Three Novel Cantharidin-Related Compounds from the Chinese Blister Beetle, *Mylabris phalerata* PALL.

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Three novel cantharidin analogues were isolated from the Chinese blister beetle, *Mylabris phalerata* PALL. (Meloidae), which has been used in traditional Chinese medicine for the treatment of cancer. Their structures were determined on the basis of heteronuclear multiple-bond connectivity and nuclear Overhauser effect spectroscopy experiments, and chemical data confirmed them to be so-called cantharimides, in which the anhydride oxygen atoms are replaced by the basic amino acid L-lysine, L-ornithine, and L-arginine moieties.

Key words Chinese blister beetle; *Mylabris phalerata*; cantharimide; Meloidae; cantharidin

The dried body of the Chinese blister beetle is sold in China as Mylabris, a concoction has been used in traditional Chinese medicine for the treatment of cancer.[1—3] The species used in medicine are *Mylabris phalerata* PALL. and *M. cichorii* LINN. (Meloidae). Among the chemical constituents in these species, cantharidin[4,5] is a well-known active principle, but no other compounds have been isolated. In our search for biologically active compounds from animal crude drugs originating from invertebrates,[6,7] we examined the constituents of the Chinese blister beetle, *Mylabris phalerata* PALL., and isolated three compounds (**1**—**3**). Their chemical and spectral data revealed that they are so-called cantharimides, in which the anhydride oxygen atoms are replaced by the basic amino acid moieties. This paper deals with the isolation and structural elucidation of these compounds.

Whole bodies of *M. phalerata* PALL. (3.4 kg) were extracted with MeOH, and the methanolic extract was shaken with $CHCl_3$–MeOH–H_2O (1 : 1 : 1) to give an upper and a lower layer. The former was subjected to a combination of Sephadex LH-20, Diaion HP-20, and normal or reverse-phase column chromatography with various solvent systems to give compounds **1** (64.0 mg), **2** (9.1 mg), and **3** (57.2 mg).

The high-resolution negative electrospray ionization (HR-ESI) MS of **1** exhibited a $(M-H)^-$ ion peak at *m*/*z* 323.1607, consistent with the molecular formula $C_{16}H_{24}N_2O_5$. The ^{1}H-NMR spectrum of **1** showed six methylene (δ 1.33, 1.63, 1.64, 1.91, 2.15, 2.86) and three methine signals (δ 4.42, 4.46, 4.46), together with two tertiary methyl signals (δ 1.17, 1.21). The ^{13}C-NMR spectrum exhibited 16 signals, including those of two quaternary (δ 55.0, 55.3) and three carbonyl carbon signals (δ 175.4, 183.3, 183.8). All proton and carbon signals were assigned on the basis of 2D-NMR spectra [^{1}H–^{1}H and ^{1}H–^{13}C shift correlation spectroscopy (COSY) and heteronuclear multiple-bond connectivity (HMBC)]. The COSY spectrum of **1** showed a series of correlation peaks from H-2 (δ 4.42) to H-6 (δ 2.86). In the HMBC spectrum, the 8′-CH_3 proton signal gave a long-range correlation with three carbon signals, C-1′ (δ 183.3), C-3′a (δ 55.0) and C-7′ (δ 85.4). Likewise, significant correlation peaks between the 9′-CH_3 proton signal and three carbon signals, C-3′ (δ 183.8), C-7′a (δ 55.3), and C-4′ (δ 85.3) were observed. Nuclear Overhauser effect spectroscopy (NOESY) gave NOE correlations between 8′-CH_3 and H-7′ (δ 4.46) and Hα-6′ (δ 1.91), and between 9′-CH_3 and H-4′ (δ 4.46) and Hα-5′ (δ 1.91). These observations revealed that compound **1** consists of two parts, a cantharidin framework and an amino acid lysine residue. Moreover, in its HMBC spectrum, H-2 of the lysine moiety (δ 4.42) showed diagnostically important correlations with C-1′ (δ 183.3) and C-3′ (δ 183.8) of the cantharidin framework (Fig. 1). These findings indicate that **1** is a so-called cantharimide, in which the anhydride oxygen atom in cantharidin is replaced by the nitrogen atom of the α-amino group in the lysine moiety. Confirmation of the absolute configuration was achieved after the synthesis of compound **1** as follows (Chart 1).

