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Short communication

Synthesis and biological evaluation of dialkylsubstituted maleic anhydrides as novel inhibitors of Cdc25 dual specificity phosphatases

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Abstract

An efficient synthesis of dialkylsubstituted maleic anhydrides **1a–j** is described. The inhibitory potential of these original anhydride derivatives was tested toward the three human isoforms A, B and C of dual specific phosphatases Cdc25. A micromolar range inhibition of Cdc25s was observed with the maleic anhydrides bearing simple alkyl side chains longer than C_9, to reach the optimal activity with a C_{17} chain length.

Keywords: Barton decarboxylation; Maleic anhydride; Cdc25; Inhibition

1. Introduction

Dual specificity phosphatases Cdc25 play a crucial role in the cell cycle progression [1]. They allow activation of cyclin/CDK complexes by dephosphorylating the phospho-Thr14 and phospho-Tyr15 residues of the CDK subunit. The Cdc25 family consists of three members: Cdc25A, Cdc25B and Cdc25C, each of them acting on distinct phases of the cell cycle. Whereas the G2-M transition is regulated through CDK1 dephosphorylation by Cdc25B and Cdc25C, Cdc25A mainly activates CDK2, allowing in particular progression from G1 to S phase [2–4]. Interestingly, Cdc25A has been described as an oncogene since this protein is over expressed in a wide variety of human tumors [5] and is also implicated in radioresistance [6]. Hence, Cdc25 inhibitors constitute attractive agents with potential anticancer activity and therefore an increasing number of natural and synthetic small molecules has been reported as inhibitors of Cdc25s [7–10]. Among them, we have recently synthesized coscinosulfate which exhibits a significant inhibition of Cdc25A with an IC_{50} of 3 μM [11]. Continuing our program devoted to the search of Cdc25 phosphatase inhibitors, we investigated the effect of dialkylsubstituted maleic anhydride toward Cdc25s. It has been suggested that in dysidiolide, the γ-hydroxybutenolide residue likely serves as a phosphate surrogate, while the long side chain occupies a hydrophobic binding pocket [12,13]. Based on this consideration, and given that our derivatives and dysidiolide analogues possess the same constrained furan unit (maleic anhydride and γ-hydroxybutenolide), we postulated that the anhydride moiety might also act as phosphate mimic toward Cdc25s. Therefore, we synthesized a series of maleic anhydrides, bearing various simple alkyl side chains at C-4 position to examine their inhibitory activity toward Cdc25s. Herein we report an efficient

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synthesis of anhydrides **1a–j** using Barton radical decarboxylation, which led to the discovery of a novel class of Cdc25 inhibitors.

