UCN-01 AND UCN-02, NEW SELECTIVE INHIBITORS OF PROTEIN KINASE C

II. PURIFICATION, PHYSICO-CHEMICAL PROPERTIES, STRUCTURAL DETERMINATION AND BIOLOGICAL ACTIVITIES

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A new inhibitor of protein kinase C (PKC), UCN-01, was isolated from the culture broth of *Streptomyces* sp. N-126. We have found that this strain also produces UCN-02 which is a stereoisomer of UCN-01. The inhibitors have the molecular formula $C_{28}H_{26}N_4O_4$ and have an indolo[2,3-*a*]carbazole chromophore. Their structures have been elucidated by mass and NMR spectra. UCN-01 has been shown to inhibit PKC and protein kinase A (PKA) with IC_{50} values of 0.0041 and 0.042 μM, respectively, and UCN-02 has been shown to inhibit PKC and PKA with IC_{50} values of 0.062 and 0.25 μM, respectively. UCN-01 and UCN-02 also showed the cytotoxic effect on the growth of HeLa S3 cells.

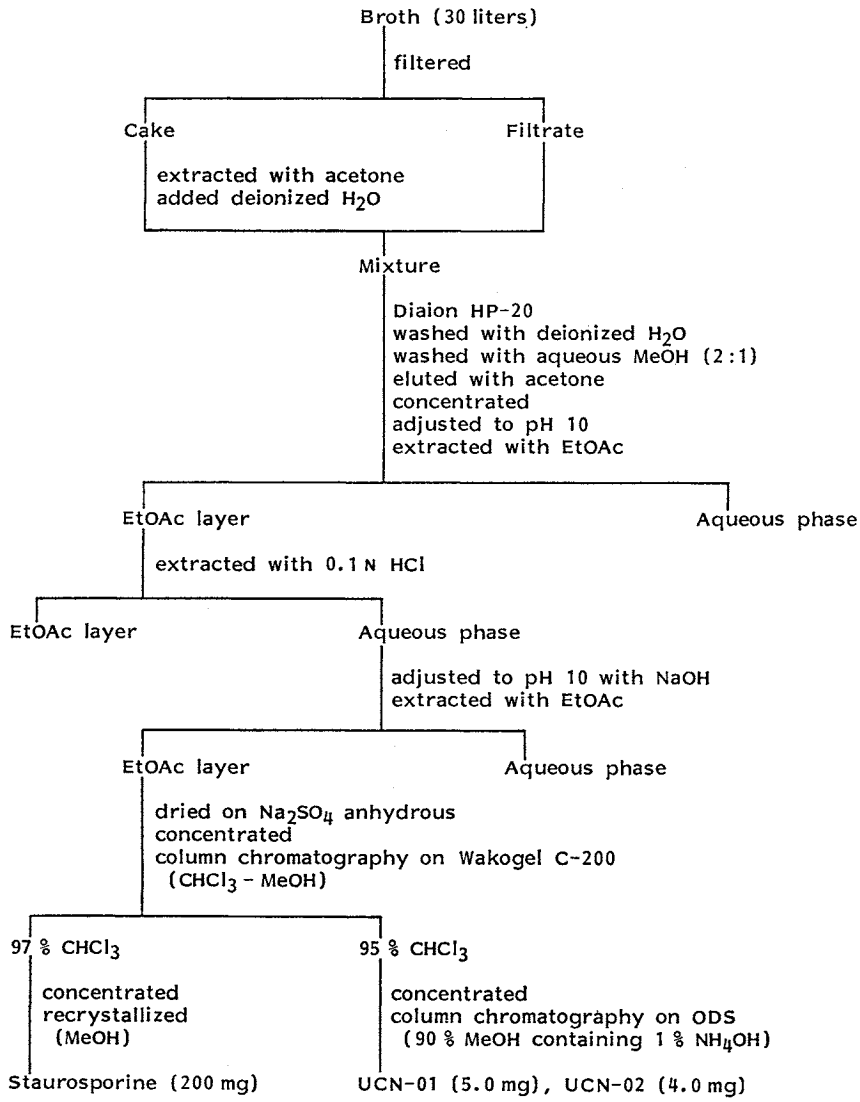
In the course of our screening program for new selective inhibitors of protein kinase C (PKC)[1~3], a *Streptomyces* strain N-126[4] was found to produce new selective inhibitors of PKC, UCN-01 and UCN-02. UCN-01 is a novel compound that has a hydroxyl group at C-7 of staurosporine[5,6], and UCN-02 is a stereoisomer at C-7 of UCN-01. They have been shown to inhibit PKC at extremely low concentration and showed the cytotoxic effect on the growth of HeLa S3 cells.

