Tetrahedron Vol. 41, No. 6, pp. 1007 to 1017, 1985
Printed in Great Britain.

0040–4020/85 $3.00 + .00
Pergamon Press Ltd.

STUDIES ON PALYTOXINS

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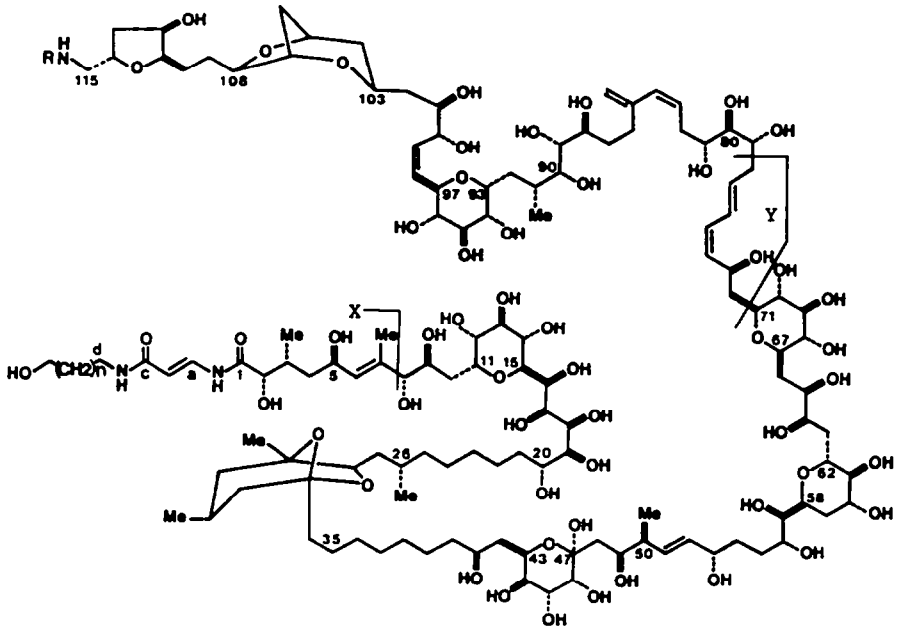
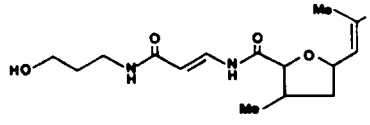
(*Received in USA* 21 *October* 1983)

Abstract—The complete structure of palytoxin (1) was elucidated by us in 1982.[1] Our continuous interests in palytoxin led us to examine minor constituents of Okinawan *Palythoa tuberculosa*. In this paper, we describe successful isolation and structural elucidation of four minor toxins, which were named homopalytoxin (2), bishomopalytoxin (3), neopalytoxin (4) and deoxypalytoxin (5).

Animals were collected in Ishigaki Island during May through September, and frozen and stored over Dry Ice. The extraction procedure is summarized in Scheme 1. Frozen specimens were crushed in a blender in 75% aqueous ethanol. After removal of the solid residue by filtration, the orange filtrate was concentrated to about one twentieth of its volume under reduced pressure below 40°. The remaining aqueous solution was charged on a TSK G3000S polystyrene gel column. After inorganic salts were washed out with water crude toxins were eluted with 75% ethanol. After ethanol removal, the residual





1 n= 1, R= H

2 n= 2, R= H

3 n= 3, R= H

13 n= 2, R= p-bromobenzoyl

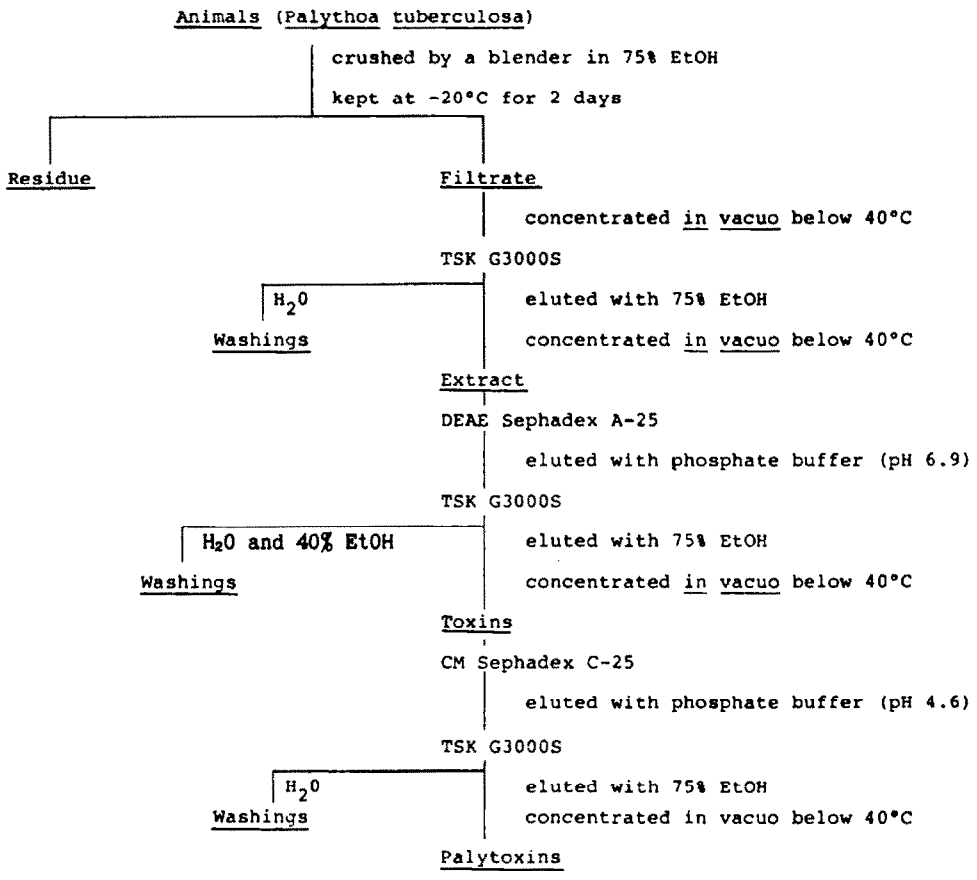
14 n= 3, R= p-bromobenzoyl

4 n=1, R= H, X=

5 n= 1, R= H
Y= ^{78}-CH$_2$CH=CHCH=CHCH$_2$CH$_2$-

26 n= 1, R= H
Y= ^{78}-CH$_2$CH=CHCH=CHCHOHCH$_2$-

Chart A.



