Structures of New Indoloditerpenes, Possible Biosynthetic Precursors of the Tremorgenic Mycotoxins, Penitrems, from *Penicillium crustosum*[1)]

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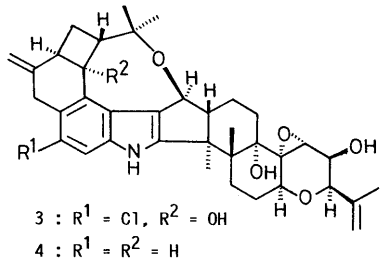
Along with the tremorgenic mycotoxins, penitrems A (3), B (4), D (6), E (7), and F (8), the minor metabolites 1 and 2 were isolated from the mycelia of *Penicillium crustosum* THOM, which was found contaminating bread intended for school lunches in Tokyo city. The structures of 1 and 2 were determined on the basis of spectroscopic and chemical investigations. These compounds (1 and 2) might be biosynthetic precursors of penitrems.

Keywords *Penicillium crustosum*; mycotoxin; indoloditerpene; penitrem A; penitrem B; penitrem C; penitrem D; penitrem E; penitrem F; PC-M5′; PC-M6; biosynthetic precursor

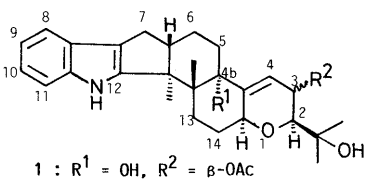
The tremorgenic mycotoxin, penitrem A (**3**), was first extracted from two strains of *Penicillium cyclopium* WESTLING that were the principal contaminants of feedstuffs causing mycotoxicoses in livestock.[2)] Later, these toxigenic isolates were assigned as *P. crustosum* THOM.[3)] Compound **3** has apparently been implicated in three different tremorgenic intoxications involving dogs, *i.e.* intoxication caused by a discarded package of moldy cream cheese in the U.S.A.,[4,5)] the "moldy walnut" toxicosis, which has been recognized for several years in walnut-producing areas of California,[6,7)] and intoxication involving a dog that had consumed a moldy hamburger bun in Australia.[8)] In the above three cases, the fungus was identified as *P. crustosum*, and the toxin was **3**. A natural human intoxication characterized by several symptoms including tremor has been recognized as being associated with the consumption of moldy beer.[9)] Thus, *P. crustosum* and penitrem A (**3**) were considered to be responsible for the mycotoxicosis.

The structure of penitrem A was established in 1983 as **3**.[10)] Penitrems B (**4**), C (**5**), D (**6**), E (**7**), and F (**8**) were isolated from *P. crustosum* as the minor metabolites.[11)] *P. crustosum* is ubiquitous as one of the food-borne Penicillia and occurs commonly in dairy products including butter and cheese.[12)] In 1983—1984, Udagawa and Sato isolated several strains of *P. crustosum* from retailed cocoa and bread collected at Shinagawa Ward Institute of Public Health Research, Tokyo. In the course of mycotoxicological examination for penitrems and related compounds, two new indoloditerpenes, PC-M5′ (**1**) and PC-M6 (**2**), were isolated along with penitrems A (**3**), B (**4**), D (**6**), E (**7**), and F (**8**) from the mycelia of one of the above strains (NHL 6491). The structures of **1** and **2** are reported in this paper.

