Synthesis and Anti-tubulin Activity of Ustiloxin D Derivatives

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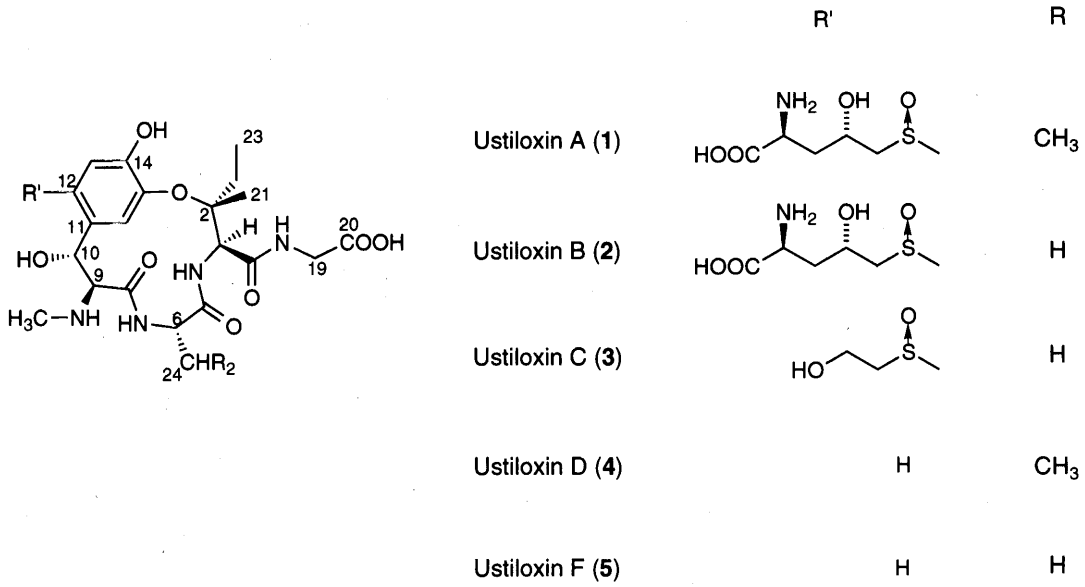
Ustiloxin D, produced by the rice plant pathogen *Ustilaginoidea virens*, exhibits potent anti-tubulin activity. In order to elucidate the effects of functional groups in ustiloxin D on its activity, several derivatives were synthesized and their anti-tubulin activities were estimated. The *N*,*N*-dimethylamino derivative and the 14-*O*-methyl derivative were inactive ($IC_{50} > 50\ \mu M$). 20-Hydroxymethylated ustiloxin D showed decreased inhibitory activity compared with ustiloxin D.

There are a number of natural and synthetic compounds that interfere with microtubule functions by binding to tubulin.[1] Maytansine, rhizoxin, dolastatin 10, phomopsin A, ustiloxin A and arenastatin A are known to share the same binding site (RZX-MAY site) on tubulin.[2~4] Their structural diversity makes it difficult to identify the common structural elements required for binding to this site. However, a common structural feature was found in ustiloxins (**1**~**5**) (Figure 1)[5~7] and phomopsins (**6**, **7**) (Figure 2)[8], and it was suggested that the 13-membered cyclic core involving an aromatic ring may play an essential role in the binding to tubulin. We were therefore interested in using ustiloxin D, the simplest rhizoxin site ligand with potent anti-tubulin activity[5,7], as a probe to examine the structural requirements for binding to the rhizoxin site. In the preceding paper,[7] we described the preparation of ustiloxin D (**4**) by platinum (PtO_2)-catalyzed desulfurization of ustiloxin A (**1**), the

Fig. 1. Structures of ustiloxins A~D (**1**~**4**) and F (**5**).



