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Original article

Synthesis, in vitro antiproliferative activities, and Chk1 inhibitory properties of pyrrolo[3,4-*a*]carbazole-1,3-diones, pyrrolo[3,4-*c*]carbazole-1,3-diones, and 2-aminopyridazino[3,4-*a*]pyrrolo[3,4-*c*]carbazole-1,3,4,7-tetraone

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Abstract

The synthesis of substituted pyrrolo[3,4-*a*]carbazole-1,3-diones, pyrrolo[3,4-*c*]carbazole-1,3-diones, and 2-aminopyridazino[3,4-*a*]pyrrolo[3,4-*c*]carbazole-1,3,4,7-tetraone is reported. Their inhibitory properties toward Checkpoint 1 kinase (Chk1) have been evaluated and their in vitro antiproliferative activities toward three tumor cell lines: murine leukemia L1210, human colon carcinoma HT29 and HCT116 have been determined. From the biological results, it appears that, in contrast with the upper E heterocycle, the lower D heterocycle is not absolutely required for Chk1 inhibition. The ATP binding pocket of Chk1 seems to be adaptable to substitution of the nitrogen of the imide E heterocycle with a hydroxymethyl group, allowing the fundamental hydrogen bond with the Glu^{85} residue of the enzyme.

Keywords: Pyrrolo[3,4-*a*]carbazole-1,3-diones; 2-Amino-pyridazino[3,4-*a*]pyrrolo[3,4-*c*]carbazole-1,3,4,7-tetraone; Antitumor agents; Chk1 inhibitors

1. Introduction

Chk1 kinase represents a relevant target for the conception of new antitumor agents [1,2]. Chk1 plays a major role in the G2 checkpoint that is activated in response to DNA damage. In more than 50% of tumors cells, the p53 protein is mutated leading to an inactive G1 checkpoint. In the p53-mutated cells, only the G2 checkpoint is activated in response to DNA damage and provokes a cell cycle arrest allowing time for DNA repair [3]. Therefore, the combination of a DNA damaging agent and a Chk1 inhibitor should drive, selectively cancer cells, toward a lethal mitosis. Inhibitors of Chk1 kinase have triggered considerable interest over the past ten years [2,4]. Granulatimide and isogranulatimide are natural products isolated from the ascidian *Didemnum granulatum* [5–7]. These compounds inhibit Chk1 with IC_{50} values of 2 and 3 μM respectively [8]. Several isomers and derivatives, in which the imidazole heterocycle is conserved, have been synthesized [7–9]. Like staurosporine and UCN-01, isogranulatimide (Fig. 1) is an ATP-competitive Chk1 inhibitor [9,10]. In the crystal structures of Chk1 in complex with staurosporine or UCN-01 or isogranulatimide, two hydrogen bonds are observed between the E lactam or imide heterocycle and the ATP binding site of the enzyme, the first one between NH and the carbonyl oxygen of Glu^{85}, and the second one between the oxygen of the carbonyl group on the left and the amide nitrogen of Cys^{87}.

In the course of structure–activity relationship studies on granulatimide, we synthesized granulatimide analogues in which the imidazole moiety was replaced by an imide or pyrrole heterocycle [11–17]. Other granulatimide analogues in which the imide E ring was replaced by a lactam ring and the imidazole D heterocycle was replaced by an imide ring were also synthesized [18]. The IC_{50} values toward Chk1 of

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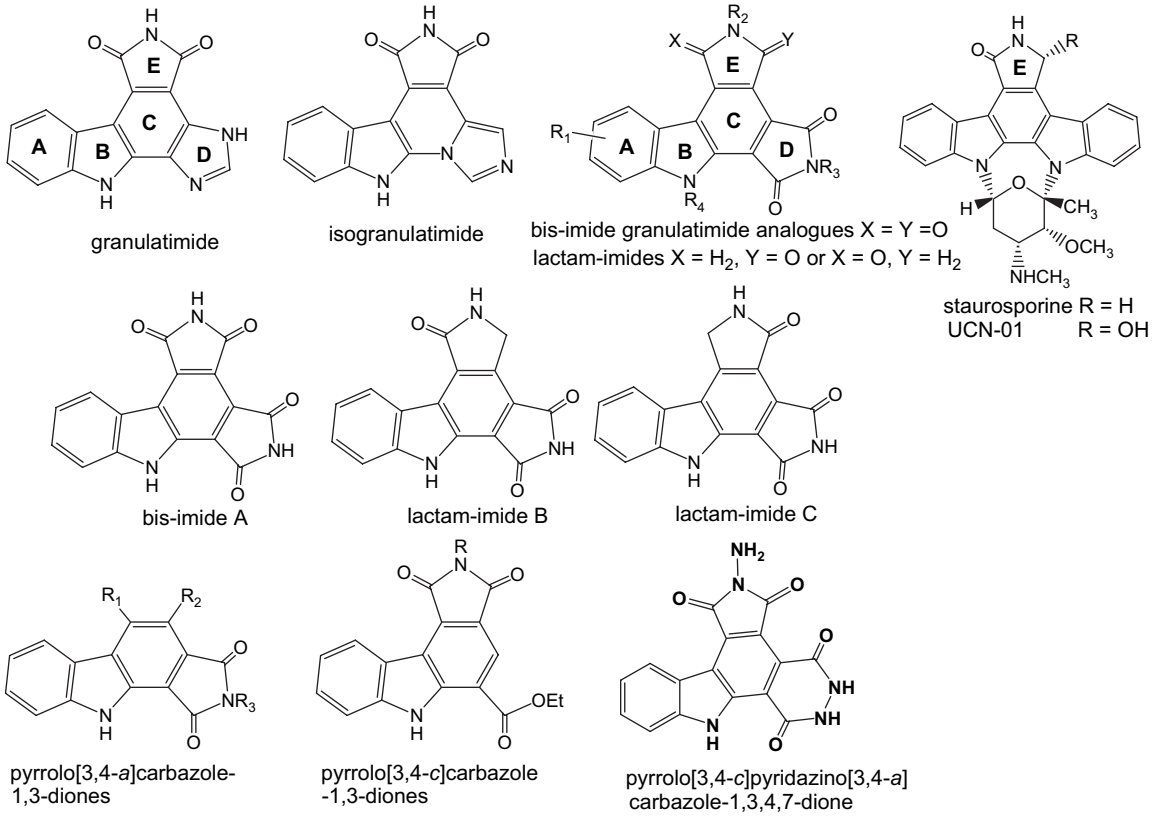


Fig. 1. Structures of granulatimide, isogranulatimide, bis-imides and lactam-imide analogues, staurosporine and UCN-01, pyrrolo[3,4-*a*]carbazole-1,3-diones, pyrrolo[3,4-*c*]carbazole-1,3-diones, and 2-aminopyridazino[3,4-*a*]pyrrolo[3,4-*c*]carbazole-1,3,4,7-tetraone.

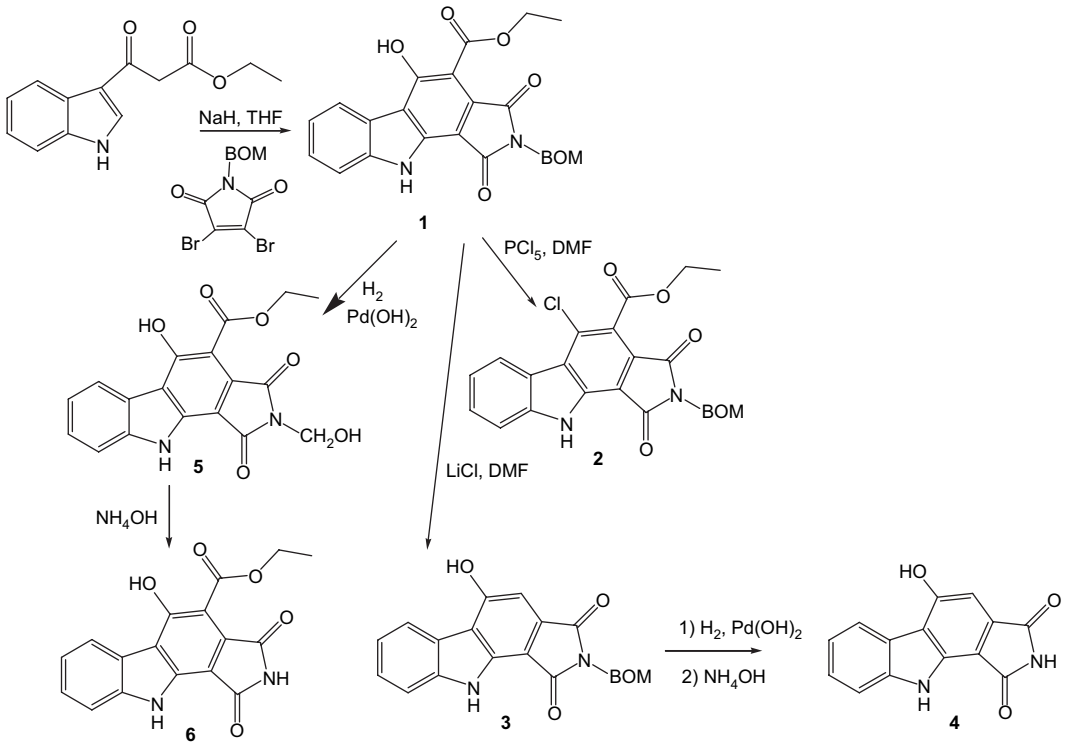
some bis-imides and lactam-imides are in the nanomolar range [15–18]. To investigate the role of the C, D, and E rings, indolylpyrazolones and an indolylpyridazinedione have been synthesized [19]. The indolylpyridazinedione was found to be inactive toward Chk1 and the indolylpyrazolones were poor Chk1 inhibitors, suggesting that a more rigid system is required for Chk1 inhibition. Accordingly, we have investigated the Chk1 inhibitory capacities of compounds bearing the A, B and C rings but in which the D or E heterocycles are missing. In this connection, substituted pyrrolo[3,4-*a*]carbazole-1,3-diones, in which the E upper imide heterocycle is missing and pyrrolo[3,4-*c*]carbazole-1,3-diones, in which the D imide heterocycle is missing, were synthesized. Moreover, 2-aminopyridazino[3,4-*a*]pyrrolo[3,4-*c*]carbazole-1,3,4,7-tetraone, in which the D heterocycle is a 6-membered pyridazine dione ring was also synthesized (Fig. 1). The inhibitory activities of the new compounds toward Chk1 were determined and their cytotoxicities toward three tumor cell lines: murine leukemia L1210, human colon carcinoma HT29 and HCT116 were evaluated.

