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Coscinosulfate, a CDC25 Phosphatase Inhibitor from the Sponge *Coscinoderma Mathewsi*

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Abstract—The dual specificity CDC25 phosphatases dephosphorylate two inhibitory phospho-amino acids of cyclin-dependent kinases, a major family of cell cycle regulators. CDC25 inhibitors constitute new anti-mitotic agents with potential anticancer activity. While screening through a collection of natural products derived from marine organisms for CDC25A inhibitors, we purified and identified coscinosulfate **1**, a sesquiterpene sulfate from the New Caledonian sponge *Coscinoderma matthewsi*, along with **4**. The purified compound **1** displayed significant inhibitory activity towards CDC25A (IC_{50}: 3 μM).

Introduction

The search for compounds, which are able to act on the specific enzymes, that regulates the cell division cycle has considerably developed during last years. This method of screening molecules can indeed lead to the detection of anti-mitotic compounds of interest as anticancer drug candidates with a novel mechanism of action.

Of particular interest are the enzymes which regulate the entry of the cell in mitotic and meiotic M phase. In eucaryotic cells entry into M phase is initiated by activation of MPF (M phase promoting factor) which is constituted of a catalytic sub-unit, CDK1 (cyclin-dependent kinase 1) and a regulatory sub-unit, cyclin B (reviews in refs 1 and 2).

In late prophase, the CDK1/cyclin B complex is inactive, due to the phosphorylation of two amino acid residues of CDK1, Thr-14 and Tyr-15, which are located just at the border of the kinase ATP-binding pocket. Phosphorylation of these residues is catalyzed by the Myt1 and Wee1 kinases, respectively. Activation of MPF results from the dephosphorylation of these inhibitory sites by a dual specificity phosphatase, the CDC25 phosphatase (reviews in refs 3 and 4).

The human genome encodes three CDC25 homologues, CDC25A, B and C, the precise functions of which have yet to be ascribed. CDC25A is thought to activate CDK2/cyclin E and thereby trigger the G1/S transition of the cell cycle. CDC25B appears to play a role in G2 (by regulating CDK2/cyclin A and CDK1/cyclin A), while CDC25C specifically dephosphorylates CDK1/cyclin B, thereby triggering the G2/M transition. Through their unique substrate selectivity and their essential functions in cell cycle control, CDC25 phosphatases constitute attractive screening target to identify new anti-mitotic compounds of potential therapeutic interest.[5,6] A few inhibitors have been reported to date: dephostatin,[7] sulfircin,[8] dnacin A1 and B1,[9] vitamin K3 and analogues[10,11] and other naphthoquinone analogues,[12] azido-homo-oxa steroid,[13] alkyllysophospholipid analogues,[14] substituted quinolin-4-one and 1,7-naphthyidin-4-one analogues,[15] 5-substituted 2-bromoindolo-[3,2]-quinoxalines,[16] tetrahydrisoquinolines[17] (review in ref 6). Dysidiolide was initially found active,[18] but later the natural as well as the synthetic material revealed inactive.[19]

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Results and Discussion

The CDC25A homologue has been cloned and expressed as a GST fusion protein in *Escherichia coli* and a straightforward colorimetric assay directed towards the search for inhibitors of this key enzyme has been proposed.[5] During our investigation of marine invertebrates for the search of biologically active compounds, both the dichloromethane and the methanol extracts of the dictyoceratid sponge collected in New Caledonia, *Coscinoderma mathewsi*[20] were found to be active in this bioassay. These extracts exhibited also antimicrobial activity towards *Staphylococcus aureus*. Purification monitored by bioassay using the CDC25A phosphatase led to the isolation of active sesterterpene sulfates. We wish to report here the isolation of coscinosulfate **1** as a selective inhibitor of the CDC25 phosphatase and of analogue **4**.

The freeze-dried sponge material was extracted successively with dichloromethane and then dichloromethane/methanol 1:1. The combined extracts were partitioned between water and dichloromethane and the dichloromethane soluble fraction partitioned between methanol and cyclohexane. The methanol soluble fraction retained the maximum of activity towards *S. aureus* and CDC25A. This fraction was first separated on a silicagel 60 silanized column eluted with acetonitrile/water (7:3) to furnish seven fractions. Active fractions were permeated through a Sephadex LH 20 column, to give three fractions. Fraction B submitted to chromatography on a C-18 column (CH_3CN/H_2O 4:6) gave pure **1**, and fraction A separated on a C-8 column (CH_3CN/H_2O 3:7) gave pure **4**. Compound **1**, an amorphous solid, mp 129–130 °C, $[\alpha]_D^{22}$: +5 (*c* 1.4), methanol), 0.1% dry weight. HRFAB-MS furnished the molecular formula $C_{31}H_{47}SO_6Na$.