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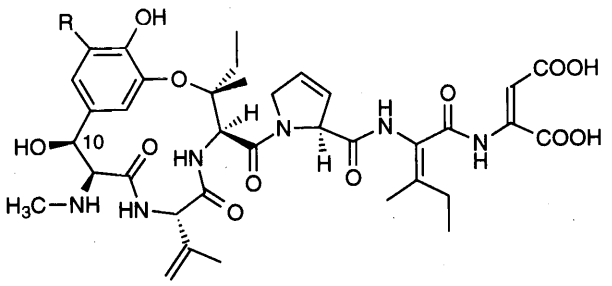
most abundant analog isolated from false smut balls. In this paper, we report on the chemical modification of ustiloxin D and the activities of the derivatives thus obtained against microtubule assembly.

Fig. 2. Structures of phomopsins A (**6**) and B (**7**).



Phomopsin A (**6**) R=Cl

Phomopsin B (**7**) R=H

Results and Discussion

To find the essential functional groups required for binding to tubulin, several derivatives were synthesized from ustiloxin D (**4**) as shown in Scheme 1. First, **4** was esterified with ethanol/0.2 N hydrochloric acid to give the ethyl ester **8** in 63% yield. *N*-Methylation of **8** to yield **9** was performed with H_2/Pd-C in methanol (20% yield).[9] The ester **9** was also prepared by using CH_3I/KF-Al_2O_3[10] in CH_3CN, in 45% yield from **8**. *O*-Methylation of the phenolic hydroxyl group of **8** was carried out with CH_3I/Cs_2CO_3 in DMF to give **10** in 80% yield. The alcohol **11** was prepared by borane reduction of **4** in 70% yield. The ^1H NMR data for compounds **8**~**11** are shown in Table 1. Modifications of the hydroxy group at the C(10) position, including its removal, were also attempted, but without success.

The inhibitory activity towards microtubule assembly was evaluated for these compounds. The IC_{50} value (concentration required for 50% inhibition of tubulin

Scheme 1.

