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Cdc25A Protein phosphatase inhibitors from anomalous ozonolysis of 5,6-*seco*-5-oxo-3-cholesten-6-oic acid

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Abstract—Ozonolysis of the α,β-unsaturated keto acid, 5,6-*seco*-5-oxo-3-cholesten-6-oic acid (**1**), in ethyl acetate/acetic acid/water (15:15:1) at −20°C, followed by addition of piperidine, led to several novel lactone products (**2**–**6**), presumably formed through rearrangements of ozonide intermediates. The major product **2** was a lactone containing a dioxolane-4-one moiety, which exhibited a carbonyl stretching frequency at 1815 cm^{-1}. Three of the five products resulted from loss of two carbon atoms from the starting material rather than one carbon atom as commonly observed in the 'anomalous ozonolysis' of α,β-unsaturated ketones. Some of these compounds possess moderate inhibitory activities against human Cdc25A protein phosphatase, an enzyme overexpressed in several human tumor cell lines.

1. Introduction

We recently reported[1] the discovery of a family of novel anticancer agents, which act presumably by inhibition of the protein phosphatase Cdc25A, obtained by pyrolysis of 3α-azido-B-homo-6-oxa-4-cholesten-7-one. The minimum structural requirements for such inhibitors were a hydrophobic portion arising from the C, D rings and attached C8 side chain, and fragmented A and B rings containing a carboxyl group or lactone. We were therefore led to consider the readily available 5,6-*seco*-5-oxo-3-cholesten-6-oic acid (**1**) as a substrate for further transformations and sought initially to subject it to ozonolysis.

The ozonolysis of allylic compounds or α,β-unsaturated carbonyl compounds is known to lead to anomalous results.[2–4] In most anomalous ozonolyses, both the carbon–carbon double bond and one of the carbon–carbon single bonds next to it are cleaved, resulting in a product (or products) with one carbon atom less. In the present case, the starting material contains not only an α,β-unsaturated ketone group but also an appropriately situated acetic acid residue which could be expected to exhibit neighboring group participation.

2. Result and discussion

As shown in Scheme 1, α,β-unsaturated keto acid **1**[5] was treated with ozone in ethyl acetate/acetic acid/water (15:15:1) at −20°C for 40 min. Then piperidine was added to the reaction mixture, which was allowed to stir at room temperature overnight. After an aqueous work up, separation of the crude ozonolysis reaction mixture by chromatography on silica gel yielded a novel dioxolane-4-one containing lactone, **2**, in 26% yield. Further separation of the higher polarity elutes from the silica gel column by reverse phase HPLC (Phenomenex® LUNA 5μ C18, 100% acetonitrile) resulted in isolation of the abnormal cleavage products **3**–**6** in yields of 8–15% in each case.

It is generally accepted that the ozonolysis reaction of olefins proceeds in three steps, each of which is a 1,3-dipolar cycloaddition or reversion.[2,6] Thus, the 'primary ozonide' results from addition of ozone to the double bond as seen in the formation of A (Scheme 2). In the second step, the primary ozonide decomposes into a carbonyl compound and a carbonyl oxide; trapping of the latter intermediate had been reported.[7] In a cyclic olefin, as in the case of **1**, the newly formed carbonyl group and carbonyl oxide are part of the same molecule, as seen in B. In the third step, the carbonyl oxide adds to the carbonyl group to give the normal ozonide, as in C. In the present case, the intermediate carbonyl oxide B contains an additional carbonyl group α to the newly generated aldehydic group, both of which would be expected to undergo cycloaddition with the carbonyl oxide. Criegee,[6] already in 1975, reviewed the matter of competition between inter and intramolecular ozonide formation and intramolecular competing reactions.

