CJ-12,371 and CJ-12,372, Two Novel DNA Gyrase Inhibitors

Fermentation, Isolation, Structural Elucidation and Biological Activities

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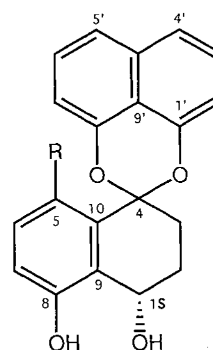
A fermentation broth of an unidentified fungus (N983-46) was found to produce DNA gyrase inhibitors, CJ-12,371 (**1**) and CJ-12,372 (**2**). Following isolation by solvent extraction and silica gel and ODS (reverse phase) chromatographies, the structures were determined to be novel spiro-ketal compounds with *S*-configuration at position C-1. CJ-12,371 and CJ-12,372 inhibit both DNA supercoiling and relaxation mediated by *Escherichia coli* DNA gyrase. The interaction of these compounds with DNA gyrase appears to be novel in that the compounds inhibit supercoiling and relaxation without blocking religation; thus, no cleavage intermediate of double strand DNA is observed. Both compounds have antibacterial activity against several species of pathogenic Gram-positive bacteria, with MICs between 25 and 100 μg/ml. These results suggest that the antibacterial potency of CJ-12,371 and CJ-12,372 is attributed to the inhibition of DNA gyrase. However, the compounds did not inhibit DNA gyrase selectively, as they also inhibited eukaryotic topoisomerase II-mediated relaxation. Semi-synthetic modifications to the dihydroxy motif in CJ-12,371 altered both gyrase- and topoisomerase II-inhibitory activities, but did not enhance selectivity.

Type II topoisomerases are key enzymes involved in transcription, DNA replication and nucleoid segregation[1,2]. Procaryotic topoisomerase II (DNA gyrase) is an important target for antibacterial agents as exemplified most recently by the quinolone antibiotics[3,4]; the eukaryotic topoisomerase II is a clinically proven target for anticancer drugs[5,6]. There are two well-characterized classes of DNA gyrase inhibitors: the quinolone class and the coumarin class. Quinolones are unusual antibiotics in that they are totally synthetic derivatives rather than secondary metabolites; compounds of this structural type interact with the A subunit of DNA gyrase, disrupting the DNA breakage-reunion reactions of supercoiling[3,4,7]. The coumarins such as novobiocin and coumermycin A_1 are microbially derived and interact with the B subunit, inhibiting ATPase activity[3,8,9]. Coumarins are not widely used to treat infectious diseases due to the rapid selection of resistance and toxicity; quinolone antibacterial agents are currently used for treatment of skin/soft tissue, respiratory tract, sexually transmitted diseases and urinary tract infections[10]. However, new classes of DNA gyrase inhibitors are required due to emergence of resistance in important clinical strains[11] and the toxicity of quinolone antibacterial agents[12]. In a screening program designed to discover new DNA gyrase inhibitors from microbial secondary metabolites, a fungal strain N983-46 was found to produce two novel compounds, CJ-12,371 (**1**) and CJ-12,372 (**2**) (Fig. 1). In this paper, we report the fermentation, structural elucidation and biological activities of CJ-12,371 and CJ-12,372.

Producing Organism

The producing strain was provided from MYCO-

Fig. 1. Absolute structures of CJ-12,371 (**1**), CJ-12,372 (**2**) and their *p*-*N*,*N*-dimethylaminobenzoates (**3** and **4**).



1: R= H, **2**: R=OH, **3**: 1,8-Di-*O*-*p*-*N*,*N*-dimethylaminobenzoyl-CJ-12,371 **4**: 1,5,8-Tri-*O*-*p*-*N*,*N*-dimethylaminobenzoyl-CJ-12,372.

search Laboratory, U.S.A. and deposited as FERM BP-3993 at National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology (Tsukuba, Japan). The taxonomical details of the strain were studied by KATZ[13].

Fermentation

The fungal strain N983-46 was maintained on potato dextrose agar slant. A vegetative cell suspension from a slant of the fungal strain N983-46 was inoculated into a 500-ml flask (first seed culture) containing 100 ml of seed medium, consisting of 2% glucose, 0.5% Polypepton (Nihon Pharmaceutical Co., Ltd.), 0.5% yeast extract, 0.5% wheat gluten, 0.3% beef extract, 0.3% blood meal and 0.3% $CaCO_3$ (adjusted to pH 7.0 before sterilization). The flask was incubated on a rotary shaker (220 rpm) at 26°C for 4 days. The first seed culture (30 ml) was transferred to four 500-ml flasks (second seed culture), each containing 150 ml of the seed medium and incubated on a rotary shaker at 26°C for 4 days. The second seed culture (600 ml) was transferred to four 6-liter jar fermentors with a small propeller-type impeller (7 cm diameter), each containing 3 liters of production medium, consisting of 2% glucose, 3% dextrin, 0.5% Polypepton, 1% Polypepton-S (Nihon Pharmaceutical Co., Ltd.), 1% corn steep liquor, 0.3% NaCl, 0.3% $CaCO_3$ and 0.0001% $CoCl_2 \cdot 6H_2O$ (adjusted to pH 6.5 before sterilization). The fermentation was carried out at 26°C for 3 days with an aeration rate of 3 liters/minute and an agitation rate of 1,700 rpm.