An extensive ^1H and ^{13}C NMR study (1-D and 2-D) indicated the presence of a sesquiterpene moiety, corroborated in MS by the fragment ion at *m/z* 191, associated with a prenyl hydroquinone (Table 1). These data showed similarities with those given for halisulfate-1,[21] a compound previously isolated from an *Halichondria* sp., for which the 12*R**, 13*R** configuration was proposed. However optical rotation of halisulfate-1 was −27.3 when the rotation of **1** was +5. In addition, the coupling constant given for H-12 in halisulfate-1 (dd, $J = 5.4$, 1 Hz) differed from that observed in **1** (dd, $J = 11.1$, 1.6 Hz). These observations led us to thoroughly investigate the stereochemistry of **1**.

NOE difference experiments showed effects between methyl-21 and methyl-23 and between methyl-20 and H-5, hence suggesting a *trans*-decaline structure, in accordance with the chemical shift value of Me-23 (δ 13.5) with data given for a *trans* ring junction.[22] Finally, irradiation of proton H-12 caused enhancement of H-13 (14%) and Me-22 (2%).

To determine the absolute stereochemistry at C-12 and C-13 we turned to the Mosher method.[23] Hydrolysis of the sulfate group was carried out by passing **1** through a silicagel column eluted with acetone/dichloromethane/acetic acid (6:4:0.5) and the free alcohol **2** was obtained as an oil, $[\alpha]_D$ +5 (*c* 0.3 methanol), and −3 (*c* 0.3 chloroform). ^1H and ^{13}C NMR data of **2** were close to that of **1**, except the highfield shift of proton H-12 (dd, $J = 9.4$, 5.4 Hz) (from δ 4.44 to 3.48 ppm) and C-12 (from δ 82 to 77.4 ppm). Direct and long-range heteronuclear correlations allowed to assign all carbons and protons of the molecule. NOE difference experiments, however, allowed to observe NOE between H-12 and H-22 and H-13: irradiation of H-12 caused enhancement of H-9 (6%) and H-24 (2.5%) which suggested that **2** is epimeric at C-12 of the sulfate **1**.

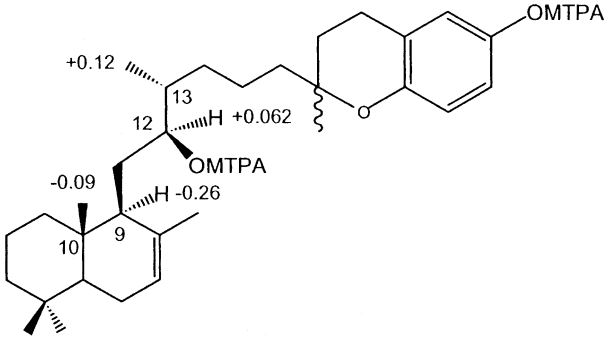

Figure 1. Δδ values [Δδ (in ppm) = $\delta_S - \delta_R$] obtained for the (*S*)- and (*R*)-MTPA esters (**3** and **3′**) of **2**.

Table 1. ^1H (300, 13 MHz) and ^{13}C (75 MHz) NMR of compound **1** (CD_3COCD_3, δ ppm) and long-range correlations (HMBC)