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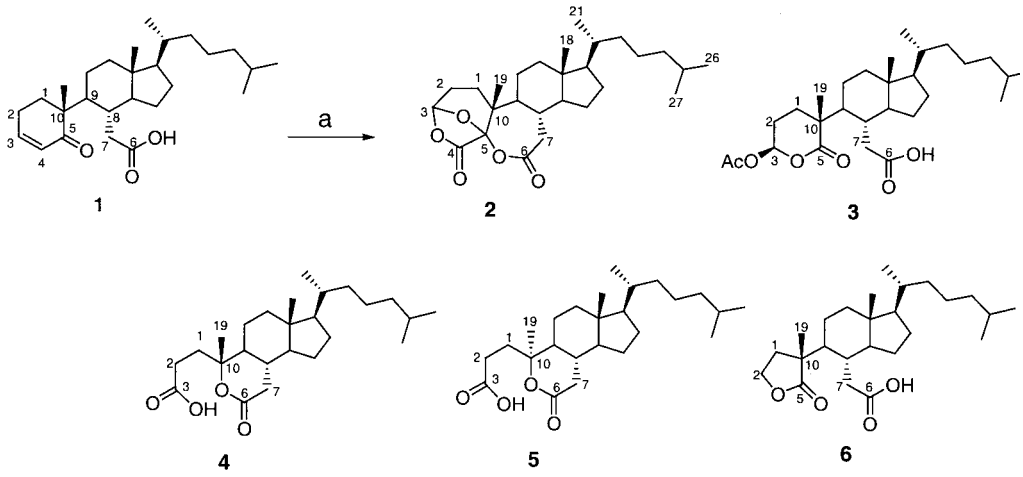
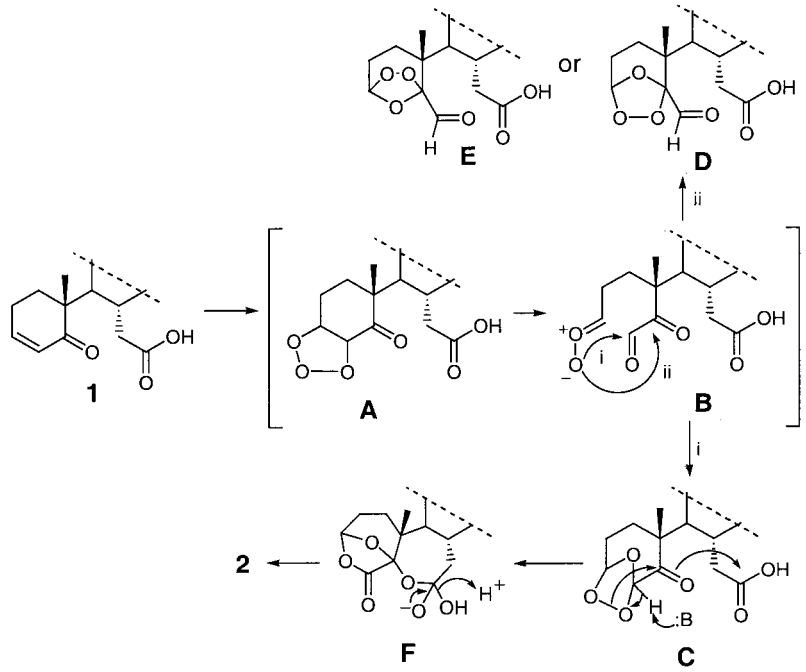


Scheme 1. Abnormal ozonolysis of 5,6-*seco*-5-oxo-3-cholesten-6-oic acid. (a) O_3, −20°C, EtOAc/AcOH/H_2O; piperidine, overnight.

As shown in Scheme 2, in addition to C, it would be expected that the cycloaddition products D or E would be produced and, indeed, the formation of all of the unusual products obtained can be explained by further rearrangements of these two ozonides.