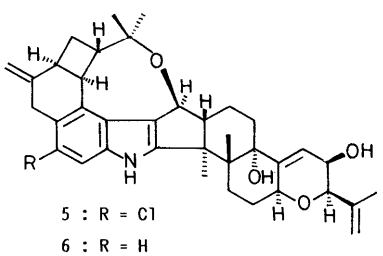
The molecular formulae of PC-M5′ (**1**) and PC-M6 (**2**) were confirmed as $C_{29}H_{37}NO_5$ and $C_{27}H_{35}NO_3$, respectively, by high-resolution electron impact ionization (EI) mass spectrometry. A positive coloration with van Urk's reagent (yellowish green or green)[13)] and the fragmentation ion at *m/z* 130 $[(C_9H_8N)^+]$[14)] in the EI-mass spectra (MS) suggested the presence of an indole moiety in both **1** and **2**. The proton nuclear magnetic resonance (^{1}H-NMR) signals of four aromatic protons of the indole moiety appeared at δ 6.94 (2H), 7.28 (1H), and 7.32 (1H) in **1**, and δ 6.90 (1H), 6.94 (1H), and 7.27 (2H) in **2**. The indole NH signal was observed at δ 9.85 in **1** and at δ 10.70 in **2**. The above results and carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectra (Table I) were explicable by assuming the presence of a 2,3-disubstituted indole moiety in PC-M5′ (**1**) and PC-M6 (**2**).



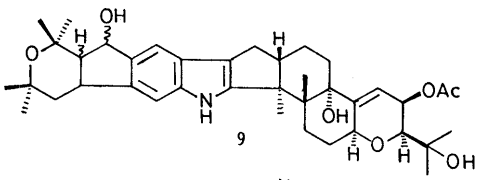
3 : R^1 = Cl, R^2 = OH
4 : R^1 = R^2 = H
7 : R^1 = H, R^2 = OH
8 : R^1 = Cl, R^2 = H



1 : R^1 = OH, R^2 = β-OAc
2 : R^1 = H, R^2 = β-OH
11 : R^1 = OH, R^2 = α-OAc



5 : R = Cl
6 : R = H



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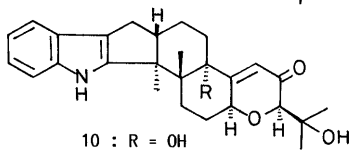

10 : R = OH
12 : R = H

TABLE I. ^{13}C-NMR Chemical Shifts of Indoloditerpenes in $(CD_3)_2CO$

Carbon[a] No.	1	2	6	9	10[b]	12[c]
2	82.56	80.91	80.39[d]	82.44	83.16	82.69
3	66.01	62.48	64.28	66.00	199.50	198.24
4	115.21	117.54	119.59	115.14	119.16	121.86
4a	151.29	144.11	148.44	151.26	169.45	168.27
4b	77.52	40.50	77.48	77.42	76.78	42.69
5	34.71	31.32	35.08	34.50	33.62	32.86
6	22.01	24.17	19.11	21.88	20.98	24.15
6a	50.67	48.94	58.87	50.27	49.57	49.01
7	30.56	29.15	72.17	27.85	28.58	30.05
7a	116.71	115.61	119.16	116.84	116.68	118.56
7b	126.11	124.32	123.18	127.67	124.94	125.07
8	118.66	118.34	128.80	114.08	118.18	118.64
9	119.54	119.13	128.09	139.74	119.16	119.82
10	120.36	119.27	120.91	131.57	120.02	120.87
11	112.45	111.75	110.22	103.36	111.61	111.64
11a	141.23	139.99	139.27	142.02	139.92	140.19
12a	153.84	150.73	153.49	155.64	152.15	149.25
12b	51.61	49.62	49.77	51.72	50.80	50.57
12b-Me	16.73	14.50	21.32	16.60	16.20	14.65
12c	43.53	40.37	43.71	43.37	43.04	42.48
12c-Me	19.93	15.41	20.11	19.87	19.37	16.30
13	27.87	26.15	27.68	27.70	27.16	25.61
14	29.13	26.87	29.24	29.14	27.34	27.35
14a	75.06	76.13	74.40[d]	75.03	72.79	74.84
1′	71.58	71.35	143.92	71.61	72.38	72.53
2′	25.99	26.46	110.75	25.95	24.19	24.27
3′	27.95	27.24	19.98	27.81	26.61	26.80
MeCOO	21.28			21.37		
MeCOO	170.69			170.86		

a) Numberings of related compounds correspond to those of **1**. *b*) The spectrum of **10** was measured in $CDCl_3$ containing a little $(CD_3)_2SO$. *c*) The spectrum of **12** was measured in $CDCl_3$. *d*) Assignments were revised.

