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Synthesis of aryl phosphates based on pyrimidine and triazine scaffolds

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ABSTRACT

The syntheses of the triazinyl-based bis-aryl phosphates **2** and **3**, and of the aminopyrimidyl-based aryl phosphate **4** are described. Each compound contains a diaryl ether-phosphate structural motif. The synthetic route to bis-aryl phosphates **2** and **3** consisted in two nucleophilic substitution reactions with amines from cyanuric chloride, followed by a Suzuki coupling with the resulting 2,4-diamino-6-chloro-1,3,5-triazine derivative **12** to introduce the diaryl ether functionality. Aryl phosphate **4** was obtained via condensation of aryl guanidine **34** with aryloxyphenyl butenone **31**. These de novo-designed aryl phosphates were evaluated as potential inhibitors of the Grb2-SH2 domain using an ELISA assay. The water-soluble sodium salt **26** of **3** gave an IC_{50} value in the high micromolar range. Molecular modeling studies were subsequently performed upon modifying the 1,3,5-trisubstituted triazine scaffold of **3**. Non-phosphate derivatives encompassing cyclopropane, pyrrole, keto-acid, and IZD fragments were thus step-wise designed and their Grb2-SH2 complexes were modeled by molecular dynamics. Some derivatives gave rise to an enriched pattern of H-bonds and cation–π interactions with Grb2-SH2.

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1. Introduction

The 2-arylaminopyrimidine and 1,3,5-triazine motifs are important as scaffolds in drug discovery chemistry. 2-Arylaminopyrimidine derivatives have exhibited biological activities such as antitumor activities involving different targets (e.g. CDKs, VEGF–TKRs, Bcr–Abl kinase) [1–6], and antiinflammatory activity by inhibition of Lck [7]. 1,3,5-Triazine derivatives [8] have displayed a broad range of biological activities including cytotoxic activities [9–11], antiangiogenic activity by targeting either VEGF-R2 (KDR) [12] or direct modulation of Tie-2 tyrosine kinase phosphorylation [13], antiparasitic activities [14,15], and glucocerebrosidase inhibition with potential as chemical chaperones for Gaucher disease [16]. To our knowledge, the syntheses of aryl phosphates based on pyrimidine and triazine scaffolds have not been reported to date. Importantly, the aryl phosphate group or its mimics are key pharmacophores in the design of cell signalling inhibitors as potential anticancer agents [17–22].

As part of our efforts in the synthesis of non-peptidic inhibitors of the SH2 domain (Src homology) of Grb2 [23] (growth factor receptor-bound protein-2) which mediates protein–protein interactions in tyrosine kinase signal transduction pathways [17–22], we have focused on the design and synthesis of aryl phosphates based on heterocycles allowing diverse functionalization [24]. Structure-based de novo design was carried out using **1** as a reference ligand [25–27], encompassing three pharmacophores [i.e., pTyr, (αMe)pTyr, $CONH_2$] for binding to Grb2 ($IC_{50} = 11$ nM, ELISA assay). The complexes of two pseudopeptides with the Grb2-SH2 domain were recently reported by Martin and co-workers [28–30].

The structure of the best MD [25] pose of **1** with Grb2-SH2 is presented in the Supporting Information. Using this structure, **2** and **3** were designed by computer graphics upon replacing the peptide scaffold by a 1,3,5-trisubstituted triazine one while retaining a satisfactory overlap of the phosphate groups at the two extremities with those of **1**. Within this context, we describe here the syntheses of the triazinyl-containing bis-aryl phosphates **2** and **3**, and the synthesis of the 2-arylaminopyrimidyl-containing aryl phosphate **4** to mimic [31,32] the phosphate moiety of **2**. These

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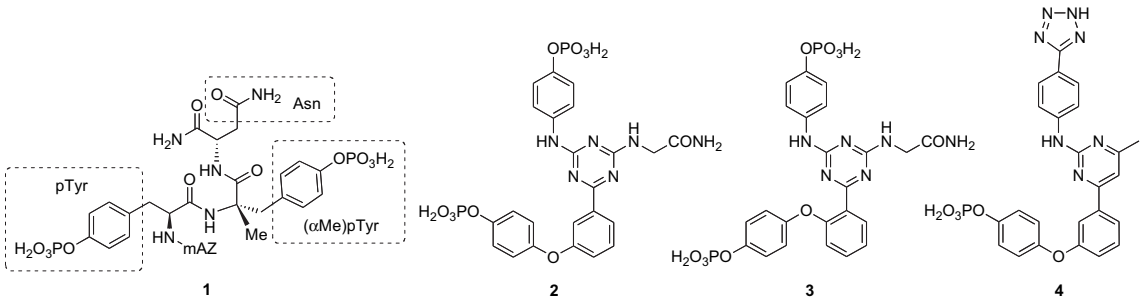


Fig. 1.

novel compounds have in common a diaryl ether-phosphate structural motif (Fig. 1).

Importantly, the common substructure of **2** and **3** [33,34], namely 4-(4-amino-1,3,5-triazin-2-ylamino)phenyl phosphate, was only published in recent patents (**5**–**7**) [35] reporting inhibition of smooth cell proliferation, treatment of inflammation, hyperproliferation, and modulation of glycosidase (Fig. 2). As for the substructure of **4**, namely *N*-(4-(2*H*-tetrazol-5-yl)phenyl)pyrimidin-2-amine, it also appeared in the three patented compounds **8**–**10** [36–38], in the context of the syntheses of protein kinase inhibitors useful in treatment of diseases like cancer.

2. Results and discussion

The syntheses of the triazine scaffold-based bis-aryl phosphates **2** and **3** were carried out starting from 2,4,6-trichloro-1,3,5-triazine (cyanuric chloride) [8]. The latter and its derivatives (i.e., mono- or dichloride) are known to react very easily with various nucleophiles such as amines [9–11,15,39–46], alcohols [47,48], thiols [49,50], and cyanide [51,52]. It can also undergo organometallics couplings such as the Stille [53,54], Suzuki–Miyaura [55–59] or Negishi [54,60] reactions, as well as the Grignard alkylation [61].

The trifunctionalization of cyanuric chloride to prepare the triazinyl-based bis-aryl phosphates **2** and **3** is shown in Fig. 3. First, 2-aminoacetamide and 4-aminophenol would be introduced by a stepwise nucleophilic substitution of two chloro groups. Then, installation of the meta- or ortho-diaryl ether unit would be carried out via Suzuki coupling [62–68].

Thus, glycinamide hydrochloride was coupled to cyanuric chloride at 0 °C, the resulting product **11** was then reacted with 4-aminophenol at room temperature to give the 2,4-diamino-6-chloro-1,3,5-triazine **12** (Scheme 1). In both cases, diisopropylethylamine (DIEA) was used as a base. This reaction sequence could be performed in the reverse order (i.e., 4-aminophenol with cyanuric chloride and then glycinamide hydrochloride) but was less satisfying in terms of yields. As expected, the phenol group of 4-aminophenol is unreactive under these reaction conditions [69].

In order to install the diaryl ether fragment on **12** via Suzuki coupling, the meta- and ortho-substituted boronic acids **15** and **18** were prepared from the corresponding brominated derivatives. The direct S_NAr displacement [70] of 1-bromo-3-fluorobenzene with hydroquinone, using sodium methoxide as base, gave aryloxyphenol **13** [71] in 43% yield [72] (Scheme 2). After benzylation, halogen–lithium exchange of 1-(4-benzyloxyphenoxy)-3-bromobenzene **14**, subsequent reaction of the anion with triisopropylboron and acidic hydrolysis led to **15** [73] in 81% yield. The ortho-substituted boronic acid **18** was prepared following the same procedure.

The Suzuki coupling of **12** with **15** is depicted in Scheme 3. Palladium tetrakis(triphenylphosphine) was chosen as the catalyst as it is the most commonly used for Suzuki couplings with chlorotriazines [56–59]. It appeared that the best solvent was a mixture of acetonitrile and water thoroughly degassed under high vacuum. Those conditions afforded **19** in 67% yield. It is worth pointing out that the same solvent degassed only via argon bubbling gave mostly homocoupling product and a very small amount of the desired Suzuki product. Toluene/water [56] and DME/water mixtures did not lead either to the expected results. The same reaction conditions were used for the ortho-substituted boronic acid **18**, giving compound **21** in 51% yield. Both Suzuki adducts **19** and **21** were debenzylated by hydrogenation with H_2 over Pd/C to give, respectively, **20** and **22** in good yields.

As outlined in Scheme 4, the resulting diphenols **20** and **22** were then converted [74,75], respectively, into the corresponding bis-dibenzyl phosphates **23** (79%) and **24** (69%). The latter were hydrogenated over Pd/C to give the non-water soluble bis-aryl phosphates **2** and **3**, of very low solubility in usual solvents except in DMSO. However, their limited solubilities necessited a quantity of DMSO of 10% final volume in PBS buffer. For the range of concentrations to be studied, this does not enable reliable ELISA-

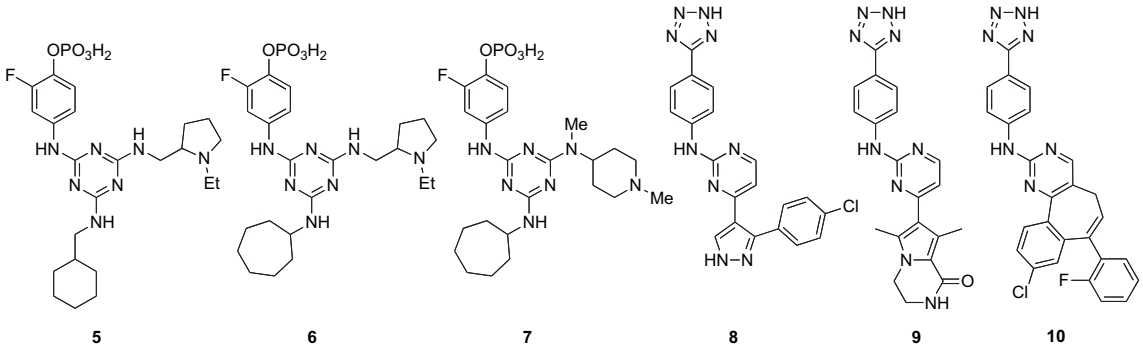


Fig. 2. Patented structures **5**–**10**.

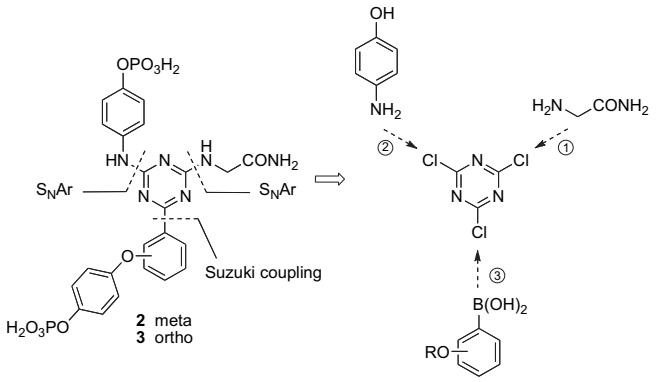


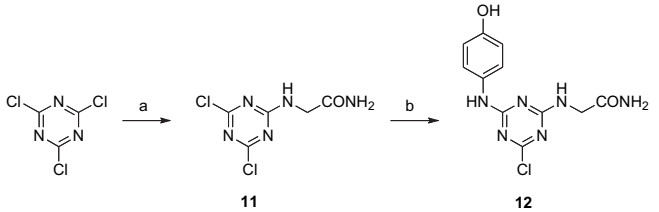
Fig. 3. Triazinyl-based bis-aryl phosphates **2** and **3** via trifunctionalization of cyanuric chloride.

based extracellular Grb2-SH2 domain binding assays (vide infra). Indeed, it was noted that only at concentrations less than 5%, did DMSO not alter ELISA assays (i.e., no effect on peroxidase activity). Water insoluble compounds **2** and **3** were subsequently transformed into the water-soluble sodium salts **25** and **26** (resp.) required for this test, since their phosphate groups bear a dianionic charge at physiological pH [76].

