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Small-molecular, non-peptide, non-ATP-competitive polo-like kinase 1 (Plk1) inhibitors with a terphenyl skeleton

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ABSTRACT

Polo-like kinase (Plk) 1 is a serine-threonine protein kinase that plays a role in cell division, and its overexpression is highly correlated with aggressiveness and prognosis of many cancers. We have designed, synthesized and evaluated a series of terphenyl compounds as inhibitors of the kinase activity of Plk1. Some of them act as non-ATP-competitive Plk1 inhibitors.

1. Introduction

Polo-like kinases (Plk) function in mitosis and in maintaining DNA integrity. There are four family members, that is, Plk1, Plk2 (also called Snk), Plk3 (also called Prk or Fnk) and one more distantly related member, Plk4 (also called Sak).[1] Plk1 regulates diverse cellular and biochemical events at multiple stages of M phase, including centrosome maturation, bipolar spindle formation, DNA damage adaptation, mitotic entry, activation of anaphase-promoting complex, and cytokinesis.[2–5] Plk2 appears to contribute to S phase entry[6] and plays a role in maintaining cell viability after spindle poisoning.[7] Plk3 regulates mitotic entry and cytokinesis.[8,9] In contrast to the role of Plk1–3 during the cell cycle, Plk4 appears to be a key regulator of centriole duplication.[10–13] Among the Plk family members, Plk1 has been especially well studied, because it is thought to be an attractive anticancer drug target.[14]

Plk1 is a serine-threonine protein kinase that is expressed only in dividing cells, with peak expression during G2/M.[15] Plk1 is essential for almost every step of mitosis through its different protein-binding and phosphorylation activities, and its cellular localization changes during mitotic progression.[14] Overexpression of Plk1 is strongly correlated with aggressiveness and prognosis of many cancers.[16] Thus, Plk1 has been studied as a potential drug target for anticancer therapy, and many small-molecular ATP-competitive Plk1 inhibitors have been reported (Fig. 1).[17–23]

On the other hand, it is known that Plk1 has a unique non-catalytic domain called the polo-box domain (PBD) in the C-terminal region, in addition to the catalytic kinase domain in the N-terminal region. The PBD recognizes phosphorylated peptide motifs of interacting proteins and contributes to PBD-dependent subcellular localization of Plk1,[1] which is important for correct mitosis. Recently, several compounds, such as poloxipan,[24] purpurogoline[25] and phosphorylated pentapeptide PLHSpT analogs (pT indicates phosphorylated threonine) (Fig. 2),[26–28] have been shown to inhibit interactions between PBD of Plk1 and the corresponding phosphorylated peptide motif, causing delocalization of Plk1, which results in dysfunction of mitosis. The binding modes of PLHSpT peptide analogs of PBD were analyzed by X-ray crystallography.[26] In this report, based on the X-ray crystallographical data, we designed, synthesized and evaluated a series of terphenyl compounds as mimetics of the PLHSpT peptide analogs, and generated non-peptide and ATP non-competitive Plk1 inhibitors, which unexpectedly works in the different mechanism of PLHSpT peptide analogs.

2. Results and discussion

The X-ray structure of the complex of Plk1 PBD and PLHSpT pentapeptide indicated that the Plk1 PBD binding pocket is stabilized by four types of binding,[26] involving (i) the core SpT motif, (ii) the N-terminal hydrophobic residue, (iii) glycerol (a buffer component) and (iv) a network of contacting water molecules. Based on this information, we designed non-peptide templates corresponding to the Plk1-bound conformation of the phosphorylated pentapeptide PLHSpT (Fig. 3). We choose a terphenyl

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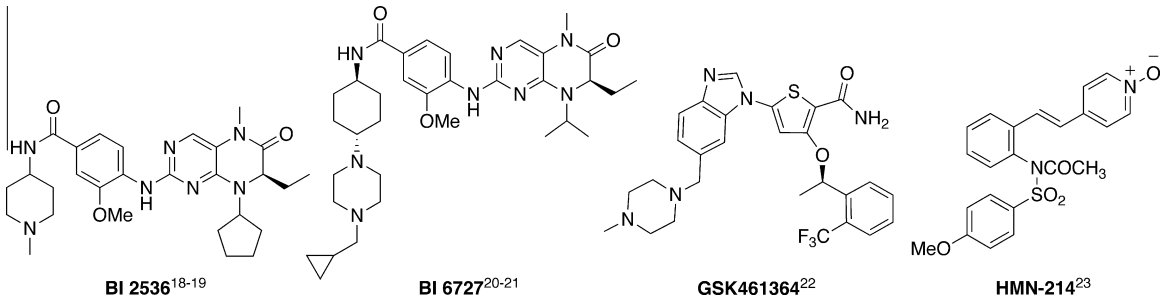


Figure 1. Plk1 kinase inhibitors under clinical trial.

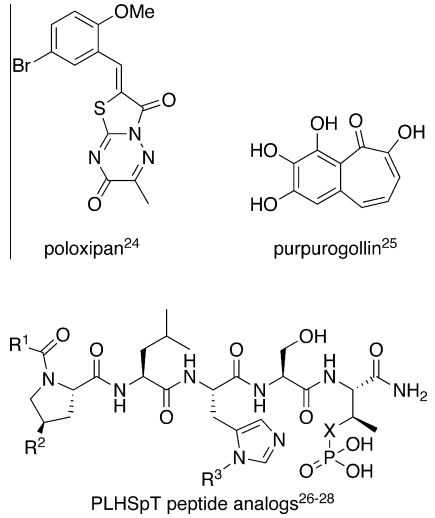


Figure 2. Compounds known to inhibit the interaction between Plk1 PBD and phosphorylated peptide.

skeleton as the scaffold because the terphenyl skeleton would be able to fix the pharmacophore rigidly, and synthesize substituted analogs easily. We thought that terphenyl compounds bearing two hydrophilic groups and a hydroxyalkyl group might mimic PLHSpT peptide. In other words, we thought that the hydrophilic groups would correspond to phosphoric acid and the carbonyl oxygen atom of the Pro residue interacting with the guanidinium moiety of Arg516 of Plk1 PBD, and the hydroxyalkyl group would bind to Plk1 PBD like glycerol.

We synthesized terphenyl compound **5**, which contains two carboxyl groups as hydrophilic groups, in four steps from *para*-crezole **1** (Scheme 1). Introduction of iodine atoms with ICl-pyridine, followed by nucleophilic substitution of 2-iodoethanol, afforded **3**. Suzuki coupling reaction of **3** and commercially available 4-cyanophenylboronic acid pinacol ester gave terphenyl compound **4**, which contains two nitrile groups. Hydrolysis of the nitrile groups afforded terphenyl compound **5** with two carboxylic groups. Terphenyl analogs **6** and **7** bearing tetrazole moieties (bioisoster of carboxylic acid) as the hydrophilic groups were synthesized by cyclization of sodium azide and dinitrile **4**.

To investigate the inhibitory activity on the interaction between Plk1 PBD and the phosphorylated peptide motif, we constructed a modified ELISA-based inhibition assay based on a previously reported assay system.[26] In this assay, biotinylated phosphopeptide (biotin-GGGGG-PLHSpT) was fixed on an avidin-coated plate via avidin–biotin interaction. Then, test samples were incubated with HeLa cell lysate, which contains Plk1 protein, on the phosphopeptide-fixed plate. Non-bound Plk1, other cell components and samples were washed out, then the 1st antibody for Plk1, 2nd antibody conjugated with HRP, 3,3′,5,5′-tetramethylbenzidine (TMB) solution and stop solution were added to the plate. The amount of oxidized TMB was determined by measuring the absorbance at 450 nm, as a measure of the intensity of the interaction between Plk1 PBD and phosphorylated peptide. PLHSpT pentapeptide, used as a positive control, inhibited the interaction between Plk1 PBD and phosphorylated peptide fixed on the plate with an IC_{50} value of 5.27 μM, as expected. Dicarboxylic acid **5** showed no effect on the interaction between Plk1 PBD and phosphorylated peptides (Fig. 4). However, terphenyl compounds bearing nitrile group(s) or tetrazole group(s), especially monotetrazole **6**, unexpectedly resulted in enhanced interaction between Plk1 PBD and phosphorylated peptide (Fig. 4). We could not investigate the inhibitory activity of compound **4** in higher concentration range because the maximum solubility of **4** was about 100 μM under the buffer

