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Ring-fused pyrazole derivatives as potent inhibitors of lymphocyte-specific kinase (Lck): Structure, synthesis, and SAR

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ABSTRACT

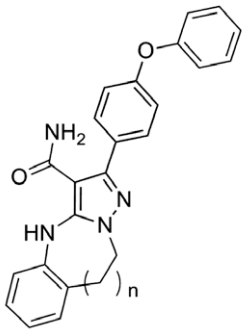
We have identified a novel series of ring-fused pyrazole derivatives as lymphocyte-specific kinase (Lck) inhibitors. The most potent analogs exhibited good enzyme inhibitory activity (IC_{50}s <1 nM) as well as excellent cellular activity against mixed lymphocyte reaction (MLR) (IC_{50}s <1 nM).

Lymphocyte-specific kinase (Lck), a Src family tyrosine kinase predominantly expressed in T lymphocytes, plays a critical role in development and activation of T cells, including T-cell antigen receptor signaling.[1–3] Lck activity leads to production of cytokines such as interleukin-2 and IFNγ. Therefore, an inhibitor against Lck could be a potential immunosuppressive agent for treatment of inflammatory diseases such as rheumatoid arthritis, atopic dermatitis, asthma, and organ transplant rejection.

Several groups have previously reported the synthesis and characterization of Lck inhibitors.[4–12] We recently described the discovery of a novel series of pyrrole derivatives as potent inhibitors of Lck. As another central scaffold, we also discovered ring-fused pyrazole derivative **1** as a potent Lck inhibitor (Fig. 1). In this Letter, we describe the synthesis, enzyme inhibitory activity,[13] cellular activity against mixed lymphocyte reaction (MLR[14]), structure–activity relationships (SAR), and molecular modeling of the ring-fused pyrazole derivatives in the Lck binding site.

The synthesis of ring-fused pyrazole derivatives is outlined in Scheme 1. Commercially available 4-phenoxybenzoic acid **3** as a starting material was reacted with thionyl chloride under reflux to give the acid chloride analog. This compound was subsequently treated with malononitrile in the presence of diisopropylethylamine (DIPEA) to give compound **4**. Methylation of the hydroxyl group in compound **4** with dimethyl sulfate gave compound **5**. This compound was treated with hydrazine monohydrate to give compound **6**. The resulting intermediate **6** was reacted with the appropriate Ar-$(CH_2)_n$–CH_2Br in the presence of K_2CO_3 to give compounds **7**–**10**.[15] These compounds were treated with $Pd_2(dba)_3$, *rac*-BINAP, and Cs_2CO_3 to give the ring-fused pyrazole derivatives. Hydrolysis of the cyano group in these compounds with H_2O_2 and K_2CO_3 gave compounds **1**, **2**, **11**, and **12**.[16] Demethylation of the methoxy group in compounds **11** and **12** was achieved using BBr_3 to give hydroxyl derivatives. These compounds were subsequently treated with various alkyl halides. Deprotection of the introduced substituent was achieved as needed to give ring-fused pyrazole derivatives **13**–**33**.

With compound **1** as the initial lead, the effect of substituents on the phenyl ring of the pyrazolobenzodiazepine skeleton was



1: n = 1 Lck IC_{50} = 1.8 nM, MLR IC_{50} = 110 nM
2: n = 2 Lck IC_{50} = 22 nM, MLR IC_{50} >1000 nM

Figure 1. Activity of initial lead **1** and **2**.

