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Coprisamides A and B, New Branched Cyclic Peptides from a Gut Bacterium of the Dung Beetle *Copris tripartitus*

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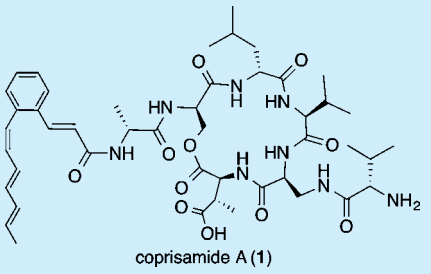
Supporting Information

ABSTRACT: Coprisamides A and B (**1** and **2**) were isolated from a bacterium in the gut of the dung beetle *Copris tripartitus*. Spectroscopic analysis revealed that the planar structures of **1** and **2** are novel cyclic heptapeptides bearing unusual units, such as β-methylaspartic acid and 2,3-diaminopropanoic acid branched to valine and 2-heptatrienyl cinnamic acid. Absolute configurations were established by chemical derivatization and chiroptical spectroscopy. The coprisamides displayed significant activity for induction of quinone reductase.



coprisamide A (**1**)

Bacteria associated with insect symbiotic systems are a strategic source for the discovery of new bioactive small molecules.[1] Structurally novel compounds from the symbiotic bacteria of insects include the polyene peroxide mycangimycin from a *Streptomyces* symbiont of the southern pine beetle *Dendroctonus frontalis*[2] and the cyclic peptide dentigerumycin from *Pseudonocardia* sp. on the exoskeleton of the fungus-growing ant *Apterostigma dentigerum*.[3] Because the biological diversity of insects is tremendously high,[4] it could be promising to study diverse insects and associated bacteria for chemical discovery. Our recent studies have demonstrated that bacteria in the dung beetle ecosystem are also a promising source of novel organic compounds.[5−8] For example, we have reported tripartilactam, a cyclobutane-bearing macrocyclic lactam, as a Na^+/K^+ ATPase inhibitor[5] and tripartin, a dichlorinated indanone, as a specific histone demethylase inhibitor[6] from actinomycete strains isolated from the ecosystem of the dung beetle *Copris tripartitus* Waterhouse. However, chemical investigations of insect gut bacteria have not been performed. The insect gut is very densely colonized, and gut microbiota play crucial roles in an insect's life, such as in digestion.[9] To investigate the potential of insect gut microbiota to harbor chemically interesting bacteria, we isolated bacterial strains from the dung beetle *Copris tripartitus*. Then, the secondary metabolites produced by the cultivated bacterial strains were analyzed by LC/MS. In the chemical analysis, we identified a *Streptomyces* strain, SNU533, isolated from the gut of a male adult specimen of *C. tripartitus* collected in Jeju Island, Republic of Korea, that produced a previously unreported compound based on UV (λ_{max} 282 nm) and mass spectra ($[M + H]^+$ at m/z 907). Further scale-up of the culture of SNU533 and isolation of the compound and its analog resulted in the discovery of the structurally novel branched cyclic peptides coprisamides A and B (**1** and **2**), which incorporate unusual amino acids and a new 2-alkenyl cinnamic acid acyl chain.

Coprisamide A (**1**) was obtained as a yellow powder with the molecular formula $C_{46}H_{66}N_8O_{11}$ and 18 degrees of unsaturation, as determined by ^1H and ^{13}C NMR spectroscopy (Table 1) and high-resolution FAB mass spectrometry ($[M + H]^+$ m/z 907.4927, calculated 907.4929). The ^1H NMR spectrum (in CD_3OH) included seven exchangeable amide proton signals [δ_H 8.78, 8.55, 8.54, 8.50, 8.43, 8.37, and 7.49] and seven α-amino proton signals [δ_H 4.80, 4.52, 4.36, 4.32, 4.28, 3.87, and 3.66], consistent with a peptide-derived compound. The ^{13}C NMR spectrum also consistently showed nine amide/ester/carboxylic acid carbonyl carbon signals [δ_C 178.5, 176.3, 174.5, 172.8, 171.3, 170.9, 170.9, 169.6, and 168.7] and seven α-carbon resonances [δ_C 60.2, 60.0, 56.6, 54.9, 52.9, 52.5, and 51.3]. Further analysis of the ^1H NMR spectrum revealed that coprisamide A possesses eight olefinic signals [δ_H 7.75, 7.05, 6.52, 6.39, 6.35, 6.24, 6.01, and 5.77] and *ortho*-substituted aromatic ring protons [δ_H: 7.62 (d, J = 7.5), 7.34 (dd, J = 7.5, 7.5), 7.28 (d, J = 7.5), and 7.25 (dd, J = 7.5, 7.5)], which are atypical for an ordinary peptide.

