New Dimeric Morphine from Opium Poppy (*Papaver somuniferum*) and Its Physiological Function

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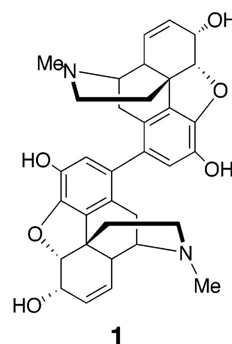
Together with bismorphine A (**1**), a new compound bismorphine B (**2**) was identified in the wounded capsules of *Papaver somniferum*. On the basis of analyses of the various spectral data, bismorphine B (**2**) was determined as a novel morphinan alkaloid, in which two morphine units are coupled through a biphenyl ether bond. When the physiological function of bismorphine B (**2**) in opium poppy was investigated, it was evident that this alkaloid more effectively cross-links cell wall polysaccharide pectins than bismorphine A (**1**) and that such cross-linking reaction leads to resistance against hydrolysis by pectinase.

Morphine is a medicinally important alkaloid found only in opium poppy (*Papaver somniferum* L.) and related species (e.g., *P. setigerum* DC.). Various studies on morphine have been conducted, and the physiological roles of this alkaloid in opium poppy have been revealed. For example, opium alkaloids including morphine are known to act as chemical defense compounds to protect opium poppy against various herbivores.[1] In addition, we recently established the metabolic mechanism of morphine in opium poppy and demonstrated the physiological importance of morphine metabolism.[2] In response to stress, the capsules of opium poppies immediately metabolize morphine to dimeric morphine, called bismorphine A (**1**). The bismorphine A (**1**) then binds ionically to the cell wall polysaccharide pectins, resulting in cross-linking pectins to each other through bismorphine bridges. Because such cross-linking reaction promotes resistance against enzymatic hydrolysis of pectins, we concluded that morphine metabolism is involved in the defense reaction of opium poppy.

Further investigation of morphine metabolism revealed that, together with bismorphine A (**1**), a new alkaloid bismorphine B (**2**) accumulates in the wounded capsules of opium poppies. This paper deals with structure elucidation of this new alkaloid. In addition, its cross-linking ability for pectins is also described.

The capsules of opium poppies were wounded using blades and then incubated at 25 °C for 48 h. When the alkaloid fraction prepared from the wounded capsules was analyzed using HPLC, we confirmed accumulation of an unknown compound, named bismorhine B (**2**). This compound was isolated by a combination of open-column chromatography and preparative HPLC, and the various spectral data were obtained to characterize its structure.

The FABMS of bismorphine B (**2**) showed an $(M + H)^+$ ion peak at *m*/*z* 569, which matched a formula of $C_{34}H_{36}N_2O_6$. The UV spectrum was similar to that of isoquinoline alkaloid with λ_{max} 284 nm.[3] The ^{1}H NMR spectrum revealed two methyl signals (δ 2.28), six methylene signals (δ 1.60−3.00), eight methine signals (δ 2.50−4.80), and four olefinic signals (δ 5.20−5.60). These signals were assigned as shown in Table 1 by analysis of the COSY spectrum and by comparison with the ^{1}H NMR signals of morphine. This suggested that bismorphine B (**2**) consisted of two morphine units. However, in the aromatic region, only one set of AB



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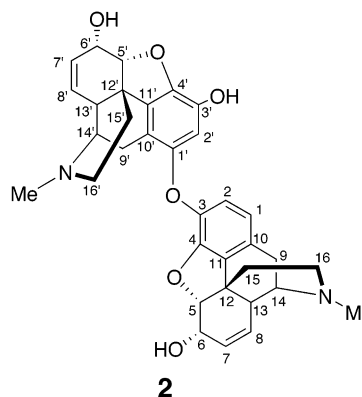

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signals (δ 6.50 and 6.57) was observed, while the remaining aromatic signal appeared as one proton singlet (δ 5.87). This singlet signal, which was assigned to H-2′, was located significantly upfield as compared to the H-2 signal (δ 6.34) in morphine. Moreover, the chemical shift (δ 151.9) of the C-1′ signal in the ^{13}C NMR spectrum indicated that the carbon at this position was oxygenated. Therefore, two morphine units in bismorphine B (**2**) were considered to be linked through a bond formed between the carbon at C-1′ and the oxygen at C-3 or C-6. However, judging from the fact that the chemical shift (δ 4.08) of the H-6 signal is almost identical with that (δ 4.09) in morphine, it was not likely that the C-6 position is phenoxylated. Further confirmation of the linkage position was provided by NOESY experiment. Observation of NOE correlation between the H-2 and H-2′ signals indicated that oxygen at C-3 is involved in connection to the counterpart unit.