We next turned our attention to the synthesis of 4-aryl-2-anilinopyrimidine **4**. Among the traditional four synthetic routes (A–D) [77–81] envisioned for the preparation of the target structure, we chose the Wendelin's procedure involving the condensation of an aryl guanidine with aryloxyphenyl butenone **31** (Route C, Scheme 5) [81]. This latter would be obtained from the Heck coupling reaction between methylvinylketone and 1-(4-benzyloxyphenoxy)-3-bromobenzene. Interestingly, a recent report described the mono-arylation of 2,4,6-trichloropyrimidine **33** via Suzuki coupling, allowing possible access to **4** (Route E) [82].

First, Heck coupling [83] of **14** with methylvinylketone (MVK), according to the protocole of Xu et al. [84], furnished the required aryloxyphenyl butenone **31** (Scheme 6). Note that the yield of the coupling compound was low (45%) when 2 equivalents of MVK were used, probably due to the polymerization of MVK [85].

The 2-arylaminopyrimidinyl-based phosphate **4** was prepared as shown in Scheme 7. High-temperature condensation [86] of aryl guanidine **34** [87] and aryloxyphenyl butenone **31** provided the desired heterocyclic structure **35**. It is noteworthy that under milder conditions – less equivalents of amidine and lower temperature – the yield was much reduced. Subsequently, the nitrile group of **35** was transformed into a tetrazole with trimethylsilyl azide in the presence of catalytic tetrabutylammonium fluoride under solventless conditions [88]. The yield of **36** was only 25% at 85 °C but increasing the temperature to 120 °C afforded a significantly enhanced 89% isolated yield. Hydrogenation of **36** over 10% Pd/C afforded phenol **37**.



Scheme 1. Synthesis of triazine **12**. Reagents and conditions: (a) Glycinamide hydrochloride (1 eq), DIEA (2.2 eq), THF, 0 °C, 1.5 h, 74%; (b) 4-Aminophenol (1 eq), DIEA (1 eq), THF, 0 °C, 30 min then 25 °C, 1 h, 87%.

The latter was converted into the corresponding dibenzyl phosphate **38** with in situ formed chlorodibenzylphosphite. Debenzylation was performed by catalytic hydrogenation over Pd/C to give aryl phosphoric acid **4** also of low solubility in usual solvents, which was subsequently treated with sodium methoxide, for the reason mentioned above, thus providing the desired sodium salt **39**.

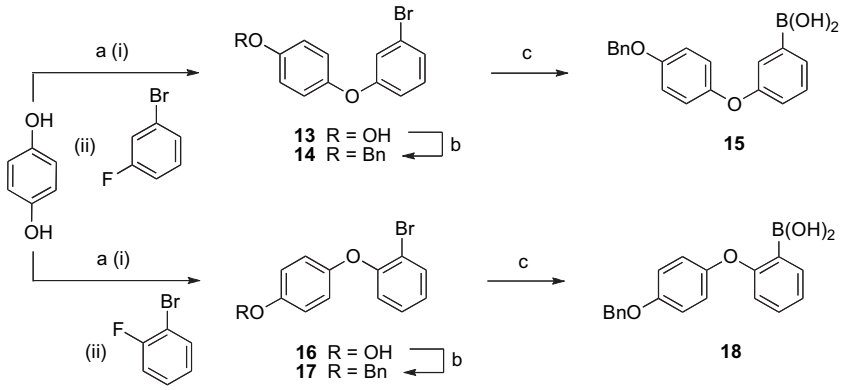
Grb2-SH2 binding data for compounds **25**, **26**, and **39** were determined using an ELISA assay [89] that measures their ability to inhibit the binding of the phosphorylated peptide PSpYVNVQN (Kd = 9 nM) to the SH2 domain. Compounds **25** and **39** were found to be inactive, whereas **26** binds to Grb2 with an IC_{50} value in the high micromolar range [90] (IC_{50} = 265 μM) (Fig. 4).

Superimposition of the docked compounds **1**, **25** and **26** shows that, while the two phosphate groups occupy similar binding positions, the carboxamide groups adopt different conformations [91]. In agreement with the ELISA results, docking experiments by molecular dynamics (MD) [92] predicted an improved pattern of interactions for compound **26** compared to compound **25**. Thus, the carboxamide NH_2 of **26** can form hydrogen bonds with the Lys 109 and Leu 120 residues in the specificity pocket (Fig. 5). For both **25** and **26**, the phosphate group occupies the pTyr-binding pocket with the expected polar interactions (Arg 86, Ser 88, Ser 90 and Ser 96). Neither **25** nor **26**, however, gave rise to significant interactions with Arg 67 while by contrast **1** closely interacts with it [91]. In the second pTyr pocket, and for both compounds, the phosphate interacts with Arg 142, Asn 143, and Ser 141.

We have then retained the scaffold of compound **26**, and, with the aid of MD simulations, we have attempted to evolve it to design compounds fitting better into the Grb2 recognition site. Thus we have considered in consecutive fashion compounds **40**–**44** (Fig. 6).

Thus **40**, bearing both the malonic acid [17–22] and keto-acid [93,94] groups as phosphate mimics, gives rise to more interactions with Grb2 than **26**. Indeed, the phenyl D-ring forms the desired cation–π interaction with Arg 67. In the pTyr pocket, one malonate carboxylate also binds to this residue by hydrogen bonding, and the interactions that involved the phosphate group take place as well with the malonate group. In the second pTyr pocket, however, the keto functionality is not well oriented for binding [91]. To improve its orientation in this pocket, we have next replaced the aminophenyl fragment of **40** by the aminopyrrole fragment of **41**. While this indeed leads to a flip of the keto group, no improvement in the polar interactions takes place. The consecutive introduction of the (R,S)-trans cyclopropane ring (**42**) instead of the phenyl C-ring enables a better anchoring to the protein (Fig. 7). The malonate is hydrogen-bonded to Arg 86, Ser 88, Ser 90, Ser 96 and Lys 109 in the pTyr-binding pocket, the carboxamide group satisfies hydrogen bonding requirements with Lys 109 and Leu 120 in the specificity pocket. In the second phosphate pocket, polar interactions take place between the carboxylate group and Arg 142, Ser 141, and Asn 143. Cation–π interactions are also observed between Arg 142 and the keto-acid-pyrrole junction. To the best of our knowledge, the use of the keto-acid group as a phosphate mimic has not been yet reported in the design of Grb2-SH2 inhibitors.

Recently, Combs et al. [95] reported a structure-based design of potent protein phosphatase 1B (PTP1B) inhibitors that incorporate the novel 1,1-dioxido-isothiazolidin-3-one (IZD) pTyr mimetic containing the highly delocalized anion of the cyclic *N*-acylsulfonamide heterocycle at physiological pH. Such a heterocycle is unexplored for the search of inhibitors of the Grb2-SH2. It was therefore adopted to replace the keto-acid group of **42** thus leading to **43**. MD reveals the stabilizing role of this group via cation–π interactions but at the cost of the interaction of the carboxamide [91]. As a further step, we have shifted the IZD group to the next position into the pyrrole ring, giving compound **44**. MD now shows



Scheme 2. Preparation of the meta- and ortho-substituted boronic acid **15** and **18**. Reagents and conditions: (a) (i) MeONa (1.1 eq), MeOH, 25 °C, 3 h, (ii) 1-Bromo-3-fluorobenzene (1 eq) or 1-bromo-2-fluorobenzene (1 eq), NMP, 180 °C, 15 h, **13** (43%), **16** (16%); (b) K_2CO_3 (1.5 eq), BnBr (1.5 eq), DMF, 25 °C, 2 h, **14** (84%), **17** (95%); (c) (i) 2 M *n*-BuLi in THF (1.2 eq), −78 °C, 45 min, (ii) $B(OiPr)_3$ (2.5 eq), −78 °C, 1 h then 25 °C, 1 h, (iii) 1 M HCl, 0 °C → 25 °C, 1 h, THF, **15** (81%), **18** (74%).

that its three pharmacophores are involved in Grb2 binding, while retaining the cation–π interactions between, on the one hand, the phenyl D-ring and Arg 67, and, on the other hand, the IZD ring and Arg 142 (Fig. 8).

3. Conclusion

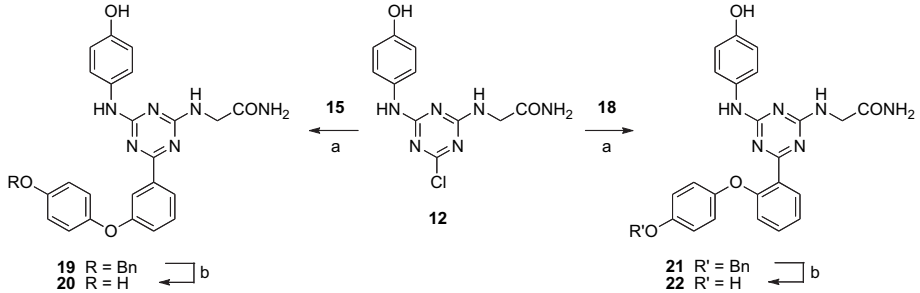
In summary, we have illustrated the syntheses of novel compounds, i.e., the triazinyl-based bis-aryl phosphates **2** and **3**, and the 2-arylaminopyrimidyl-based aryl phosphate **4**. Trifunctionalization of cyanuric chloride allowed us to prepare **2** and **3**. The key step was the Suzuki cross-coupling between the required diaryl ether boronic acid (**15**, **18**) and the 2,4-diamino-6-chloro-1,3,5-triazine derivative **12**. Bis-phosphorylation completed the syntheses. The aminopyrimidine scaffold in **4** was prepared by condensation of aryl guanidine **34** with aryloxyphenyl butenone **31**, this latter being obtained via a Heck reaction. Introduction of the tetrazole and phosphate groups was then successively performed. These designed compounds were evaluated for their ability to bind to the SH2 domain of Grb2. Thus, the water-soluble sodium salt **26** of **3** was found to be a ligand for Grb2 in the high micromolar range ($IC_{50} = 265$ μM). Subsequently, computer-aided step-wise modifications of its 1,3,5-trisubstituted triazine scaffold led to novel potential ligands bearing exclusively phosphate mimics, as exemplified by compounds **42** and **44**. Promising fragments in their construction are a cyclopropane ring, a keto-acid group, and a 1,1-dioxido-isothiazolidin-3-one, for which there are no precedents, to our knowledge, in the design of Grb2-SH2 inhibitors. MD simulations have predicted an enriched pattern of H-bonds and cation–π interactions with this protein. Such preliminary results could open new opportunities in the design of non-peptidic, non-phosphate inhibitors of Grb2-SH2.