The major product of the ozonolysis, compound **2**, can be seen to arise from the postulated ozonide C by breaking the weak O–O bond followed by lactonization and loss of a molecule of water from F. Mass and elemental analysis established the molecular formula of **2** as $C_{27}H_{42}O_5$. NMR studies, including DEPT, COSY, HMBC and HMQC, were used to elucidate the structure, and assign carbon and hydrogen atoms. The observed HMBC correlations between H_3 (δ 6.02, s, 1H) and C_4 (δ 167.5, C), C_5 (δ 101.9, C), C_2 (δ 25.8, CH_2) and C_1 (δ 28.6, CH_2); between H_{19} (δ 0.88, s, 3H) and C_5 (δ 101.9, C); as well as between H_7 (δ 2.59, m, 2H) and C_6 (δ 170.2, C) confirm the structure of compound **2** (Fig. 1).



Scheme 2. Proposed mechanism for the formation of ozonides.

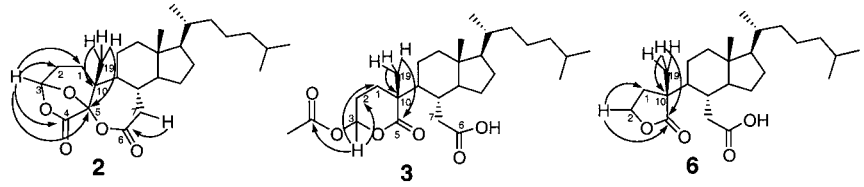


Figure 1. HMBC correlations observed in compounds **2**, **3** and **6**.

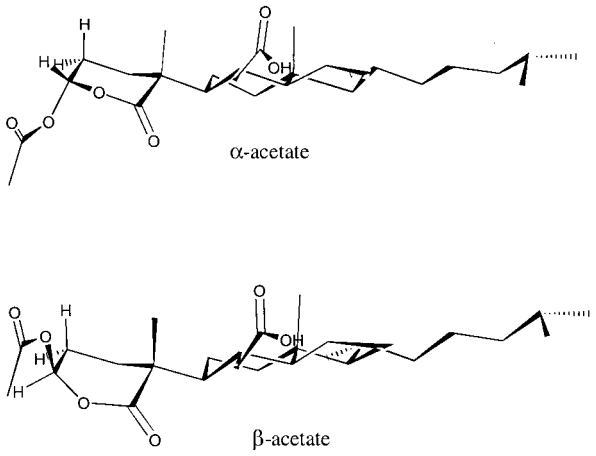


Figure 2. Energy minimized conformations for two possible isomers of **3**.

However, the configurations at C_3 and C_5 have not been determined. The IR spectrum of **2** shows sharp absorption bands at 1815 and 1769 cm^{-1}. The high frequency band at 1815 cm^{-1} has been assigned to the stretching vibrations of the carbonyl group in the five membered 1,3-dioxolane-4-one moiety. In the literature, such carbonyls exhibited absorptions at around 1800 cm^{-1}.[8–9] The absorptions at 1769 cm^{-1} has been assigned to the C=O stretch of the lactone carbonyl, which usually appears at 1735 cm^{-1}. The increased frequency may arise from substitution and strain introduced at C_5.

Compounds **3**–**6** all contain carboxyl groups, as evidenced from their IR spectra, which exhibited absorptions in the 3400–2500 and 1700 cm^{-1} regions. The structure of compound **3** was suggested from its exact mass, which revealed the molecular formula, $C_{28}H_{46}O_6$, from additional IR absorptions at 1742 and 1200–1000 cm^{-1}, from its ^1H NMR spectrum which showed signals at δ 2.09 (s, 3H) and 6.44 (m, dd, 1H) corresponding to protons on an acetoxy group and at C_3, respectively, and its ^{13}C NMR spectrum, where signals corresponding to these carbons appeared at δ 168.8 ($-OCOCH_3$), 20.9 ($-OCOCH_3$) and δ 175.8 (C_5) as assigned by DEPT, COSY, HMBC and HMQC experiments. The structure was confirmed by the correlations between H_3 (δ 6.44) and the acetoxy carbonyl (δ 168.8), C_2, C_1 (δ 24.697, 24.696), C_5 (δ 175.8), and between H_{19} (1.39, s, 3H) and C_5 in the HMBC spectrum (Fig. 1).

