

Discovery of pyrido[3,4-*g*]quinazoline derivatives as CMGC family protein kinase inhibitors: Design, synthesis, inhibitory potency and X-ray co-crystal structure

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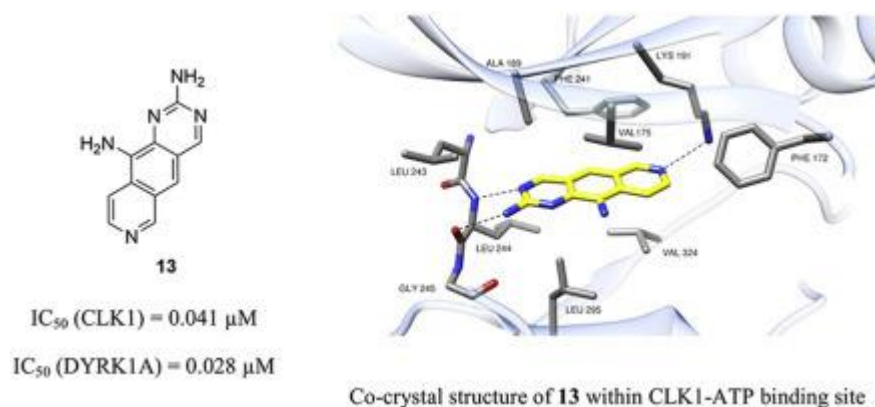
Highlights

- New pyrido[3,4-*g*]quinazoline derivatives were synthesized.
- Compounds **12** and **13** are nanomolar CLK1 and/or DYRK1A inhibitors.
- Co-crystal structures of **13** and **14** in CLK1 ATP-binding site were obtained.

Abstract

The design and synthesis of new pyrido[3,4-*g*]quinazoline derivatives is described as well as their protein kinase inhibitory potencies toward five CMGC family members (CDK5, CK1, GSK3, CLK1 and DYRK1A). The interest for this original tricyclic heteroaromatic scaffold as modulators of CLK1/DYRK1A activity was validated by nanomolar potencies (compounds **12** and **13**). CLK1 co-crystal structures with two inhibitors revealed the binding mode of these compounds within the ATP-binding pocket.

Graphical abstract



Keywords

- Pyrido[3,4-*g*]quinazoline;

- Kinase inhibitors;
- Ser/Thr kinases;
- CMGC family;
- CLK1 binding mode

1. Introduction

Alzheimer's disease (AD) is expanding dramatically in association with population ageing. This neurodegenerative disorder is characterized by the apparition of intracellular neurofibrillary tangles containing microtubule associated Tau proteins and extracellular amyloid plaques that accumulate in brain. The control of microtubule stabilization is mediated by Tau interaction with tubulin and phosphorylation; abnormal phosphorylation of Tau could lead to neuronal cytoskeleton disruption. Additionally, increased phosphorylation of Tau leads to its aggregation into filaments that could be responsible for neuronal death [1] and [2]. Various protein kinases participate in the regulation of Tau by site-specific phosphorylation. CDK5, CK1, GSK3 and DYRK1A are involved in the formation of neurofibrillary tangles by abnormal Tau phosphorylation on AD-specific sites leading to the formation of filaments [3], [4], [5], [6] and [7]. Moreover, CLK1 and DYRK1A participate in alternative pre-mRNA splicing, a physiological process that is altered in AD [8] and [9]. Due to the important physiological functions of these kinases, the development of multi-target directed ligands (MTDLs), inhibitors that could target more than one of them, is of high interest and may allow the development of new biological tools/therapies to better understand/treat AD [10].

Meridianins A–G (Fig. 1A), indolic derivatives substituted by a 2-aminopyrimidine ring, are natural products isolated from *Aplidium meridianum* ascidiae [11], [12] and [13]. Recently, we described the synthesis of diversely substituted meridianin G analogues. In order to identify new tools to study AD, these compounds were evaluated on a panel of five AD-relevant members of the Ser/Thr kinases CMGC family (CDK5, CK1, GSK3, CLK1, DYRK1A) (Fig. 1B) [14], [15], [16] and [17].

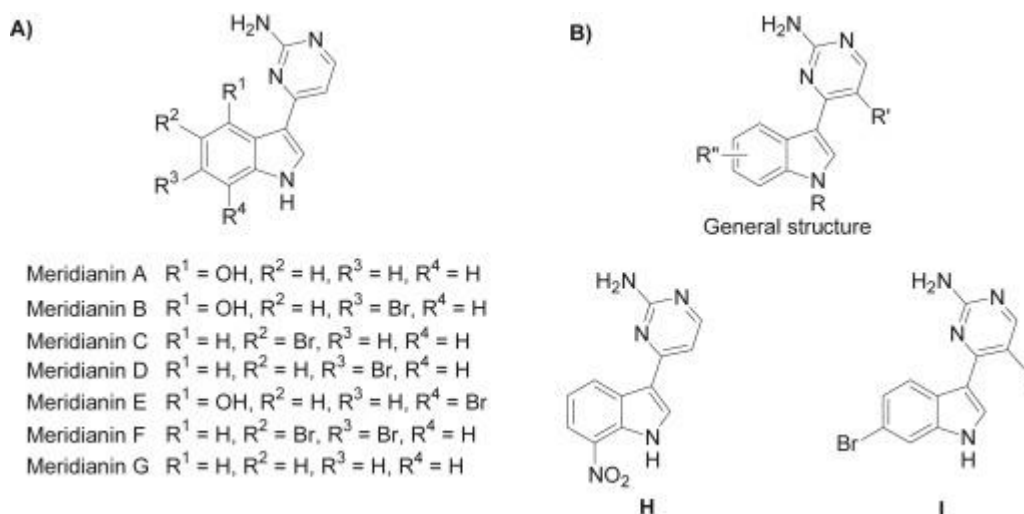


Fig. 1.

A) Chemical structure of meridianins A–G, B) General structure of meridianin analogues and two DYRK1A/CLK1 inhibitors (**H**, **I**) described by our group.

