Graphiumins I and J, New Thiodiketopiperazines from the Marine-derived Fungus *Graphium* sp. OPMF00224

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Abstract – Two new thiodiketopiperazines (TDKPs), designated graphiumins I (**1**) and J (**2**), were isolated from the culture broth of the marine-derived fungus *Graphium* sp. OPMF00224 by solvent extraction, silica gel column chromatography, and HPLC. Their absolute structures were elucidated by spectroscopic analyses (1D and 2D NMR data, ROESY correlations, and CD data) and chemical methods. They were found to be structurally rare TDKPs with a phenylalanine-derived indolin substructure. Compounds **1** and **2** inhibited yellow pigment production by methicillin-resistant *Staphylococcus aureus* (MRSA) with IC_{50} values of 63.5 and 76.5 µg/ml, respectively, without inhibiting its growth, even at 250 µg/ml.

Keywords – Thiodiketopiperazines, Marine-derived fungus, MRSA, Yellow pigment inhibitor

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major nosocomial pathogen that is resistant to many other antibiotics including currently used *β*-lactams.[1] Although vancomycin is widely used to treat MRSA infections, vancomycin-resistant *S. aureus* (VRSA) has been identified and reported.[2] Therefore, it has become increasingly important and necessary to discover new antibiotics effective against MRSA infections. *S. aureus* strains including MRSA produce a yellow pigment called staphyloxanthin (STX), which is one of the important virulence factors of *S. aureus*.[3-8] In 2005, Liu *et al.* reported that the a dehydrosqualene synthase (CrtM)-deficient mutant, which lacked the ability to produce STX, failed to survive in a mouse host, indicating that compounds that inhibit the production of STX have the potential to become new anti-infectious agents against MRSA. BPH-652,[7] zaragozic acid,[9] 7-benzyloxyindoles,[10] and flavones[11] have been shown to inhibit STX production and are a lead for new anti-infective agents targeting the biosynthesis of the STX virulence factor in MRSA. We started to search for such inhibitors from natural sources using our established screening system,[12] and subsequently discovered

tylopilusins from the fruiting bodies of *Tylopilus eximius*,[13] citridones A from *Penicillum* sp. FKI-1938,[14] and graphiumins A to H from the marine-derived fungus *Graphium* sp. OPMF00224.[15] Graphiumins were the first thiodiketopiperazine (TDKP) to have one or two octanoyl side chains. Further investigations of the culture broth of the graphiumin-producing fungus led to the discovery of two new graphiumins I (**1**) and J (**2**), with a rare phenylalanine-derived indolin substructure. In the present study, we described the isolation, structural elucidation, and biological activities of compounds **1** and **2**.

Experimental

General experimental procedures – Optical rotations were recorded with a DIP-1000 digital polarimeter (JASCO, Tokyo, Japan). ESI-MS spectrometry was conducted on a JMS-T1000LP spectrometer (JEOL, Tokyo, Japan). UV and IR spectra were measured with a U-2800 spectrophotometer (HITACHI, Tokyo, Japan) and FT/IR-460 plus spectrometer (JASCO), respectively. NMR spectra were measured with an INOVA 600 spectrometer (Agilent Technologies Inc., California, USA). Reversed-phase HPLC separation was performed using a Senshu Pak preparative C18 PEGASIL ODS SP100 column (10 × 250 mm, Senshu Scientific Co., Ltd., Tokyo, Japan) and DAICEL CHIRALCEL OJ-H column (150 × 4.6 mm, DAICEL CHEMICAL INDUSTRIES, Ltd., Tokyo, Japan) with the

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SHIMAZU LS20AT pump and SHIMAZU LS20AS UV detector (Kyoto, Japan).