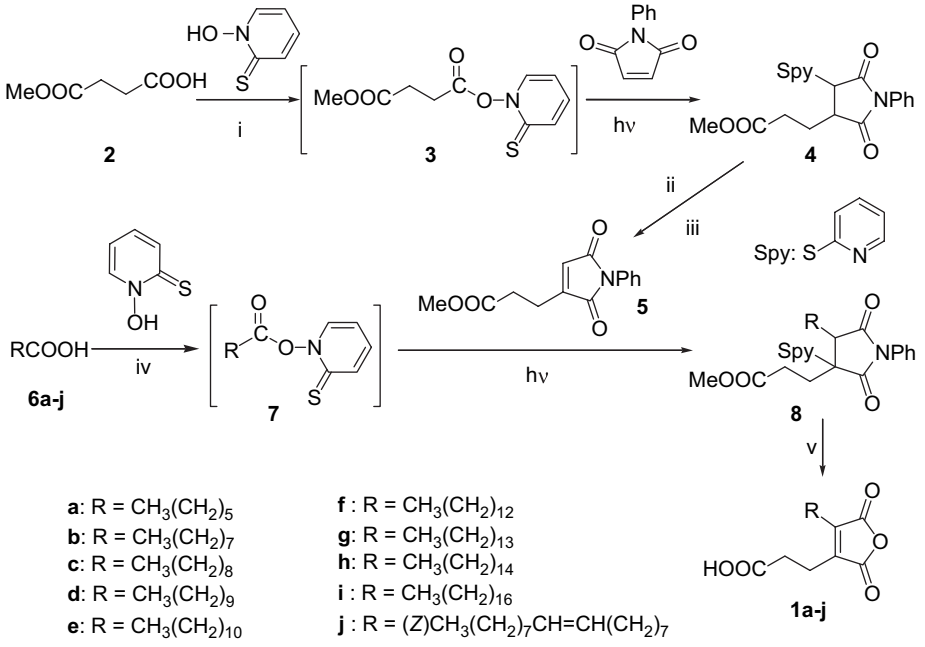
2. Chemistry

The dialkylsubstituted maleic anhydrides **1a–j** were synthesized based on a two-step radical addition to phenyl maleimide by means of Barton decarboxylation [14–16]. In the first step, the readily available succinic acid monomethyl ester **2** was transformed into its thiohydroxamic ester **3**, in the presence of 2-mercaptopyridine-*N*-oxide using the DCC coupling method. Irradiation of **3** in situ with tungsten light, in the presence of *N*-phenyl maleimide, gave the addition product **4** as a mixture of isomers. Treatment of **4** with *m*CPBA, followed by elimination of the resulting sulfoxide produced the monosubstituted anhydride **5** in 90% yield. Compound **5** was subjected as the olefin trap to a second step radical decarboxylation. Thus acid **6** was transformed into its thiohydroxamic ester **7** as was described for **2**, and irradiation in situ with tungsten light in the presence of olefin **5**, produced the intermediate addition product **8** as a mixture of isomers, which was further treated with KOH in MeOH–THF to give the desired dialkylsubstituted maleic anhydride **1** as the sole isomer (Scheme 1).

3. Biology

The dialkylsubstituted maleic anhydrides **1a–j** were assessed for in vitro inhibition of Cdc25s and horse liver alkaline phosphatase using 4-nitrophenyl phosphate (*p*-NPP) as substrate. As shown in Table 1, the screening of these derivatives indicates that none of these compounds inhibited alkaline phosphatase, whereas an inhibitory potential was found toward Cdc25s and was dependent upon the chain length. The inhibitory activity was detected for compounds **1d–j** bearing an alkyl side chain of C_{10} and higher. In contrast, the anhydrides **1a–c** possessing aliphatic groups shorter than C_{10} were inactive toward Cdc25s. The highest activity was found for compounds **1h–j** that displayed $IC_{50} < 10$ μM toward Cdc25s. Compounds **1g**, **1h** and **1j** exhibited a higher inhibitory activity toward Cdc25A than Cdc25B (ratio ≥ 1.5) and Cdc25C (ratio ≥ 1.8). Interestingly, the presence of the double bond in anhydride **1j** bearing a C_{17} chain length similar to **1i**, seemed to reduce its activity toward Cdc25B and Cdc25C. This indicates that not only the chain length but also its conformation may be involved in activity toward Cdc25s. Our results highlight, for these derivatives, the importance of the alkyl side chain, which could interact with hydrophobic residues of the enzymes as already mentioned in the literature for another Cdc25 inhibitor presenting an alkyl chain [8] or an inhibitor bearing a benzene ring [17].

We further examined the kinetics of inhibition of human Cdc25A by the potent derivative **1i** (Fig. 1). The inhibition profiles best fit with a mixed inhibition kinetic model ($R^2 = 0.945 - 0.987$). This model implies that the inhibitor combines with the free enzyme (dissociation constant K_I) and with the enzyme–substrate complex (dissociation constant K_I'). Values of 0.58 ± 0.07 μM for K_I and 5.50 ± 0.66 μM for K_I' were deduced from four independent experiments. This inhibition model was already proposed for structurally different Cdc25 inhibitors [9,18].



Scheme 1. (i) DCC, CH_2Cl_2, rt, 2 h, then phenyl maleimide (5 equiv), *hν*, 15 °C, 30 min (82%); (ii) *m*CPBA, CH_2Cl_2, 0 °C, 1 h; (iii) toluene, 110 °C, 1 h (90% from **4**); (iv) DCC, CH_2Cl_2, rt, 2 h, then 5 equiv of **5**, *hν*, 15 °C, 30 min; and (v) KOH, THF–MeOH, reflux, 3 h, **1a** (44%); **1b** (42%); **1c** (43%); **1d** (46%); **1e** (48%); **1f** (51%); **1g** (49%); **1h** (50%); **1i** (53%); **1j** (45%), two steps from **6**.