2. Results and discussion

2.1. Chemistry

To obtain pyrrolo[3,4-*a*]carbazole-1,3-diones diversely substituted in 4 and 5 positions, compound **1** was firstly synthesized (Scheme 1). Ethyl 3-oxo-3-(1*H*-indol-3-yl)propionate was prepared from indole and ethyl 1-chloromalonate in the presence of $AlClEt_2$ as previously described [19]. Reaction of ethyl 3-oxo-3-(1*H*-indol-3-yl)propionate with NaH then with *N*-BOM-dibromomaleimide gave compound **1**. The nucleophilic substitution leading to the coupling of the maleimide was followed by HBr elimination yielding pyrrolocarbazole **1**. By reaction of **1** with PCl_5 in DMF, chlorinated compound **2** was obtained in 94% yield, whereas compound **3** without the ethoxycarbonyl group was prepared from compound **1** in 96% yield via a Krapcho decarboxylation [20] by reaction with LiCl in DMF. Hydrogenolysis of **3** followed by aminolysis led to compound **4** in 33% yield. Hydrogenolysis of **1** using $Pd(OH)_2$ as a catalyst led to compound **5** in 80% yield. Aminolysis of **5** afforded imide **6** in 30% yield.

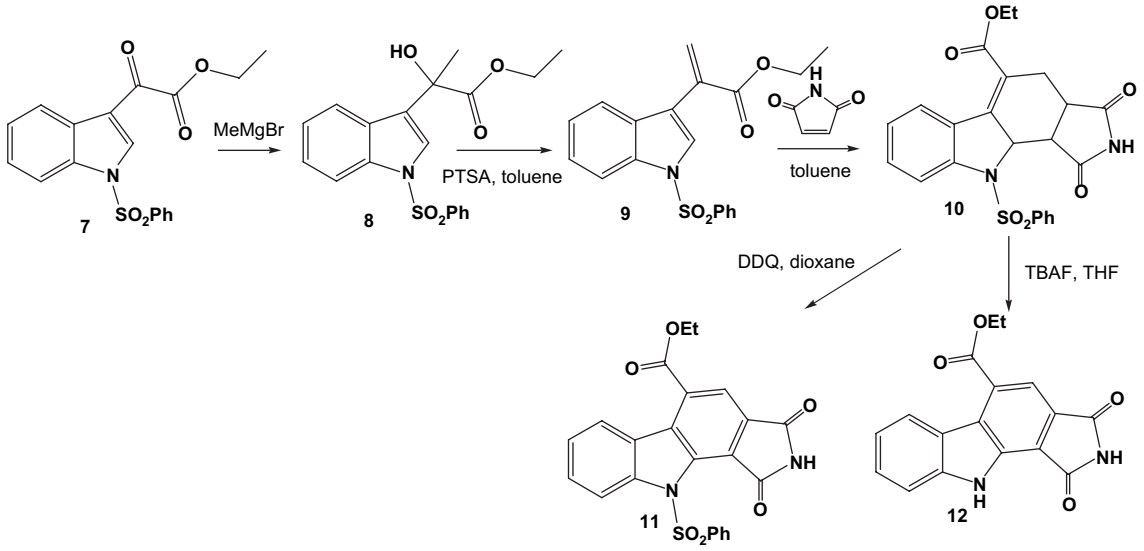
The sequence of reactions for the synthesis of a pyrrolo[3,4-*a*]carbazole-1,3-dione with the ethoxycarbonyl group in the 5-position is outlined in Scheme 2. Reaction of ethyl 1-(phenylsulfonyl)indole-3-glyoxylate [21] **7** with methyl magnesium bromide provided intermediate **8** in 77% yield. Compound **8** was dehydrated in the presence of *p*-toluenesulfonic acid to give compound **9** in 95% yield. Compound **9** was isolated as two conformers. The conformers' ratio (6:1 in $CDCl_3$) was determined from the ^1H NMR spectrum on the signals of the ethylenic CH_2 at 6.02 and 6.45 ppm for the major conformer and at 6.14 and 6.26 ppm for the minor conformer. In DMSO, the conformers' ratio (4:1) was determined on the signals of the ethylenic CH_2 at 6.27 and 6.49 ppm for the major conformer and at 6.42 and 6.56 ppm for the minor



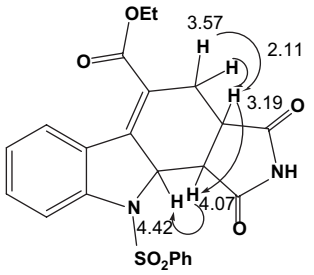
Scheme 1. Synthetic scheme for compounds **1**–**6**.

conformer. A Diels–Alder cycloaddition between compound **9** and maleimide carried out in xylene led to the indoline isomer **10** of the cycloadduct in 44% yield. The structure of the indoline isomer **10** was assigned from NMR $^1H-^1H$ COSY correlations (Fig. 2). The two protons of the methylene group at 2.11 (m) and 3.57 ppm (dd) are coupled with the proton at 3.19 ppm (ddd) which is coupled with the proton at 4.07 ppm (dd) which is coupled with the proton at 4.42 ppm (dd). Oxidation using DDQ led to *N*-phenylsulfonyl carbazole **11** in a poor yield (20%). To improve the yield of the oxidation, deprotection of the indole nitrogen was carried out using tetrabutylammonium fluoride (15 equiv.) in refluxing THF. Concomitant with the removal of the phenylsulfonyl protective group, oxidation into carbazole was observed leading directly to compound **12** in 83% yield.

To get an insight into the influence of the D imide heterocycle, compound **13** (Scheme 3), in which this heterocycle is missing, was prepared from indolylmaleimide and ethyl acrylate, followed by oxidation of the Diels–Alder cycloadduct intermediate. The position of ethyloxycarbonyl substituent was



Scheme 2. Synthetic scheme for compounds **8**–**12**.



10

Fig. 2. $^{1}H-^{1}H$ COSY correlations in compound **10**.

assigned from NMR experiments (COSY $^{1}H-^{1}H$, HSQC, and HMBC). The singlet at 8.23 ppm was coupled with the tertiary carbon at 120.2 ppm. The HMBC spectrum showed that the proton at 8.23 ppm was coupled with two C=O at 164.4, and 169.3 ppm (Fig. 3).

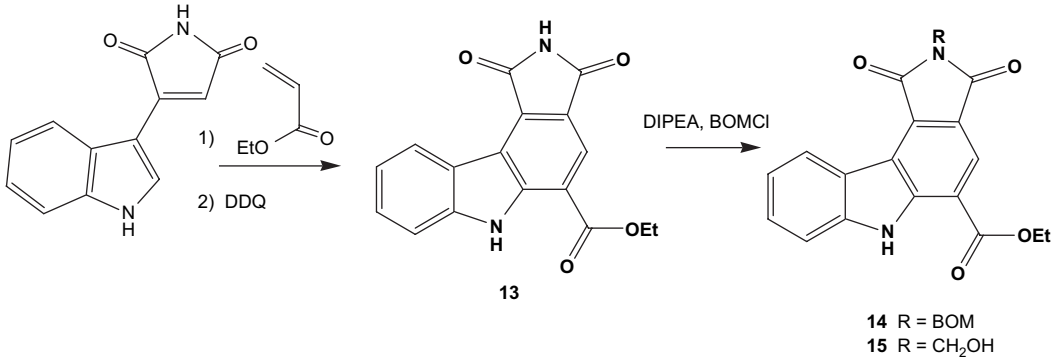
With the aim of converting the ester function into a hydrazide function, the imide nitrogen of compound **13** was protected with a benzyloxymethyl substituent. Reaction of **13** with benzyloxymethylchloride in the presence of diisopropylethylamine as a base led to compounds **14** and **15** bearing a benzyloxymethyl and a hydroxymethyl substituent on the imide nitrogen, in 76% and 17% yields, respectively.