Isolation of CJ-12,371 (1) and CJ-12,372 (2)

The fermentation broth (15 liters) was freeze-dried, extracted with 70% aqueous acetone (5 liters) and then filtered. The filtrate was evaporated under reduced pressure to an aqueous suspension and extracted with ethyl acetate (2 liters × 2). The ethyl acetate layer was dried over anhydrous Na_2SO_4 and evaporated. The resultant oily residue (11 g) was loaded on a silica gel column (350 ml, Merck Kieselgel 60, 70 ~ 230 mesh) and a step-gradient of *n*-hexane - ethyl acetate - methanol mixture was applied. The active fractions (1.80 g, eluted with *n*-hexane - ethyl acetate, 1 : 1) were applied onto a Sephadex LH-20 column (1 liter) and eluted with methanol to give yellowish amorphous solids (949 mg). A part (600 mg) of the amorphous substance was subjected to reverse phase chromatography (Kusano Kagakukikai Co., C.I.G. prepacked column C-18) with methanol - water (7 : 1), and pure CJ-12,371 (**1**, 363 mg) and CJ-12,372 (**2**, 196 mg) were obtained. Recrystallization of each compound was also performed with *n*-hexane - ethyl acetate to give colorless plates.

Physico-chemical Properties

The physico-chemical properties of CJ-12,371 (**1**) and CJ-12,372 (**2**) are summarized in Table 1. They are moderately soluble in methanol, pyridine, ethyl acetate, chloroform and benzene and extremely soluble in dimethyl sulfoxide (DMSO) and in a mixture of chloroform and methanol. Both compounds melted around 180°C when the temperature was increased at a rate faster than 3°C/minute. However, they appeared to partially sublime at temperatures higher than 170°C, with mist forming around the crystals if temperature was increased slowly (1°C/minute). Decomposition was complete at the temperatures specified in Table 1.

Structural Elucidation

Structural Elucidation of CJ-12,371 (1)

The molecular formula of CJ-12,371 was determined to be $C_{20}H_{16}O_4$ by HREI-MS (*m/z* 320.1053, Δ0.4 mmu); the formula corresponds to the number of protons and carbons observed by NMR. ^1H NMR data (Table 2) indicated a total of 16 protons: 9 aromatic methines between 7.6 and 6.9 ppm, 1 oxymethine at 5.01 ppm, 4 aliphatic protons between 2.3 and 1.8 ppm and 2 D_2O-exchangeable protons at 9.72 and 5.17 ppm. The signal at 9.72 ppm apparently belongs to the phenolic-OH, which is visualized by $FeCl_3$. The other exchangeable proton at 5.17 ppm is an aliphatic-OH which is coupled to oxymethine at 5.01 ppm with 4.9 Hz. Examination of coupling constants, decoupling experiments and COSY revealed 4 fragments: $-CH_2-CH_2-CH(OH)$ system and 3 sets of 1,2,3-trisubstituted benzene. ^{13}C NMR data (Table 2) showed a total of 20 resonances. The DEPT spectra revealed 7 sp^2 singlets (including 3 oxygen-bearing sp^2 singlets between 156 and 147 ppm), 9 sp^2 doublets, 1 ketal sp^3 singlet (100 ppm), 1 oxygen-bearing sp^3 doublet and 2 sp^3 triplets. The one bond HETCOR experiment allowed to assign the carbons

Table 1. Physico-chemical properties of CJ-12,371 (**1**) and CJ-12,372 (**2**).

	CJ-12,371 (**1**)	CJ-12,372 (**2**)
Formula	$C_{20}H_{16}O_4$	$C_{20}H_{16}O_5$
HREI-MS (*m/z*, M^+)		
Found:	320.1053	336.1001
Calcd:	320.1049	336.0998
Appearance	Colorless plates	Colorless plates
MP (°C)	>265 (dec)	>238 (dec)
$[\alpha]_D^{24}$ (MeOH)	−46.8° (*c* 0.23)	−82.0° (*c* 0.22)

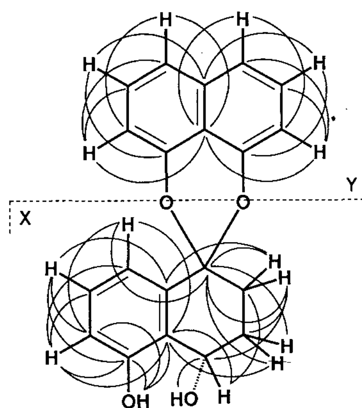
^1H and ^{13}C NMR: See Table 2.
UV and CD: See Table 3.
IR and EI-MS: See Experimental section.

Table 2. ^{1}H and ^{13}C NMR assignments of CJ-12,371 (**1**) and CJ-12,372 (**2**) in DMSO-d_6.

