1.86	1.78	36.83	t	1.84	1.78
107	79.60	d	4.23		79.62	d	4.21	
108	82.67	d	4.35		82.74	d	4.35	
109	26.48	t	1.76	1.63	26.59	t	1.78	1.67
110	32.08	t	1.47		32.30	t	1.47	
111	83.45	d	3.86		83.81	d	3.89	
112	73.24	d	4.24		73.27	d	4.27	
113	39.53	t	2.04	1.84	39.78	t	2.10	1.86
114	77.02	d	4.28		75.31	d	4.36	
115	44.63	t	3.30	3.25	45.13	t	2.99	2.87
a	134.5	d	7.78		134.82	d	7.79	
b	106.91	d	5.96		106.82	d	5.95	

Table 1. (Continued)

No.	*N*-Acetylpalytoxin				Palytoxin			
	^{13}C (δ)	Mult.	^{1}H (δ)	^{1}H (δ)	^{13}C (δ)	Mult.	^{1}H (δ)	^{1}H (δ)
c	169.59	s	–		169.66	s	–	
d	37.37	t	3.34		37.42	t	3.33	
e	33.10	t	1.75		33.28	t	1.74	
f	60.33	t	3.61		60.40	t	3.60	
C=O (Ac)	173.85	s	–		–		–	
CH_3 (Ac)	22.71	q	1.98		–		–	

N-Acetylpalytoxin was solved in carbon-13 depleted and deuterated methanol, and palytoxin was solved in deuterated metanol with one drop of deuterium oxide. TMS was used as an internal or external chemical shift reference of 0 ppm in proton NMR spectra. The solvent peak was used as an internal chemical shift reference of 49.00 ppm in carbon-13 NMR spectra. The 'mult.' column shows a number of proton attached to the carbon as a multiplicity of peak (s: singlet, d: doublet, t: triplet, q: quartet). The marked rows from *1 to *4 show a group of carbons which are exchangeable each other.

Table 2. ^{15}N NMR chemical shift data of *N*-acetylpalytoxin.

No.	^{15}N (δ)
N–A	117.36
N–B	133.17
N–C	120.01

Nitrogen peak of nitromethane was used as an external chemical shift reference of 379.6 ppm.

play an important role in the interaction between the membrane protein and the toxin. Furthermore, methanol is thought to mimic a cell membrane surface, and thus it may yield important information about biological functions.

We achieved complete assignment of palytoxins using 750 MHz NMR (Bruker DMX-750 spectrometer was operated on 750.13 MHz for proton at 298 K). The 3D TOCSY–HSQC,[6] digital filtering and linear prediction techniques were used in the measurement of the restricted region of the aliphatic portion, because high digital resolution was necessary for the analysis of 3D TOCSY–HSQC with a small data matrix. Signal assignment was carried out as follows: first, the protons and carbons indicated by bold lines in Fig. 1 were assigned by the DQF–COSY, TOCSY, editing-HSQC[7], and

