Cantharimide Dimers from the Chinese Blister Beetle, *Mylabris phalerate* PALLAS

Takafumi NAKATANI, Katsuaki JINPO, and Naoki NODA*

Faculty of Pharmaceutical Sciences, Setsunan University; 45–1 Nagaotoge-cho, Hirakata, Osaka 573–0101, Japan.
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Five cantharidin-related compounds were isolated from the Chinese blister beetle, *Mylabris phalerate* PALLAS (Meloidae). Their structures were determined based on spectroscopic and chemical evidence. Three of them were identified as cantharimide dimers, which consist of two units of cantharimide combined with a tri-, tetra-, or penta-methylene group.

Key words Chinese blister beetle; *Mylabris phalerate*; cantharimide dimer; Meloidae; cantharidin

The Chinese blister beetle (*Mylabris phalerate* PALLAS) has been used in traditional Chinese medicine for the treatment of cancer.[1—3] Among the constituents of this insect, cantharidin is a well-known active principle.[4,5] Recently, Chinese researchers have reported the isolation of two fatty acid amides from this drug.[6] Our previous study revealed that this beetle contains so-called cantharimides, in which the anhydride oxygen atoms in cantharidin are replaced by the basic amino acid moieties.[7] We have continued to study the constituents of this insect and have isolated five cantharidin-related compounds. Three of them are cantharimide dimers, which consist of two units of cantharimide combined with a tri-, tetra-, and penta-methylene group. This paper deals with the isolation and structural elucidation of these compounds.

The H_2O-eluted fraction (fr. III) described in the previous paper[7] was chromatographed over silica gel to give fractions 1—3. Each of fr. 1—3 was further purified by HPLC to give compounds **1** and **2**. The lower layer obtained by the treatment of the MeOH extract with $CHCl_3$–MeOH–H_2O was separated by the method described in the experimental section to give three compounds **3**, **4**, and **5** together with remarkable amounts of cantharidin.

The molecular formula, $C_{10}H_{12}NO_3$, of compound **1** was established by high-resolution (HR) FAB-MS (*m*/*z* 194.0813 $[M-H]^-$). The ^{1}H-NMR spectrum of **1** exhibited characteristic signals due to a cantharidin skeleton, *i.e.*, two signals each of methyl (6H, δ_H 1.14), methylene (2H, δ_H 1.63; 2H, δ_H 1.87), and methine protons (2H, δ_H 4.48). The ^{13}C-NMR spectrum showed, in contrast to that of cantharidin, a remarkable downfield shift (7.0 ppm) of C-7 and C-8, while the other signals remain almost unshifted. These findings indicate that **1** is a cantharimide, in which the anhydride O atom in cantharidin is replaced by NH.[8] Compound **1** has been synthesized,[9] although no comparable spectroscopic data have been reported.

Treatment of cantharidin with 28% ammonium hydroxide at 180 °C gave a product of which the spectroscopic data were identical with those of **1**; the above suggestion was thus confirmed, and the structure of compound **1** was characterized as (1*S*,2*R*,3*S*,6*R*)-1,2-dimethyl-3,6-epoxycyclohexane-1,2-dicarboximide (Fig. 1).

The HR-FAB-MS of **2** exhibited an $[M-H]^-$ ion peak at *m*/*z* 210.0764, consistent with the molecular formula $C_{10}H_{12}NO_4$. The ^{1}H-NMR spectrum of **2**, when compared with that of **1**, showed the absence of one methyl group and the appearance of unequivalent methylene signals (δ_H 3.66, 3.93, each 1H, d, J=11.0 Hz). In the ^{13}C-NMR spectrum, signals of methine (C-3, C-6), methylene (C-4, C-5), and carbonyl (C-7, C-8) were observed separately. From these findings, compound **2** was considered to be an asymmetric cantharimide, and one of the two tertiary methyl groups in **1** is replaced by a hydroxymethyl group. The heteronuclear multiple-bond correlation (HMBC) spectrum showed diagnostic correlations between H_2-1/C-2, C-6, and C-7, and H_3-2/C-1, C-3, and C-8. Compound **2** showed specific rotation, $[\alpha]_D$ $<\pm 1°$ (c=0.3, MeOH) and no CD maximum, indicating that it is a racemate. The ^{1}H-NMR spectrum of its (−)-MTPA derivative exhibited two sets of signals due to optical isomers in a ratio of *ca.* 1 : 1. These observations revealed that **2** was a racemate, (1*R**,2*R**,3*S**,6*R**)-1-hydroxymethyl-2-methyl-3,6-epoxycyclohexane-1,2-dicarboximide (Fig. 1).