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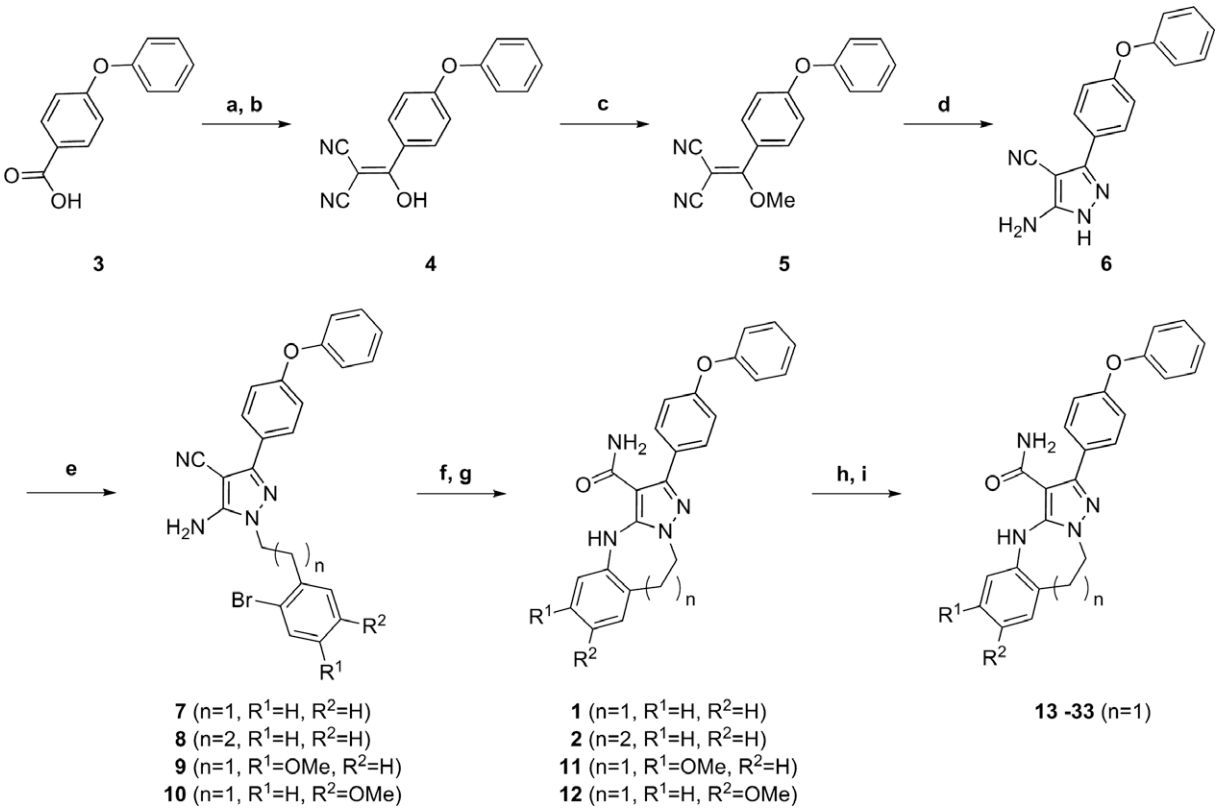


Scheme 1. Synthesis of ring-fused pyrazole derivatives. Reagents and conditions: (a) $SOCl_2$, reflux; (b) malononitrile, DIPEA, toluene, THF, rt; (c) dimethyl sulfate, $NaHCO_3$, dioxane, H_2O, 100 °C; (d) hydrazine monohydrate, EtOH, rt; (e) Ar-$(CH_2)_n$–CH_2Br, K_2CO_3, DMF, 80 °C; (f) Cs_2CO_3, $Pd_2(dba)_3$, *rac*-BINAP, dioxane or diglyme, 100–160 °C; (g) K_2CO_3, H_2O_2, DMSO, 50–80 °C; (h) BBr_3, $CHCl_3$, −78 °C–rt; (i) various alkyl halides, K_2CO_3, DMF, 80 °C. The deprotection reaction of the introduced substituent was achieved as needed.

