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Novel Naphthoquinones from a *Streptomyces* sp.

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Cdc25A assay-guided fractionation of a fermentation broth derived from a *Streptomyces* sp. resulted in the isolation of four novel naphthoquinones **1**~**4**. Structures of these compounds were deduced by NMR and mass spectrometry. Two of them, **3** and **4**, incorporate a modified cysteine residue which is observed for the first time in this class of natural products. Naphthoquinones **1**~**4** showed weak activity against cdc25A phosphatase.

Cyclin/cdk (cyclin-dependent kinase) complexes serve as regulatory checkpoints for cell cycle progression.[1] Cell cycle progression is delayed when the activities of cdks are suppressed through phosphorylation of their critical threonine and tyrosine residues. Cdc25, a family of dual specificity phosphatases, activate cdks by dephosphorylation of threonine and tyrosine residues and thus play a significant role in cell cycle progression. Cdc25A is a member of this family of phosphatases that appears to function early in the cell cycle.[2] Furthermore, it has been shown that abnormal expression of cdc25A in the presence of RAS mutants or RB deletion mutants can lead to oncogenic transformation.[3,4] Therefore, cdc25A appears to be an attractive target for the discovery of novel cancer therapeutic agents.

During the screening of microbial fermentations for cdc25A phosphatase inhibitors, an extract derived from a *Streptomyces* sp. was discovered to be active. We herein report the production, purification, structure determination and cdc25A phosphatase activity of four novel napthoquinones.

Experimental

General Methods

HPLC separations were performed on Rainin SD-1 and HPXL systems with an UV-1 absorbance detector ($\lambda = 240$ nm) using Dynamax 60A Microsorb phenyl (41.4 × 250 mm, flow rate 45 ml/minute) and Dynamax 60A C18 (21.4 × 250 mm, flow rate 12 ml/minute) column. UV absorption spectra were measured as the compounds eluted in 0.5% NH_4OAc-CH_3CN gradient using a Waters 717 system equipped with a Waters 996 photodiode array detector. IR spectra were determined on a Nicolet 510 P spectrophotometer. Optical rotations were measured on a JASCO model DIP-70 polarimeter. FAB-MS and HRFAB-MS were obtained on a VG Analytical ZAB-2-SE mass spectrometer. NMR spectra were determined on a Varian Unity (^{1}H: 500 MHz; ^{13}C: 125.7 MHz) spectrometer using a Z-SPEC microdual microsample probe. Carbon multiplicities were assigned by DEPT experiments. Standard pulse sequences were employed for all 2D NMR experiments.

Fermentation

The producing organism was isolated from a soil sample collected in Curacao. Stock cultures were kept as a suspension in 10% glycerol - 5% lactose in the vapor phase of liquid nitrogen. Thawed aliquots (1 ml each) were inoculated into 3 × 50 ml portions of medium containing 3% Trypticase Soy Broth (BBL), 0.5% glucose, 0.4% maltose, 0.3% yeast extract, and 0.2% $MgSO_4 \cdot 7H_2O$ in de-ionized water. Each portion of inoculated medium was contained in a 250 ml Erlenmeyer flask and was incubated at 250 rpm for 48 hours at 30°C

in a rotary shaker with a 5.1 cm throw. The resulting culture (8.0 ml aliquots each) was transferred to 3× 600 ml of the same medium in 2 liter Erlenmeyer flasks. After incubating for 43 hours, the contents of 3 flasks were combined to inoculate a 150 liter bioreactor containing 115 liters of production medium. The production medium consisted of 3% potato dextrin, 1% glucose, 0.5% cane molasses, 0.5% enzyme-hydrolyzed casein (Amber EHC), 0.5% yeast extract, 0.2% $CaCO_3$, 0.15% K_2HPO_4, 0.1% $MgSO_4 \cdot 7H_2O$, and 0.0001% each of $MnCl_2 \cdot 4H_2O$, $FeCl_2 \cdot 4H_2O$, $CoCl_2$, and $ZnCl_2$ in tap water (pH 6.4 after steam sterilization). During fermentation, temperature was controlled at 30°C and dissolved oxygen was maintained at or above 30% of air saturation. The pH of the culture dropped to 5.8 after 24 hours of fermentation then steadily rose to a value of 7.3 at 141 hours when the culture was harvested.