Table 1
Phosphatases inhibitory profile of dialkylsubstituted maleic anhydrides

Compound	IC_{50} (μM)[a]			
	Cdc25A	Cdc25B	Cdc25C	AP
1a	NA[b]	NA	NA	NA
1b	NA	NA	NA	NA
1c	NA	NA	NA	NA
1d	44.4 ± 1.4	55.2 ± 1.4*	53.9 ± 1.7*	NA
1e	44.3 ± 2.4	47.7 ± 3.0	NA	NA
1f	8.1 ± 0.3	8.7 ± 0.8	5.7 ± 0.2*	NA
1g	6.3 ± 0.2	11.6 ± 0.4*	12.6 ± 0.3*	NA
1h	4.7 ± 0.1	7.6 ± 0.6*	8.6 ± 1.5*	NA
1i	5.1 ± 0.3	3.6 ± 0.4*	4.3 ± 1.1	NA
1j	4.5 ± 1.7	6.7 ± 1.9	8.8 ± 1.3	NA

AP: horse liver alkaline phosphatase. *: Significantly different from Cdc25A inhibitory activity at $p < 0.05$.

[a] IC_{50} values are means ± SD calculated using a multiple comparison test with the Bonferroni's correction for at least two independent experiments. Each of them was performed with four determinations for all inhibitor concentrations (0–100 μM).

[b] NA: not active, $IC_{50} > 100$ μM.

4. Conclusion

In summary, a series of novel maleic anhydride derivatives were synthesized and evaluated as inhibitors of Cdc25 dual specificity phosphatases. The inhibitory activity of these compounds may result from the presence of two distinct structural features. Their anhydride moiety could serve as a phosphate surrogate as proposed for the γ-hydroxybutenolide residue of dysidiolide analogs [13]. The results obtained herein indicate that the hydrophobic alkyl chain length contributes to the inhibitory potency of these molecules, since a chain length of at least 13 carbons is necessary to reach a significant activity. This is in good agreement with the enhanced Cdc25B inhibitory activity when tetronic acid derivatives are substituted with an increasing alkyl chain length [19]. Considering the compounds synthesized and tested, the results described herein indicate that maleic anhydride derivatives could be regarded as attractive Cdc25 inhibitors.

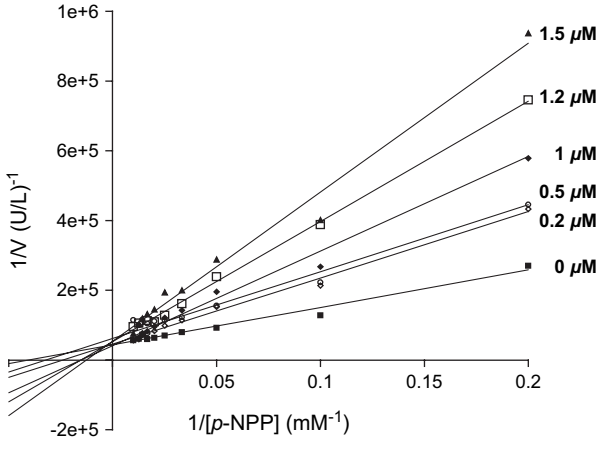


Fig. 1. Kinetic analysis of Cdc25A inhibition by **1i**. Lineweaver–Burk plot illustrating a mixed inhibition.

5. Experimental section

5.1. Chemistry

All reactions were carried out under an argon atmosphere. All reagents were obtained from commercial suppliers and used without further purification. THF and toluene were freshly distilled from sodium/benzophenone. Methylene chloride was distilled from CaH_2. Flash chromatography was carried out using silica gel 60 (Merck) with mixtures of ethyl acetate and cyclohexane as eluent unless specified otherwise. TLC analyses were performed on thin-layer analytical plates 60F254 (Merck).