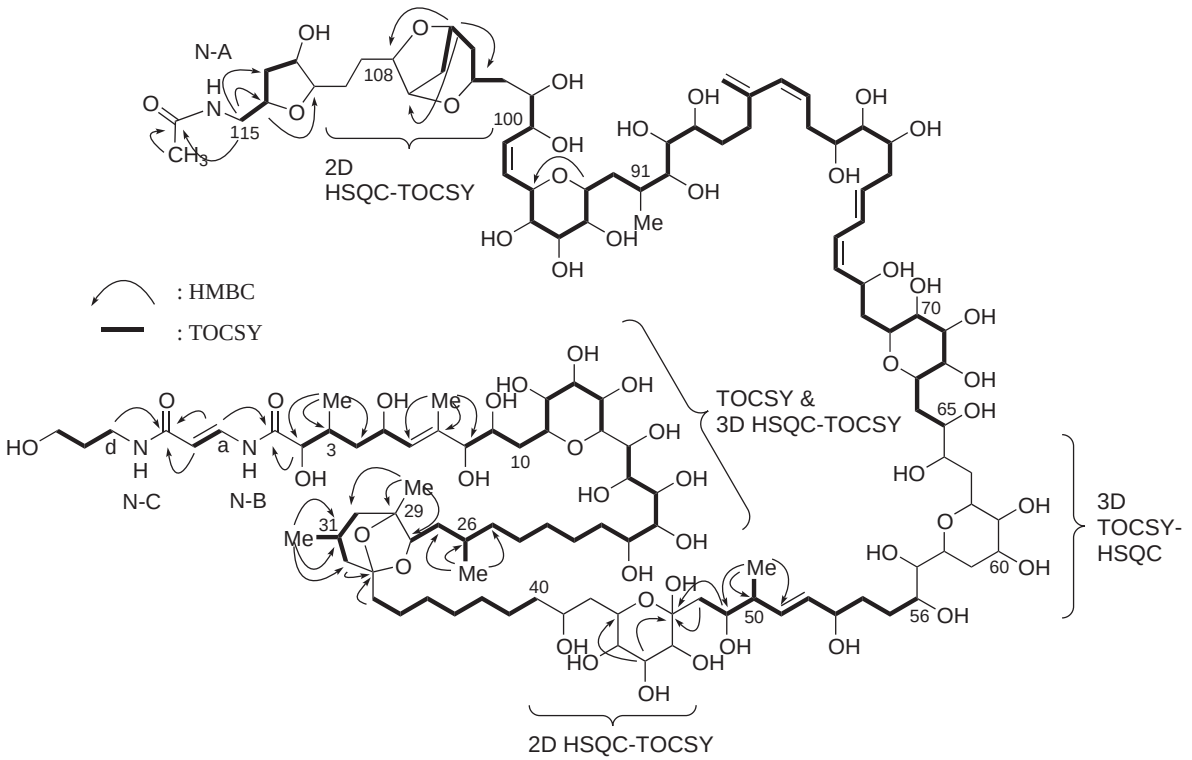

Figure 1. Summary of NMR signal assignment of *N*-acetyl palytoxin. Bold lines show connectivities elucidated by TOCSY. Curves with arrow denote the long rang spin–spin coupling between proton and carbon detected by HMBC. The connectivities from C-40 to C-48, C-101 to C-112 were mainly ascertained by the 2D HSQC–TOCSY. For the region of C-12 to C-19 and C-57 to C-65, some slice data of the 3D TOCSY–HSQC spectrum gave useful information.

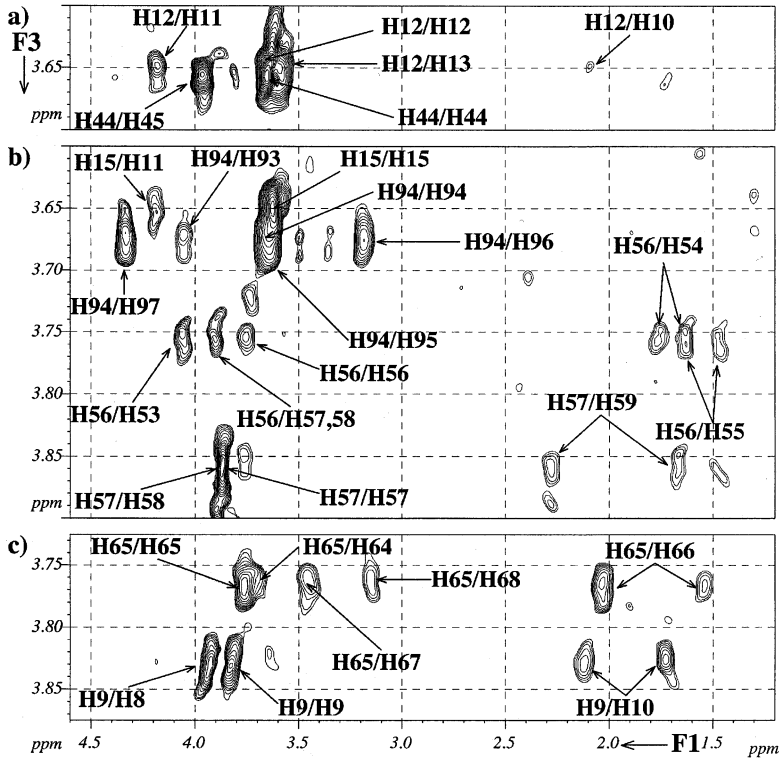

Figure 2. The 3D TOCSY–HSQC spectrum of *N*-acetyl palytoxin was used for the analysis of the most crowded oxymethine and aliphatic regions. The sliced spectra at (a) 73.8 ppm (C-12, 44); (b) 72.9 ppm (C-15, 56, 57, 94) and (c) 72.2 ppm (C-9, 65) are shown as 2D TOCSY representation.