4. Experimental section

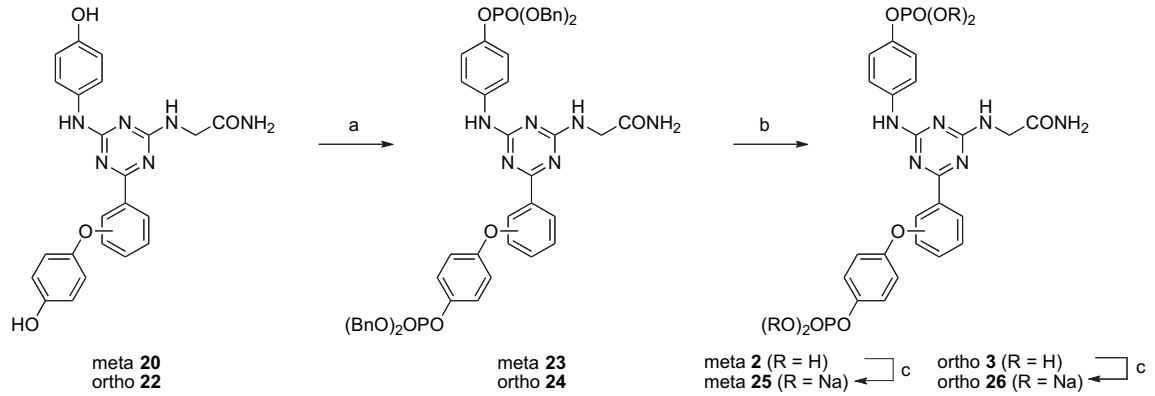
4.1. General information

All commercial reagents were used without purification and all solvents were reaction grade. When necessary, solvents were previously dried over molecular sieves. Tetrahydrofuran was also dried over molecular sieves 4 Å unless otherwise stated (distilled from sodium/benzophenone under argon). All reactions were performed under an inert atmosphere of argon unless otherwise stated. All reaction mixtures were stirred magnetically and monitored by thin-layer chromatography using Merck silica gel 60 F_{254}, visualized with UV light. Flash chromatography were performed using SDS silica gel 60 (35–70 μm). Melting points (uncorrected) were determined on a Kofler bench. Mass spectra were recorded on a Nermag R10-10C MS (CI) or a ZQ 2000 MS (ES) or a MS Station JMS-700 JEOL (FAB).

^1H, ^{13}C and ^{31}P NMR spectra were recorded on a Bruker AM 300 spectrometer. Chemical shift values are reported in parts per million (ppm) and coupling constants in Hertz (Hz). In order to simplify the reading of the NMR spectra interpretations (COSY, HMQC, HMBC), the following attributions were chosen (Fig. 9): Letter "A" will always refer to the heterocyclic core – whether it is a pyrimidine or a triazine – and letter "B" to the aryl group linked to this heterocycle through an NH bridge. "C" and "D" will refer to the diaryl ether and finally "E" will refer to the benzyl groups, either benzyloxy or dibenzylphosphates. Two AA′BB′ systems were



Scheme 3. Suzuki coupling. Reagents and conditions: (a) **15** (1.4 eq) or **18** (1.2 eq), $Pd(PPh_3)_4$ (0.02 eq), K_2CO_3 (3 eq), CH_3CN/H_2O (1:1), reflux, 6 h, **19** (67%), **21** (51%); (b) H_2, Pd/C 10% (10% w/w), MeOH/AcOH (4:1), 25 °C, 4 days, **20** (81%), **22** (80%).



Scheme 4. Reagents and conditions: (a) $HPO(OBn)_2$ (5 eq), DIEA 8 eq, DMAP (1 eq), CH_3CN/CCl_4 5:1, −15 °C, 3 h, **23** (79%) and **24** (69%) from **20** and **22**, respectively; (b) H_2, Pd/C 10% (10% w/w), MeOH, 25 °C, 5 days, **2** (55%), **3** (44%); (c) MeONa (4 eq), MeOH, 0 °C, 15 min then 25 °C, 1 h, **25** (99%), **26** (99%).

observed for the two para-substituted aromatic rings B and D. Compounds **19**, **23** and **35** are described below as examples.

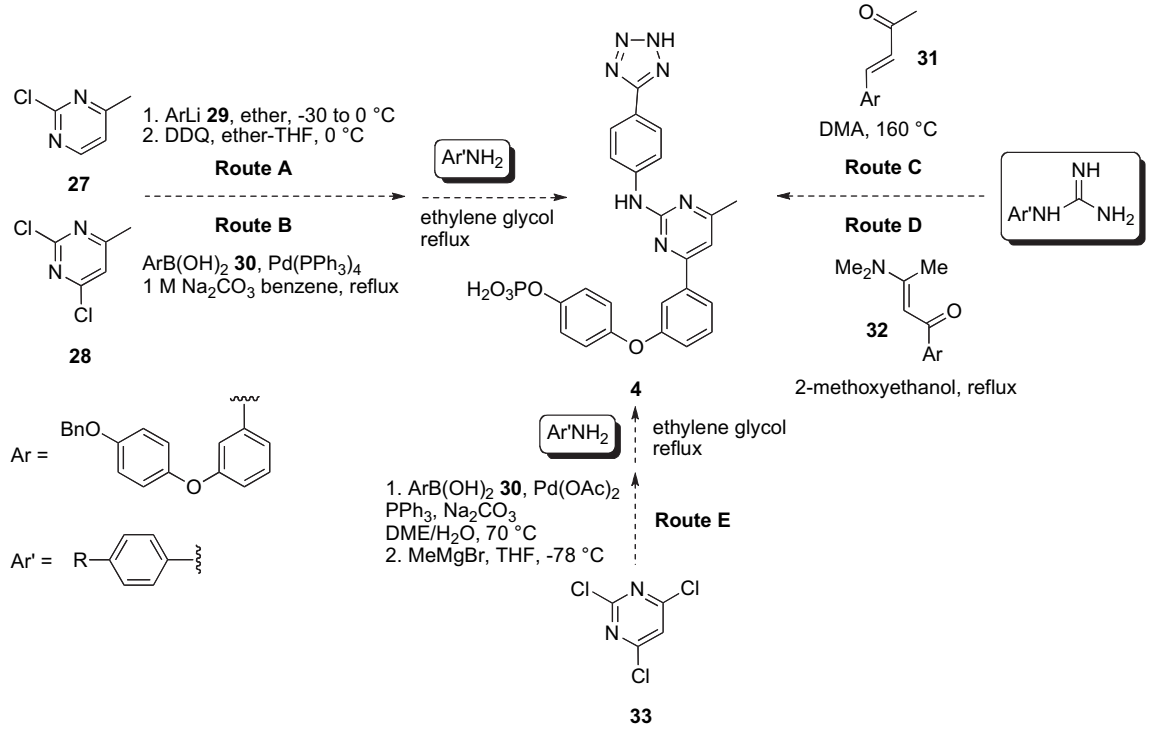
4.2. Experimental procedures

4.2.1. 2-(4,6-Dichloro-1,3,5-triazin-2-ylamino)-acetamide (*11*)

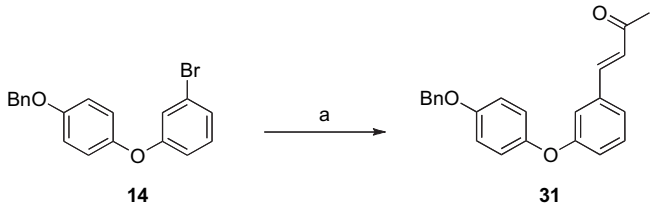
Diisopropylethylamine (700 μL, 4.01 mmol) was added to a solution of cyanuric chloride (334 mg, 1.81 mmol) and glycinamide hydrochloride (200 mg, 1.81 mmol) in dry THF (15 mL) at 0 °C. The mixture was stirred at 0 °C for 1.5 h and the solvent removed under reduced pressure. Purification by flash chromatography with cyclohexane/ethyl acetate (gradient from 50:50 to 0:100) and then methanol/ethyl acetate (5:95) furnished **11** as an off-white powder (296 mg, 74%). Mp 188–190 °C; ^{1}H NMR (300 MHz; THF-d_8): δ 4.01 (s, 1H, CH_2), 4.02 (s, 1H, CH_2), 6.72 (s, 1H, $CONH_2$), 6.92 (s, 1H, $CONH_2$), 9.32 (s, 1H, NH); ^{13}C NMR (75 MHz; THF-d_8): δ 43.4 (CH_2), 166.3 (2_A), 169.6 ($CONH_2$), 170.3 (4_A, 6_A); MS (ES^+) *m*/*z*: 222 $[M+H]^+$, 244 $[M+Na]^+$.

4.2.2. 2-[4-Chloro-6-(4-hydroxyphenylamino)-1,3,5-triazin-2-ylamino]acetamide (*12*)

Diisopropylethylamine (1 mL, 5.74 mmol) was added to a solution of **11** (1.29 g, 5.81 mmol) and 4-aminophenol (634 mg, 5.81 mmol) in dry THF (15 mL) at 0 °C. The mixture was stirred at 0 °C for 30 min and then at room temperature for 1 h. The precipitate was filtered, washed with THF and water and then dried over P_2O_5, yielding to **36** as a strongly insoluble white powder (1.33 g, 77%). Furthermore, the filtrate was extracted with ethyl acetate and



Scheme 5. Synthetic routes envisioned to the pyrimidinyl-based aryl phosphate **4**. **Route A** (see refs [77–79]): Addition of aryloxyphenyllithium **29** to 2-chloro-4-methylpyrimidine **27** followed by DDQ oxidation. **Route B** (see refs [77] and [80]): Suzuki coupling of 2,4-dichloro-6-methylpyrimidine **28** with aryloxyphenyl boronic acid **30**. **Route C** (see ref. [81] and refs cited therein): Condensation of an aryl guanidine with aryloxyphenyl butenone **31**. **Route D** (see ref. [77]): Condensation of an aryl guanidine with β-dimethylamino-butenone **32**. **Route E** (see ref. [82]): Suzuki coupling of 2,4,6-trichloropyrimidine **33** with aryloxyphenyl boronic acid **30**.



Scheme 6. Synthesis of the diaryl ether-substituted α-enone **31**. (a) methylvinylketone (4 eq), $Pd(OAc)_2$ (0.1 eq), PPh_3 (0.2 eq), NEt_3 (8 eq), sealed tube, DMF, 150 °C, 4 h, 83%.

the combined extracts were washed with water and brine, dried over magnesium sulfate and evaporated under reduced pressure to give an additional portion of **12** as a white powder (168 mg, overall yield: 87%). Mp 256 °C with decomposition (recryst. from ethanol/DMF); ^{1}H NMR (300 MHz; DMSO-d_6): δ 3.78 (s, 2H, CH_2), 6.67–6.69 (m, 2H, 2_B, 6_B), 7.06 (br s, 1H, NH), 7.42–7.45 (m, 2H, 3_B, 5_B), 8.08 (br s, 1H, NH), 9.20 (br s, 1H, NH), 9.84 (br s, 1H, NH); ^{13}C NMR (75 MHz; DMSO-d_6): δ 43.5 (CH_2), 115.1 (2_B, 6_B), 121.8 (3_B, 5_B), 130.3 (1_B), 153.2 (4_B), 163.1 (4_A or 6_A), 165.9 (2_A or $CONH_2$), 167.6 (4_A or 6_A), 170.6 (2_A or $CONH_2$); MS (ES^+) m/z: 295 $[M+H]^+$, 317 $[M+Na]^+$ MS (ES^-) m/z: 293 $[M-H]^-$.