Atom No.	CJ-12,371 (1)		CJ-12,372 (2)	
	^{13}C	^{1}H	^{13}C	^{1}H
1	61.0 d	5.01 (br q, J=4.5 Hz) 5.17 (d, J=4.9 Hz, -OH)	61.5 d	4.97 (br q, J=4.5 Hz) 5.11 (d, J=4.9 Hz, -OH)
2	27.7 t	1.99 (m), 1.83 (m)	27.6 t	1.90 (m), 1.79 (m)
3	25.4 t	2.26 (m), 1.99 (m)	26.9 t	2.30 (m), 2.01 (m)
4	100.0 s		101.0 s	
5	117.5 d	7.18 (dd, J=7.9, 1.5 Hz)	149.2 s	8.37 (s, -OH)
6	128.5 d	7.25 (t, J=7.9 Hz)	116.8 d	6.74 (d, J=8.4 Hz)
7	116.1 d	6.95 (dd, J=7.4, 1.5 Hz)	117.4 d	6.83 (d, J=8.4 Hz)
8	155.4 s	9.72 (s, -OH)	147.7 s	9.01 (s, -OH)
9	126.4 s		127.3 s	
10	135.5 s		120.4 s	
1′	147.8 s		147.9 s	
2′	109.1 d	6.99 (dd, J=7.4, 1.0 Hz)	108.8 d	6.93 (dd, J=7.4, 1.0 Hz)
3′	127.6 d	7.48 (t, J=7.4 Hz)	127.6 d	7.47 (t, J=7.4 Hz)
4′	120.2 d	7.55 (br d, J=8.4 Hz)	119.7 d	7.52 (br d, J=7.4 Hz)
5′	120.2 d	7.55 (br d, J=8.4 Hz)	119.7 d	7.52 (br d, J=7.4 Hz)
6′	127.6 d	7.45 (t, J=7.4 Hz)	127.6 d	7.45 (t, J=7.4 Hz)
7′	109.1 d	6.92 (dd, J=7.4, 1.0 Hz)	108.9 d	6.89 (dd, J=7.4, 1.0 Hz)
8′	147.5 s		147.7 s	
9′	113.0 s		112.7 s	
10′	133.7 s		133.8 s	

δ, DMSO-d_6: 2.50 and 39.5 ppm for ^{1}H and ^{13}C, respectively.

Fig. 2. Long range C-H correlations of CJ-12,371 (**1**) by HETCOR.



in the 4 fragments deduced from ^{1}H NMR. Further, the long range HETCOR experiment was carried out to connect the 4 fragments, all 8 quaternary carbons and phenolic-OH group, yielding partial structures **X** and **Y** (Fig. 2). In this experiment, no correlation was observed between **X** and **Y**. However, considering that **X** and **Y** contain all chemical elements in the molecular formula, the only way to satisfy the remaining one unsaturation is to connect **X** and **Y** through the oxygens to form a 6-membered spiro-ketal ring as shown in structure **1**.

Structural Elucidation of CJ-12,372 (2)

The structure of CJ-12,372 was determined by the similar procedure used to elucidate CJ-12,371 (**1**). The molecular formula $C_{20}H_{16}O_5$ was deduced from HREI-MS (m/z 336.1001, Δ0.3 mmu) and supported by the number of ^{1}H and ^{13}C resonances observed in NMR (Table 2). When compared to **1**, CJ-12,372 has an additional oxygen substituting for one of the aromatic protons, resulting in a phenolic-OH. This conclusion is supported by the appearance of an additional D_2O-exchangeable singlet at 8.37 ppm and an sp^2 singlet at 149.2 ppm, with the loss of an sp^2 doublet in ^{1}H and ^{13}C NMR. The long range HETCOR experiment confirmed the position of the second phenolic-OH and provided the structure (**2**), with full assignments of ^{1}H and ^{13}C signals.

Absolute Stereochemistry at C-1

The absolute stereochemistry at C-1 in CJ-12,371 (**1**) and CJ-12,372 (**2**) was determined as S-configuration by applying the CD exciton chirality method[14] to the O-p-N,N-dimethylaminobenzoyl derivatives (**3** and **4**). The UV and CD data are summarized in Table 3.

The CD spectra of **3** and **4** clearly showed a positive first Cotton effect (at 329 and 331 nm for **3** and **4**, respectively) and a negative second Cotton effect (at 306 and 309 nm for **3** and **4**, respectively). These strong split Cotton effects are apparently due to the CD exciton coupling between O-p-N,N-dimethylaminobenzoyl

groups at C-1 and C-8. This positive exciton chirality indicates that C-1 has *S*-configuration in **3** and **4**. The exciton interaction between *O*-benzoyl groups at C-1 and C-5 does not affect the final assignment of the absolute configuration in **4** because the 1,5-interaction is relatively weak due to the remote distance compared to 1,8-interaction. The other exciton interactions among benzoates, naphthalene and benzene moieties in **3** and **4** are also small enough to be ignored.

Table 3. UV and CD data of CJ-12,371 (**1**), CJ-12,372 (**2**) and their *p*-*N*,*N*-dimethylaminobenzoates (**3** and **4**).