Assignment	δ ^1H (m, *J* in Hz)	^{13}C	HMBC (^1H–^{13}C)
1′		128.8	
2′		147.9	
3′	6.65 (d, 1H, 8.6)	115.8	1′, 5′
4′	6.44 (dd, 1H, 8.6)	113.2	6′, 2′, 5
5′		150.9	
6′	6.68 (d, 1H, 2.7)	116.4	4′, 2′, 19
2′ OH	7.63 (s, 1H)		1′, 2′
5′ OH	7.83 (s, 1H)		5′, 6′, 4′
1	1.77 (m, 1H), 1.18 (m, 1H)	38.8	
2	1.5 (m, 1H), 1.4 (m, 1H)	19.0	
3	1.32 (m, 1H), 1.16 (m, 1H)	42.5	
4		32.8	
5	1.21 (m, 1H)	50.2	23, 21, 20, 10, 9, 6
6	1.87 (m, 2H)	24.1	
7	5.3 (brs, 1H)	121.6	
8		136.7	
9	2.3 (m, 1H)	48.5	11
10		36.7	
11	1.5 (m, 1H), 1.18 (m, 1H)	27.0	12, 9, 10, 13, 8
12	4.44 (dd, 1H, $J = 11.1$, 1.6)	82.0	
13	2.3 (m, 1H)	36.9	
14	1.42 (m, 1H), 1.16 (m, 1H)	38.2	
15	1.37 (m, 2H)	25.8	14
16	2 (m, 2H)	39.6	17, 17, 15, 25
17		135.5	
18	5.38 (t, 1H, 7.1)	123.4	16, 19, 25
19	3.24 (d, 2H, 7.1)	28.3	6′, 18, 1′, 17, 2′
20	0.82 (s, 3H)	33.1	21, 4, 3, 5
21	0.86 (s, 3H)	21.9	20, 4, 3, 5
22	1.65 (s, 3H)	22.5	7, 8
23	0.72 (s, 3H)	13.6	9, 5, 1, 10
24	0.88 (d, 3H, 7.1)	13.1	13, 12
25	1.65 (s, 3H)	15.7	18, 17, 16

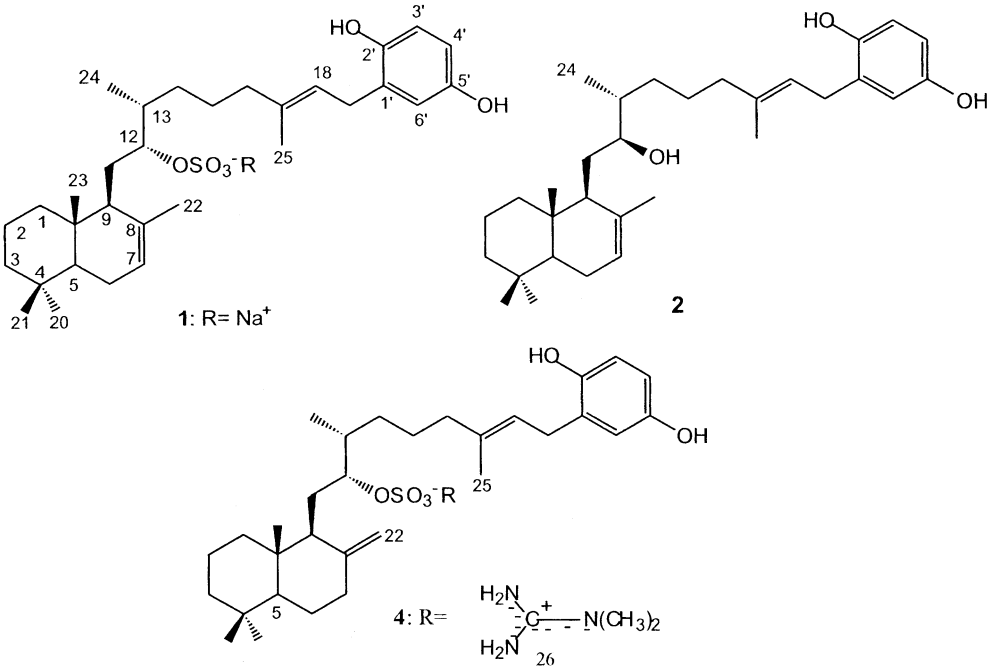

Figure 2. Structure of coscinosulfate **1**, dimethyl-guanidine analogue **4** and alcohol **2**.

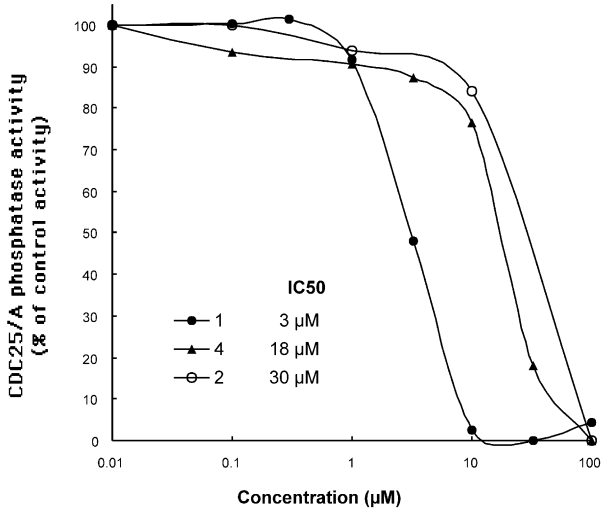

Figure 3. Effects of coscinosulfate (**1**), and compounds **2** and **4** on the activity of recombinant CDC25A, dose–response curve.

