

Steroidal Derived Acids as Inhibitors of Human Cdc25A Protein Phosphatase

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Abstract—A group of steroidal derived acids were synthesized and found to be human Cdc25A inhibitors. Their potency ranged from 1.1 to >100 μ M; the best ones compare very favorably with that of the novel cyano-containing 5,6-*seco*-cholesteryl acid **1** (IC₅₀ = 2.2 μ M) reported by us recently (Peng, H.; Zalkow, L. H.; Abraham, R. T.; Powis, G. J. *Med. Chem.* **1998**, *41*, 4677). Structure–activity relationships of these compounds revealed that a hydrophobic cholesteryl side chain and a free carboxyl group are crucial for activity. The distance between these two pharmacophores is also important for the potency of these compounds. Several of the compounds showed selective growth inhibition effects in the NCI in vitro cancer cell line panel. © 2000 Elsevier Science Ltd. All rights reserved.

Introduction

Accumulating evidence suggests that cell cycle-related oncogenes contribute to the neoplastic transformation of normal cells. Cdc25A is now considered as such an oncogene^{1,2} that was found overexpressed in a number of human cancers, including head and neck cancer, colon cancer, non-small cell lung cancer and breast cancer.^{3–5} It is also found significantly associated with lymphomas of more aggressive phenotypes.⁶ Cdc25A is a protein phosphatase expressed early in the G1 phase of the cell cycle, and it acts to remove inhibitory phosphate groups from the cyclin/CDK complexes which, in turn, trigger cell cycle progression from G1 to S phase.^{7,8} Cdc25A protein phosphatase is considered as a promising target for the development of potential chemotherapeutic anti-cancer agents because of its high specificity toward substrate and its unique phase specificity in the cell cycle.⁹ However, the mechanisms that lead to Cdc25A dysregulation, and the resulting neoplastic transformations in human tumors are not clear yet.

A number of Cdc25A phosphatase inhibitors (Fig. 1) have been reported, including vanadate,¹⁰ the natural

products dysidiolide,¹¹ menadione (vitamin K₃) and its naphthoquinone analogues,^{12,13} SC- $\alpha\alpha\delta 9$ and its analogues,¹⁴ and our recently published novel cyano-containing 5,6-*seco*-cholesteryl derivatives,¹⁵ represented by **1**, the most potent Cdc25A inhibitor reported (IC₅₀ = 2.2 μ M) (Fig. 1). Menadione and its naphthoquinone analogues were considered to inactivate Cdc25A irreversibly by modifying its active site. Vanadate and SC- $\alpha\alpha\delta 9$ were reported to be competitive to the Cdc25A substrate binding,^{10,14} presumably by mimicking the phosphate group on the substrate with the carboxyl or vanadate moiety. Compound **1**, which contains a free carboxyl group, may inhibit Cdc25A with a similar mechanism as SC- $\alpha\alpha\delta 9$.

To identify unique chemical templates for the design of inhibitors active against oncogenic components of cell cycle and intracellular signaling pathways, unusual chemical transformations of readily available natural product scaffolds were investigated. As a result, a group of novel cyano-containing 5,6-*seco*-cholesteryl derivatives represented by **1** were discovered as potent reversible Cdc25A phosphatase inhibitors, possessing antitumor activities. In search of new Cdc25A inhibitors with simpler structures and ready availability, a series of steroidal derived acids were synthesized and evaluated in the Cdc25A enzyme inhibition assay. The potencies of the best inhibitors in this group compared very favorably

Keywords: antitumor compds; antiproliferatives agents; enzyme inhibitors; steroids and sterols.

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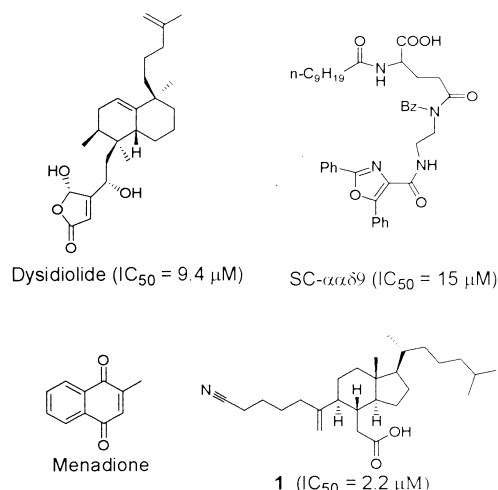


Figure 1. Chemical structures for dysidiolide, SC- $\alpha\alpha\delta$, menadione, and compound **1**.

with that of compound **1**. Some of these also showed significant growth inhibition activities in human tumor cell cultures.

Chemistry

The synthesis of **7** is illustrated in Scheme 1. Ozonolysis of pregnenolone acetate in ethyl acetate:petroleum ether at $-60^\circ C$ afforded 3 β -acetoxy-5-oxo-5,6-*seco*-pregnane-6-oic acid **5** in 60% yield. Reductive amination of **5** using 4 equiv of *N,N*-dimethylethylenediamine and 1.5 equiv of sodium cyanoboro hydride gave **7** in a ratio of 20*R*:20*S*=3:1, the major isomer was assigned the 20*R* configuration based on literature precedent.^{16,17}

The syntheses of compounds **11**¹⁸–**14** are illustrated in Scheme 2. Compounds **13**¹⁸ and **14** were synthesized from 4-cholesten-3-one in a ratio of 5:1 (**13**:**14**), and the relative configurations were determined by the NOE observed between Me-19 and H-28 in the NOESY

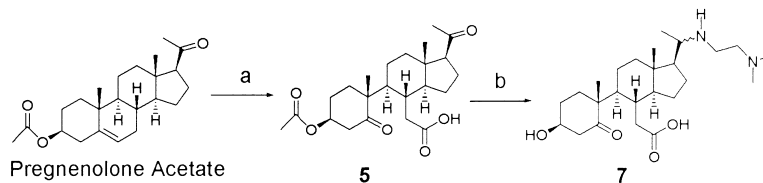
spectrum of compound **14**. The β -isomer **14** was easily epimerized to the α -isomer when treated with KOH. Compounds **11** and **12** were obtained from treatment of a mixture of **13** and **14** (5:1) with saturated KOH solution in water and THF (v/v, 5:2).