In this paper, their purification, physico-chemical properties, structures and biological activities are described.

Isolation and Purification

TLC and HPLC analysis were used to monitor UCN-01 and UCN-02 during isolation from the culture broth. The whole broth (30 liters) was filtered and the solid cake was extracted with acetone. Acetone extract was diluted with equivalent volume of water and then the extract was mixed with the filtrate of the broth. The mixed solution was applied on a column (2 liters) of non-ionic porous resin, Diaion HP-20 (Mitsubishi Chemical Industries Limited). After washing with water and then with aqueous methanol (2 : 1) to remove impurities, the column was eluted with acetone. The acetone eluate was evaporated to remove acetone. The aqueous solution was adjusted to pH 10 and extracted with ethyl acetate. The resulting organic phase was extracted with 0.1 N hydrochloric acid and after the separation the aqueous phase was immediately adjusted to pH 10 by addition of NaOH and extracted with ethyl acetate. The ethyl acetate extract was applied to silica gel (Wakogel C-200) column chromatography with chloroform - methanol as a elution solvent. The 97%-chloroform eluate was concentrated to give a pale yellow syrup, which was recrystallized from methanol to obtain 200 mg of

Fig. 1. Isolation and purification of UCN-01 and UCN-02.



staurosporine. The 95%-chloroform eluate was evaporated to dryness and the residue was applied to silica gel (LiChroprep Si 60) column chromatography with chloroform - methanol followed by reversed phase HPLC (ODS, 90% methanol containing 1% NH_4OH) to yield 5.0 mg of UCN-01 and 4.0 mg of UCN-02. The isolation and purification procedures are summarized in Fig. 1.

Physico-chemical Properties and Structure

In the preceding communication[3], we have reported the planar structure of UCN-01 (**1**) which differs from staurosporine (**3**) in C-7 hydroxyl group. In this paper, we describe the structure elucidation by 2D NMR spectroscopy in further detail, where the assignments of all signals in ^1H and ^{13}C NMR are presented.

Physico-chemical properties, ^1H and ^{13}C NMR data of **1** are summarized in Tables 1, 2 and 3, respectively. ^1H and ^{13}C NMR and proton homodecoupling experiments of **1** showed the presence

Table 1. Physico-chemical properties of UCN-01 and UCN-02.

	UCN-01	UCN-02
Appearance	Pale yellow needles	Pale yellow needles
MP (°C, dec)	245~250	245~250
Molecular formula	$C_{28}H_{26}N_4O_4$	$C_{28}H_{26}N_4O_4$
Elemental analysis		
Calcd for:	$+\frac{3}{4}$MeOH; C 68.17, H 5.77, N 11.06	$+\frac{1}{2}$MeOH; C 68.66, H 5.66, N 11.23
Found:	C 68.20, H 5.38, N 10.63	C 68.62, H 5.52, N 10.96
EI-MS (*m/z*)	482 (M^+), 353, 156	482 (M^+), 353, 156
FAB-MS (*m/z*)	483 $(M+H)^+$, 505 $(M+Na)^+$	483 $(M+H)^+$, 505 $(M+Na)^+$
Optical rotation $[\alpha]_D^{22}$	+132.0° (*c* 0.3, MeOH)	−38.6° (*c* 0.35, MeOH)
UV λ_{max}^{MeOH} nm (ε)	240 (29,000), 264 (sh, 20,000), 274 (sh, 21,000), 300 (55,000), 326 (sh, 9,600), 338 (sh, 8,300), 358 (7,500), 374 (8,500)	240 (29,000), 264 (sh, 20,000), 274 (sh, 21,000), 300 (55,000), 326 (sh, 9,600), 338 (sh, 8,300), 358 (7,500), 374 (8,500)
IR ν^{KBr} cm^{-1}	3500~3300, 1690, 1580, 1450, 1320, 1115, 1020, 750	3500~3300, 1670, 1580, 1450, 1310, 1100, 1020, 730
Rf value[a]	0.30	0.34
Color reaction		
Rydon Smith	+	+
Ninhydrin	+	+

FAB-MS: Fast atom bombardment mass spectra.