Attempts to protect the phenolic groups of **2** remained unsuccessful and we therefore condensed unprotected **2,** respectively, with the *R* and *S* α-methoxy-α-trifluoromethyl-phenyl acetic chloride[24] to obtain two esters **3** and **3′**.

^{1}H and ^{13}C NMR of esters **3** and **3′** showed the absence of the double bond at C-17, and the highfield chemical shift of the methyl-25 signal from δ 1.64 1.25 ppm indicated that a cyclisation occurred between one of the phenolic group and the double bond at C-17 leading to the two esters with the benzopyrane structures **3** and **3′**. Careful attribution of the chemical shifts of all protons was achieved by combination of 2-D homo and heteronuclear correlations. Calculation of the Δδ of the *R* and *S* MTPA derivatives: $\Delta\delta = \Delta S - \Delta R$ allowed to assign the 12*S*, 13*R* configuration for alcohol **2** and therefore the 12*R*, 13*R* configuration for **1**. Comparison of the chemical shifts of the H-12 vicinal protons in **3** and **3′** is given in Figure 1.

To confirm this proposal, a stereo-controlled synthesis of 1 was achieved[25] which led to a compound identical in all respects with natural **1**.

Compound **4**, amorphous powder $[\alpha]_D^{22}$ +8 (*c* 0.01, methanol), HR-MS 723.4797 provided the empirical formula $C_{37}H_{67}N_6SO_6$. The NMR data were close to those of **1** except for the presence of signals at δ 3.09 (s 6H) and δ 7.33 (4H, D_2O exchangeable), ^{13}C δ 158.0 and 38.3, assigned to a dimethylguanidine and presence of an exo-methylene: two protons at 4.68 and 4.35 ppm, ^{13}C δ 105.6 and 150.5 ppm. Chemical shifts of all protons and carbons were interpreted through HMBC and HMQC experiments and led to assign structure **4**.

Interpretation of NMR spectra allowed to analyze results of FAB-MS and led to the conclusion that the ion at *m/z* 723 corresponds to the M+ 88 adduct as it was observed in MS analysis of suvanin.[26]

Biological activity

Coscinosulfate **1** inhibits the CDC25A phosphatase with an IC_{50} of 3 μM, while **4** and the non-sulfated compound **2** were less active (IC_{50} values of 18 and 30 μM, respectively) (Figs 2 and 3). In a cytotoxic assay using KB cells both **1** and **4** were inactive at 10 μg/mL. Coscinosulfate (1–20 μM) was also unable to inhibit Jurkat and HBL100 cell proliferation (data not shown). However alcohol **2** was cytotoxic towards KB cells with an ID_{50} of 0.25 μg/mL. **1**, **2** and **4** displayed antimicrobial activity towards *S. aureus* with inhibition

zones, respectively: 12 mm (50 μg/disk), 11 and 10 mm (100 μg/disk).

Experimental

General experimental procedures

^{1}H and ^{13}C NMR spectra were obtained on a Brüker AC 300 spectrometer with standard pulse sequences operating at 300.13 and 75.47 MHz, respectively. The chemical shift values are reported as ppm units, and the coupling constants are in Hz. The programs used for J_{mod}, HMQC, HMBC ($J = 7$ Hz) experiments were those furnished in the Brüker manual. HRFABMS were measured on a ZAB-SEQ spectrometer in a thioglycerine matrix at the 'Service Central d'Analyses du CNRS' (Lyon), and EI-MS of compound on a Kratos MS 50 at 70 eV. UV spectra were obtained in MeOH, using a Kontron type Uvikon 930 spectrophotometer, and IR spectra were taken as KBr pellets on a Nicolet (Impact 400D) FT-IR spectrophotometer. Optical rotations were measured with a Perkin-Elmer 141 polarimeter with a sodium lamp operating at $\lambda = 589$ nm in a 10-cm microcell. Silica gel column chromatography was carried out using Kieselgel 60 (230–400 mesh, Merck), C-2 and C-18. Fractionations were monitored by TLC using Aluminum-backed TLC sheets (Si gel 60 F-254, 0.25-mm thick) with visualization under UV (254 and 365 nm) and Liebermann spray reagent.