Isolation of Naphthoquinones **1**~**4**

Fermentation broth (215 liters) was filtered using a tangential flow ceramic filter (Memralox, 0.2 μm pore size). The mycelium was discarded. The filtrate was applied to a 10 liter Diaion HP20SS resin (Mitsubishi Chemical America) column and was eluted with 0~100% H_2O-MeOH gradient over 60 minutes with a flow rate of 1 liter/minute. Fractions 1~15, 4 liter each, were collected during the gradient. The column was finally stripped with 20 liters of MeOH (fraction 16). The active fraction 13 was concentrated *in vacuo* to dryness and resuspended in 9 ml of MeOH and filtered. The clear filtrate was purified in 3 ml portions over a Dynamax phenyl column (41.4×250 mm; mobile phase 30~100% H_2O–CH_3CN gradient) to afford 101 mg of **3**. The active fraction 16 was concentrated *in vacuo* to dryness and resuspended in 12 ml of MeOH and after filtration was purified in 3 ml portions on a Dynamax phenyl column (41.4×250 mm; mobile phase 30~100% H_2O–CH_3CN gradient) to give two groups of fractions. Fractions containing **1** were repurified as detailed above to yield 114 mg of **1**. Fractions containing **2** and **4** were rechromatographed on a Dynamax C-18 column (21.4×250 mm; mobile phase 54~90% 0.5% NH_4OAc–CH_3CN gradient) to yield **2** (9.9 mg) and impure **4** which upon repurification as above with 20~60% 0.5% NH_4OAc–MeOH gradient gave 6.3 mg of **4**.

Preparation of **5** from **3**

To a solution of **3** (8.1 mg) in 1 ml of MeOH was added trimethylsilyldiazomethane (300 μl of 2 M solution in hexane). The mixture was stirred for 5 minutes. Excess reagent and MeOH were evaporated, and the resulting residue was purified on a Dynamax column (21.4×250 mm; mobile phase 30~100% H_2O–CH_3CN gradient) to furnish 8 mg of **5**.

Physico-chemical Properties of **1**~**5**

1: Red amorphous powder; $[\alpha]_D$ −408.7 (*c* 0.53, MeOH); UV λ_{max} nm 211, 231, 269, 320, 424 br, 484 br; IR ν_{max} (KBr) cm^{-1} 1714, 1679, 1616; FAB-MS *m/z* 388 $(M+H)^+$; HRFAB-MS *m/z* 388.1768 $(M+H)^+$ calcd for $C_{21}H_{26}NO_6$ *m/z* 388.1760; ^1H and ^{13}C NMR data are listed in Tables 1 and 2, respectively.

2: Red amorphous powder; $[\alpha]_D$ −436.4 (*c* 0.11, MeOH); UV λ_{max} nm 209, 229, 267, 318, 422 br, 483 br; FAB-MS *m/z* 374 $(M+H)^+$; HRFAB-MS *m/z* 374.1639 $(M+H)^+$ calcd for $C_{20}H_{24}NO_6$ *m/z* 374.1604; ^1H and ^{13}C NMR data are listed in Tables 1 and 2, respectively.

3: Dark red amorphous powder; $[\alpha]_D$ −63.0 (*c* 0.51, MeOH); UV λ_{max} nm 214, 252, 284, 414 br, 503 br; IR ν_{max} (KBr) cm^{-1} 1724, 1678, 1605; FAB-MS *m/z* 549 $(M+H)^+$, 420 $(M-C_5H_6NO_3)$, 388 $(M-C_5H_6NO_3S)$; HRFAB-MS *m/z* 549.1934 $(M+H)^+$ calcd for $C_{26}H_{33}N_2O_9S$ *m/z* 549.1907; ^1H and ^{13}C NMR data are listed in Tables 1 and 2, respectively.