Finally, to obtain a compound with a pyridazinedione upper E heterocycle, with which the fundamental hydrogen bonds with the Glu^{85} and Cys^{87} in the ATP binding site of the enzyme should be conserved but with a six-membered ring heterocycle, a Diels–Alder reaction between 4-(indol-3-yl)pyridazin-3,6-dione [19] and maleimide was tried. Since the cycloaddition did not occur, 1,3,4,6-tetrahydrofuro[3,4-*a*]pyrrolo-[3,4-*c*]carbazole-1,3,4,6-tetraone **17** (Scheme 4) was prepared from 3-(3-indolyl)furane-2,5-dione [11]. A Diels–Alder cycloaddition with maleimide afforded indole **16** in 90% yield. The indole structure of compound **16** was assigned from its two exchangeable protons shifted at about 11.5 ppm (in indolines, the NH proton is shifted at about 8 ppm). Oxidation of **16** with DDQ led to carbazole **17** in 86% yield. Reaction of compound **17** with hydrazine hydrate followed by acidification led to compounds **18** and **19** in 3% and 37% yields, respectively. Compound **19** was not obtained as the hydrochloride, probably due to the electron withdrawing effect of the maleimide. It was not surprising to isolate compound **19** from the reaction with hydrazine hydrate, because in rebeccamycin and indolocarbazole series, the amino group on the maleimide nitrogen could never be converted into a hydrochloride [22].

In rebeccamycin and indolocarbazole series, we previously observed the conversion of a maleic anhydride or a maleimide into a *N*-amino-maleimide or pyridazine dione (Fig. 4) [23,24]. It seems that the pyridazine dione 6-membered heterocycle is favoured when the overall structure is more rigid. In compound **19**, the heterocycles D and E could be inversed. Indeed, in the presence of hydrazine hydrate, the maleic anhydride of compound **17** could lead to a pyridazine dione whereas the D maleimide cycle could lead to a *N*-amino maleimide [23,25], but the isolation of intermediate **18** is in the favour of the formation of pyridazine dione as the D heterocycle. In the ^{1}H NMR spectrum of compound **18**, the signals of the exchangeable protons are shifted at 12.29 ppm as a broad signal (3H) which is in agreement with the presence of a pyridazine ring. In the ^{1}H NMR spectrum of compound **19**, two exchangeable protons appear as a singlet shifted at 5.30 ppm which is in agreement with $N-NH_2$. Three other exchangeable protons, shifted at chemical shifts >12 ppm, correspond to the indolic NH and NH–NH of the hydrazide. The isolation of compound **18** shows that the maleimide ring reacts more quickly than the maleic anhydride ring with hydrazine hydrate. Compound **18** should be an intermediate which in the presence of a second molecule of hydrazine hydrate would lead to compound **19** bearing a *N*-amino-maleimide as the E heterocycle.

2.2. Chk1 inhibitory activities

Unfortunately, compound **19** was highly insoluble, therefore its biological activities could not be evaluated. In the other newly synthesized structures, the D or E ring is missing. In compounds **5** and **12**, the position of the ethyl carboxylate is inverted on the C ring, which should have an influence on the Chk1 inhibitory activities. The Chk1 inhibitory properties have been evaluated and compared with those of granulatimide, isogranulatimide, bis-imide **A**, lactam-imides **B** and **C**



13

14 R = BOM
15 R = CH_2OH

Scheme 3. Synthetic scheme for compounds **13–15**.

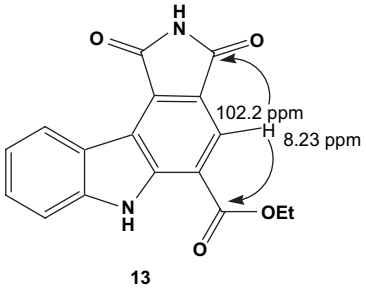


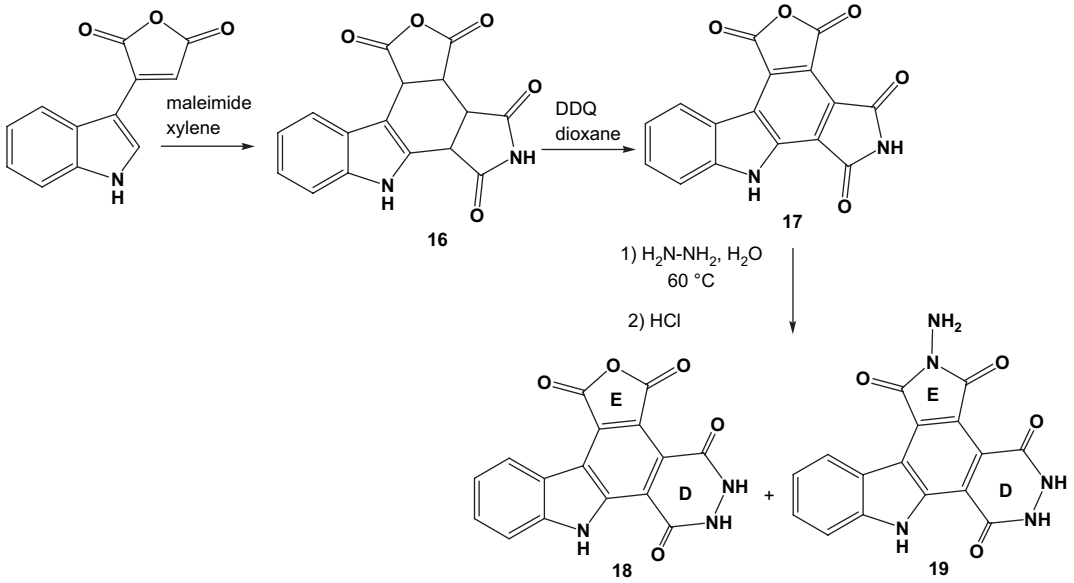
Fig. 3. $^{13}C-^{1}H$ correlations in compound **13**.

shown in Fig. 1 (Table 1). The percentage of Chk1 inhibition were determined at a compound concentration of 10 μM. For the potent Chk1 inhibitors, the IC_{50} values were determined. Compound **4** in which the E ring is missing and which does not bear a carbonyl group attached to the C ring was a poor Chk1 inhibitor. Compounds **5** and **6** in which the E ring is missing and in which a carbonyl group is attached to the C ring at the 4-position were inactive. Compound **12**, in which the E ring is missing and in which a carbonyl group is attached to the C ring at the 5-position, was a modest Chk1 inhibitor whereas compounds **5** and **6** do not inhibit this kinase. These results are in agreement with what observed in the lactam-imide series [18]. When the E ring was a lactam, the Chk1 inhibitory activities of lactam-imides in which the carbonyl of the lactam is oriented on the left (toward the indole) were always stronger than those of lactam-imides in which the carbonyl of the lactam is oriented on the right (toward the imide) [18]. These results might be explained by the possibility of the first ones to establish the fundamental hydrogen bonds with Glu^{85} and Cys^{87} residues in the ATP binding pocket of Chk1.

Anhydride **16** was a poor Chk1 inhibitor and anhydride **17** did not inhibit Chk1 suggesting that the hydrogen bond with Glu^{85} is necessary to stabilize the molecules inside the ATP binding site. Compound **13**, possessing the E imide heterocycle but lacking the D heterocycle inhibited Chk1 although the IC_{50} value was superior than 5 μM. Compound **15**, in which the D heterocycle is missing but bearing a hydroxymethyl substituent on the imide nitrogen of the E heterocycle, was a strong Chk1 inhibitor with an IC_{50} value of 0.21 μM. This result was somewhat surprising because in the previous series, a free nitrogen at the imide E heterocycle was required for Chk1 inhibitory activity. Molecular modelling studies have been carried out to investigate the stabilization of compounds **13** and **15** in the ATP binding pocket of Chk1.