*4.2.3. 3-(4-Hydroxyphenoxy)-1-bromobenzene (**13**)*

A 4.6 M solution of sodium methoxide (2.17 mL, 9.98 mmol) was added to a solution of hydroquinone (1 g, 9.08 mmol) in dry methanol (2 mL). The mixture was stirred at room temperature for 3 h and the methanol was removed under reduced pressure. Dry *N*-methylpyrrolidinone (8 mL) and 1-bromo-3-fluorobenzene (1 mL, 8.92 mmol) were added and the mixture was heated at 180 °C for 15 h. Water was then added and the mixture was extracted with ether. The combined organic extracts were washed with water and brine, dried over magnesium sulfate and evaporated under reduced

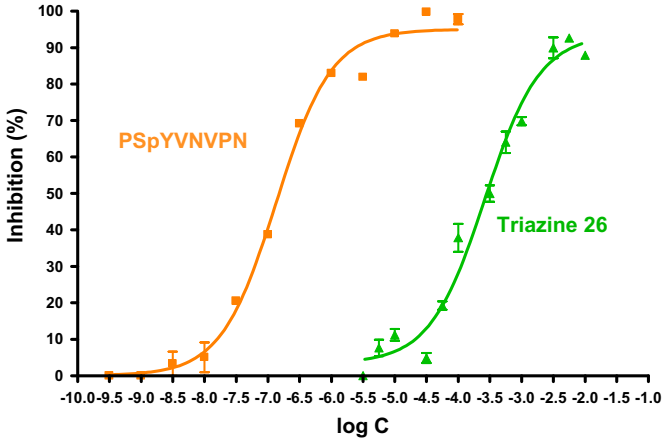
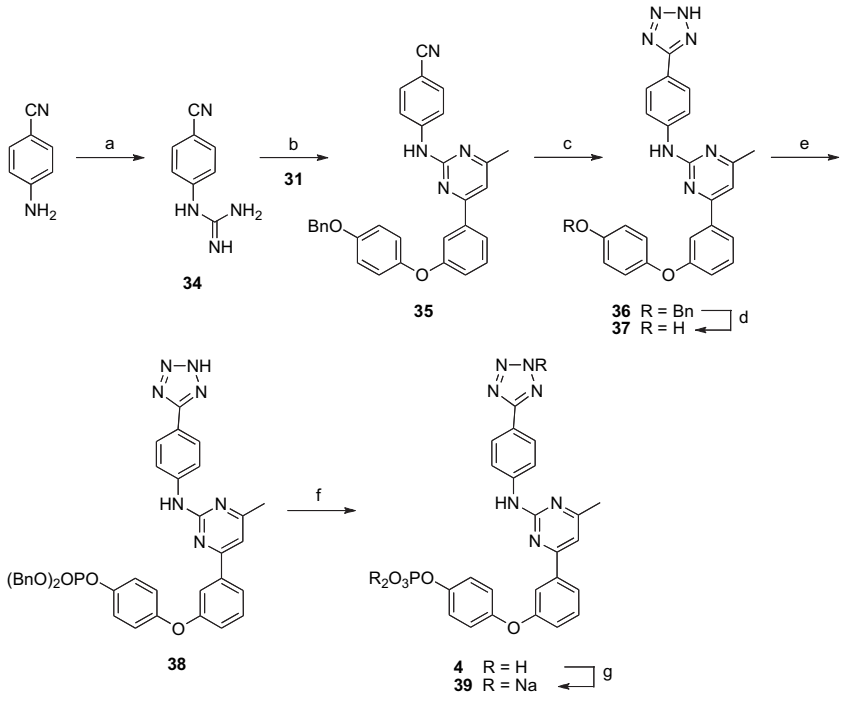


Fig. 4. Competitive ELISA assay (PSpYVNVPN as the reference peptide).

pressure. After a purification by flash chromatography with cyclohexane/ethyl acetate (gradient from 99:1 to 90:10), 4-aryloxyphenol **13** was obtained as a colourless oil (1.02 g, 43%). ^{1}H NMR (300 MHz; $CDCl_3$): δ 4.96 (s, 1H, OH), 6.82–6.86 (m, 2H, 3_D, 5_D), 6.86–6.88 (m, 1H, 4_C), 6.92–6.95 (m, 2H, 2_D, 6_D), 7.05–7.07 (m, 1H, 2_C), 7.15–7.17 (m, 2H, 5_C, 6_C); ^{13}C NMR (75 MHz; $CDCl_3$): δ 116.1 (4_C), 116.5 (3_D, 5_D), 120.5 (2_C), 121.4 (2_D, 6_D), 122.8 (1_C), 125.4 (6_C), 130.7 (5_C), 149.3 (1_D), 152.2 (4_D), 159.4 (3_C); MS (ES^-) m/z: 263 $[M-H]^-$, 265 $[M-H]^-$.

*4.2.4. 3-(4-Benzyloxyphenoxy)-1-bromobenzene (**14**)*

Potassium carbonate (2.4 g, 17.4 mmol) was added to a solution of **13** (3 g, 11.3 mmol) in dry DMF (50 mL). Benzyl bromide (2.1 mL, 17.6 mmol) was then added and the mixture was stirred at room



Scheme 7. Synthesis of the 2-arylaminopyrimidinyl-based phosphate **4** and its sodium salt **39**. Reagents and conditions: (a) (i) H_2NCN (1 eq), HNO_3 (1.6 eq), EtOH, reflux, 6 h, (ii) NaOH (1 eq), H_2O, reflux, 5 min, 15%; (b) **34** (3 eq) and **31** (1 eq), DMA, 160 °C, 6 h, 81% based on compound 31; (c) Me_3SiN_3 (1.5 eq), $Bu_4NF.3H_2O$ (0.5 eq), solventless, 120 °C, 6 h, 89%; (d) H_2, Pd/C 10% (10% w/w), MeOH/CH_2Cl_2 (1:1), 25 °C, 24 h, 90%; (e) $HPO(OBn)_2$ (5 eq), DIEA (7 eq), DMAP (0.5 eq), CH_3CN/CCl_4 (5:1), −15 °C, 2.5 h, 61%; (f) H_2, Pd/C 10% (10% w/w), MeOH, 25 °C, 10 days, 49% (g) MeONa (3 eq), MeOH, 0 °C, 1 h, 100%.

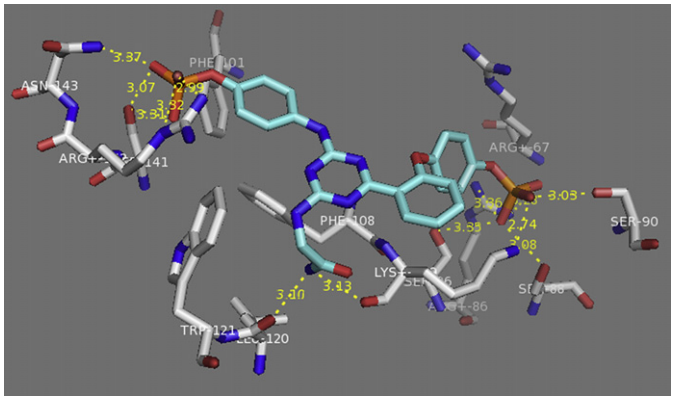


Fig. 5. Predicted binding mode of compound **26** to Grb2-SH2. The atoms of **26** are colored with carbon in cyan, oxygen in red, nitrogen in blue, and phosphorus in orange. Amino acid residues important for binding are labeled in grey. Intermolecular hydrogen bonds are shown by yellow dashed lines. The amino acid residues involved in interaction are Asn 143, Arg 142, Ser 141, Leu 120, Lys 109, Ser 96, Arg 86, Ser 88 and Ser 90 (from left to right) This illustration was prepared by PyMOL (Delano Scientific, San Carlos, CA).

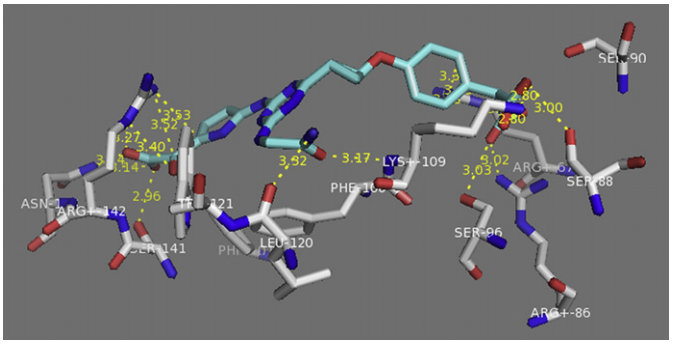


Fig. 7. Predicted binding mode of compound **42**. The amino acid residues involved in interaction are Asn 143, Arg 142, Ser 141, Leu 120, Lys 109, Ser 96, Arg 86, Ser 88 and Arg 67 (from left to right).

temperature for 2 h. Water was added and the mixture was extracted with ether. The combined extracts were washed with water and brine, dried over magnesium sulfate and evaporated under reduced pressure. Purification by flash chromatography with cyclohexane/ethyl acetate (100:1) gave **14** as a white powder (3.39 g, 84%). Mp 96 °C (recryst. from ethyl acetate/pentane). ^{1}H NMR (300 MHz; $CDCl_3$): δ 5.06 (s, 2H, CH_2), 6.87–6.91 (m, 1H, 4_C), 6.99 (s, 4H, 2_D, 3_D, 5_D, 6_D), 7.08–7.09 (m, 1H, 2_C), 7.15–7.17 (m, 2H, 5_C, 6_C), 7.34–7.47 (m, 5H, 2_E, 3_E, 4_E, 5_E, 6_E); ^{13}C NMR (75 MHz; $CDCl_3$): δ 70.4 (CH_2), 116.1 (3_D, 5_D), 116.2 (4_C), 120.5 (2_C), 121.2 (2_D, 6_D), 122.8 (1_C), 125.4 (6_C), 127.5 (2_E, 6_E), 128.1 (4_E), 128.6 (3_E, 5_E), 130.7 (5_C), 136.9 (1_E), 149.5 (1_D), 155.6 (4_D), 159.4 (3_C). Anal. Calcd for $C_{19}H_{15}BrO_2$: C, 64.24; H, 4.26. Found: C, 64.17; H, 4.37.

4.2.5. 3-(4-Benzyloxyphenoxy)phenylboronic acid (**15**)

A solution of 2 M *n*-butyllithium in THF (3.8 mL, 7.8 mmol) was added dropwise to a solution of compound **14** (2.49 g, 7.01 mmol) in dry THF (Na/benzophenone) (25 mL) at −78 °C. The resulting mixture was stirred at −78 °C for 45 min and triisopropyl borate (4.0 mL, 17.3 mmol) was added. The mixture was again stirred at −78 °C for 1 h and then at room temperature for a further 1 h. Ether (25 mL) and 1 M HCl (25 mL) were added at 0 °C. The mixture was stirred at 0 °C for 10 min, at room temperature for 1 h and then the mixture was extracted with ether. The combined extracts were washed with water and brine, dried over magnesium sulfate and evaporated under reduced pressure to give a white solid. Purification by flash chromatography with cyclohexane/ethyl acetate (gradient from 80:20 to 0:100) and then methanol/ethyl acetate (5:95) afforded **15** as a white powder (1.81 g, 81%). Mp 162 °C; ^{1}H NMR (300 MHz; $CDCl_3$): δ 5.06 (s, 2H, CH_2), 6.98–7.01 (m, 4H, 2_D, 3_D, 5_D, 6_D), 7.15 (dd, $J_o = 8.0$ Hz, $J_m = 1.7$ Hz, 1H, 4_C), 7.34–7.46 (m, 6H, 2_E, 3_E, 4_E, 5_E, 6_E, 5_C), 7.86 (m, 1H, 2_C), 7.89–7.91 (m, 1H, 6_C); ^{13}C NMR (75 MHz; $CDCl_3$): δ 70.6 (CH_2), 116.0 (3_D, 5_D), 120.5 (2_D, 6_D), 122.2 (4_C), 125.1 (2_C), 127.6 (2_E, 6_E), 128.0 (4_E), 128.7 (3_E, 5_E), 129.5 (5_C), 130.0 (6_C), 131.9 (1_C), 137.0 (1_E), 150.7 (1_D), 155.1 (4_D), 158.0 (3_C); MS (CI) *m*/*z*: 321 $[M+H]^+$, 338 $[M+NH_4]^+$.