Scheme 1. Procedure of extraction and separation of palytoxins.

crude extract was passed through a DEAE Sephadex A-25 column, equilibrated with phosphate buffer at pH 6.9 before use. Toxins, which are weakly basic,[2] appeared in the solvent front without adsorption. The toxic fraction was freed from the phosphate buffer by adsorbing on a TSK G3000S column, washed thoroughly with water and 40% aqueous ethanol, and then eluted with 75% ethanol. After removal of solvent, the resulting residue was applied to a CM Sephadex C-25 column (equilibrated with phosphate buffer at pH 4.6 before use), and toxins were eluted with phosphate buffer (pH 4.6) monitoring by a UV detector at 263 nm (Fig. 1).

The toxic fractions were desalted by a TSK G3000S column as before, and concentrated under reduced pressure below 40° to give a glassy brown

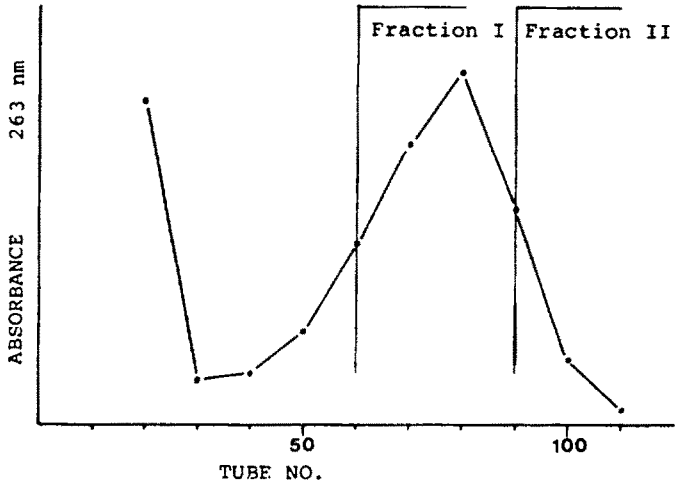


Fig. 1. Chromatographic separation of palytoxins with CM Sephadex C-25. Eluent: pH 4.6 phosphate buffer. Column: 80 cm × 2.4 cm (i.d.).

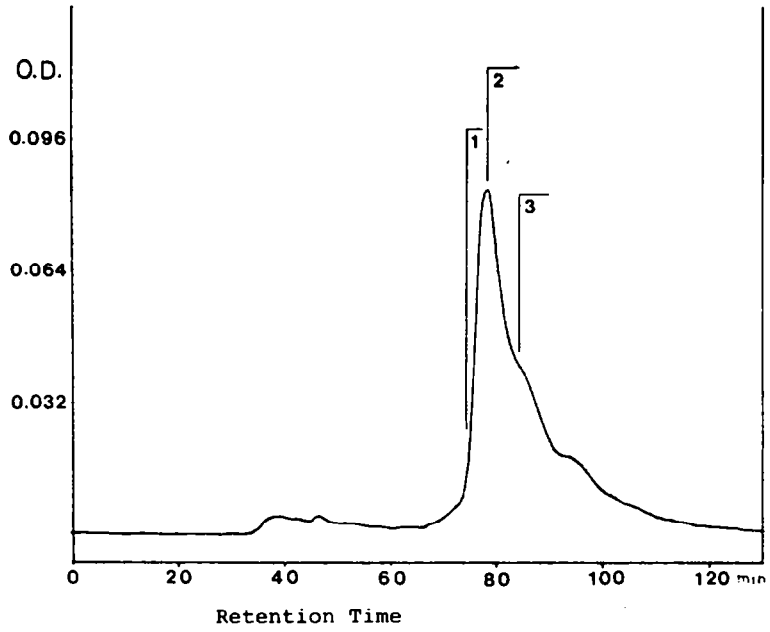


Fig. 2. HPLC chromatogram of fraction II (Fig. 1). Column: Mitsubishi MCI gel CQP-30, 50 cm × 2 cm (i.d.); eluent: pH 6.9 phosphate buffer; flow rate: 3 ml/min and detector: UV (263 nm).

amorphous solid. Fraction I was enriched in palytoxin (1) whereas fraction II was enriched in the minor toxins. The crude toxins were further purified by HPLC and high performance TLC (HPTLC). The glassy brown amorphous solid was dissolved in 75% ethanol and injected into a Mitsubishi MCI CQP-30 column (equilibrated with phosphate buffer at pH 6.9 before use) and eluted with the same eluent. A typical chromatogram pattern is shown in Fig. 2. Fraction 3 (Fig. 2) was collected, desalted with a TSK G3000S column, and concentrated under reduced pressure below 40° to give the residue which was separated again by HPLC (Fig. 3). Three peaks were observed. The first peak corresponds to palytoxin (1) contaminated with a small amount of neopalytoxin; the second peak corresponds to deoxypalytoxin (5); and the third peak corresponds to a 1:1 mixture of homopalytoxin (2) and bishomopalytoxin (3). By

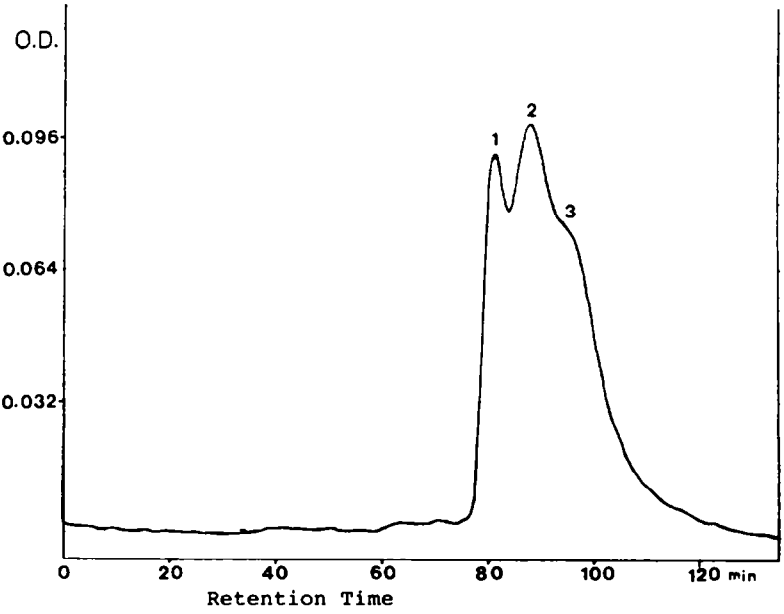


Fig. 3. HPLC pattern of fraction 3 in Fig. 2. Column: Mitsubishi MCI gel CQP-30, 50 cm × 2 cm (i.d.); eluent: pH 6.9 phosphate buffer; flow rate: 3 ml/min; detector: UV (263 nm). Peak 1: palytoxin and neopalytoxin, peak 2: deoxypalytoxin and peak 3: homopalytoxin and bishomopalytoxin.

using the same procedure, fraction 1 (Fig. 2) was shown to be free from peaks of deoxy-, homo- and bishomopalytoxins (Fig. 3). Similarly, fraction 2 (Fig. 2) was shown to contain a small amount of **2**, **3** and **4**. Separation of neopalytoxin from palytoxin is possible by using preparatively HPTLC plate (Merck, NH_2F_{254} pre-coated, No. 15647). The separation of homo- and bishomopalytoxins was not possible chromatographically, but they were distinguishable in the 500 MHz ^{1}H-NMR spectrum as shown in Fig. 4 (*vide infra*).