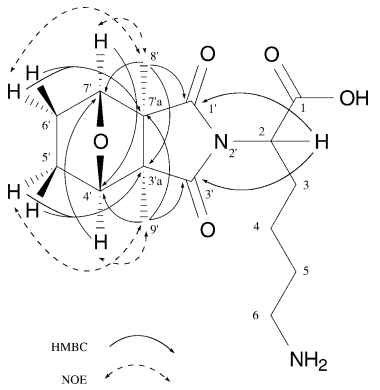

Fig. 1. NOE and HMBC Correlations of **1**

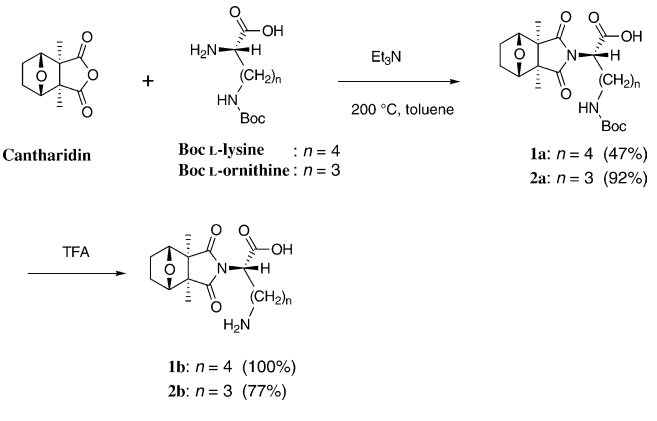

Chart 1

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Treatment of cantharidin with N^{ε}-(*tert*-butoxycarbonyl)-L-lysine in the presence of triethylamine in toluene at 200 °C for 48 h gave **1a**. Deprotection of **1a** with trifluoroacetic acid gave **1b**. The ^{1}H- and ^{13}C-NMR spectra of **1b** were in good accord with those of **1**, and its optical rotation showed the same sign as that of **1**. On the basis of all the above chemical and spectral data, the structure of **1** was determined to be (2*S*)-6-amino-2-[(3a*R**,4*S**,7*R**,7a*S**)-3a,7a-dimethyl-1,3-dioxo-4,7-epoxyoctahydroisoindol-2-yl]-hexanoic acid (Fig. 2).

Compound **2** provided a $(M-H)^{-}$ ion peak at *m*/*z* 309.1450 in the negative HR-ESI-MS, corresponding to the molecular formula $C_{15}H_{22}N_2O_5$, which was one methylene group less than that of **1**. The ^{1}H- and ^{13}C-NMR spectra of compound **2** were closely related to those of **1**, except for the absence of the methylene proton (δ 1.33) and the carbon (δ 24.4) signals observed in **1**. These findings suggest that **2** is an analogue of **1** in that the lysine moiety of **1** is replaced by the ornithine residue. This was confirmed by its HMBC and NOESY spectral data.

Condensation of cantharidin and N^{δ}-(*tert*-butoxycarbonyl)-L-ornithine, followed by deprotection in the same manner as described for **1**, gave product **2b**. The ^{1}H- and ^{13}C-NMR spectra of **2b** were almost identical to those of **2**, and its optical rotation showed the same sign as that of **2**. Accordingly, the configuration at C-2 of the amino acid residue was concluded to be an L-form, and thus compound **2** has the structure depicted in Fig. 2.