The syntheses of compounds **15**^{19,20}–**19** are illustrated in Scheme 3. Compound **15** was obtained by treatment of the potassium salt of cholestanol with carbon disulfide. Alkyl *O*-cholestanyl xanthates **16**–**19** were synthesized by treatment of compound **15** with 1 equivalent of alkyl halide in DMSO.

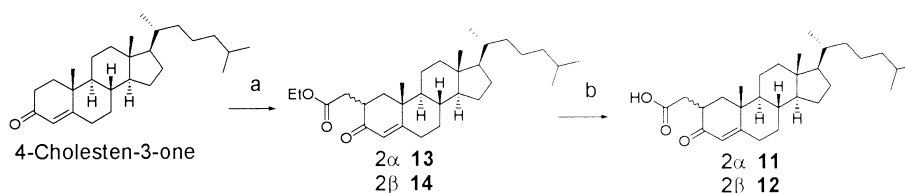
Results and Discussion

Compounds **2**–**16** were evaluated as inhibitors of the recombinant human Cdc25A phosphatase at $25^\circ C$, using fluorescein diphosphate (FDP) as the substrate. The inhibitory activities of these compounds are shown in Chart 1. The B ring opened acid (**2**), 3 β -acetoxy-5,6-*seco*-5-oxo-cholestan-6-oic acid, resembles the previously reported compound **1**, but the A ring of the cholestane skeleton remains intact. It showed fairly good inhibitory activity against Cdc25A ($IC_{50} = 9.3 \mu M$). However, when the free carboxyl group was methylated, the resulting compound **3** was found to be inactive, suggesting that the free carboxylic acid functionality of compound **2** is necessary for activity. The carboxyl group may serve as a surrogate phosphate group which interacts with the active site arginine (Arg-436), thus, competing with substrate binding. Compound **4** inhibited Cdc25A at the same level ($IC_{50} = 9.5 \mu M$) as compound **2** suggesting that the 3-acetoxy group was not critical for activity.

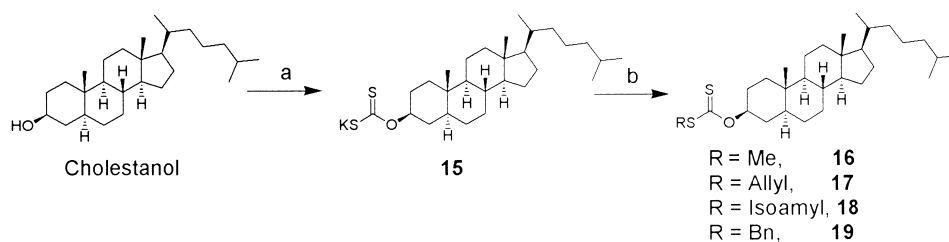
In contrast, when the 5,6-*seco*-6-acid moiety was present in the pregnenolone nucleus, **5** and **6**, essentially all activity was lost, confirming the hypothesis that a hydrophobic pocket exists close to the active site of this enzyme. Replacement of carbon atoms in the side chain by more polar atoms, as in compound **7**, likewise led to



Scheme 1. Reagents: (a) O_3 , $-60^\circ C$, petroleum ether:ethyl acetate 1:1; piperidine; 2 M HCl, 60%; (b) *N,N*-dimethylethylenediamine, $CH_3OH:THF$, $NaB(CN)H_3$, 25%.



Scheme 2. Reagents: (a) LDA, THF; ethyl bromoacetate; 93%; (b) KOH, $H_2O:THF$; 64%.



Scheme 3. Reagents: (a) K, $(\text{CH}_3\text{OCH}_2\text{CH}_2)_2\text{O}$, CS_2 , 85%; (b) RX, DMSO (X = Cl, Br, or I), 80–85%.

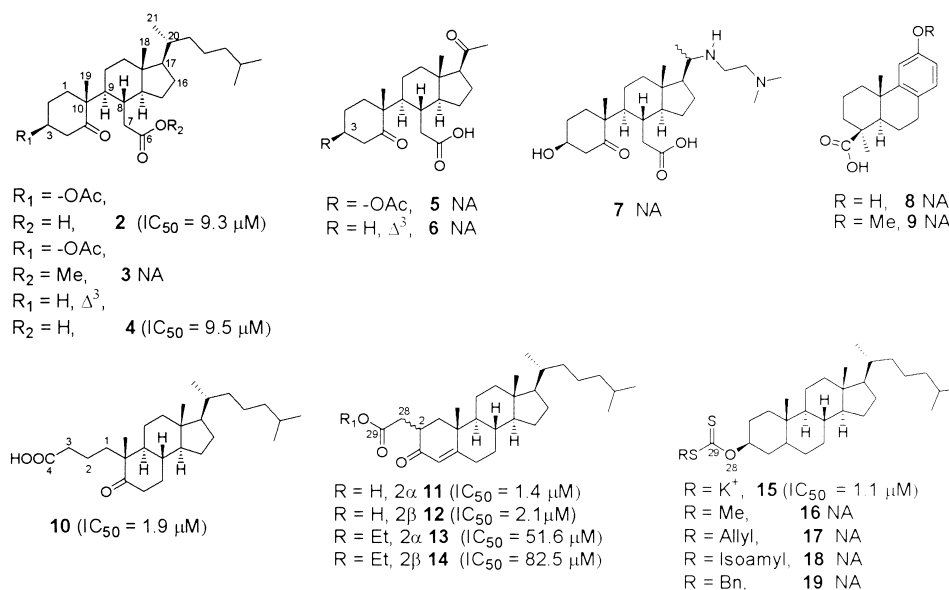


Chart 1. IC_{50} values were determined from at least two independent determinations, each run in triplicate, where the variation from the mean is $\pm 20\%$ or less. NA stands for IC_{50} value larger than $100 \mu\text{M}$. ND stands for not determined.

loss of activity. Lacking the steroidal skeleton, the natural product podocarpic acid **8** and its methyl ester **9** were found to be inactive.