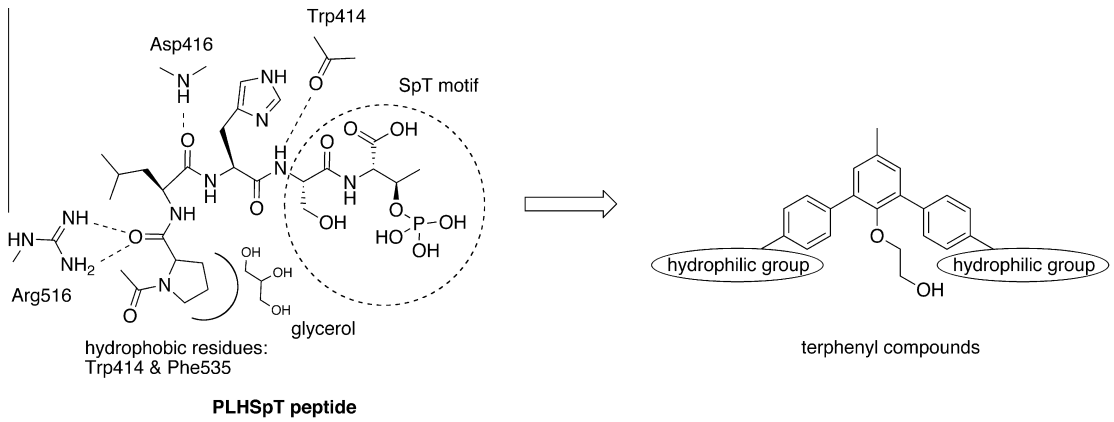
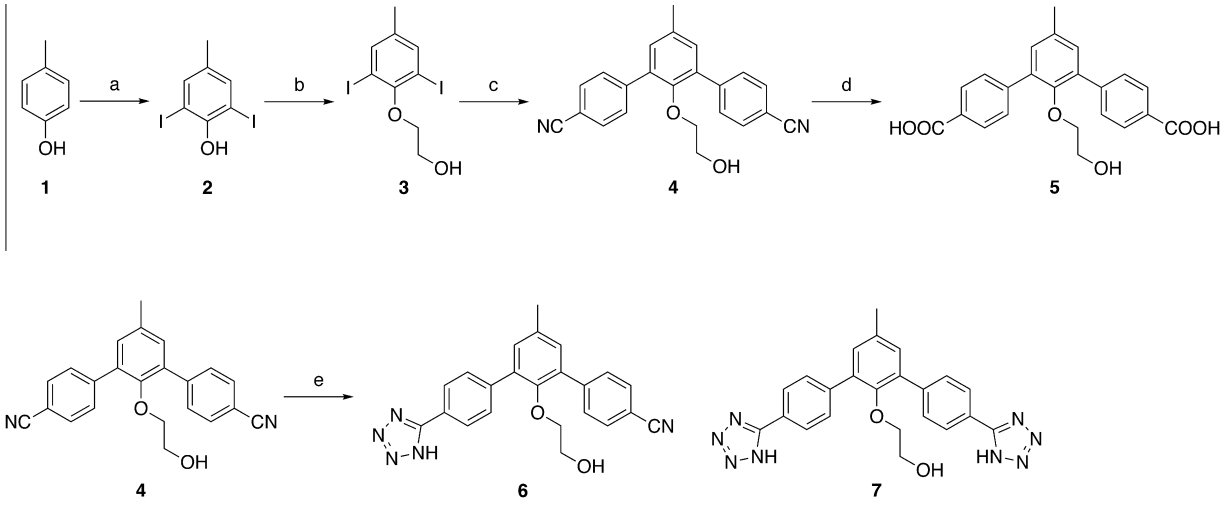


Figure 3. Binding mode of phosphorylated pentapeptide PLHSpT and Plk1 PBD, and illustration of the designed terphenyl compounds.



Scheme 1. Reagents and conditions: (a) ICl-pyridine, CH_2Cl_2/H_2O = 2/1, 0 °C to rt; (b) 2-iodoethanol, Cs_2CO_3, DMF, 0 °C to 80 °C; (c) 4-cyanophenylboronic acid pinacol ester, $PdCl_2$(dppf), K_3PO_4, DMF, 80 °C; (d) KOH, EtOH, reflux; (e) NH_4Cl, LiCl, NaN_3, DMF, 100 °C.

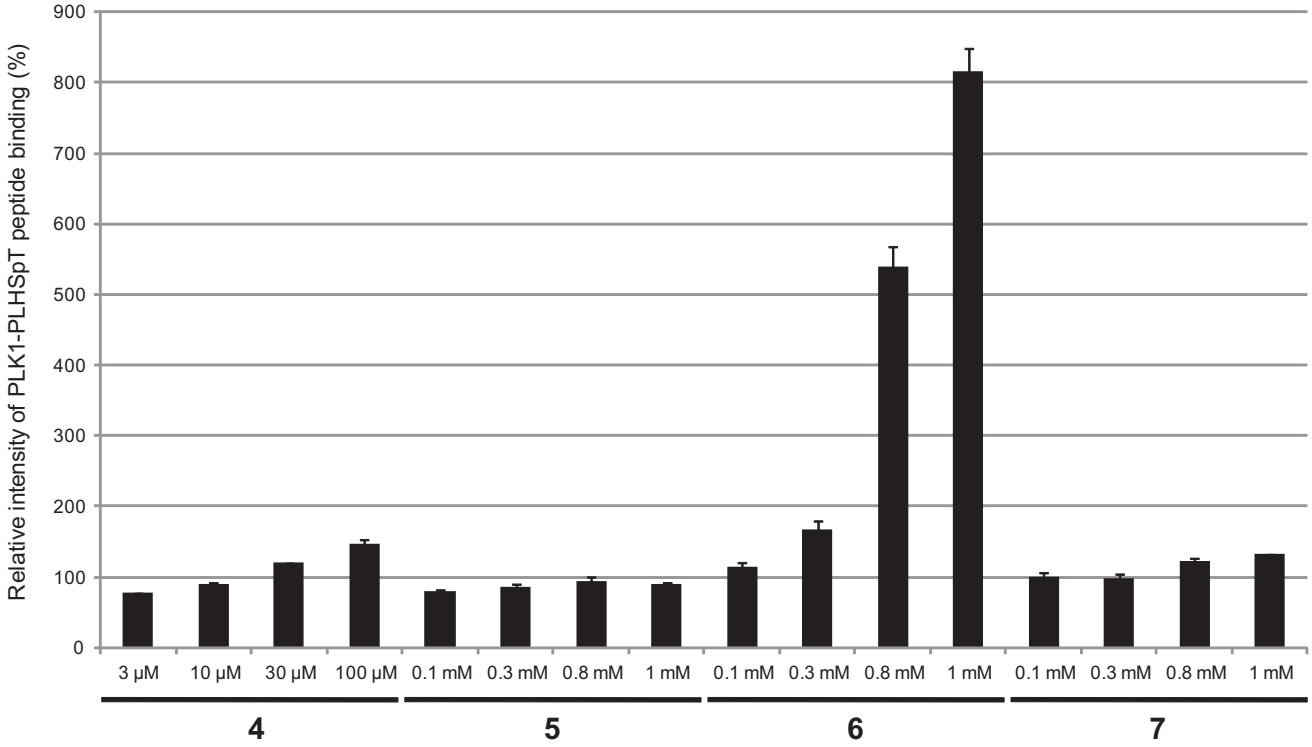


Figure 4. ELISA-based assay for evaluating the binding of Plk1 PBD and phosphorylated peptide. Plk1 kinase activity was calculated based on the value of the DMSO-treated sample, taken as 100%. The maximum solubility of compound **4** was about 100 μM under the buffer conditions for our assay system.

conditions for our ELISA-based assay system. It is unknown why terphenyl compounds increase the interaction between Plk1 PBD and phosphorylated peptide substrate in our assay system. One possible interpretation would be that they bind Plk1 and change the conformation of Plk1 in a way that favors recognition of the phosphorylated peptide motif. This idea led us evaluate whether terphenyl compounds inhibit Plk1 kinase activity.

To investigate inhibition of Plk1, we used a CycLex Polo-like kinase 1 Assay/Inhibitor Screening Kit (CycLex Co. Ltd), which employs recombinant Plk1. The phospho-threonine specific polyclonal antibody used in this assay kit has been demonstrated to recognize the Plk1-related phospho-threonine residue in the plate-fixed protein. Interestingly, the assay showed that **6** dose-dependently inhibited the kinase activity of Plk1, and the IC_{50} of **6** was calculated to be 151 μM. On the other hand, PLHSpT peptide did not inhibit the kinase activity of Plk1 (Fig. 5). These results suggested that **6**, which has a monotetrazole moiety, might interact with a domain of Plk1 other than the PLHSpT-binding site to inhibit the kinase activity of Plk1. In order to examine the structure–activity relationship (SAR) of the terphenyl compounds, we further designed and synthesized a range of terphenyl analogs. These were synthesized by Suzuki coupling reaction of halide **3** and appropriate boronic acid pinacol esters. The boronic acid pinacol esters were synthesized as described below.