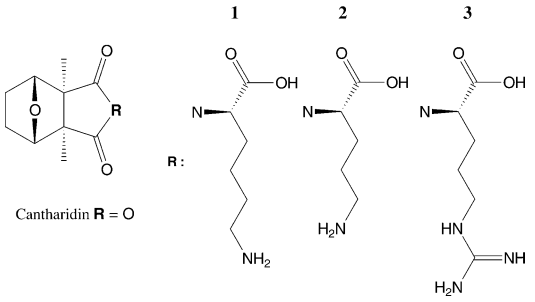

Fig. 2. Structures of **1**, **2**, and **3**

Compound **3** showed a $(M-H)^{-}$ ion peak at *m*/*z* 351.1666 in its negative HR-ESI-MS, consistent with the molecular formula $C_{16}H_{24}N_4O_5$. This was confirmed by the ^{13}C-NMR spectrum presenting signals for all 16 carbons of the molecular formula including three carbonyl carbons (δ 177.3, 186.3, 186.7), together with an additional nitrogen-substituted carbon (δ 159.2). In the HMBC spectrum of **3**, similar to those of **1** and **2**, significant correlations, including cross peaks between H-2 methine of the amino acid group and C-1′ and C-3′ of the cantharidin skeleton were observed. From these findings, compound **3** is considered to be an analogue of **1** and **2** differing only in the amino acid residue, that is, the lysine unit in **1** is replaced by the arginine moiety.

To determine the absolute stereochemistry, we attempted to prepare compound **3** under the same conditions as described for **1** and **2**. However, no product could be obtained due to the poor solubility of N^{ω}-(*tert*-butoxycarbonyl)-L-arginine in toluene. The configuration at C-2 of the arginine moiety remained unclear, but this is also considered to have an L-form because it has optical rotation similar ($[\alpha]_D$ −21.1°) to those of **1** ($[\alpha]_D$ −23.3°) and **2** ($[\alpha]_D$ −26.9°).

Compounds **1**—**3** isolated in this study could be artifacts formed by condensation between cantharidin and the basic amino acids L-lysine, L-ornithine, and L-arginine. This possibility was excluded, however, since these compounds were detected by TLC of the MeOH extract.

Recently, many 8,9-dinorcantharidin analogues with various amino acid moieties, called norcantharimids, have been synthesized and tested for inhibitory activity against protein phosphatases 1 and 2A by McCluskey *et al.*[8] They reported that these compounds with a basic amino acid D- or L-histidine residue are more potent inhibitors, whereas those with a neutral or an acidic amino acid residue have poor activity. This information leads us to expect that the three cantharimides obtained in this study have strong inhibitory activity against protein phosphatases 1 and 2A, because they consist of basic amino acid moieties. The biological activities of these compounds will be examined in future investigations.

Table. ^{1}H- and ^{13}C-NMR Spectral Data of **1**—**3**

Position	**1**		**2**		**3** (D_2O)	
	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
Cantharidin-1′	183.3		183.1		186.3	
3′	183.8		183.7		186.7	
4′	85.3	4.46 (dd, 5.4, 6.0)	85.5	4.47 (dd, 4.2, 4.2)	86.7	4.63 (dd, 6.0, 6.0)
5′	24.6	1.91 (m), 1.63 (m)	24.8	1.92 (m), 1.63 (m)	25.9	1.97 (m), 1.63 (m)
6′	24.8	1.91 (m), 1.63 (m)	25.1	1.92 (m), 1.63 (m)	26.2	1.97 (m), 1.63 (m)
7′	85.4	4.46 (dd, 5.4, 6.0)	85.6	4.47 (dd, 4.2, 4.2)	86.8	4.63 (dd, 6.0, 6.0)
3′a	55.0		55.3		56.7	
7′a	55.3		55.6		56.9	
8′	12.4	1.17 (s)	12.6	1.17 (s)	14.1	1.20 (s)
9′	12.6	1.21 (s)	12.9	1.21 (s)	14.3	1.24 (s)
1	175.4		174.9		177.3	
2	56.5	4.42 (dd, 4.8, 10.8)	56.6	4.42 (dd, 7.8, 7.8)	57.8	4.50 (dd, 8.4, 8.4)
3	29.1	2.15 (2H, m)	27.3	2.16 (2H, m)	27.8	2.08 (2H, m)
4	24.4	1.33 (2H, m)	26.2	1.62 (2H, m)	28.4	1.48 (2H, m)
5	27.9	1.64 (2H, m)	40.4	2.90 (2H, m)	43.2	3.21 (m), 3.18 (m)
6	40.6	2.86 (2H, m)				
C=NH					159.2	

δ (ppm) from TMS as an internal standard in CD_3OD or D_2O [coupling constants (Hz) in parentheses].