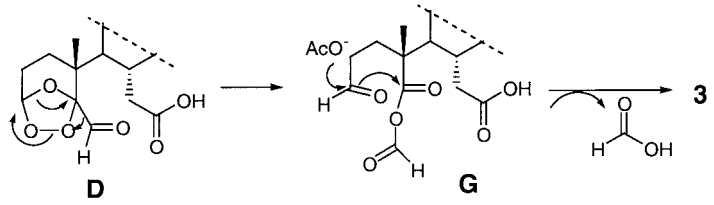
The anomeric acetate at C_3 is very likely at a β position although it could not be unequivocally determined without a crystal structure. Anomeric protons, such as those in pyranoses, typically show ax–ax coupling of 9.0 Hz and eq–eq coupling of 4.0 Hz. As shown in Fig. 2, the A ring of the energy minimized conformation (Chem 3D) of the α-acetate has a chair conformation and its anomeric proton should give eq–ax and eq–eq coupling both at around 4 Hz. The β-acetate, on the other hand, shows a half chair conformation for A ring, with the acetate positioning in an axial orientation. The *endo–endo* coupling and *endo–exo* coupling should be close to those in norbornene at 9.0 and 3.9 Hz, respectively.[10] The coupling constants observed for H_3 in compound **3** are 7.2 and 3.9 Hz. Therefore, it is very likely that **3** has an axial positioned β-acetate at C_3. The axial orientation of the acetate is supported by the chemical shift (δ 93.5) of the anomeric C_3. In pyranoses, anomeric carbons with equatorial hydroxyl groups typically appear at δ 96–97, while those with axial hydroxyl groups at δ 92–93.[10]

The formation of compound **3** is proposed in Scheme 3. Such a rearrangement as illustrated for D–G was long ago suggested in the anomalous ozonolysis of cholest-4-en-3-one to account for the loss of a carbon atom as formic acid.[2] In the present case, we suggest that **3** arises by attack of acetate, from the reaction media, at the C_3 aldehydic carbon in G with simultaneous lactonization and loss of formic acid.

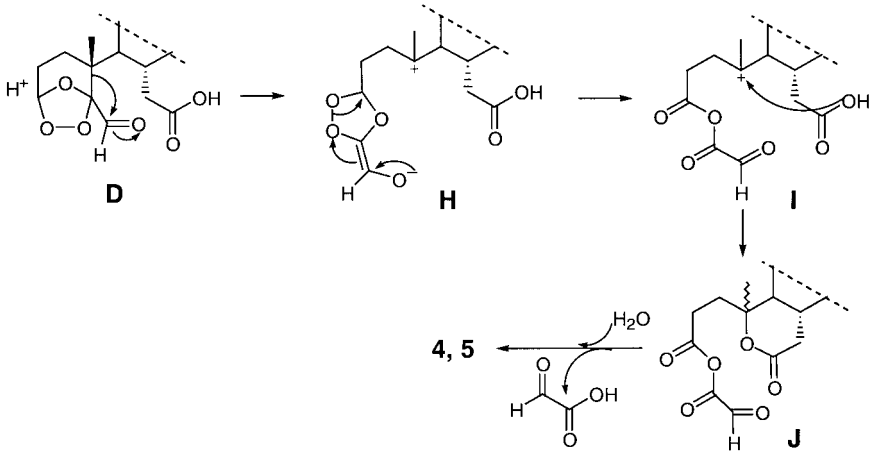
The exact masses and elemental analyses of compounds **4**–**6** revealed that each possessed a molecular formula of $C_{25}H_{43}O_4$, which surprisingly, is two carbons less than the parent compound **1**. Compounds **4** and **5** were assigned as two 6-membered lactone epimers based on their IR absorptions at 1733 and 1720 cm^{-1}, respectively, and the chemical shift positions of their C_{10} quaternary carbon atoms (δ 86.4 and 86.0, respectively). The structures and the configurations at C_{10} were assigned by the comparisons of their chemical shifts to those reported by us for closely analogous compounds, which were unequivocally assigned based on a single-crystal X-ray analysis.[1a]