Fungal strain – Fungus OPMF00224, isolated from marine sediments collected at a depth of −17 m on Ishigaki Island, Okinawa, Japan, was used in the production of graphiumins as previously reported.[15]

Fermentation – The strain was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of seed medium (2.0% glucose, 0.2% yeast extract, 0.05% $MgSO_4 \cdot 7H_2O$, 0.5% Polypeptone, 0.1% KH_2PO_4, and 0.1% agar, pH 6.0). The flask was shaken on a rotary shaker at 27 °C for 3 days. The seed culture (2.0 ml) was transferred into a 1000-ml culture box containing 150 ml of production medium (5% oat meal, 0.2% yeast extract, 0.1% Na tartrate, 0.1% KH_2PO_4, and 0.8% DAIGO authentic seawater). Fermentation was carried out at 27 °C for 24 days under static conditions.

Isolation – The culture broth (150 ml × 30) was extracted with ethanol (4.5 L) for two hours. After the extract had been evaporated to an aqueous solution, the residue was partitioned between water and EtOAc to yield a crude extract (600 mg) after evaporation of the EtOAc fraction. The crude extract was dissolved in a small volume of chloroform, applied to a silica gel column (30 g, 3.4 × 15 cm, 0.04 - 0.063 mm), and eluted stepwise with 100% chloroform, 50:1, 25:1, 10:1, 5:1, and 1:1 (v/v) of chloroform-methanol solvent and 100% methanol (200 ml each). Graphiumins were observed in the last fraction eluted with 50:1 chloroform-methanol. This fraction was further purified by reversed-phase C-18 HPLC (10 × 250 mm; PEGASIL ODS, Senshu Scientific Co., Tokyo, Japan) under the following conditions: solvent, a 40-min linear gradient from 60% to 80% aqueous acetonitrile, at a flow rate of 3.0 ml/min with UV detection at 210 nm. Under this condition, compounds **1** and **2** were eluted as peaks with retention times of 29.0 and 27.0 min, respectively. These peaks were collected and concentrated to yield 4.7 and 2.0 mg as pale yellow solids, respectively.

Analysis of absolute configuration of the hydroxyacyl side chain in graphiumins – Hydrolysis of 1 or 2 - A total of 0.1 N NaOH (1 ml) was added to a solution of compounds **1** and **2** (0.5 mg) in a 1:3 mixture of water (250 μl) and methanol (750 μl). Hydrolysis was carried out at room temperature by stirring for 4 h to release hydroxyoctanoic acid. The mixture was evaporated to give a colorless solid. Cesium carbonate (1 mg, 5 mmol) was added to a solution of the colorless solid prepared from compounds **1** and **2** and 1 mg authentic (±)-3-hydroxyloctanoic acid (SIGMA-ALDRICH, St. Louis, USA) in water (100 μl), and then stirred at room tem-

perature for 30 minutes. After removal of the solvent, the cesium salt was dissolved in DMF (100 μl).

Benzoylation of hydroxyoctanoic acid – Benzyl bromide (10 μl) was added to each cesium salt of hydroxyoctanoic acid in DMF (100 μl) to prepare benzyl-hydroxyoctanoate. After stirring at room temperature for 20 h, the mixture (110 μl) was poured into a 1:1 mixture (2 ml) of water and ethyl acetate. The phases were separated to collect the organic layer, and the aqueous layer was then extracted with ethyl acetate (1 ml) three times. The combined organic layer (4 ml) was dried over Na_2SO_4, filtrated, and concentrated under reduced pressure. The residue was dissolved in MeOH (40 μl) for a chiral HPLC analysis.

Physicochemical properties graphiumins I (1) and J (2).

Graphiumin I (1, 4.7 mg) – Pale yellow solid; $[\alpha]_D^{24.9}$ −266.7 (C 0.1 CH_3OH); CD (MeOH) $\lambda_{ex\ termum}$ (Δ) 303 (+2.3), 271 (−5.0), 254 (−7.4), 226 (−16.8); IR (KBr) ν_{max} 3472, 2930, 1733, 1700, 1660 (sh), 1603 (sh), 1485 cm^{-1}; UV (MeOH) λ_{max} (log ε) 268 (3.86), 202 (4.30); ^1H and ^{13}C NMR, see Table 1; ESI-MS $[M+Na]^+$ *m/z* 551.1288, ($C_{26}H_{28}N_2O_6S_2Na$, calcd 551.1286).