Experimental

Melting points (mp) were determined on Yanaco MP-S3 apparatus and are uncorrected. Optical rotations were measured at 25 °C with a JASCO DIP-140 polarimeter. ^{1}H- and ^{13}C-NMR spectra were recorded on a JEOL JNM GX400 and a GE Omega 600 spectrometers, respectively, using tetramethylsilane (TMS) as an internal reference. ESI-MS, including high-resolution MS, were recorded on a JEOL JMS-700T spectrometer. TLC was carried out on silica gel precoated Al sheets (Merck art. 5554). Spots were visualized with 5% H_2SO_4 in MeOH or 0.3% ninhydrin in BuOH (by heating). 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 Cosmosil 75C_{18}-OPN (Nacalai Tesque, Inc.) colums. Preparative HPLC was conducted on Mightysil RP-18 GP Aqua (5 μm, 20×250 mm, Kanto Chemical Co., Inc.) on a JASCO 880-PU equipped with a JASCO 830-RI.

Material The commercial crude drug "Mylabris," *M. phalerata* Pall. 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—3 Whole bodies (3.4 kg) of Mylabris were extracted with MeOH (4 l) at room temperature. The solvent was removed *in vacuo* to give a extract (450.0 g). This was shaken with $CHCl_3$–MeOH–H_2O (1 : 1 : 1) to give an upper and a lower layer. After removal of the solvent, the upper layer was shaken with *n*-BuOH–H_2O to give an *n*-BuOH and a 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. Fraction II was subjected to Diaion HP20 column chromatography using H_2O and MeOH successively to give fr. III (155.9 g) and fr. IV (16.6 g). Fraction IV was chromatographed on a Sephadex LH-20 column (MeOH) to give fr. 1 (5.5 g), fr. 2 (5.4 g), and fr. 3 (2.3 g). Part of fr. 2 (2.7 g) was successively chromatographed on silica gel ($CHCl_3$–MeOH–H_2O, 8 : 2 : 0.1→7 : 3 : 0.5→6 : 4 : 1→MeOH) to yield four fractions, fr. 4 (543 mg), fr. 5 (819 mg), fr. 6 (651 mg), and fr. 7 (276 mg). Fraction 6 was chromatographed on silica gel ($CHCl_3$–MeOH–H_2O, 6 : 4 : 1), to give fr. 8 (90 mg), fr. 9 (158 mg), fr. 10 (373 mg), and fr. 11 (25 mg). Fraction 10 was subjected to preparative HPLC with Mightysil RP-18 GP Aqua (size, 20×250 mm; 5 μm) using 10% CH_3CN to give **1** (64.0 mg), **2** (9.1 mg), **3** (24.6 mg), and fr. 12 (250 mg). Fraction 9 was subjected to preparative HPLC with Mightysil RP-18 GP Aqua (size, 2×250 mm; 5 μm) using 10% CH_3CN to give **3** (32.6 mg).

(2*S*)-6-Amino-2-[(3a*R**,4*S**,7*R**,7a*S**)-3a,7a-dimethyl-1,3-dioxo-4,7-epoxyoctahydroisoindol-2-yl]-hexanoic Acid (**1**): Powder, mp 159.0—162.0 °C. $[\alpha]_D$ −23.3° (*c*=0.30, MeOH). HR-ESI-MS *m/z*: 323.1607 (Calcd for $C_{16}H_{23}N_2O_5$: 323.1607) $[M-H]^-$. IR (KBr) cm^{-1}: 2980, 1697, 1644, 1588, 1404, 1358, 1236, 993, 817, 675. ^{1}H- and ^{13}C-NMR δ: see Table 1.