The ^{1}H-NMR signal at δ 1.99, the ^{13}C-NMR signals at δ 21.28 and 170.69, and the infrared (IR) absorption at 1720 cm^{-1} of PC-M5′ (**1**) suggested the presence of an acetoxyl group in the molecule. The ^{1}H- and ^{13}C- (Table I) NMR spectra of the diterpene moiety of **1** were closely similar to those of janthitrem F (**9**), originally isolated from *P. janthinellum* BIOURGE.[15] Therefore PC-M5′ (**1**) was assumed to have a 3-*O*-acetoxyl group instead of the 3-oxo group of paxilline (**10**), the tremorgenic mycotoxin isolated from *P. paxilli* BAINIER[16] and *Emericella striata* (RAI, TEWARI & MUKERJI) MALLOCH & CAIN.[17]

In order to determine the absolute structure of PC-M5′ (**1**), **10** was reduced with sodium borohydride followed by acetylation to give two compounds (major, **1**; minor, **11**). The major compound was identical with PC-M5′, including the circular dichroism (CD) spectrum, whereas the minor one was determined to be the 3-epimer of **1** by comparison of the ^{1}H-NMR signals of the dihydropyran ring of **1** and **11**. The ^{1}H-NMR signal at δ 5.20 assigned at 3-H in **1** was coupled with 2-H (δ 3.32) with J=1.8 Hz, whereas the ^{1}H-NMR signal at δ 5.48 (3-H) in **11** was coupled with the signal at δ 3.25 assigned to 2-H with J=9.0 Hz. From the above results, it was confirmed that **1** has a 3β-hydroxy group (axial) and **11** has a 3α-hydroxy group (equatorial), as judged from a molecular model of the dihydropyran ring of indoloditerpene. The absolute structure of PC-M5′ (**1**) was, consequently, confirmed as **1**.

The four ^{1}H-NMR signals at δ 3.00, 3.92, 4.04, and 5.45 of PC-M6 (**2**), together with their coupling patterns, suggested the presence of a dihydropyranol ring in the indoloditerpene moiety. The ^{13}C-NMR signal at δ 40.50, assigned to C-4b in **2**, was markedly shifted upfield compared with those of PC-M5′ (**1**) and paxilline (**10**). Furthermore, the ^{1}H-NMR signal at δ 3.92 (14a-H) in **2** was shifted upfield compared with those in **1** and **10** (δ 4.65 and 4.63, respectively). The above results suggested the structure of PC-M6 to be **2**, *i.e.*, the dihydro derivative of dehydroxypaxilline (**12**), originally isolated from *E. striata*.[14] The reduction of **12** with sodium borohydride gave two compounds, the major one of which was identical with naturally occurring PC-M6 (**2**) by comparison of the spectral data including the CD curve. The new hydroxy group in **2** derived from **12** was determined to have β-configuration, because the protons at C-2 (δ 3.00) and at C-3 (δ 4.04) were coupled with each other with J=1.8 Hz, as in the case of PM-5′ (**1**). Therefore, the structure of PC-M6 (**2**), including the absolute stereochemistry, was confirmed.

It was proposed that penitrems are biosynthesized through dehydroxypaxilline (**12**) or its derivative dehydrated on the side chain, followed by reduction at C-3 and the condensation of two isoprene units. Mantle and Penn reported that exogenous **10** was directly incorporated to penitrem A (**3**) and PC-M6 (**2**), though the data were not given in their paper, in *P. jaczewskii* ZALESKI.[18] In our work, paxilline-type indoloditerpenes having a 3-hydroxy or 3-acetoxyl group, PC-M5′ (**1**) and PC-M6 (**2**), were isolated as minor metabolites along with penitrems (**3**—**8**) from the mycelium of *P. crustosum*, whereas no 3-oxoindoloditerpene such as paxilline (**10**) and **12** was detected in the extract of the above fungus. The above result suggested that penitrems (**3**—**8**) may be biosynthesized directly through 3-hydroxyindoloditerpene such as PC-M6 (**2**), but not directly through 3-oxoindoloditerpenes such as paxilline (**10**) and dehydroxypaxilline (**12**).