Note: The *N*-hydroxy-2-thiopyridone derivatives are somewhat sensitive to daylight. It is advisable to cover the reaction flask with an aluminum foil.

5.1.1. Methyl 3-(2,5-dioxo-1-phenyl-4-(pyridin-2-ylthio) pyrrolidin-3-yl)propanoate (*4*)

To a solution of acid **2** (1.320 g, 10 mmol) and 2-mercaptopyridine *N*-oxide (1.466 g, 12 mmol) in dry CH_2Cl_2 (50 mL) was added DCC (2.475 g, 12 mmol) under argon and the mixture was stirred at room temperature for 2 h. Phenyl maleimide (8.658 g, 50 mmol) was added, the aluminum foil was removed, and the mixture was irradiated with a tungsten lamp (500 W) at 10–15 °C for 30 min. CH_2Cl_2 was evaporated under reduced pressure and the residue was dissolved in ether. The solution was filtered to remove the urea and the filtrate was washed with saturated $NaHCO_3$, water, brine, dried over $MgSO_4$, and concentrated in vacuo. The residue was purified over silica gel using cyclohexane–EtOAc (80:20) as an eluent to yield compound **4** (3.047 g, 82%) as a white solid. Mp 128–129 °C; IR (KBr) 1733, 1714, 1176 cm^{-1}; ^1H NMR ($CDCl_3$), δ 8.30 (d, $J = 5$ Hz, 1H), 7.53–7.00 (m, 8H), 3.96 (d, $J = 6$ Hz, 1H), 3.68 (s, 3H), 3.30 (q, $J = 6$ Hz, 1H), 2.68 (t, 2H), 2.37 (m, 1H), 2.25 (m, 1H); ^{13}C NMR ($CDCl_3$), δ 176.5, 173.7, 173.0, 155.8, 149.0, 136.7, 132.4, 129.1, 128.5, 126.4, 122.1, 120.3, 51.8, 47.0, 46.4, 31.2, 25.6.

5.1.2. Methyl 3-(2,5-dioxo-1-phenyl-2,5-dihydro-1H-pyrrol-3-yl)propanoate (*5*)

To a solution of **4** (3.047 g, 8.198 mmol) in CH_2Cl_2 (40 ml) was added *m*CPBA (2.230 g, 9.018 mmol) at 0 °C. The reaction mixture was stirred at 0 °C for 1 h, diluted with CH_2Cl_2, washed with saturated $NaHCO_3$, water, brine, dried over $MgSO_4$, and concentrated in vacuo. The crude sulfoxide was dissolved in dry toluene (40 mL) and the solution was refluxed for 1 h. The solvent was evaporated under reduced pressure and the residue was purified over silica gel using cyclohexane–EtOAc (60:40) as an eluent to yield compound **5** (1.890 g, 90%) as a white solid. Mp 71–72 °C; IR (KBr) 1737, 1708, 1698, 1409, 873 cm^{-1}; ^1H NMR ($CDCl_3$), δ 7.49–7.32 (m, 5H), 6.48 (t, $J = 1.5$ Hz, 1H), 3.73 (s, 3H), 2.87 (t, $J = 7$ Hz, 2H), 2.72 (t, $J = 7$ Hz, 2H); ^{13}C NMR ($CDCl_3$), δ 172.1, 170.0, 169.3, 148.0, 131.5, 129.1, 127.8, 127.2, 125.9, 52.0, 31.2, 21.0. Anal. calcd for $C_{14}H_{13}NO_4$: C, 64.86; H, 5.05. Found: C, 64.61; H, 5.36.