4: Dark red amorphous powder; $[\alpha]_D$ −360 (*c* 0.15, MeOH); UV λ_{max} nm 212, 250, 282, 413 br, 505 br; FAB-MS *m/z* 549 $(M+H)^+$, 420 $(M-C_5H_6NO_3)$, 388 $(M-C_5H_6NO_3S)$; HRFAB-MS *m/z* 549.1939 $(M+H)^+$ calcd for $C_{26}H_{33}N_2O_9S$ *m/z* 549.1907, *m/z* 388.1779 $(M-C_5H_6NO_3S)$ calcd for $C_{21}H_{26}NO_6$ *m/z* 388.1760; ^1H and ^{13}C NMR data are listed in Tables 1 and 2, respectively.

5: Dark red amorphous powder; $[\alpha]_D$ −180 (*c* 0.1, MeOH); FAB-MS *m/z* 577 $(M+H)^+$, 434 $(M-C_6H_8NO_3)$, 402 $(M-C_6H_8NO_3S)$; HRFAB-MS *m/z* 577.2215 $(M+H)^+$ calcd for $C_{28}H_{37}N_2O_9S$ *m/z* 577.2220; ^1H and ^{13}C NMR data are listed in Tables 1 and 2, respectively.

Cdc25A Assay

Preparation of cdc25A-Glutathione-*S*-transferase Fusion Protein

Recombinant cdc25A-glutathione-*S*-transferase fusion protein was produced in *E. coli* BL 21 cells. Cells were harvested by centrifugation and were suspended in three volumes of TED buffer (50 mM Tris-HCl, pH 8.0, buffer containing 1 mM EDTA, 10 mM DTT, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 5 mg/ml aprotinin, 5 mg/ml leupeptin). Lysozyme (0.25 mg/ml) was

added to this cell suspension and the slurry was incubated for 1 hour at room temperature. The cells were broken by sonication and any unbroken cells and cell debris were removed by centrifugation at 30,000 *g* for 30 minutes. The supernatant containing the fusion protein typically had a specific activity of 0.017 unit/mg (1 unit of activity = the amount of enzyme required to convert 1 μmole of *p*-nitrophenylphosphate to *p*-nitrophenyl in 1 minute.

This enzyme preparation was subjected to anion exchange chromatography using DEAE-Sepharose (Pharmacia). Unbound proteins were removed by a TED buffer wash and the activity was eluted with a linear gradient of 0 to 1 M KCl in TED buffer. The high activity fractions were pooled, concentrated to ~50 ml and dialyzed against 2 × 2 liter TED buffer. The dialyzed pool was further purified by hydroxyapatite chromatography using Ultra Gel HA (IBF Biotechniques). Unbound proteins were again removed by a TED buffer wash and the bound activity was eluted with a linear gradient of 0 to 0.4 M potassium phosphate in TED buffer. SDS-PAGE analysis of the high activity pool indicated the cdc25A-glutathione-*S*-transferase protein to be about 60% pure and this preparation was used for the enzyme assay.

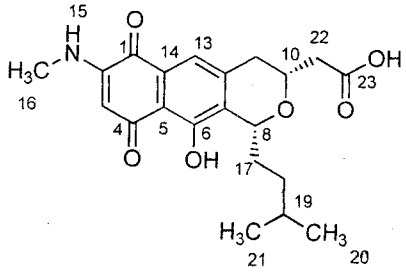
Enzyme Assay

Stock solutions of test compounds were prepared in DMSO at 30 mM. From this stock 0.1, 1 and 10 μM final inhibitor concentrations were prepared by diluting with de-ionized water. The maximum concentration of DMSO in the assay was 0.03%. A 46.23 mM stock solution of the substrate, fluorescein diphosphate (Molecular Probes Inc.), was prepared in de-ionized water. The final concentration of fluorescein diphosphate used in the assay was 20 μM. Sodium orthovanadate at 1 mM was used as a control inhibitor.