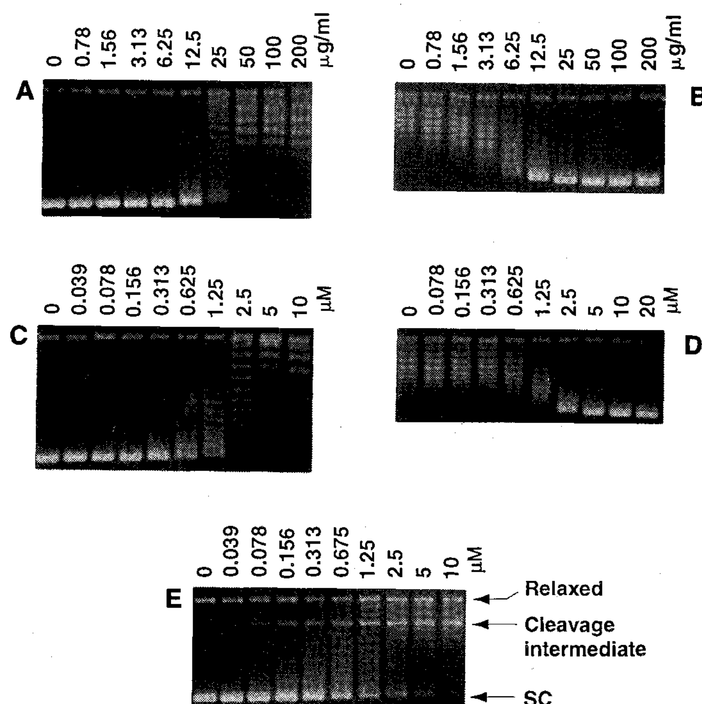
CJ-12,371 (**1**)	**3**	CJ-12,372 (**2**)	**4**
	UV: λ_{max} nm (MeOH, ε)		
227.2 (63,700)	226.5 (50,600)	227.0 (65,400)	225.5 (59,400)
288.6 (11,800)	314.0 (49,400)	288.0 (sh)	318.5 (76,700)
301.0 (11,500)		300.8 (20,400)	
314.2 (8,600)		312.6 (17,200)	
328.4 (6,100)		327.2 (7,600)	
	CD: λ_{ext} nm (MeOH, $\Delta\varepsilon$)		
229 (−8.8)	229 (−35.3)	230 (−10.6)	229 (−32.9)
264 (−0.1)	259 (+ 0.6)	259 (− 0.1)	256 (0)
300 (−0.7)	306 (−59.0)	295 (− 1.4)	285 (+ 3.1)
329 (−0.5)	318 (0)	327 (− 0.9)	290 (0)
	329 (+75.8)		309 (−55.8)
			322 (0)
			331 (+54.0)

Biological Properties

DNA Gyrase and Eukaryotic Topoisomerase II Inhibitory Activities

The two well-characterized classes of DNA gyrase inhibitors, quinolone and coumarin antibacterials, are different in their interaction with the DNA gyrase subunits. Quinolones, like ciprofloxacin, forms the double strand (ds) DNA cleavage intermediate[3,4,10], which can be visualized as linear dsDNA in agarose gels when sodium dodecyl sulfate (SDS) is included in the stop solution (Fig. 3). Novobiocin, on the other hand, interacts with the B subunit of DNA gyrase that binds ATP[8,9]; this compound only inhibits the supercoiling reaction since relaxation mediated by DNA gyrase occurs in the absence of ATP[1,2]. CJ-12,371 and CJ-12,372 distinguish themselves from the quinolone and coumarin antibiotics in that they inhibit both DNA gyrase-mediated supercoiling and relaxation without the formation of a cleavage intermediate (see Fig. 3 for CJ-12,372). This mechanism of action was also seen with atrovenetinones[15], another microbially-derived DNA gyrase inhibitors.

Fig. 3. The effects of CJ-12,372 on DNA gyrase-mediated supercoiling and relaxation.

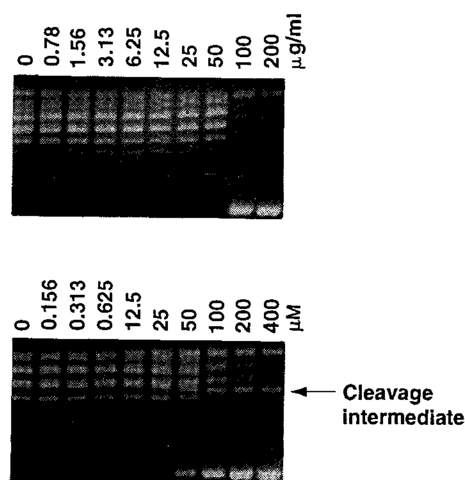


Different concentrations (noted on top of the gels) of CJ-12,372 were incubated with DNA gyrase in a reaction mixture designed to monitor supercoiling (panel A) or relaxation (panel B). Novobiocin (panel C), an inhibitor of supercoiling, and ciprofloxacin, an inhibitor of relaxation (panel D) and supercoiling (panel E), were incubated as control compounds. The position of relaxed DNA, supercoiled (SC) DNA and cleavage intermediate are shown in panel E. The cleavage intermediate that forms in the presence of ciprofloxacin is only visualized if SDS is used to stop the reaction (the condition for panels A, C and E, but not panels B and D).

To determine the selectivity of CJ-12,371 and CJ-12,372, the compounds were tested in a relaxation assay mediated by eukaryotic (Drosophila) topoisomerase II. Both compounds inhibited ATP-dependent relaxation (Table 4) and, unlike etoposide (specific topoisomerase II inhibitor), no cleavage intermediate was noted (Fig. 4). Thus, CJ-12,371 and CJ-12,372 are not specific for DNA gyrase and their mechanism of interaction with either the prokaryotic or eukaryotic enzyme appears to be unique from the classical inhibitors of the respective enzymes.