To determine if the structural features introduced by opening of the B ring are critical for activity, an A ring opened cholesteryl acid, 4,5-*seco*-5-oxo-cholestan-4-oic acid (**10**), was synthesized and found to be more potent ($\text{IC}_{50} = 1.9 \mu\text{M}$) than the B ring cleaved acid **2** ($\text{IC}_{50} = 9.2 \mu\text{M}$) in the Cdc25A inhibition assay. In addition, compound **11**, which was envisioned to hold the carboxyl group at the same position as in compound **10**, but has an intact cholesteryl ring system, was found to have the same potency level ($\text{IC}_{50} = 1.4 \mu\text{M}$) as **10**, suggesting that one of the most important features for Cdc25A inhibition is the relative positioning of the cholesteryl side chain and the free carboxylic acid, while opening of either the A or B ring is not necessary. The kinetic characteristic of Cdc25A inhibition with compound **11** was studied using FDP as substrate. The inhibition pattern is most consistent with a competitive inhibition model ($K_i = 0.57 \mu\text{M}$), suggesting that the carboxyl group mimics a phosphate of the substrate. Compound **12**, the 2β isomer of **11**, exhibited similar inhibition potency at $\text{IC}_{50} = 2.1 \mu\text{M}$.

In light of this consideration, the dithiocarboxylic acid derivative (**15**) of cholesterol was synthesized and found

to be slightly more potent ($\text{IC}_{50} = 1.1 \mu\text{M}$). This may be due to the better interaction between the larger negatively charged sulfur atoms and the active site arginine group, or to the improved binding affinity associated with the longer distance between the hydrophobic side chain and the surrogate phosphate group. Again, when the carboxyl group of **11** was protected as in compound **13**, or when the xanthate group of **15** was alkylated as in compounds **16–19**, inhibition was lost. The fact that more lipophilic compounds, such as **13**, **16–19** were inactive as compared to their acid counterparts **11** and **15** demonstrated that the inhibitory activities of these compounds are not due to the lipophilicity of the steroidal structure. Lipophilic compounds sometime disrupt enzyme activity by aggregating or stacking effects.

In summary, three crucial features emerged from analysis of the structure–activity relationship of the compounds described above. (1) A free alkyl carboxyl or xanthate functionality is required for inhibitory activity. (2) A completely hydrophobic alkyl chain, such as the cholesteryl side chain contributes greatly to the potency. (3) The inhibitory activity is sensitive to the distance between the carboxyl or xanthate functionality and the alkyl side chain.

Only the crystal structure of the catalytic domain of Cdc25A, which lies at the edge of the crystal structure,

has been reported;²¹ therefore, it is difficult, at this time, to verify the SAR by molecular modeling. In attempted modeling (data not shown), when the carboxyl group forms a reasonable interaction with the active site arginine (Arg 436), then the cholesteryl side chain hangs outside the reported structure, making it impossible to obtain any useful information. We therefore, for comparison purposes, simply determined the distances between the carboxyl group and the end of the side chain in the lowest energy conformations of compounds **2**, **10**, **11** and **15**, to see if this information could be related to the activities of the various compounds. As illustrated in Figure 2, the distances between the carbon atom (labeled as A) that holds the two oxygen atoms of the carboxylate, or the carbon atom (labeled as A) that holds the two sulfur atoms of the xanthate functionality, and the atom (labeled as B) that holds the two methyl groups on the cholesteryl side chain were measured. Interestingly, the distances appeared to be well correlated with their inhibitory activities. In compound **15**, the most potent Cdc25A inhibitor ($IC_{50} = 1.1 \mu M$) in this series, a distance of 17.7 Å was observed between A and B (Fig. 2). This series of compounds are either A or B-ring cleaved, or with an intact steroidal skeleton. The correlation is however not applicable to compounds like **1**, in which both the A and B rings are opened, and the cyano-containing side chain contributes additionally to its inhibitory activity.¹⁵ Of course, it is recognized that the gas phase modeling of distances may not relate to conditions in solution.

Compound **2** was tested for its growth inhibition effects on HT-29 colon cancer in cell culture²² and showed an IC_{50} of 16.2 μM .²³ The in vitro cytotoxicity of compounds **4**, **10–12** and **15** was evaluated at the National Cancer Institute against a panel of 60 human tumor cell lines representing nine different cancer types. The GI_{50} values

(micromolar concentrations required to produce 50% growth inhibition) are shown in Table 1 for sensitive subpanels.

Compound **4** showed a mean GI_{50} value (MG MID) at 3.95 μM for 54 tumors. It was more selective against the HOP-92 non-small cell lung cancer ($GI_{50} = 0.66 \mu M$), the OVCAR-8 ovarian cancer ($GI_{50} = 1.62 \mu M$), two breast cancer cell lines NCI/ADR-RES ($GI_{50} = 1.37 \mu M$) and BT549 ($GI_{50} = 1.12 \mu M$), and the leukemia HL-60 ($GI_{50} = 56 nM$).