Analysis of the HSQC NMR spectrum readily assigned all the one-bond $^1H-^{13}C$ correlations. The subsequent interpretation of the COSY and HMBC NMR correlations resulted in eight partial structures dissected by amide or ester carbonyl carbons. These included five common amino acids: two valines, a leucine, a serine, and an alanine. The other three partial structures were uncommon amino acids and an unusual acyl chain.

First, the COSY NMR spectrum showed one spin system composed of 2-NH [δ_H 8.43], H-2 [δ_H 3.87], H-3 [δ_H 3.10],

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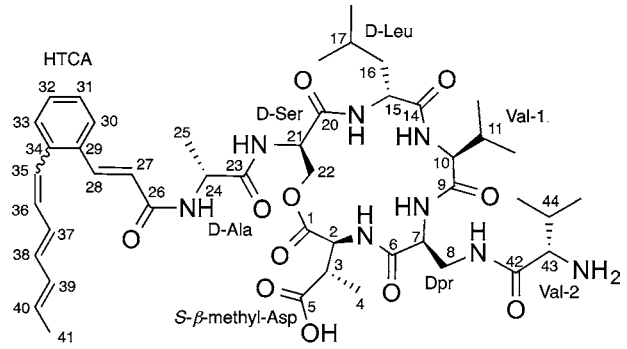
Table 1. NMR Data for Coprisamide A (1) in CD_3OH[a]

no.	δ_H	mult (J in Hz)	δ_C	type
1			170.9	C
2	3.87	dd (10.0, 7.0)	56.6	CH
2-NH	8.43	d (7.0)		
3	3.10	qd (10.0, 7.0)	40.0	CH
4	1.05	d (7.0)	15.4	CH_3
5			178.5	C
6			170.9	C
7	4.80	m[b]	51.3	CH
7-NH	8.50	d (9.5)		
8a	4.39	m	41.0	CH_2
8b	2.95	ddd (12.5, 6.0, 3.0)		
8-NH	8.54	d (6.0)		
9			172.8	C
10	4.36	m	60.0	CH
10-NH	8.55	d (6.0)		
11	2.48	m	29.5	CH
12	0.94	d (7.0)	19.7	CH_3
13	0.87	d (7.0)	16.9	CH_3
14			176.3	C
15	4.28	m	54.9	CH
15-NH	8.78	d (3.0)		
16a	1.64	m	40.1	CH_2
16b	1.50	m		
17	1.57	m	25.6	CH
18	0.95	d (6.5)	22.3	CH_3
19	0.91	d (6.5)	23.1	CH_3
20			171.3	C
21	4.52	br dd (7.5, 2.0)[b]	52.9	CH
21-NH	7.49	d (7.5)		
22a	5.29	dd (11.0, 2.0)	64.4	CH_2
22b	3.94	br d (11.0)		
23			174.5	C
24	4.32	qd (7.0, 6.5)	52.5	CH
24-NH	8.37	d (6.5)		
25	1.45	d (7.0)	16.8	CH_3
26			168.7	C
27	7.05	d (16.0)	122.3	CH
28	7.75	d (16.0)	140.3	CH
29			134.3	C
30	7.62	d (7.5)	127.5	CH
31	7.25	dd (7.5, 7.5)	128.5	CH
32	7.34	dd (7.5, 7.5)	130.3	CH
33	7.28	d (7.5)	131.6	CH
34			139.1	C
35	6.52	d (11.0)	127.7	CH
36	6.39	dd (11.0, 11.0)	133.4	CH
37	6.24	dd (15.0, 11.0)	127.0	CH
38	6.35	dd (15.0, 11.0)	137.3	CH
39	6.01	dd (15.0, 11.0)	132.9	CH
40	5.77	qd (15.0, 7.0)	132.2	CH
41	1.73	d (7.0)	18.1	CH_3
42			169.6	C
43	3.66	d (4.5)	60.2	CH
44	2.19	m	31.7	CH
45	1.07	d (7.0)	18.7	CH_3
46	1.02	d (7.0)	17.2	CH_3

[a] ^1H and ^{13}C NMR were recorded at 600 and 150 MHz, respectively. [b] Measured in CD_3OD.



1: 35*Z* **2**: 35*E*

and H_3-4 [δ_H 1.05]. HMBC correlations from H_3-4 to the carboxylic acid carbonyl carbon C-5 [δ_C: 178.5] and from H-2 to C-1 [δ_C 170.9] identified β-methylaspartic acid (Figure 1a).