Structure of homopalytoxin and bishomopalytoxin. As chromatographic separation of homo- and bishomopalytoxins was not feasible, their structures were studied by using a 1:1 mixture of the two components. The 500 MHz ^{1}H-NMR spectrum of the 1:1 mixture is shown in Fig. 4. The signals around δ7.8 ppm are useful for the determination of the ratio of homo- and bishomopalytoxins. Homopalytoxin gives a resonance at δ7.790 ppm, whereas bishomopalytoxin gives a resonance at δ7.788 ppm. The chemical shift of the corresponding resonance, assigned to the proton at C-a in palytoxin, is observed at δ7.792 ppm. These data suggest that the structural difference among homo-, bishomo-, and palytoxin is in the proximity of the C-a proton. In order to confirm this, periodate oxidation was attempted to isolate degradation products corresponding to the α,β-unsaturated amide **6** obtained in the palytoxin series.[3] A 1:1 mixture of **2** and **3** was adsorbed on a TSK G3000S polystyrene gel column, and oxidation was performed by passing an aqueous solution of $NaIO_4$ (excess) through the column. The products were eluted by gradually increasing the concentration of ethanol. The 40% ethanol eluate contained the fragments corresponding to **6**. After acetylation, the desired segments **7** and **8** were isolated by preparative TLC. Structures **7** and **8** were suggested by their spectral data. Particularly, the ^{1}H-NMR spectra gave valuable information; except for the difference in the chemical shift of the amide NH (**6**: δ6.30, **7**: δ4.89, and **8**: δ4.58), the ^{1}H-NMR spectra of **7** and **8** were extremely similar to each other and to the ^{1}H-NMR spectrum of the α,β-unsaturated amide **6** obtained from palytoxin. Desorption/chemical ionization mass spectra (D/CI/MS)[4] showed the molecular weight of **7** to be larger by 14 mass units than that of **6**, and the molecular weight of **8** to be larger by 28 mass units than that of **6**. Oxidation of **6** with OsO_4 and $NaIO_4$ in THF–H_2O gave two aldehydes, **9** and **10**. On the other hand, **7** yielded **10** and **11**, and **8** yielded **10** and **12**. Optical rotation of the aldehyde **10** obtained from **7** and **8** was found to be identical with that obtained from palytoxin, allowing to assign the stereochemistry of **7** and **8**.

In order to confirm that the remaining parts of molecule of homo- and bishomopalytoxins are the same as that of palytoxin, the following degradation reaction was performed. By using known procedures,[5] a 1:1 mixture of homo- and bishomopalytoxins was converted to a 1:1 mixture of *p*-bromobenzamides **13** and **14**. This 1:1 mixture of **13** and **14** was ozonized in methanol at −40°, and reduced with $NaBH_4$. After removal of solvent, the crude products were separated on a TSK G3000S column. The aqueous eluates were combined and then treated with Dowex 50W × 4 (H^+ form). After acetylation, three products, **15**, **16** and **17**, were isolated. On comparison of the ^{1}H-NMR spectra and the specific rotation, these products were identical with the corresponding products obtained from palytoxin. From the 60% ethanol eluate of the TSK G3000S column, a tetraol was obtained, which was acetylated to give a tetraacetate **18**. On comparison of the ^{1}H-NMR spectrum and the rotation, this product was identical with an authentic sample obtained from palytoxin. From the 75% aqueous eluate of a TSK G3000S column, polyol **19** was obtained, which was further converted to a mixture of spiroketals by treatment with 50% aqueous acetic acid at room temperature. After acetylation of this mixture, four peracetates **20**, **21**, **22** and **23** in approx. 5:2:1:1 ratio were isolated by careful separation with preparative TLC (SiO_2, 2.5% MeOH–$CHCl_3$). The 5,6-spiro compound **20** was the major product, and was identical with an authentic sample obtained from palytoxin on comparison with the ^{1}H-NMR spectrum and the optical rotation. On treatment with aqueous NaOH in MeOH, followed by acid equilibration (50% aqueous acetic acid) and then acetylation, the 5,6-spiroperacetate **20** gave a mixture of **20**, **21**, **22** and **23** in about the same ratio as before.

The stereochemistry of the trisubstituted double bond at C-6 was evident from the structure of **10**. The stereochemistry of all remaining double bonds was deduced from the values of spin–spin coupling constants in the 500 MHz ^{1}H-NMR spectrum of **2** and **3**: $J_{a,b}$ = 14 Hz, $J_{51,52}$ = 16 Hz, $J_{74,75}$ = 11 Hz, $J_{76,77}$ = 15 Hz, $J_{83,84}$ = 11 Hz and $J_{98,99}$ = 11 Hz. Based on the evidence presented, the structures of homo- and bishomopalytoxins are **2** and **3**.

Structure of neopalytoxin. Separation of neopalytoxin from palytoxin was not feasible by HPLC, but was possible by HPTLC (*vide supra*). The 360 MHz ^{1}H-NMR spectrum of neopalytoxin was indistinguishable from that of palytoxin. On periodate oxidation followed by acetylation, neopalytoxin gave an aldehyde **24**. The molecular formula was determined to be $C_{18}H_{26}N_2O_6$ by high resolution mass spectrum. The ^{1}H-NMR spectrum of **24** suggested the presence of one acetoxyl group [δ1.69 (3H, s)] and two protons attached on C atoms bearing an ether O [δ4.21 (1H, d, J = 6.0 Hz) and 4.62 (1H, ddd, J = 1.0, 7.6 and 7.6 Hz)]. Although conclusive evidence is not available, the stereochemistry of the α,β-unsaturated moiety seems to be *trans*, since the chemical shift of the β-proton of the α,β-unsaturated aldehyde group is very close to that of triacetate **6** obtained from palytoxin. Since ozonolysis of N-(*p*-bromobenzoyl)-neopalytoxin, followed by reduction with $NaBH_4$ and then acetylation, gave degradation products **15**, **16**, **17**, **18** and **20**, the remaining part of neopalytoxin must be identical with that of palytoxin. From the ^{1}H-NMR spectrum, the stereochemistry of all double bonds except the one at C-6 position is as shown in structure **4**. However, the stereochemistry at C-2, C-3 and C-5 is under investigation.