On the day of the experiment, the cdc25A-glutathione-*S*-transferase fusion protein was diluted with assay buffer (60 mM Tris-HCl buffer, pH 8.5, containing 150 mM NaCl, 1.33 mM EDTA, 0.066% BSA, 2 mM DTT) and incubated with fluorescein diphosphate and test compounds, or appropriate controls, for 1 hour at room temperature in the dark. Immediately after incubation, the fluorescence emission (excitation at 485 nm and emission at 530 nm) was measured using a Millipore Cytofluor 2350. The ability of test compounds to inhibit the cdc25a activity is expressed as % inhibition at 10 μM concentration of test compounds.

Results and Discussion

The molecular formula of **1** was established to be $C_{21}H_{25}NO_6$ by HRFAB-MS. In agreement with this, the ^{13}C NMR (broad-band decoupled and DEPT) spectra exhibited 21 resonances—three methyls, four methylenes, five methines and nine quaternaries. Characteristic UV absorptions (*vide supra*) and the presence of two quaternary carbons at δ 189.1 and 180.9 ppm and eight additional aromatic-type carbons (Table 2) indicated a naphthoquinone moiety in **1**. The ^1H NMR spectrum exhibited resonances for one acidic hydrogen, two aromatic protons, and three isolated spin systems. The three spin systems were easily delineated in the COSY and TOCSY spectra as related to substructures NH–CH_3, CH_2–CH–CH_2 (C-11 ~ C-22) and CH–CH_2–CH_2–CH–$(CH_3)_2$ (C-8 ~ C-21). In the latter two substructures the proton chemical shifts of H-8 (δ 5.07) and H-10 (δ 3.97) suggested that these methine carbons were oxygenated; the HMQC spectrum confirmed this by correlation of these protons to carbon signals at δ 74.9 and 69.1, respectively. The positioning of these substructures on the naphthoquinone core was accomplished through an HMBC experiment (optimized for $J_{C-H} = 8$ Hz). Fig. 1 depicts selected HMBC correlations



1

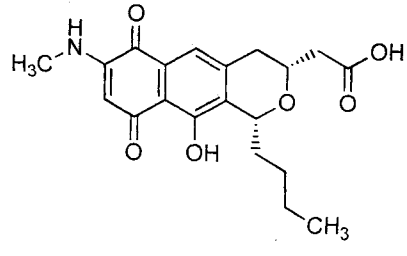


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Table 1. ^{1}H NMR (500 MHz) chemical shifts of **1**~**5**.