*5.1.3. General procedure for synthesis of dialkylsubstituted maleic anhydrides **1a–j***

To a solution of acid **6** (1 mmol) and 2-mercaptopyridine-*N*-oxide (147 mg, 1.2 mmol) in dry CH_2Cl_2 (10 mL) was added DCC (248 mg, 1.2 mmol) under argon. The mixture was stirred at room temperature for 2 h. Olefin **5** (1.296 g, 5 mmol) was then added, the aluminum foil was removed, and the mixture irradiated with a tungsten lamp (500 W) at 10–15 °C for 30 min. CH_2Cl_2 was evaporated under reduced pressure and the residue was dissolved in ether. The solution was filtered to remove the urea and the filtrate was washed with saturated $NaHCO_3$, water, brine, dried over $MgSO_4$, and concentrated in vacuo. The residue was purified over silica gel using cyclohexane–EtOAc (80:20) as an eluent to yield compound **8** which was used in the next step without further characterization. To a solution of imide **8** in a THF–MeOH (6 mL, 1:2) was added a solution of KOH (0.6 g) in water (2 mL), and the reaction was refluxed for 3 h with stirring. The solvent mixture was removed under reduced pressure, and the residue was dissolved in water and extracted with ether. The aqueous phase layer was acidified with diluted HCl and extracted with ether. The organic layer was washed with water, brine, dried over $MgSO_4$ and concentrated. The residue was purified by column chromatography on silica gel using cyclohexane–EtOAc as an eluent to yield compound **1**.

*5.1.3.1. 3-(4-Hexyl-2,5-dioxo-2,5-dihydrofuran-3-yl)propanoic acid (**1a**) [20,21].* Oil; IR (neat) 2915, 2848, 1768, 1712, 1257, 910 cm^{-1}; ^1H NMR ($CDCl_3$), δ 2.77 (s, 4H), 2.49 (t, $J = 7$ Hz, 2H), 1.57 (m, 2H), 1.28 (m, 6H), 0.88 (t, $J = 6.5$ Hz, 3H); ^{13}C NMR ($CDCl_3$), δ 176.1, 165.6, 165.4, 146.3, 141.4, 31.3, 30.8, 29.2, 27.9, 24.6, 22.4, 19.6, 13.9.

*5.1.3.2. 3-(4-Octyl-2,5-dioxo-2,5-dihydrofuran-3-yl)propanoic acid (**1b**) [20,21].* Oil; IR (neat) 2915, 2848, 1770, 1714, 1259, 910 cm^{-1}; ^1H NMR ($CDCl_3$), δ 2.77 (s, 4H), 2.50 (t, $J = 7$ Hz, 2H), 1.57 (m, 2H), 1.28 (m, 10H), 0.88 (t, $J = 6.5$ Hz, 3H); ^{13}C NMR ($CDCl_3$), δ 176.8, 165.6, 165.4, 146.3, 141.4, 31.7, 30.9, 29.6, 29.1, 29.0, 27.9, 24.6, 22.6, 19.6, 14.0.

*5.1.3.3. 3-(4-Nonyl-2,5-dioxo-2,5-dihydrofuran-3-yl)propanoic acid (**1c**).* Oil; IR (neat) 2917, 2850, 1768, 1712, 1257, 910 cm^{-1}; ^1H NMR ($CDCl_3$), δ 2.76 (s, 4H), 2.49 (t, $J = 7$ Hz, 2H), 1.58 (m, 2H), 1.28 (m, 12H), 0.88 (t, $J = 6.5$ Hz, 3H); ^{13}C NMR ($CDCl_3$), δ 176.2, 165.4, 165.2, 146.1, 141.2, 31.6, 30.8, 29.5, 29.4, 29.2, 29.0, 27.7, 24.4, 22.4, 19.4, 13.9. Anal. calcd for $C_{16}H_{24}O_5$: C, 64.84; H, 8.16. Found: C, 64.65; H, 8.30.

*5.1.3.4. 3-(4-Decyl-2,5-dioxo-2,5-dihydrofuran-3-yl)propanoic acid (**1d**) [22].* Oil; IR (neat) 2917, 2850, 1768, 1712, 1257, 910 cm^{-1}; ^1H NMR ($CDCl_3$), δ 2.77 (s, 4H), 2.49 (t, $J = 7$ Hz, 2H), 1.57 (m, 2H), 1.28 (m, 14H), 0.88 (t, $J = 6.5$ Hz, 3H); ^{13}C NMR ($CDCl_3$), δ 176.1, 165.4, 165.2, 146.0, 141.4, 31.7, 30.9, 29.5, 29.4, 29.3, 29.2, 29.1, 27.9, 24.6, 22.4, 19.6, 13.9.