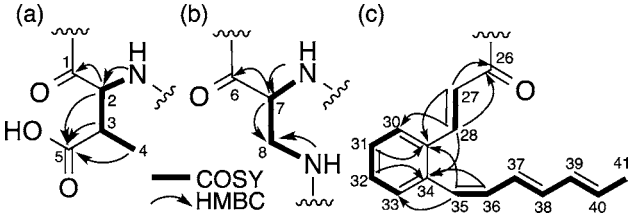


Figure 1. Determination of the unusual partial structures in **1**. (a) β-Methylaspartic acid, (b) 2,3-diaminopropanoic acid, (c) 2-heptatrienyl cinnamic acid (HTCA).

Moreover, the COSY correlations from H-7 [δ_H 4.80] to 7-NH [δ_H 8.50] and H_2-8 [δ_H 4.39, 2.95] identified 7-NH-C-7-C-8 connectivity. This unit was further extended to 8-NH [δ_H 8.54] by the $^1H-^1H$ coupling between H_2-8 and 8-NH and to C-6 by the HMBC correlation from H-7 to C-6 [δ_C 170.9], assigning the 2,3-diaminopropanoic acid (Dpr) (Figure 1b). The initially identified olefinic and aromatic signals suggested an unusual acyl chain. The COSY correlations of H-27 to H-28 and H-35 to H-41 indicated two olefinic moieties. The $^1H-^1H$ coupling of the aromatic protons H-30, H-31, H-32, and H-33 and their HMBC correlations identified an *ortho*-substituted aromatic ring and assigned this ring to the middle of the two olefinic partial structures. Thus, the last unit was revealed as 2-heptatrienyl cinnamic acid (HTCA), which has not been previously reported (Figure 1c).

The geometry of the double bond at C-27 in HTCA in **1** was assigned as *E* by the $^1H-^1H$ coupling constant (16.0 Hz) between H-27 and H-28. The *cis*-coupling constant (11.0 Hz) between H-35 and H-36 assigned the 35*Z* geometry. The large vicinal $^1H-^1H$ coupling constants (C-37 and C-38, J_{HH} = 15.0 Hz; C-39 and C-40, J_{HH} = 15.0 Hz) confirmed the geometries of 37*E* and 39*E*. All geometry assignments were further supported by ROESY correlations.

The connectivity of the eight partial structures (two valines, a leucine, a serine, an alanine, 2,3-diaminopropanoic acid, β-methylaspartic acid, and HTCA) was established by analysis of the HMBC and ROESY correlations (Figure 2). The HMBC correlation from the 2-NH [δ_H 8.43] of β-methyl-Asp to the C-6 [δ_C 170.9] belonging to Dpr established the connectivity between β-methyl-Asp and Dpr. The connectivity between Dpr and Val-1 was deduced by long-range heteronuclear couplings from the 7-NH [δ_H 8.50] of Dpr and the α-proton [H-10; δ_H 4.36] of Val-1 to C-9 [δ_C 172.8]. The long-range $^1H-^{13}C$

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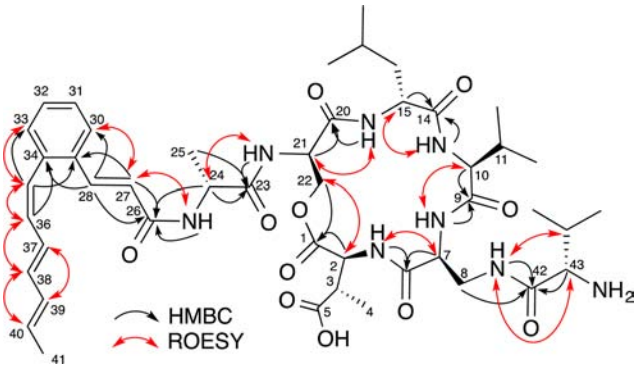

Figure 2. Key HMBC and ROESY correlations of **1**.