Graphiumin J (2, 2.0 mg) – Pale yellow solid; $[\alpha]_D^{24.9}$ −117.9 (C 0.1 CH_3OH); CD (MeOH) $\lambda_{ex\ termum}$ (Δ) 297 (+1.2), 255 (−1.9), 226 (−15.9); IR (KBr) ν_{max} 3489, 2932, 1739, 1682, 1603, 1477 cm^{-1}; UV (MeOH) λ_{max} (log ε) 294 (3.57), 221 (4.14); ^1H and ^{13}C NMR, see Table 1; ESI-MS $[M+Na]^+$ *m/z* 597.1702, ($C_{28}H_{34}N_2O_7S_2Na$, calcd 597.1705).

Analysis of benzyl-3R/3S-hydroxyoctanoate by HPLC – In order to determine the stereochemistry of the hydroxyoctanoyl side chain in compounds **1** and **2**, 20 μl of the methanol solution (40 μl) containing benzyl-hydroxyoctanoate was analyzed using a HPLC system under the following conditions: pump, LS20AT; column, CHIRALCEL OJ-H column (150 × 4.6 mm); flow rate, 1.0 ml/minute; detection, UV at 254 nm; solvent, 35% CH_3CN in 0.05% TFA. Under these conditions, authentic benzyl-3S-hydroxyoctanoate and benzyl-3R-hydroxyoctanoate prepared from (±)-3-hydroxyloctanoic acid were eluted as peaks with retention times of 29.2 and 31.4 minutes.

Assay for yellow pigment production by MRSA.

Paper disk method – The assay method of yellow pigment production by MRSA was established and reported previously by our group.[12] In brief, the MRSA K-24 strain, a clinical isolate, was cultured in Mueller-Hinton broth at 37 °C for 20 h and adjusted to 1×10^8 colony-forming units (CFU)/ml. The inoculum (100 μl) was spread on 25 ml of TYB agar (tryptone 1.7%, yeast extract 1.0%, NaCl 0.5%, K_2HPO_4 0.25%, agar 1.5%, and glycerol monoacetate 1.5%) in a plate (100 × 140 mm).

Paper disks (8 mm i.d.) containing each sample (25 or 50 μg) were placed on the plate and incubated at 37 °C for 72 h. The inhibition of yellow pigment production by a sample was expressed as the diameter (mm) of the white zone on the plate.

Liquid culture method – A mixture containing TYB (980 μl), a sample (10 μl), and MRSA (10 μl of 1×10^7 CFU/ml) was incubated on a rotary shaker at 210 rpm for 72 h at 37 °C. In this assay, MRSA growth was first determined at 600 nm using a Power Wave × 340 (BIO-TEK Instruments Inc., Winooski, VT, USA). The culture was then centrifuged to remove the supernatant, and

Table 1. ^{13}C and ^{1}H NMR spectroscopic data for graphiumins I (**1**) and J (**2**) in CDCl$_3$