To optimize the alkyl chain length of the carboxylic acid, compounds **12b–c** (and **12a** = **5**) were synthesized as shown in

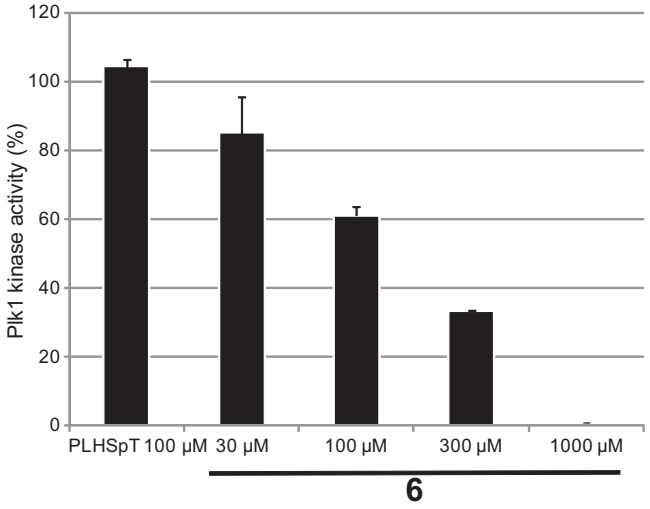
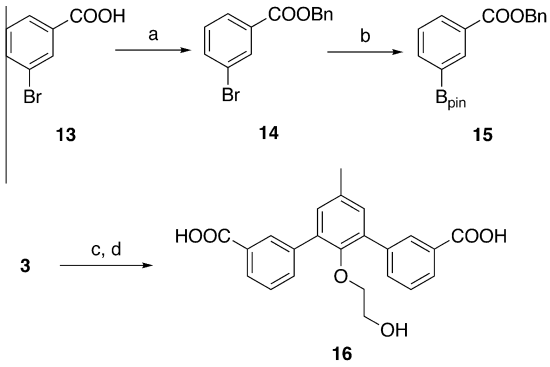


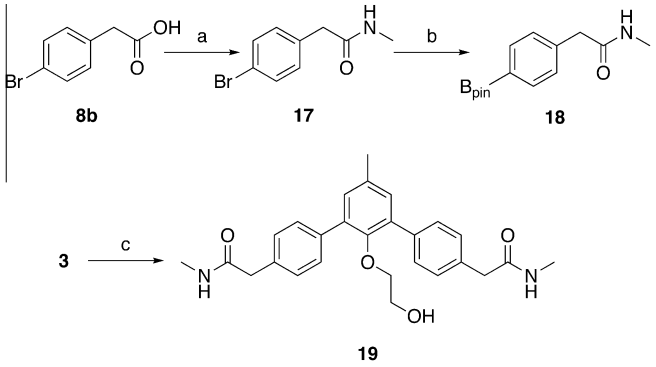
Figure 5. Terphenyl compound **6** dose-dependently inhibited Plk1 kinase activity. Plk1 kinase activity was calculated based on the value of the DMSO-treated sample, taken as 100%.

Scheme 2. Protection of the carboxyl group of **8a–c** by benzyl ester, followed by substitution of the bromine atom with boronic acid pinacol ester gave **10a–c**. Terphenyl compound **16** which had carboxylic acids at the *meta*-positions was similarly synthesized (Scheme 3). Condensation reaction of 2-(4-bromophenyl)ethanoic acid (**8b**) (Scheme 4) and methylamine, followed by substitution of the bromine atom with boronic acid pinacol ester gave **18**, which is a precursor of **19**. Bromination of 2,6-difluorophenol (**20**) (Scheme 5) followed by substitution of the bromine atom with boronic acid pinacol ester and Suzuki coupling reaction with **3** afforded **22**, which has two difluorophenyl groups (bioisoster of carboxylic acid).[29] Terphenyl compound **24** (Scheme 6) without alkyl alcohol was synthesized in two steps, that is, Suzuki coupling reaction and deprotection of the carboxylic acids.

Table 1 shows the results of evaluation of the inhibition ratio at 300 μM terphenyl analogs. Compounds **6**, **7**, **12b** and **22** showed moderately strong inhibition of Plk1 kinase activity, while compounds **4**, **5**, **12c**, and **24** showed weak inhibition. Compounds **11a**, **16**, and **19** showed no inhibition of Plk1 kinase activity at the investigated concentrations. These results suggest that hydrophilic groups are necessary for Plk1-inhibitory activity, and lipophilic acidic groups, such as tetrazole and difluorophenol, might be preferable to inhibit Plk1 activity.

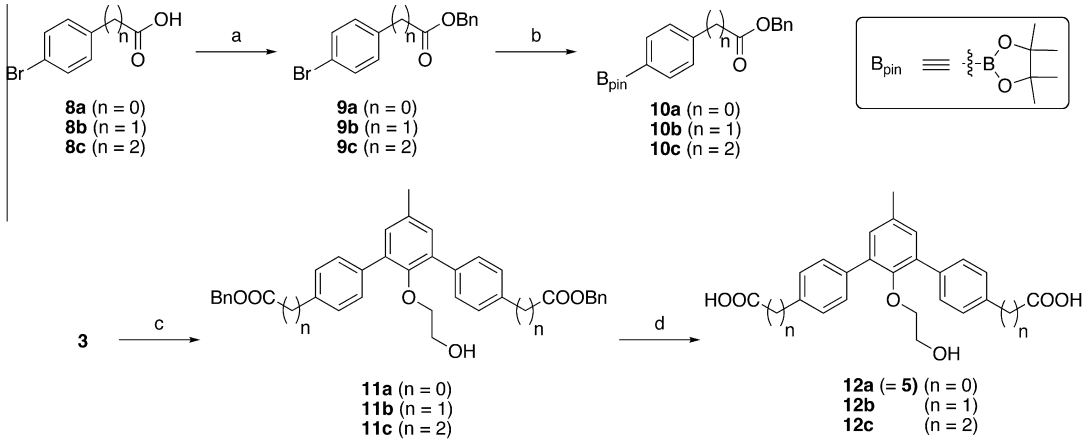


Scheme 3. Reagents and conditions: (a) benzyl bromide, K_2CO_3, DMF, 100 °C; (b) $(Ph_3)_2PdCl_2$, bis(pinacolate)diboron, KOAc, 1,4-dioxane, 100 °C; (c) compound **15**, $PdCl_2$(dppf), K_3PO_4, DMF, 80 °C; (d) 2 N NaOH aq, MeOH, rt.

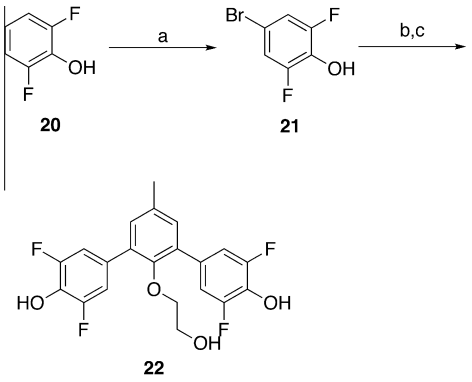


Scheme 4. Reagents and conditions: (a) $MeNH_2$ in MeOH, $HOBt{\cdot}H_2O$, DIPEA, EDCI, CH_2Cl_2, rt; (b) $(Ph_3)_2PdCl_2$, bis(pinacolate)diboron, KOAc, 1,4-dioxane, 100 °C; (c) compound **18**, $PdCl_2$(dppf), K_3PO_4, DMF, 80 °C.

In order to obtain insights into the mechanism of Plk1 inhibition, we determined the IC_{50} values of **6** and staurosporine, the latter of which is an ATP-competitive kinase inhibitor, for Plk1 in the presence of various concentrations of ATP. A typical data set is shown in Figure 6 and Table 2. The measured IC_{50} values of **6** were independent of the ATP concentration, indicating a non-ATP-competitive mechanism of action. On the other hand, a high concentration of ATP resulted in a dramatic decrease of the inhibitory efficacy of staurosporine, that is, the IC_{50} value of staurosporine



Scheme 2. Reagents and conditions: (a) benzyl bromide, K_2CO_3, DMF, 0 °C to100 °C; (b) $(Ph_3)_2PdCl_2$, bis(pinacolate)diboron, KOAc, 1,4-dioxane, 100 °C; (c) compound **10a–c**, $PdCl_2$(dppf), K_3PO_4, DMF, 80 °C; (d) 2 N NaOH aq, MeOH, rt, or H_2, Pd/C, 1,4-dioxane, rt.