Collection, extraction and isolation procedures

The sponge *C. mathewsi* Lendenfield (Dyctioceratida)[20] was collected along the Eastern coast of New Caledonia. The freeze-dried sponges (148 g) were extracted twice with dichloromethane, and then twice with methanol/dichloromethane 1:1. The solvents of the combined extracts were evaporated in vacuum and the residue submitted to solvent partitions. A partition between water and dichloromethane led to the bioactive dichloromethane fraction, which was partitioned, between cyclohexane and methanol. Only this last fraction was found biologically active. The methanolic fraction was first chromatographied on a silica gel 60 silanised (C-2) column at atmospheric pressure, eluted with a water/acetonitrile gradient. Fractions 3 to 5 eluted respectively with acetonitrile/water 8:2, 7:3, 6:4, retaining maximum activity were pooled and submitted to a filtration on a L1210 gel eluted with methanol-dichloromethane 1/1 to give three active fractions A, B, C. Fraction A was separated on a C-18 column, under atmospheric pressure, eluted with acetonitrile/water (3/7) to give compound **4** (25 mg). Fraction B, after separation by HPLC on a C18 column (CH_3CN/H_2O 4:6) gave **1** (14 mg). ^{1}H and ^{13}C NMR see Table 1. HRFAB-MS m/z 593.2888 $[M+Na]^+$, calcd for $C_{31}H_{47}SO_6Na_2$: 593.2889.

Alcohol 2. ^{1}H NMR (300 MHz), $CDCl_3$, δ ppm 0.71 (s, 3H, H-23), 0.82 (s, 3H, H-20), 0.84 (s, 3H, H-21), 0.92 d, 1H, 6.7, H-24), 1.64 (s, 3H, H-25), 3.27 (d, 2H, 7.2, H-19), 3.48 (dd, $J = 9.4$, 5.4 Hz, 1H, H-12), 5.29 (t, 1H, 7.2, H-19), 6.54 (dd, 1H, 2.9, 8.5) (H-4′), 6.62 (d, 1H, 8.5, H-3′), 6.64 (d, 1H, 2.9, H-2′).

^{13}C NMR (75 MHz) $CDCl_3$, δ ppm 13.5 (C-23), 15.4 (C-25), 15.5 (C-24), 18.7 (C-2), 21.8 (C-21), 22.5 (C-22), 23.8 (C-6), 24.5 (C-15), 28.3 (C-19), 31.2 (C-11), 32.2 (C-14), 32.9 (C-4), 33.1 (C-20), 36.2 (C-10), 39.2 (C-1), 39.5 (C-16), 40.2 (C-13), 42.1 (C-3), 49.8 (C-5), 51.1 (C-9), 77.4 (C-12), 113.4 (C-4′), 116.2 (C-3′), 122.1 (C-18), 122.7 (C-7), 128.2 (C-1′), 137.7 (C-17), 147.3 (C-2′), 134.9 (C-8), 147.3 (C-2′), 149.8 (C-5′).

(*S*)-MTPA ester 3 of alcohol 2. To a solution of **2** (6 mg, 10 mmol) in 3 mL of freshly distilled dichloromethane (on P_2O_5), 4.9 mg (40 mmol) of triethylamine and 5.6 mL (30 mmol) of (*S*)-(+)-α-methoxy-α-trifluoromethyl) phenylacetic acid chloride were added. The mixture was kept 15 h at room temperature, 3.7 mL (29 mmol) of 3-[(dimethylamino) propyl] amine are added. After 10 min, solvent and reagents were removed under reduced pressure. The residue was purified on a silica gel column (CH_2Cl_2/MeOH, 99:1) to furnish **3** (3.8 mg, 33%).