Position	1		2	
	$\delta_C{}^a$	δ_H *mult (J in Hz)*^{b}	$\delta_C{}^a$	δ_H *mult (J in Hz)*^{b}
1	160.4, s		169.2, s	
2	75.7, s		69.9, s	
3	34.2, t	4.15, dq (18.0, 1.0)	39.3, t	3.23, dt (17.0, 1.0)
		3.10, dq (18.0, 1.5)		3.13, dt (17.0, 1.5)
4	113.1, s		108.9, s	
5	139.7, d	6.69, q (2.0)	138.2, d	6.64, t (2.0)
6	141.6, d	6.35, dd (8.0, 2.0)	139.9, d	6.34, dd (8.0, 2.0)
7	105.0, d	4.61, dd (8.0, 1.5)	105.5, d	4.69, dd (8.0, 2.0)
8	69.9, d	5.86, dt (8.0, 1.5)	71.9, d	5.93, dt (8.5, 2.0)
9	63.0, d	5.18, dq (8.0, 1.5)	60.5, d	5.21, br d (8.5)
10				
11	171.7, s		171.6, s	
12	41.9, t	2.56, dd (17.0, 3.0)	42.1, t	2.56, dd (17.0, 3.0)
		2.46, dd (17.0, 9.0)		2.47, dd (17.0, 9.0)
13	67.6, d	4.09, m	67.5, d	4.07, m
14	36.5, t	1.44, m	36.5, t	1.43, m
		1.52, m		1.51, m
15	25.3, t	1.37, m	25.2, t	1.34, m
		1.45, m		1.44, m
16	31.7, t	1.31, m	31.7, t	1.29, m
17	22.6, t	1.32, m	22.6, t	1.30, m
18	14.1, q	0.91, t (8.0)	14.0, q	0.89, t (8.0)
1'	162.8, s		164.3, s	
2'	76.5, s		73.6, s	
3'	35.6, t	4.32, br d (18.0)	39.9, t	3.74, d (17.0)
		3.25 d (18.0)		3.44, d (17.0)
4'	128.1, s		131.4, s	
5'	125.3, d	7.33, d (7.0)	116.2, d	6.82, d (7.0)
6'	125.9, d	7.21, t (7.0)	129.3, d	7.17, t (7.0)
7'	128.8, d	7.34, t (7.0)	118.4, d	6.92, d (7.0)
8'	115.8, s	7.93, d (7.0)	146.4, s	
9'	138.1, s		126.7, s	
2-SMe			15.1, q	2.36, s
2'-SMe			14.2, q	2.20, s
8'-OH			10.03, s	
13-OH	ND^c		ND^c	

a) Chemical shifts are shown with reference to CDCl$_3$ as 77.0 ppm.
b) Chemical shifts are shown with reference to CDCl$_3$ as 7.26 ppm.
c) ND: not detected

yellow pigment in MRSA mycelia was extracted with methanol (500 μl) at 60 °C for 2 h in the dark. Yellow pigment production was determined at 450 nm using a Power Wave × 340. The inhibition of MRSA growth and yellow pigment production by a sample (% of control) was defined as (absorbance-sample/absorbance-control) × 100. The IC_{50} value was defined as a sample concentration that caused 50% inhibition of MRSA growth or yellow pigment production.

Results and Discussion

Structure elucidation of graphiumins I and J. – Graphiumin I (**1**): Absorptions at approximately 1733, 1700, and 1660 cm^{-1} in IR spectra suggested the presence of three kinds of carbonyl groups. The molecular formula $C_{26}H_{28}N_2O_6S_2$ ([M+Na]$^+$ *m/z* 551.1288) indicated that **1** contained 13 degrees of unsaturation. ^{1}H and ^{13}C NMR data (in CDCl$_3$) supported the molecular formula (Table 1). The ^{13}C NMR spectrum showed 26 resolved signals, which were classified into one methyl, seven methylenes, ten (seven sp^2) methines, and eight (three carbonyl) quaternary carbons. The ^{1}H NMR spectrum of **1** showed one methyl signal, seven olefinic methine signals, two oxygenated methine signals, and many methylene signals. The connectivity of all proton and carbon atoms was established by HMQC experiments (Table 1). An analysis of ^{1}H-^{1}H COSY data allowed three linkages: C-6 to C-9, C-5' to C-8', and C-12 to C18, to be assigned (Fig. 2). An analysis of HMBC spectroscopic data gave further structural information on **1**. 1) The cross peaks from H$_2$-3 (δ 4.15, 3.10) to C-1 (δ 160.4), C-2 (δ 75.7), C-4 (δ 113.1), C-5 (δ 139.7), and C-9 (δ 63.0), from H-5 (δ 6.69) to C-3 (δ 34.2), C-4, and C-6 (δ 141.6), from H-6 (δ 6.35) to C-5, from H-9 (δ 5.18) to C-2, C-3, and C-4, from H$_2$-3' (δ 4.32, 3.25) to C-1' (δ 162.8), C-2' (δ 76.5), C-4' (δ 128.1), C-5' (δ 125.3), and C-9' (δ 138.1), from H-5' (δ 7.33) to C-3' (δ 35.6), C-4', and C-9', and from H-8' (δ 7.93) to C-4' and C-9' built up the partial structure I. 2)