The absolute configuration of bismorphine B (**2**) was determined by synthesis from optically active morphine.

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Table 1. NMR Data for Bismorphine B (**2**) in DMSO-d_6 [a]

position	δ_C	δ_H
1	119.5	6.57 (d, 8.2)
2	119.1	6.50 (d, 8.2)
3	137.2	
4	146.6	
5	92.4	4.68 (d, 6.2)
6	66.3	4.08 (m)
7	133.3	5.27 (ddd, 9.5, 3.0, 2.9)
8	128.4	5.54 (brd, 9.5)
9	20.2	2.79 (d, 17.5), 2.07 (dd, 17.5, 6.5)
10	130.5	
11	125.1	
12	42.8	
13	40.3	2.50 (m)
14	57.9	3.19 (dd, 6.5, 3.0)
15	35.1	1.93 (ddd, 11.2, 11.0, 3.8), 1.61 (brd, 11.2)
16	45.8	2.44 (dd, 11.2, 3.8), 2.25 (ddd, 11.2 11.0, 3.8)
N-Me	42.3	2.28(s)
1′	151.9	
2′	106.3	5.87 (s)
3′	137.6	
4′	148.3	
5′	92.4	4.74 (d, 6.2)
6′	66.3	4.13 (m)
7′	133.3	5.31 (ddd, 9.5, 3.0, 2.9)
8′	128.4	5.57 (brd, 9.5)
9′	20.2	2.94 (d, 17.6), 2.27 (dd, 17.6, 6.5)
10′	124.3	
11′	124.4	
12′	42.4	
13′	40.6	2.58 (m)
14′	57.9	3.29 (dd, 6.5, 3.0)
15′	35.1	2.01 (ddd, 11.2, 11.0, 3.8), 1.66 (br d, 11.2)
16′	45.8	2.48 (dd, 11.2, 3.8), 2.28 (ddd, 11.2 11.0, 3.8)
N-Me	42.3	2.28 (s)

[a] ^{1}H NMR, 500 MHz, coupling constants (J in Hz) in parentheses; ^{13}C NMR, 125 MHz.

In this study, enzyme extracts from the capsules of opium poppies were used to synthesize this alkaloid. The fresh capsules were homogenized, and the homogenate was then centrifuged. The supernatant was used as crude enzyme extracts. When bismorphine B-forming activity was investigated using various conditions, the crude enzyme extracts catalyzed formation of the product corresponding to bismorphine B (**2**) only in the presence of H_2O_2. This enzymatic product was purified, and its optical rotation was measured. Since the optical rotation ($[\alpha]^{28}_D$ −67.7°) of the enzymatic product derived from (−)-morphine was almost identical with that ($[\alpha]^{28}_D$ −68.3°) of bismorphine B (**2**) isolated from the wounded capsules, we unequivocally confirmed that two morphine units in bismorphine B (**2**) have the same absolute configuration as (−)-morphine.

Previously, we reported that bismorphine A (**1**) acts as a cross-linker of the galacturonic acid-containing polysaccharide pectins by forming ionical bonds between the two amino groups of this alkaloid and the carboxyl groups of the galacturonic acid residues.[2] We assumed that bismorphine B (**2**) also has such function (Figure 1), because two amino groups exist in its molecule. To confirm this hypothesis, cross-linking ability for pectins was measured according to the previously described method.[2] Bismorphine B (**2**) was applied to an affinity matrix, which was prepared by coupling galacturonic acid to Sepharose 6B, and pectins were then loaded onto this matrix. After the pectins bound to the matrix through bismorphine bridges were eluted with HCl solution, the pectin amounts in the HCl eluate were determined using phenol/sulfuric acid reagent. Surprisingly, the amounts of pectins cross-linked to the matrix by bismorphine B (**2**) were much higher than those by bismorphine A (**1**) or Ca^{2+} (Figure 2), which is well known as a cross-linker for pectins.[4] These results demonstrated that bismorphine B (**2**) has the ability to more effectively cross-link pectins to each other than bismorphine A (**1**).