(2*S*)-5-Amino-2-[(3a*R**,4*S**,7*R**,7a*S**)-3a,7a-dimethyl-1,3-dioxo-4,7-epoxyoctahydroisoindol-2-yl]-pentanoic Acid (**2**): Powder, mp 157.0—160.0 °C. $[\alpha]_D$ −26.9° (*c*=0.26, MeOH–H_2O, 1 : 1). HR-ESI-MS *m/z*: 309.1450 (Calcd for $C_{15}H_{21}N_2O_5$: 309.1451) $[M-H]^-$. IR (KBr) cm^{-1}: 2979, 1701, 1583, 1404, 1235, 990, 903, 812. ^{1}H- and ^{13}C-NMR δ: see Table 1.

(2*S*)-2-[(3a*R**,4*S**,7*R**,7a*S**)-3a,7a-Dimethyl-1,3-dioxo-4,7-epoxy-octahydroisoindol-2-yl]-5-guanidino Pentanoic Acid (**3**): Powder, mp 195.0—197.0 °C. $[\alpha]_D$ −21.1° (*c*=2.2, MeOH–H_2O, 1 : 1). HR-ESI-MS *m/z*: 351.1666 (Calcd for $C_{16}H_{23}N_4O_5$: 351.1667) $[M-H]^-$. IR (KBr) cm^{-1}: 3373, 2978, 1767, 1693, 1628, 1461, 1402, 1359. ^{1}H- and ^{13}C-NMR δ: see Table 1.

Preparation of 1a A mixture of cantharidin (Sigma, 20 mg, 0.102 mmol), N^{ε}-Boc-L-lysine (Aldrich, 25 mg, 0.102 mmol), and triethylamine (0.02 ml) was dissolved in toluene (5 ml) and heated at 200 °C for 48 h in a sealed tube. After being cooled to room temperature, the reaction mixture was diluted with EtOAc (50 ml) and extracted with 10% $NaHCO_3$ (3×50 ml). The aqueous layer was acidified with HCl and extracted with EtOAc (3×50 ml). The organic layer was washed with H_2O and brine, dried over $MgSO_4$, and then concentrated *in vacuo* to give a product (21 mg, pale yellow oil). This product was subjected to silica gel column chromatography ($CHCl_3$–MeOH, 4 : 1) to give **1a** (20.1 mg, 47%) as a colorless solid. $[\alpha]_D$ −1.5° (*c*=1.0, MeOH). IR (KBr) cm^{-1}: 1695, 1598, 1549, 1384. ^{1}H-NMR (400 MHz, CD_3OD) δ: 1.16 (3H, s, H_3-8′), 1.19 (3H, s, H_3-9′), 1.19—1.29 (2H, m, H_2-4), 1.35—1.55 (2H, m, H_2-5), 1.42 (9H, s, Boc C$\underline{H}_3$×3), 1.67 (2H, m, H_β-5′, 6′), 1.91 (2H, m, H_α-5′, 6′), 2.13 (2H, m, H_2-3), 2.99 (2H, m, H_2-6), 4.48 (1H, m, H-2), 4.50 (2H, br s, H-4′, 7′). ^{13}C-NMR (100 MHz, CD_3OD) δ: 12.6 (C-8′), 12.8 (C-9′), 24.6 (C-5′), 24.6 (C-4), 24.8 (C-6′), 28.8×3 (Boc $\underline{C}H_3$), 29.0 (C-5), 30.2 (C-3), 41.1 (C-6), 55.0 (C-3′a), 55.1 (C-7′a), 56.5 (C-2), 79.7 (Boc $\underline{C}$-$(CH_3)_3$), 85.1×2 (C-4′, 7′), 158.2 (Boc $\underline{C}$O), 176.5 (C-1), 183.1 (C-1′), 183.2 (C-3′).