Scheme 5. Reagents and conditions: (a) NBS, DMF, 0 °C to rt; (b) $(Ph_3)_2PdCl_2$, bis(pinacolate)diboron, KOAc, 1,4-dioxane, 100 °C; (c) compound **3**, $PdCl_2$(dppf), K_3PO_4, DMF, 80 °C.

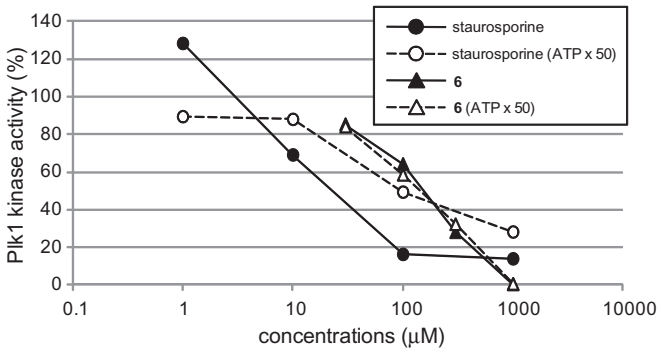
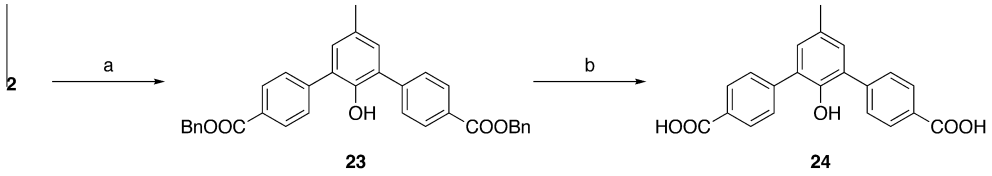
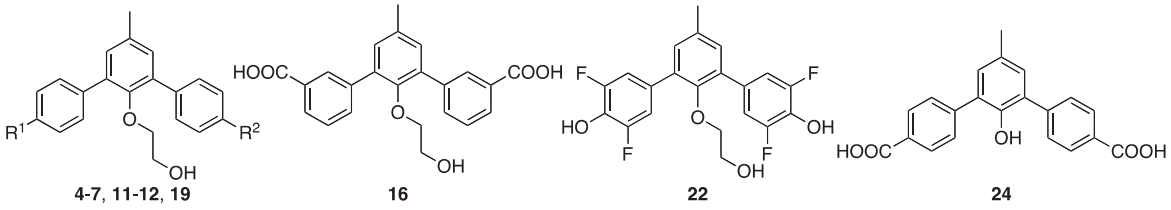


Figure 6. Terphenyl compound **6** inhibited Plk1 kinase activity in a non-ATP-competitive manner. Plk1 kinase activity was calculated based on the value of the DMSO-treated sample, taken as 100%.



Scheme 6. Reagents and conditions: (a) **9a**, $PdCl_2$(dppf), K_3PO_4, DMF, 80 °C, (b) H_2, Pd/C, 1,4-dioxane, rt.

Table 1
Inhibition ratio of Plk1 kinase activity



Compound	R^1	R^2	Inhibition ratio[a] (%)
6		–CN	66
4	–CN	–CN	11
5	–COOH	–COOH	14
7			47
11a	–COOBn	–COOBn	0
12b	$–CH_2COOH$	$–CH_2COOH$	53
12c	$–CH_2CH_2COOH$	$–CH_2CH_2COOH$	26
19	$–CH_2CONHCH_3$	$–CH_2CONHCH_3$	0[b]
16			0
22			61
24			36

[a] Inhibition ratio at 300 μM.
[b] Inhibition ratio at 250 μM (300 μM could not be tested because of poor solubility).

was over five times higher (129 μM) in the presence of 3.12 mM ATP as compared to that observed in the presence of 62.5 μM ATP (22.4 μM). These results suggested that terphenyl compounds might interact with Plk1 protein at a domain other than the PLHSpT peptide-binding site, and inhibit Plk1 kinase activity in a non-ATP-competitive manner.

3. Conclusion

Plk1 is a serine-threonine protein kinase that plays a critical role in cell division. Overexpression of Plk1 is strongly correlated with aggressiveness and prognosis of many cancers, so Plk1 is a potential target for anticancer therapy. Here, we designed a series of

Table 2
IC_{50} values of **6** and staurosporine at normal and high ATP concentrations

Compound	IC_{50} (μM)	
	ATP (62.5 μM)	ATP (3.12 mM)
6	146	139
Staurosporine	22.4	129

terphenyl compounds based on the X-ray structure of the complex of Plk1 PBD and PLHSpT pentapeptide, anticipating that they would inhibit the interaction between Plk1 PBD and phosphorylated protein substrate, and worked as non-ATP-competitive inhibitors. Contrary to our expectation, these compounds did not inhibit but rather increased the interaction between Plk1 PBD and the phosphorylated peptide motif. Investigation of the mechanism of Plk1 kinase inhibition showed that the terphenyl compounds are non-ATP-competitive inhibitors of Plk1 kinase. The phosphorylated peptide did not inhibit Plk1 kinase activity under the same assay conditions. These results suggested that terphenyl compounds interact with Plk1 protein at a domain other than the PLHSpT peptide-binding site, consequently inhibiting Plk1 kinase activity in a non-ATP-competitive manner.

4. Experimental section

4.1. Synthesis

4.1.1. General

Melting points were determined by using a Yanagimoto hot-stage melting point apparatus and are uncorrected. ^{1}H NMR spectra were recorded on a JEOL JNM-GX500 (500 MHz) spectrometer. Chemical shifts are expressed in parts per million relative to tetramethylsilane. Mass spectra were recorded on a JEOL JMS-DX303 spectrometer. Elemental analyses were carried out in the Microanalytical Laboratory, Faculty of Pharmaceutical Sciences, University of Tokyo, and were within ±0.4% of theoretical values.

4.1.2. General procedure A (GP-A)

Under an Ar atmosphere, DMF was added to a mixture of **3** (or **2**), the corresponding boronic acid pinacol ester, K_3PO_4, and Pd(dppf)Cl_2. The reaction mixture was stirred for an appropriate time at 80 °C, then filtered through Celite. The filtrate was diluted with H_2O and extracted with AcOEt. The organic layer was washed with H_2O, dried over $MgSO_4$, and concentrated. The resulting residue was purified by silica gel chromatography to afford the target material.

4.1.3. General procedure B (GP-B)

Benzyl bromide and K_2CO_3 were added to a solution of carboxylic acid **8** in DMF at 0 °C, then the atmosphere was replaced with Ar. The reaction mixture was stirred for an appropriate time at 100 °C, then the reaction was quenched with H_2O and the whole was extracted with AcOEt. The organic layer was washed with H_2O and brine, dried over $MgSO_4$, and concentrated. The resulting residue was purified by silica gel chromatography to afford the target material.

4.1.4. General procedure C (GP-C)

Under an Ar atmosphere, 1,4-dioxane was added to a mixture of aryl bromide, bis(pinacolate)diboron, KOAc, and $(PPh_3)_2PdCl_2$. The reaction mixture was stirred for an appropriate time at 100 °C, then filtered through filter paper or Celite. The filtrate was diluted with H_2O and brine, and extracted with AcOEt. The organic layer was washed with H_2O and brine, dried over $MgSO_4$, and concentrated. The resulting residue was purified by silica gel chromatography to afford the target material.

4.1.5. 2,6-Diiodo-4-methylphenol (2)

ICl–pyridine complex (5.46 g, 22.6 mmol) was added to a stirred solution of *p*-cresol (1.11 g, 10. 3 mmol) in CH_2Cl_2 (66 mL) and H_2O (33 mL) at 0 °C. The reaction mixture was stirred for 7 h at rt, then the reaction was quenched with 0.3 M $Na_2S_2O_5$ aq at rt, and the mixture was stirred until its color changed to yellow. At that point, it was extracted with CH_2Cl_2. The organic layer was dried over $MgSO_4$ and concentrated. The resulting residue was purified by silica gel chromatography (hexane/AcOEt = 20/1) to afford **2** (2.54 g, 7.06 mmol, 69%) as a white solid.