[a] Plate: Silica gel 60 (Merck, Art. No. 5554), solvent: $CHCl_3$ - MeOH (10 : 1).

Table 2. ^1H NMR data of UCN-01, UCN-02 and staurosporine (400 MHz, DMSO-d_6).

Position	Chemical shift[a] (*J*, Hz)		
	UCN-01	UCN-02	Staurosporine
1-H	7.60 (d, 8.2)	7.58 (d, 8.1)	7.56 (d, 8.1)
2-H	7.47 (br t)	7.46 (br t)	7.45 (ddd, 8.1, 7.6, 1)
3-H	7.28 (br t)	7.28 (br t)	7.27 (d, 7.6)[b]
4-H	9.22 (d, 7.9)	9.22 (d, 7.9)	9.30 (br d, 7.6)
6-H	8.72 (d, 1.1)	8.74 (br s)	8.51 (s)
7-H	6.39 (dd, 9.8, 1.1)	6.41 (br)	4.95 (s)
7-OH	6.44 (d, 9.8)	6.41 (br)	
8-H	8.43 (d, 7.9)	8.37 (d, 7.9)	7.96 (dd, 7.1, 1.2)
9-H	7.23 (br t)	7.25 (br t)	7.27 (t, 7.1)[b]
10-H	7.40 (br t)	7.41 (br t)	7.41 (ddd, 7.6, 7.1, 1.2)
11-H	7.96 (d, 8.6)	7.96 (d, 8.5)	7.97 (d, 7.6)
2′-CH_3	2.29 (d, 0.8)	2.29 (d, 0.8)	2.29 (s)
3′-H	4.09 (d, 3.6)	4.07 (d, 3.6)	4.03 (d, 3.7)
3′-OCH_3	3.37 (s)	3.34 (s)	3.33 (s)
4′-H	3.26 (m)	3.25 (m)	3.24 (m)
4′-NCH_3	1.53 (s)	1.45 (s)	1.44 (s)
4′-NH	0.93 (br)	0.80 (br)	0.76 (br)
5′-H	2.53 (m)[c]	2.49 (m)[c]	2.50 (m)[c]
6′-H	6.69 (dd, 4.3, 1.3)	6.69 (dd, 4.3, 1.3)	6.68 (dd, 4.9, 2.2)

[a] Chemical shift in ppm from TMS as an internal reference.

[b] These signals are overlapped. [c] These signals are overlapped with DMSO.

of two 1,2-disubstituted benzenes, amide carbonyl, hydroxymethine which proton coupled to amide proton, six quaternary carbons, one hexopyranose ring, one methoxy and one methylamino group. ^1H-^{13}C long range coupling were observed between aromatic protons (δ_H 8.43 and 9.22 ppm) and quaternary carbons (δ_C 114.5 and 113.8 ppm), respectively, amide proton (δ_H 8.72 ppm) and two

quaternary carbons (δ_C 118.1 and 134.1 ppm), and 6′-H of sugar moiety (δ_H 6.69 ppm) to another quaternary carbon (δ_C 127.2 ppm) by correlation spectroscopy *via* long range coupling (COLOC) experiment. These results revealed the existence of four structural moieties, that is two benzene rings bonded by one quaternary carbon (**Ia** and **Ib**), γ-hydroxy-γ-lactum containing two quaternary carbons (**Ic**) and sugar moiety bonded by quaternary carbon through heteroatom (**II**) as shown in Fig. 2. Observation of weak correlation peaks in COLOC spectrum between amide proton and two quaternary carbons (δ_C 114.5 and 113.8 ppm) belonging to partial structures **Ia** and **Ib** showed connectivity of partial structures **Ia**, **Ib** and **Ic**, which suggested the partial structure **I**.

Considering a molecular formula and building units of **1**, it is apparent that five quaternary carbons belonging to partial structures **I** and **II**, and remaining quaternary carbon (δ_C 130.1 ppm) constitute another aromatic ring system.