Compound **11**, which is a more potent Cdc25A inhibitor than **4**, showed an average GI_{50} of 7.07 μM . It exhibited selectivity over HOP-92 non-small cell lung cancer ($GI_{50} = 3.56 \mu M$), HCC2998 colon cancer ($GI_{50} = 3.96 \mu M$), OVCAR8 ovarian cancer ($GI_{50} = 4.63 \mu M$), and two breast cancer cell lines, NCI/ADR-RES ($GI_{50} = 4.20 \mu M$) and BT-549 ($GI_{50} = 4.08 \mu M$). Compound **12**, the 2 β epimer of **11** showed a similar sensitivity pattern to that of **11**, but was less active in each panel. In contrast to their potent Cdc25A inhibition activities, the MG MIDs of compounds **10** and **15** were at 19.5 and 18.2 μM , respectively. Compound **10** showed good selectivity for leukemia SR (1.89 μM) and compound **15** exhibited good selectivity for HT-29 colon cancer (1.22 μM). The reasons that the more potent Cdc25A inhibitors **10**, **11**, and **15** were less active in the in vitro cytotoxicity screening are unknown at this time. Poor penetration of plasma membrane is possible, and we can not rule out the possibility that the observed cytotoxicity is not related to Cdc25A inhibition. The finding that compounds **4**, **11**, **12** and **15** were more selective for the subpanels of non-small cell lung cancer, colon cancer, and breast cancer as compared to other tumor cell lines, is consistent with reports that Cdc25A phosphatase is overexpressed in these cancer cell lines.^{3–5}

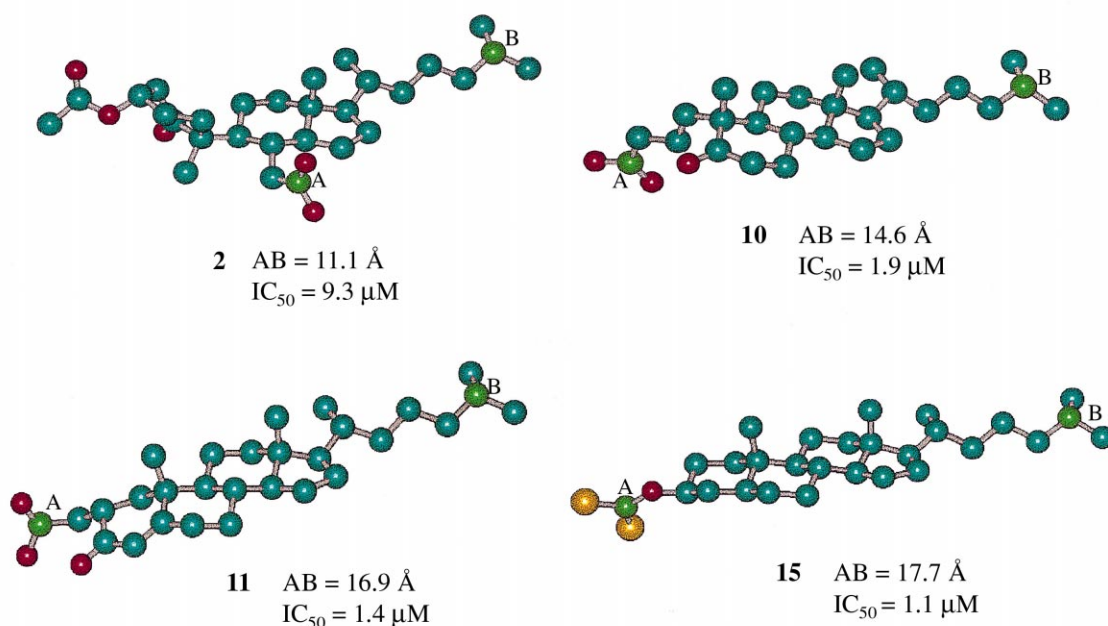


Figure 2. Calculated lowest-energy conformations for compounds **2**, **10**, **11** and **15**.

Table 1. Inhibition of in vitro cancer cell lines by compounds **4**, **10**–**12** and **15**

Cell lines	GI ₅₀ (μM) ^a				
	4	10	11	12	15
Leukemia					
HL60TB	0.056	6.27	8.28	6.11	>25
SR	5.55	1.89	6.16	5.73	>25
Non-small cell lung cancer					
EKVX	4.21	>25	4.86	6.91	6.67
HOP-92	0.66	>25	3.56	6.52	>25
NCI-H23	4.51	>25	>25	12.3	>25
Colon cancer					
COLO205	ND ^c	>25	5.19	5.36	11.7
HCC2998	4.36	ND ^c	3.96	5.88	3.92
HT29	4.44	19.4	4.87	6.94	1.22
Ovarian cancer					
OVCAR8	1.62	>25	4.63	8.90	>25
Prostate cancer					
PC-3	3.65	9.32	7.75	10.7	5.75
Breast cancer					
NCI/ADR-RES	1.37	>25	4.20	16.5	>25
BT-549	1.12	>25	4.08	10.7	>25
T-47D	3.90	9.18	7.35	11.2	>25
MG-MID ^b	3.95	19.5	7.07	12.0	18.2

^aGI₅₀ represents the compound concentration (μM) required to achieve 50% inhibition of tumor cell growth.

^bMG MID represents the calculated mean GI₅₀ for all panels.

^cNot determined.

Conclusion

In summary, a group of steroidal derived acids were found to be potent Cdc25A inhibitors (IC₅₀ = 1.1–9.5 μM). Structure–activity relationships of these compounds revealed that potency depended on the presence of a free carboxyl group and an alkyl side chain. Potassium xanthate **15**, with a distance of about 17.7 Å between the surrogate phosphate group (xanthate) and the hydrophobic side chain, was found to be the most potent Cdc25A inhibitor to date. The lead compounds identified in this study provide readily available Cdc25A inhibitors (**10**–**12**, **15**) and control compounds (**3**, **5** and **6**) for Cdc25A and cell cycle studies. Although the selectivities of these inhibitors over the other two isoforms (B, C) of Cdc25 phosphatase or other protein tyrosine phosphatases have not been evaluated at this time, it is important to have selective inhibitors since Cdc25C, a normal gene product, is a critical component for DNA damage induced cell cycle check points.²⁴ It is noteworthy that compounds **4**, **10**–**12** showed selective antitumor activities for non-small cell lung, colon, breast and ovarian cancers in in vitro tumor cell screening.