In an attempt to improve potency and/or enhance selectivity, several semi-synthetic derivatives of CJ-12,371 were made (Table 5). Substitution of one or both of the hydroxyl groups with ethyl, acetyl or methyl groups yielded more potent compounds than CJ-12,371 without improving the selectivity. The best compound, A in Table 5, was equipotent to CJ-12,372.

Fig. 4. The effect of CJ-12,372 on relaxation mediated by eukaryotic topoisomerase II.

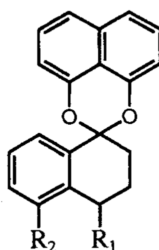


Different concentrations (noted on top of the gels) of CJ-12,372 (upper panel) and etoposide (bottom panel) were incubated with topoisomerase II in a reaction mixture designed to monitor ATP-dependent relaxation. Etoposide blocks religation of the cleavage intermediate formed during relaxation: the position of this intermediate is indicated in the bottom panel.

Table 4. Inhibitory activities of CJ-12,371 and CJ-12,372 against DNA gyrase and eukaryotic topoisomerase II (Topo II).

Compound	IC_{50} (μg/ml)			Selectivity ratio (Topo II/DNA gyrase)
	DNA gyrase-mediated			
	Supercoiling	Relaxation	Eukaryotic Topo II	
CJ-12,371	100	200	140	1.4
CJ-12,372	12.5	12.5	75	6.0
Novobiocin	0.62	300	400	645

Table 5. Effects of CJ-12,371 derivatives on DNA gyrase-mediated supercoiling and eukaryotic topoisomerase II (Topo II)-mediated relaxation activities.



Compound	R_1	R_2	Inhibitory activity (IC_{50}, μg/ml)		Selectivity ratio (Topo II/ DNA gyrase)
			DNA gyrase	Topo II	
CJ-12,371 (**1**)	OH	OH	100	144	1.4
Derivative A	OCH_2CH_3	OH	6	13	2.0
B	OCH_3	OH	19	34	1.8
C	OH	OCH_3	38	13	0.3
D	$OCOCH_3$	$OCOCH_3$	13	13	1.0
E	$OCOC_6H_4OCH_3$	$OCOC_6H_4OCH_3$	50	50	1.0
3	$OCOC_6H_4N(CH_3)_2$	$OCOC_6H_4N(CH_3)_2$	100	140	1.4

Antimicrobial Activities

The antimicrobial activities of CJ-12,371 and CJ-12,372 against various bacterial species were determined according to the standard procedure[16]. Both compounds were predominantly active against Gram-positive bacteria, including ciprofloxacin-resistant and -susceptible *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus pyogenes*, *Streptococcus agalactiae*, and *Enterococcus faecalis*, with minimum inhibitory concentrations ranging from 25 to 100 μg/ml (data not shown).

Discussion

CJ-12,371 (**1**) and CJ-12,372 (**2**) inhibit DNA gyrase and have antibacterial activity against Gram-postive bacteria, suggesting that their antibacterial potency is attributed to the inhibition of DNA gyrase. It appears that CJ-12,371 and CJ-12,372 are a new class of DNA gyrase inhibitors, structurally different from other microbially-derived DNA gyrase inhibitors: novobiocin[3,8], coumermycin A_1[3,8], cinodine[17], microcin[18], clerocidin[19], atrovenetinones[15] and cyclothialidine[20,21]. Mechanistically, CJ-12,371 and CJ-12,372 appear to resemble atrovenetinones in that both structural classes inhibit DNA gyrase-mediated supercoiling and relaxation at a stage distinct from the religation step.

The structures of CJ-12,371 and CJ-12,372 are closely related to the antibiotics: diepoxins[22], Sch 49209 (=diepoxin σ)[23], MK3018[24] and bipendensin[25]. These compounds have naphthalene and decalin moieties which are connected through spiro-ketal linkages. The spectral data of these antibiotics correspond well with those of CJ-12,371 and CJ-12,372. Unusual bis-spiro-ketal compounds, preussomerins A to F[26], were also previously reported with the same carbon skeleton. It is worthwhile to examine whether these reported antibiotics have DNA gyrase inhibitory activities.

Experimental

General

Spectral and physico-chemical data were obtained on the following instruments: UV, JASCO Ubest-30; CD, JASCO J-720; IR, Shimadzu IR-470; NMR, JEOL JNM-GX270 updated with a LSI-11/73 host computer, TH-5 tunable probe and version 1.6 software; LREI-MS, JEOL Automass 120 equipped with a direct inlet module; HREI-MS, Hitachi M-80 with an M-003 data processing system; mp (uncorrected), Yanako Micro Melting Point Apparatus; Optical rotations, JASCO DIP-370 with a 10 cm cell. Merck Kieselgel $60F_{254}$ pre-coated TLC plates (0.5 or 1 mm thickness) were used for preparative purposes.