^{1}H NMR ($CDCl_3$, δ ppm) 7.65 (m, 2H), 7.55 (m, 2H), 7.4 (m, 3H), 7.35 (m, 3H), 6.81 (m, 1H, H-6′), 6.8 (m, 1H, H-3′); 6.75 (m, 1H, H-4′), 5.35 (sl, 1H, H-7), 5.28 (m, 1H, H-12), 3.66 (s, 3 h, OMe-MTPA), 3.6 (s, 3H, OMe-MTPA), 2.75 (t, 2H, $J = 6.5$ Hz, H-19), 1.9 (m, 1H, H-13); 1.8 (m, 2H, H-18), 1.67, (s, 3H, H-22), 1.6 (m, 2H, H-11), 1.25 (s, 3H, H-25), 1.24 (m, 1H, H-9), 0.92 (d, 3H, H-24), 0.77 (s, 6H, H-21 and 20), 0.7 (dd, 1H, H-5), 0.6 (s, 3H, H-23).

(*R*)-MTPA ester 3′ of alcohol 2. ^{1}H NMR ($CDCl_3$, δ ppm) 7.65 (m, 2H), 7.55 (m, 2H), 7.4 (m, 3H), 7.35 (m, 3H), 6.81 (m, 1H, H-6′) 6.8 (m, 1H, H-3′), 6.75 (m, 1H, H-4′), 5.37 (sl, 1H, H-7), 5.22 (m, 1H, H-12), 3.66 (s, 3H, OMe-MTPA), 3.4 (s, 3H, OMe-MTPA), 2.72 (t, 2H, $J = 6.5$ Hz, H-19), 1.9 (m, 1H, H-13), 1.79 (m, 2H, H-18), 1.69 (m, 2H, H-11), 1.5 (m, 1H, H-9), 1.22 (s, 3H, H-25), 0.91 (m, 1H, H-5), 0.82 (d, 3H, H-24), 0.8 (s, 6H, H-20 and 21), 0.6 (s, 3H, H-23).

Compound 4. HR-MS: m/z 723.4797, calcd for $C_{37}H_{67}N_6SO_6$ 723.4802. ^{1}H NMR (300 MHz, $(CD_3)_2CO$-d_6) δ ppm (m, J, Hz): 0.62 (s, 3H, H-23), 0.76 (s, 3H, H-21), 0.81 (s, 3H, H-20), 0.82 (d, 3H, 7, H-24), 3.09 (s, 6H, $N(Me)_2$), 3.22 (d, 2H, 7.2, H-19), 4.2 (dd, 1H, 11.1, 1.6, H-12), 4.35 (s, 1H), 4.68 (s, 1H), H_2-22, 6.4 (dd, 1H, 8.5, 2.9, H-4′), 6.6 (d, 1H, 8.5, H-3′), 6.65 (d, 1H, 2.9, H-6′), 7.3 (brs, 4H, NH_2), 7.5 (s, 1H, OH-2′), 7.65 (s, 1H, OH-5′).

^{13}C NMR (75 MHz, $(CD_3)_2CO$-d_6) δ ppm 13.6 (C-24), 14.8 (C-23), 15.8 (C-25), 19.1 (C-2), 21.9 (C-21), 24.0 (C-15), 24.1 (C-6), 26.1 (C-11), 28.7 (C-19), 32.7 (C-14), 33.1 (C-4), 33.6 (C-20), 37.0 (C-10), 37.1 (C-13), 37.7 (C-1), 38.3 (N $(Me)_2$), 38.5 (C-7), 39.9 (C-16), 42.7 (C-3), 51.6 (C-9), 55.6 (C-5), 80.7 (C-12), 105.6 (C-22), 113.2 (C-4′), 115.9 (C-3′), 116.6 (C-6′), 123.5 (C-18), 128.9 (C-1′), 135.8 (C-17), 148.1 (C-2′), 150.5 (C-8), 151.0 (C-5′), 158 (C-26).

Bioassays

Antibacterial activity was estimated using the Petri dish bioassay. Dishes were inoculated by *S. aureus*, and disks (6 mm) impregnated with **1** displayed inhibition zone of 12 mm (50 μg/disk), **2** and **4** inhibition zones, respectively, of 11 and 10 mm (100 μg/disk).