4.2.6. 2-(4-Hydroxyphenoxy)-1-bromobenzene (**16**)

Compound **16** was prepared as **13,** from hydroquinone (12 g, 109 mmol) and 1-bromo-2-fluorobenzene (12 mL, 110 mmol). Purification by flash chromatography with cyclohexane/ethyl acetate (gradient from 98:2 to 90:10) gave **16** as a white powder (4.6 g, 16%). Mp 85 °C (recryst. from ethyl acetate/pentane); ^{1}H NMR (300 MHz; $CDCl_3$): δ 4.92 (br s, 1H, OH), 6.80–6.84 (m, 3H, 3_D, 5_D, 3_C), 6.88–6.93 (m, 2H, 2_D, 6_D), 6.85 (dd, $J_o = 7.9$ Hz, $J_m = 1.5$ Hz, 1H, 5_C), 7.21 (ddd, $J_o = 8.1$ Hz, $J_o = 7.9$ Hz, $J_m = 1.5$ Hz, 1H, 4_C), 7.60 (dd, $J_o = 7.8$ Hz, $J_m = 1.5$ Hz, 1H, 6_C); ^{13}C NMR (75 MHz; $CDCl_3$): δ 113.7 (1_C), 116.4 (3_D, 5_D), 118.8 (3_C), 120.4 (2_D, 6_D), 124.2 (5_C), 128.5 (4_C), 133.7 (6_C), 150.1 (1_D), 151.8 (4_D), 154.8 (2_C); MS (ES^-) *m*/*z*: 263 $[M-H]^-$; Anal. Calcd for $C_{12}H_9BrO_2$: C, 54.27; H, 3.42. Found C, 54.57; H, 3.39.

4.2.7. 2-(4-Benzyloxyphenoxy)-1-bromobenzene (**17**)

Potassium carbonate (3.2 g, 23.1 mmol) was added to a solution of **16** (4.09 g, 15.4 mmol) in dry DMF (50 mL). Benzyl bromide

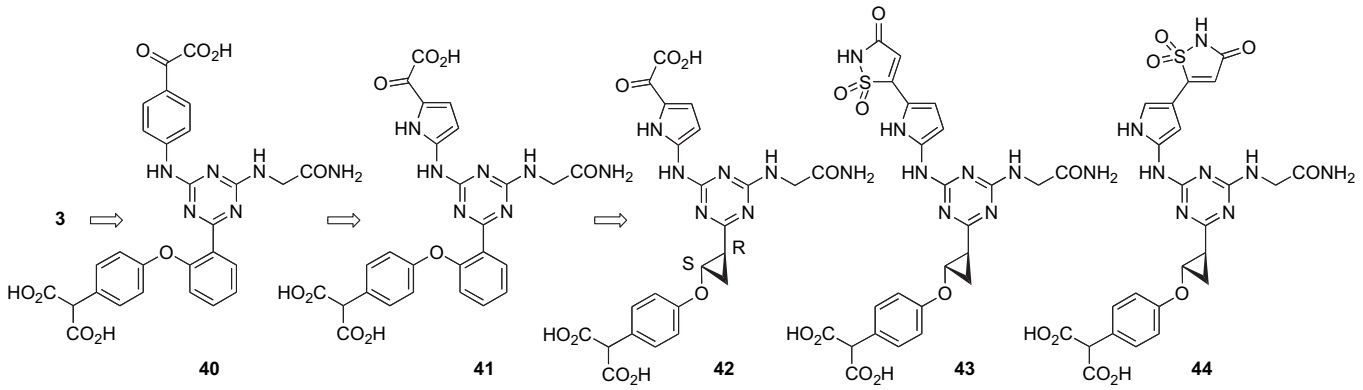


Fig. 6. De novo design from the heterocycle scaffold of **3**: generation of compounds **40**–**44**.

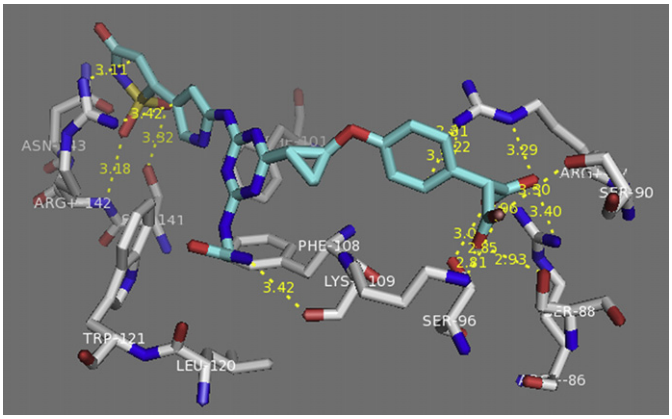


Fig. 8. Predicted binding mode of compound **44**. The amino acid residues involved in interaction are Arg 142, Ser 141, Lys 109, Ser 96, Arg 86, Ser 88, Arg 67 and Ser 90.

(2.7 mL, 23.1 mmol) was then added and the mixture was stirred at room temperature for 2 h. Water was added and the mixture was extracted with ether. The combined extracts were washed with water and brine, dried over magnesium sulfate and evaporated under reduced pressure to give an off-white solid. Purification by flash chromatography with cyclohexane/ethyl acetate (100:0.5) furnished **17** as a white powder (5.18 g, 95%). Mp 71 °C (recryst. from ethyl acetate/pentane); ^{1}H NMR (300 MHz; $CDCl_3$): δ 5.07 (s, 2H, CH_2), 6.89 (dd, $J_o = 8.1$ Hz, $J_m = 1.4$ Hz, 1H, 3_C), 6.96–7.00 (m, 5H, 5_C, 2_D, 3_D, 5_D, 6_D), 7.23 (ddd, $J_{o\ 4-3} = 8.1$ Hz, $J_{o\ 4-5} = 7.4$ Hz, $J_m = 1.5$ Hz, 1H, 4_C), 7.36–7.48 (m, 5H, 2_E, 3_E, 4_E, 5_E, 6_E), 7.64 (dd, $J_o = 7.9$ Hz, $J_m = 1.5$ Hz, 1H, 6_C); ^{13}C NMR (75 MHz; $CDCl_3$): δ 70.6 (CH_2), 113.9 (1_C), 116.0 (3_D, 5_D), 119.0 (3_C), 120.2 (2_D, 6_D), 124.2 (5_C), 127.5 (2_E, 6_E), 128.1 (4_E), 128.4 (4_C), 128.6 (3_E, 5_E), 133.4 (6_C), 137.0 (1_E), 150.3 (1_D), 154.9 (2_C), 155.3 (4_D); MS (ES^+) m/z: 377 $[M + Na]^+$, 393 $[M + K]^+$; Anal. Calcd for $C_{19}H_{15}BrO_2$: C, 64.24; H, 4.26. Found: C, 64.14; H, 4.23.

4.2.8. 2-(4-Benzyloxyphenoxy)phenylboronic acid (**18**)

Compound **18** was prepared as **15**, i.e. from **17** (4.4 g, 12.4 mmol) instead of **14**. Purification by flash chromatography with cyclohexane/ethyl acetate (2:1) afforded **18** as a white powder (2.94 g, 74%). Mp 100 °C (recryst. from ethyl acetate/pentane); ^{1}H NMR (300 MHz; $CDCl_3$): δ 5.08 (s, 2H, CH_2), 6.67 (d, $J_o = 8.3$ Hz, 1H, 3_C), 7.02 (m, 4H 2_D, 3_D, 5_D, 6_D), 7.09 (ddd, $J_o = 7.3$ Hz, $J_o = 7.3$ Hz, $J_m = 0.8$ Hz, 1H, 5_C), 7.33 (m, $J_o = 7.3$ Hz, $J_m = 1.7$ Hz, 1H, 4_C), 7.35–7.47 (m, 5H, 2_E, 3_E, 4_E, 5_E, 6_E), 7.92 (dd, $J_o = 7.3$ Hz, $J_m = 1.7$ Hz, 1H, 6_C); ^{13}C NMR (75 MHz; $CDCl_3$): δ 70.5 (CH_2), 116.1 (3_C), 116.1 (3_D, 5_D), 121.9 (2_D, 6_D), 122.6 (5_C), 127.5 (2_E, 6_E), 128.1 (4_E), 128.7 (3_E, 5_E), 132.7 (4_C), 136.8 (1_C), 136.8, 136.9 (6_C, 1_E), 148.6 (1_D), 156.0 (4_D), 164.3 (2_C); MS (ES^-) m/z: 319 $[M - H]^-$; Anal. Calcd for $C_{19}H_{17}BO_4$: C, 71.28; H, 5.35. Found: C, 70.59; H, 5.36.

4.2.9. 2-[4-[3-(4-Benzyloxyphenoxy)-phenyl]-6-(4-hydroxyphenylamino)-1,3,5-triazin-2-ylamino]-acetamide (**19**)

Triazine **12** (304 mg, 1.03 mmol), boronic acid **15** (463 mg, 1.45 mmol) and potassium carbonate (409 mg, 3.09 mmol) were placed into an acetonitrile/water mixture (30 mL, 1:1). The solvent was then degassed as follows: the mixture was frozen in liquid nitrogen, placed under high vacuum for 5 min to remove dissolved gases, and then purged with argon. The operation was reiterated twice before addition of palladium tetrakis(triphenylphosphine) (24 mg, 0.021 mmol). The mixture was then refluxed for 6 h, filtrated upon celite to remove the catalyst and evaporated under reduced pressure to give a brown solid. Purification by flash chromatography with ethyl acetate/methanol (gradient from 100:0 to 92:8) gave **19** as a beige powder (370 mg, 67%). Mp 101–103 °C; ^{1}H NMR (300 MHz; MeOD): δ 4.02 (s, 2H, CH_2–$CONH_2$), 5.06 (s, 2H, CH_2–Ph), 6.72–6.75 (m, 2H, 3_B, 5_B), 6.99–7.00 (m, 4H, 2_D, 3_D, 5_D, 6_D), 7.09–7.11 (m, 1H, 4_C), 7.27–7.46 (m, 8H, 2_E, 3_E, 4_E, 5_E, 6_E, 2_B, 6_B, 5_C), 7.89 (s, 1H, 2_C), 8.04 (br d, $J_o = 7.4$ Hz, 1H, 6_C); ^{13}C NMR (75 MHz; MeOD): δ 44.9 (CH_2–$CONH_2$), 71.5 (CH_2–Ph), 116.1 (3_B, 5_B), 117.1 (3_D, 5_D), 118.2 (2_C), 121.9 (2_D, 6_D, 4_C), 123.3 (2_B, 6_B), 123.5 (6_C), 127.3 (5_C), 128.7 (2_E, 6_E), 128.9 (4_E), 129.5 (3_E, 5_E), 132.6 (1_B), 138.7 (1_E), 140.1 (1_C), 151.5 (1_D), 154.3 (4_B), 156.7 (4_D), 160.0 (3_C), 165.9 (4_A or 6_A), 167.8 (2_A), 171.7 (4_A or 6_A), 176.0 ($CONH_2$); MS (CI) m/z: 535 $[M + H]^+$.

4.2.10. 2-(4-(3-(4-Hydroxyphenoxy)phenyl)-6-(4-hydroxyphenylamino)-1,3,5-triazin-2-ylamino)-acetamide (**20**)

10% Palladium on carbon (24 mg), was added to a solution of **19** (242 mg, 0.453 mmol) in methanol/acetic acid (25 mL, 4:1). The mixture was stirred under H_2 at room temperature for 4 days, filtrated upon celite to remove the catalyst and evaporated under reduced pressure to give a yellow oil. Purification by flash chromatography with a gradient from ethyl acetate/cyclohexane (75:25) to ethyl acetate/methanol (95:5) gave **20** as white solid (162 mg, 80%). Mp 236–238 °C; ^{1}H NMR (300 MHz; MeOD): δ 4.02 (s, 2H, CH_2–$CONH_2$), 6.72–6.75 (m, 2H, 3_B, 5_B), 6.79–6.82 (m, 2H, 3_D, 5_D), 6.88–6.91 (m, 2H, 2_D, 6_D), 7.06 (d, $J_o = 8.4$ Hz, 1H, 4_C), 7.33–7.37 (m, 1H, 5_C), 7.42–7.45 (m, 2H, 2_B, 6_B), 7.87 (s, 1H, 2_C), 8.02 (d, $J_o = 7.5$ Hz, 1H, 6_C); ^{13}C NMR (300 MHz; MeOD): δ 44.9 (CH_2–$CONH_2$), 116.2 (3_B, 5_B), 117.3 (3_D, 5_D, 2_C), 121.5 (4_C), 122.1 (2_D, 6_D), 123.3 (2_B, 6_B, 6_C), 130.4 (5_C), 132.6 (1_B), 140.0 (1_C), 150.3 (1_D), 154.3 (4_B), 155.0 (4_D), 160.4 (3_C), 165.8 (4_A or 6_A), 167.8 (2_A), 171.7 (4_A or 6_A), 175.9 ($CONH_2$); MS (ES^-) m/z: 443 $[M - H]^-$; MS (ES^+) m/z: 445 $[M + H]^+$.