coupling from the amide proton 10-NH [Val-1; δ_H 8.55] to C-14 [Leu; δ_C 176.3] connected Val-1 to Leu. Both the 15-NH [δ_H 8.78] of Leu and H-21 [δ_H 4.52], the α-proton of Ser, exhibited HMBC correlations with C-20 [δ_C 171.3], establishing the connection of Leu to Ser. Based on the HMBC correlations of 21-NH [δ_H 7.49] and H-24 [δ_H 4.32] to C-23, the connectivity of Ser to Ala was assigned. The HTCA was positioned next to Ala by the $^1H-^{13}C$ long-range correlations from the NH-24 [δ_H 8.37] of Ala and the H-28 [δ_H 7.75] of HTCA to C-26 [δ_C 168.7]. The HMBC correlations from H-22b [β-proton of Ser; δ_H 3.94] and H-2 [α-proton of β-methyl-Asp; δ_H 3.87] to C-1 [δ_C 170.9] secured the ring closure, satisfying the 18 degrees of unsaturation deduced from the molecular formula. Finally, the NH proton [8-NH; δ_H 8.54] and the β-proton [H-8b; δ_H 2.95] of Dpr exhibited heteronuclear correlations to C-42 [δ_C 169.6], connecting the branched amino acid unit Val-2 to Dpr. The ROESY correlation between 8-NH and H-43 [δ_H 3.66] also supported the linkage of these two units.

The absolute configurations at the α-carbons of the amino acids in coprisamide A (**1**) were established by the advanced Marfey's method with FDAA (1-fluoro-2,4-dinitrophenyl-5-alanine amide).[10] After acid hydrolysis of **1** for 2 h, the free amino acids in the hydrolysate were derivatized with L- and D-FDAA. LC/MS analysis of the derivatives revealed two L-Val, β-methyl-L-Asp, D-Ser, D-Ala, and D-Leu. In addition, by comparison of the retention times of the authentic standard *S*-Dpr coupled with L- and D-FDAA (*di*, $[M + H]^+$ m/z 609), Dpr in **1** was assigned as *S* (see Supporting Information Table S1).

To establish the absolute configuration of the additional stereogenic center at C-3 of the β-methyl-Asp-bearing carboxylic acid, **1** was subjected to phenylglycine methyl ester (PGME) derivatization.[11] The derivatization was performed in the dark at rt for 40 min to avoid degradation. After careful analysis of the ^1H and COSY NMR data of the *S*- and *R*-PGME amides (**3a** and **3b**, $[M - H]^-$ m/z 1052), the absolute configuration at the β-carbon of β-methyl-L-Asp in coprisamide A (**1**) was determined as *S* (Figure 3a). This configuration was further supported by *J*-based configuration analysis of the C-2 and C-3 consecutive stereogenic centers.[12] The large coupling constant (10.0 Hz) between H-2 and H-3 established their *anti*-relationship. The H_3-4/2-NH ROESY correlation established the rotamer in Figure 3b. Because the absolute configuration of the α-carbon of β-methyl-L-Asp was already determined as *S* by the advanced Marfey's method (*vide supra*), the established rotamer possesses a 3*S* configuration, supporting the PGME analysis result.

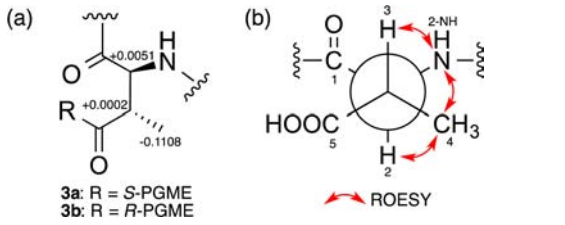

Figure 3. (a) $\Delta\delta_{S-R}$ values in ppm for the *S*- and *R*-PGME amide products (**3a** and **3b**) in CD_3OD. (b) *J*-based configuration analysis of C-2 and C-3.

Coprisamide B (**2**) was isolated as a yellow powder along with coprisamide A (**1**) during HPLC purification. Its molecular formula was determined as $C_{46}H_{66}N_8O_{11}$, which is identical to that of **1**. The HR-FAB mass spectrum ($[M + H]^+$ m/z 907.4919, calculated 907.4929) and ^1H and ^{13}C NMR data (Table S2) were obtained. The analysis of the 1D and 2D NMR data revealed that the gross structure of **2** is identical to that of **1**. Further analysis of the $^1H-^1H$ coupling constants revealed that coprisamide B is a geometric isomer of **1** possessing the 35*E* configuration (J_{H35H36} = 15.0 Hz), in contrast to the 35*Z* geometry of **1**. Comparison of the CD spectra of **1** and **2** indicated that the absolute configuration of **2** was identical to that of **1** (see Supporting Information Figure S14).