The structure–activity relationship study performed on meridianin derivatives showed that the introduction of a bromine atom or a nitro group at the 6- or 7-position of the indolic moiety could result in DYRK1A/CLK1 inhibitors with nanomolar potencies (Fig. 1B, compounds **H**, **I**). Because of these interesting results, we decided to extend the structure–activity relationship study in this aminopyrimidine series. The putative binding mode between the ATP binding site of CLK1/DYRK1A and most active compounds of the series revealed that the aminopyrimidine moiety was oriented toward the bottom of the ATP-binding pocket, establishing two hydrogen bonds with the hinge [17]. In order to develop a second generation of inhibitors, we first decided to restrict the conformation between the aminopyrimidine and indole moieties. For this purpose these rings were connected by an additional phenyl ring. Next, we noticed that the 3-aminopyrimidinylindole chemical scaffold could be simplified by removing the pyrrole moiety that is not directly involved in the molecular interaction within the ATP-binding pocket. Finally, a pyridine moiety was introduced to reinforce the H-bonding network within the ATP-binding site (Fig. 2).

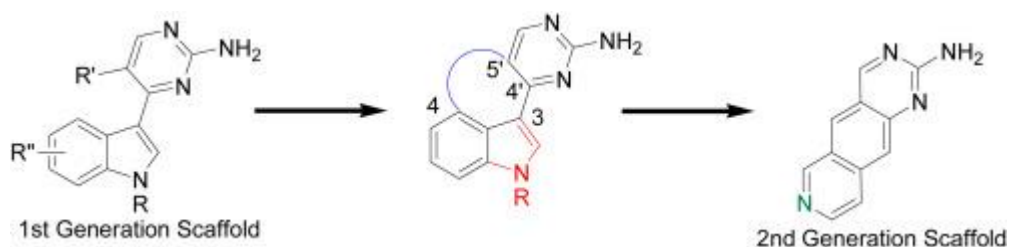


Fig. 2.

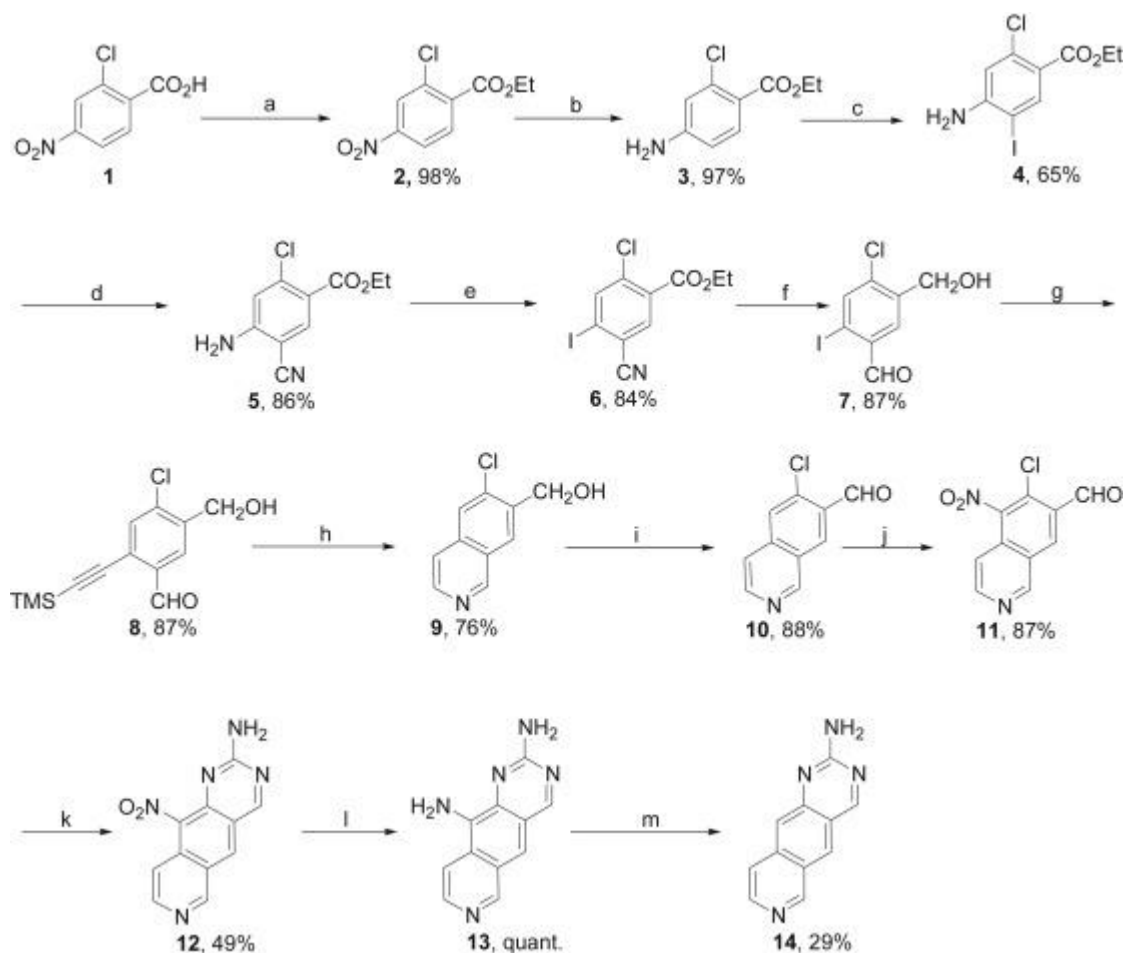
Design of the second generation scaffold.

To validate this model, the synthesis of this novel pyrido[3,4-*g*]quinazoline series was undertaken. The inhibitory potency of new compounds on a panel of five kinases (CDK5/p25, CK1 δ/ϵ , GSK-3 α/β , DYRK1A and CLK1) was examined. To interact with targeted kinases, potential inhibitors should cross the blood–brain barrier (BBB) therefore physical properties that influence BBB permeability were also calculated. Finally the binding mode of this series within the CLK1 ATP-binding pocket was elucidated by X-ray crystallography.

2. Results and discussion

The preparation of the targeted molecules (Scheme 1) starts from commercially available 2-chloro-4-nitrobenzoic acid **1** that was first esterified using ethanol in the presence of sulfuric acid to give **2** in 98% yield. The reduction of the nitro group [18] of **2** led to amino derivative **3** that was iodinated by treatment with iodine and silver sulfate [19] to give **4**, as the major regioisomer, in 65% yield. The CuCN-mediated cyanation of **4** was performed in good yield utilizing a modified Rosenmund-von Braun procedure in the presence of l-proline according to Wang *et al.* [20]. Next, the amino group of **5** was substituted using a Sandmeyer type reaction to give its iodo analogue **6** in 84% yield [21]. The treatment of **6** by DIBAL-H led to **7** in 87% yield by ester and nitrile reduction [22]. Then, Sonogashira cross-coupling reaction of **7** with TMS-acetylene gave compound **8** in 87% yield [23] and [24]. The isoquinoline moiety was formed under microwave irradiation of **8** in the presence of ammonia in methanol

[25]. Finally, oxidation of **9** using MnO₂ led to 6-chloroisoquinoline-7-carbaldehyde **10** in 88% yield (Scheme 1) [26].