Experimental

Starting materials were purchased from Aldrich. Thin-layer chromatography analysis (TLC) was performed on aluminum sheets precoated with 0.2 mm of silica gel containing 60F254 indicator. Flash chromatography

was run using 230–400 mesh silica gel. Reverse phase high performance liquid chromatography (HPLC) was run on a Phenomenex[®] LUNA 5 μ C18 semi-preparative column (Phenomenex, Torrance, CA). The homogeneity of all the compounds was routinely checked by TLC on silica gel plates, and also by HPLC. Fourier transformed infrared spectra were obtained on a Nicolet 520 FTIR spectrometer. ¹H (300 or 400 MHz), ¹³C (75 or 100 MHz) NMR and DEPT spectra were recorded on either a Varian Gemini-300 or on a Varian XL-400 spectrometer. High-resolution mass spectra (EI or FAB) were recorded on a VG Analytical 70-SE mass spectrometer equipped with a 11-250J data system. Elemental analyses were performed by Atlantic Microlab, Norcross, GA.

Synthesis and spectral data for steroidal inhibitors 2–19

The syntheses of compounds **2**,²⁵ **3**,²⁶ **4**,²⁷ **5**,²⁸ **7**,^{16,17} **10**,²⁴ **11**, **13**,¹⁸ **16** and **17**^{19,20} were performed as previously reported except as indicated.

3β-Acetoxy-5,6-seco-5-oxo-cholest-6-oic acid (2). Compound **2** was obtained as colorless crystals: mp 114–116 °C (Lit.²⁴ mp 115–117 °C); FTIR (neat film) 3400–2500 (br), 2960, 2868, 1736, 1716, 1249 cm^{−1}; ¹H NMR (CDCl₃, 300 MHz) δ 9.89 (broad), 5.29 (brs, 1H), 3.11 (dd, *J* = 14.4, 4.3 Hz, 1H), 2.33 (d, *J* = 14.5 Hz, 1H), 2.08 (s, 3H), 0.99 (s, 3H), 0.88 (d, *J* = 6.4 Hz, 3H), 0.83 (d, *J* = 6.6 Hz, 6H), 0.65 (s, 3H); ¹³C NMR and DEPT (CDCl₃, 100 MHz) δ 216.4 (C), 178.6 (C), 170.3 (C), 73.5 (CH), 55.9 (CH), 54.4 (CH), 52.3 (C), 43.1 (CH₂), 42.5 (C), 41.5 (CH), 39.7 (CH₂), 39.4 (CH₂), 35.9 (CH₂), 35.7 (CH), 35.5 (CH), 34.4 (CH₂), 34.1 (CH₂), 27.9 (CH), 27.9 (CH₂), 25.1 (CH₂), 24.3 (CH₂), 23.7 (CH₂), 23.0 (CH₂), 22.8 (CH₃), 22.5 (CH₃), 21.5 (CH₃), 18.5 (CH₃), 17.6 (CH₃), 11.6 (CH₃); EIMS *m/z* (relative intensity) 416.4 (M⁺-AcOH, 43), 398.4 (M⁺-H₂O, 21), 306.3 (34), 247.3 (97), 110.1 (100); CIMS *m/z* (relative intensity) 477.4 (M⁺ + 1, 4), 417.4 (M⁺-AcOH + 1, 88), 399.3 (100), 357.3 (40), 331.3 (45).

3β-Acetoxy-5,6-seco-5-oxo-pregnan-6-oic acid (5). Ozone was passed into a stirred and cooled (chloroform/dry ice bath) solution of 5 g pregnenolone acetate in a 300 mL mixture of ethyl acetate:petroleum ether (1:1) for 40 min, until the solution turned light blue. To the cold solution was added 5 mL piperidine. The mixture was stirred for 2 h at −60 °C. The resulting white precipitate was isolated by filtration, then dissolved in 80 mL chloroform which was washed with 2 M HCl (3 × 10 mL), then with water, dried over anhydrous Na₂SO₄, and the solution was evaporated. The residue was subjected to flash chromatography (petroleum ether:ethyl acetate 3:1) to yield 3β-acetoxy-5-oxo-5,6-seco-pregnan-6-oic acid (**5**), 3.1 g (60%), as an oil: IR (neat film) 3400–2500 (br), 2951, 1735, 1710, 1703, 1697 cm^{−1}; ¹H NMR (CDCl₃, 300 MHz) δ 5.35 (br, 1H), 3.16 (dd, *J* = 14.3, 4.5 Hz, 1H), 2.09 (s, 3H), 1.97 (s, 3H), 1.06 (s, 3H), 0.61 (s, 3H); ¹³C NMR (CDCl₃, 100 MHz) δ 212.1, 209.2, 176.5, 170.3, 73.3, 63.4, 53.9, 52.2, 43.9, 43.2, 41.1, 38.7, 35.4, 34.4, 33.2, 31.4, 25.2, 24.6, 23.0, 22.6, 21.2, 17.7, 13.0; EIMS *m/e* (relative intensity) 346

($M^+ - CH_3COOH$, 30), 328 ($M^+ - CH_3COOH - H_2O$, 10), 110 (100).