Coprisamides A and B (**1** and **2**) were primarily tested for growth inhibition activity against human pathogenic bacteria (*Staphylococcus aureus*, *Bacillus subtilis*, *Kocuria rhizophila*, *Salmonella enterica*, *Proteus hauseri*, and *Escherichia coli*) and fungi (*Candida albicans* and *Aspergillus fumigatus*). However, these compounds did not exhibit antibacterial or antifungal activity. The cytotoxicities of **1** and **2** were evaluated against various cancer cell lines (HCT 116, A549, SNU-638, SK-HEP-1, and MDA-MB-231), but no significant cytotoxicity was observed.

The induction of phase II detoxification enzymes is an attractive, safe, and promising strategy for decreasing the risk of developing cancer. Because quinone reductase (QR), a representative phase II detoxification enzyme, is widely distributed in mammalian tissues and is easily measured, QR is considered to have significance in cancer chemoprevention. The biological activity of the coprisamides was evaluated using a QR induction assay. In the assay, the Hepa-1c1c7 murine hepatoma cell line was utilized because the induction of QR in this cell line is highly associated with the induction of phase II enzymes *in vivo*.[13]

Coprisamide A (**1**) significantly induced QR activity by 2.4-, 2.6-, and 3.3-fold at concentrations of 5, 10, and 20 μM, respectively (Figure 4a), while coprisamide B enhanced QR activity by 1.7-, 2.1-, 2.8-, and 3.2-fold at concentrations of 5, 10, 20, and 40 μM, respectively (Figure 4b). The significant effects of **1** and **2** QR induction suggest that the coprisamides may represent a chemotype for cancer chemopreventive agents.

The effects of coprisamides A and B on melanogenesis were also evaluated in cell-based assay systems. Coprisamides A and B inhibited the production of melanin content in mouse melan-a cells by 51.3% and 41.9%, respectively, at 20 μM. These data indicate that coprisamides A and B have inhibitory activity in melanin formation (see Supporting Information Figure S15). Therefore, coprisamides A and B may be applicable as potential candidates for treating or preventing hyperpigmentation and as skin whitening agents.

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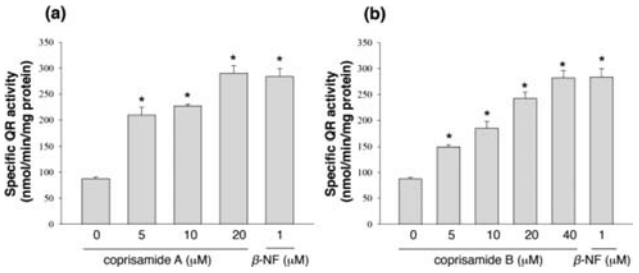

Figure 4. Effects of (a) coprisamide A and (b) coprisamide B on the induction of quinone reductase in murine Hepa-1c1c7 cells. Cells were cultured for 24 h and then exposed to coprisamide A or B for 24 h. Quinone reductase activities in the cell lysates were measured by reduction of a tetrazolium dye and expressed as nmol/min/mg protein. β-NF (β-naphthoflavone) was used as a positive control (*P < 0.001).

The structures of the coprisamides are unique in several aspects. First, the amino acid sequence incorporating mostly the unusual amino acids *S*-β-methyl-L-Asp and *S*-Dpr as well as D-Leu, D-Ser, and D-Ala is unprecedented among the numerous reported nonribosomal peptides.[14] In particular, β-methyl-L-Asp is infrequently encountered, with the rare examples of the friulimicin class of peptides[15] and the skyllamycins.[16] The branched feature of **1** and **2** at Dpr extended to Val-2 is also unique in nature. Moreover, the acyl chain, which is composed of a 2-heptatrienyl cinnamic acid (HTCA), has not been previously reported. Such 2-alkenyl cinnamic acids are extremely rare. Based on our exhaustive literature search, only four classes of natural products bearing a 2-alkenyl cinnamic acid have been reported. WS9326s, the mohangamides, and the pepticinnamins incorporate a 2-pentenyl cinnamic acid,[17] whereas the skyllamycins possess a 2-propenyl cinnamic acid.[16] Based on the study of skyllamycin biosynthesis,[18] the HTCA unit could be biosynthesized from the putative precursor C_{16}-polyene by an enzymatic 6π-electrocyclization (see Supporting Information Figure S18).

The coprisamides are the first secondary metabolites to be isolated from insect gut symbionts. Insect gut microbiota are likely determined by the environmental habitat, diet, developmental stage, and phylogeny of the host, resulting in the tremendous biodiversity of insect gut-associated microbes.[19] Our discovery of the coprisamides from a dung beetle gut symbiont signifies the huge chemical potential of insect microbiota for bioactive small molecules.

■ ASSOCIATED CONTENT

Supporting Information

The detailed experimental procedures and NMR data for compounds **1** and **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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