The positive ion FAB-MS of **3** exhibited an $[M+H]^+$ ion peak at *m*/*z* 431, and its mass was approximately two-fold greater than those of compounds **1** and **2**. The ^{13}C-NMR spectrum showed two methylene signals (δ_C 25.3, 36.1), together with the five typical signals due to a cantharimide unit (δ_C 12.6, 23.6, 53.8, 83.3, 181.1). In the ^{1}H-NMR spectrum, two new methylene signals were observed at δ_H 1.91 (quintet, H_2-2′) and δ_H 3.48 (t, H_2-1′, 3′) in a ratio of 1 : 2. Based on the above information combined with its molecular weight, compound **3** was considered to be a dimer, which consists of two units of a cantharimide moiety combined with a trimethylene group. Diagnostically important fragment ion peaks at *m*/*z* 194 and 236 in its FAB-MS support the above suggestion (Fig. 2).

Confirmation of the structure was achieved by the synthesis of compound **3**. Treatment of cantharidin with 1,3-diaminopropane at 180 °C for 5 h gave a product that was shown to be identical to **3** by the ^{13}C- and ^{1}H-NMR spectro-

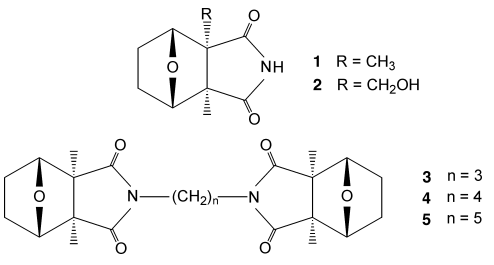

Fig. 1. Structures of Compounds **1**—**5**

* To whom correspondence should be addressed. e-mail: noda@pharm.setsunan.ac.jp

scopic data (Chart 1). Based on the results obtained above, the structure of compound **3** was characterized as bis[(1*S*,2*R*,3*S*,6*R*)-1,2-dimethyl-3,6-epoxycyclohexane-1,2-dicarboximido]-trimethylene (Fig. 1). Compound **3** has been synthesized by Chinese researchers,[10] but its isolation from natural sources has not previously been reported.

The positive-ion FAB-MS of compound **4** gave an $[M+H]^+$ ion peak at *m/z* 445 which was 14 mass units more than that of **3**. The ^{13}C-NMR spectrum exhibited the same seven signals as those of **3**, but in its ^{1}H-NMR spectrum, unlike **3**, two methylene signals appeared at δ_H 1.55 (H_2-2′, 3′) and 3.48 (H_2-1′, 4′) in a ratio of 1 : 1. These findings, together with the significant fragment ion peaks at *m/z* 194 and *m/z* 250 in its FAB-MS (Fig. 2), suggest that **4** is an analogue of **3** with a different alkyl chain; the trimethylene group in **3** is replaced by a tetramethylene group in **4**. Treatment of cantharidin with 1,4-diaminobutane under the same conditions as described for **3** gave a product of which the ^{1}H- and ^{13}C-NMR spectra were good accordance with those of **4** (Chart 1). Therefore **4** has the structure shown in Fig. 1.

Compound **5** exhibited a $[M+H]^+$ ion peak at *m/z* 457 which was 28 mass units more than that of **3**. By analyses of the NMR spectroscopic data, **5** was found to be another cantharimide dimer that differed from **3** in the linkage alkyl chain. The trimethylene group in **3** was replaced by a pentamethylene residue. The structure was confirmed by condensation of cantharidin with 1,5-diaminopentane, which yielded **5** (Chart 1). Thus the structure of **5** was deduced to be bis[(1*S*,2*R*,3*S*,6*R*)-1,2-dimethyl-3,6-epoxycyclohexane-1,2-dicarboximido]-pentamethylene (Fig. 1).