Scheme 1.

Synthesis of compounds **12–14**. Reagents and conditions: (a) EtOH, H₂SO₄ (b) Fe, NH₄Cl, *i*PrOH/H₂O (c) I₂, Ag₂SO₄, EtOH (d) CuCN, *l*-proline, DMF (e) Isoamylnitrite, CH₂I₂ (f) DIBAL-H, toluene (g) TMS-acetylene, PdCl₂(PPh₃)₂, CuI, Et₃N (h) NH₃, MeOH, μ w (i) MnO₂, CHCl₃ (j) HNO₃, H₂SO₄ (k) Guanidine. H₂CO₃, DMF (l) H₂, Pd/C, CH₂Cl₂/MeOH (m) NaNO₂, H₃PO₂, HCl/H₂O.

The formation of the aminopyrimidine part was initially envisaged by reacting chloroaldehyde **10** with a guanidine salt. However, under the conditions tested, we never managed to get the expected aminopyrimidine. Therefore, in order to get a more activated product for the cyclization, a nitro group was regioselectively introduced on compound **10** leading to **11**.

Then, as anticipated the coupling reaction between **11** and guanidine carbonate was successful and led to the tricyclic derivative **12** in an acceptable yield [27]. The removal of the nitro group was performed in two steps by catalytic hydrogenation of **12** leading to **13** that was diazotized to produce the corresponding diazonium salt. This latter was quenched by hypophosphorous acid to give derivative **14** [28].

The inhibitory potency toward CDK5/p25, CK1 δ/ϵ , GSK-3 α/β , DYRK1A and CLK1 of the first three representatives of this tricyclic series (compounds **12–14**) were evaluated ([Table 1](#)). As indicated in [Table 1](#), DYRK1A and CLK1 are the most efficiently inhibited kinases. Contrarily to **14**, compounds **12** and **13** substituted by a nitro or amino function exhibited nanomolar potencies toward one or two kinases tested. This result indicated that the presence of a nitro or amino substitution is particularly important for CLK1/DYRK1A inhibition. Compound **13** bearing an amino function is the most potent DYRK1A/CLK1 inhibitor of the series. We have previously discussed on these two CMGC kinases family members that exhibit sequence homology (32.8%) and a considerable ATP binding site sequence identity (70.4%) [[17](#)]. Compound **12** showed a 10-fold higher activity toward CLK1 compared to DYRK1A and thus constitute an interesting tool to distinguish between CLK1 and DYRK1A functions.

Table 1.

Physicochemical properties and kinases inhibitory potencies (IC₅₀ in μM) for compounds **12–14**.

Cpds	PSA (\AA^2)	HBD	clogP	clogD pH 7.4	MW	Kinase inhibition – IC ₅₀ in μM ^a				
						CDK5/p25	CK1 δ/ϵ	GSK-3 α/β	CLK1	DYRK1A
12	211	2	0.64	0.99	241	>10	0.78	2.2	0.069	0.62
13	182	4	0.67	0.22	211	4.8	5.8	9.1	0.041	0.028
14	132	2	0.90	1.05	196	>10	0.8	>10	0.19	0.18
Har ^b	–	–	–	–	–	8	1.8	>10	0.072	0.056
L41 ^b	–	–	–	–	–	>10	>10	0.41	0.071	0.040

a) Values are means of triplicate experiments.

b) Data given in Ref. [[29](#)] for harmine (Har) and leucettine 41 (L41).

To evaluate their ability to cross the BBB, compounds **12–14** physicochemical properties were calculated using Sybylx2.1 (Polar Surface Area (PSA), cLogP) [[30](#)] and MarvinSketch (clogD). The calculated values ([Table 1](#)) were compared to the required one for an optimized BBB penetration [[31](#)]. Molecular weights of **12–14** (<500) are acceptable as well as the number of hydrogen bond donors (HBD). Actually, this number is preferred to be < 3 to increase the BBB penetration. However, PSA of **12–14** are superior to the usually required values (<90 \AA^2) while their clogP and clogD values are inferior to the requested ones (2–5). Altogether, these results indicated that compounds **12–14** could be too hydrophilic, according to the usually admitted physicochemical properties for an optimal BBB crossing. This could be changed by the introduction of hydrophobic substituents such as alkyl, aryl groups or halogens.

Finally, to get structural data on the binding mode of this pyrido[3,4-g]quinazoline series, X-ray crystal structures of compounds **13** and non-substituted analogue **14** were solved at 2.52 \AA and 2.42 \AA , respectively. As shown in [Fig. 3](#), compounds **13** and **14** adopt the same pose within the ATP-binding site of CLK1. The aminopyrimidine part is located in the hinge region and is H-bonded to Leu244 backbone NH and carbonyl groups. A third H-bond is established between the pyridine nitrogen atom and the Lys191 side chain amino group. In

addition, hydrophobic interactions involve residues Val175, Leu295 and Val324. The 5-fold better activity of **13** over its unsubstituted analogue **14** could be partly explained by the amino group interactions within the kinase ATP-binding pocket. Indeed, the amino group of compound **13** is directed toward the ribose pocket and is probably involved in a large stabilizing H-bonds network with water molecules.

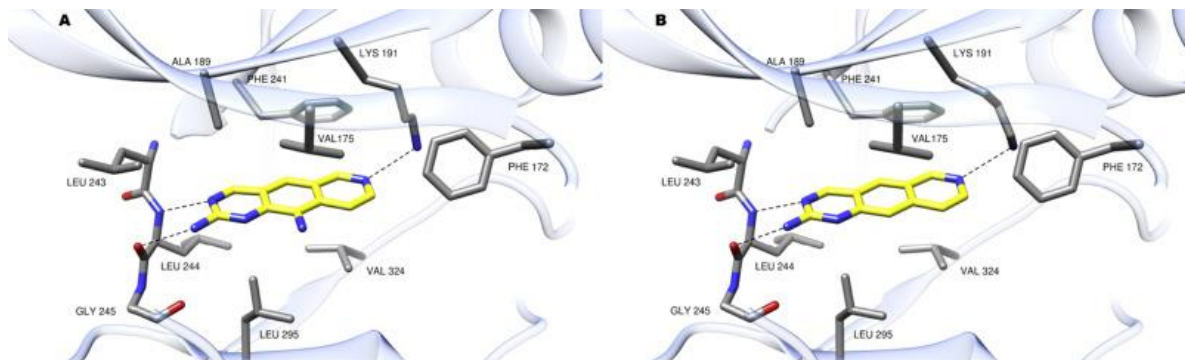


Fig. 3. : Co-crystal structures of compounds **13** (Fig. 3A) and **14** (Fig. 3B) with CLK1-ATP binding site. PDB codes: [5J1V](#) (**13**) and [5J1W](#) (**14**). The hydrogen bonds are shown by dashed lines. The images were produced using UCSF Chimera [\[32\]](#).