The cross peaks from H$_2$-12 (δ 2.56, 2.46) to C-11 (δ 171.7) indicated the partial structure II. In addition, the cross peaks from H-8 (δ 5.86) to C-11 and chemical shift of C-8 (δ 69.9) and C-11 indicated that the partial structures I and II were linked between C-8 and C-11 via one oxygen atom. The chemical shifts of C-1 and C-2 and the number of nitrogen and sulfur atoms supported the presence of an epidithiodiketopiperazine skeleton in **1**.[15] Based on these data, the planar structure of **1** was elucidated as shown in Fig. 2a.

The stereochemistry of **1** was determined by comparing with all data of known graphiumin A (**3**) including ROESY, H-H coupling constants, and CD. NOE correlations between 3-H and 5-H as well as between 8-H and 9-H (Fig. 2b) and the coupling constants J_{7-8} (1.5 Hz) and J_{8-9} (8.0 Hz) (Fig. 2C) indicated that **1** had the same configuration 8S*/9S* as **3**.[15] In addition, the CD spectrum of **1** exhibited a typical positive Cotton effect (CE) at 254 nm for a disulfide bridge-containing diketopiperazine (DKP) ring, indicating that **1** had the 2R/2'R configurations.[16] The absolute configuration of the 3-hydroxyoctanoyl side chain was then determined by a chiral HPLC analysis of the benzyl derivative of the base hydrolysate of **1**.[15,17] The authentic benzyl-3S/3R-hydroxyoctanoate were eluted at 29.2/31.4 minutes by chiral HPLC. The derivative from **1** was observed at 29.2 minutes, revealing that the configuration of C-13 was oriented as S. Taken together, the absolute structure of **1** was elucidated as shown in Fig. 1.

Graphiumin J (**2**): The structure of **2** was elucidated by comparing all spectroscopic data with those of **1**. The molecular formula of **2** was established as $C_{28}H_{34}N_2O_7S_2$ ([M+Na]$^+$ *m/z* 597.1702), indicating that **2** was 46 mass units (C$_2$H$_6$O) larger than **1**. The difference between **1** and **2** was that two additional methyl signals, 2-SCH$_3$ (δ 15.1, δ 2.36) and 2'-SCH$_3$ (δ 14.2, δ 2.20), were observed in the ^{13}C and ^{1}H NMR spectra of **2** (Table 1). In addition, one aromatic proton (8'-H) in **1** was missing in the ^{1}H NMR spectra of **2**. Cross peaks from 2-SCH$_3$ to C-2 (δ 69.9) and from 2'-SCH$_3$ to C-2' (δ 73.6) were observed in
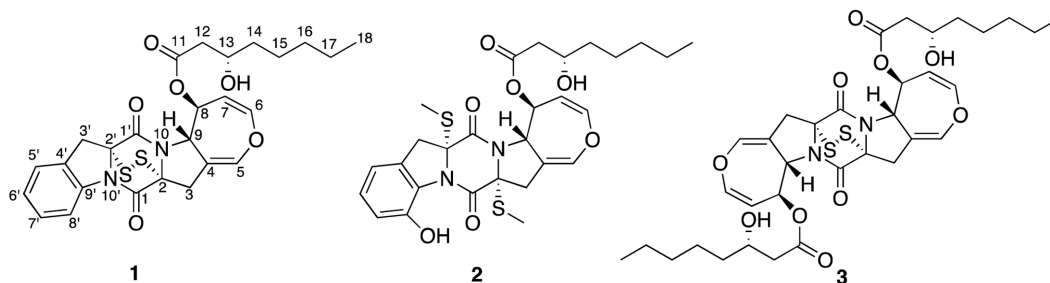