3 β -Acetoxy-5,6-*seco*-5-oxo-3-pregnen-6-oic acid (6). Compound **6** was prepared from pregnenolone acetate by ozonolysis in ethyl acetate, as described for **5**, and isolated as a colorless oil (65%): IR (neat film) 3400–2500 (br), 2972, 2939, 1753, 1706, 1673 cm^{-1} ; 1H NMR ($CDCl_3$, 300 MHz) δ 6.76 (m, 1H), 5.89 (m, 1H), 2.50 (m, 2H), 2.08 (s, 3H), 1.20 (s, 3H), 0.62 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 209.4, 208.2, 178.0, 147.1, 28.5, 63.5, 54.7, 47.7, 43.8, 41.6, 38.7, 35.1, 34.8, 34.6, 31.4, 21.7, 24.7, 23.2, 22.5, 18.1, 12.9; CIMS m/z (relative intensity) 347 ($M^+ + 1$, 80), 329 ($M^+ - H_2O$, 50), 110 (100); HRMS (CI) calcd for $C_{21}H_{31}O_4$ ($M + 1$) $^+$ m/z 347.2222, found 347.2234.

3 β -Hydroxy-5-oxo-5,6-*seco*-22, 25-diaza-cholestan-6-oic acids (7). A solution 1 g of acid **5** and **4** equiv *N,N*-dimethylethylenediamine in 20 mL methanol was adjusted to pH 6 by the addition of glacial acetic acid. The solution was diluted with 20 mL THF and treated with 1.5 equiv $NaB(CN)H_3$. The resulting mixture was heated at reflux for 18 h. After cooling, saturated Na_2CO_3 solution was added, the solution stirred at room temperature for 2 h, adjusted to pH 7–8 by adding saturated NH_4Cl solution. After evaporation of the solution, the residue was extracted with chloroform, which was washed with aq $FeSO_4$ and dried over anhyd Na_2SO_4 . The residue of the evaporated solution was washed with ethyl acetate:petroleum ether (1:1) to give a epimeric mixture of 3 β -hydroxy-5-oxo-5,6-*seco*-22, 25-diaza-cholestan-6-oic acids 20R and 20S (ca. 3:1 by 1H NMR analysis) as a brown solid: 1H NMR ($CDCl_3$, 300 MHz) δ 3.46 (m, 6H), 2.28, 2.26 [1/3, (2s, 6H, $-N(CH_3)_2$], 0.97 (s, 3H), 0.72 (s, 3H); CIMS m/z (relative intensity) 419 ($M^+ + 1 - H_2O$, 55), 402 ($M^+ - CH_3$, 100), 362 (80). Anal. ($C_{25}H_{44}N_2O_4 \cdot 2H_2O$) C, H, N.

3-Oxo-4-cholesten-2-acetic acid ethyl esters (13, 14). To a solution of 4-cholesten-3-one (1.8 g) in THF (15 mL) was added 3.5 mL of LDA (2 M) at $-10^\circ C$ under argon and the solution was stirred for 3 h. HMPA (2.4 mL) was added to the solution, and then ethyl bromoacetate (780 μL) was added at $-10^\circ C$. After stirring for 2 h, the mixture was quenched with H_2O and the aqueous layer was washed with ethyl acetate three times. The combined organic layer was dried ($MgSO_4$), filtered and concentrated. The concentrate was chromatographed on silica gel, and elute with EtOAc:hexane (1:10) to afford ethyl 3-oxo-4-cholesten-2-acetic acids **13** (1.71 g, 79%) and **14** (0.34 g, 14%) with a ratio of 5:1. Compound **13**: 1H NMR ($CDCl_3$, 300 MHz) δ 5.69 (s, 1H), 4.14 (m, 2H), 2.86 (m, 2H), 2.39–2.12 (m, 3H), 2.00 (m, 2H), 1.80 (m, 2H), 1.68–0.89 (m, 20H), 1.24 (t, $J = 6.9$ Hz, 3H), 1.21 (s, 3H), 0.87 (d, $J = 6.6$ Hz, 3H), 0.83 (d, $J = 6.6$ Hz, 6H), 0.67 (s, 3H); HRMS (EI) calcd for $C_{31}H_{50}O_3$ 470.3760, found 470.3725; Compound **14**: HPLC (acetonitrile, 4 mL/min, 205 nm) 22.13 min; 1H NMR ($CDCl_3$, 300 MHz) δ 5.71 (s, 1H), 4.13 (m, 2H), 2.86 (m, 2H), 2.46 (dt, $J = 4.8$ Hz, 13.2 Hz, 1H), 2.17–1.74 (m, 6H), 1.65–0.94 (m, 20H), 1.24 (t, $J = 6.9$ Hz, 3H), 1.10 (s, 3H), 0.87 (d, $J = 6.6$ Hz, 3H),

0.83 (d, $J = 5.4$ Hz, 6H), 0.66 (s, 3H); HRMS (FAB) m/z calcd for $C_{31}H_{51}O_3$ 471.3838, found 471.3873.

3-Oxo-4-cholesten-2 α -acetic acid (11). A mixture of **13** and **14** (5:1, 120 mg) was dissolved in THF (2 mL) and H_2O (5 mL). A saturated KOH solution (5 drops) was added and the solution was stirred for 12 h at room temperature. The solution was adjusted to pH 2 with 10% HCl and diluted with H_2O . Aqueous layer was washed with ethyl acetate several times and the combined organic layer was dried ($MgSO_4$), filtered and concentrated. The concentrate was chromatographed and eluted with MeOH:EtOAc:hexane: CH_2Cl_2 1:3:7:10 to afford 72 mg of 3-oxo-4-cholesten-2 α -acetic acid **11**¹⁸ (64%) as colorless crystals: mp 174–176 $^\circ C$; $[\alpha] + 41.0^\circ$ (c 0.5, $CHCl_3$); HPLC (acetonitrile, 4 mL/min, 205 nm) 17.27 min; FTIR (neat film) 3500–2500 (br), 2948, 2874, 1715, 1681 cm^{-1} ; 1H NMR ($CDCl_3$, 300 MHz) δ 5.73 (s, 1H), 2.90 (m, 2H), 2.34 (dt, $J = 4.5$ Hz, 13.2 Hz, 1H), 2.27 (m, 2H), 2.01 (dt, $J = 3.6$ Hz, 9.9 Hz, 2H), 1.81 (m, 2H), 1.65–0.96 (m, 20H), 1.22 (s, 3H), 0.88 (d, $J = 6.6$ Hz, 3H), 0.83 (d, $J = 6.6$ Hz, 6H), 0.67 (s, 3H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 200.0, 178.1, 172.3, 123.1, 56.5, 56.2, 54.5, 42.8, 42.7, 39.9 (3C, 39.92, 39.90, 39.85), 39.6, 36.5, 36.1, 35.8, 35.1, 33.1, 32.3, 28.6, 28.4, 24.6, 24.2, 23.2, 23.0, 21.3, 19.0, 17.8, 12.4; HRMS (EI) calcd for $C_{29}H_{46}O_3$ m/z 442.3447, found 442.3446.