As illustrated in Scheme 4, fragmentation of the O–C bond to C_{10} yields intermediate tertiary carbocation H. Fragmentation of the enolate ozonide in H, give intermediate I, which by lactonization, expulsion of glyoxylic acid, give the abnormal cleavage products **4** and **5**. To our best knowledge, loss of two carbon atoms in the form of glyoxylic acid is unprecedented in ozonolysis of α,β-unsaturated ketones. A unique factor that facilitates loss of glyoxylic acid during the formation of **4** and **5** (scheme 4) may be the ease of breaking C_6–C_{10} bond to give a tertiary carbocation which readily cyclizes with neighboring carboxylate to form a lactone.

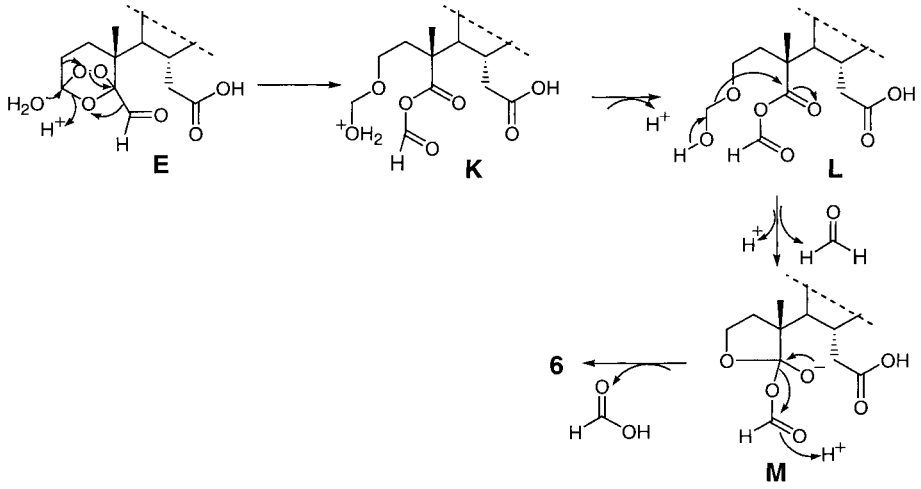
Compound **6** shows in its IR spectrum, an absorption band at 1766 cm^{-1} corresponding to a 5-membered lactone ring,



Scheme 3. Proposed mechanism for the formation of **3**.



Scheme 4. Proposed mechanism for the formation of **4** and **5**.



Scheme 5. Proposed mechanism for the formation of **6**.

rather than a 6-membered lactone as seen in **4** and **5**. The structure of **6** was confirmed by 2D NMR experiments, especially by the correlations between H_2 (δ 4.24, m, 2H) and C_5 (δ 182.2, C), C_1 (δ 32.4, CH_2); as well as between H_{19} (δ 1.35, s, 3H) and C_5 in its HMBC spectrum (Fig. 1). In Scheme 5, we suggest a mechanism by which ozonide E could fragment and rearrange with the C_2 and aldehydic group migration to their adjacent oxygen atoms to give intermediate K. Upon lactonization and expulsion of formaldehyde and formic acid consecutively, compound **6** could be formed.