2.3. Molecular modelling studies

Molecular modelling with compounds **13** and **15** were carried out using as model the complex structure of Chk1/staurosporine [10] downloaded from the Protein Data Bank. Interestingly, in both cases, the two fundamental hydrogen bonds were conserved between Glu^{85} and Cys^{87} residues of the enzyme and the D heterocycle. In both cases, there is a hydrogen bond between NH of Cys^{87} and the carbonyl group on the left of the D imide heterocycle. The hydrogen bond with the carbonyl of Glu^{85} was formed either with the imide NH of compound **13** (Fig. 5B) or with the hydroxy group of compound **15** (Fig. 5A). In both models, a hydrogen bond between the carbonyl of the ethyl carboxylate and the indole NH stabilized the conformation of the ethyl carboxylate. The two models were superimposed to visualize the modification of the orientation in the ATP binding site of Chk1 (Fig. 5C). It can be observed that when compound **15** lies in the ATP binding pocket, a slight displacement of Glu^{85} allows a hydrogen bond with the hydroxy group.



Scheme 4. Synthetic scheme for compounds **16–19**.

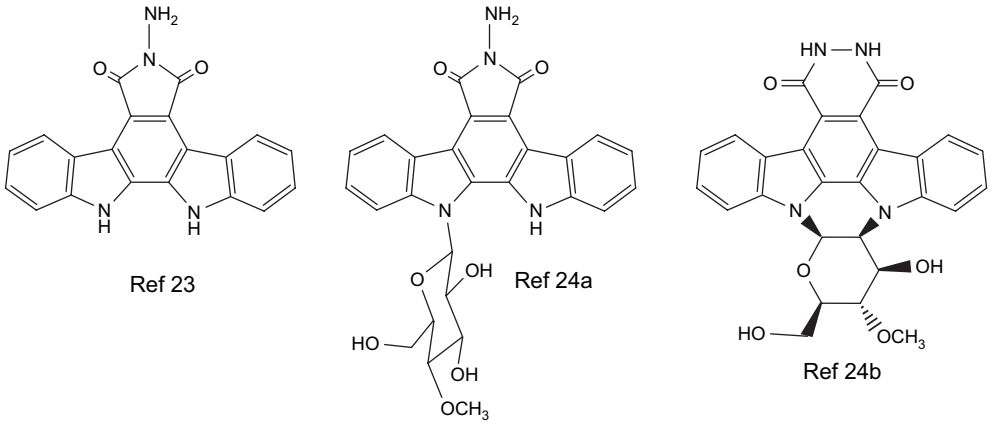


Fig. 4. *N*-Amino maleimides and pyridazine heterocycles in indolocarbazole series.

2.4. In vitro antiproliferative activities

The in vitro antiproliferative activities against three tumor cell lines: murine leukemia L1210, human HT29 and HCT116 colon carcinoma were evaluated. The IC_{50} values in μM are reported in Table 1. Compounds **4** and **6** were poorly cytotoxic toward the tumor cell lines tested. The IC_{50} values for compounds **12**, **13**, **15** and **16** were in the micromolar range and these compounds were not selective toward the tumor cell lines tested. Checkpoint inhibitors are not expected to be cytotoxic by themselves. They should be cytotoxic in association with DNA damaging agents. However, the cytotoxicity observed with compounds **12**, **13**, **15** and **16** could be due to the inhibition of other kinases than Chk1. To get a first insight into the kinase selectivity, the inhibitory activity of compound **15** toward the tyrosine kinase Src has been evaluated. Compound **15** did not behave as a Src inhibitor (percentage of Src inhibition at 10 μM: 11.5%).

3. Conclusion

In conclusion, this work reports the synthesis granulatimide analogues in which the D and E rings are modified or absent. The biological activities of compound **19** in which the heterocycles D + E are modified could not be evaluated due to its insolubility. Compared with bis-imide **A** and lactam-imide **B** the newly synthesized compounds **4**, **5**, **6** and **12** in which the E ring is missing were poor Chk1 inhibitors suggesting that this ring is required for Chk1 inhibition. Compound **15** in which the D ring is missing and in which the nitrogen of the E heterocycle bears a hydroxymethyl group was found to be a potent Chk1 inhibitor. At first sight, the D ring is not absolutely required. Moreover, a substitution of the imide nitrogen with a hydroxylmethyl group is compatible with Chk1 inhibitory activity and allows hydrogen bonding with Glu^{85} in the ATP binding pocket of Chk1. This result is especially interesting and provides a new avenue to the design of Chk1 inhibitors.

Table 1
Percentages of Chk1 inhibition at a drug concentration of 10 μM. IC_{50} values (μM) toward Chk1

Compound	% Chk1 inhibition at 10 μM	IC_{50} Chk1 (μM)	L1210	HCT116	HT29
Granulatimide	93.9	0.08	2.8	6.1	5.7
Isogranulatimide	89.7	0.44	10	13	13.7
Bis-imide A	94.4	0.02	32.7	nd	9.7
Lactam-imide B	85.4	0.05	45.8	10.2	8.2
Lactam-imide C	71.7	0.37	47.0	58.9	>100
4	11		54.4	34.4	46.7
5	Inactive		10.6	11.5	31.7
6	Inactive		57.5	33.6	84.9
12	45.3		4.9	3.8	6.4
13	71.7	>5	5.8	2.3	4.0
15	72.4	0.21	5.5	2.9	3.9
16	10.6		5.7	nd	6.2
17	Inactive		11.9	10.7	33.0

In vitro antiproliferative activities against three tumor cell lines: murine leukemia L1210, human HT29 and HCT116 colon carcinoma (IC_{50} μM). nd: not determined.

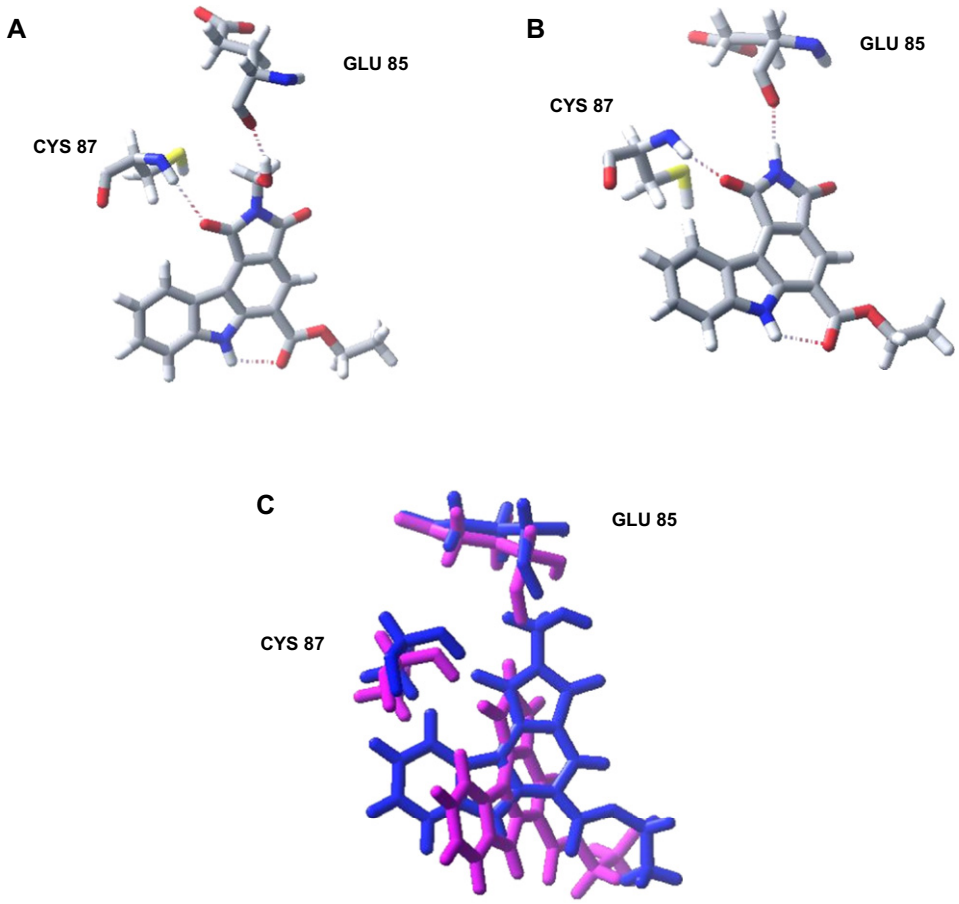


Fig. 5. Molecular modelling of compounds **13** and **15** in the ATP binding site of Chk1. 5A: compound **15**, 5B: compound **13**. 5C: superimposition of the two models: compounds **13** (in pink) and **15** (in blue). (For interpretation of the references to colours in figure legends, the reader is referred to the web version of this article.)

4. Experimental section

4.1. Chemistry

IR spectra were recorded on a Perkin–Elmer 881 spectrometer (ν in cm^{-1}). NMR spectra were performed on a Bruker AVANCE 400 (chemical shifts δ in parts per million, the following abbreviations are used: singlet (s), broad singlet (br s), doublet (d), doubled doublet (dd), triplet (t), doubled triplet (dt), multiplet (m), quadruplet (q), tertiary carbons (C tert), quaternary carbons (C quat)). Low resolution mass spectra (ESI+) and HRMS were determined on a MS Hewlett Packard engine, mass spectra (FAB+) were determined at CESAMO (Talence, France) on a high resolution Fisons Autospec-Q spectrometer. Chromatographic purifications were performed by flash silicagel Geduran SI 60 (Merck) 0.040–0.063 mm column chromatography.