Experimental

Melting points were determined on a Yanagimoto micro-melting point apparatus and are uncorrected. EI-MS were taken with a JEOL JMD-D-300 spectrometer. Ultraviolet (UV) and IR spectra were recorded on a Hitachi U-3210 spectrophotometer and a JASCO IR-810 spectrophotometer, respectively. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a JEOL JNM-GX-400 spectrometer at 399.78 MHz and at 100.43 MHz, respectively, or ^{1}H-NMR spectra were taken with a JEOL JNM-GX-270 spectrometer at 270.17 MHz, using tetramethylsilane as an internal standard. The coupling patterns are indicated as follows: singlet = s, doublet = d, triplet = t, multiplet = m, and broad = br. CD curves were determined on a JASCO J-600 spectropolarimeter. Column chromatography was performed using Kieselgel 60 (Art. 7734, Merck). Low pressure liquid chromatography (LPLC) was performed on a Chemco Low-Prep 81-M-2 pump and glass column (10 i.d. × 150 mm) packed with Silica gel CQ-3 (30—50 μm; Wako). High speed liquid chromatography (HPLC) was performed on a Nihon Seimitsu NSP-800-15DX pump and pre-packed column [Senshu Pak Silica-430-N (10 i.d. × 300 mm). Thin layer chromatography (TLC) was conducted on pre-coated Kieselgel 60 F_{254} plates (Art. 5715, Merck). Spots on TLC plates were detected on the basis of their absorption of UV light, and/or by spraying van Urk's reagent and then heating.[12]

Isolation of Metabolites from *P. crustosum* *P. crustosum*, strain NHL 6491, isolated from contaminated bread which had been intended for school lunches in Shinagawa-ku, Tokyo, in 1983, was used throughout this investigation. *P. crustosum* was cultivated at 25 °C for 14 d in 200 Roux flasks containing 250 ml of Czapek–Dox medium supplemented with 0.2% yeast extract in each flask. The fresh mycelium was extracted with CH_2Cl_2 at room temperature.

The residue (9.2 g) obtained by evaporation of the extract was

chromatographed on silica gel with benzene–acetone (100 : 1) to give ergosterol (500 mg), with benzene–acetone (50 : 1) followed by purification by LPLC using benzene to obtain penitrems F (**8**) (30 mg) and B (**4**) (14 mg), with benzene–acetone (20 : 1) followed by purification by LPLC using the solvent system of benzene–acetone (100 : 1) to give penitrems A (**3**) (300 mg) and E (**7**) (40 mg), with benzene–acetone (10 : 1) followed by further purification by LPLC [benzene–acetone (50 : 1)] to obtain penitrem D (**6**) (100 mg), with benzene–acetone (5 : 1) followed by repeated purification by LPLC [benzene–acetone (20 : 1) and/or hexane–AcOEt–EtOH (12 : 6 : 1)] to give cyclopenin (38 mg) and PC-M5′ (**1**) (12 mg), with benzene–acetone (3 : 1) followed by purification by HPLC using the solvent system of benzene–AcOEt (5 : 2) to give PC-M6 (**2**) (4 mg), and with EtOH followed by alumina column chromatography [$CHCl_3$–MeOH–NH_4OH (200 : 1 : 1)] to give roquefortine (190 mg).

The culture filtrate was extracted with CH_2Cl_2 after acidification. After evaporation of the solvent, the residue (6.0 g) was chromatographed on silica gel with benzene–acetone (5 : 1) followed by purification by LPLC using the solvent system of benzene–acetone (20 : 1) to give cyclopenin (32 mg).