CJ-12,371 (1)

IR (KBr, cm^{-1}): 3440, 3145, 3110, 3060, 1633, 1607, 1586, 1504, 1474, 1462, 1442, 1411, 1378, 1348, 1325, 1271, 1208, 1180, 1156, 1115, 1064, 1042, 1023, 1012, 955, 923, 885, 846, 821, 803, 793, 755, 663, 645, 627, 600, 568, 547, 535 and 509. EI-MS: m/z 320 (M^+, 11% rel. int.), 302 (43), 285 (7), 284 (6), 273 (2), 255 (3), 189 (2), 160 (8), 159 (7), 144 (8), 131 (57), 115 (100), 114 (40), 113 (26), 103 (39), 102 (29), 89 (22), 77 (52) and 55 (56).

CJ-12,372 (2)

IR (KBr, cm^{-1}): 3455, 3205 (sh), 1634, 1608, 1585, 1471, 1442, 1412, 1380, 1345, 1302, 1266, 1221, 1177, 1121, 1098, 1053, 1023, 999, 955, 915, 891, 867, 821, 793, 767, 754, 737, 649, 624, 565 and 549. EI-MS: m/z 336 (M^+, 17% rel. int.), 318 (49), 301 (4), 300 (5), 289 (2), 273 (3), 271 (2), 189 (3), 175 (10), 160 (35), 159 (14), 147 (17), 144 (16), 131 (58), 115 (100), 114 (46), 113 (28), 103 (46), 102 (31), 91 (38), 89 (25), 77 (60), 65 (33), 58 (35) and 55 (48).

1,8-Di-*O*-*p*-*N*,*N*-dimethylaminobenzoyl-CJ-12,371 (3)

CJ-12,371 (**1**, 5.1 mg) was heated at 80°C with *p*-*N*,*N*-dimethylaminobenzoyl chloride[14] (7.5 mg) in 0.5 ml of pyridine for 21 hours. The resulting mixture was concentrated *in vacuo*, followed by the separation on preparative TLC ($CHCl_3$ - Me_2CO, 20:1). Pure 1,8-di-*O*-*p*-*N*,*N*-dimethylaminobenzoyl-CJ-12,371 (**3**, 7.4 mg) was obtained as colorless glass: $[\alpha]_D$ +74.8° (*c* 0.21, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 7.84 (1H, dd, J=7.7, 1.1 Hz), 7.77 (2H, d, J=9.2 Hz), 7.72 (2H, d, J=9.2 Hz), 7.57 (1H, dd, J=8.1, 7.7 Hz), 7.49 (2H, dd, J=8.4, 1.1 Hz), 7.42 (1H, dd, J=8.4, 7.3 Hz), 7.41 (1H, dd, J=8.4, 7.3 Hz), 7.37 (1H, dd, J=8.1, 1.1 Hz), 6.98 (1H, dd, J=7.3, 1.1 Hz), 6.90 (1H, dd, J=7.3, 1.1 Hz), 6.54 (2H, d, J=9.2 Hz), 6.49 (2H, d, J=9.2 Hz), 6.40 (1H, br t, J=3.3 Hz), 3.01 (6H, s), 2.99 (6H, s), 2.43~2.21 (3H, m) and 2.14 (1H, m).

1,5,8-Tri-*O*-*p*-*N*,*N*-dimethylaminobenzoyl-CJ-12,372 (4)

CJ-12,372 (**2**, 5.5 mg) was stirred at 80°C with 11.9 mg of *p*-*N*,*N*-dimethylaminobenzoyl chloride[14] in 0.5 ml of pyridine for 18 hours. The reaction mixture was concentrated *in vacuo* and components were separated by preparative TLC (*n*-hexane - $CHCl_3$ - EtOAc, 3:2:1). A total of 11.2 mg of 1,5,8-tri-*O*-*p*-*N*,*N*-dimethylaminobenzoyl-CJ-12,372 (**4**) was obtained as colorless glass $[\alpha]_D$ −62.6° (*c* 0.50, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 7.79 (2H, d, J=9.2 Hz), 7.78 (2H, d, J=9.2 Hz), 7.57 (2H, d, J=8.8 Hz), 7.43 (2H, s), 7.34 (2H, br d, J=4.5 Hz), 7.25 (1H, br d, J=8.1 Hz), 7.16 (1H, dd, J=8.1, 7.7 Hz), 6.88 (1H, br t, J=4.5 Hz), 6.64 (1H, dd, J=7.7, 1.1 Hz), 6.58 (2H, d, J=9.2 Hz), 6.49 (2H, d, J=9.2 Hz), 6.36 (1H, br s), 6.03 (2H, d, J=8.8 Hz), 3.01 (12H, s), 2.83 (6H, s), 2.30 (3H, m) and 2.15 (1H, m).