In conclusion, three cantharimide dimers together with two cantharimides were isolated. All of them are the first examples of naturally occurring cantharimides isolated from an insect. In 1967, during the isolation of cantharidin from another blister beetle, *Epicauta pestifera*, Walter and Cole reported that cantharidin exists partly free and partly combined in the insect.[11] However, the structures of the latter are as yet unclear. It seems likely that the cantharimides isolated in this study correspond to the above complex. The biological activities of these compounds will be examined in future investigations.

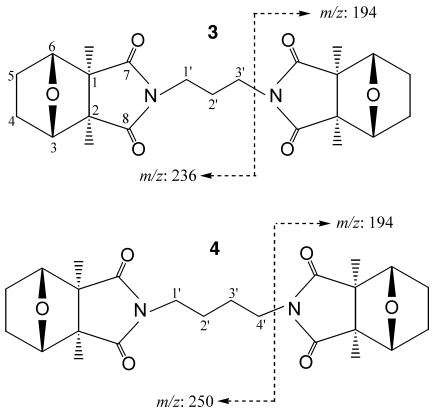

Fig. 2. Fragmentation Pattern of Compounds **3** and **4**

Experimental

^{1}H- and ^{13}C-NMR spectra were recorded on a JEOL ECA 600SN and a JEOL JNM GX400 spectrometer, respectively, using tetramethylsilane (TMS) as an internal reference. FAB-MS, including high-resolution MS, were recorded on a JEOL JMS-700T spectrometer (accelerating voltage, 5 kV; matrix, glycerin; collision gas, Xe). Column chromatography was carried out on Diaion HP-20 (Mitsubishi Chemical Co.), Sephadex LH-20 (Amersham Pharmacia Biotech AB), Merck silica gel (230—400 mesh, art. 9385), and Bio-beads S-X2 (Bio-Rad) columns. Preparative HPLC was conducted on Mightysil RP-18 GP Aqua (5 μm, 20×250 mm, Kanto Chemical Co., Inc.), and Inertsil ODS-3 Jet (5 μm, 10×50 mm, GL Sciences) on a JASCO 880-PU equipped with a JASCO 830-RI unit.

Material The commercial crude drug "Mylabris," *M. phalerata* PALLAS was purchased from Matsuura Yakugyo Co. (lot no. HS000126C). A voucher specimen was deposited in the Faculty of Pharmaceutical Sciences, Setsunan University.

Isolation of Compounds 1—5 Whole bodies of Mylabris (3.4 kg) were extracted with MeOH (4 l) at room temperature. The solvent was removed *in vacuo* to give an extract (450.0 g). This was shaken with $CHCl_3$–MeOH–H_2O (1 : 1 : 1) to give an upper and a lower layer. The upper layer was concentrated to give an extract, fr. A (200 g). It was shaken with *n*-BuOH–H_2O to give an *n*-BuOH and an H_2O layer. The *n*-BuOH and H_2O layers were concentrated to give fr. I (30.8 g) and fr. II (175.0 g), respectively. Fr. II was subjected to Diaion HP-20 column chromatography using H_2O and MeOH successively to give fr. III (155.9 g) and fr. IV (16.6 g). Part (45 g) of fr. III was placed on a silica gel column and eluted successively with $CHCl_3$–MeOH (10 : 1→8 : 2)→$CHCl_3$–MeOH–H_2O (7 : 3 : 0.5→6 : 4 : 1) to give five fractions, fr. 1—5. Among them, the selected fr. 3 (3.6 g) was separated by HPLC with a Mightysil RP-18 GP Aqua using MeOH–H_2O (2 : 8) as an eluent to give compounds **1** (2.6 mg) and **2** (3.2 mg). The lower layer was concentrated to give fr. B (250 g). Part (101 g) of fr. B was subjected to silica gel column chromatography using *n*-hexane–AcOEt (2 : 1) to give cantharidin (394 mg) (positive to 0.1% bromocresol green in isopropanol with NaOH until appearance of blue color.[11]) Part (28 g) of fr. B was partitioned between NaOH 1 M and Et_2O. The ether-soluble fraction was concentrated *in vacuo* to give an oily fraction (7.0 g). This was applied to a Bio-beads S-X2 column using AcOEt as an eluent to give fr. 6—8 together with cantharidin (8.4 mg). Fr. 7 was further separated by HPLC with an Inertsil ODS-3 Jet column (×2) using MeOH–H_2O (1 : 1) as a mobile phase to give compounds **3** (14.0 mg), **4** (16.6 mg), and **5** (6.4 mg).