Structure of deoxypalytoxin. The most characteristic feature in the ^{1}H-NMR spectrum of deoxypalytoxin is the appearance of a broad double triplet (J = 10 and 6 Hz) at δ5.36 assigned to C-74 proton when compared with the ^{1}H-NMR spectrum of palytoxin. This piece of data suggested the absence of a hydroxy group at C-73. The results of the following

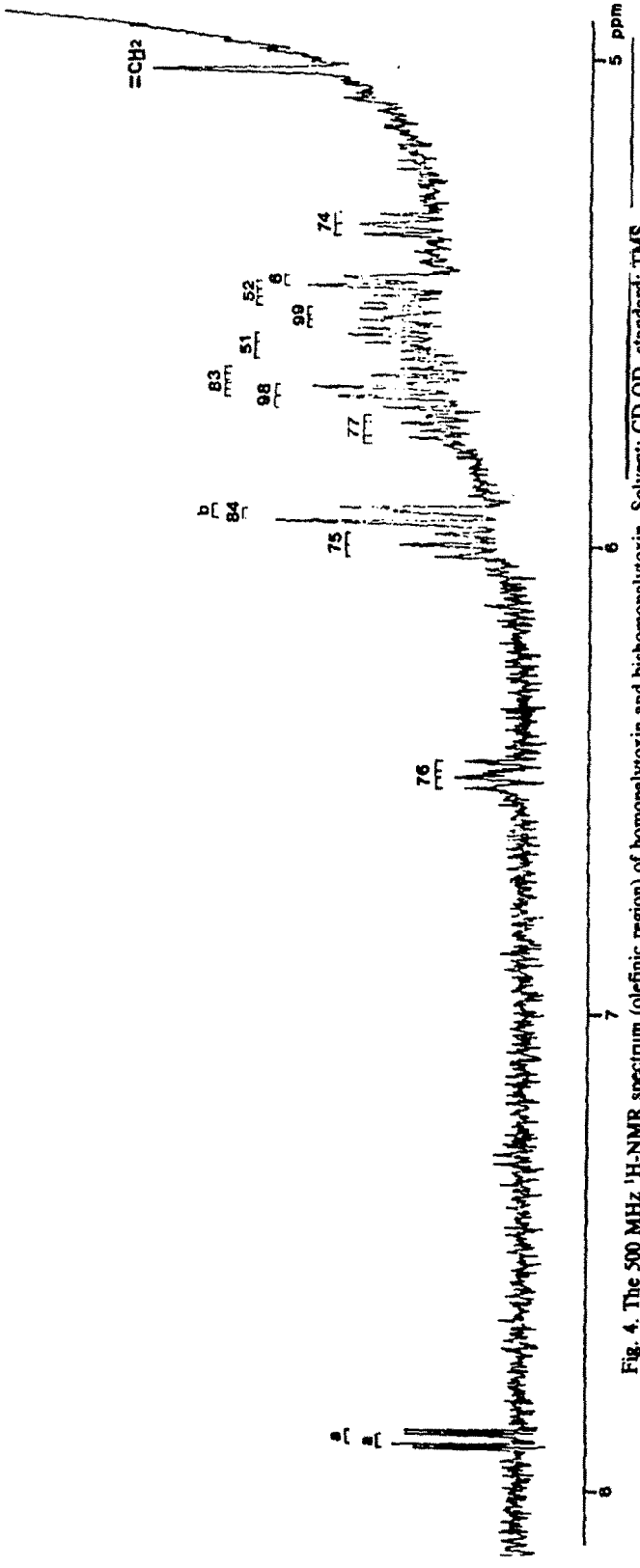
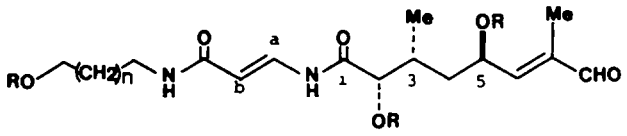


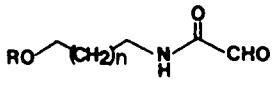
Fig. 4. The 500 MHz ^{1}H-NMR spectrum (olefinic region) of homopalytoxin and bishomopalytoxin. Solvent: CD_3OD, standard: TMS.



6 n= 1, R= $COCH_3$

7 n= 2, R= $COCH_3$

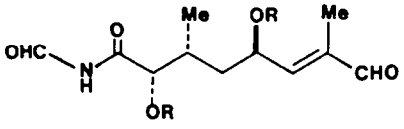
8 n= 3, R= $COCH_3$



9 n= 1, R= $COCH_3$

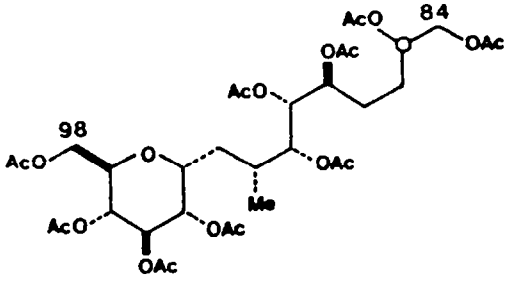
11 n= 2, R= $COCH_3$

12 n= 3, R= $COCH_3$

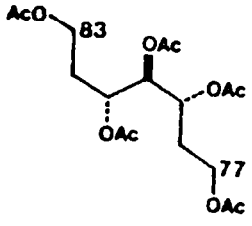


10 R= $COCH_3$

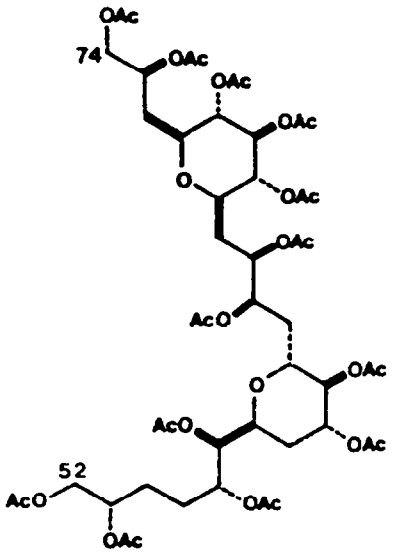
Chart B.



15



16



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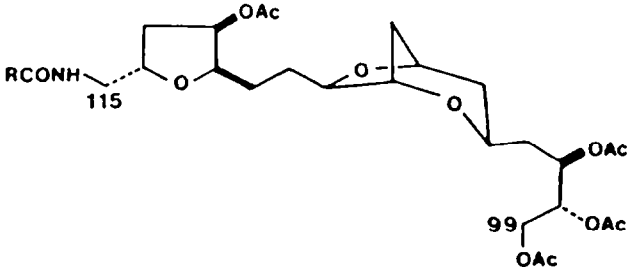

18 R = p-bromophenyl

Chart C.