Cell culture

Human T lymphoblastic Jurkat cells (ECACC) were cultured in RPMI 1640 medium (Gibco-BRL) containing 10% FCS, 2 mM L-glutamine and 25 μg/mL gentamycin. Cells were maintained in a humidified atmosphere of 5% CO_2 at 37 °C. For the proliferation assay and the cell cycle analysis exponentially growing Jurkat cells were seeded at a cell density of 3.5×10^5 cells/mL into a 12 well-plate and incubated in complete medium in the presence of coscinosulfate at concentrations ranging from 0 to 20 μM for 30 h. SV-40 immortalized human breast epithelial cell line HBL-100 were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL) supplemented with 10% FCS, 2 mM L-glutamine and 50 μg/mL gentamycin (Gibco-BRL). Adherent human breast epithelial cells were cultured in a 24-well plate at a density of 5×10^4 cells per 400 μL complete medium per well. Cells were grown to 50% confluence and then treated for 30 h with medium containing coscinosulfate (1–20 μM) prepared in DMSO.

Cell counts and flow cytometry cell cycle analysis

Cell proliferation was evaluated by a direct counting with a hemocytometer. Simultaneously, cell cycle distribution was analyzed by flow cytometry. Briefly, cell samples were collected by centrifugation, washed once with PBS and fixed in cold 70% ethanol for 4 h. Fixed cells were washed with PBS, incubated with 5 μg/mL RNAse A (Sigma Chemical Co.) and stained for 30 min at 37 °C with 25 μg/mL propidium iodide (Aldrich). The stained cells were analysed on a FACSort cytofluorimeter (Becton-Dickinson) for relative DNA content based on red fluorescence levels. Cell cycle analyses were done using the MultiCYCLE (P. Rabinowich). This program models DNA histogram following the algorithm of Dean and Jett as the sum of two gaussian peaks for G1 and G2 cells and a polynomial function broadened by gaussian variability for S cells. Initially, the fractions of cells in G1 (position of G1 peak, variation coefficient of G1 peak) are estimated interactively and then fitted nonlinearly using the Marquardt algorithm.

Preparation and purification of GST-CDC25A

Bacterial growth and fusion protein induction. An *E. coli* strain was transformed by a plasmid encoding the gene fusion constructs of Glutathione-S-Transferase (GST) and human CDC25A. Bacteria were first grown overnight at 37 °C in the presence of 100 μg/mL ampicilin in LB medium. 4 mL of this preculture were inoculated per litre of LB containing 100 μg/mL ampicillin. Incubation was continued at 30 °C until the culture O.D. at 600 nm reached 0.8–1. At this time, 0.4 mM IPTG was added and the culture was incubated at 25 °C for at least 7 h (GST-CDC25A). Cells were then harvested by a 3000*g* centrifugation for 15 min at 4 °C. Pellets were kept frozen at −80 °C until extraction.

Purification of the fusion protein. The bacterial pellet was homogenised by sonication at 4 °C in lysis buffer (1% Nonidet-P40, 1 mM EDTA, 1 mM DTT, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 10 μg/mL soybean trypsin inhibitor, 100 mM benzamidine in Phosphate Buffered Saline). The homogenate was centrifuged at 100,000*g* for 30 min at 4 °C and the supernatant was stored in 10 mL aliquots at −80 °C. Fusion proteins were purified by affinity chromatography on glutathione-agarose beads. 10 mL of bacterial extract were incubated with 400 μL of GSH-beads (equilibrated in lysis buffer) for 30 min at 4 °C under constant rotation. The beads were washed four times with 10 mL of lysis buffer, followed by four washes with 10 mL of Tris buffer B (50 mM Tris, pH 8, 50 mM NaCl, 1 mM EDTA, 20 mM DTT). Fusion proteins were eluted by incubation with 4×1 mL 20 mM glutathione in Tris buffer B. Efficiency of the elution was monitored by a phosphatase assay and by SDS-PAGE. The GSH-beads were recycled by a wash with 1 M NaCl followed by equilibration in lysis buffer.

Assay of GST-CDC25A phosphatase activity. Assays were performed in microtitration plates as described by Baratte et al.[5] 20 μL of GST-CDC25A were added to 20 μL of 100 mM DTT in Tris buffer A (50 mM Tris, pH 8, 50 mM NaCl, 1 mM EDTA, 1 mM DTT) and 140 μL of Tris buffer A. Plates were preincubated at 37 °C for 15 min in a Denley Wellwarm 1 microplate incubator. Reactions were initiated by addition of 20 μL of 500 mM *p*-nitrophenylphosphate in Tris buffer A. After a 30 min incubation at 37 °C, absorbance at 405 nm was measured in a Biorad microplate reader.

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