CJ-12,371-derivative A (1-*O*-Ethyl-CJ-12,371)

1,8-Di-*O*-acetyl-CJ-12,371 (2.0 mg), of which preparation will be mentioned later, was treated with K_2CO_3 (7 mg) in 0.5 ml of absolute EtOH at room temperature. After overnight reaction, the excess EtOH was evaporated with a flow of N_2. The resulting reaction mixture was filtered through a small silica gel column (1×2 cm) with EtOAc (8 ml) to provide 1.7 mg of 1-*O*-ethyl-CJ-12,371 as colorless glass: EI-MS *m/z* 348 (M^+, 20% rel. int.), 302 (100), 285 (7), 284 (4), 255 (4), 144 (5), 131 (16) and 115 (19); ^1H NMR ($CDCl_3$) δ 8.49 (1H, s), 7.48 (2H, dd, J=8.1, 1.0 Hz), 7.42 (1H, dd, J=8.1, 7.3 Hz), 7.41 (1H, dd, J=8.1, 7.3 Hz), 7.40 (1H, dd, J=7.8, 1.5 Hz), 7.33 (1H, t, J=7.8 Hz), 6.97 (1H, dd, J=7.8, 1.5 Hz), 6.92 (1H, dd, J=7.3, 1.0 Hz), 6.90 (1H, dd, J=7.3, 1.0 Hz), 4.98 (1H, dd, J=8.6, 6.6 Hz), 3.78 (1H, dq, J=9.0, 7.1 Hz), 3.63 (1H, dq, J=9.0, 7.1 Hz), 2.41 (1H, m), 2.18 (2H, m), 1.89 (1H, m) and 1.32 (3H, t, J=7.1 Hz).

CJ-12,371-derivative B (1-*O*-Methyl-CJ-12,371)

1,8-Di-*O*-acetyl-CJ-12,371 (0.5 mg), of which preparation will be mentioned later, was treated with K_2CO_3 (5 mg) in 0.25 ml of MeOH at 0°C. After 1-hour reaction, the excess MeOH was evaporated with a flow of N_2. The resulting reaction mixture was filtered through a small silica gel column (1×2 cm) with EtOAc to provide 0.3 mg of 1-*O*-methyl-CJ-12,371 as colorless glass: EI-MS *m/z* 334 (M^+, 19% rel. int.), 302 (100), 285 (7) and 284 (5); ^1H NMR ($CDCl_3$) δ 8.24 (1H, s), 7.48 (2H, dd, J=8.4, 0.7 Hz), 7.45~7.36 (3H, m), 7.34 (1H, dd, J=8.1, 7.7 Hz), 6.98 (1H, dd, J=8.1, 1.4 Hz), 6.91 (1H, dd, J=7.4, 1.1 Hz), 6.90 (1H, dd, J=7.3, 1.1 Hz), 4.91 (1H, dd, J=8.1, 6.2 Hz), 3.49 (3H, s), 2.41 (1H, m), 2.19 (2H, m) and 1.90 (1H, m).

CJ-12,371-derivative C (8-*O*-Methyl-CJ-12,371)

CJ-12,371 (**1**, 0.8 mg) was reacted with CH_3I (0.1 ml) and K_2CO_3 (5 mg) in 0.3 ml of tetrahydrofuran at 60°C for 3 hours. After excess CH_3I and tetrahydrofuran were evaporated with a flow of N_2, the reaction mixture was passed through a small silica gel column (1×2 cm) with 3 ml of $CHCl_3$ to elute 8-*O*-methyl-CJ-12,371 (1.0 mg, colorless glass): EI-MS *m/z* 334 (M^+, 24% rel. int.), 316 (100), 301 (19), 284 (5) and 273 (5); ^1H NMR ($CDCl_3$) δ 7.53~7.36 (6H, m), 6.99 (1H, dd, J=8.1, 1.1 Hz), 6.93 (1H, dd, J=7.3, 1.1 Hz), 6.87 (1H, dd, J=7.3, 1.1 Hz), 5.15 (1H, br q, J=2.2 Hz), 3.94 (3H, s), 3.02 (1H, br s) and 2.40~1.90 (4H, m).

CJ-12,371-derivative D (1,8-Di-*O*-acetyl-CJ-12,371)

CJ-12,371 (**1**, 3.8 mg) was treated overnight with Ac_2O (0.3 ml) and pyridine (0.3 ml) at room temperature. The solvent was evaporated and pure 1,8-di-*O*-acetyl-CJ-12,371 (4.7 mg) was obtained as colorless glass: EI-MS *m/z* 404 (M^+, 15% rel. int.), 344 (29), 302 (100), 285 (6), 273 (4), 255 (5), 160 (9), 159 (10), 144 (6), 131 (17), 115 (24), 103 (5) and 43 (47); ^1H NMR ($CDCl_3$) δ 7.81 (1H, dd, J=8.1, 1.4 Hz), 7.52 (1H, t, J=8.1 Hz), 7.50 (2H, br d, J=8.4 Hz), 7.43 (1H, dd, J=8.4, 7.3 Hz), 7.41 (1H, dd, J=8.4, 7.3 Hz), 7.20 (1H, dd, J=8.1, 1.4 Hz), 6.95 (1H, dd, J=7.3, 1.1 Hz), 6.87 (1H, dd, J=7.3, 0.7 Hz), 6.23 (1H, br t, J=3.3 Hz), 2.28 (3H, s), 2.25 (3H, m), 2.02 (3H, s) and 1.95 (1H, m).