HMBC spectra. The proton and carbon signals in this region were easily identifiable from the starting points of the characteristic olefin and methyl groups. Even when the proton signals had overlapped, the carbon signals could be separated. The HMBC spectrum supported the connection of the assigned fragments, which were represented by curves with arrowhead as shown in Fig. 1. In the second step, the connectivities from C-40 to C-48, C-101 to C-112 were primarily ascertained by the 2D HSQC–TOCSY and HMBC spectra. The combination of 2D HSQC–TOCSY and HMBC spectra demonstrated the connectivity from C-101 to C-115. For example, the C-103, C-107, C-108 and C-111 carbon signals showed cross peaks with protons H-102, H-104, H-105, H-108, H-109, H-110, H-111 and H-112 in the 2D HSQC–TOCSY[8] spectrum. The long-range conectivities, H-105/C-108, H-105/C-107 and H-105/C-102, were obtained from the HMBC spectrum. In the third step, the 3D TOCSY–HSQC spectrum was used for the analysis of the most crowded oxymethine and aliphatic regions as shown in Fig. 2. For C-56 and C-65, the corresponding protons were observed at the same chemical shift of 3.76–3.77 ppm. The F1–F3 plane correlated with C-56, which showed the cross peaks H-56/H-53 ~ 58 in Fig. 2b, whereas the F1–F3 plane of C-65 showed H-65/H-64 ~ 66 and H-65/H-68 in Fig. 2c. For other carbons, slice data of the 3D TOCSY–HSQC spectrum yielded useful information in spite of its low digital resolution in F1 axis (3.13 Hz/point). Accurate chemical shift values were obtained from the F3 axis of the 3D TOCSY–HSQC spectrum (1.00 Hz/point) or 2D TOCSY spectrum (1.71 Hz/point).

In the editing-HSQC spectrum, the cross peak of methylene carbon C-48/H-48 was found to be oriented in the same direction (positive) as methine in the CD_3OD solution. This result could be explained by hydrogen–deuterium exchange for the active methylene, because C-47 carbon was acetonide which is equivalent to the ketone group. The C-47 carbon was revealed as a broad peak in the DEPT90 spectrum caused by carbon–deuterium coupling. The CD_3OH solution of palytoxin showed the negative cross peak of C-48/H-48 in the editing-HSQC spectrum, which was oriented in the same direction as methylene.

Both palytoxin and *N*-acetylpalytoxin cause contraction of the isolated guinea-pig vas deferens, but the potency of *N*-acetylpalytoxin is approximately 100 times weaker than that of palytoxin.[9] However, the full assignment data of the ^1H and ^{13}C NMR spectra of palytoxin and *N*-acethylpalytoxin have identified a slight difference only around the N-terminal part of palytoxin, and thus the three dimensional conformations of both compounds are probably nearly identical. This similarity suggests that they may bind in an analogous manner to a protein target compound, which is thought to be a key event in promoting toxicity. Now that the ^1H and ^{13}C NMR assignments are complete, the stage is set for studies of structurally related paly-

toxin compounds, and the analysis of the biosynthetic pathways involved in their biogenesis.

Supplementary material

2D $^{1}H\{^{13}C\}$ HSQC and $^{1}H\{^{13}C\}$ HMBC spectra of palytoxin and *N*-acetylpalytoxin. 3D $^{1}H\{^{1}H, ^{13}C\}$ HSQC–TOCSY (partial, deduced to 2D spectra) and 2D $^{1}H\{^{15}N\}$ HMBC of *N*-acetylpalytoxin.

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