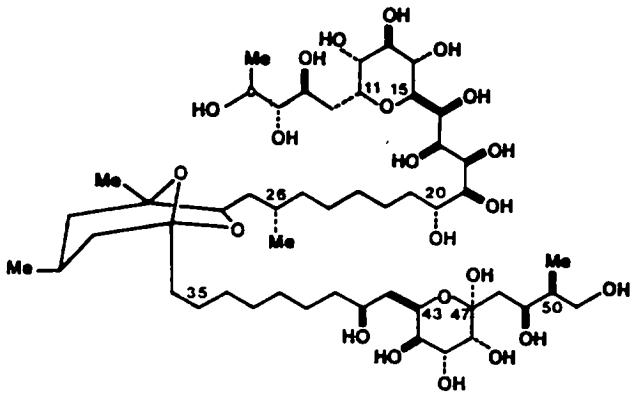

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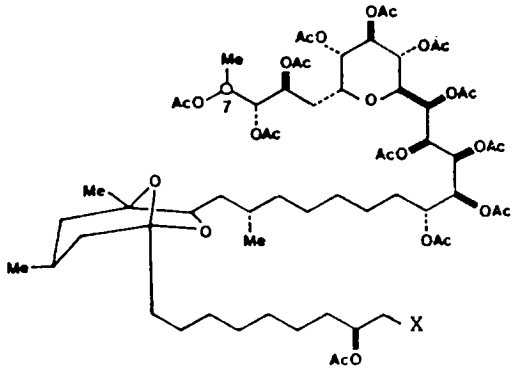
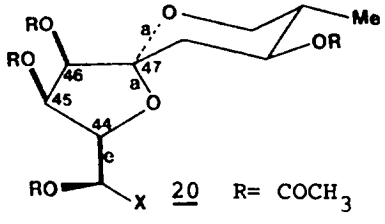
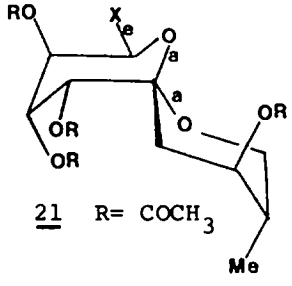
Chart D.

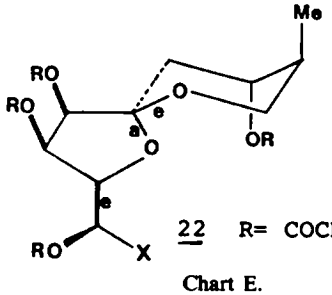




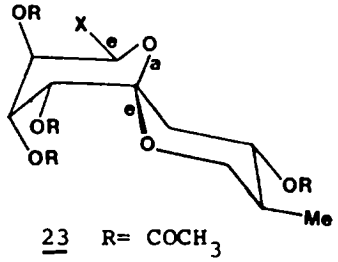
20 R= $COCH_3$



21 R= $COCH_3$

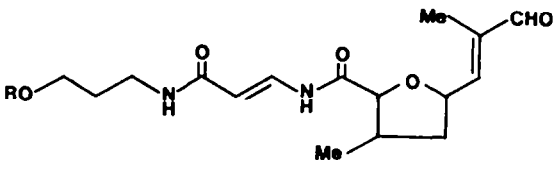


22 R= $COCH_3$



23 R= $COCH_3$

Chart E.



24 R= $COCH_3$

Chart F.

degradation reactions support this assignment. Periodate oxidation followed by acetylation gave product **6**. On the other hand, ozonolysis of N-(*p*-bromobenzoyl)deoxypalytoxin, followed by reduction with $NaBH_4$ and acetylation, gave degradation products **15**, **16**, **19** and **20** (by acetylation after treatment with 50% aqueous acetic acid concerning **20**). A new product **25** was isolated by preparative TLC. The ^{1}H-NMR spectrum of this compound coupled with the COSY method[6] (Fig. 5) was consistent with structure **25**. The stereochemistry of the double bonds was deduced from values of spin–spin coupling constants: $J_{a,b} = 14$ Hz, $J_{51,52} = 15$ Hz, $J_{74,75} = 11$ Hz, $J_{76,77} = 15$ Hz, $J_{83,84} = 11$ Hz and $J_{98,99} = 11$ Hz. The structure of deoxypalytoxin was found to be **5**. However, in the ^{1}H-NMR spectrum of deoxypalytoxin, two additional olefinic protons were easily observed at δ6.13 (br dd, J = 10, 15Hz, H-75) and δ6.23 (br dd, J = 10, 15 Hz, H-76). This result indicated contamination by a further minor component. The presence of the typical *trans,trans*-conjugated diene was further suggested by the isolation of a small amount of **17**, accompanied by product **25**, after the ozonolysis reaction

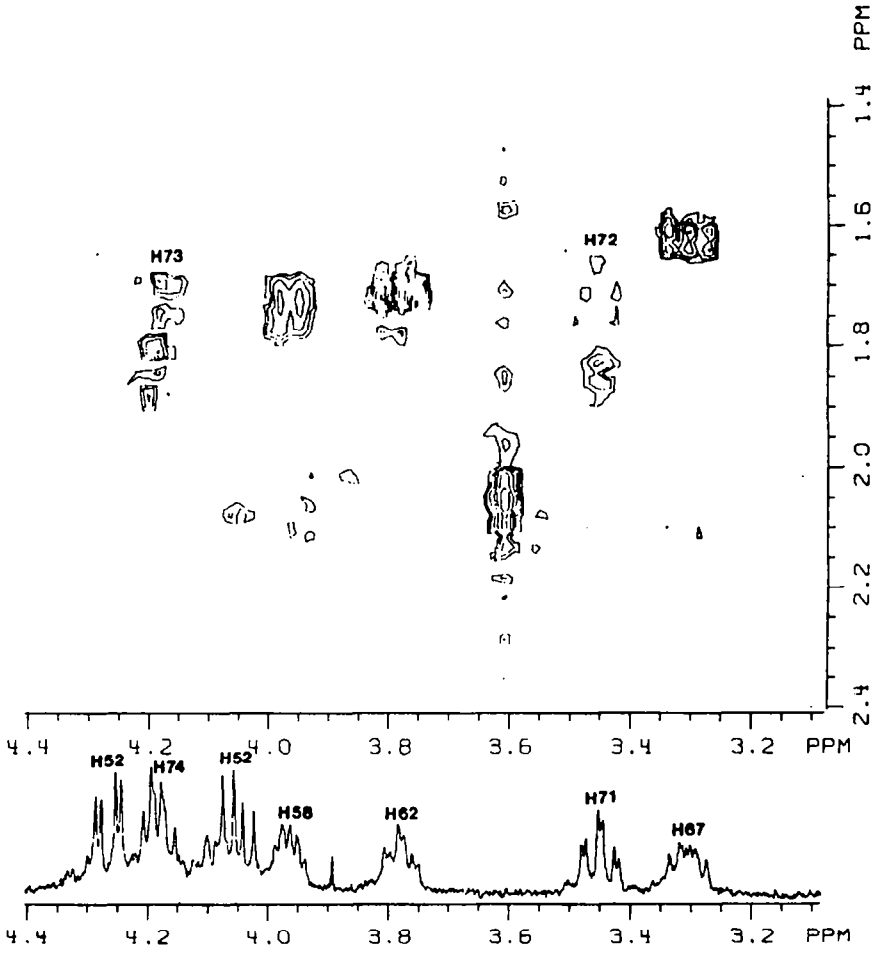
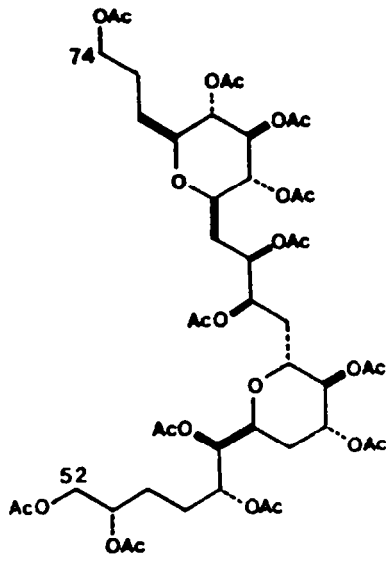