(1*S*,2*R*,3*S*,6*R*)-1,2-Dimethyl-3,6-epoxycyclohexane-1,2-dicarboximide (**1**): Colorless needles from EtOH, mp 205—206 °C. HR-FAB-MS *m/z*: 194.0813 $[M-H]^-$ (Calcd for $C_{10}H_{12}NO_3$: 194.0817). ^{1}H-NMR (CD_3OD, 600 MHz) δ: see Table 1. ^{13}C-NMR (CD_3OD, 150 MHz) δ: see Table 2.

(1*R**,2*R**,3*S**,6*R**)-1-Hydroxymethyl-2-methyl-3,6-epoxycyclohexane-1,2-dicarboximide (**2**): White powder, mp 195—197 °C. FAB-MS *m/z*: 210.0764 $[M-H]^-$ (Calcd for $C_{10}H_{12}NO_4$: 210.0766). ^{1}H-NMR (CD_3OD, 600 MHz) δ: see Table 1. ^{13}C-NMR (CD_3OD, 150 MHz) δ: see Table 2.

Bis[(1*S*,2*R*,3*S*,6*R*)-1,2-dimethyl-3,6-epoxycyclohexane-1,2-dicarboximido]-trimethylene (**3**): White powder, mp 156—157 °C. HR-FAB-MS *m/z*: 431.2190 $[M+H]^+$ (Calcd for $C_{23}H_{31}N_2O_6$: 431.2182). ^{1}H-NMR ($CDCl_3$, 600 MHz) δ: see Table 1. ^{13}C-NMR ($CDCl_3$, 150 MHz) δ: see Table 2.

Bis[(1*S*,2*R*,3*S*,6*R*)-1,2-dimethyl-3,6-epoxycyclohexane-1,2-dicarboximido]-tetramethylene (**4**): White powder, mp 246—247 °C. HR-FAB-MS *m/z*: 445.2343 $[M+H]^+$ (Calcd for $C_{24}H_{33}N_2O_6$: 445.239). ^{1}H-NMR ($CDCl_3$, 600 MHz) δ: see Table 1. ^{13}C-NMR ($CDCl_3$, 150 MHz) δ: see Table 2.