*5.1.3.5. 3-(4-Undecyl-2,5-dioxo-2,5-dihydrofuran-3-yl)propanoic acid (**1e**).* Mp 54–55 °C; IR (KBr) 2917, 2850, 1768, 1712, 1257, 910 cm^{-1}; ^1H NMR ($CDCl_3$), δ 2.76 (s, 4H), 2.49 (t, $J = 7$ Hz, 2H), 1.58 (m, 2H), 1.28 (m, 16H), 0.88 (t, $J = 6.5$ Hz, 3H); ^{13}C NMR ($CDCl_3$), δ 177.1, 165.6, 165.4, 146.3, 141.4, 31.9, 30.9, 29.6, 29.5, 29.4, 29.32, 29.28, 29.2, 27.9, 24.6, 22.7, 19.6, 14.1; HRMS *m/z*: calcd for $C_{18}H_{29}O_5$ [MH] 325.2015, found 325.2022.

*5.1.3.6. 3-(4-Tridecyl-2,5-dioxo-2,5-dihydrofuran-3-yl)propanoic acid (**1f**).* Mp 63–64 °C; IR (KBr) 2915, 2848, 1768, 1712, 1259, 910 cm^{-1}; ^1H NMR ($CDCl_3$), δ 2.77 (s, 4H), 2.50 (t, $J = 7$ Hz, 2H), 1.57 (m, 2H), 1.28 (m, 20H), 0.88 (t, $J = 6.5$ Hz, 3H); ^{13}C NMR ($CDCl_3$), δ 176.4, 165.6, 165.4, 146.3, 141.4, 31.9, 30.9, 29.7, 29.66, 29.6, 29.58, 29.4, 29.3, 29.2, 27.9, 24.6, 22.7, 19.6, 14.1; HRMS *m/z*: calcd for $C_{20}H_{33}O_5$ [MH] 353.2328, found 353.2315.

*5.1.3.7. 3-(4-Tetradecyl-2,5-dioxo-2,5-dihydrofuran-3-yl)propanoic acid (**1g**).* Mp 61–62 °C; IR (KBr) 2917, 2850, 1768, 1712, 1259, 910 cm^{-1}; ^1H NMR ($CDCl_3$), δ 2.77 (s, 4H), 2.50 (t, $J = 7$ Hz, 2H), 1.57 (m, 2H), 1.27 (m, 22H), 0.88 (t, $J = 6.5$ Hz, 3H); ^{13}C NMR ($CDCl_3$), δ 176.1, 165.6, 165.4, 146.2, 141.5, 31.9, 30.9, 29.7, 29.63, 29.6, 29.5, 29.4, 29.3, 29.2, 27.9, 24.6, 22.7, 19.6, 14.1; HRMS *m/z*: calcd for $C_{21}H_{35}O_5$ [MH] 367.2484, found 367.2519.

*5.1.3.8. 3-(4-Pentadecyl-2,5-dioxo-2,5-dihydrofuran-3-yl)propanoic acid (**1h**).* Mp 68–69 °C; IR (KBr) 2917, 2848, 1768, 1712, 1257, 910 cm^{-1}; ^1H NMR ($CDCl_3$), δ 2.77 (s, 4H), 2.49 (t, $J = 7$ Hz, 2H), 1.57 (m, 2H), 1.26 (m, 24H), 0.88 (t, $J = 6.5$ Hz, 3H); ^{13}C NMR ($CDCl_3$), δ 176.2, 165.6, 165.4, 146.2, 141.5, 31.9, 30.9, 29.7, 29.66, 29.63, 29.6, 29.44, 29.4, 29.2, 27.9, 24.6, 22.7, 19.6, 14.1; HRMS *m/z*: calcd for $C_{22}H_{37}O_5$ [MH] 381.2641, found 381.2625.