4.2.11. 2-(4-(2-(4-Benzyloxyphenoxy)phenyl)-6-(4-hydroxyphenylamino)-1,3,5-triazin-2-ylamino)-acetamide (**21**)

Compound **21** was prepared as **19**, from triazine **12** (54 mg, 0.183 mmol) and boronic acid **18** (70 mg, 0.219 mmol). Purification

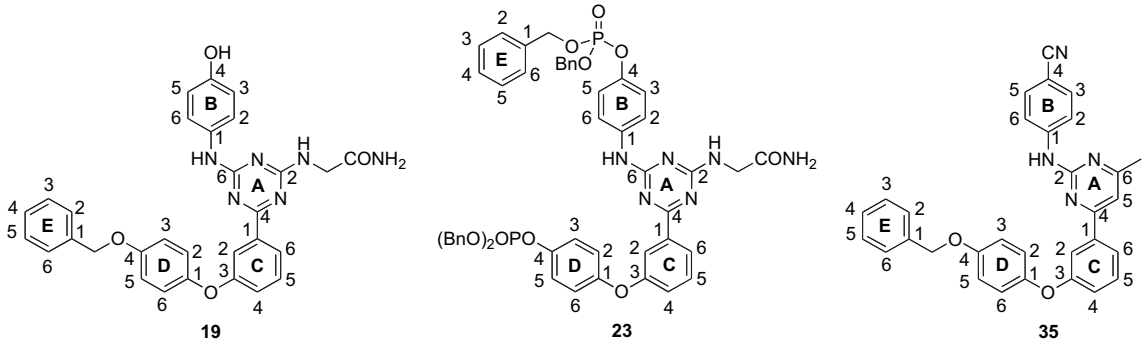


Fig. 9.

by flash chromatography with ethyl acetate afforded **21** as a beige powder (50 mg, 51%). Mp 115–118 °C; ^{1}H NMR (300 MHz; MeOD): δ 3.95 (s, 2H, CH_2–CONH$_2$), 4.99 (s, 2H, CH_2–Ph), 6.68 (br s, 2H, 3_B, 5_B), 6.86–6.92 (m, 5H, 2_D, 3_D, 5_D, 6_D, 3_C), 7.14 (m, 1H, 5_C), 7.27–7.41 (m, 8H, 2_E, 3_E, 4_E, 5_E, 6_E, 2_B, 6_B, 4_C), 7.72–7.74 (m, 1H, 6_C); ^{13}C NMR (75 MHz; MeOD): δ 44.8 (CH_2–CONH$_2$), 71.5 (CH_2–Ph), 116.1 (3_B, 5_B), 117.0 (3_D, 5_D), 119.6 (3_C), 121.5 (2_D, 6_D), 123.7 (2_B, 6_B, 5_C), 128.6 (2_E, 6_E), 128.9 (4_E), 129.5 (3_E, 5_E), 130.4 (1_C), 132.1 (1_B, 6_C), 132.5 (4_C), 138.7 (1_E), 152.1 (1_D), 154.6 (4_B), 156.4 (4_D), 157.6 (2_C), 165.1 (6_A), 167.0 (2_A), 173.0 (4_A), 175.4 (CONH$_2$); MS (ES$^+$) *m/z*: 535 [M + H]$^+$, 557 [M + Na]$^+$; MS (ES$^-$) *m/z*: 533 [M − H]$^-$.

4.2.12. 2-(4-(2-(4-Hydroxyphenoxy)phenyl)-6-(4-hydroxyphenylamino)-1,3,5-triazin-2-ylamino)-acetamide (**22**)

10% Palladium on carbon (22 mg), was added to a solution of **21** (223 mg, 0.417 mmol) in acetic acid (22 mL). The mixture was stirred under H_2 at room temperature for 48 h, filtrated upon celite to remove the catalyst and evaporated under reduced pressure to give a yellow oil. Purification by flash chromatography with ethyl acetate/methanol (gradient from 99:1 to 95:5) gave **22** as a white solid (149 mg, 80%). Mp 148 °C; ^{1}H NMR (300 MHz; MeOD): δ 3.95 (s, 2H, CH_2–CONH$_2$), 6.69–6.77 (m, 4H, 3_B, 5_B, 3_D, 5_D), 6.83–6.89 (m, 3H, 2_D, 6_D, 3_C), 7.11 (br t, J_o = 7.4 Hz, 1H, 5_C), 7.32–7.41 (m, 3H, 2_B, 6_B, 4_C), 7.70–7.74 (m, 1H, 6_C); ^{13}C NMR (75 MHz; MeOD): δ 44.8 (CH_2–CONH$_2$), 116.1 (3_B, 5_B), 117.1 (3_D, 5_D), 118.9 (3_C), 121.7 (2_D, 6_D), 123.4 (5_C), 123.7 (2_B, 6_B), 130.0 (1_C), 132.1 (1_B, 6_C), 132.5 (4_C), 150.5 (1_D), 154.6 (4_B), 156.8 (4_D), 158.1 (2_C), 165.0 (6_A), 167.0 (2_A), 173.1 (4_A), 175.6 (CONH$_2$); MS (ES$^+$) *m/z*: 445 [M + H]$^+$; MS (ES$^-$) *m/z*: 443 [M − H]$^-$.

4.2.13. 2-[4-(3-(4-Dibenzylphosphoryloxyphenoxy)phenyl)-6-(4-dibenzylphosphoryloxyphenylamino)-1,3,5-triazin-2-ylamino]acetamide (**23**)

Compound **23** was prepared from **20** (84 mg, 0.189 mmol), diisopropylethylamine (265 μL, 1.52 mmol), *N*,*N*-dimethylaminopyridine (23 mg, 0.188 mmol) and dibenzyl phosphite (210 μL, 0.951 mmol). Purification by flash chromatography with ethyl acetate afforded **23** as a colourless oil (144 mg, 79%). ^{1}H NMR (300 MHz; CDCl$_3$): δ 3.99 (s, 2H, CH_2–CONH$_2$), 5.09–5.14 (m, 8H, 4 × O–CH_2–C$_6$H$_5$), 6.96–7.08 (m, 6H, 2_D, 3_D, 5_D, 6_D, 3_B, 5_B), 7.30–7.32 (m, 21H, 4 × 2_E, 4 × 3_E, 4 × 4_E, 4 × 5_E, 4 × 6_E, 5_C), 7.50–7.53 (m, 2H, 2_B, 6_B), 7.74 (m, 1H, 4_C), 7.90 (s, 1H, 2_C), 8.06 (m, 1H, 6_C); ^{13}C NMR (75 MHz; CDCl$_3$): δ 44.3 (CH_2–CONH$_2$), 69.9 (O–CH_2–C$_6$H$_5$), 70.0 (O–CH_2–C$_6$H$_5$), 117.0 (2_C), 118.2 (4_C), 120.1, 120.3, 121.0 (3_D, 5_D, 3_B, 5_B, 2_D, 6_D), 121.5 (2_B, 6_B), 123.2 (6_C), 128.0 (2_E, 6_E), 128.6 (4_E), 128.8 (3_E, 5_E), 129.8 (5_C), 135.4 (1_E), 135.9 (1_B), 138.4 (1_C), 145.9 (4_B), 146.2 (1_D), 153.6 (4_D), 157.5 (3_C), 164.4, 166.1 (2_A, 6_A), 170.6 (4_A), 172.3 (CONH$_2$); MS (ES$^+$) *m/z*: 987 [M + Na]$^+$; MS (ES$^-$) *m/z*: 963 [M − H]$^-$.

4.2.14. 2-(4-(2-(4-Dibenzylphosphoryloxyphenoxy)phenyl)-6-(4-dibenzylphosphoryloxyphenylamino)-1,3,5-triazin-2-ylamino)acetamide (**24**)

Compound **24** was prepared as **23**, from **22** (126 mg, 0.284 mmol), diisopropylethylamine (300 μL, 1.72 mmol), *N*,*N*-dimethylaminopyrimidine (35 mg, 0.287 mmol) and dibenzyl phosphite (250 μL, 1.13 mmol). Purification by flash chromatography with ethyl acetate afforded **24** as a colourless oil (189 mg, 69%). ^{1}H NMR (300 MHz; CDCl$_3$): δ 3.43 (s, 2H, CH_2–CONH$_2$), 5.09 (s, 4H, 2 × O–CH_2–C$_6$H$_5$), 5.12 (s, 4H, 2 × O–CH_2–C$_6$H$_5$), 6.84–6.86 (m, 2H, 3_D, 5_D), 7.01–7.08 (m, 5H, 2_D, 6_D, 3_B, 5_B, 5_C), 7.31–7.35 (m, 21H, 4 × 2_E, 4 × 3_E, 4 × 4_E, 4 × 5_E, 4 × 6_E, 3_C), 7.48–7.54 (m, 3H, 2_B, 6_B, 4_C), 7.94–7.96 (m, 1H, 6_C); ^{13}C NMR (75 MHz; CDCl$_3$): δ 42.7 (CH_2–CONH$_2$), 69.9 (O–CH_2–C$_6$H$_5$), 70.0 (O–CH_2–C$_6$H$_5$), 117.7 (3_D, 5_D), 118.8 (3_C), 120.4 (3_B, 5_B), 121.0 (2_D, 6_D), 121.4 (2_B, 6_B), 122.9 (5_C), 125.1 (1_C), 128.0 (2_E, 6_E), 128.7 (4_E), 128.9 (3_E, 5_E), 131.5 (6_C), 132.0 (4_C), 135.4, 135.5 (1_E, 1_B), 144.5 (1_D), 146.1 (4_B), 153.9 (2_C), 157.1 (4_D), 164.1 (6_A), 165.4 (2_A), 171.2 (4_A), 171.6 (CONH$_2$); MS (ES$^+$) *m/z*: 965 [M + H]$^+$, 987 [M + Na]$^+$.

4.2.15. 2-(4-(3-(4-Phosphoryloxyphenoxy)phenyl)-6-(4-phosphoryloxyphenylamino)-1,3,5-triazin-2-ylamino)acetamide (**2**)

10% Palladium on carbon (9 mg) was added to a solution of compound **23** (90 mg, 0.093 mmol) in methanol (10 mL), and the mixture was stirred under H_2 for 3 days at room temperature. The catalyst was filtered using two filter papers that were washed with methanol and THF – giving only impurities – and then with warm water, for the solubility of compound **2** was very low. The latter filtrate was then evaporated under reduced pressure to give a white solid (41 mg, 73%). A portion of this product (25 mg) was purified via semi-preparative HPLC (X-Terra C$_{18}$ MS 5 μm silica, 150 × 19 mm column, NH$_{3\ aq}$ buffer pH 9/CH$_3$CN, gradient from 90:10 to 50:50) and compound **2** was obtained as a white solid (19 mg, 55%). Mp > 250 °C; MS (ES$^+$) *m/z*: 605 [M + H]$^+$, 627 [M + Na]$^+$; HRMS (ES$^-$) *m/z*: calcd for C$_{23}$H$_{22}$N$_6$O$_{10}$P$_2$ 603.0794 [M − H]$^-$; found 603.0821.