4.1.1. 10H-2-Benzyloxymethyl-4-ethoxycarbonyl-5-hydroxy-1,3-dihydropyrrolo[3,4-a]carbazole-1,3-dione ***1***

To a solution of ethyl 3-oxo-3-(1*H*-indol-3-yl)propionate (250 mg, 1.08 mmol) in THF (15 mL) was added NaH (60% in oil, 85 mg, 2.16 mmol) at 0 °C. The mixture was stirred at 0 °C for 30 min, before dropwise addition to a solution of *N*-benzyloxymethyl-dibromomaleimide (490 mg, 1.31 mmol) in THF (10 mL). The mixture was stirred at 0 °C for 2 h before addition of MeOH to remove the excess of NaH. After extraction with EtOAc, the organic phase was dried over $MgSO_4$ and the solvent was removed. The solid residue was purified by flash chromatography (eluent: AcOEt/cyclohexane from 1:9 to 3:7) to give **1** (144 mg, 0.32 mmol, 30% yield) as a yellow solid.

Mp 193 °C. IR (KBr) $\nu_{C=O}$ 1697, 1725, 1760 cm^{-1}, $\nu_{NH,OH}$ 3100–3600 cm^{-1}. HRMS (ESI+) $[M+Na]^+$ calcd for $C_{25}H_{20}N_2O_6Na$ 467.1219, found 467.1243. ^1H NMR (400 MHz, DMSO-d_6): 1.40 (3H, t, $J = 7.0$ Hz), 4.45 (2H, q, $J = 7.0$ Hz), 4.65 (2H, s), 5.10 (2H, s), 7.26–7.41 (6H, m), 7.55 (1H, t, $J = 7.0$ Hz), 7.70 (1H, d, $J = 8.0$ Hz), 8.35 (1H, d, $J = 8.0$ Hz), 11.55 (1H, s, OH), 12.36 (1H, s, NH). ^{13}C NMR (100 MHz, DMSO-d_6): 13.7 (CH_3), 61.5, 66.6, 70.3 (CH_2), 105.5, 108.6, 116.7, 121.0, 127.9, 135.2, 137.7, 141.3, 155.9 (C quat), 112.1, 120.8, 122.8, 127.0, 127.5 (3C), 128.2 (2C) (C tert), 165.5, 166.6, 166.7 (C=O).

4.1.2. 10H-2-Benzyloxymethyl-5-chloro-4-ethoxycarbonyl-1,3-dihydropyrrolo[3,4-a]carbazole-1,3-dione ***2***

To a solution of **1** (40 mg, 0.090 mmol) in DMF (3 mL) was added PCl_5 (19 mg, 0.090 mmol). The mixture was refluxed for 18 h. Water was added, the precipitate was filtered

off and washed with water to give **2** (39 mg, 0.084 mmol, 94% yield) as an orange solid.

Mp > 280 °C. IR (KBr) $\nu_{C=C}$ 1612, 1642 cm^{-1}, $\nu_{C=O}$ 1710, 1774 cm^{-1}, ν_{NH} 3380 cm^{-1}. HRMS (ESI+) $[M + Na]^+$ calcd for $C_{25}H_{19}N_2O_5ClNa$ 485.0880, found 485.0898. ^1H NMR (400 MHz, DMSO-d_6): 1.43 (3H, t, $J = 7.0$ Hz), 4.52 (2H, q, $J = 7.0$ Hz), 4.67 (2H, s), 5.15 (2H, s), 7.26–7.38 (5H, m), 7.46 (1H, t, $J = 8.0$ Hz), 7.72 (1H, t, $J = 7.0$ Hz), 7.81 (1H, d, $J = 8.0$ Hz), 8.61 (1H, d, $J = 8.0$ Hz), 12.82 (1H, s, NH). Due to its insolubility, the ^{13}C NMR spectrum could not be recorded.

*4.1.3. 10H-2-Benzyloxymethyl-5-hydroxy-1,3-dihydropyrrolo[3,4-a]carbazole-1,3-dione **3***

To a solution of **1** (20 mg, 0.045 mmol) in DMF (1.5 mL) was added LiCl (10 mg, 0.22 mmol). The mixture was refluxed for 18 h. Water was added. The precipitate was filtered off and then washed with water to give **3** (16 mg, 0.043 mmol, 96% yield) as a yellow solid.

Mp > 250 °C (decomposition). IR (KBr) $\nu_{C=C}$ 1610 cm^{-1}, $\nu_{C=O}$ 1689, 1756 cm^{-1}, $\nu_{NH,OH}$ 3200–3650 cm^{-1}. HRMS (ESI+) $[M + Na]^+$ calcd for $C_{22}H_{16}N_2O_4Na$ 395.1008, found 395.1024. ^1H NMR (400 MHz, DMSO-d_6): 4.65 (2H, s), 5.12 (2H, s), 7.09 (1H, s), 7.27–7.42 (6H, m), 7.51 (1H, t, $J = 7.5$ Hz), 7.66 (1H, d, $J = 8.0$ Hz), 8.28 (1H, d, $J = 8.0$ Hz), 11.76 (1H, s), 12.16 (1H, s, NH). ^{13}C NMR (100 MHz, DMSO-d_6): 66.4, 70.2 (CH_2O), 100.1, 111.8, 120.4, 122.6, 126.4, 127.5 (3C), 128.2 (2C) (C tert), 104.0, 115.8, 121.0, 131.4, 135.6, 137.8, 141.0, 159.4 (C quat), 167.3, 168.5 (C=O).

*4.1.4. 2H,10H-5-Hydroxy-1,3-dihydropyrrolo[3,4-a]-carbazole-1,3-dione **4***

A solution of **3** (40 mg, 0.107 mmol) in THF/MeOH (4/1 mL) was hydrogenated (1 bar) in the presence of 20% $Pd(OH)_2/C$ (206 mg) for 3 h at room temperature. After filtration over Celite, the solvents were removed. The solid residue was dissolved into THF/NH_4OH (11/23 mL) and the mixture was stirred at room temperature for 24 h. After evaporation, AcOEt was added to the residue and the mixture was filtered off to give **4** (9 mg, 0.036 mmol, 33% yield) as a yellow solid.

Mp > 280 °C. IR (KBr) $\nu_{C=C}$ 1617, 1636 cm^{-1}, $\nu_{C=O}$ 1704, 1746 cm^{-1}, $\nu_{NH,OH}$ 3416, 3479 cm^{-1}. HRMS (FAB+) $[M + H]^+$ calcd for $C_{14}H_9N_2O_3$ 253.0613, found 253.0620. ^1H NMR (400 MHz, DMSO-d_6): 6.99 (1H, s), 7.28 (1H, t, $J = 8.0$ Hz), 7.47 (1H, t, $J = 8.0$ Hz), 7.62 (1H, d, $J = 8.0$ Hz), 8.26 (1H, d, $J = 8.0$ Hz), 10.88 (1H, s, NH), 11.56 (1H, s, NH), 12.01 (1H, s, NH). ^{13}C NMR (100 MHz, DMSO-d_6): 99.5, 111.7, 120.1, 122.5, 126.1 (C tert arom), 105.2, 115.4, 121.1, 132.8, 135.3, 140.9, 159.0 (C quat arom), 169.2, 170.1 (CO).

*4.1.5. 10H-4-Ethyloxycarbonyl-2-hydroxymethyl-5-hydroxy-1,3-dihydropyrrolo[3,4-a]carbazole-1,3-dione **5***

A solution of compound **1** (60 mg, 0.14 mmol) in THF/MeOH (4.0/1.0 mL) was hydrogenated (1 bar) in the presence of $Pd(OH)_2$ (66 mg) for 5 h at room temperature. After filtration over Celite, the solvents were removed and the residue was purified by flash chromatography (eluent: EtOAc/cyclohexane 5:5) to give **5** (39 mg, 0.11 mmol, 80% yield) as a yellow solid.

Mp > 280 °C. IR (KBr) $\nu_{C=C}$ 1619, 1644 cm^{-1}, $\nu_{C=O}$ 1706, 1720, 1750 cm^{-1}, $\nu_{NH,OH}$ 3300–3650 cm^{-1}. HRMS (ESI+) $[M + H]^+$ calcd for $C_{18}H_{15}N_2O_6$ 355.0930, found 355.0939. ^1H NMR (400 MHz, DMSO-d_6): 1.40 (3H, t, $J = 7.0$ Hz), 4.44 (2H, q, $J = 7.0$ Hz), 5.00 (2H, s), 6.36 (1H, br s, OH), 7.34 (1H, t, $J = 7.5$ Hz), 7.54 (1H, t, $J = 8.0$ Hz), 7.70 (1H, d, $J = 8.0$ Hz), 8.35 (1H, d, $J = 8.0$ Hz), 11.58 (1H, br s), 12.30 (1H, s). ^{13}C NMR (100 MHz, DMSO-d_6): 13.8 (CH_3), 59.9, 61.4 (CH_2–O), 105.4, 108.7, 116.6, 121.1, 128.2, 135.2, 141.3, 156.1 (C quat), 112.1, 120.7, 122.8, 126.9 (C tert), 165.6, 166.5, 166.6 (CO).