3-Oxo-4-cholesten-2 β -acetic acid (12). Compound **14** (25 mg) was dissolved in THF (1 mL) and H_2O (2.5 mL). A saturated KOH solution (5 drops) were added to the solution and stirred for 4 h at room temperature. The mixture was adjusted to pH 2 with 10% HCl and diluted with H_2O . The aq layer was washed with ethyl acetate by several times and the combined organic layer was dried ($MgSO_4$), filtered and concentrated. The concentrate was chromatographed on silica gel and eluted with MeOH:EtOAc:hexane: CH_2Cl_2 1:3:7:10 to afford 8.1 mg (34%) of **11** and 6.0 mg (26%) of **12**, 3-oxo-4-cholesten-2 β -acetic acid: HPLC (acetonitrile, 4 mL/min, 205 nm) 17.09 min; 1H NMR ($CDCl_3$, 300 MHz) δ 5.77 (s, 1H), 2.85 (m, 2H), 2.48 (dt, $J = 4.5$ Hz, 12.6 Hz, 1H), 2.20 (m, 3H), 1.97 (m, 2H), 1.81 (m, 1H), 1.66–0.92 (m, 20H), 1.12 (s, 3H), 0.88 (d, $J = 6.6$ Hz, 3H), 0.84 (d, $J = 6.6$ Hz, 6H), 0.67 (s, 3H); HRMS (EI) calcd for $C_{29}H_{46}O_3$ m/z 442.3447, found 442.3456.

Potassium O-cholestanyl xanthate (15).¹⁹ 3 β -Cholestanol (5 g, 12.9 mmol) was dissolved in 120 mL of 2-methoxyethyl ether, then 500 mg (1.0 equiv) of potassium metal was added. The reaction mixture was stirred under reflux, for 1 h. The reaction mixture was cooled to 50 $^\circ C$, then 0.9 mL (1.1 equiv) of carbon disulfide was added and the solution was stirred for an additional hour. The reaction mixture was concentrated to give the crude product as a brown powder. The residue was washed with petroleum ether and dried to give 5.7 g (85%) of **15** as yellow powder: mp 279–281 $^\circ C$; 1H NMR (DMSO) δ 0.61 (s, 3H), 0.77 (s, 3H), 0.82 (d, $J = 6.6$ Hz, 6H), 0.85 (d, $J = 5.1$ Hz, 3H), 5.19 (m, 1H); CIMS m/z (relative intensity) 502 (M^+ , 5), 388 ($M^+ + 1 - (S)CSK$, 45), 387 ($M^+ - (S)CSK$, 45), 371 ($M^+ - O(S)CSK$, 100).

Alkyl *O*-cholestanyl xanthates (16–19). General procedures for the syntheses of compounds **16–19**: Compound **15** (100 mg, 0.20 mmol) was dissolved in 4 mL warm DMSO, and 1.0 equiv of alkyl halide was added. After stirring for 2 h at room temperature, the reaction solution was poured into water and extracted with ethyl acetate. The organic layer was washed with brine and then dried over anhydrous sodium sulfate. Evaporation gave the corresponding alkyl *O*-cholestanyl xanthate in 80–85% yield. Using methyl iodide, *O*-cholestanyl methyl xanthate (**16**) was obtained: mp 86–87 °C (Lit.¹⁹ 87.5–88 °C); ¹H NMR (CDCl₃) δ 0.62 (s, 3H), 0.82 (s, 3H), 0.83 (d, *J* = 6.6 Hz, 6H), 0.87 (d, *J* = 5.4 Hz, 3H), 5.47 (m, 1H); FABMS *m/z* (relative intensity) 479 (*M*⁺ + 1, 5), 371 (*M*⁺ – *O*(S)CSMe, 100); HRMS (FAB): calcd for (*M*⁺ + 1) *m/z* 479.3381, found 479.3381. Using benzyl bromide, benzyl *O*-cholestanyl xanthate (**19**) was obtained: mp 99–101 °C (Lit.¹⁹ 102–102.5 °C); ¹H NMR (CDCl₃) δ 0.60 (s, 3H), 0.80 (s, 3H), 0.81 (d, *J* = 7.5 Hz, 6H), 0.85 (d, *J* = 5.4 Hz, 3H), 4.26 (s, 2H), 5.47 (m, 1H), 7.26 (m, 5H); FABMS *m/z* (relative intensity) 555 (*M*⁺ + 1, 6), 478 (*M*⁺ + 1 – C₆H₅, 5), 371 (*M*⁺ – *O*(S)CSBn, 100); HRMS (FAB) calcd for (*M*⁺ + 1) *m/z* 555.3694, found 555.3701. Using allyl bromide, allyl *O*-cholestanyl xanthate (**17**) was obtained: mp 90–91 °C; ¹H NMR (CDCl₃) δ 0.62 (s, 3H), 0.82 (s, 3H), 0.83 (d, *J* = 6.6 Hz, 12H), 0.87 (d, *J* = 5.4 Hz, 3H), 3.72 (d, *J* = 6.6 Hz, 2H), 5.13 (d, *J* = 9.6 Hz, 1H), 5.25 (d, *J* = 16.8 Hz, 1H), 5.47 (m, 1H), 5.85 (m, 1H); FABMS *m/z* (relative intensity) 505 (*M*⁺ + 1, 4), 464 (*M*⁺ + 1 – C₃H₅, 3), 371 (*M*⁺ – *O*(S)CSC₃H₅, 100); HRMS (FAB) calcd for (*M*⁺ + 1) *m/z* 505.3540, found 505.3526. Using isoamyl bromide, *O*-cholestanyl isoamyl xanthate (**18**) was obtained: mp 105–107 °C; ¹H NMR (CDCl₃) δ 0.62 (s, 3H), 0.82 (s, 3H), 0.83 (d, *J* = 6.6 Hz, 12H), 0.87 (d, *J* = 5.4 Hz, 3H), 3.06 (m, 2H), 5.47 (m, 1H); FABMS *m/z* (relative intensity) 535 (*M*⁺ + 1, 4), 507 (*M*⁺ + 1 – C₂H₄, 3), 371 (*M*⁺ – *O*(S)CSC₅H₁₁, 100); HRMS (FAB) calcd for (*M*⁺ + 1) *m/z* 535.4009, found 535.3970.