CJ-12,371-derivative E (1,8-Di-*O*-*p*-methoxybenzoyl-CJ-12,371)

CJ-12,371 (**1**, 5.5 mg) was reacted with *p*-methoxybenzoyl chloride (9.1 mg) in 0.5 ml of pyridine at 80°C for 21 hours. The reaction mixture was concentrated *in vacuo*, then applied to preparative TLC ($CHCl_3$-acetone, 20:1), providing pure 1,8-di-*O*-*p*-methoxybenzoyl-CJ-12,371 (7.3 mg) as colorless glass: $[\alpha]_D$ −32.9° (*c* 0.21, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 7.88 (1H, dd, J=8.1, 1.1 Hz), 7.82 (2H, d, J=9.2 Hz), 7.74 (2H, d, J=8.8 Hz), 7.60 (1H, t, J=8.1 Hz), 7.50 (2H, dd, J=8.4, 1.1 Hz), 7.43 (1H, dd, J=8.4, 7.3 Hz), 7.42 (1H, dd, J=8.4, 7.3 Hz), 7.35 (1H, dd, J=8.1, 1.1 Hz), 6.98 (1H, dd, J=7.3, 1.1 Hz), 6.91 (1H, dd, J=7.3, 1.1 Hz), 6.78 (2H, d, J=8.8 Hz), 6.75 (2H, d, J=9.2 Hz), 6.42 (1H, dd, J=3.7, 3.0 Hz), 3.83 (3H, s), 3.82 (3H, s), 2.45~2.23 (3H, m) and 2.14 (1H, m).

DNA Gyrase Purification

DNA gyrase subunits A and B were purified from *E. coli* strains constructed to overproduce GyrA (JM*tacA*) and GyrB (JM*tacB*)[27]. GyrA was purified by conventional methodology[28] while GyrB protein was obtained by an FPLC procedure. Briefly, the latter protocol involved the addition of a concentrated ammonium sulfate precipitate (resuspended in 20 mM Tris-HCl, pH 7.5, 1 mM EDTA and 5 mM dithiothreitol [TED]) to a Phenyl Superose column equilibrated in TED/1 M ammonium sulfate. The crude GyrB protein (2 ml, representing *ca.* 80 mg total protein) was loaded on the column, which was then washed with TED/1 M ammonium sulfate until the A_{280} baseline returned to zero. A 30-minute linear gradient of 1~0 M ammonium sulfate in TED was applied and GyrB protein was eluted at 0.5 M ammonium sulfate, free from contaminating nucleases. Active fractions were identified by enzyme activity, after the addition of GyrA protein to reconstitute holoenzyme. To the final holoenzyme stock, KCl was added to a final concentration of 0.1 M, the mixture was diluted in half with glycerol, and aliquots were stored at −20°C. One unit of holoenzyme is defined as the amount of enzyme required to completely supercoil 0.125 μg of relaxed pBR322 DNA at 30°C in 2 hours under the conditions specified below.

DNA Gyrase Supercoiling and Relaxation Assays

The supercoiling reaction mixture[29] (25 μl) contained 40 mM Tris-HCl (pH 7.5), 6.5% glycerol (w/v), 1.4 mM ATP (pH 7.0), 0.64 mM EDTA, 365 μg/ml bovine serum albumin, 4 mM $MgCl_2$, 24 mM KCl, 0.5 mM spermidine, 0.05 mM dithiothreitol and 0.125 μg relaxed pBR322

plasmid DNA (prepared from supercoiled pBR322 by Nippon Gene Co., Ltd.). Each sample solubilized in DMSO was added to the reaction mixture at a final concentration of 1.44%. All enzyme reactions were assembled on ice, with the addition of DNA gyrase holoenzyme (1 unit) last to the reaction mixture. Reaction mixtures were incubated at 30°C for 2 hours; the enzyme reaction was terminated by the addition of a tenth volume of stop solution: 5% bromophenol blue (w/v), 50% glycerol (w/v), 200 mM Tris-acetate, 5 mM EDTA (pH 7.5) and 2.33% SDS. Relaxed, linear and supercoiled DNA were separated by agarose gel electrophoresis (0.8% agarose in Tris-borate-EDTA) and detected by UV irradiation after staining with 1 μg/ml ethidium bromide in 40 mM EDTA, pH 7.5.

Reactions measuring relaxation mediated by DNA gyrase holoenzyme were carried out in the same fashion as the supercoiling reaction, with 4 important exceptions: 1) ATP was omitted from the reaction mixture, 2) 2 units of holoenzyme were added per reaction, 3) the substrate was supercoiled pBR322 and 4) the stop reagent did not contain SDS.

Eukaryotic Topoisomerase II Relaxation Assay

Drosophila topoisomerase II was purchased from United States Biochemicals Corporation. The assay procedure was slightly modified from that suggested by the manufacturer. To start the enzyme reaction, topoisomerase II (1 unit) was added to a mixture (25 μl) containing 15 μl of sample in 2.4% DMSO, 10 mM Tris-HCl (pH 7.9), 0.45 mM Na_2HPO_4 (pH 7.1), 71 mM NaCl, 50 mM KCl, 5 mM $MgCl_2$, 7.5% glycerol (w/v), 1.0 mM ATP (pH 7.0), 0.08 mM dithiothreitol, 0.12 mM EDTA, 15 μg/ml BSA and 1.25 μg of supercoiled plasmid DNA pBR322 (Nippon Gene Co., Ltd.). The mixture was incubated at 30°C for 30 minutes. The enzyme reaction was stopped by adding 2.5 μl of SDS (2.2%) and 2.5 μl of proteinase K (1.25 mg/ml). DNA was detected by the same procedure described above.

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