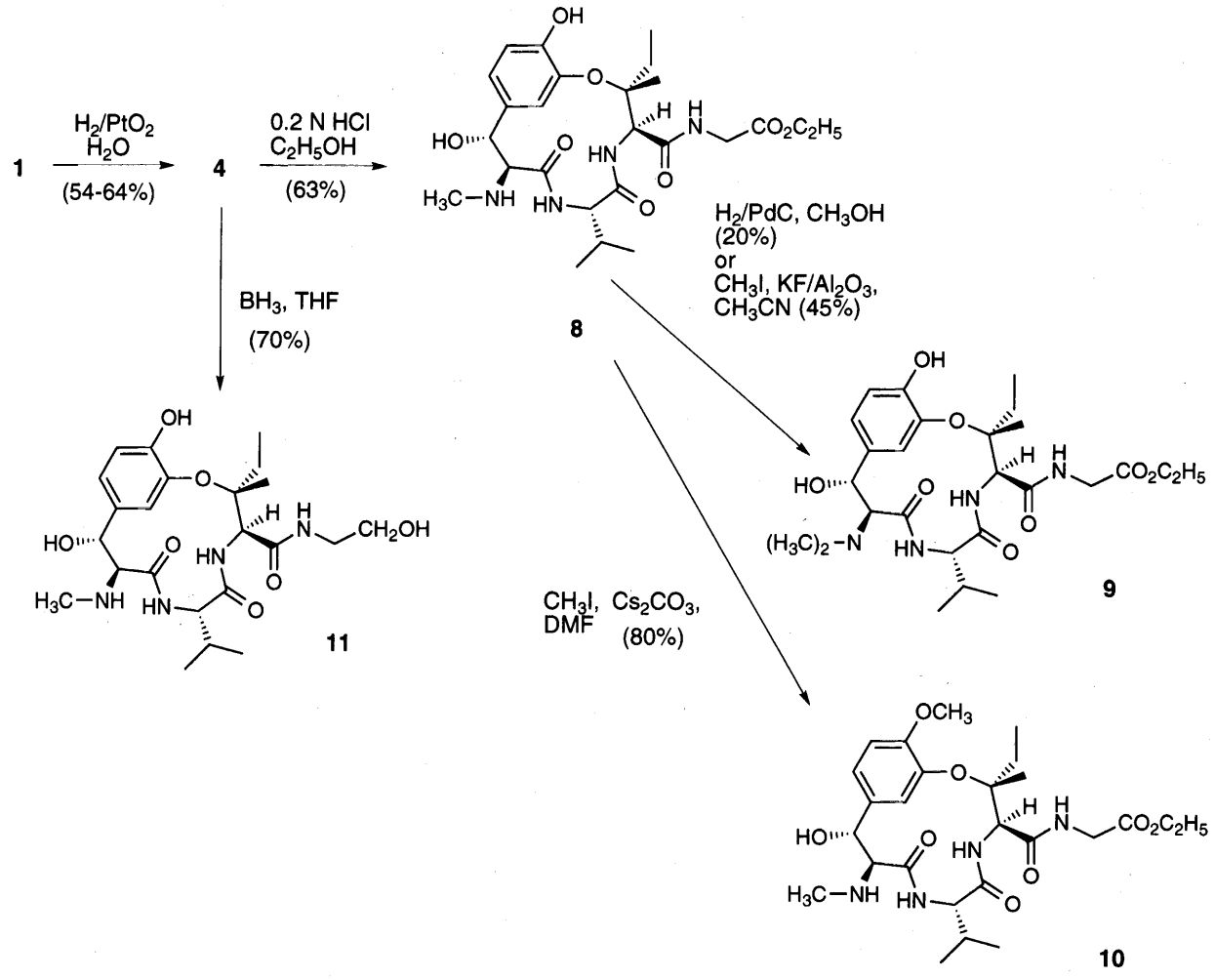


Table 1. ^{1}H NMR chemical shifts (δ_H ppm), multiplicities[a] and coupling constants (J, Hz) of ustiloxin D derivatives (**8**~**11**) and FGHMBC correlation of **8**.

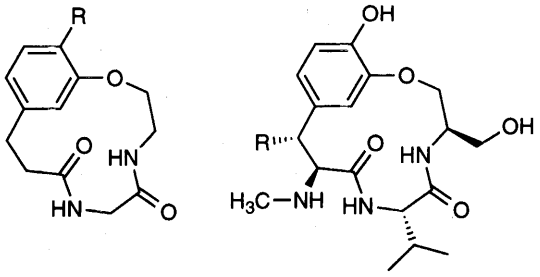
H No.	**8**[b]		**9**[b]	**10**[b]	**11**[c]
3	4.77 s	2, 17, 21	4.59 s	4.82 s	4.63 s
6	4.02 d, 8.0	5, 24, 25, 26	3.98 d, 8.0	4.01 d, 9.0	3.83 d, 9.5
9	3.26 d, 8.0	8, 10, 11, N-CH_3	3.23 d, 10.0	3.22 d, 7.5	3.13 d, 9.0
10	4.54 d, 8.0	8, 9, 11, 12, 16	*ca.* 4.8[d]	4.55 d, 8.5	4.34 d, 9.0
12	7.06 dd, 2.0, 8.0	16	7.25 drd, 8.8	7.12 dd, 8.5, 2.0	6.97 dd, 8.5, 2.0
13	6.84 d, 8.0	11, 14, 15	6.89 d, 8.8	6.94 d, 8.5	6.83 d, 8.5
16	7.07 brs	12, 14, 15	7.25 brs	6.99 brs	6.76 d, 2.0
19	3.91 d, 17.5	17, 20	3.96 d, 17.5	3.89 d, 17.5	3.20 m
	3.96 d, 17.5	17, 20	4.05 d, 17.5	3.93 d, 17.5	—
20	—		—	—	3.51 m
21	1.64 s	2, 3, 22	1.74 s	1.55 s	1.52 s
22	1.74 m	2, 3, 21, 23	1.66 m	1.71 m	1.50 dq, 14.0, 7.5
	2.05 m	2, 3, 21, 23	2.09 m	2.01 m	1.94 dq, 14.0, 7.5
23	1.01 t 7.0	2, 22	0.89 t, 7.5	1.07 t, 7.5	0.92 dd, 7.5, 7.5
24	2.05 m	6, 25, 26	2.09 m	2.01 m	1.72 m
25	0.84 d, 6.8	6, 24, 26	0.83 d, 7.0	0.82 d, 6.8	0.60 d, 6.5
26	0.86 d, 6.8	6, 24, 25	0.86 d, 7.0	0.86 d, 6.8	0.69 d, 6.5
N-CH_3	2.36 s	9	—	2.34 s	2.16 s
N-$(CH_3)_2$	—		2.44 s	—	—
OCH_2CH_3	1.25 t, 7.0	CH_2CH_3	1.24 t, 7.0	1.25 t, 7.0	—
OCH_2CH_3	4.16 q, 7.0	20, CH_2CH_3	4.17 q, 7.0	4.16 q, 7.0	—
ph-OCH_3	—		—	3.81 s	—