^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.49 (s, 2H), 5.57 (s, 1H), 2.22 (s, 3H). FAB-MS *m*/*z*: 360 (M^+).

4.1.6. 2-(2,6-Diiodo-4-methylphenoxy)ethanol (3)

2-Iodoethanol (467 μL, 6.00 mmol) was added to a solution of **2** (1.08 g, 3.00 mmol) and Cs_2CO_3 (3.04 g, 9.33 mmol) in DMF (10 mL) at 0 °C. The reaction mixture was stirred for 9.5 h at 80 °C, then the reaction was quenched with H_2O, and the mixture was extracted with AcOEt. The organic layer was washed with H_2O, dried over $MgSO_4$, and concentrated. The resulting residue was purified by silica gel chromatography (hexane/AcOEt = 3/1) to give **3** (1.02 g, 2.51 mmol, 84%) as a white solid.

^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.60 (s, 2H), 4.15–4.12 (m, 2H), 4.06–4.02 (m, 2H), 2.33–2.29 (m, 1H), 2.25 (s, 3H). FAB-MS *m*/*z*: 404 (M^+).

4.1.7. 2′-(2-Hydroxyethoxy)-5′-methyl-(1,1′:3′,1-terphenyl)-4,4″-dinitrile (4)

This compound was prepared by means of GP-A, with **3** (198 mg, 490 μmol), 4-cyanophenylboronic acid pinacol ester (228 mg, 995 μmol), K_3PO_4 (447 mg, 2.11 mmol), Pd(dppf)Cl_2 (39.6 mg, 54.1 μmol) and DMF (10 mL). Purification by silica gel chromatography (hexane/AcOEt = 4/1 to 2/1) afforded **4** (113 mg, 319 μmol, 65%) as a pale yellow solid.

Mp 210–212 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.76–7.70 (m, 8H), 7.20 (s, 2H), 3.32–3.25 (m, 4H), 2.42 (s, 3H), 1.07–1.03 (m, 1H). FAB-MS *m*/*z*: 354 (M^+), 355 ($M+H^+$). FAB-HRMS *m*/*z*: 354.1373 (Calcd for $C_{23}H_{18}N_2O_2$: 354.1368).

4.1.8. 2′-(2-Hydroxyethoxy)-5′-methyl-[1,1′:3′,1″-terphenyl]-4,4″-dicarboxylic acid (5 = 12a)

KOH (438 mg, 7.81 mmol) was added to a solution of **4** (47.0 mg, 133 μmol) in EtOH (2.5 mL). The reaction mixture was refluxed for 6.5 h, then acidified with 2 N HCl aq to pH 1. The mixture was extracted with AcOEt and the organic layer was washed with H_2O, dried over $MgSO_4$, and concentrated. The resulting residue was purified by PTLC ($CHCl_3$/MeOH = 5/1) to afford **5** (=**12a**) (6.90 mg, 17.6 μmol, 13%) as a white solid.

Mp 276–279 °C. ^{1}H NMR (500 MHz, CD_3OD) δ: 8.09 (d, *J* = 8.6 Hz, 4H), 7.74 (d, *J* = 8.6 Hz, 4H), 7.26 (s, 2H), 3.24–3.22 (m, 4H), 2.42 (s, 3H), 1.20–1.15 (m, 1H). FAB-MS *m*/*z*: 392 (M^+), 393 ($M+H^+$). FAB-HRMS *m*/*z*: 392.1255 (Calcd for $C_{23}H_{20}O_6$: 392.1260).

4.1.9. 2′-(2-Hydroxyethoxy)-5′-methyl-(1,1′:3′:1″-terphenyl)-4″-(1*H*-tetrazol-5-yl)-4-nitrile (6) and 2-[5′-methyl-4,4″-ditetrazol-5-yl-(1,1′:3′,1″-terphenyl)-2′- oxy]ethanol (7)

Under an Ar atmosphere, DMF (1.0 mL) was added to a mixture of **4** (33.0 mg, 93.1 μmol), NH_4Cl (18.9 mg, 353 μmol), LiCl (2.50 mg, 59.0 μmol), NaN_3 (20.7 mg, 318 μmol). The reaction mixture was stirred for 18 h at 100 °C, then the reaction was quenched with H_2O and the mixture was extracted with AcOEt. The organic layer was dried over $MgSO_4$ and concentrated. The resulting residue was purified by PTLC ($CHCl_3$/MeOH = 3/1) to afford **6** (15.9 mg, 40.0 μmol, 43%, white solid) and **7** (4.50 mg, 10.2 μmol, 11%, colorless oil).

Compound **6**: Mp 218–221 °C. ^{1}H NMR (500 MHz, CD_3OD) δ: 8.11 (d, J = 8.0 Hz, 2H), 7.84 (d, J = 8.6 Hz, 2H), 7.79 (d, J = 8.6 Hz, 2H), 7.76 (d, J = 8.0 Hz, 2H), 7.30 (s, 1H), 7.24 (s, 1H), 3.26–3.23 (m, 4H), 2.43 (s, 3H). FAB-MS m/z: 397 (M^+), 398 ($M+H^+$). FAB-HRMS m/z: 397.1542 (Calcd for $C_{23}H_{19}N_5O_2$: 397.1539). Anal. C, 66.72; H, 5.00; N, 16.70 (Calcd for $C_{23}H_{19}N_5O_2 \cdot H_2O$: C, 66.49; H, 5.09; N, 16.86).

Compound **7**: ^{1}H NMR (500 MHz, CD_3OD) δ: 8.12 (d, J = 7.9 Hz, 4H), 7.80 (d, J = 7.9 Hz, 4H), 7.27 (s, 2H), 3.28–3.25 (m, 4H), 2.44 (s, 3H). FAB-MS m/z: 440 (M^+), 441 ($M+H^+$). FAB-HRMS m/z: 440.1706 (Calcd for $C_{23}H_{20}N_8O_2$: 440.1709).

4.1.10. Benzyl 4-bromobenzoate (9a)

This compound was prepared by means of GP-B, with 4-bromobenzoic acid (4.33 g, 21.5 mmol), benzyl bromide (2.58 mL, 21.7 mmol), K_2CO_3 (6.11 g, 44.2 mmol), and DMF (25 mL). Purification by silica gel chromatography (hexane/AcOEt = 10/1 to 5/1) afforded **9a** (5.13 g, 17.6 mmol, 82%) as a white solid.

^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.93 (d, J = 8.5 Hz, 2H), 7.58 (d, J = 8.5 Hz, 2H), 7.45–7.33 (m, 5H), 5.35 (s, 2H). FAB-MS m/z: 183, 185 (M-OBn$^+$).

4.1.11. Benzyl 2-(4-bromophenyl)acetate (9b)

This compound was prepared by means of GP-B, with 4-bromophenylacetic acid (1.07 g, 4.99 mmol), benzyl bromide (564 μL, 5.00 mmol), K_2CO_3 (1.39 g, 10.0 mmol), and DMF (10 mL). Purification by silica gel chromatography (hexane/AcOEt = 10/1) afforded **9b** (1.11 g, 3.62 mmol, 73%) as a pale yellow oil.

^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.44 (d, J = 8.6 Hz, 2H), 7.37–7.29 (m, 5H), 7.16 (d, J = 8.6 Hz, 2H), 5.13 (s, 2H), 3.62 (s, 2H). FAB-MS m/z: 304, 306 (M^+), 305, 307 ($M+H^+$).

4.1.12. Benzyl 3-(4-bromophenyl)propionate (9c)

This compound was prepared by means of GP-B, with 3-(4-bromophenyl)propionic acid (1.15 g, 5.02 mmol), benzyl bromide (594 μL, 5.00 mmol), K_2CO_3 (1.43 g, 10.4 mmol), and DMF (10 mL). Purification by silica gel chromatography (hexane/AcOEt = 10/1) afforded **9c** (1.34 g, 4.18 mmol, 83%) as a colorless oil.

^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.38 (d, J = 8.6 Hz, 2H), 7.36–7.32 (m, 3H), 7.30–7.27 (m, 3H), 7.05 (d, J = 8.6 Hz, 2H), 5.10 (s, 2H), 2.92 (t, J = 7.9 Hz, 2H), 2.67 (t, J = 7.9 Hz, 2H). FAB-MS m/z: 319, 321 (M^+).