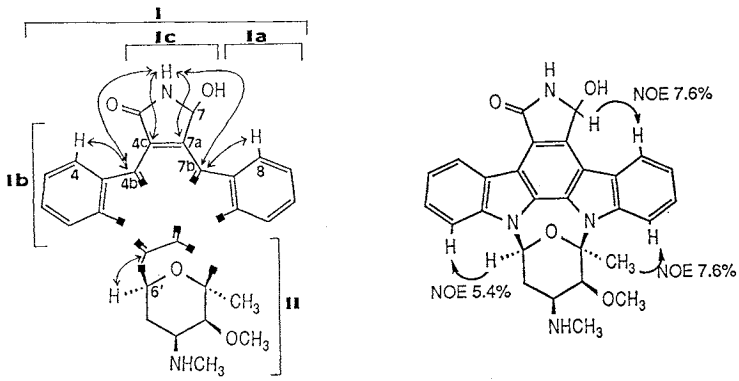
Sugar moiety **II** has five substituents. C-2′ and C-6′ were thought to be aminal carbon from their chemical shifts (δ_C 91.0 and 79.9 ppm, respectively) in ^{13}C NMR. The other three were methylamino, methoxyl and methyl group, the bonding position of which were confirmed to be

Table 3. ^{13}C NMR data of UCN-01, UCN-02 and staurosporine (100 MHz, DMSO-d_6).

Position	Chemical shift (multiplicity)		
	UCN-01	UCN-02	Staurosporine
C-1	108.2 (d)	108.2 (d)	108.2 (d)
C-2	125.0 (d)	125.0 (d)	124.8 (d)
C-3	119.0 (d)	119.0 (d)	118.9 (d)
C-4	125.4 (d)	125.3 (d)	125.5 (d)
C-4a	122.3 (s)	122.3 (s)	122.4 (s)
C-4b	113.8 (s)	113.7 (s)	113.4 (s)
C-4c	118.1 (s)	118.0 (s)	118.7 (s)
C-5	170.7 (s)	170.7 (s)	172.2 (s)
C-7	78.4 (d)	78.4 (d)	45.3 (t)
C-7a	134.1 (s)	134.2 (s)	131.9 (s)
C-7b	114.5 (s)	114.4 (s)	114.0 (s)
C-7c	123.4 (s)	123.4 (s)	123.8 (s)
C-8	122.9 (d)	122.5 (d)	120.7 (d)
C-9	119.3 (d)	119.3 (d)	119.6 (d)
C-10	124.3 (d)	124.4 (d)	124.2 (d)
C-11	114.9 (d)	114.9 (d)	115.1 (d)
C-11a	139.7 (s)	139.6 (s)	139.4 (s)
C-12a	130.1 (s)	130.1 (s)	129.9 (s)
C-12b	127.2 (s)	127.2 (s)	126.6 (s)
C-13a	136.7 (s)	136.5 (s)	136.3 (s)
C-2′	91.0 (s)	91.0 (s)	91.0 (s)
C-3′	82.7 (d)	82.7 (d)	82.7 (d)
C-4′	50.2 (d)	50.0 (d)	50.0 (d)
C-5′	29.3 (t)	29.2 (t)	29.3 (t)
C-6′	79.9 (d)	79.8 (d)	79.8 (d)
2′-CH_3	29.8 (q)	29.7 (q)	29.7 (q)
3′-OCH_3	57.2 (q)	57.1 (q)	57.2 (q)
4′-NCH_3	33.4 (q)	33.2 (q)	33.2 (q)

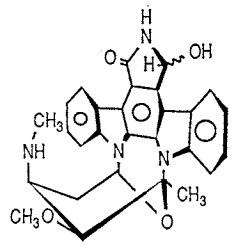
Chemical shift in ppm from TMS as an internal reference.

Fig. 2. ^1H-^{13}C Long range coupling and NOE experiments of UCN-01.

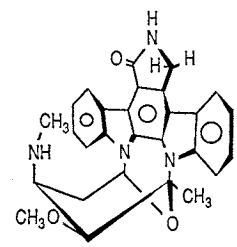


^1H-^{13}C Long range coupling

Fig. 3. The structures of UCN-01 (1), UCN-02 (2) and staurosporine (3).