PC-M5′ (**1**): Colorless crystalline powder, mp 162—164 °C. EI-MS *m/z* (%): 479.2669 (M^+, 479.2670 for $C_{29}H_{37}NO_5$, 63), 464.2433 [$(M-Me)^+$, 464.2433 for $C_{28}H_{34}NO_5$, 100], 182 (87), 130 (68). UV λ_{max}^{MeOH} nm (log ε): 229 (4.74), 279 (4.05). IR ν_{max}^{KBr} cm^{-1}: 3500, 3460, 3400 (NH, OH), 1720 (AcO). ^{1}H-NMR [$(CD_3)_2CO$] δ: 1.00 (3H, s, 12c-Me), 1.20 (3H, s, 2′-Me), 1.26 (3H, s, 3′-Me), 1.36 (3H, s, 12b-Me), 1.70 (3H, m), 1.82 (1H, dddd, *J* = 13.5, 13.5, 8.5, 5.2 Hz), 1.95 (1H, ddd, *J* = 13.5, 13.5, 4.5 Hz), 1.99 (3H, s, 3-OCOMe), 2.10 (1H, m), 2.39 (1H, dd, *J* = 13.0, 10.8 Hz, 7β-H), 2.67 (1H, dd, *J* = 13.0, 6.3 Hz, 7α-H), 2.69 (1H, ddd, *J* = 13.3, 13.3, 4.8 Hz), 2.85 (1H, m), 3.32 (1H, d, *J* = 1.8 Hz, 2-H), 3.48 (1H, s, OH), 3.49 (1H, s, OH), 4.65 (1H, br dd, *J* = 10.5, 7.6 Hz, 14a-H), 5.20 (1H, ddd, *J* = 5.8, 1.8, 1.8 Hz, 3-H), 5.69 (1H, dd, *J* = 5.8, 1.8 Hz, 4-H), 6.94 (2H, m, 9-H, 10-H), 7.28 (1H, m, 8-H or 11-H), 7.32 (1H, m, 11-H or 8-H), 9.85 [1H, br s, 12-H (NH)]. CD ($c = 4.55 \times 10^{-5}$, MeOH) $[\theta]^{20}$ (nm): −61000 (232), +4900 (286), +5000 (294).

PC-M6 (**2**): Colorless crystalline powder, mp (dec.) 260—263 °C. EI-MS *m/z* (%): 421.2616 (M^+, 421.2616 for $C_{27}H_{35}NO_3$, 64), 406 [$(M-Me)^+$, 100], 182 (82), 130 (47). UV λ_{max}^{MeOH} nm (log ε): 229 (4.64), 280 (3.98). IR ν_{max}^{KBr} cm^{-1}: 3560, 3420, 3270 (NH, OH). ^{1}H-NMR [$(CD_3)_2SO$] δ: 0.88 (3H, s, 12b-Me), 0.98 (3H, s, 12c-Me), 1.17 (3H, s, 2′-Me), 1.20 (3H, s, 3′-Me), 1.46 (1H, ddd, *J* = 11.5, 11.5, 4.6 Hz), 1.54 (1H, br d, *J* = 12.7 Hz), 1.61 (1H, m), 1.71 (2H, m), 1.80 (1H, ddd, *J* = 11.5, 11.5, 3.5 Hz), 1.84 (1H, ddd, *J* = 12.7, 12.7, 3.5 Hz), 2.03 (2H, m), 2.19 (1H, br d, *J* = 11.5 Hz), 2.31 (1H, dd, *J* = 12.7, 10.4 Hz, 7α-H), 2.60 (1H, dd, *J* = 12.7, 6.9 Hz, 7β-H), 2.70 (1H, m), 3.00 (1H, d, *J* = 1.8 Hz, 2-H), 3.92 (1H, br dd, *J* = 10.2, 7.2 Hz, 14a-H), 4.04 (1H, dd, *J* = 6.0, 1.8 Hz, 3-H), 5.45 (1H, d, *J* = 6.0 Hz, 4-H), 6.90 (1H, br t, *J* = 7.0 Hz, 9-H or 10-H), 6.94 (1H, br t, *J* = 7.0 Hz, 10-H or 9-H), 7.27 (2H, dd, *J* = 7.0, 1.2 Hz, 8-H, 11-H), 10.70 (1H, br s, NH). CD ($c = 4.84 \times 10^{-5}$, MeOH) $[\theta]^{20}$ (nm): −28000 (227), +5000 (276), +3900 (295).