4.1.13. Benzyl 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzoate (10a)

This compound was prepared by means of GP-C, with **9a** (510 mg, 1.75 mmol), bis(pinacolate)diboron (488 mg, 1.92 mmol), KOAc (700 mg, 7.13 mmol), $(PPh_3)_2PdCl_2$ (63.5 mg, 90.5 μmol) and 1,4-dioxane (18 mL). Purification by silica gel chromatography (hexane/AcOEt = 1/0 to 1/1) afforded **10a** (381 mg, 1.13 mmol, 64%) as a yellow solid.

^{1}H NMR (500 MHz, $CDCl_3$) δ: 8.05 (d, J = 7.6 Hz, 2H), 7.86 (d, J = 7.6 Hz, 2H), 7.45 (d, J = 7.3 Hz, 2H), 7.40 (dd, J = 7.3, 7.3 Hz, 2H), 7.35 (d, J = 7.3 Hz, 1H), 5.37 (s, 2H), 1.35 (s, 12H). FAB-MS m/z: 339 ($M+H^+$).

4.1.14. Benzyl 2-(4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)acetate (10b)

This compound was prepared by means of GP-C, with **9b** (630 mg, 2.06 mmol), bis(pinacolate)diboron (536 mg, 2.11 mmol), KOAc (788 mg, 8.03 mmol), $(PPh_3)_2PdCl_2$ (39.5 mg, 56.3 μmol) and 1,4-dioxane (20 mL). Purification by silica gel chromatography (hexane/AcOEt = 1/0 to 5/1) afforded **10b** (410 mg, 1.16 mmol, 56%) as a colorless oil.

^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.77 (d, J = 7.3 Hz, 2H), 7.35–7.28 (m, 7H), 5.12 (s, 2H), 3.68 (s, 2H), 1.34 (s, 12H). FAB-MS m/z: 352 (M^+), 353 ($M+H^+$).

4.1.15. Benzyl 3-(4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)propionate (10c)

This compound was prepared by means of GP-C, with **9c** (637 mg, 2.00 mmol), bis(pinacolate)diboron (534 mg, 2.10 mmol), KOAc (791 mg, 8.06 mmol), $(PPh_3)_2PdCl_2$ (47.8 mg, 68.1 μmol) and 1,4-dioxane (20 mL). Purification by silica gel chromatography (hexane/AcOEt = 1/0 to 5/1) afforded **10c** (392 mg, 1.07 mmol, 54%) as a colorless oil.

^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.73 (d, J = 7.9 Hz, 2H), 7.37–7.28 (m, 5H), 7.20 (d, J = 7.9 Hz, 2H), 5.10 (s, 2H), 2.98 (t, J = 7.3 Hz, 2H), 2.68 (t, J = 7.3 Hz, 2H), 1.34 (s, 12H). FAB-MS m/z: 366 (M^+), 367 ($M+H^+$).

4.1.16. 2′-(2-Hydroxyethoxy)-5′-methyl-[1,1′:3′,1″-terphenyl]-4,4″-dicarboxylic acid, dibenzyl ester (11a)

This compound was prepared by means of GP-A, with **3** (189 mg, 469 μmol), **10a** (348 mg, 1.03 mmol), K_3PO_4 (404 mg, 1.90 mmol), Pd(dppf)Cl_2 (34.6 mg, 47.2 μmol) and DMF (5.0 mL). Purification by silica gel chromatography (toluene/AcOEt = 10/1) afforded **11a** (186 mg, 325 μmol, 69%) as a pale yellow oil.

^{1}H NMR (500 MHz, $CDCl_3$) δ: 8.15 (d, J = 8.3 Hz, 4H), 7.69 (d, J = 8.3 Hz, 4H), 7.47 (d, J = 7.3 Hz, 4H), 7.41 (dd, J = 7.3, 7.3 Hz, 4H), 7.36 (d, J = 7.3 Hz, 2H), 7.19 (s, 2H), 5.39 (s, 4H), 3.29–3.25 (m, 2H), 3.25–3.21 (m, 2H), 2.41 (s, 3H), 1.08–1.04 (m, 1H). FAB-MS m/z: 572 (M^+), 573 ($M+H^+$). FAB-HRMS m/z: 572.2200 (Calcd for $C_{37}H_{32}O_6$: 392.2199).

4.1.17. 2′-(2-Hydroxyethoxy)-5′-methyl-[1,1′:3′,1″-terphenyl]-4,4″-diacetic acid, dibenzyl ester (11b)

This compound was prepared by means of GP-A, with **3** (102 mg, 252 μmol), **10b** (190 mg, 539 μmol), K_3PO_4 (237 mg, 1.12 mmol), Pd(dppf)Cl_2 (20.3 mg, 27.7 μmol) and DMF (2.5 mL). Purification by silica gel chromatography (toluene/AcOEt = 8/1) afforded **11b** (94.7 mg, 158 μmol, 63%) as a colorless oil.

^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.57 (d, J = 8.0 Hz, 4H), 7.38–7.31 (m, 14H), 7.15 (s, 2H), 5.16 (s, 4H), 3.73 (s, 4H), 3.30–3.28 (m, 2H), 3.22–3.17 (m, 2H), 2.40 (s, 3H), 1.09–1.05 (m, 1H). FAB-MS m/z: 600 (M^+), 601 ($M+H^+$).

4.1.18. 2′-(2-Hydroxyethoxy)-5′-methyl-[1,1′:3′,1″-terphenyl]-4,4″-dipropionic acid, dibenzyl ester (11c)

This compound was prepared by means of GP-A, with **3** (102 mg, 252 μmol), **10c** (223 mg, 609 μmol), K_3PO_4 (243 mg, 1.14 mmol), Pd(dppf)Cl_2 (23.3 mg, 31.8 μmol) and DMF (2.5 mL). Purification by silica gel chromatography (toluene/AcOEt = 8/1) afforded **11c** (94.7 mg, 158 μmol, 63%) as a colorless oil.

^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.52 (d, J = 8.0 Hz, 4H), 7.38–7.32 (m, 10H), 7.27–7.24 (m, 4H), 7.13 (s, 2H), 5.13 (s, 4H), 3.29–3.26 (m, 2H), 3.21–3.17 (m, 2H), 3.02 (t, J = 7.9 Hz, 4H), 2.73 (t, J = 7.9 Hz, 4H), 2.39 (s, 3H), 1.08–1.04 (m, 1H). FAB-MS m/z: 628 (M^+), 629 ($M+H^+$).

4.1.19. 2′-(2-Hydroxyethoxy)-5′-methyl-[1,1′:3′,1″-terphenyl]-4,4″-dicarboxylic acid (5 = 12a)

Under an Ar atmosphere, 10% Pd/C (21.5 mg) was added to a solution of **11a** (75.8 mg, 132 μmol) in 1,4-dioxane (3.0 mL). The Ar atmosphere was replaced with H_2. The reaction mixture was stirred for 18 h at rt, then filtered through Celite. The filtrate was concentrated to afford **5** (=**12a**) (41.8 mg, 107 μmol, 81%) as a white solid. See data for **5**.

4.1.20. 2′-(2-Hydroxyethoxy)-5′-methyl-[1,1′:3′,1″-terphenyl]-4,4″-diacetic acid (12b)

2 N NaOH aq (60.0 μL, 120 μmol) was added to a stirred solution of **11b** (18.0 mg, 30.0 μmol) in MeOH (1.0 mL) at rt. The reaction mixture was stirred for 1 day at rt, then concentrated. The

resulting residue was dissolved in AcOEt/MeOH (10/1), and 2 N HCl aq was added to the solution. The organic layer was partitioned and the water layer was extracted with AcOEt/MeOH (10/1). The combined organic layer was washed with H_2O, dried over $MgSO_4$, and concentrated. The resulting residue was purified by PTLC ($CHCl_3$/EtOH/H_2O/NH_4OH = 4/4/1/1) to afford **12b** (R_f 0.00; 9.80 mg, 23.3 μmol, 78%) as a yellow oil.

^{1}H NMR (500 MHz, CD_3OD) δ: 7.53 (d, J = 7.6 Hz, 4H), 7.36 (d, J = 7.6 Hz, 4H), 7.12 (s, 2H), 3.55 (s, 4H), 3.25–3.20 (m, 4H), 2.37 (s, 3H). FAB-MS m/z: 420 (M^+), 421 ($M+H^+$). FAB-HRMS m/z: 420.1568 (Calcd for $C_{25}H_{24}O_6$: 420.1568).