Conformational search

Compounds **2**, **10**, **11** and **15** were computer-built using ISIS/Draw (version 2.1; MDL Information System, Inc., San Leandro, CA) and input into Hyperchem Molecular Modeling Package (version 5.1; Hypercube Inc., Gainesville, FL) with Chemplus. Conformation searches were performed by varying torsion angles defined by atoms in the side chains as well as those in the ring system using usage directed searching method. The acyclic torsion angles were varied between ±60 and 180°, the torsion angles in the ring system were varied between ±30 and 120°. The conformations obtained were geometry optimized to reach a rms gradients of <0.01 kcal/(Å mol) with the MM⁺ force field, and with the Polak-Ribiere optimizer. A total of 500 optimizations were performed for each compound.

For compound **2**, the torsion angles varied were 21-20-17-16, 9-8-7-6, 1-10-9-8, 29-28-3-4, as well as those in the A, C and D rings. Seven low-energy conformations were obtained. For compound **10**, the torsion angles varied were 21-20-17-16, 2-1-10-5, 3-2-1-10, 4-3-2-1, as

well as those in the B, C and D ring system. Ninety-two low-energy conformations were obtained. For compound **11**, the torsion angles varied were 21-20-17-16, 29-28-2-1, as well as those in the A, B, C, and D ring system. Fifteen low-energy conformations were obtained. For compound **15**, the torsion varied were 21-20-17-16, 29-28-3-2, as well as those in the A, B, C, and D ring system. Five low energy conformations were obtained. The lowest-energy conformations obtained for compounds **2**, **10**, **11** and **15** are shown in Figure 2, and the distances between the atom labeled as A and the atom labeled as B were measured and given in Figure 2.

Cdc25A inhibition assay

The full-length human Cdc25A cDNA (a kind gift of Dr. Laurent Meijer, CNRS, Roscoff, France) was cloned into the pGEX-KG expression vector. The resulting pGEX-cdc25A plasmid was transformed into *E. coli* strain BL21(DE3). The bacteria were grown in a medium containing 100 µg per mL ampicillin at 30 °C until the cell suspension reached an optical density (OD_{600 nm}) of 0.7. The incubation temperature was then decreased to 25 °C, and after 1 h, production of the fusion protein was induced by addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After 4 h, the bacteria were harvested by centrifugation. The bacterial pellet was resuspended in phosphate-buffered saline (PBS), pH 7.2, containing 1% (wt/vol) Triton X-100, 1 mM EDTA, 5 mM dithiothreitol, 10 µg per mL leupeptin, 10 µg per mL aprotinin, and 10 µg per mL soybean trypsin inhibitor. The bacteria were disrupted by sonication on ice, and the cell lysates were cleared by centrifugation for 30 min at 100,000 g. The glutathione *S*-transferase (GST)-Cdc25A fusion protein was purified from the cleared supernatant by chromatography over glutathione-agarose. The eluted protein was dialyzed into assay buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, and 5 mM dithiothreitol), and aliquots of purified enzyme were brought to 40% (wt/vol) glycerol for storage at –70 °C.

The Cdc25A phosphatase assay was performed in 96-well plates. The substrate, fluorescein diphosphate (FDP; Molecular Probes, Inc.), was dissolved in water to yield a final concentration of 46.2 µM. The FDP stock solution was aliquoted and stored at –70 °C. Each well received 100 µL assay buffer modified to contain 20 mM dithiothreitol. The indicated compounds were dissolved in dimethylsulfoxide and 1–2 µL of the concentrated stock solutions were added per well. Control incubations contained an equivalent volume of dimethylsulfoxide only. Purified GST-Cdc25A was diluted in assay buffer, and 20 µL of the diluted enzyme was added per well. In preliminary studies, each batch of enzyme was calibrated such that the quantity added to each well dephosphorylated FDP in a linear fashion over a 20-min reaction time course. After 5 min at room temperature, the reactions were initiated by addition of 65 µL of FDP solution. The plates were incubated for 15 min at 25 °C in the dark. The reactions were terminated with 35 µL of sodium orthovanadate (285 mM) dissolved in distilled water. The fluorescence emission of the reaction product

(fluorescein monophosphate)^{14,29} was measured with a Millipore Cytofluor 2350 fluorimeter (excitation wavelength, 485 nm; emission wavelength, 530 nm).

Cytotoxicity assays

The in vitro cytotoxicity assays were carried out at the National Cancer Institute. Details of the assay procedures have been reported previously.³⁰

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