[a] Multiplicities, s: singlet, d: doublet, t: triplet, q: quartet, m: multiplet, br: broad.
[b] Solvent: CD_3OD. Reference value: δ_H 3.30 ppm of methanol.
[c] Solvent: D_2O. Reference value: δ_H 4.65 ppm of water.
[d] Overlapped with other signals.

Table 2. Inhibition of porcine brain microtubule assembly.

Compound No.	IC_{50} (μM)
1	1.0[7]
2	1.8[7]
3	4.4[5]
4	2.5[7]
5	10.3[7]
8	5.6
9	>50
10	>50
11	14.0[11]

Fig. 3. Structures of synthesized 13-membered peptides (**12**~**15**).



12 R=H
13 R=OH

14 R=H
15 R=OH

polymerization) of **8** was found to be 5.6 μM (Table 2). Although the ester **8** exhibits potent anti-tubulin activity, the *N*,*N*-dimethylamino derivative **9** and *O*-methyl derivative **10** were inactive (IC_{50} > 50 μM). Interestingly, the alcohol **11** showed moderate inhibitory activity (IC_{50} = 14 μM).[11]

In addition to the chemical modification of natural ustiloxins, we have synthesized several ustiloxin analogs with simplified core structures (**12**~**15**) (Figure 3)[12,13]. However, the compounds so far synthesized are all inactive (IC_{50} > 100 μM).

To summarize the present results: (1) the CH_3NH group and the phenolic hydroxy group play important roles in the binding to tubulin. (2) The carboxylic acid group of the glycine residue may have some positive effect

on the activity, but is not essential, since the ester **8** and the alcohol **11** showed strong to moderate activity. (3) Alkyl substituents at the C(2) and C(6) positions on the 13-membered cyclic core seem to be important, because ustiloxin F (**5**) has much less activity than ustiloxin D (**4**)[7], and the synthetic analog **15** showed no activity[13].

Experimental

General

^1H and ^{13}C NMR spectra were measured on a JEOL ALPHA-500 NMR spectrometer at 500 and 125 MHz, respectively. FAB-MS and HRFAB-MS were measured on a JEOL JMS-HX110 instrument with the EBE arrangement. Thin layer chromatography was carried out on Merck Kieselgel 60F-254 plates, and HPLC was performed on a Shimadzu LC-10A apparatus.

Preparation of Microtubule Protein and Microtubule Assembly Assay

The procedures were the same as described in the previous paper.[5]

Ustiloxin D Ethyl Ester (8)