3. Conclusion

In our efforts to design original aza-heterocyclic compounds as potent protein kinase inhibitors, a new series of pyrido[3,4-*g*]quinazolines was synthesized and evaluated on five members of the CMGC family. Two derivatives (**12** and **13**) demonstrated nanomolar potencies toward CLK1 and/or DYRK1A. This preliminary work identified a new scaffold to target CLK1/DYRK1A kinases. The binding mode of this series with CLK1 was elucidated by X-ray crystallography. Taking into account these structural information and in order to reach an improved physicochemical profile in this series, the enlargement of the structure–activity relationship study is currently under investigation in our group. In addition complementary cellular assays will be undertaken to further examine the biological profile of this series.

4. Experimental section

4.1. Chemistry

4.1.1. General

Starting materials were obtained from commercial suppliers and used without further purification. Experiments under microwave irradiation were performed using a CEM Discover Benchmate apparatus. IR spectra were recorded on a Shimadzu FTIR-8400S or Perkin–Elmer Spectrum 65 FT-IR spectrometers ($\bar{\nu}$ in cm^{-1}). NMR spectra, performed on a Bruker AVANCE 400 (^1H : 400 MHz, ^{13}C : 100 MHz), or a Bruker AVANCE 500 (^1H : 500 MHz), are reported in ppm using the solvent residual peak as an internal standard; the following abbreviations are used: singlet (s), doublet (d), triplet (t), quadruplet (q), doublet of doublet (dd), doublet of triplet (dt), broad signal (br s). High resolution mass spectra (ESI+) were determined on a high-resolution Waters Micro Q-ToF or Thermo Scientific Q Exactive Q-Orbitrap apparatus (UBP-START, Université Blaise Pascal, Clermont-Ferrand, France).

Chromatographic purifications were performed by column chromatography using 40–63 μm silica gel. Reactions were monitored by TLC using fluorescent silica gel plates (60 F254 from Merck). Melting points were measured on a Stuart SMP3 apparatus and are uncorrected.

The purity of compounds **12–14** was established to be $\geq 95\%$ by HPLC analysis using a Dionex liquid chromatograph (TTC-100, 30 $^{\circ}\text{C}$; P580; UVD340U) and a C18 Uptisphere ODB Interchim column (4.6 mm \times 250 mm, 5 μm , 120 \AA). Detection wavelength was 280 nm, and flow rate 0.5 mL/min. Solvents were (A) water; (B) water/0.1% TFA; (C) acetonitrile; (D) methanol; three methods were used: method I, 95:5 B/C for 5 min then 95:5 B/C to 5:95 B/C in 20 min and then 5:95 B/C for 5 min; method II, 40:60 A/D in an isocratic mode. For method III, HPLC analysis was performed using an Agilent HP 1100 liquid chromatograph (ambient temperature) and a C18 Uptisphere ODB Interchim column (4.6 mm \times 250 mm, 5 μm , 120 \AA). Detection wavelength was 254 nm, flow rate 0.5 mL/min and 40:60 B/C in an isocratic mode.

4.1.2. Ethyl 2-chloro-4-nitrobenzoate (**2**)

To a solution of 2-chloro-4-nitrobenzoic acid (20 g, 99.2 mmol, 1.0 equiv) in anhydrous ethanol (160 mL) was added H_2SO_4 (8 mL, 1.5 equiv). After stirring overnight at reflux, water was added, the reaction mixture was concentrated and the product was extracted with EtOAc. The combined organic layers were washed with an aqueous solution of NaOH (1 M), dried over MgSO_4 , filtered and concentrated under reduced pressure. Without any purification, compound **2** (22.4 g, 97.5 mmol, 98%) was obtained as a pale yellowish oil. $R_f = 0.8$ (EtOAc/cyclohexane 1:2). IR (ATR): 1733, 1526, 1350, 1246 cm^{-1} ; ^1H NMR (400 MHz, $\text{DMSO}-d_6$): 1.33 (3H, t, $J = 7.2$ Hz), 4.38 (2H, q, $J = 7.2$ Hz), 8.03 (1H, d, $J = 8.4$ Hz), 8.27 (1H, dd, $J_1 = 8.4$ Hz, $J_2 = 2.4$ Hz), 8.38 (1H, d, $J = 2.4$ Hz); ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$): 13.9 (CH_3), 62.2 (CH_2), 122.4, 125.4, 131.8 (CH_{arom}), 132.3, 136.2, 149.3 (C_{arom}), 164.0 (CO); HRMS (ESI $^+$) calcd for $\text{C}_9\text{H}_8\text{ClNaNO}_4$ ($\text{M} + \text{Na}$) $^+$ 252.0040, found 252.0052.

4.1.3. Ethyl 4-amino-2-chlorobenzoate (**3**)

To a solution of compound **2** (5.0 g, 21.8 mmol, 1 equiv) in a 10:1 isopropanol/water mixture (50 mL) were successively added iron powder (7.3 g, 131.2 mmol, 6 equiv) and ammonium chloride (0.46 g, 8.6 mmol, 0.4 equiv). The reaction mixture was heated at 80 $^{\circ}\text{C}$ for 45 min then filtered through Celite and washed with EtOAc and water. The filtrate was then extracted with EtOAc and the combined organic layers were dried over MgSO_4 , filtered and concentrated under reduced pressure. Without any purification, compound **3** (4.2 g, 21.2 mmol, 97%) was obtained as a yellowish powder. $R_f = 0.3$ (EtOAc/cyclohexane 1:3). Mp 109–111 $^{\circ}\text{C}$; IR (ATR): 3500–3200, 1698, 1597, 1244 cm^{-1} ; ^1H NMR (400 MHz, $\text{DMSO}-d_6$): 1.26 (3H, t, $J = 6.8$ Hz), 4.19 (2H, q, $J = 6.8$ Hz), 6.16 (2H, br s), 6.50 (1H, dd, $J_1 = 8.8$ Hz, $J_2 = 2.4$ Hz), 6.62 (1H, d, $J = 2.4$ Hz), 7.63 (1H, d, $J = 8.8$ Hz); ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$): 14.2 (CH_3), 59.9 (CH_2), 111.5, 114.5, 133.5 (CH_{arom}), 114.1, 134.7, 153.4 (C_{arom}), 164.3 (CO); HRMS (ESI $^+$) calcd for $\text{C}_9\text{H}_{11}\text{ClNO}_2$ ($\text{M} + \text{H}$) $^+$ 200.0478, found 200.0484.