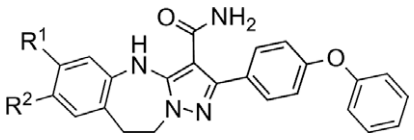
studied, and these results are summarized in Table 1. The methoxy analog **11** displayed poor cellular activity, even though it showed potent enzyme inhibitory activity. We concentrated on introducing polar moieties to the phenyl ring of the pyrazolobenzodiazepine skeleton with the objective of improving cellular potency. Introduction of the terminally aminated alkoxy groups resulted in an increased potency against MLR (**13**–**15**, **24**–**26**). Introduction of a terminally hydroxylated group also resulted in the same potency (**16**, **27**). In general, R^2-substitution was more favored over R^1-substitution for MLR. Introduction of a hydrophobic alkoxy group to R^1 led to a large decrease in potency compared with the corresponding terminally aminated analog (**17** vs **15**). The terminally carbamoyl analogs displayed modest potency (**18**, **28**). Introduction of a terminally carboxylated group to R^1 was tolerable for Lck activity but led to a large loss in cellular activity (**19**). The terminally pyridinated analogs showed modest cellular potency (**21**, **29**–**31**). All of the terminally cyclicaminated analogs also displayed excellent potency against Lck but modest cellular activity (**22**, **23**, **32**, **33**).

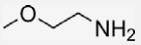
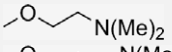
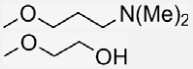
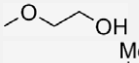
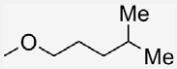
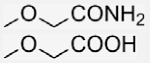
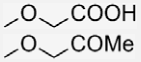
We then focused on the optimization of the phenoxy moiety. Compound **36** was prepared according to Scheme 2. Compound **34** was synthesized by a method similar to that described in Scheme 1, using commercially available 4-benzyloxybenzoic acid. Deprotection of the benzyl group followed by tosylation afforded compound **35**. Demethylation of the methoxy group with BBr_3 resulted in an intermediate phenol analog. This compound was coupled with 2-dimethylaminoethyl chloride and hydrolysis gave compound **36**. Compounds **40** and **41** were prepared as shown in Scheme 3. Reduction of the nitro group in compound **37** gave an intermediate aniline analog. This compound was treated with triphenylbismuth, $PhI(OAc)_2$, and $Cu(OAc)_2$ to give compound **38**. Alternatively, compound **37** was reacted with 2,6-dichlorobenzyl bromide to give compound **39**. Compounds **38** and **39** were deprotected and coupled with 2-dimethylaminoethyl chloride to give the corresponding compounds **40** and **41**. Compounds **44**–**46** were prepared according to Scheme 4. Reduction of the nitro group in compound **42** gave compound **43**. This compound was reacted with benzyl chloroformate to give compound **44**. Alternatively, compound **43** was treated with corresponding isocyanates to give compounds **45** and **46**.

Table 2 outlines the SAR observed when a phenyl group was substituted at the 2-position of pyrazolobenzodiazepine. Replacement of the phenoxy group with a hydroxyl group resulted in decreased cellular activity, even though there was excellent activity against Lck (**36** vs **25**). The amino analog **43** displayed a significant loss in cellular potency. Replacement of the phenoxy group with an aniline group resulted in no change in Lck activity but led to a 10-fold loss in cellular activity (**40** vs **25**). The 2,6-dichlorobenzylamino analog **41** showed good potency and cellular activity against Lck. However, the benzyloxycarbonylamino analog **44** as well as the phenylureido analog **45** displayed decreased potency against MLR, even though they both showed comparable inhibitory activity against Lck. The 2,6-dichlorophenylureido analog **46** also showed decreased potency against MLR.