4.1.21. 2′-(2-Hydroxyethoxy)-5′-methyl-[1,1′:3′,1″-terphenyl]-4,4″-dipropionic acid (12c)

2 N NaOH aq (92.5 μL, 185 μmol) was added to a stirred solution of **11c** (29.1 mg, 46.3 μmol) in MeOH (1.0 mL) at rt. The reaction mixture was stirred for 1 day at rt, then concentrated. The resulting residue was dissolved in Et_2O, and 2 N HCl aq was added to the solution. The organic layer was partitioned and the water layer was extracted with Et_2O. The combined Et_2O layer was washed with H_2O, dried over $MgSO_4$, and concentrated. The resulting residue was recrystallized (EtOH/hexane) to afford **12c** (7.80 mg, 17.4 μmol, 38%) as white needles.

Mp 176–179 °C. ^{1}H NMR (500 MHz, CD_3OD) δ 7.52 (d, J = 8.0 Hz, 4H), 7.29 (d, J = 8.0 Hz, 4H), 7.11 (s, 2H), 3.22–3.18 (m, 4H), 2.96 (t, J = 7.3 Hz, 4H), 2.64 (t, J = 7.9 Hz, 4H), 2.37 (s, 3H). FAB-MS m/z: 448 (M^+), 449 ($M+H^+$). FAB-HRMS m/z: 448.1885 (Calcd for $C_{27}H_{28}O_6$: 448.1886).

4.1.22. Benzyl 3-bromobenzoic acid (14)

This compound was prepared by means of GP-B, with 3-bromobenzoic acid (5.03 g, 25.0 mmol), benzyl bromide (3.00 mL, 25.3 mmol), K_2CO_3 (6.96 g, 50.4 mmol), and DMF (25 mL). Purification by silica gel chromatography (hexane/AcOEt = 10/1) afforded **14** (6.87 g, 23.9 mmol, 96%) as a colorless oil.

^{1}H NMR (500 MHz, $CDCl_3$) δ: 8.20 (s, 1H), 8.00 (d, J = 8.0 Hz, 1H), 7.68 (d, J = 6.6 Hz, 1H), 7.46–7.29 (m, 6H), 5.63 (s, 2H). FAB-MS m/z: 291, 293 ($M+H^+$).

4.1.23. Benzyl 3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzoate (15)

This compound was prepared by means of GP-C, with **14** (1.03 g, 3.54 mmol), bis(pinacolate)diboron (902 mg, 3.55 mmol), KOAc (1.06 g, 10.8 mmol), $(PPh_3)_2PdCl_2$ (47.8 mg, 68.1 μmol) and 1,4-dioxane (35 mL). Purification by silica gel chromatography (hexane/AcOEt = 15/1 to 10/1) afforded **15** (998 mg, 2.95 mmol, 83%) as a colorless oil.

^{1}H NMR (500 MHz, $CDCl_3$) δ: 8.50 (s, 1H), 8.16 (d, J = 8.0 Hz, 1H), 7.99 (d, J = 7.3 Hz, 1H), 7.48–4.32 (m, 6H), 5.38 (s, 2H), 1.35 (s, 12H). FAB-MS m/z: 338 (M^+), 339 ($M+H^+$).

4.1.24. 2′-(2-Hydroxyethoxy)-5′-methyl-[1,1′:3′,1″-terphenyl]-3,3″-dicarboxylic acid (16)

Under an Ar atmosphere, DMF (4.5 mL) was added to a mixture of **3** (169 mg, 418 μmol), **15** (315 mg, 931 μmol), K_3PO_4 (362 mg, 1.71 mmol), and Pd(dppf)Cl_2 (30.7 mg, 41.9 μmol). The reaction mixture was stirred for 6 h at 80 °C, then the reaction was quenched with H_2O and brine, and the mixture was extracted with AcOEt. The organic layer was dried over $MgSO_4$, and concentrated. The resulting residue was purified by silica gel chromatography (toluene/AcOEt = 10/1) to afford a mixture of dicarboxylic acid and dibenzyl ester (203 mg) as a pale yellow oil. In the next reaction, 2 N NaOH aq (600 μL, 1.20 mmol) was added to a solution of the mixture (203 mg) in MeOH (3.0 mL) at rt. The reaction mixture was stirred for 23 h at rt, then the reaction was quenched with H_2O, and the whole was acidified with 2 N HCl aq at 0 °C. The resulting solution was extracted with AcOEt, and the water layer was extracted with AcOEt/MeOH (10/1). The organic layers were combined and washed with H_2O. The organic layer was dried over $MgSO_4$ and concentrated. The resulting residue was purified by reprecipitation (EtOH/hexane/CH_2Cl_2) to afford **16** (70.8 mg, 180 μmol, 43% (two steps)) as a white solid.

Mp 240–242 °C. ^{1}H NMR (500 MHz, CD_3OD) δ: 8.28 (s, 2H), 8.02 (d, J = 7.3 Hz, 2H), 7.89 (d, J = 7.3 Hz, 2H), 7.55 (dd, J = 7.3, 7.3 Hz, 2H), 7.24 (s, 2H), 3.24–3.15 (m, 4H), 2.43 (s, 3H). FAB-MS m/z: 393 ($M+H^+$). FAB-HRMS m/z: 392.1256 (Calcd for $C_{23}H_{20}O_6$: 392.1260).

4.1.25. 2-(4-Bromophenyl)-*N*-methylethanamide (17)

Methylamine (40% in MeOH) (600 μL, 5.88 mmol) was added to a solution of 4-bromophenylacetic acid (1.01 g, 4.69 mmol), HOBt·H_2O (836 mg, 5.46 mmol), DIPEA (2.50 mL, 14.3 mmol), and EDCI (1.09 g, 5.76 mmol) in CH_2Cl_2 (25 mL) at rt. The reaction mixture was stirred for 23 h, then the reaction was quenched with satd $NaHCO_3$ aq and the mixture was partitioned. The organic layer was washed with satd $NaHCO_3$ aq and H_2O, dried over $MgSO_4$, and concentrated. The resulting residue was purified by silica gel chromatography ($CHCl_3$/MeOH = 10/1 to 5/1) to afford **17** (344 mg, 1.51 mmol) as a white crystals.

^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.48 (d, J = 8.6 Hz, 2H), 7.14 (d, J = 8.6 Hz, 2H), 5.45–5.20 (m, 1H), 3.52 (s, 2H), 2.78 (d, J = 4.9 Hz, 3H). FAB-MS m/z: 228, 230 ($M+H^+$).

4.1.26. 2-[4-(4,4,5,5-Tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl]-*N*-methylethanamide (18)

This compound was prepared by means of GP-C, with **17** (229 mg, 1.00 mmol), bis(pinacolate)diboron (264 mg, 1.04 mmol), KOAc (392 mg, 3.99 mmol), $(PPh_3)_2PdCl_2$ (33.3 mg, 47.4 μmol) and 1,4-dioxane (10 mL). Purification by silica gel chromatography (hexane/AcOEt = 1/1 to 1/2) afforded **18** (127 mg, 462 μmol, 64%) as a colorless oil.

^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.80 (d, J = 7.3 Hz, 2H), 7.26–7.25 (m, 2H), 5.35–5.16 (m, 1H), 3.60 (s, 2H), 2.73 (d, J = 4.9 Hz, 3H), 1.33 (s, 12H). FAB-MS m/z: 275 (M^+), 276 ($M+H^+$).

4.1.27. 2′-(2-Hydroxyethoxy)-5′-methyl-N^4,$N^{4\prime\prime}$-dimethyl-[1,1′:3′,1″-terphenyl]-4,4″-diacetamide (19)

This compound was prepared by means of GP-A, with **3** (88.6 mg, 219 μmol), **18** (127 mg, 462 μmol), K_3PO_4 (186 mg, 876 μmol), Pd(dppf)Cl_2 (15.3 mg, 20.9 μmol) and DMF (2.5 mL). Purification by silica gel chromatography ($CHCl_3$/MeOH = 10/1), PTLC ($CHCl_3$/MeOH = 10/1) and reprecipitation (AcOEt) afforded **19** (13.4 mg, 30.0 μmol, 14%) as a white solid.

Mp 234–236 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.56 (d, J = 7.9 Hz, 4H), 7.34 (d, J = 7.9 Hz, 4H), 7.13 (s, 2H), 3.53 (s, 4H), 3.22–3.18 (m, 4H), 2.73 (s, 6H), 2.67 (s, 3H); MS (FAB) 446 (M^+), 447 ($M+H^+$). FAB-HRMS m/z: 446.2202 (Calcd for $C_{27}H_{30}N_2O_4$: 446.2206).