4.1.4. Ethyl 4-amino-2-chloro-5-iodobenzoate (**4**)

To a solution of compound **3** (3.02 g, 15.1 mmol, 1 equiv) in anhydrous ethanol (150 mL) at 0 $^{\circ}\text{C}$ were added silver sulfate (4.68 g, 15.1 mmol) and iodine (3.81 g, 15.1 mmol). The

solution was stirred for 45 min at 0 °C and additional 90 min at room temperature. After completion, a saturated aqueous sodium thiosulfate solution was added. The reaction mixture was filtered through Celite, washed with ethanol and filtrate was concentrated to dryness. Water was added and the product was extracted with EtOAc, the combined organic layers were dried over MgSO₄, filtered and concentrated under reduced pressure. The crude product was triturated with cyclohexane and pentane yielding the compound **4** (3.18 g, 9.77 mmol, 65%) as a brown powder. *R*_f = 0.3 (EtOAc/cyclohexane 1:9). Mp 125–127 °C; IR (ATR): 3470–3325, 1702, 1573, 1242 cm⁻¹; ¹H NMR (400 MHz, DMSO-*d*₆): 1.27 (3H, t, *J* = 6.8 Hz), 4.21 (2H, q, *J* = 6.8 Hz), 6.23 (2H, br s), 6.78 (1H, s), 8.08 (1H, s); ¹³C NMR (100 MHz, DMSO-*d*₆): 14.1 (CH₃), 60.4 (CH₂), 122.9, 142.2 (CH_{arom}), 114.3, 116.5, 134.3, 153.1 (C_{arom}), 163.0 (CO); HRMS (ESI⁺) calcd for C₉H₁₀ClINO₂ (M + H)⁺ 325.9445, found 325.9443.

4.1.5. Ethyl 4-amino-2-chloro-5-cyanobenzoate (**5**)

To a solution of compound **4** (4.13 g, 12.7 mmol, 1 equiv) in *N,N*-dimethylformamide (25 mL) were added copper(I) cyanide (2.27 g, 25.4 mmol, 2 equiv) and *l*-proline (1.46 g, 12.7 mmol). After stirring overnight at 80 °C, water and EtOAc were added. The resulting suspension was filtered through Celite and filtrate was extracted with EtOAc. The combined organic layers were dried over MgSO₄, filtered and concentrated under reduced pressure. Trituration of the crude material with small amount of diisopropyl ether yielded the pure product **5** (2.45 g, 10.9 mmol, 86%) as a pale brown powder. *R*_f = 0.5 (EtOAc/cyclohexane 1:2). Mp 208–210 °C; IR (ATR): 3750–3200, 2220, 1705, 1643, 1599, 1265 cm⁻¹; ¹H NMR (400 MHz, DMSO-*d*₆): 1.28 (3H, t, *J* = 7.2 Hz), 4.22 (2H, q, *J* = 7.2 Hz), 6.88 (1H, s), 7.04 (2H, br s), 7.99 (1H, s); ¹³C NMR (100 MHz, DMSO-*d*₆): 14.1 (CH₃), 60.7 (CH₂), 116.1, 137.7 (CH_{arom}), 92.2, 116.3, 138.6, 154.1 (C_{arom}), 115.5 (CN), 162.9 (CO); HRMS (ESI⁺) calcd for C₁₀H₁₀ClN₂O₂ (M + H)⁺ 225.0431, found 225.0442.

4.1.6. Ethyl 2-chloro-5-cyano-4-iodobenzoate (**6**)

To a solution of compound **5** (1.60 g, 7.1 mmol, 1 equiv) in diiodomethane (5 mL) was added isoamyl nitrite (1.8 mL, 13.4 mmol, 1.88 equiv) under argon atmosphere. The reaction mixture was stirred at room temperature for 45 min, warmed to 80 °C and stirred for 3 h. The mixture was diluted with CH₂Cl₂ and washed with water and a saturated NaCl aqueous solution. The organic layer was dried over MgSO₄, filtered and concentrated under reduced pressure. Residue was purified by column chromatography (pure cyclohexane then EtOAc/cyclohexane 1:99 to 5:95) to give compound **6** (2.01 g, 6.0 mmol, 84%) as a white powder. *R*_f = 0.5 (EtOAc/cyclohexane 1:5). Mp 108–110 °C; IR (ATR): 2232, 1727, 1575, 1449, 1245 cm⁻¹; ¹H NMR (400 MHz, DMSO-*d*₆): 1.32 (3H, t, *J* = 7.6 Hz), 4.33 (2H, q, *J* = 7.6 Hz), 8.23 (1H, s), 8.36 (1H, s); ¹³C NMR (100 MHz, DMSO-*d*₆): 13.9 (CH₃), 62.1 (CH₂), 135.9, 140.8 (CH_{arom}), 105.4, 118.3, 130.7, 136.9 (C_{arom}), 118.7 (CN), 163.2 (CO); HRMS (ESI⁺) calcd for C₁₀H₈ClINO₂ (M + H)⁺ 335.9288, found 335.9302.