	1[a]	2[b]	3[b]	4[c]	5[a]
H-3	5.58 br s	5.55 br s			
H-8	5.07 br d (6)	4.99 br d	4.99 br d	4.96 br d	5.04 br d
H-10	3.97 m	3.96 m	3.96 m	3.92 m	3.94 m
H-11a	2.78[d]	2.85 br d (16)	2.86 br d	2.81 br d	2.74[d]
H-11b		2.76 dd (16, 10.5)	2.76 dd	2.71 dd	
H-13	7.29 br s	7.29 br s	7.30 br s	7.26 br s	7.32 br s
H-15	6.13 q (5.5)	7.21 br q			
H-16	2.94 d (5.5)	2.97 d	3.51 br s	3.46 br s	3.52 br s
H-17a	2.15 m	2.12 m	2.14 m	2.11 m	2.10 m
H-17b	1.90 m	1.90 m	1.91 m	1.89 m	1.91 m
H-18a	1.31 m	1.39 m	1.33 m	1.31 m	1.29 m
H-18b	1.08 m	1.25 m	1.13 m	1.09 m	1.02 m
H-19	1.51 m	1.30 m	1.51 m	1.48 m	1.49 m
H-20	0.83 d (6.5)	0.84 t (7)	0.83 d	0.81 d	0.81 d
H-21	0.85 d (7)		0.84 d	0.83 d	0.84 d
H-22a	2.78[d]	2.63 ABq	2.63 ABq	2.59 ABq	2.72[d]
H-22b	2.68 dd (15.5, 5)				2.59 dd (15, 6)
H-25a			3.17 ABq	3.18 m	3.31 dd (14,5)
H-25b				3.09 dd (13.5, 7)	2.98 dd (14, 4.5)
H-26			4.63 dt (8, 6)	4.46 m	4.80 dt (7.5, 5)
H-28			7.68 d (8)	7.75 br m	7.39 br m
H-30			1.93 s	1.90 s	2.06 s
OH-6	13.57 s	14.07 s	14.01 s		13.55 s
OMe-23					3.72 s
OMe-27					3.64 s

Coupling constants (Hz) are in parentheses and are not repeated if identical with those in the preceding column.
[a] Measured in $CDCl_3$; chemical shifts reported are relative to solvent signal (7.26 ppm).
[b] Measured in CD_3COCD_3; chemical shifts reported are relative to solvent signal (2.04 ppm).
[c] Measured in $CD_3COCD_3 + CD_3OH$; chemical shifts reported are relative to solvent signal (2.04 ppm).
[d] Partly obscured.

observed for **1**.

The HMBC correlations from C-1 (δ 180.9) to H-13 (δ 7.29) and H-15 (δ 6.13) suggested that the secondary amine moiety was connected to C-2 rather than C-3 of the quinone ring. This was also apparent from the strong three bond correlations observed from C-5 (δ 112.6) to H-3 (δ 5.58) and the hydroxyl proton at C-6 (δ 13.57). One end (C-11) of the three carbon residue was attached to C-12 as indicated by the HMBC cross peak from C-11 (δ 35.5) to H-13 (δ 7.29) and the significant NOE interaction observed between H_2-11 and H-13 (Table 3). The chemical shifts of H_2-22 (δ 2.78 and 2.68) and HMBC correlations from a carbonyl carbon (δ 175.5) to H-10 and H_2-22 implied that C-22, the other end of this residue, was juxtaposed on a carboxylic acid group (IR: acid carbonyl 1714 cm^{-1}). The position of the third substructure was located on C-7, the only remaining carbon to be substituted, by the HMBC correlation observed between C-7 (δ 135.5) and H-8 (δ 5.07). Finally, the HMBC correlation observed between C-10 (δ 69.1) and H-8 (δ 5.07) and consideration of the molecular formula indicated that C-10 and C-8 must be linked through an ether bridge leading to a planar structure as shown in **1**.

As regards the relative stereochemistry at C-8 and C-10, a strong NOE observed between H-8 and H-10 (Table 3) implied that they were *cis* and had to be oriented axially.

Compound **2** revealed a molecular formula of $C_{20}H_{23}NO_6$ by HRFAB-MS—*i.e.*, 14 amu less than compound **1**. Comparison of the ^{1}H and ^{13}C NMR data (Tables 1 and 2) of **2** with those of **1** revealed that they differed only in the nature of the alkyl chain attached at C-8; the *iso*-pentyl group in **1** was replaced by a *n*-butyl group in **2**.

Naphthoquinone **3** and its congener **4**, had identical molecular formulas of $C_{26}H_{32}N_2O_9S$ as revealed by

Table 2. ^{13}C NMR (125.7 MHz) chemical shifts of **1**~**5**.