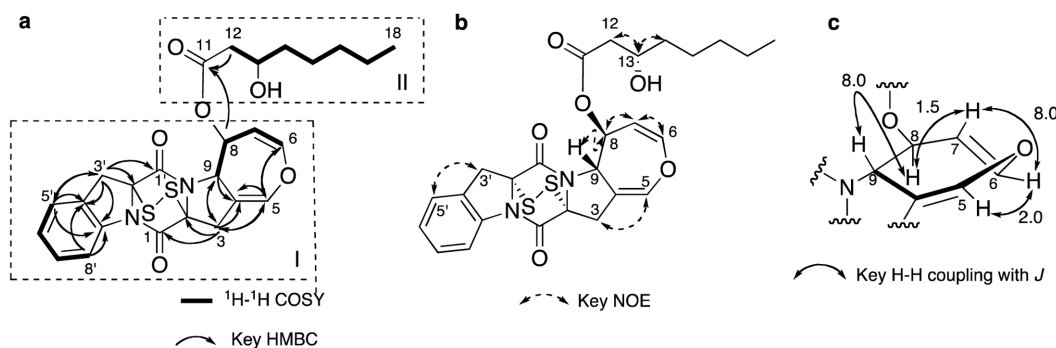**Fig. 1.** Structures of graphiumins I (**1**), J (**2**) and A (**3**).

Fig. 2. ^{1}H-^{1}H COSY (a), Key HMBC (a), NOE (b) correlations, and H-H coupling (c) of **1**.
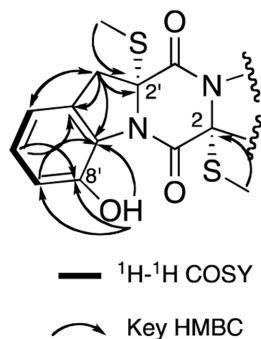

Fig. 3. ^{1}H-^{1}H COSY and Key HMBC correlations of **2**.

Table 2. Summary of biological activities of graphiumins I (**1**) and J (**2**) against MRSA

Compound	White zone (mm)[a]		Growth[b]	Y. P. prod.[b,c]
	50 μg	25 μg		(IC$_{50}$ μg/ml)
1	24	22	>250	63.5
2	23	20	>250	76.5
Citridone A[d]	23	18	>30	11.1

[a]Paper disk method (8 mm disk).
[b]Liquid culture method.
[c]Y. P. prod.; Yellow pigment production.
[d]Positive control.[15]

the HMBC data of **2** (Fig. 3). Cross peaks from 8'-OH (δ 10.03) to C-7' (δ 118.4), C-8' (δ 146.4), and C-9' (δ 126.7) were also observed (Fig. 3). These data indicated that **2** was di-*S*-methylated 8'-hydroxyl **1**. Considering the biogenetic mechanisms, ROESY experiments, and the value of optical rotation, **2** was deduced to have the same absolute configurations as **1**.

The effects of compounds **1** and **2** on yellow pigment production by MRSA were tested using the paper disk method. Compounds **1**, **2**, and citridone A[14] (positive compound) showed white zones (24, 23 and 23 mm, respectively) with no inhibition zone at 50 μg/8 mm paper disk, suggesting that they selectively inhibited the production of yellow pigment without affecting the growth of MRSA. Thus, **1** and **2** appeared to be more potent inhibitors of MRSA yellow pigment production than citridone A by the paper disk method.[15] In order to confirm this result, they were evaluated by the liquid culture method (Table 2), and the results obtained showed that **1** and **2** inhibited yellow pigment production with IC$_{50}$ values of 63.5 and 76.5 μg/ml (vs 11.1 μg/ml for citridone A), respectively. Although the discrepancy between the two assays is not clear, it may be worth comparing the efficacies of these inhibitors in an *in vivo* infection assay.[8]