4.1.7. 4-Chloro-5-(hydroxymethyl)-2-iodobenzaldehyde (**7**)

To a stirred solution of compound **6** (2.00 g, 5.9 mmol, 1 equiv) in toluene (40 mL) at –78 °C under argon atmosphere, was added dropwise a solution of DIBAL-H in toluene (1 M, 19.1 mmol, 3.2 equiv). The reaction mixture was stirred at –78 °C for 1 h 30 min, warmed to room temperature and stirred for additional 1 h 30, then quenched by the slow addition of an aqueous HCl solution (1 M). The solution was extracted with EtOAc, the organic layers were washed with brine, dried over MgSO₄ and concentrated under reduced pressure. The crude

product was triturated with cyclohexane yielding the compound **7** (1.54 g, 5.2 mmol, 87%) as a pale yellowish powder. $R_f = 0.3$ (EtOAc/cyclohexane 1:4). Mp 102–104 °C; IR (ATR): 3524–3050, 1678, 1579, 1371, 1043 cm^{-1} ; ^1H NMR (400 MHz, DMSO- d_6): 4.54 (2H, d, $J = 6.0$ Hz), 5.65 (1H, t, $J = 6.0$ Hz, OH), 7.94 (1H, s), 8.12 (1H, s), 9.94 (1H, s, CHO); ^{13}C NMR (100 MHz, DMSO- d_6): 59.7 (CH_2), 128.6, 139.6 (CH_{arom}), 98.8, 133.8, 137.6, 140.9 (C_{arom}), 194.7 (CO); HRMS (ESI $^+$) calcd for $\text{C}_9\text{H}_9\text{ClIO}_2$ ($\text{M} - \text{H}_2\text{O} + \text{CH}_3\text{OH} + \text{H}$) $^+$ 310.9336, found 310.9333.

4.1.8. 4-Chloro-5-(hydroxymethyl)-2-(2-(trimethylsilyl)ethynyl)benzaldehyde (**8**)

To a solution of compound **7** (3.65 g, 12.4 mmol, 1 equiv) in triethylamine (60 mL), under argon atmosphere, were successively added CuI (96 mg, 0.5 mmol, 4 mol%), $\text{PdCl}_2(\text{PPh}_3)_2$ (176 mg, 0.25 mmol, 2 mol%) and trimethylsilylacetylene (4.5 mL, 31 mmol, 2.5 equiv). The mixture was stirred at 50 °C for 3 h. The solvent was removed in vacuo and the crude material was purified by filtration over silica gel with 300 mL of EtOAc/cyclohexane 2:8. The filtrate was concentrated under reduced pressure and the residue was triturated with pentane yielding compound **8** (2.87 g, 10.8 mmol, 87%) as a pale brown powder. $R_f = 0.4$ (EtOAc/cyclohexane 1:4). Mp 91–93 °C; IR (ATR): 3650–3273, 1697, 1595, 1235 cm^{-1} ; ^1H NMR (400 MHz, DMSO- d_6): 0.27 (9H, s, 3 CH_3), 4.60 (2H, d, $J = 7.2$ Hz), 5.69 (1H, t, $J = 7.2$ Hz, OH), 7.72 (1H, s), 8.00 (1H, s), 10.35 (1H, s, CHO); ^{13}C NMR (100 MHz, DMSO- d_6): -0.4 (3 CH_3), 59.8 (CH_2), 126.1, 133.1 (CH_{arom}), 98.9, 102.9, 124.6, 134.4, 136.7, 141.7 ($\text{C}_{\text{alkyne}} + \text{C}_{\text{arom}}$), 190.2 (CO); HRMS (ESI $^+$) calcd for $\text{C}_{13}\text{H}_{16}\text{ClO}_2\text{Si}$ ($\text{M} + \text{H}$) $^+$ 267.0608, found 267.0606.

4.1.9. 6-Chloro-7-(hydroxymethyl)isoquinoline (**9**)

A 10 mL CEM Discover microwave tube was charged with compound **8** (500 mg, 1.87 mmol, 1 equiv) and 5 mL of a solution of ammonia in methanol (7 M). The reaction flask was sealed and irradiated for 15 min (Discover mode, Control Type = Standard, $P = 75$ W, $T = 130$ °C). The solvent was removed under reduced pressure. Residue was purified by flash chromatography (EtOAc/cyclohexane 7:3 to 10:0) yielding the isoquinoline **9** (276 mg, 1.42 mmol, 76%) as a purple powder. $R_f = 0.3$ (EtOAc/cyclohexane 7:3). Mp 138–140 °C; IR (ATR): 3500–2965, 1626, 1436, 1177 cm^{-1} ; ^1H NMR (400 MHz, DMSO- d_6): 4.73 (2H, d, $J = 5.2$ Hz), 5.68 (1H, t, $J = 5.2$ Hz, OH), 7.80 (1H, d, $J = 6.0$ Hz), 8.12 (1H, s), 8.25 (1H, s), 8.51 (1H, d, $J = 6.0$ Hz), 9.37 (1H, s); ^{13}C NMR (100 MHz, DMSO- d_6): 60.5 (CH_2), 119.2, 125.9, 126.1, 143.3, 152.2 (CH_{arom}), 126.7, 134.2, 134.8, 139.3 (C_{arom}); HRMS (ESI $^+$) calcd for $\text{C}_{10}\text{H}_9\text{ClNO}$ ($\text{M} + \text{H}$) $^+$ 194.0373, found 194.0366.

4.1.10. 6-Chloroisoquinoline-7-carbaldehyde (**10**)

To a solution of compound **9** (3.73 g, 19.3 mmol, 1 equiv) in CHCl_3 (175 mL) was added MnO_2 (5.02 g, 57.8 mmol, 3 equiv). The mixture was stirred at reflux for 36 h, filtered through a pad of Celite and washed with EtOAc. The filtrate was concentrated in vacuo. The crude product was triturated with cyclohexane and diisopropyl ether yielding compound **10** (3.28 g, 17.1 mmol, 88%) as a purple powder. $R_f = 0.2$ ($\text{CH}_2\text{Cl}_2/\text{EtOAc}$ 9:1). Mp 155–157 °C; IR (ATR): 1692, 1617, 1410 cm^{-1} ; ^1H NMR (400 MHz, DMSO- d_6): 7.90 (1H, d, $J = 6.0$ Hz), 8.30 (1H, s), 8.68 (1H, d, $J = 6.0$ Hz), 8.78 (1H, s), 9.57 (1H, s), 10.44 (1H, s, CHO); ^{13}C NMR (100 MHz, DMSO- d_6): 119.5, 127.9, 132.5, 146.2, 154.4 (CH_{arom}), 126.0, 131.0, 135.1, 138.0 (C_{arom}), 189.6 (CO); HRMS (ESI $^+$) calcd for $\text{C}_{10}\text{H}_7\text{ClNO}$ ($\text{M} + \text{H}$) $^+$ 192.0216, found 192.0225.