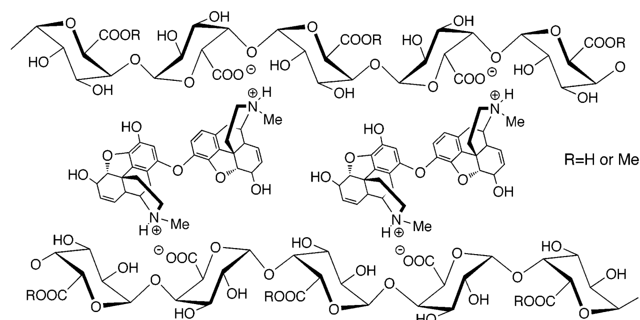

Figure 1. Possible model for interaction between bismorphine B (**2**) and pectins.

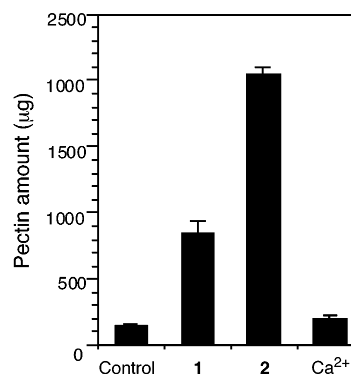

Figure 2. Amount of pectin bound to galacturonic acid-conjugated Sepharose 6B. Pectins were loaded onto galacturonic acid-conjugated Sepharose 6B pretreated bismorphine A (**1**, 12 μmol), bismorphine B (**2**, 12 μmol), and Ca^{2+} (11 μmol). Pectins bound to the matrix were quantified by phenol/sulfuric acid analysis. Control experiments were conducted using the same matrix without any pretreatment.[11] The data are the means of five replicated assays.

We also investigated the effect of bismorphine B (**2**) on pectinase reaction, because cross-linking of the cell wall polysaccharide pectins by bismorphine A (**1**) results in inhibition of their hydrolysis by pectinase.[2] Crude cell wall prepared from opium capsules was treated with bismorphine B (**2**) and incubated in the presence of pectinases. The reducing groups of pectin fragments that were produced from the cell wall by pectinase reaction were determined. The amounts (10.1 nmol/mg cell walls) of the reducing groups from the bismorphine B-treated cell wall were lower than those (73.2 nmol/mg cell walls) from the untreated cell wall, indicating that treatment of the cell wall with bismorphine B (**2**) inhibits hydrolysis of pectins by pectinases (∼86% inhibition). These properties were similar to those of bismorphine A (**1**), but bismorphine A treatment displayed a somewhat lower inhibitory effect (∼66% inhibition).[2]

In this study, we identified a new alkaloid, bismorphine B (**2**), in the wounded capsules of opium poppies. Bismorphine B (**2**) was not detectable in the intact capsules, but in response to stress opium poppies immediately biosynthesized this alkaloid by oxidative coupling of morphine. This biosynthetic reaction was considered to be catalyzed by peroxidases, because the crude enzyme extracts from opium capsules required H_2O_2 for its formation. Bismorphine B (**2**) effectively cross-linked pectins, resulting in strengthening of these polysaccharides. From these results, we concluded that formation of bismorphine B (**2**) in opium poppy is also a defense response. Pectins are cell wall polysaccharides which play important roles in cell adhesion

and assembly of cell wall components.[5−7] Therefore, the strengthening system for pectins is important.

Experimental Section

General Experimental Procedures. Optical rotations were determined on a DIP-4 digital polarimeter (JASCO). UV spectra were measured on a U-2001 spectrophotometer (Hitachi). ^{1}H and ^{13}C NMR spectra were obtained on a Varian Unity 500P spectrometer at 500 and 125 MHz, respectively, and all NMR spectra were measured in DMSO-d_6 using TMS as an internal standard. Mass spectra were recorded on a TMS-D300 instrument (JEOL). HPLC was conducted using a CCPM pump and a UV-8000 absorbance detector (Tosoh) equipped with a column (0.46 × 15.0 cm) of Cosmosil $5C_{18}$ AR-II (Nacalai). The eluate was monitored by absorption at 280 nm.

Plant Material. *P. somniferum* L. (opium poppy) was grown in the herbal garden of the Graduate School of Pharmaceutical Sciences, Kyushu University. This plant was identified by S.M., and a voucher specimen is on deposit at the Herbarium of the same Graduate School.