Fig. 5. The 360 MHz COSY spectrum (contour plot of compound **25**). The presence of $AcOCH_2{}^{73}CH_2{}^{72}CH_2CH$–O– is confirmed.



25

Chart G.

sequence. Consequently, it is proposed that the second peak of Fig. 3 contains a further minor component **26** (isopalytoxin).

Thus, the structures of minor toxins, co-occurring with palytoxin in *Palythoa tuberculosa*, have been elucidated. As seen in the structures of palytoxin (**1**), homopalytoxin (**2**) and bishomopalytoxin (**3**), variation of the ω-aminoalcohol moiety is often observed in natural products[7] produced by micro-organisms. This may suggest that palytoxin might be biosynthesized by symbiotic micro-organisms,[8] and further taxonomical studies would be interesting. Related to this, it is worth to note that we observed another species of the genus *Palythoa* in Ishigaki Island; although the taxonomical classification is not established, this species grows as a thin body, whereas *Palythoa tuberculosa* grows as a round body. Specimens of the unclassified *Palythoa* sp. were collected during May through September, and palytoxins were extracted by using the same procedure as described before. Except for palytoxins as described before, no new palytoxin was detected in the extract. The major toxin was confirmed to be palytoxin, but the content of homo-, bishomo- and deoxypalytoxins was higher than that in *Palythoa tuberculosa*.

EXPERIMENTAL

UV spectra were recorded on a Hitachi double beam spectrophotometer Model 200-10. Optical rotations were recorded on a JASCO DIP-360 polarimeter. Mass spectra were taken on a Hitachi RMU-6C mass spectrometer and a JOEL-O1SG mass spectrometer, operating with an ionization energy of 70 eV. ^{1}H-NMR spectra were recorded on a JEOL FX-270 and a Nicolet NT-360 NMR spectrometer. Chemical shifts are given in ppm relative to TMS, and coupling constants in Hz. Data processing for a COSY spectrum was carried out with Nicolet software. The HPLC experiments were carried out on a JASCO HPLC system (BIP-I pump, UVIDEC 100-III UV spectrophotometer).

Extraction and separation of **1**, **2**, **3**, **4** *and* **5**. The frozen animals (approx. 45 kg) were warmed to room temp for 4 hr. After contaminating corals and stones were carefully excluded, the animals were cut into small pieces with scissors and then crushed by a blender in 75% aq EtOH. 500 ml of 75% aq EtOH was used to treat approx. 0.5 kg of animals. Resulting extracts were stored in a freezer for 2 days and filtered by the aid of Hyflo Super-Cel. Solid residue was twice washed with 75% aq EtOH. Combined orange filtrates were concentrated to about one twentieth by vacuum evaporator *in vacuo* below 40°. The brown aq residue was charged on a TSK G3000S polystyrene gel (prepared from Toyo Soda Manufacturing Co., Ltd., Tokyo, Japan) column [10 × 17 (i.d.) cm], which was well washed with EtOH and then H_2O before use. By washing with H_2O, inorganic salts and non-lipophilic compounds were removed, and 75% aq EtOH was added with stirring onto the column. Eluate was collected until no more toxin was eluted, monitoring by interperitoneal injection into mice. About 10 L of 75% aq EtOH was used. This procedure was repeated five times in order to treat all extracts. After EtOH was removed by evaporation *in vacuo* below 40°, the aq residue (1:1) was charged on a DEAE Sephadex A-25 column [40 × 9 (i.d.) cm] equilibrated with pH 6.9 phosphate buffer before use. Checking the toxicity, eluate was combined and then desalted by the use of a column [20 × 5 (i.d.) cm] packed with TSK G3000S gel. After washing with H_2O and then 40% aq EtOH, crude toxin was effectively desorbed with 75% aq EtOH and desalted. The EtOH soln was concentrated *in vacuo* below 40°. The resulting residue (5.8 g) was dissolved in 10 ml of pH 4.6 phosphate buffer. This soln was applied to a CM Sephadex C-25 column [80 × 2.4 (i.d.) cm] which was equilibrated with pH 4.6 phosphate buffer before use. The chromatogram monitored by UV is shown in Fig. 1. Fractions I and II were individually desalted with a TSK G3000S column [9 × 3 (i.d.) cm]. Two 75% aq EtOH eluates (1:1 each) were carefully concentrated to give glassy toxins: fraction I, 352 mg and fraction II, 93 mg. Fraction I was enriched in palytoxin (**1**), and used for the purpose of its structural elucidation. On the other hand, fraction II was enriched in minor palytoxins, and further analyzed as shown in Fig. 2. Purification was performed by the use of this system. Fractions 1, 2 and 3 were also separately collected and desalted with a TSK G3000S column [6 × 2 (i.d.) cm]. Evaporation of 75% aq EtOH eluate gave glassy toxins: fraction 1 (26 mg), fraction 2 (20 mg) and fraction 3 (16 mg). Fraction 3 was further purified by HPLC (see Fig. 3). Each

pure peak was obtained as a colorless solid (4 mg each) after desalting. The components corresponding to peaks 2 and 3 were also found in fraction 2.

Peak 3: homopalytoxin **2** and bishomopalytoxin **3**; ^{1}H-NMR (500 MHz, CD_3OD) Fig. 4; UV (H_2O) 233 and 263 nm.