4.1.11. 6-Chloro-5-nitroisoquinoline-7-carbaldehyde (**11**)

To a solution of compound **10** (1.35 g, 7.05 mmol, 1 equiv) in concentrated sulfuric acid (15 mL) was added dropwise a 65% nitric acid solution (0.6 mL, 8.45 mmol, 1.2 equiv). After stirring overnight at room temperature the reaction mixture was poured into ice/water, and filtered through a pad of Celite. Portions of solid NaHCO₃ were added to the filtrate (until pH > 7) before extraction with EtOAc. The organic layers were washed with brine, dried over MgSO₄ and concentrated under reduced pressure. The crude material was triturated with diethyl ether yielding compound **11** (1.45 g, 6.13 mmol, 87%) as a pale brown powder. *R*_f = 0.4 (CH₂Cl₂/EtOAc 9:1). Mp 186–187 °C; IR (ATR): 1692, 1613, 1523, 1268 cm⁻¹; ¹H NMR (500 MHz, DMSO-*d*₆): 7.73 (1H, dt, *J*₁ = 6.0 Hz; *J*₂ = 0.8 Hz), 8.86 (1H, d, *J* = 6.0 Hz), 9.07 (1H, d, *J* = 0.8 Hz), 9.78 (1H, d, *J* = 0.8 Hz), 10.39 (1H, s, CHO); ¹³C NMR (100 MHz, DMSO-*d*₆): 113.3, 134.5, 148.6, 154.9 (CH_{arom}), 125.7, 127.2, 129.0, 130.7, 145.2 (C_{arom}), 187.8 (CO); HRMS (ESI⁺) calcd for C₁₀H₆ClN₂O₃ (M + H)⁺ 237.0067, found 237.0078.

4.1.12. 10-nitropyrido[3,4-*g*]quinazolin-2-amine (**12**)

A 10 mL CEM Discover microwave tube was charged with compound **11** (100 mg, 0.42 mmol, 1 equiv) and guanidine carbonate (100 mg, 0.52 mmol, 1.3 equiv) in 4 mL of *N,N*-dimethylformamide (99.8%; for peptide synthesis) resulting in a suspension. The reaction flask was purged with argon for 30 min, sealed and irradiated for 45 s (Discover Mode, Control Type = Standard, P = 300 W, T = 166 °C). After cooling, the reaction was diluted with EtOAc and filtered through a pad of Celite. The organic layer was washed with water, dried over MgSO₄ and concentrated under reduced pressure. Residue was purified by flash chromatography using CH₂Cl₂/MeOH (99:1 to 97:3) as eluant, yielding the pure compound **12** (50 mg, 0.21 mmol, 49%) as an orange powder. *R*_f = 0.3 (EtOAc). Mp > 245 °C (decomposition); IR (ATR): 3300-3000, 1700, 1623, 1608, 1576, 1507 cm⁻¹; ¹H NMR (500 MHz, DMSO-*d*₆): 7.52 (1H, d, *J* = 6.0 Hz, H₉), 8.00 (1H, br s, NH), 8.09 (1H, br s, NH), 8.56 (1H, d, *J* = 6 Hz, H₈), 8.99 (1H, s, H₅), 9.53 (1H, s, H₆), 9.58 (1H, s, H₄); ¹³C NMR (100 MHz, DMSO-*d*₆): 112.2 (C₉), 134.2 (C₅), 146.1 (C₈), 155.0 (C₆), 165.8 (C₄), 119.5 (C_{4a}), 121.8 (C_{5b}), 128.5 (C_{9b}), 136.2 (C₁₀), 141.7 (C_{10a}), 161.2 (C₂); HRMS (ESI⁺) calcd for C₁₁H₈N₅O₂ (M + H)⁺ 242.0673, found 242.0682. HPLC (method I): purity ≥ 98%, t_R = 15.8 min.

4.1.13. Pyrido[3,4-*g*]quinazoline-2,10-diamine (**13**)

To a suspension of compound **12** (50 mg, 0.21 mmol) in 40 mL of 1:1 mixture of anhydrous dichloromethane and anhydrous methanol was added palladium on charcoal (10% wt, 9 mg, 0.008 mmol). The mixture was stirred under 1 atm. H₂ for 3 h, and filtered through Celite. The Celite pad was washed with a mixture of CH₂Cl₂/MeOH 1:1. Combined filtrates were concentrated under reduced pressure to give the title compound without further purification (43 mg, 0.21 mmol, quantitative yield) as a red powder. *R*_f = 0.2 (EtOAc). Mp > 250 °C (decomposition); IR (ATR): 3400-3000, 1646, 1614 cm⁻¹; ¹H NMR (500 MHz, DMSO-*d*₆): 6.24 (2H, br s, NH₂), 7.02 (2H, br s, NH₂), 7.85 (1H, s), 7.97 (1H, d, *J* = 6.2 Hz), 8.20 (1H, d, *J* = 6.2 Hz), 9.23 (1H, s), 9.36 (1H, s); ¹³C NMR (100 MHz, DMSO-*d*₆): 113.6, 115.4, 139.4, 154.8, 164.8 (CH_{arom}), 118.9, 121.0, 124.4, 135.1, 136.0, 158.7 (C_{arom}); HRMS (ESI⁺) calcd for C₁₁H₁₀N₅ (M + H)⁺ 212.0936, found 212.0941. HPLC (method II): purity ≥ 95%, t_R = 8.1 min.