Extraction and Isolation. The capsules of opium poppies at 10−15 days after flowering were used in this study. Wounding of the capsules was carried out as previously reported. The opium poppies of which capsules were wounded were incubated for 48 h at 25 °C in the greenhouse. The wounded capsules (1250 g) were homogenized in 20 mM HCl, and the insoluble materials were removed by centrifugation at 20000*g* for 5 min. The pH of the HCl extracts was adjusted to 8.0 using 0.1 M NaOH. The resulting precipitates, which contain about 60 mg of bismorphine A (**1**), were removed by filtration, and the filtrate was concentrated under vacuum. The residue was subjected to a Si gel column ($CHCl_3$−MeOH−H_2O, 4:4:1), followed by an ODS column (Cosmosil $75C_{18}$-OPN, 1% acetic acid). The fractions containing bismorphine B (**2**) were further applied to preparative HPLC (50% aqueous acetonitrile containing 5 mM sodium di-2-ethylhexyl sulfosuccinate[8]) and then Si chromatography ($CHCl_3$−MeOH−H_2O, 4:4:1) to afford bismorphine B (**2**) (1.5 mg, 0.00012%).

Bismorphine B (2): off-white amorphous powder; $[\alpha]^{28}_{D}$ −68.3° (*c* 0.15, MeOH); UV (MeOH) λ_{max} (log ϵ) 284 (4.12) nm; ^{1}H NMR (DMSO-d_6), see Table 1; ^{13}C NMR (DMSO-d_6), see Table 1; FABMS *m/z* 569 $(M + H)^+$; *anal.* C 69.53%, H 6.24%, N 4.99%, calcd for $C_{34}H_{36}N_2O_6 \cdot H_2O$, C 69.61%, H 6.53%, N 4.77%.

Binding of Pectins to Galacturonic Acid-Conjugated Sepharose 6B. The 60 μM bismorphine B (**2**) solution (300 mL) was loaded onto the column (1.0 × 5.0 cm) containing galacturonic acid-conjugated Sepharose 6B. After 0.2% pectin solution (5 mL) was applied to the same column, the gel was washed with water (20 mL). The bound pectins were eluted with 20 mM HCl (20 mL), and the amounts of pectins in the HCl eluate were quantified by phenol/sulfuric acid analysis.

Enzymatic Synthesis of Bismorphine B (2). The fresh capsules of opium poppies (100 g) were homogenized in 100 mM phosphate buffer (pH 7.0, 100 mL) containing 3 mM mercaptoethanol, and the homogenate was filtered through Nylon filters. The filtrate was centrifuged at 100000*g* for 15 min. The supernatant was dialyzed against 10 mM phosphate buffer (pH 7.0, 5000 mL) and then used as crude enzyme extracts. These procedures for preparation of the enzyme extracts were carried out at 4 °C. The substrate solution (300 mL) containing 5 mM morphine, 50 mM Tris-HCl (pH 8.5), and 5 mM H_2O_2 was incubated at 30 °C for 24 h with the crude enzyme extracts. The resulting precipitates, which mostly consist of bismorphine A (**1**), were removed by centrifugation at 20000*g* for 10 min, and the supernatant was directly subjected to a Cosmosil $75C_{18}$-OPN column. After elution with 1% acetic acid solution, the fractions containing bismorphine B (**2**) were applied to preparative HPLC (50% aqueous acetonitrile containing 5 mM sodium di-2-ethylhexyl sulfosuccinate) to afford bismorphine B (**2**) (7.0 mg).

Pectinase Treatment of Crude Cell Wall. The crude cell wall (50 mg), which was prepared from the opium capsules as previously described,[2] was incubated at 30 °C for 30 min in 50 mL of 10 mM citrate buffer (pH 5.5) containing bismorphine B (**2**) (30 μg). *Aspergillus* pectinase (Sigma, 1 unit) was added to the bismorphine B-treated cell wall and incubated at 30 °C for 5 min. To extract pectins and their fragments from the cell wall, ammonium oxalate (100 mg) was added to the enzymatic reaction mixture and then heated at 85 °C for 1 h.[9,10] After centrifugation at 20000*g* for 5 min, the reducing groups in the supernatant were quantified by a dinitrosalicylic acid reagent.

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(11) A small amount of pectins bound to the matrix was observed in the control experiments. We assumed that this may have been due to interaction between pectins and a few charged groups in Sepharose 6B.

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