Thirty mg of ustiloxin D (**4**) was dissolved in 0.6 ml of 0.2 N HCl ($(C_2H_5)_2O/C_2H_5OH=1/4$), and the solution was stirred for 18 hours at room temperature. After addition of $NaHCO_3$ (36 mg), followed by stirring for a few minutes, the solid was filtered off, and the filtrate was concentrated with a rotary evaporator. The residue was purified on a silica gel column ($CH_3Cl/CH_3OH=4/1$), and 20 mg of **8** was obtained (63% yield). **8**: FAB-MS *m/z*: 523 (M+H); HRFAB-MS *m/z*: 523.2767. Calcd for $C_{25}H_{39}N_4O_8$ (M+H), 523.2768. ^1H NMR and FGHMBC correlation data are shown in Table 1. ^{13}C NMR (CD_3OD, reference value: δ_C 49.0 ppm of methanol) δ 85.4 (C-2), 60.6 (C-3), 171.9 (C-5), 61.6 (C-6), 172.6 (C-8), 71.7 (C-9), 74.8 (C-10), 133.1 (C-11), 124,6 (C-12), 118.2 (C-13), 152.2 (C-14), 144.0 (C-15), 123.1 (C-16), 171.5 (C-17), 42.1 (C-19), 170.8 (C-20), 22.0 (C-21), 32.5 (C-22), 8.7 (C-23), 30.0 (C-24), 19.4 (C-25), 18.5 (C-26), 34.1 (N-CH_3), 14.5 (CH_2CH_3), 62.2 (CH_2CH_3).

N-Methyl Ustiloxin D Ethyl Ester (9)

(i) Ustiloxin D ethyl ester (**8**) (1.5 mg, 3 mmoles) was dissolved in 0.15 ml of methanol, and 3 mg of 10% PdC was added to the solution with stirring. Stirring was continued under H_2 at room temperature, and the *N*-methylation was completed within 16 hours. The catalyst was filtered off, and the filtrate was concentrated with a rotary evaporator. The residue was chromatographed on silica gel ($CHCl_3/CH_3OH=4/1$) to give **9** (0.3 mg, 20% yield). (ii) A mixture of **8** (2 mg), KF-Al_2O_3[10] (3 mg, KF: 5 equiv. to **8**), and 0.5% CH_3I in CH_3CN (0.1 ml) (CH_3I: 2 equiv. to **8**) was stirred for 4 hours at room temperature. The solid was filtered off, and the filtrate was concentrated. Compound **9** was purified by silica gel TLC ($CH_3Cl/CH_3OH=4/1$). The yield of **9** was determined to be 45% from the ^1H NMR spectrum. **9**: FAB-MS *m/z*: 537 (M+H). HRFAB-MS *m/z*: 537.2889, Calcd for $C_{26}H_{41}N_4O_8$ (M+H), 537.2924. ^1H NMR data are shown in Table 1.

14-*O*-Methyl Ustiloxin D Ethyl Ester (10)

Cs_2CO_3 (73 mg) and 0.25% CH_3I/dimethylformamide (DMF) (0.1 ml, CH_3I: 1 equiv.) were added to a solution of **8** (2 mg, 4 mmoles) in DMF (0.1 ml), and the mixture was stirred for 30 minutes at room temperature. After filtration, the organic solvent was removed with an N_2 stream. The reaction product **10** was purified by TLC. The yield was 80% as judged from the ^1H NMR spectrum. A by-product of this reaction was the *N*-methylated compound **9**. **10**: FAB-MS *m/z*: 537 (M+H). HRFAB-MS *m/z*: 537.2885, Calcd for $C_{26}H_{41}N_4O_8$ (M+H), 537.2924. ^1H NMR data are shown in Table 1.

20-Hydroxymethylated Ustiloxin D (11)

Ustiloxin D (**1**) (5 mg, 0.01 mmole) was dissolved in tetrahydrofuran (THF) (0.5 ml) and the solution was cooled to 0°C. To this solution, 0.5 ml of 1 M BH_3-THF (0.5 mmoles) was added with stirring over a period of 10 minutes. After 30 minutes, the ice-bath was removed, and stirring was continued for 1 hour at room temperature. The reaction was quenched with saturated aqueous $NaHCO_3$, and the THF was removed with a rotary evaporator. Compound **11** was purified on a CHP 20P column (50% aqueous CH_3OH). 3.5 mg (70% yield). **11**: FAB-MS *m/z*: 481 (M+H). HRFAB-MS *m/z*: 481.2660, Calcd for $C_{23}H_{37}N_4O_7$ (M+H), 481.2662. ^1H NMR data are shown in Table 1.

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