Reduction of Paxilline (10) with Sodium Borohydride Followed by Acetylation $NaBH_4$ (50 mg) was slowly added to a stirred solution of paxilline (**10**) (97 mg) in MeOH (25 ml). After being stirred for 30 min at room temperature, the reaction mixture was poured into ice-water, acidified, and extracted with CH_2Cl_2. The residue obtained by evaporation of the extract was dissolved in a mixture of Ac_2O (2 ml) and pyridine (2 ml), and the solution was kept overnight at room temperature. The reaction mixture was poured into ice-water and extracted with CH_2Cl_2. The residue obtained by evaporation of the extract was purified by LPLC using hexane–AcOEt–MeOH (12 : 6 : 1) to give **1** (25 mg) and the 3-epimer of PC-M5′ (**11**) (5 mg). The above compound **1** was identical with PC-M5′ on the basis of a comparison of the IR, UV, EI-MS, ^{1}H-NMR, and CD spectra, and TLC behavior.

The 3-Epimer of PC-M5′ (**11**): Colorless crystalline powder. EI-MS *m/z* (%): 479 (M^+, 95), 464 [$(M-Me)^+$, 89], 182 (100), 130 (59). UV λ_{max}^{MeOH} nm (log ε): 229 (4.54), 280 (3.86). IR ν_{max}^{KBr} cm^{-1}: 3530, 3460, 3400 (NH, OH), 1730 (OAc). ^{1}H-NMR [$(CD_3)_2CO$] δ: 1.01 (3H, s, 12b-Me), 1.15 (3H, s, 2′-Me), 1.19 (3H, s, 3′-Me), 1.37 (12c-Me), 1.71 (2H, m), 2.00 (3H, s, 3-OAc), 2.04 (5H, m), 2.40 (1H, dd, *J* = 13.3, 10.5 Hz, 7β-H), 2.67 (1H, m), 2.67 (1H, dd, *J* = 13.3, 5.7 Hz, 7α-H), 2.84 (1H, m), 3.25 (1H, d, *J* = 9.0 Hz, 2-H), 4.68 (1H, ddt, *J* = 7.6, 2.4, 2.4 Hz, 14a-H), 5.31 (1H, dd, *J* = 2.4, 2.4 Hz, 4-H), 5.48 (1H, ddd, *J* = 9.0, 2.4, 2.4 Hz, 3-H), 6.94 (2H, m, 9-H, 10-H), 7.30 (2H, m, 8-H, 11-H), 9.85 (1H, s, NH). CD ($c = 5.30 \times 10^{-5}$, MeOH) $[\theta]^{20}$ (nm): −37000 (232), +2900 (275), +2600 (288).

Reduction of Dehydroxypaxilline (12) with $NaBH_4$ $NaBH_4$ (10 mg) was slowly added to a stirred solution of dehydroxypaxilline (**12**) (20 mg) in MeOH (5 ml). After being stirred for 30 min at room temperature, the reaction mixture was poured into ice-water, acidified, and extracted with CH_2Cl_2. The residue obtained after evaporation of the solvent was purified by LPLC using benzene–acetone (20 : 1) as the solvent to give **2** (5 mg). This compound was identical with PC-M6 on the basis of a comparison of the IR, UV, EI-MS, ^{1}H-NMR, and CD spectra, and TLC and HPLC behavior.

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