*5.1.3.9. 3-(4-Heptadecyl-2,5-dioxo-2,5-dihydrofuran-3-yl)propanoic acid (**1i**).* Mp 76–77 °C; IR (KBr) 2915, 2850, 1770, 1712, 1259, 906 cm^{-1}; ^1H NMR ($CDCl_3$), δ 2.76 (s, 4H), 2.49 (t, $J = 7$ Hz, 2H), 1.57 (m, 2H), 1.26 (m, 28H), 0.88 (t, $J = 6.5$ Hz, 3H); ^{13}C NMR ($CDCl_3$), δ 176.1, 165.1, 164.8, 146.6, 141.1, 31.9, 30.9, 29.7, 29.63, 29.6, 29.57, 29.4, 29.3, 29.2, 27.9, 24.6, 22.7, 19.6, 14.1; HRMS *m/z*: calcd for $C_{24}H_{41}O_5$ [MH] 409.2954, found 409.3000.

*5.1.3.10. (Z)-3-(4-(Heptadec-8-enyl)-2,5-dioxo-2,5-dihydrofuran-3-yl)propanoic acid (**1j**).* Oil; IR (neat) 2927, 2856, 1768, 1714, 1274, 912 cm^{-1}; ^1H NMR ($CDCl_3$), δ 5.35 (m, 2H), 2.77 (s, 4H), 2.50 (t, $J = 7$ Hz, 2H), 2.01 (m, 4H), 1.57 (m, 2H), 1.26 (m, 20H), 0.88 (t, $J = 6.5$ Hz, 3H); ^{13}C NMR ($CDCl_3$), δ (ppm) 176.4, 165.6, 165.4, 146.3, 141.4, 130.1, 129.6, 31.9, 30.9, 30.3, 29.8, 29.7, 29.6, 29.5, 29.3, 29.1,

29.0, 27.9, 27.2, 27.1, 24.6, 22.7, 19.6, 14.1; HRMS *m/z*: calcd for $C_{24}H_{39}O_5$ [MH] 407.2797, found 407.2851.

5.2. Biological evaluations

5.2.1. Expression and purification of Cdc25s

The *Escherichia coli* BL21(DE3) strain containing a pGex-2T plasmid encoding the genes fusion construct of glutathione S-transferase (GST) and human Cdc25s whose expression was under the control of IPTG was used. These constructs correspond to the entire Cdc25A and Cdc25C, and to the catalytic domain of Cdc25B. *E. coli* was first grown in 50 μg of ampicillin/mL of LB medium. When the optical density at 600 nm reached a value between 0.4 and 0.6, IPTG (50 μg/mL) was added and the culture incubated for 5 h. Cells were then harvested by a 3000*g* centrifugation for l5 min at 4 °C. Pellets were kept frozen at −80 °C until extraction. The bacterial pellet was disrupted by sonication in lysis buffer (50 mM Tris−HCl (pH 7.5) 250 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 0.1 mM PMSF, 3% protease inhibitor cocktail (Sigma)) at 4 °C. The homogenate was centrifuged for 45 min at 4 °C at 100,000*g*. The supernatant was then frozen at −80 °C until purification. Elution was performed using a Biologic low-pressure chromatography (BioRad). Supernatant was incubated with glutathione−agarose gel previously packed in a column and equilibrated with lysis buffer for 3 h. The glutathione−agarose gel was washed with five volumes of lysis buffer, followed by a wash with five volumes of Tris buffer A (50 mM Tris−HCl (pH: 8.0), 50 mM NaCl, 1 mM EDTA, and 10 mM DTT). Elution of the fusion protein was performed by 20 mM glutathione in Tris buffer A at a flow rate of 0.5 mL/min.

5.2.2. In vitro enzyme assays

p-Nitrophenyl phosphate (*p*-NPP) was used to measure the activity of each GST−Cdc25 fusion protein and alkaline phosphatase (Roche Diagnostic) as described by Baratte et al. [23]. Initial rates at different inhibitor concentrations (0, 0.2, 0.5, 1, 1.2 and 1.5 μM) were measured. For each inhibitor concentration, measurements were performed at 10 different concentrations of *p*-NPP (0−100 mM). The inhibition pattern was evaluated and constants were determined using a curve-fitting program, Prism 4.0 (GraphPad software, Inc.) with the Lineweaver−Burk equation for a mixed inhibition: $v = V_{max}[S]/((1 + [I]/K_I)K_M + (1 + [I]/K_I')[S])$.

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