Deprotection of 1a A solution of **1a** (20.1 mg, 0.047 mmol) in trifluoroacetic acid (3 ml) was stirred at room temperature for 30 min and then evaporated *in vacuo*. The residue was dissolved in water, and the solution was neutralized with 1 M NaOH. After removal of the solvent, the reaction mixture was subjected to Diaion HP-20 column chromatography using H_2O and MeOH successively to give an MeOH eluate. The MeOH eluate was concentrated *in vacuo* to yield a pale yellow oil (18.1 mg), and this was subjected to silica gel column chromatography ($CHCl_3$–MeOH–H_2O, 6 : 4 : 1) to give **1b** (16 mg, 100%) as a white powder. $[\alpha]_D$ −5.4° (*c*=0.50, MeOH).

Preparation of N^{δ}-Boc-L-ornitine A solution of N^{δ}-Boc-N^{α}-CBZ-L-ornithine (Kokusan Chemical Works, 500 mg, 1.37 mmol) was shaken with 10% palladium carbon (50 mg) in EtOH (10 ml) for 48 h. The catalyst was removed by filtration, and the filtrate was concentrated *in vacuo* to give a product. This product was subjected to silica gel chromatography ($CHCl_3$–MeOH–H_2O, 7 : 3 : 0.5) to give N^{δ}-Boc-L-ornithine (274.7 mg, 86.4%) as a white powder. ^{1}H-NMR (400 MHz, CD_3OD) δ: 1.45 (9H, s, Boc C$\underline{H}_3$×3), 1.58 (2H, m, H_2-4), 1.80 (1H, m, H_a-3), 1.89 (1H, m, H_b-3), 3.11 (2H, m, H_2-5), 3.52 (1H, dd, *J*=5.2, 7.2 Hz, H-2). ^{13}C-NMR (100 MHz, CD_3OD) δ: 26.8 (C-4), 28.7×3 (Boc $\underline{C}H_3$), 29.6 (C-3), 40.7 (C-5), 55.7 (C-2), 79.8 (Boc $\underline{C}$-$(CH_3)_3$), 158.3 (Boc $\underline{C}$O), 174.1 (C-1).

Preparation of 2a A mixture of cantharidin (60 mg, 0.31 mmol), N^{δ}-Boc-L-ornithine (80 mg, 0.34 mmol), and triethylamine (0.06 ml) was dissolved in toluene (5 ml) and treated in the same manner as described for the preparation of **1a** to give **2a** (116.8 mg, 92%) as a white powder. $[\alpha]_D$ −2.6° (*c*=2.0, MeOH). IR (KBr) cm^{-1}: 3386, 2978, 1699, 1404, 1169, 996. ^{1}H-NMR (400 MHz, $CDCl_3$+CD_3OD, 1 : 1) δ: 1.16 (3H, s, H_3-8′), 1.18 (3H, s, H_3-9′), 1.38 (2H, m, H_2-4), 1.42 (9H, s, Boc C$\underline{H}_3$×3), 1.71 (2H, m, H_β-5′, 6′), 1.85 (2H, m, H_α-5′, 6′), 2.10 (2H, m, H_2-3), 3.06 (2H, t, *J*=6.4 Hz, H_2-5), 4.50 (1H, dd, *J*=4.8, 10.8 Hz, H-2), 4.59 (2H, br s, H-4′, 7′). ^{13}C-NMR (100 MHz, $CDCl_3$+CD_3OD) δ: 12.3 (C-8′), 12.5 (C-9′), 23.7 (C-5′), 23.8 (C-6′), 25.6 (C-4), 26.7 (C-3), 28.4×3 (Boc $\underline{C}H_3$), 39.8 (C-5), 53.8 (C-3′a), 53.9 (C-7′a), 54.5 (C-2), 79.1 (Boc $\underline{C}$-$(CH_3)_3$), 83.8 (C-4′), 83.9 (C-7′), 156.4 (Boc $\underline{C}$O), 174.9 (C-1), 181.6 (C-1′), 181.7 (C-3′).

Deprotection of 2a A solution of **2a** (100 mg, 0.244 mmol) in trifluoroacetic acid (5 ml) was treated in the same manner as described for the preparation of **1b** to give **2b** (58.4 mg, 77%) as a white powder. $[\alpha]_D$ −3.8° (*c*=1.0, MeOH–H_2O, 1 : 1).

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