4.2.16. 2-(4-(2-(4-Phosphoryloxyphenoxy)phenyl)-6-(4-phosphoryloxyphenylamino)-1,3,5-triazin-2-ylamino)acetamide (**3**)

Compound **3** was prepared as compound **2**, from **24** (87 mg, 0.090 mmol) and 10% palladium on carbon (9 mg). The aqueous filtrate was evaporated under reduced pressure to give **3** as a white solid (40 mg, 74%). A portion of this product (25 mg) was purified via semi-preparative HPLC (X-Terra C$_{18}$ MS 5 μm silica, 150 × 19 mm column, NH$_{3\ aq}$ buffer pH 9/CH$_3$CN, gradient from 90:10 to 50:50) and compound **3** was obtained as a white solid (15 mg, 44%). Mp 238–240 °C (decomposition); MS (ES$^-$) *m/z*: 523 [M − PO$_3$H$_2$]$^-$, 603 [M − H]$^-$, 625 [M + Na]$^-$.

4.2.17. Sodium 2-(4-(3-(4-phosphonatooxyphenoxy)phenyl)-6-(4-phosphonatooxyphenylamino)-1,3,5-triazin-2-ylamino)acetamide (**25**)

Sodium methoxide (6.8 mg, 0.126 mmol) was added to a suspension of compound **2** (19 mg, 0.031 mmol) in dry methanol (8 mL) at 0 °C. The mixture was stirred at 0 °C for 15 min and then at room temperature for 1 h. Methanol was removed under reduced pressure to afford **25** as a water-soluble white powder (21.5 mg, 99%). ^{1}H NMR (300 MHz; D$_2$O): δ 4.05 (s, 2H, CH_2–CONH$_2$), 7.08–7.11 (m, 2H, 3_D, 5_D), 7.24 (m, 5H, 2_D, 6_D, 3_B, 5_B, 4_C), 7.48 (m, 3H, 2_B, 6_B, 5_C), 7.75 (s, 1H, 2_C), 7.89–7.90 (m, 1H, 6_C); ^{13}C NMR (75 MHz; D$_2$O): δ 43.4 (CH_2–CONH$_2$), 117.0 (2_C), 120.5 (3_D, 5_D, 3_B, 5_B), 121.2 (4_C), 121.7 (2_D, 6_D), 122.8 (2_B, 6_B, 6_C), 130.0 (5_C), 132.0 (1_B), 137.2 (1_C), 150.3, 150.7 (1_D, 4_D, 4_B), 157.9 (3_C), 161.2 (6_A), 165.8 (2_A), 171.1 (4_A), 175.5 (CONH$_2$); ^{31}P NMR (121 MHz; D$_2$O): δ 0.67; MS (ES$^+$) *m/z*: 693 [M + H]$^+$, 714 [M + Na]$^+$, 737 [M + 2Na]$^+$.

4.2.18. Sodium 2-(4-(2-(4-phosphonatooxyphenoxy)phenyl)-6-(4-phosphonatooxyphenylamino)-1,3,5-triazin-2-ylamino)acetamide (**26**)

Compound **26** was prepared as **25**, from **3** (15 mg, 0.025 mmol) and sodium methoxide (5.4 mg, 0.100 mmol). **26** was obtained as a water-soluble white powder (17 mg, 99%). ^{1}H NMR (300 MHz; D$_2$O): δ 3.74 (s, 2H, CH_2–CONH$_2$), 6.75–6.81 (m, 2H, 3_D, 5_D), 6.94–6.97 (m, 3H, 2_D, 6_D, 3_C), 6.99–7.02 (m, 2H, 3_B, 5_B), 7.10–7.15 (m, 1H, 5_C), 7.22–7.25 (m, 2H, 2_B, 6_B), 7.34–7.39 (m, 1H, 4_C), 7.48–7.51 (m, 1H, 6_C); ^{13}C NMR (75 MHz; D$_2$O): δ 43.3 (CH_2–CONH$_2$), 118.5 (3_C), 119.3 (3_D, 5_D), 120.5 (3_B, 5_B), 121.4 (2_D, 6_D), 123.4 (2_B, 6_B), 123.7 (5_C), 127.0 (1_C), 130.5 (6_C), 130.7 (1_B), 131.7 (4_C), 149.7 (1_D), 150.7 (4_B), 151.6 (4_D), 154.8 (2_C), 161.6 (6_A), 165.0 (2_A), 171.9 (4_A), 174.8 (CONH$_2$); ^{31}P NMR (121 MHz; D$_2$O): δ 0.60. MS (ES$^+$) *m/z*: 693 [M + H]$^+$, 714 [M + Na]$^+$, 737 [M + 2Na]$^+$; HRMS (ES$^+$) *m/z*: calcd for C$_{23}$H$_{18}$N$_6$Na$_4$O$_{10}$P$_2$ 693.0228 [M + H]$^+$; found 693.0215.

*4.2.19. (E)-4-(3-(4-Benzyloxyphenoxy)phenyl)but-3-en-2-one (**31**)*

Compound **14** (200 mg, 0.563 mmol), but-2-ene-3-one (192 μL, 2.31 mol), triethylamine (570 μL, 4.9 mmol), palladium acetate (13 mg, 0.058 mmol), triphenylphosphine (30 mg, 0.112 mmol) and dry DMF (2 mL) were placed in a sealed Pyrex tube. The mixture was heated at 150 °C for 4 h and cooled to room temperature before safely opening the tube. Water was added and the mixture was extracted with ether. The combined extracts were washed with water and brine, dried over magnesium sulfate and evaporated under reduced pressure to give a yellow oil. Purification by flash chromatography with cyclohexane/ethyl acetate (3:1) furnished **31** as a yellow powder (161 mg, 83%). Mp 119 °C (recryst. from dichloromethane); ^{1}H NMR (300 MHz; $CDCl_3$): δ 2.36 (s, 3H, CH_3), 5.07 (s, 2H, CH_2), 6.63 (d, J_{trans} = 16.3 Hz, 1H, HC=C*H*–CO), 6.99 (s, 5H, 4_C, 2_D, 3_D, 5_D, 6_D), 7.08 (s, 1H, 2_C), 7.22–7.25 (m, 1H, 6_C), 7.33–7.44 (m, 7H, 5_C, *H*C=CH–CO, 2_E, 3_E, 4_E, 5_E, 6_E); ^{13}C NMR (75 MHz; $CDCl_3$): δ 27.6 (CH_3), 70.6 (CH_2), 116.2 (3_D, 5_D), 116.6 (2_C), 119.8 (4_C), 121.2 (2_D, 6_D), 122.7 (6_C), 127.7 (2_E, 6_E), 127.8 (HC=*C*H–CO), 128.2 (4_E), 128.8 (3_E, 5_E), 130.3 (5_C), 136.2 (1_C), 137.0 (1_E), 143.0 (HC=CH–CO), 149.8 (1_D), 155.6 (4_D), 159.2 (3_C), 198.5 (CO); MS (CI) *m*/*z*: 345 $[M+H]^+$; Anal. Calcd for $C_{23}H_{20}O_3$: C, 80.21; H, 5.85. Found: C, 79.52; H, 5.86.

*4.2.20. 1-(4-Cyanophenyl)guanidine (**34**)*

Aryl guanidine **34** was prepared as described by Bauer and Saffir [87]. 8 N nitric acid (5 mL, 41 mmol) was slowly added to a solution of 4-aminobenzonitrile (3 g, 25 mmol) and cyanamide (1.08 g, 25 mmol) in ethanol (13 mL) under air atmosphere. The mixture was then refluxed for 6 h. The guanidine nitrate that precipitated upon cooling was filtered and washed, giving 1.16 g of a pale orange powder (5.2 mmol, 21%). To a solution of this nitrate derivative in refluxing water (10 mL) was added dropwise a 1 M sodium hydroxide solution (5.2 mL, 5.2 mmol). The mixture was refluxed for 5 min and the precipitate obtained upon cooling was filtered, washed with water and dried to give **34** as a pale brown powder (609 mg, 15% overall yield). Mp 220–222 °C (recryst. from ethanol); ^{1}H NMR (300 MHz; MeOD): δ 7.06–7.09 (m, 2H), 7.55–7.58 (m, 2H); ^{13}C NMR (75 MHz; MeOD): δ 105.1 (4_B), 120.6 (CN), 125.5 (2_B, 6_B), 134.4 (3_B, 5_B), 154.9 (1_B), 156.9 (NH–C(NH)–NH_2); MS (CI) *m*/*z*: 161 $[M+H]^+$; Anal. Calcd for $C_{23}H_{20}O_3$: C, 59.99; H, 5.03; N, 34.98. Found: C, 59.22; H, 5.06; N, 34.15

*4.2.21. 4-{4-[3-(4-Benzyloxyphenoxy)-phenyl]-6-methylpyrimidin-2-ylamino}-benzo-1-nitrile (**35**)*

A mixture of aryloxyphenyl butenone **31** (308 mg, 0.89 mmol) and aryl guanidine **34** (430 mg, 2.68 mmol) in *N*,*N*-dimethylacetamide (10 mL) was heated at 160 °C for 6 h under air atmosphere. It was cooled overnight to room temperature. Water was added and the mixture was extracted with ether. The combined extracts were washed with water and brine, dried over magnesium sulfate and evaporated under reduced pressure to give an orange oil. Purification by flash chromatography with cyclohexane/ethyl acetate (5:2) gave **35** as a white powder (350 mg, 81%). Mp 138 °C (recryst. from ethyl acetate/pentane); ^{1}H NMR (300 MHz; THF-d_8): δ 2.43 (s, 3H, CH_3), 5.07 (s, 2H, CH_2), 7.01 (s, 4H 2_D, 3_D, 5_D, 6_D), 7.06 (ddd, J_o = 8.1 Hz, J_m = 2.5 Hz, J_m = 2.5 Hz, 1H, 4_C), 7.23–2.35 (m, 4H, 5_A, 3_E, 4_E, 5_E), 7.40–7.44 (m, 3H, 5_C, 2_E, 6_E), 7.54–7.58 (m, 2H, 3_B, 5_B), 7.77–7.79 (m, 2H, 2_C, 6_C), 7.95–7.98 (m, 2H, 2_B, 6_B), 9.23 (s, 1H, NH); ^{13}C NMR (75 MHz; THF-d_8): δ 23.3 (CH_3), 70.2 (CH_2), 103.7 (4_B), 108.4 (5_A), 115.8 (3_D, 5_D), 115.9 (2_C), 118.2 (2_B, 6_B), 118.8 (CN), 119.7 (4_C), 120.5 (2_D, 6_D), 121.0 (6_C), 127.2 (2_E, 6_E), 127.5 (4_E), 128.1 (3_E, 5_E), 129.8 (5_C), 132.6 (3_B, 5_B), 137.5 (1_E), 139.1 (1_C), 145.1 (1_B), 150.2 (1_D), 155.5 (4_D), 159.1 (3_C), 159.9 (4_A), 163.8 (6_A), 168.9 (2_A); MS (ES^+) *m*/*z*: 485 $[M+H]^+$, 507 $[M+Na]^+$. Anal. Calcd for $C_{31}H_{24}N_4O_2$: C, 76.84; H, 4.99; N, 11.56. Found: C, 76.62; H, 4.88; N, 11.39.