Compounds **1**–**6** were tested for their inhibitory activities against human Cdc25A protein phosphatase, an enzyme overexpressed in several human tumor cell lines.[11] We have discussed in our previous reports that the cholesteryl side chain and a free carboxylate group contribute greatly to the Cdc25A inhibitory activities of steroidal derived acids.[1] As shown in Table 1, compounds **3**–**5** exhibited reasonable activity (IC_{50}=8–10 μM) in inhibiting human Cdc25A protein phosphatase. However, it is unexpected that without the important carboxylate group, compound **2** also inhibited Cdc25A activity by 50% at 19.6 μM. The observed activity may arise from partial hydrolysis of **2** in assay condition. Changing the A ring into a 5-membered lactone ring, as in compound **6**, significantly reduced its Cdc25A inhibitory activity (IC_{50}=18.7 μM).

Table 1. Cdc25A inhibitory activities of compounds **1**–**6**

Compound	**1**	**2**	**3**	**4**	**5**	**6**
IC_{50} (μM)	9.5[1]	19.6	8.7	9.9	7.9	18.7

3. Conclusions

In conclusion, the 'abnormal ozonolysis' of α,β-unsaturated cholesteryl keto acid **1** was investigated. An unexpected lactone (**2**) with a dioxolane-4-one moiety fused in the cholesteryl A-ring was obtained as a major product. The structure of product **3** is consistent with the widely observed phenomenon of the loss of one carbon atom in the form of formic acid in the ozonolysis of α,β-unsaturated carbonyl compounds. The more unusual products **4**–**6** arise by the unexpected loss of two carbon atoms from the starting material, presumably as glyoxylic acid in **4** and **5**, or by consecutive loss of formaldehyde and formic acid in **6**. The solvent mixture EtOAc/AcOH/H_2O was chosen to

provide a hydrolytic media that facilitate the cleavage. Some of the compounds exhibited moderate Cdc25A inhibitory activities.

4. Experimental

4.1. General

Thin layer chromatography analysis (TLC) was performed on aluminum sheets precoated with 0.2 mm of silica gel containing 60F254 indicator. Spots were detected with shortwave UV light or Ceric sulfate spray. Flash chromatography was run using 230–400 mesh silica gel. Reverse phase high performance liquid chromatography (HPLC) was run on a Phenomenex® LUNA 5μ C18 semi-preparative column. The homogeneity of all the compounds was routinely checked by TLC on silica gel plates and also by HPLC. Melting points were measured on a Kofler hot stage apparatus attached to a digital thermometer and were uncorrected. Fourier transformed infrared spectra were obtained on a Nicolet 520 FTIR spectrometer. ^{1}H (300 or 400 MHz), ^{13}C (75 or 100 MHz) NMR and DEPT spectra were recorded on either a Varian Gemini-300 or on a Varian XL-400 spectrometer. Chemical shifts are reported relative to $CDCl_3$ (δ 7.24). 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.

A solution of 2.5 g of 5,6-seco-5-oxo-cholest-3-en-6-oic acid in a mixture of 150 mL of ethyl acetate, 150 mL of glacial acetic acid and 10 mL of water was cooled in a dry ice bath, and ozone was passed through the solution for 40 min. After standing at room temperature for 1 h, 5 mL of piperidine was added and the solution was stirred at room temperature overnight. The volume of the solution was then reduced to 50%, and poured into 500 mL water, which was extracted with ethyl ether. The extracts were combined and washed with 2×25 mL brine. The crude product was subjected to silica gel column chromatography (hexane/ethyl acetate 10:1) to afford compounds **2** (695 mg, 26%) and partially purified eluents of compounds **3**–**6** (1.3 g, 43%).