*4.1.6. 2H,10H-4-Ethoxycarbonyl-5-hydroxy-1,3-dihydropyrrolo[3,4-a]carbazole-1,3-dione **6***

To a solution of **5** (28 mg, 0.079 mmol) in THF (8 mL) was added 28% aqueous NH_4OH (17 mL). The mixture was stirred for 24 h at room temperature. After evaporation, water was added to the residue. After filtration, the solid residue was washed with water to give **6** (10 mg, 0.031 mmol, 39% yield) as a yellow solid.

Mp > 280 °C. IR (KBr) $\nu_{C=C}$ 1619, 1645 cm^{-1}, $\nu_{C=O}$ 1662, 1700, 1725 cm^{-1}, $\nu_{NH,OH}$ 3300–3600 cm^{-1}. HRMS (ESI+) $[M + Na]^+$ calcd for $C_{17}H_{12}N_2O_5Na$ 347.0644, found 347.0661. ^1H NMR (400 MHz, DMSO-d_6): 1.38 (3H, t, $J = 7.0$ Hz), 4.42 (2H, q, $J = 7.0$ Hz), 7.33 (1H, dt, $J_1 = 8.0$ Hz, $J_2 = 1.0$ Hz), 7.53 (1H, dt, $J_1 = 8.0$ Hz, $J_2 = 1.0$ Hz), 7.68 (1H, d, $J = 8.0$ Hz), 8.32 (1H, d, $J = 8.0$ Hz), 11.05 (1H, s), 11.39 (1H, s), 12.24 (1H, s). ^{13}C NMR (100 MHz, DMSO-d_6): 13.2 (CH_3), 60.8 (CH_2), 111.5, 120.0, 122.2, 126.2 (C tert arom), 106.2, 107.6, 115.8, 120.5, 128.7, 134.5, 140.7, 155.2 (C quat arom), 165.2, 167.8, 167.9 (CO).

*4.1.7. Ethyl 2-hydroxy-2-(N-phenylsulfonyl-indol-3-yl)-propanoate **8***

A 3 M solution of MeMgBr in Et_2O (0.68 mmol, 249 μL) was added dropwise to a solution of **7** (110 mg, 0.31 mmol) in THF (4 mL). The mixture was stirred at −78 °C for 6.5 h. The mixture was allowed to reach room temperature, and then water was added. After extraction with EtOAc, the organic phase was dried over $MgSO_4$. The solvent was removed and the residue was purified by flash chromatography (eluent: cyclohexane/EtOAc 7:3) to give **8** (90 mg, 0.24 mmol, 77% yield) as a yellow oil.

IR (NaCl film) $\nu_{C=O}$ 1725 cm^{-1}, ν_{OH} 3501 cm^{-1}. HRMS (ESI+) $[M + Na]^+$ calcd for $C_{19}H_{19}NO_5NaS$ 396.0882, found 396.0882. ^1H NMR (400 MHz, DMSO-d_6): 1.07 (3H, t, $J = 7.0$ Hz), 1.78 (3H, s), 4.08 (1H, q, $J = 7.0$ Hz), 6.14 (1H, s, OH), 7.27 (1H, dt, $J_1 = 8.0$ Hz, $J_2 = 1.0$ Hz), 7.37 (1H, dt, $J_1 = 7.5$ Hz, $J_2 = 1.0$ Hz), 7.63 (2H, t, $J = 8.0$ Hz), 7.71–7.74 (2H, m), 7.75 (1H, s), 7.96 (1H, d, $J = 8.0$ Hz), 8.03 (1H, d, $J = 9.0$ Hz), 8.03 (1H, d, $J = 7.0$ Hz). ^{13}C NMR (100 MHz, DMSO-d_6): 13.8, 26.1 (CH_3), 60.8 (CH_2O),

113.1, 121.8, 123.2 (2C), 124.7, 126.7 (2C), 129.8 (2C), 134.6 (C tert), 72.1, 126.1, 128.2, 136.1 (C quat), 173.5 (C=O).

4.1.8. Ethyl 2-(N-phenylsulfonyl-indol-3-yl)prop-2-enoate **9**

A solution of **8** (100 mg, 0.27 mmol) in toluene (4 mL) was refluxed for 1.5 h in a Dean–Stark apparatus in the presence of catalytic amounts of *p*-TsOH (5 mg, 0.03 mmol). After cooling to room temperature, and extraction with EtOAc, the organic phase was washed with water and then dried over $MgSO_4$. Compound **9**, a brown oil, was isolated as a mixture of two conformers (91 mg, 0.26 mmol, 95% yield).

IR (NaCl film) $\nu_{C=O}$ 1723 cm^{-1}. Mass (APCI+) $[M+H]^+$ 356. ^1H NMR (400 MHz, $CDCl_3$) of the major conformer: 1.24 (3H, t, $J_1 = 7.0$ Hz, $J_2 = 2.0$ Hz), 4.21 (2H, q, $J_1 = 7.0$ Hz, $J_2 = 2.0$ Hz), 6.02 (1H, s), 6.45 (1H, s), 7.15 (1H, dt, $J_1 = 8.0$ Hz, $J_2 = 1.0$ Hz), 7.23 (1H, dt, $J_1 = 8.0$ Hz, $J_2 = 1.0$ Hz), 7.31 (2H, dt, $J_1 = 8.0$ Hz, $J_2 = 1.0$ Hz), 7.39 (1H, dd, $J_1 = 7.5$ Hz, $J_2 = 2.0$ Hz), 7.50 (1H, d, $J = 8.0$ Hz), 7.81 (2H, d, $J_1 = 7.5$ Hz, $J_2 = 1.0$ Hz), 7.83 (1H, s), 7.93 (1H, d, $J = 8.0$ Hz). ^{13}C NMR (100 MHz, $CDCl_3$) of the major conformer: 14.2 (CH_3), 61.3 (CH_2O), 113.7, 120.4, 123.6, 124.8, 126.9, 127.0 (2C), 129.3 (2C), 133.9 (C tert), 126.7 (=CH_2), 78.6, 117.9, 120.2, 132.7, 138.1 (C quat). The signal of the carbonyl was not observed.

4.1.9. 2H-5-Ethoxycarbonyl-10-phenylsulfonyl-1,2,3,4-tetrahydropyrrolo[3,4-a]carbazole-1,3-dione **10**

A mixture of **9** (50 mg, 0.14 mmol) and maleimide (16 mg, 0.16 mmol) in toluene (4 mL) was refluxed for 48 h. The solvent was removed and the residue was purified by flash chromatography (eluent: cyclohexane/EtOAc from 7:3 to 5:5) to give **10** (21 mg, 0.046 mmol, 33% yield) as a pale yellow oil.

IR (NaCl film) $\nu_{C=O}$ 1724, 1782 cm^{-1}, ν_{NH} 3441 cm^{-1}. HRMS (ESI+) $[M+H]^+$ calcd for $C_{23}H_{21}N_2O_6S$ 453.1120, found 453.1132. ^1H NMR (400 MHz, $CDCl_3$): 1.24 (3H, t, $J = 7.0$ Hz), 2.11 (1H, m), 3.19 (1H, ddd, $J_1 = 9.0$ Hz, $J_2 = 7.0$ Hz, $J_2 = 1.0$ Hz), 3.57 (1H, dd, $J_1 = 16.0$ Hz, $J_2 = 1.0$ Hz), 4.07 (1H, dd, $J_1 = 9.0$ Hz, $J_2 = 7.0$ Hz), 4.17 (2H, dq, $J_1 = 7.0$ Hz, $J_2 = 3.0$ Hz), 4.42 (1H, dd, $J_1 = 7.0$ Hz, $J_2 = 1.0$ Hz), 7.00 (1H, t, $J = 7.0$ Hz), 7.33 (1H, t, $J = 8.0$ Hz), 7.38 (2H, t, $J = 8.0$ Hz), 7.50 (1H, t, $J = 8.0$ Hz), 7.71 (1H, d, $J = 8.0$ Hz), 7.79 (2H, d, $J = 8.0$ Hz), 8.02 (1H, s, NH), 8.45 (1H, d, $J = 8.0$ Hz). ^{13}C NMR (100 MHz, $CDCl_3$): 14.1 (CH_3), 27.4, 61.3 (CH_2), 37.5, 43.5, 63.5 (CH), 60.4 (C quat), 115.0, 124.2, 127.2 (2C), 128.4 (2C), 129.4, 132.8, 133.9 (C tert arom), 118.4, 124.6, 136.7, 144.6, 147.2 (C quat arom), 165.2, 173.5, 177.5 (C=O).