	1[a]	2[b]	3[b]	4[c]	5[a]
C-1	180.9 (s)	181.6	181.4[e]	181.8[e]	180.0[f]
C-2	149.7 (s)	151.3	[g]	[g]	151.2[f]
C-3	99.4 (d)	99.2	[g]	[g]	104.1[e] (s)
C-4	189.1 (s)	189.6	[g]	[g]	187.6[f]
C-5	112.6 (s)	113.4	113.6[f]	113.7[e]	112.5[f]
C-6	158.4 (s)	158.7	158.8[e]	158.2[f]	158.2[f]
C-7	135.5 (s)	135.6	135.6[f]	135.0[e]	136.3[f]
C-8	74.9 (d)	75.1	75.3	75.3	74.6
C-10	69.1 (d)	70.5	70.5	70.6	69.3
C-11	35.5 (t)	36.0	36.0	36.1	35.5
C-12	141.1 (s)	142.7	143.0[e]	142.9	142.2
C-13	119.2 (d)	119.5	119.8	119.8	120.0
C-14	128.1 (s)	129.4	[g]	126.9[e]	127.4
C-16	29.2 (q)	29.5	34.2[e]	33.9	33.8[f]
C-17	31.8 (t)	34.4	32.6	32.6	31.7
C-18	33.8 (t)	27.8	34.7	34.7	33.6
C-19	27.9 (d)	23.3 (t)	28.7 (d)	28.8	28.0
C-20	22.3[d] (q)	14.3	22.7[e]	22.7[d]	22.4[f]
C-21	22.8[d] (q)	—	23.2[e]	23.1[d]	22.9[f]
C-22	40.3 (t)	40.8	40.8	41.0	40.4
C-23	175.5 (s)	172.1	172.1	173.9[f]	171.4
C-25			37.7[e] (t)	38.3	39.2[f]
C-26			53.1[e] (d)	54.4[f]	51.9[f]
C-27			172.2 (s)	173.0	170.9
C-29			170.5 (s)	171.2	170.6
C-30			22.6 (q)	22.7[d]	23.0
OMe-23					51.9 (q)
OMe-27					52.7 (q)

Carbon multiplicities are in parentheses and are not repeated if identical with those in the preceding column.

[a] Measured in $CDCl_3$; chemical shifts reported are relative to solvent signal (77.0 ppm).

[b] Measured in CD_3COCD_3; chemical shifts reported are relative to solvent signal (29.8 ppm).

[c] Measured in $CD_3COCD_3 + CD_3OH$; chemical shifts reported are relative to solvent signal (29.8 ppm).

[d] Assignments in the same column may be interchanged.

[e] Observed only in the HMBC spectrum.

[f] Broad.

[g] Not observed.

Fig. 1. HMBC (C→H) data for **1**.

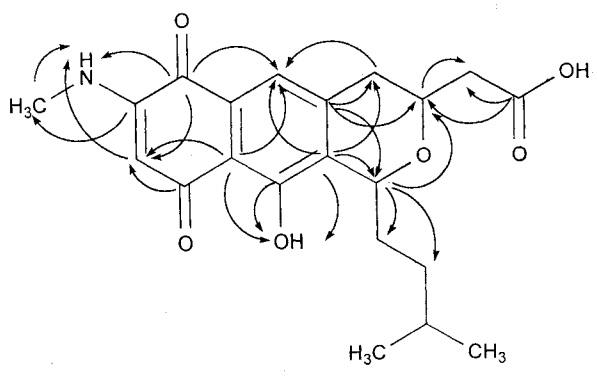


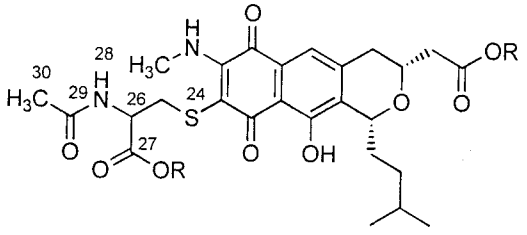
Table 3. Selected NOE difference data of **1**.