4.1.14. Pyrido[3,4-g]quinazolin-2-amine (14)

To an ice/water bath cooled solution of compound **13** (57 mg, 0.27 mmol, 1 equiv) in a 5 M aqueous solution of HCl (2 mL), was added dropwise a solution of NaNO₂ (24 mg, 0.35 mmol, 1.3 equiv) in water (2 mL) and the mixture was stirred at 0 °C for 1 h. Then, an aqueous solution of H₃PO₂ (50% wt, 0.5 mL) was added and the mixture was stirred at room temperature for 24 h. The mixture was then diluted with water and basified by addition of a saturated aqueous NaHCO₃ solution. The aqueous layer was extracted with EtOAc and the organic layers were dried over MgSO₄ and concentrated. Residue was purified by flash chromatography on Et₃N neutralized silica gel using CH₂Cl₂/MeOH/Et₃N 96:3:1 as eluant. The residue was dissolved in EtOAc and further washed with a diluted NaHCO₃ aqueous solution. The organic phase was dried over MgSO₄ and evaporated yielding the title compound **14** (15 mg, 0.08 mmol, 29%) as a yellow powder. *R*_f = 0.3 (CH₂Cl₂/MeOH/Et₃N 96:3:1). Mp > 220 °C (decomposition); IR (ATR): 3350-3100, 1653, 1624, 1579, 1558 cm⁻¹; ¹H NMR (400 MHz, DMSO-*d*₆): 7.32 (2H, br s, NH₂), 7.78 (1H, d, *J* = 6.0 Hz), 7.84 (1H, s), 8.36 (1H, d, *J* = 6.0 Hz), 8.73 (1H, s), 9.40 (1H, s), 9.49 (1H, s); ¹³C NMR (100 MHz, DMSO-*d*₆): 118.0, 119.3, 130.5, 142.7, 154.8, 165.4 (CH_{arom}), 119.7, 124.0, 137.5, 149.4, 160.3 (C_{arom}); HRMS (ESI⁺) calcd for C₁₁H₉N₄ (M + H)⁺ 197.0827, found 197.0835. HPLC (method III): purity ≥ 99%, t_R = 4.8 min.

4.2. Crystallography

Recombinant CLK1 was expressed and purified as previously described [33]. Apo crystals were obtained using sitting vapour diffusion method at 4 °C and the condition containing 30% 1,2-propanediol, 10% glycerol and 50 mM Na/K phosphate. Soaking was performed overnight using the reservoir solution supplemented with 5–10 mM compounds and 25% glycerol. Diffraction data were collected at Diamond Light Source, beamline I03 using X-ray at 0.97625 Å wavelength, and were processed and scaled with Mosflm [34] and Scala [35], respectively. Structures were solved by molecular replacement method using Phaser [36] and the coordinates of published CLK1 structure [33]. Model rebuilding was performed in COOT [37], and the structures were refined using REFMAC [38]. Geometric correctness was verified using MOLPROBITY [39]. The data collection and refinement statistics are summarized in the table below.

Complex	CLK1-13	CLK1-14
PDB accession code	5J1V	5J1W
	<i>Data Collection</i>	
Resolution ^a (Å)	35.47–2.52 (2.66–2.52)	35.59–2.42 (2.55–2.42)
Spacegroup	<i>P</i> 2 ₁	<i>P</i> 2 ₁
Cell dimensions	<i>a</i> = 56.5, <i>b</i> = 116.0, <i>c</i> = 90.1 Å <i>α</i> = <i>γ</i> = 90.0°, <i>β</i> = 99.3°	<i>a</i> = 56.6, <i>b</i> = 118.0, <i>c</i> = 90.5 Å <i>α</i> = <i>γ</i> = 90.0°, <i>β</i> = 99.6°
No. unique reflections ^a	38,700 (5,626)	44,729 (6,537)
Completeness ^a (%)	99.8 (99.8)	99.9 (99.9)
<i>I</i> /σ ^a	7.6 (2.0)	8.3 (2.0)
<i>R</i> _{merge} ^a (%)	0.119 (0.829)	0.082 (0.599)

Complex PDB accession code	CLK1-13 5J1V	CLK1-14 5J1W
Redundancy ^a	4.8 (4.5)	3.4 (3.4)
Refinement		
No. atoms in refinement (P/L/O) ^b	7,927/48/107	8,015/45/193
B factor (P/L/O) ^b (Å ²)	72/36/62	66/32/64
R _{fact} (%)	19.9	18.9
R _{free} (%)	24.4	22.8
rms deviation bond ^c (Å)	0.011	0.013
rms deviation angle ^c (°)	1.2	1.3
Molprobability Ramachandran		
Favour (%)	95.89	95.51
Allowed (%)	4.11	4.49

- a) Values in brackets show the statistics for the highest resolution shells.
b) P/L/O indicate protein, ligand molecules presented in the active sites, and other (water and solvent molecules), respectively.
c) rms indicates root-mean-square.

4.3. In vitro kinase inhibition assays

Buffer A: 10 mM MgCl₂, 1 mM ethylene glycol-bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA), 1 mM dithiothreitol (DTT), 25 mM Tris-HCl pH 7.5, 50 µg heparin/mL.

Buffer B: 60 mM β-glycerophosphate, 15 mM *p*-nitrophenylphosphate, 25 mM 3-(*N*-morpholino)propanesulfonic acid (Mops) (pH 7.2), 5 mM EGTA, 15 mM MgCl₂, 1 mM DTT, 1 mM sodium vanadate, 1 mM phenylphosphate.

Kinase activities were assayed in buffer A or B, at 30 °C, at a final adenosine triphosphate (ATP) concentration of 15 µM. Blank values were subtracted and activities expressed in % of the maximal activity, *i.e.* in the absence of inhibitors. Controls were performed with appropriate dilutions of dimethylsulfoxide (DMSO).

CDK5/p25 (human, recombinant) was prepared as previously described [40] and [41]. Its kinase activity was assayed in buffer B, with 1 mg histone H1/mL, in the presence of 15 µM [γ -³³P] ATP (3000 Ci/mmol; 10 mCi/mL) in a final volume of 30 µL. After 30 min incubation at 30 °C, 25 µL aliquots of supernatant were spotted onto 2.5 × 3 cm pieces of Whatman P81 phosphocellulose paper, and, 20 s later, the filters were washed five times (for at least 5 min each time) in a solution of 10 mL phosphoric acid/L of water. The wet filters were counted in the presence of 1 mL ACS (Amersham) scintillation fluid.

GSK-3α/β (porcine brain, native) was assayed, as described for CDK5/p25 but in buffer A and using a GSK-3 specific substrate (GS-1: YRRAAVPPSPSLSRHSSPHQSpEDEEE) (pS stands for phosphorylated serine) [42]. GS-1 was synthesized by Millegen (Labege, France).

CK1δ/ε (porcine brain, native) was assayed as described for CDK5/p25 but using the CK1-specific peptide substrate RRKHAAIGpSAYSITA [43], obtained from Millegen (Labège, France).

DYRK1A (rat, recombinant, expressed in *Escherichia coli* as a glutathione transferase (GST) fusion protein) was purified by affinity chromatography on glutathione-agarose and assayed as described for CDK5/p25 using myelin basic protein (1 mg/mL) as a substrate.

CLK1 (human, recombinant, expressed in *E. coli* as GST fusion protein) was assayed in buffer A (+0.15 mg BSA/ml) with RS peptide (GRSRSRSRSR) (1 µg/assay).

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