4.1.10. 2H-5-Ethoxycarbonyl-10-phenylsulfonyl-1,3-dihydro-pyrrolo[3,4-a]carbazole-1,3-dione **11**

A mixture of **10** (90 mg, 0.20 mmol) and DDQ (100 mg, 0.40 mmol) in dioxane (6 mL) was refluxed for three days. The solvent was removed and the residue was purified by flash chromatography (eluent: cyclohexane/EtOAc 7:3) to give **11** (18 mg, 0.04 mmol, 20% yield) as a yellow solid.

Mp 260–261 °C. IR (KBr) ν_{SO_2Ph} 1296, 1380, $\nu_{C=O}$ 1704, 1724 cm^{-1}, ν_{NH} 3329–3614 cm^{-1}. Mass (APCI+) $[M+Na]^+$ 471, $[M+K]^+$ 487. ^1H NMR (400 MHz, DMSO-d_6): 1.43 (3H, t, $J = 7.0$ Hz), 4.51 (2H, q, $J = 7.0$ Hz), 7.40 (2H, t, $J = 8.0$ Hz), 7.44 (1H, t, $J = 8.0$ Hz), 7.52 (2H, d, $J = 8.0$ Hz), 7.59 (1H, t, $J = 7.0$ Hz), 7.64 (1H, t, $J = 7.0$ Hz), 8.01 (1H, d, $J = 8.0$ Hz), 8.23 (1H, s), 8.34 (1H, d, $J = 8.0$ Hz), 11.63 (1H, s, NH). ^{13}C NMR (100 MHz, DMSO-d_6): 13.9 (CH_3), 62.2 (CH_2O), 117.4, 120.8, 124.9, 125.7, 126.8 (2C), 129.1 (2C), 130.0, 134.4 (C tert), 124.7, 124.8, 130.4, 132.1, 132.8, 134.7, 135.8, 141.9 (C quat), 165.3, 165.8, 167.8 (C=O).

4.1.11. 2H,10H-5-Ethoxycarbonyl-1,3-dihydropyrrolo-[3,4-a]carbazole-1,3-dione **12**

A 1 M solution of TBAF in THF (1.40 mL) was added dropwise to a solution of **10** (40 mg, 0.09 mmol) in THF (4 mL). The mixture was refluxed for 48 h. The solvent was removed, water was added to the residue and the mixture was filtered off. The solid residue was washed with water to give **12** (23 mg, 0.075 mmol, 83% yield) as a yellow solid.

Mp > 220 °C (degradation). IR (KBr) $\nu_{C=O}$ 1704, 1767 cm^{-1}, ν_{NH} 3355 cm^{-1}. HRMS (ESI+) $[M+H]^+$ calcd for $C_{17}H_{13}N_2O_4$ 309.0875, found 309.0888. ^1H NMR (400 MHz, DMSO-d_6): 1.44 (3H, t, $J = 7.0$ Hz), 4.52 (2H, q, $J = 7.0$ Hz), 7.29 (1H, dt, $J_1 = 8.0$ Hz, $J_2 = 1.0$ Hz), 7.59 (1H, dt, $J_1 = 8.0$ Hz, $J_2 = 1.0$ Hz), 7.72 (1H, d, $J = 8.0$ Hz), 7.97 (1H, s), 8.64 (1H, d, $J = 8.0$ Hz), 11.42 (1H, s, NH), 12.48 (1H, s, NH). ^{13}C NMR (100 MHz, DMSO-d_6): 14.0 (CH_3), 61.7 (CH_2), 112.4, 114.1, 120.3, 124.9, 128.4 (C tert arom), 116.2, 119.7, 127.2, 128.7, 129.0, 133.9, 143.3 (C quat arom), 166.3, 168.9, 169.3 (C=O).

4.1.12. 2H,6H-5-Ethoxycarbonyl-1,3-dihydropyrrolo-[3,4-c]carbazole-1,3-dione **13**

A mixture of 3-(indol-3-yl)maleimide (250 mg, 1.18 mmol) and ethyl acrylate (16.6 mmol, 1.75 mL) in toluene (25 mL) was refluxed for 36 h. After evaporation, the solid residue was purified by flash chromatography (eluent: EtOAc/cyclohexane 6:4) to give the Diels–Alder cycloadduct as a mixture of isomers (orange solid, 258 mg, 0.83 mmol, 70% yield).

The mixture of isomers (140 mg, 0.45 mmol) and DDQ (224 mg, 0.90 mmol) in dioxane (15 mL) was refluxed for 24 h. After filtration, the filtrate was evaporated and the residue was purified by flash chromatography (eluent: cyclohexane/EtOAc 5:5) to give **13** (88 mg, 0.28 mmol, 64% yield) as a yellow solid.

Mp > 300 °C. IR (KBr) $\nu_{C=O}$ 1686, 1716, 1765 cm^{-1}, ν_{N-H} 3236, 3398 cm^{-1}. HRMS (ESI+) $[M+H]^+$ calcd for $C_{17}H_{13}N_2O_4$ 309.0875, found 309.0888. ^1H NMR (400 MHz, DMSO): 1.48 (3H, t, $J = 7.0$ Hz), 4.52 (2H, q, $J = 7.0$ Hz), 7.39 (1H, dt, $J_1 = 8.0$ Hz, $J_2 = 0.5$ Hz), 7.63 (1H, dt, $J_1 = 8.0$ Hz, $J_2 = 1.0$ Hz), 7.85 (1H, d, $J = 8.0$ Hz), 8.23 (1H, s), 8.85 (1H, d, $J = 8.0$ Hz), 11.45 (1H, s, NH), 12.05 (1H, s, NH). ^{13}C NMR (100 MHz, DMSO): 14.2 (CH_3), 61.3 (CH_2), 112.8, 120.2, 121.0, 124.6, 128.7 (C tert), 114.7, 119.4,

120.6, 122.7, 129.7, 142.1, 143.0 (C quat), 164.4, 169.3, 169.4 (C=O).

*4.1.13. 6H-2-N-Benzyloxymethyl-5-ethoxycarbonyl-pyrrolo[3,4-c]carbazole-1,3-dione **14** and 6H-2-N-hydroxymethyl-5-ethoxycarbonyl-pyrrolo-[3,4-c]carbazole-1,3-dione **15***

To a mixture of **13** (50 mg, 0.16 mmol) in acetone (1 mL) was added diisopropylethylamine (70 μL, 0.40 mmol). The mixture was stirred for 15 min at room temperature before dropwise addition of benzyloxymethylchloride (45 μL, 0.32 mmol). The mixture was stirred at room temperature for 1 h, and then water was added. After extraction with EtOAc, the organic phase was dried over $MgSO_4$, the solvent was removed and the residue was purified by flash chromatography (eluent: EtOAc/cyclohexane from 3:7 to 1:0). Two compounds were isolated: **14** (52 mg, 0.12 mmol, 76% yield) and **15** (9 mg, 0.028 mmol, 17% yield) as yellow solids.

*4.1.13.1. Compound **14***. Mp 153 °C. IR (KBr) $\nu_{C=C}$ 1607 cm^{-1}, $\nu_{C=O}$ 1698, 1708, 1761 cm^{-1}, ν_{NH} 3355 cm^{-1}. HRMS (ESI+) $[M+Na]^+$ calcd for $C_{25}H_{20}N_2O_5Na$ 451.1270, found 451.1284. ^1H NMR (400 MHz, DMSO-d_6): 1.50 (3H, t, $J = 7.0$ Hz), 4.56 (2H, q, $J = 7.0$ Hz), 4.69 (2H, s), 5.21 (2H, s), 7.24–7.40 (5H, m), 7.45 (1H, t, $J = 7.5$ Hz), 7.68 (1H, t, $J = 8.0$ Hz), 7.91 (1H, d, $J = 8.0$ Hz), 8.36 (1H, d, $J = 2.0$ Hz), 8.91 (1H, d, $J = 8.0$ Hz), 12.21 (1H, s). ^{13}C NMR (100 MHz, DMSO-d_6): 14.2 (CH_3), 61.5, 66.9, 70.5 (CH_2O), 113.0, 120.7, 121.2, 124.6, 127.5 (2C), 128.2 (2C), 129.1 (C tert), 115.2, 119.3, 121.0, 121.4, 128.4, 137.7, 142.3, 143.2 (C quat), 164.8, 167.8, 167.9 (C=O).