4.1.28. 4-Bromo-2,6-difluorophenol (21)

N-Bromosuccinimide (3.60 g, 20.2 mmol) was added to a stirred solution of 2,5-difluorophenol (2.61 g, 20.1 mmol) in DMF (10 mL) at 0 °C. The reaction mixture was stirred for 24 h at rt with shielding from light, then the reaction was quenched with H_2O, and the mixture was extracted with AcOEt. The organic layer was washed with H_2O, dried over $MgSO_4$, and concentrated. The resulting residue was purified by silica gel chromatography (hexane/AcOEt = 4/1) to afford **21** (3.72 g, 17.8 mmol, 88%) as pale yellow needles.

^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.09 (d, J = 6.7 Hz, 2H), 5.23 (s, 1H). FAB-MS m/z: 256 (M^+), 257 ($M+H^+$).

4.1.29. 2′-(2-Hydroxyethoxy)-5′-methyl-[1,1′:3′,1″-terphenyl]-3,3″,5,5″-tetrafluoro-4,4″-diol (22)

Under an Ar atmosphere, 1,4-dioxane (20 mL) was added to a mixture of **21** (1.09 g, 5.23 mmol), bis(pinacolate)diboron (1.33 g,

5.24 mmol), KOAc (1.96 g, 20.0 mmol), and $(PPh_3)_2PdCl_2$ (82.1 mg, 117 μmol). The reaction mixture was stirred for 10.5 h at 100 °C, and filtered through Celite. The filtrate was diluted with H_2O and extracted with AcOEt. The organic layer was washed with H_2O, dried over $MgSO_4$, and concentrated. The resulting residue was purified by silica gel chromatography (hexane/AcOEt = 3/1) to afford a mixture of pinacol ester boronic acid (373 mg) as a white solid. This mixture was used in the next step without further purification. In the next step, DMF (7 mL) was added to pinacol ester boronic acid (373 mg, mixture), **3** (263 mg, 651 μmol), K_3PO_4 (578 mg, 2.72 mmol), and Pd(dppf)Cl_2 (47.3 mg, 64.6 μmol) under an Ar atmosphere. The reaction mixture was stirred for 9 h at 80 °C, and filtered through Celite. The filtrate was diluted with H_2O and extracted with AcOEt. The water layer was acidified with 2 N HCl aq to pH 1, and extracted with AcOEt. The combined organic layer was washed with H_2O, dried over $MgSO_4$, and concentrated. The resulting residue was purified by silica gel chromatography (hexane/AcOEt = 1/0 to 1/2) and recrystallization (CH_2Cl_2/hexane) to afford **22** (53.9 mg, 132 μmol, 3% (two steps)) as colorless crystals.

Mp 123 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.20 (d, J = 8.6 Hz, 4H), 7.11 (s, 2H), 5.17–5.14 (m, 2H), 3.42–3.34 (m, 4H), 2.39 (s, 3H), 1.28–1.25 (m, 1H). FAB-MS m/z: 408 (M^+). FAB-HRMS m/z: 408.0981 (Calcd for $C_{21}H_{16}F_4O_4$: 408.0985).

4.1.30. 2′-Hydroxy-5′-methyl-[1,1′:3′,1″-terphenyl]-4,4″-dicarboxylic acid, dibenzyl ester (23)

This compound was prepared by means of GP-A, with **2** (269 mg, 822 μmol), **9a** (556 mg, 1.64 mmol), K_3PO_4 (719 mg, 3.38 mmol), Pd(dppf)Cl_2 (62.2 mg, 85.0 μmol) and DMF (8.5 mL). Purification by silica gel chromatography (hexane/AcOEt = 1/0 to 5/1) afforded **23** (126 mg, 238 μmol, 28%) as a colorless oil.

^{1}H NMR (500 MHz, $CDCl_3$) δ: 8.17 (d, J = 8.3 Hz, 4H), 7.26 (d, J = 8.3 Hz, 4H), 7.48–7.45 (m, 4H), 7.42–7.38 (m, 4H), 7.37–7.33 (m, 2H), 7.12 (s, 2H), 5.39 (s, 4H), 5.13 (s, 1H), 2.37 (s, 3H). FAB-MS m/z: 528 (M^+), 529 ($M+H^+$).

4.1.31. 2′-Hydroxy-5′-methyl-[1,1′:3′,1″-terphenyl]-4,4″-dicarboxylic acid (24)

Under an Ar atmosphere, 10% Pd/C (21.5 mg) was added to a solution of **23** (70.2 mg, 133 μmol) in 1,4-dioxane (2.0 mL). Then atmosphere was replaced with H_2. The reaction mixture was stirred for 22 h at rt, then filtered through Celite. The filtrate was reprecipitated (EtOH/hexane) to afford **24** (5.10 mg, 14.6 μmol, 11%) as a white solid.

Mp decomposed (248 °C). ^{1}H NMR (500 MHz, CD_3OD) δ: 8.06 (d, J = 8.3 Hz, 4H), 7.64 (d, J = 8.3 Hz, 4H), 7.11 (s, 2H), 3.35 (s, 1H), 2.35 (s, 3H). FAB-MS m/z: 348 (M^+), 349 ($M+H^+$). FAB-HRMS m/z: 348.0995 (Calcd for $C_{21}H_{16}O_5$: 348.0998).

4.2. Biology

4.2.1. ELISA-based PBD-binding inhibition assay

Biotin-GGGGG-PLHSpT peptide (PH Japan) was first diluted with coating solution (KPL Inc., 50-84-01) to the final concentration of 0.1 μM, and then 100 μL of the resulting solution was immobilized onto a 96-well streptavidin-coated plate (Thermo Fisher Scientific Inc., 236004) for 90 min. To block unoccupied sites, wells were washed three times with PBST (PBS (pH 7.4) + 0.05% Tween 20) and incubated with 100 μL of blocking buffer (PBS + 1% BSA (Sigma, A2153)) for 90 min. HeLa lysate was prepared by homogenization with a sonicator (SHIMADZU, USP-600A) in homogenization buffer (20 mM Tris–maleate (pH 7.0), 150 mM NaCl, 5 mM EGTA, 1.5 mM EDTA, 0.5 mM Na_3VO_4 and 20 mM disodium 4-nitrophenyl phosphate), centrifuged at 105,000×g for 60 min at 4 °C (Beckman, Optima L-70 Ultracentrifuge) and the supernatant diluted with homogenization buffer to the assay concentration (5.0×10^6 cells/mL for HeLa cells). Then, 225 μL/well of the resulting lysate was applied a 96-well plate (IWAKI, 3860-096) and 25 μL/well of DMSO solution of 10 times test compounds was added to the plate. Pre-incubation was carried out for 30 min at rt. Blocking buffer was removed from the streptavidin-coated plate and the plate was washed three times with PBST. Then, 100 μL/well mixture of HeLa lysate and test compounds were added to the plate in duplicate and incubation was continued for 60 min at rt. To terminate the reaction, the ELISA plate was washed three times with PBST. For detection of bound biotin-GGGGG-PLHSpT peptide and Plk1, plates were incubated for 1 h with 100 μL/well of monoclonal anti-Plk1 antibody (Novus Biologicals, NB100-74502 or Thermo Fisher Scientific Inc., MA1-848) at a concentration of 1 μg/mL in the blocking buffer. The plates were washed three times, then 100 μL/well of an HRP-conjugated secondary antibody (Cell Signaling Technology, Inc., #7076) diluted to 1:500 in the blocking buffer was added to each well and incubation was continued for 1 h at rt. Afterwards, the plates were washed five times with PBST and incubated with 100 μL/well of 3,3′,5,5′-tetramethylbenzidine solution (TMB) (Cell Signaling Technology, Inc., #7004S) for 15 min at rt. The reactions were terminated by the addition of 100 μL/well stop solution (Cell Signaling Technology, Inc., #7002S) and the optical density for each sample was measured at 450 nm by using a plate reader (PerkinElmer Co., Ltd, ARVO-SX).

4.2.2. Assay for inhibitory activity of Plk1

In this assay, a CycLex Polo-like kinase 1 Assay/Inhibitor Screening Kit (CycLex Co. Ltd, CY-1163) was used and the assay was performed according to the kit instructions. We used recombinant Plk1 enzyme (CycLex Co. Ltd, CY1163E), which is the same as the constitutive active form of human full-length Plk1 with the mutation T210D in the active-site loop of kinase domain, together with an N-terminal GST tag. To investigate the mechanism of inhibition of Plk1 by terphenyl compounds, assay was performed at a normal ATP concentration (62.5 μM final concentration) and at a high ATP concentration (3.12 mM final concentration).

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