Graphiumins I (**1**) and J (**2**) have two unique characteristics; TDKPs with one/two medium-chain acyl group (s) as well as the other graphiumins A to H and SCHs[18] (certain TDKPs have acetyl group (s) at the corresponding position), and also a phenylalanine-derived indolin substructure (the first TDKP of this type is aranotin derivative reported by Neuss *et al.*)[19] Guo *et al.* recently cloned the biosynthetic gene cluster of acetylaranotin and related TDKPs in order to propose a biosynthetic pathway.[20] Based on their proposal, Fig. 4 shows a potential biosynthetic pathway of compounds **1** and **2**. Two Phe are condensed and cyclized to produce the intermediate **4**, and two glutathiones are bound to the core ring to form **5**. Gly and Glu residues of the glutathione parts are then removed from **5**, which is further converted to epidithiol **6**. Compounds **1** and **2** appear to share a similar biosynthetic pathway to this intermediate **6**. Regarding the biosynthesis of **1**, **6** was cyclized and oxidized at the dithiol parts to form **7**. Dehydroxylation and octanoylation occur to yield **1**. Regarding **2**, methylation occurred at the dithiol residues of **6** to produce **8**, then similar reactions (cyclization and oxidation) occurred to produce **9**. Dehydroxylation and octanoylation proceeded to yield **2**. The biosynthetic gene clusters of compounds **1** and **2** need to be cloned and analyzed to define the proposed pathway.
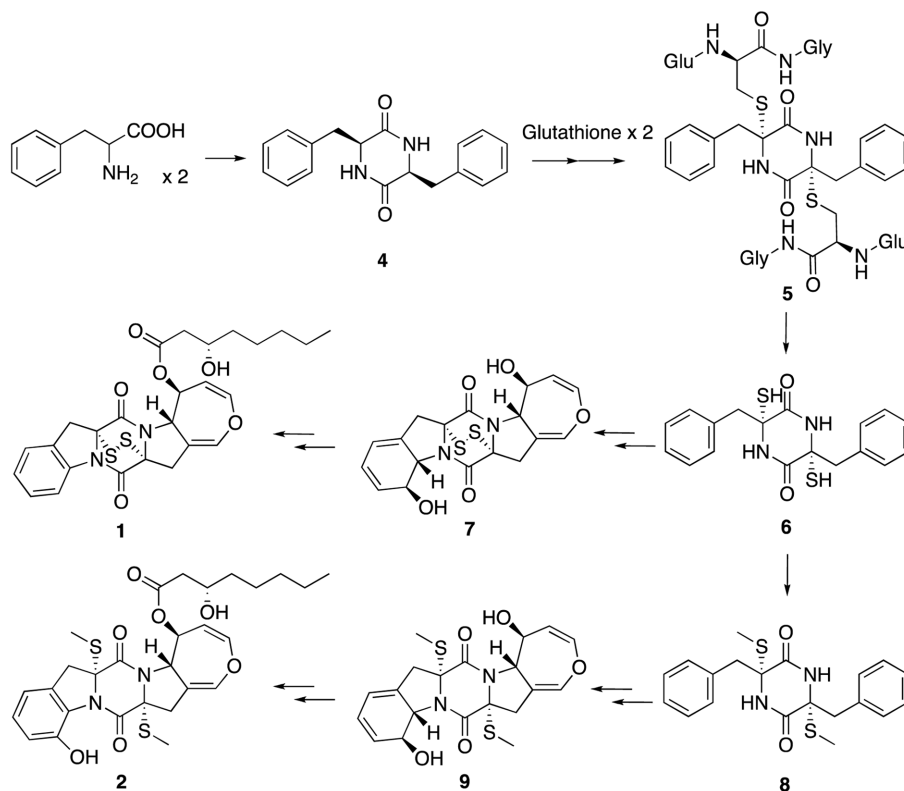
Fig. 4. Proposed biosynthetic pathway of graphiumins I (**1**) and J (**2**).

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