*4.1.13.2. Compound **15***. Mp > 230 °C (decomposition). IR (KBr) $\nu_{C=C}$1608 cm^{-1}, $\nu_{C=O}$ 1687, 1708, 1763 cm^{-1}, $\nu_{NH,OH}$ 3362, 3439 cm^{-1}. HRMS (ESI+) $[M+Na]^+$ calcd for $C_{18}H_{14}N_2O_5Na$ 361.0800, found 361.0805. ^1H NMR (400 MHz, DMSO-d_6): 1.49 (3H, t, $J = 7.0$ Hz), 4.53 (2H, q, $J = 7.0$ Hz), 5.03 (2H, d, $J = 7.0$ Hz), 6.45 (1H, t, $J = 7.0$ Hz, OH), 7.40 (1H, t, $J = 7.5$ Hz), 7.64 (1H, t, $J = 7.5$ Hz), 7.86 (1H, d, $J = 8.0$ Hz), 8.23 (1H, s), 8.82 (1H, d, $J = 8.0$ Hz), 12.09 (1H, s, NH). ^{13}C NMR (100 MHz, DMSO-d_6): 14.1 (CH_3), 60.1, 61.4 (CH_2O), 112.9, 120.4, 121.1, 124.4, 128.9 (C tert), 115.0, 119.2, 120.8, 121.3, 128.3, 124.2, 143.1 (C quat), 164.7, 167.3, 167.4 (C=O).

*4.1.14. 5H,7H-1,3,3a,3b,4,6,6a,11c-Octahydrofuro-[3,4-c]pyrrolo[3,4-a]carbazol-1,3,4,6-tetraone **16***

A solution of 3-(indol-3-yl)furane-2,5-dione (150 mg, 0.70 mmol) and maleimide (82 mg, 0.84 mmol) in xylene (15 mL) was refluxed for five days. After filtration, the solid residue was washed with water and dried to give **16** (195 mg, 0.63 mmol, 90% yield) as an orange solid.

Mp 220–222 °C. IR (KBr) $\nu_{C=O}$ 1719, 1782 cm^{-1}, ν_{NH} 3411 cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6): 4.03 (1H, t, $J = 7.5$ Hz), 4.21 (1H, t, $J = 8.0$ Hz), 4.34 (1H, d, $J = 7.5$ Hz), 4.78 (1H, d, $J = 8.0$ Hz), 7.06 (1H, m), 7.15 (1H, t, $J = 8.0$ Hz), 7.42 (1H, d, $J = 8.0$ Hz), 7.64 (1H, d, $J = 8.0$ Hz), 11.44 (1H, s, NH), 11.54 (1H, s, NH). ^{13}C NMR (100 MHz, DMSO-d_6): 39.3, 40.2, 40.5 (2C) (CH), 100.7, 125.2, 128.2, 137.0 (C quat), 111.7, 119.2, 119.5, 122.2 (C tert), 170.4, 171.2, 175.6, 177.2 (C=O).

*4.1.15. 5H,7H-1,3,4,6-Tetrahydrofuro[3,4-c]pyrrolo-[3,4-a]carbazol-1,3,4,6-tetraone **17***

A solution of anhydride **16** (196 mg, 0.63 mmol) and DDQ (575 mg, 1.39 mmol) in dioxane (10 mL) was refluxed for three days. After removal of the solvent, water was added to the residue. The mixture was filtered off and the solid residue was washed with water and EtOAc to give **17** (166 mg, 0.54 mmol, 86% yield) as a yellow solid.

Mp > 300 °C. IR (KBr) $\nu_{C=O}$ 1701, 1776, 1847 cm^{-1}, ν_{NH} 3247, 3368 cm^{-1}. HRMS (ESI+) $[M+H]^+$ calcd for $C_{16}H_7N_2O_5$ 307.0355, found 307.0360. ^1H NMR (400 MHz, DMSO-d_6): 7.51 (1H, t, $J = 7.0$ Hz), 7.75 (1H, t, $J = 7.0$ Hz), 7.82 (1H, d, $J = 7.5$ Hz), 8.83 (1H, d, $J = 7.5$ Hz), 11.78 (1H, s, NH), 13.09 (1H, s, NH). Due to its insolubility, the ^{13}C NMR spectrum could not be recorded.

*4.1.16. 5H,6H,8H-1,3,4,7-Tetrahydrofurano-[3,4-c]pyridazino[3,4-a]carbazole **18** and 2H,5H,6H,8H-2-amino-1,3,4,7-tetrahydropyridazino[3,4-a]pyrrolo-[3,4-c]carbazole-1,3,4,7-tetraone **19***

A solution of **17** (150 mg, 0.49 mmol) in hydrazine hydrate (5 mL) was stirred at 60 °C for 24 h. Water (150 mL) was added, then 12 N HCl (20 mL) was added dropwise. After stirring for 30 min at 0 °C, the precipitate was filtered off, and then washed with water and EtOAc to give **19** (60 mg, 0.18 mmol, 37% yield) as an orange solid. The filtrate was evaporated to give **18** (5 mg, 0.016 mmol, 3% yield) as a yellow solid.

*4.1.16.1. Compound **18***. Mp > 280 °C. IR (KBr) $\nu_{C=O}$ 1720, 1782 cm^{-1}, ν_{NH} 3411 cm^{-1}. Mass (ESI+) $[M+H]^+$ 322. ^1H NMR (400 MHz, DMSO-d_6): 7.35 (1H, dt, $J_1 = 7.0$ Hz, $J_2 = 1.0$ Hz), 7.61 (1H, dt, $J_1 = 8.0$ Hz, $J_2 = 1.0$ Hz), 7.99 (1H, d, $J = 8.0$ Hz), 8.16 (1H, d, $J = 8.0$ Hz), 11.90–12.60 (3H, br s, NH).

*4.1.16.2. Compound **19***. Mp > 300 °C. IR (KBr) $\nu_{C=O}$ 1736 cm^{-1}, $\nu_{N=N}$ 2173 cm^{-1}, ν_{NH} 3445 cm^{-1}. HRMS (FAB+) $[M+H]^+$ calcd for $C_{16}H_{10}N_5O_4$ 336.0732, found 336.0730. ^1H NMR (400 MHz, DMSO-d_6): 5.30 (2H, s, NH_2), 7.52 (1H, t, $J = 7.5$ Hz), 7.73 (1H, t, $J = 7.5$ Hz), 8.07 (1H, d, $J = 8.0$ Hz), 8.99 (1H, d, $J = 8.0$ Hz), 12.36 (1H, s, NH), 12.46 (1H, s, NH), 12.90 (1H, s, NH). ^{13}C NMR (100 MHz, DMSO-d_6): 113.2, 115.1, 118.8, 119.1, 119.2, 128.4, 141.4, 142.4 (C quat arom), 113.7, 121.9, 124.6, 129.3 (C tert arom), 148.5, 158.1, 166.5, 171.5 (C=O).

4.2. Kinase inhibition assays

Chk1inhibition assays: Human Chk1 full-length enzyme with an N-terminal GST sequence was either purchased

from Upstate Biochemicals (No. 14-346) or purified from extracts of Sf9 cells infected with a baculovirus encoding GST-Chk1. Assays for compound testing were based upon the method described by Davies et al. [26].

Src inhibition assays: Inhibitors were diluted with a Tecan Evo150 robot. The kinase assay was performed with 4 μL of inhibitor (10% dimethylsulfoxide (DMSO)), 10 μL of kinase assay buffer 4× concentrated (80 mM $MgCl_2$, 200 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 0.4 mM ethylenediaminetetraacetic acid (EDTA), 2 mM DL-dithiothreitol (DTT)), 10 μL substrate peptide (KVEKIGEGYYGVVYK, 370 nM) and 6 μL Src kinase (stock GTP purified diluted with 1× kinase assay buffer to 200 nM). 10 μL co-substrate (40 μM ATP with 0.2 μCi P^{33}-γ-ATP) was added with a Precision 2000 (Biotek Robotic). The assay was incubated 20 min at 30 °C then stopped by adding 200 μL 0.85% orthophosphoric acid, then transferred to a phosphocellulose filter microplate (Whatman - P81). The plate was washed three times with 200 μL 0.85% orthophosphoric acid and dried with 200 μL acetone. The remaining activity was measured on a Topcount with 25 μL scintillation solution (Packard UltimaGold).

4.3. Molecular modelling

All molecular mechanics calculations were performed by the Macromodel [27] molecular modelling software. We used as model the complex structure of Chk1/staurosporine [10] downloaded from the Protein Data Bank (1NVR file).

Energy minimisation was done with AMBER force field [28,29] using the Truncated Newton Conjugate Gradient method (TNCG).

4.4. Growth inhibition assays

Tumor cells were provided by American Type Culture Collection (Frederik, MD, USA). They were cultivated in RPMI 1640 medium (Life Science technologies, Cergy-Pontoise, France) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, and 10 mM HEPES buffer (pH = 7.4). Cytotoxicity was measured by the microculture tetrazolium assay as described [30]. Cells were continuously exposed to graded concentrations of the compounds for four doubling times, then 15 μL of 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide were added to each well and the plates were incubated for 4 h at 37 °C. The medium was then aspirated and the formazan solubilized by 100 μL of DMSO. Results are expressed as IC_{50}, concentration which reduced by 50% the optical density of treated cells with respect to untreated controls.

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