Peak 2: deoxypalytoxin **5**; ^{1}H-NMR (360 MHz, CD_3OD) 0.8–0.95 (12H, m, four secondary methyls), 1.03 (3H, d, J = 6.8 Hz, C-50-Me), 1.17 (3H, s, C-7-Me), 3.60 (2H, t, J = 6.8 Hz, H-f), 5.36 (1H, br dt, J = 10, 6 Hz, H-74), 5.50 (1H, br d, J = 8 Hz, H-6), 5.51 (1H, dd, J = 6.2, 15 Hz, H-52), 5.62 (1H, dd, J = 7, 15 Hz, H-51), 5.68–5.72 (1H, m, H-83), 5.71 (1H, dd, J = 11, 11 Hz, H-98), 5.73 (1H, dt, J = 15, 7 Hz, H-77), 5.95 (1H, d, J = 14 Hz, H-b), 5.95 (1H, d, J = 11 Hz, H-84), 6.00 (1H, dd, J = 11, 11 Hz, H-75), 6.40 (1H, br dd, J = 11, 15 Hz, H-76), 7.79 (1H, d, J = 14 Hz, H-a); UV (H_2O) 233 and 263 nm.

Peak 1 was purified by prep HPTLC (Merck, NH_2F_{254} precoated, No. 15647, n-AmOH–pyridine–H_2O 7:7:6, eluent, MeOH), giving palytoxin (**1**): 2 mg (R_f value, 0.26) and neopalytoxin (**4**): 0.7 mg (R_f value, 0.29) 0.8–1.0 (12H, m, four secondary methyls), 1.04 (3H, d, J = 6.7 Hz, C-50-Me), 1.18 (3H, s, C-29-Me), 1.73 (3H, br s, C-7-Me), 5.36 (1H, br t, J = 11 Hz, H-74), 5.95 (1H, d, J = 14 Hz, H-b), 6.00 (1H, dt, J = 11, 11 Hz, H-75), 6.46 (1H, dd, J = 11, 15 Hz, H-76), 7.80 (1H, d, J = 14 Hz, H-a); UV (H_2O) 233 and 263 nm.

N-(p-*Bromobenzoyl*)*homopalytoxin* (**13**) and N-(p-*bromobenzoyl*)*bishomopalytoxin* (**14**). A mixture (3 mg) of **13** and **14** was dissolved in H_2O (0.5 ml) and pyridine (1 ml). To the soln was added 10 mg of *p*-nitrophenyl *p*-bromobenzoate in 1 ml of pyridine with stirring. The mixture was stirred at 30° overnight. Evaporation of solvent under reduced pressure gave an oily material, which was purified by prep TLC (SiO_2, 1:4 MeOH–$CHCl_3$ and then 2:2:1 AcOEt–MeOH–H_2O). After removal of eluent (90% aq MeOH), a mixture (2.4 mg) of **13** and **14** was obtained.

Compounds **13** *and* **14**. NMR (360 MHz, CD_3OD) 0.8–1.0 (12 H, m, five secondary methyls), 1.04 (3H, d, J = 6.6 Hz, C-50-Me), 1.18 (3H, s, C-29-Me), 1.71 (3H, br s, C-7-Me), 5.36 (1H, dd, J = 10, 11 Hz, H-74), 5.94 (1H, br d, J = 14 Hz, H-b), 5.98 (1H, dd, J = 11, 11 Hz, H-75), 6.45 (1H, dd, J = 11, 15 Hz, H-76), 7.6–7.8 (4H, m), 8.01 (1H, br d, J = 14 Hz).

NaIO *oxidation of* **2** *and* **3**. A mixture (3 mg) of **2** and **3** was charged on a TSK G3000S column [4 × 2 (i.d.) cm], which was well washed with EtOH and then H_2O. An aq $NaIO_4$ (21 mg in 1.5 ml) soln was applied to this column. After washing with water and then 20% aq EtOH, 40% aq EtOH was passed through the column. After removal of solvent by vacuum evaporator, the resulting residue was immediately acetylated with Ac_2O (0.5 ml) and pyridine (1 ml) overnight. The reagents were removed by evaporation, and the residual products were separated by prep TLC (SiO_2, 2% MeOH–$CHCl_3$) to give **7** and **8**.

Compounds **7**. D/CI/MS, *m/z* 500 [$(M + NH_4)^+$], 441; ^{1}H-NMR (270 MHz, $CDCl_3$) 0.94 (3H, d, J = 6.6 Hz, C-7-Me), 1.66, 1.67, 1.69, 1.82 (3H, each), 2.30 (1H, m, H3), 3.14 (2H, dt, J = 9.0, 6.0 Hz, H-d), 3.90 (2H, t, J = 5.9 Hz, H-g), 4.89 (1H, br s, NH), 5.14 (1H, d, J = 4.0 Hz, H-2), 5.80 (2H, m, H-5 and H-6), 5.85 (1H, d, J = 14 Hz, H-b), 7.76 (1H, dd, J = 12, 14 Hz, H-a), 8.65 (1H, br d, J = 12 Hz).

Compound **8**. D/CI/MS, *m/z* 514 [$(M + NH_4)^+$], 497 [$(M + H)^+$], 456; ^{1}H-NMR (270 MHz, $CDCl_3$) 0.95 (3H, d, J = 6.6 Hz), 1.75, 1.76, 1.81, 1.85 (3H each), 2.30 (1H, m), 3.12 (2H, q, J = 6.2 Hz), 3.95 (2H, t, J = 6.3 Hz), 4.58 (1H, br s, NH), 5.15 (1H, d, J = 3.6 Hz), 5.78 (2H, m), 7.68 (1H, dd, J = 12, 14 Hz), 8.70 (1H, br d, J = 12 Hz), 9.20 (1H, s).

OsO_4–$NaIO_4$ *oxidation of* **7** *and* **8**. To a soln of **7** (1.8 mg) in THF (1 ml) was added OsO_4 (excess) and then a soln of $NaIO_4$ (excess) in H_2O (1 ml). After a period of 1 hr, H_2O (5 ml) was added and the aq soln was extracted with three 5 ml portions of $CHCl_3$. The combined $CHCl_3$ layers were dried over Na_2SO_4 and concentrated to give a residue, which was separated by prep TLC (SiO_2, 5% MeOH–$CHCl_3$). Compounds **10** and **11** were obtained. The same procedure of **8** gave **10** and **12**. **10**: EIMS, *m/z* 327 (M^+), 298; ^{1}H-NMR (270 MHz, $CDCl_3$) 1.06 (3H, d, J = 6.6 Hz), 1.85 (3H, d, J = 1.3 Hz), 2.09 (3H, s), 2.39 (3H, s), 5.25 (1H, d, J = 3.6 Hz), 5.70 (1H, ddd, J = 4.0, 8.2, 7.9 Hz), 6.25 (1H, dq, J = 8.2, 1.3 Hz), 8.56 (1H, br d, J = 10 Hz), 9.14 (1H, d, J = 10 Hz), 9.42 (1H, s); $[\alpha]_D$ = −15 (c 0.12, $CHCl_3$); High resolution mass measurement (Found: 327.1250. Calc for $C_{15}H_{21}NO_7$: 327.1318).