4.1.1. Compound 2. This compound was obtained as colorless needles: mp 193–195°C; FTIR (neat film) 2953, 2910, 2890, 2868, 1815, 1769, 1256, 1178, 1128, 1084, 910 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 Hz) δ 6.02 (s, 1H), 2.59 (m, 2H), 2.12 (dd, 1H, J=14.9, 4.9 Hz), 2.08–1.90 (m, 4H), 1.08 (s, 3H), 0.88 (d, 3H, J=6.6 Hz), 0.84 (d, 6H, J=6.6 Hz), 0.68 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 170.2 (C), 167.5 (C), 102.6 (CH), 101.9 (C), 56.3 (CH), 55.4 (CH), 43.3 (CH), 42.5 (C), 41.3 (C), 39.6 (CH_2), 39.4 (CH_2), 38.8 (CH_2), 35.9 (CH_2), 35.6 (CH), 33.5 (CH), 28.6 (CH_2), 28.0 (CH), 27.6 (CH_2), 25.8 (CH_2), 25.2 (CH_2), 23.7 (CH_2), 22.8 (CH_3), 22.7 (CH_2), 22.5 (CH_3), 18.5 (CH_3), 14.9 (CH_3), 11.8 (CH_3); FAB MS m/z (relative intensity) 447 (M^++1, 100), 429 (M^++1−H_2O, 30), 401 (M^++1−2H_2O, 32), 373 (48); Anal. calcd for $C_{27}H_{42}O_5$: C, 72.61; H, 9.48. Found: C, 72.36; H, 9.44.

4.1.2. Compound 3. This compound was obtained as colorless oil by further purification of the eluents of hexane/ethyl acetate 5:1 to 1:1 on HPLC (Phenomenex® LUNA 5μ C18, acetonitrile, flow rate=4 mL/min). FTIR (neat film) 2951, 2868, 1741, 1706, 1465, 1373, 1215, 1118, 1088, 940 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 Hz) δ 6.44 (dd, 1H, J=7.2, 3.9 Hz), 2.58 (dd, 1H, J=17.6, 2.8 Hz), 2.46 (dd, 1H, J=17.6, 5.5 Hz), 2.09 (s, 3H), 1.39 (s, 3H), 0.86 (d, 3H, J=7.1 Hz), 0.83 (d, 6H, J=6.6 Hz), 0.66 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 177.6 (C), 175.8 (C), 168.8 (C), 93.5 (CH), 55.9 (CH), 55.2(CH), 46.1 (CH), 45.8 (C), 42.2 (C), 39.4 (CH_2), 39.1 (CH_2), 36.5 (CH_2), 35.9 (CH_2), 35.8 (CH), 34.1 (CH), 28.0 (CH), 27.7 (CH_2), 26.5 (CH_2), 25.2 (CH_2), 24.7 (CH_2), 24.7 (CH_2), 24.6 (CH_2), 23.8 (CH_2), 22.8 (CH_3), 22.5 (CH_3), 20.9 (CH_3), 18.5 (CH_3), 11.6 (CH_3); FAB MS m/z (relative intensity) 479 (M^++1, 3), 419 (M^++1−H_2O, 100), 401 (M^++1−2H_2O, 25), 373 (95); HRMS (FAB) calcd for $C_{28}H_{47}O_6$ 479.3372. Found 479.3369.

4.1.3. Compound 4. This compound was obtained as a colorless oil: FTIR (neat film) 3400–2500, 2952, 2873, 1733, 1700 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 Hz) δ 2.62–2.46 (m, 3H), 2.06–2.00 (m, 2H), 1.96–1.80 (m, 4H), 1.53–1.47 (m, 3H), 1.31 (s, 3H), 0.89 (d, 3H, J=6.6 Hz), 0.84 (d, 6H, J=6.6 Hz), 0.67 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 177.6 (C), 170.9 (C), 86.4 (C), 55.8 (CH), 55.6 (CH), 45.5 (CH), 42.5 (C), 39.4 (CH_2), 38.9 (CH_2), 36.0 (CH_2), 35.7 (CH_2), 35.6 (CH), 35.0 (CH_2), 30.8 (CH), 28.0 (CH_2), 28.0 (CH_2), 27.7 (CH_2), 23.8 (CH_2), 23.5 (CH_2), 22.8 (CH_2), 22.8 (CH_3), 22.5 (CH_3), 22.4 (CH_3), 18.6 (CH_3), 11.9 (CH_3). FAB MS m/z (relative intensity) 407 (M^++1, 25), 389 (M^++1−H_2O, 100), 371 (21); HRMS (FAB) calcd for $C_{25}H_{43}O_4$ 407.3161. Found 407.3157.