1 and 2



3

4′, 3′ and 2′, respectively, by COLOC experiment. Stereochemistry of the substituents was determined as follows. 2′-N and 6′-N should be *cis*-diaxial relationship, because they constitute an oxadiazepine ring with chromophore. 4′-Amino proton (δ_H 0.93 ppm) and 4′-*N*-methyl protons (δ_H 1.53 ppm) were observed in extraordinary high field, due to anisotropy of the chromophore. Examination of Dreiding model shows that such an anisotropy effect could be observed only in the case when the 4′-methylamino group is β-axial conformation with chair conformation. This conformation was also assisted by that 4′-H proton appeared as multiplet with small coupling constants.

Table 4. IC_{50} value of UCN-01 and UCN-02 against protein kinases.

	IC_{50} (μM)		
	UCN-01	UCN-02	Staurosporine
PKC	0.0041	0.062	0.0027
PKA	0.042	0.25	0.0082

Then the partial structures **I** and **II** were connected. There were observed the nuclear Overhauser effects (NOE's) from 7-H to 8-H (7.6%), 2′-methyl to 11-H (7.6%) and 6′-H to 1-H (5.4%), which confirmed the attachment of N-12 to C-2′ and N-13 to C-6′. Thus the whole structure of **1** was determined as shown in Fig. 3 except the configuration of 7-hydroxy group.

UCN-02 (**2**), pale yellow needles from methanol, showed the same molecular formula as **1**, $C_{28}H_{26}N_4O_4$, determined by electron impact mass spectrum (EI-MS) and elemental analysis data. Physico-chemical properties, listed in Table 1, are similar to those of **1**, except for their optical rotation and Rf value on TLC.

All resonances in ^1H and ^{13}C NMR spectra of **2** were assigned as shown in Tables 2 and 3, by means of 2D NMR techniques. The difference in ^1H and ^{13}C NMR spectra between **1** and **2** is only in a 7 position, indicating **2** is a diastereomer of **1** at 7 position. Althogh in neutral buffer solution **1** and **2** were hard to be converted each other, **1** or **2** gave an equilibrium mixture of **1** and **2** in acidic or alkaline buffer solution. Thus it was confirmed that **2** is a stereoisomer at 7-OH of **1**.

Biological Activities

UCN-01 has been shown to inhibit PKC with IC_{50} value of 0.0041 μM and to inhibit protein kinase A (PKA) with IC_{50} value of 0.042 μM. While UCN-02 has also been shown to inhibit PKC and PKA with IC_{50} values of 0.062 and 0.25 μM, respectively, as shown in Table 4. Thus UCN-01 and UCN-02 selectively inhibited PKC activity. Therefore they are new selective inhibitors of PKC and will be valuable research tool to study the role of PKC in the regulation of cellular function. There are dif-

ferences in the IC_{50} value of PKC inhibition between UCN-01 and UCN-02, which will be of great interest to see how the configuration at C-7 contributes to the inhibition of PKC.

UCN-01 and UCN-02 exhibited no antimicrobial activity against *Candida albicans*, *Streptococcus faecium*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis*, *Proteus vulgaris*, *Shigella sonnei*, *Salmonella typhi* or *Klebsiella pneumoniae* at 100 μg/ml. UCN-01 and UCN-02 showed the cytotoxic effect on the growth of HeLa S3 cells with IC_{50} value of 0.0041 and 0.010 μg/ml, respectively, under the condition of 72 hours exposure. The LD_{50} value of UCN-01 is 25 mg/kg and no acute toxicity of UCN-02 was observed at 100 mg/kg in mice injected intraperitoneally.

UCN-01 and UCN-02 did not intercalate into DNA, nor cause single-strand or double-strand DNA breaks *in vitro* with agarose gel electrophoresis experiments whereas rebeccamycin and AT2433 clearly intact with DNA (data not shown). These results suggest that the cytotoxic effects of UCN-01 and UCN-02 are appeared by raising a disturbance of cell growth control through the inhibition of protein kinase. We are now investigating the antitumor activity of UCN-01 and UCN-02 against various tumor panels.

Experimental

General

NMR spectra were measured on a Jeol FX100 and Bruker AM400 spectrometer. Mass spectra were obtained on a Hitachi M-80B spectrometer. IR spectra were measured with a Shimadzu IR-27G spectrometer. UV spectra were taken with a Hitachi 200-20 spectrometer. Optical rotations were measured with a Perkin-Elmer 141 polarimeter. MP's were taken with a Yanagimoto micro melting point apparatus and were not corrected. TLC was performed on precoated plates, Merck Kieselgel 60 F_{254} and detected with UV light (254 nm).

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