Compounds **11**. EIMS, *m/z* 187 (M^+), 128; ^{1}H-NMR (270 MHz, $CDCl_3$) 2.05 (3H, s) 3.38 (2H, q, J = 6.8 Hz), 4.05 (2H, t, J = 6.6 Hz), 6.60 (1H, br s), 9.65 (1H, s); High resolution mass measurement (Found: 187.0925. Calc for $C_8H_{13}NO_4$: 187.0844).

Compound **12**. EIMS, *m/z* 201 (M^+), 173; ^{1}H-NMR (270 MHz, $CDCl_3$) 2.03 (3H, s), 3.35 (2H, q, J = 6.6 Hz), 6.21 (1H, br s), 9.63 (1H, s); High resolution mass measurement (Found: 201.1100. Calc for $C_9H_{15}NO_4$: 201.1002).

Complete ozonolysis of the 1:1 *mixture of* **13** *and* **14**. A soln of the mixture (8.3 mg) of N-(*p*-bromobenzoyl)homo- and bishomopalytoxins in MeOH (30 ml) was cooled to −40°. O_3 was passed through the soln for 2 hr. After removal of excess O_3, $NaBH_4$ (excess) was added slowly. The soln was allowed to stand for 4 hr at room temp, and MeOH was evaporated under reduced pressure to give semi-solid which was dissolved in 2 ml of H_2O. The aq soln was applied to a TSK G3000S column [3 × 2 (i.d.) cm]. The aq eluates were further treated with Dowex 50W × 4. Evaporation of H_2O and then methyl borate gave a residue, which was acetylated with Ac_2O (1 ml) and pyridine (2 ml) overnight. Reagents were removed under reduced pressure to give an oily mixture. Separation was performed by prep TLC (SiO_2, 4:6 EtOAc–$CHCl_3$) to give compounds **15**, **16** and **17**. The 60% aq EtOH eluate was also concentrated under reduced pressure. The resulting residue was immediately acetylated with Ac_2O (1 ml) and pyridine (2 ml). After removal of acetylating reagents, compound **18** was obtained. Finally, the 75% aq EtOH eluate was concentrated to give **19**, which was treated with 50% aq AcOH (3 ml). After standing overnight at room temp, aq AcOH was removed by vacuum evaporator and the resulting residue was acetylated with Ac_2O and pyridine. After evaporation of reagents, polyacetates were purified by prep TLC (SiO_2, 2.5% EtOH–$CHCl_3$); R_f values of each compound, **20**: 0.37, **21**: 0.34, **22**: 0.28 and **23**: 0.42.

$NaIO_4$ *oxidation of* **3**. Neopalytoxin (1.8 mg) was oxidized with $NaIO_4$ (excess) on a TSK G3000S gel column [3 × 2 (i.d.) cm]. The 40% aq EtOH was concentrated to dryness under reduced pressure. The remaining residue was acetylated with Ac_2O (0.5 ml) and pyridine (2 ml) overnight. Evaporation of reagents afforded the crude acetate, which was purified by prep TLC (SiO_2, 2% MeOH–$CHCl_3$), giving **24** (0.3 mg): EIMS, *m/z* 366 (M^+), 337, 307; ^{1}H-NMR (270 MHz, $CDCl_3$) 0.75 (3H, d, J = 7.3 Hz), 1.58 (3H, br s), 1.69 (3H, s), 3.14 (2H, t, J = 6.6 Hz), 3.89 (2H, t, J = 6.6 Hz), 4.21 (1H, d, J = 6.0 Hz), 4.62 (1H, ddd, J = 1.0, 7.6, 7.6 Hz), 4.82 (1H, br s, NH), 5.71 (1H, d, J = 13 Hz), 5.84 (1H, br d, J = 7.6 Hz), 8.06 (1H, d, J = 13 Hz), 8.93 (1H, br s), 9.27 (1H, s); High resolution mass measurement (Found: 366.1821. Calc for $C_{18}H_{26}N_2O_6$: 366.1790).

Complete ozonolysis of N-(p-*bromobenzoyl*)*neopalytoxin*. N-(*p*-Bromobenzoyl)neopalytoxin was prepared by the same procedure from neopalytoxin (2.5 mg). The same operation in the case of complete ozonolysis of **13** and **14** gives **15**, **16**, **17**, **18** and **20**.

$NaIO_4$ *oxidation of* **5**. The $NaIO_4$ oxidation of **5** (0.5 mg) on a TSK G3000S column was done. The 20% aq EtOH eluate was concentrated to give an oily residue, which was acetylated with Ac_2O (1 ml) and pyridine (3 ml) to give **6** (trace).

Complete ozonolysis of N-(p-*bromobenzoyl*)*deoxypalytoxin*. By the same procedure, deoxypalytoxin (3.8 mg) was converted to N-(p-bromobenzoyl)deoxypalytoxin (3.2 mg), which was ozonized at −40° and reduced with $NaBH_4$ (excess). The same separation as described before was performed

with a TSK G3000S column [3 × 2 (i.d.) cm]. The aq eluate gave crude polyols which were acetylated by the same operation to give **15**, **16** and **25** (0.5 mg). The 75% aq fraction was also concentrated under reduced pressure to give a glassy residue, which was treated with 50% aq AcOH and then acetylated with Ac_2O–pyridine to give **18** and **20**.

Compound **25**. EIMS, *m/z* 975 ($M^+ - CH_2OAc$), 961 ($M^+ - CH_2CH_2OAc$), 931, 915, 812 (base peak); ^{1}H-NMR (360 MHz, $CDCl_3$) 4.71 (1H, t, J = 8.0 Hz, H-61), 4.82 (1H, t, J = 10 Hz, H-67), 4.85 (1H, t, J = 10 Hz, H-70), 4.80 (1H, m, H-56), 4.97 (1H, m, H-60), 5.09 (1H, m, H-53), 5.13 (1H, t, J = 10 Hz, H-69), 5.18 (2H, m, H-64 and H-65), 5.42 (1H, dd, J = 2.3, 9.0 Hz, H-57), and the remaining signals are shown in Fig. 5; High resolution mass measurements (Found: 657.2350. Calc for $C_{30}H_{41}O_{16}$ ($M^+ - C_{17}H_{27}O_{10}$) ($M^+ - CH_2OCOCH_3$): 657.2394).

The physical and spectral data of **1**, **6**, **9**, **15**, **16**, **17**, **18**, **19**, **20**, **21**, **22** and **23** will be reported in another full paper.

Acknowledgements—This work was supported by the Naito Foundation. We are also grateful to Prof. Y. Kishi (Harvard University) for the measurements of the 500 MHz ^{1}H-NMR spectrum and desorption/chemical ionization mass spectra.

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