Irradiation	Enhancement
H-3	H-16
H-8	H-10, H-18a and H-19
H-10	H-8
H-11	H-13
H-13	H-11
OH-6	H-8, H-17a and 17b

HRFAB-MS. Structures of these compounds were elucidated by the analysis of NMR data coupled with the interpretation of FAB-MS data. In addition to showing nearly all of those resonances observed in **1**, the ^{1}H and ^{13}C NMR spectra of **3** and **4** displayed resonances reminiscent of an *N*-acetylated cysteine residue (δ_H 4.63 and 4.46, δ_C 53.1 and 54.4; δ_H 3.17 and 3.18 and 3.09, δ_C 37.7 and 38.3; δ_C 172.2 and 173.0; δ_C 170.5 and 171.2; δ_H 1.93 and 1.90; δ_C 22.6 and 22.7 for **3** and **4**, respectively). Many of the ^{13}C NMR signals, irrespective of solvents, were broad, and some carbons were not visible (Table 2). Therefore, a dimethyl ester **5** was prepared by brief exposure of **3** in the presence of trimethylsilyldiazomethane. The ^{13}C NMR spectrum of **5**, in spite of signal broadening, displayed all carbons except C-3 which was observable in the HMBC spectrum. The conspicuous absence of the H-3 resonance in **3** and **4** compared to **1** and the chemical shift of C-3 (~104 ppm) implied that the cysteine residue was attached to C-3 through the sulfur atom. This was consistent with the mass spectral fragments observed in the FAB-MS at *m/z* 420 and 388 for **3** and **4** and *m/z* 434 and 402 for **5** (Fig. 2).

A marked difference in the α-hydrogen resonances (δ 4.63 in **3** and δ 4.46 in **4**) and subtle differences in the β-hydrogen chemical shifts of the *N*-acetylated cysteine residue (Table 1) suggested that **3** and **4** may be epimeric at the α-carbon. The stereochemical distinction between **3** and **4** and the absolute configuration of **1**~**4** remain to be established.

There are a number of fused pyranonaphthoquinone metabolites reported from microbial[5~7] and plant[8] origin. However, to our knowledge, **3** and **4** are the first members of this class of natural products incorporating an amino acid residue. In cdc25A assays naphthoquinones **1**~**4** showed reasonable activity. However, in the presence of $2 \times Km$ (20 μM) of the substrate, fluorescein diphosphate, they failed to produce significant inhibition of the enzyme at 10 μM (Table 4).

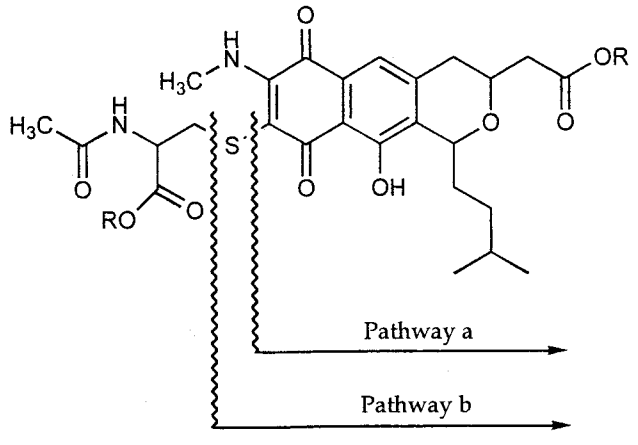


3 R = H
4 C-26 isomer of **3**
5 R = CH_3

Table 4. Cdc25A activity of **1**~**4**.

Compound	% Inhibition at 10 μM
1	8.3
2	15.5
3	12.5
4	13.7

Fig. 2. Mass spectral fragmentation of **3**, **4** and **5**.



3 and **4** (R = H) *m/z* 388 (a+2H)
m/z 420 (b+2H)

5 (R = CH_3) *m/z* 402 (a+2H)
m/z 434 (b+2H)

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