4.1.4. Compound 5. This compound was obtained as a colorless oil: FTIR (neat film) 3400–2500, 2950, 2875, 1720, 1700 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 Hz) δ 2.64–2.48 (m, 3H), 2.12–2.03 (m, 2H), 2.00–1.77 (m, 4H), 1.35 (s, 3H), 0.88 (d, 3H, J=6.6 Hz), 0.83 (d, 6H, J=6.6 Hz), 0.69 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 177.9 (C), 170.9 (C), 86.0 (C), 55.9 (CH), 55.8 (CH), 49.2 (CH), 42.6 (C), 39.4 (CH_2), 39.1 (CH_2), 36.0 (CH_2), 35.6 (CH_2), 35.6 (CH), 30.7 (CH_2), 30.6 (CH), 28.1 (CH_2), 28.0 (CH_2), 28.0 (CH), 25.7 (CH_3), 23.8 (CH_2), 23.4 (CH_2), 22.8 (CH_2), 22.7 (CH_3), 22.5 (CH_3), 18.6 (CH_3), 12.0 (CH_3); EI MS m/z (relative intensity) 406 (M^+, 10), 388 (M^+−H_2O, 28), 333 (25), 348 (35), 135 (100); HRMS (CI) calcd for $C_{25}H_{43}O_4$ 407.3161. Found 407.3161.

4.1.5. Compound 6. This compound was obtained as a colorless oil: FTIR (neat film) 2939, 2867, 1766, 1706, 1461, 1382, 1196, 1170, 1030 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 Hz) δ 4.24 (m, 2H), 2.64 (dd, 1H, J=18.2, 2.2 Hz), 2.47 (dd, 1H, J=18.2, 5.8 Hz), 2.40 (m, 1H), 1.35 (s, 3H), 0.86 (d, 3H, J=6.6 Hz), 0.83 (d, 6H, J=6.6 Hz), 0.67 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 182.2 (C), 178.8 (C), 65.2 (CH_2), 55.9 (CH), 55.0 (CH), 46.4 (C), 44.7 (CH), 42.2 (C), 39.4 (CH_2), 39.2 (CH_2), 37.0 (CH_2), 35.9 (CH_2), 35.7 (CH), 35.6 (CH), 32.4 (CH_2), 28.0 (CH), 27.7 (CH_2), 25.0 (CH_2), 24.6 (CH_2), 24.4 (CH_3), 23.8 (CH_2), 22.8 (CH_3), 22.5 (CH_3), 18.5 (CH_3), 11.7 (CH_3); FAB MS m/z (relative intensity) 407 (M^++1, 8), 389 (M^++1−H_2O, 100); Anal.

calcd for $C_{25}H_{44}O_4$: C, 73.84; H, 10.41. Found: C, 73.66; H, 10.46.

4.1.6. Cdc25A inhibition assay. The procedures for the preparation of GST-Cdc25A fusion protein and Cdc25A inhibition assay have been described in detail previously.[1] Briefly, each batch of GST-Cdc25A fusion protein was calibrated, such that the quantity added to each well dephosphorylated FDP in a linear fashion over a 20 min reaction time course in the preliminary study. The phosphatase assay was performed in 96-well plates. The standard reaction time and conditions were 15 min at 25°C in a darkened chamber. The reactions were terminated with 15 μL of sodium orthovanadate (285 mM) dissolved in distilled water. The fluorescence emission of the reaction product (fluorescein monophosphate) was measured with a Millipore Cytofluor 2350 fluorimeter (excitation wavelength, 485 nm; emission wavelength, 530 nm). The IC_{50} values were determined from at least two independent determinations, each run in triplicate, where the variation from the mean did not exceed ±20%.

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