Bis[(1*S*,2*R*,3*S*,6*R*)-1,2-dimethyl-3,6-epoxycyclohexane-1,2-dicarbox-

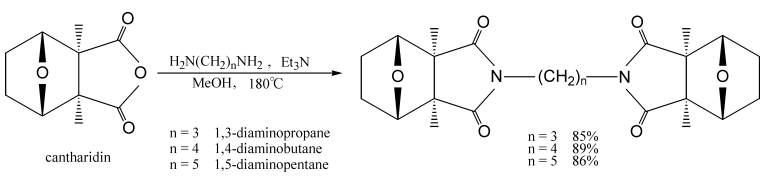

Chart 1

Table 1. ^{1}H-NMR Data[a,b] for **1**—**5**

Position	**1**	**2**	**3**	**4**	**5**
3	4.48 (2H, dd, 2.1, 1.2)	4.47 (1H, d, 4.0)	4.57 (2H, dd, 2.2, 0.8)	4.54 (2H, dd, 2.4, 0.6)	4.55 (2H, m)
4	1.63 (1H, m), 1.87 (1H, m)	1.65 (2H, m)	1.68 (2H, m), 1.78 (2H, m)	1.68 (2H, m), 1.79 (2H, m)	1.68 (2H, m), 1.79 (2H, m)
5	1.63 (1H, m), 1.87 (1H, m)	1.65 (1H, m), 1.93 (1H, m)	1.68 (2H, m), 1.78 (2H, m)	1.68 (2H, m), 1.79 (2H, m)	1.68 (2H, m), 1.79 (2H, m)
6	4.48 (1H, dd, 2.1, 1.2)	4.50 (1H, d, 3.2)	4.57 (2H, dd, 2.2, 0.8)	4.54 (2H, dd, 2.4, 0.6)	4.55 (2H, m)
1-CH_3	1.14 (3H, s)		1.15 (6H, s)	1.13 (6H, s)	1.13 (6H, s)
2-CH_3	1.14 (3H, s)	1.30 (3H, s)	1.15 (6H, s)	1.13 (6H, s)	1.13 (6H, s)
1-C$\underline{H_2}$OH		3.66, 3.93 (each 1H, d, 11.0)			
1′			3.48 (2H, t, 7.2)	3.51 (2H, m)	3.47 (2H, t, 7.2)
2′			1.91 (2H, quint., 7.2)	1.55 (2H, m)	1.58 (2H, quint., 7.2)
3′			3.48 (2H, t, 7.2)	1.55 (2H, m)	1.26 (2H, m)
4′				3.51 (2H, m)	1.58 (2H, quint., 7.2)
5′					3.47 (2H, t, 7.2)

a) **1** and **2** were dissolved in CD_3OD and **3**—**5** were dissolved in $CDCl_3$. *b*) Chemical shift values are given in ppm, and *J* values in parentheses are given in Hz.

Table 2. ^{13}C-NMR Data[a] for **1**—**5**

Position	**1**	**2**	**3**	**4**	**5**
1	56.5	63.0	53.8	53.8	53.8
2	56.5	55.9	53.8	53.8	53.8
3	85.1	85.3	83.3	83.5	83.6
4	24.5	24.6[b]	23.6	23.7	23.7
5	24.5	24.9[b]	23.6	23.7	23.7
6	85.1	83.1	83.3	83.5	83.6
7	184.9	182.8	181.0	181.4	181.4
8	184.9	184.9	181.0	181.4	181.4
1-CH_3	12.6		12.6	12.6	12.7
2-CH_3	12.6	12.3	12.6	12.6	12.7
1-CH_2OH		59.2			
1′			36.1	38.3	38.9
2′			25.3	24.4	27.1
3′			36.1	24.4	23.7
4′				38.3	27.1
5′					38.9

a) **1** and **2** were dissolved in CD_3OD and **3**—**5** were dissolved in $CDCl_3$. *b*) Interchangeable within a column.

imido]-pentamethylene (**5**): White powder, mp 148—149 °C. HR-FAB-MS *m/z*: 459.2487 $[M+H]^+$ (Calcd for $C_{25}H_{35}N_2O_6$: 459.2495). ^{1}H-NMR ($CDCl_3$, 600 MHz) δ: see Table 1. ^{13}C-NMR ($CDCl_3$, 150 MHz) δ: see Table 2.

Preparation of 1 Cantharidin (50 mg) was dissolved in 28% ammonium hydroxide (2 ml) and heated at 180 °C for 2 h. After removal of the solvent, the reaction mixture was recrystallized from EtOH to give **1** (45.7 mg, 91%).

Preparation of 3 A mixture of cantharidin (37.6 mg), 1,3-diaminopropane (7.1 mg), and triethylamine (0.027 ml) was dissolved in MeOH (3 ml) and heated at 180 °C for 24 h in a sealed tube. After being cooled to room temperature, the reaction mixture was concentrated, and the residue was column chromatographed on silica gel to give a product (35 mg, 85%), which was identical to compound **3**.

Preparation of 4 A mixture of cantharidin (19.6 mg), 1,4-diaminobutane (4.4 mg), and triethylamine (0.014 ml) was dissolved in MeOH (3 ml) and heated at 180 °C for 24 h and purified in the same manner as describe for **3** to give **4** (19.7 mg, 89%).

Preparation of 5 A mixture of cantharidin (38.9 mg), 1,5-diaminopentane (10 mg), and triethylamine (0.027 ml) was dissolved in MeOH (3 ml) and heated at 180 °C for 24 h and purified in the same manner as describe for **3** to give **5** (39.2 mg, 86%).

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