*4.2.22. N-[4-(2H-Tetrazol-5-yl)phenyl]-4-[3-(4-benzyloxyphenoxy)phenyl]-6-methylpyrimidin-2-amine (**36**)*

Compound **35** (40 mg, 0.083 mmol), trimethylsilyl azide (16 μL, 0.125 mmol) and tetrabutylammonium fluoride trihydrate (13 mg, 0.041 mmol) were introduced into a Pyrex tube. The tube was sealed, heated at 120 °C for 6 h and then cooled to room temperature. Water was added and the mixture was extracted with dichloromethane. The combined extracts were washed with water and brine, dried over magnesium sulfate and evaporated under reduced pressure to give **36** as a white powder (39 mg, 89%). Mp 129–131 °C; ^{1}H NMR (300 MHz; THF-d_8): δ 2.45 (s, 3H, CH_3), 5.06 (s, 2H, CH_2), 7.03 (s, 4H, 2_D, 3_D, 5_D, 6_D), 7.05–7.06 (m, 1H, 4_C), 7.22 (s, 1H, 5_A), 7.25–7.34 (m, 3H, 3_E, 4_E, 5_E), 7.38–7.41 (m, 3H, 2_E, 6_E, 5_C), 7.80–7.82 (m, 2H, 2_C, 6_C), 7.95–7.98 (m, 2H, 3_B, 5_B), 8.02–8.05 (m, 2H, 2_B, 6_B), 9.13 (s, 1H, NH); ^{13}C NMR (75 MHz; THF-d_8): δ 23.3 (CH_3), 69.9 (CH_2), 107.7 (5_A), 115.8 (3_D, 5_D), 116.1 (2_C), 117.6 (4_B), 118.4 (2_B, 6_B), 119.4 (4_C), 120.5 (2_D, 6_D), 120.9 (6_C), 127.2, 127.3, 127.4 (2_E, 4_E, 6_E, 3_B, 5_B), 128.1 (3_E, 5_E), 129.7 (5_C), 137.5 (1_E), 139.3 (1_C), 143.6 (1_B), 146.2 (1_D), 155.5 (4_D), 156.4 (CN_4H), 159.2 (3_C), 160.2 (2_A), 163.7 (4_A), 168.7 (6_A); MS (CI) *m*/*z*: 528 $[M+H]^+$, 550 $[M+Na]^+$.

*4.2.23. 4-(3-{6-Methyl-2-[4-(2H-tetrazol-5-yl)-phenylamino]-pyrimidin-4-yl}-phenoxy)-phenol (**37**)*

10% Palladium on carbon (10 mg) was added to a solution of **36** in methanol/dichloromethane (3 mL, 1:1). The mixture was stirred under H_2 at room temperature for 24 h, filtrated upon celite to remove the catalyst and evaporated under reduced pressure to give a yellow solid. Purification by flash chromatography with dichloromethane/methanol (93:7) gave **37** as a yellow powder (30 mg, 90%). Mp 162 °C; ^{1}H NMR (300 MHz; THF-d_8): δ 2.46 (s, 3H, CH_3), 6.80–6.83 (m, 2H, 3_D, 5_D), 6.91–6.94 (m, 2H, 2_D, 6_D), 7.06 (dd, J_o = 8.0 Hz, J_m = 1.5 Hz, 1H, 4_C), 7.25 (s, 1H, 5_A), 7.40 (br t, J_o = 8.0 Hz, 1H, 5_C), 7.77–7.78 (m, 2H, 2_C, 6_C), 7.93–8.00 (m, 4H, 2_B, 3_B, 5_B, 6_B,), 9.65 (s, 1H, NH); ^{13}C NMR (75 MHz; THF-d_8): δ 22.5 (CH_3), 107.6 (5_A), 115.4 (2_C), 116.2 (3_D, 5_D), 117.8 (4_B), 118.7 (2_B, 6_B), 119.5 (4_C), 120.7 (6_C), 120.9 (2_D, 6_D), 127.3 (3_B, 5_B), 129.6 (5_C), 138.7 (1_C), 143.1 (1_B), 148.6 (1_D), 154.2 (4_D), 156.8 (CN_4H), 159.1 (3_C), 159.7 (2_A), 164.6 (4_A), 167.6 (6_A); HRMS (CI) *m*/*z*: calcd for $C_{24}H_{20}N_7O_2$ 438.1678 $[M+H]^+$; found 438.1675.

*4.2.24. 4-(3-(2-(4-(2H-Tetrazol-5-yl)phenylamino)-6-methylpyrimidin-4-yl)phenoxy)phenyl dibenzyl phosphate (**38**)*

Dry tetrachloromethane (2 mL) was added to a solution of **37** (67 mg, 0.153 mmol) in dry acetonitrile (10 mL) cooled at −15 °C and the resulting mixture was stirred at −15 °C for 5 min. Diisopropylethylamine (200 μL, 1.15 mmol) and *N*,*N*-dimethylaminopyrimidine (10 mg, 0.082 mmol) were then added and the mixture was stirred at −15 °C for 30 min. Dibenzyl phosphite (180 μL, 0.815 mmol) was added dropwise over 45 min, the mixture was stirred at −15 °C for 1 h and then quenched with a 0.5 M solution of potassium dihydrogen phosphate (4 mL). The mixture was warmed up to room temperature and extracted with ethyl acetate. The combined extracts were then washed with water and brine, dried over magnesium sulfate and evaporated under reduced pressure to give a yellow oil. Purification by flash chromatography with dichloromethane/methanol (98:2) afforded **38** as a pale yellow solid (65 mg, 61%). Mp 77–80 °C; ^{1}H NMR (300 MHz; $CDCl_3$): δ 2.47 (s, 3H, CH_3), 5.16 (s, 2H, CH_2), 5.19 (s, 2H, CH_2) 7.06–7.09 (m, 2H, 3_D, 5_D), 7.12 (s, 1H, 5_A), 7.16–7.18 (m, 2H, 2_D, 6_D), 7.31 (m, 11H, 2 × 2_E, 2 × 3_E, 2 × 4_E, 2 × 5_E, 2 × 6_E, 4_C), 7.47–7.52 (m, 1H, 5_C), 7.64–7.66 (m, 3H, 2_B, 6_B, 6_C), 7.86–7.89 (m, 3H, 3_B, 5_B, 2_C); ^{13}C NMR (75 MHz; $CDCl_3$): δ 24.0 (CH_3), 70.7 (CH_2), 70.8 (CH_2), 109.1 (5_A), 117.1 (2_C), 117.4 (4_B), 118.5 (2_B, 6_B), 119.3 (3_D, 5_D), 121.2 (2_D, 6_D), 122.3 (6_C), 122.4 (4_C), 128.1 (2_E, 6_E), 128.2 (3_B, 5_B), 128.7 (4_E), 128.9 (3_E, 5_E), 130.4 (5_C), 134.6 (1_E), 139.1 (1_C), 142.2 (1_B), 145.5 (1_D),

154.5, 154.7 (4_D, CN_4H), 157.2 (3_C), 159.3 (2_A), 164.2 (4_A), 168.7 (6_A); ^{31}P NMR (121 MHz, $CDCl_3$): δ −5.60; MS (FAB+) *m/z*: 698 $[M+H]^+$.

*4.2.25. 4-(3-(2-(4-(2H-Tetrazol-5-yl)phenylamino)-6-methylpyrimidin-4-yl)phenoxy)phenyl dihydrogen phosphate (**4**)*

10% Palladium on carbon (3 mg) was added to a solution of **38** (30 mg, 0.043 mmol) in methanol (5 mL). The mixture was stirred under H_2 for 10 days at room temperature, then filtered through celite and rinsed with methanol and water. The combined filtrates were evaporated under reduced pressure. Purification by flash chromatography with acetonitrile/water/acetic acid (100:8:2) gave **4** as a white solid (11 mg, 49%). Mp 168–170 °C; ^1H NMR (300 MHz; DMSO-d_6): δ 2.41 (s, 3H, CH_3), 6.93–6.96 (m, 2H, 3_D, 5_D), 7.07–7.09 (m, 1H, 4_C), 7.17–7.19 (m, 2H, 2_D, 6_D), 7.28 (s, 1H, 5_A), 7.47–7.52 (m, 1H, 5_C), 7.75–7.78 (m, 3H, 2_B, 6_B, 2_C), 7.83–7.85 (m, 3H, 3_B, 5_B, 6_C), 9.61 (s, 1H, NH); ^{13}C NMR (75 MHz; DMSO-d_6): δ 24.3 (CH_3), 108.0 (5_A), 116.0 (2_C), 118.9 (2_B, 6_B), 120.1 (3_D, 5_D), 120.2 (4_C), 121.5 (2_D, 6_D), 121.7 (6_C), 125.4 (4_B), 126.7 (3_B, 5_B), 130.9 (5_C), 139.3 (1_C), 140.4 (1_B), 150.2 (4_D), 151.5 (1_D), 158.8 (3_C), 160.4 (2_A), 160.6 (CN_4H), 163.2 (4_A), 169.1 (6_A); ^{31}P NMR (121 MHz; DMSO-d_6): δ −5.06; MS (ES^-) *m/z*: 516 $[M-H]^-$, 436 $[M-PO_3H_2]^-$; HRMS (ES^-) *m/z*: calcd for $C_{24}H_{20}N_7O_5P$ 516.1185 $[M-H]^-$; found 516.1185.

*4.2.26. Sodium 5-(4-(4-methyl-6-(3-(4-(phosphonatooxy)phenoxy)phenyl)pyrimidin-2-ylamino)phenyl)-tetrazol-2-ide (**39**)*

A 1 M sodium methoxide solution (80 μL, 0.080 mmol) was added to a suspension of **4** (13 mg, 0.025 mmol) in dry methanol (3 mL) at 0 °C. The mixture was stirred at 0 °C for 15 min and then at room temperature for 1 h. The methanol was removed under reduced pressure to afford **39** as a water-soluble white powder (15 mg, quant.). ^1H NMR (300 MHz; D_2O): δ 2.24 (s, 3H, CH_3), 6.88–6.91 (m, 2H, 3_D, 5_D), 6.95 (s, 1H, 5_A), 7.00 (d, $J_o = 8.1$ Hz, 1H, 4_C), 7.05–7.07 (m, 2H, 2_D, 6_D), 7.30–7.36 (m, 1H, 5_C), 7.41 (s, 1H, 2_C), 7.54–7.60 (m, 3H, 2_B, 6_B, 6_C), 7.76–7.79 (m, 2H, 3_B, 5_B); ^{13}C NMR (75 MHz; D_2O): δ 21.2 (CH_3), 108.6 (5_A), 116.0 (2_C), 118.9 (2_B, 6_B), 119.6 (4_C), 120.4 (3_D, 5_D), 121.7 (2_D, 6_D), 122.0 (6_C), 122.2 (4_B), 126.8 (3_B, 5_B), 130.1 (5_C), 137.8 (1_C), 140.2 (1_B), 150.2 (1_D), 150.7 (4_D), 157.9 (3_C), 158.7 (2_A), 161.6 (CN_4H), 163.5 (4_A), 169.2 (6_A); ^{31}P NMR (121 MHz; D_2O): δ −0.60; MS (ES^+) *m/z* 584 $[M+H]^+$, 562 $[M-Na+H]^+$; MS (ES^-) *m/z*: 582 $[M-H]^-$, 538 $[M-2Na-H]^-$.

4.3. Competition assay

Precoated streptavidin plates (Boehringer) were incubated with 100 mL/well of biotin-Ahx-PSpYVNVQN peptide (100 nM in PBS buffer) overnight at 4 °C. Nonspecific binding was blocked with PBS/3% BSA for 4 h at 4 °C. Competitors were incubated at the appropriate concentrations in PBS/3% milk containing 40 nM GST-Grb2 protein (100 μL/well) overnight at 4 °C. Revelation is made after anti-GST (Transduction Laboratories; 1/500 in PBS/milk/0.05% Tween 20) and peroxidase-coupled antimouse (Amersham; 1/1000 in PBS/milk/0.05% Tween 20) incubations, using TMB solution (Interchim). After coloration was stopped with H_2SO_4 (10% v/v), the optical density (OD) was read at 550 nm. Dose–response relationships were constructed by nonlinear regression of the competition curves with Origin 40 software.

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Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.ejmech.2009.10.003.

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