The most potent analog **25** was docked into the molecular model, which was developed based on the published X-ray co-crystal structure of Lck and imatinib (PDB code: 2PL0).[17,18] As shown in Figure 2, several hydrogen bonding interactions are thought to be involved: the 4-NH of the pyrazolobenzodiazepine skeleton is in H-bond contact with the Met319 main-chain carbonyl; the carbonyl of the 3-carboxamide is in H-bond contact with the

Table 1
In vitro data of ring-fused pyrazole derivatives with R^1- and R^2-substitution modifications



Compound	R^1	R^2	IC_{50} (nM)	
			Lck[a]	MLR[b]
11	OMe	H	4.8	300
12	OMe	H	2.3	—
13		H	0.30	25
14		H	1.2	6.4
15		H	0.51	12
16		H	1.5	58
17		H	>100	620
18		H	1.5	16
19		H	1.0	>1000
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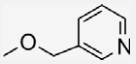
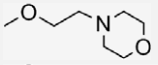
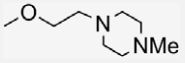
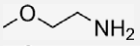
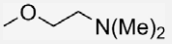
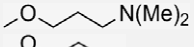
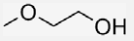
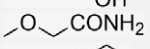
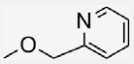
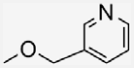
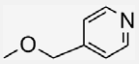
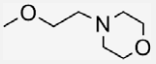
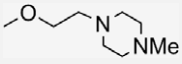
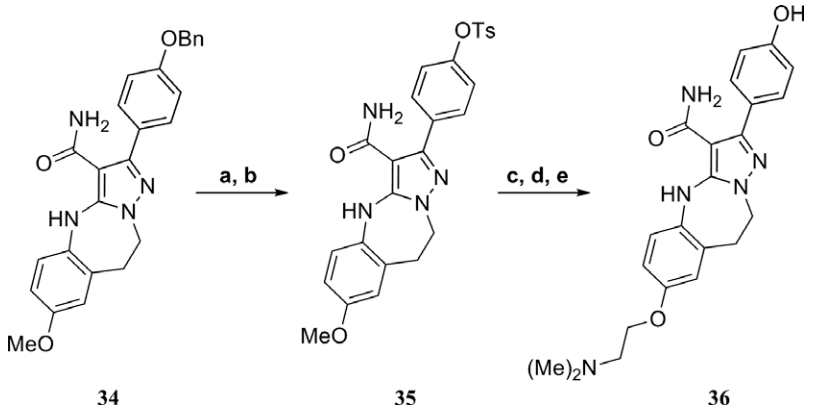
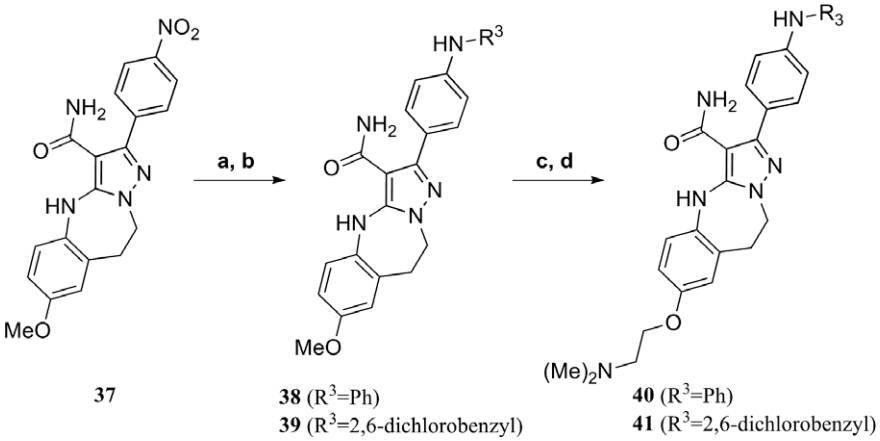
21		H	8.2	52
22		H	0.77	15
23		H	0.55	12
24	H		0.41	1.3
25	H		0.72	0.74
26	H		1.0	0.85
27	H		0.75	1.2
28	H		1.2	88
29	H		5.4	8.4
30	H		2.8	1.5
31	H		10	4.4
32	H		0.56	1.1
33	H		1.1	6.7

[a] Mean values from at least two independent experiments. IC_{50} values were determined from full eight-point, half-log concentration–response curves.
[b] Mean values from at least two independent experiments. IC_{50} values were determined from full nine-point, half-log concentration–response curves.

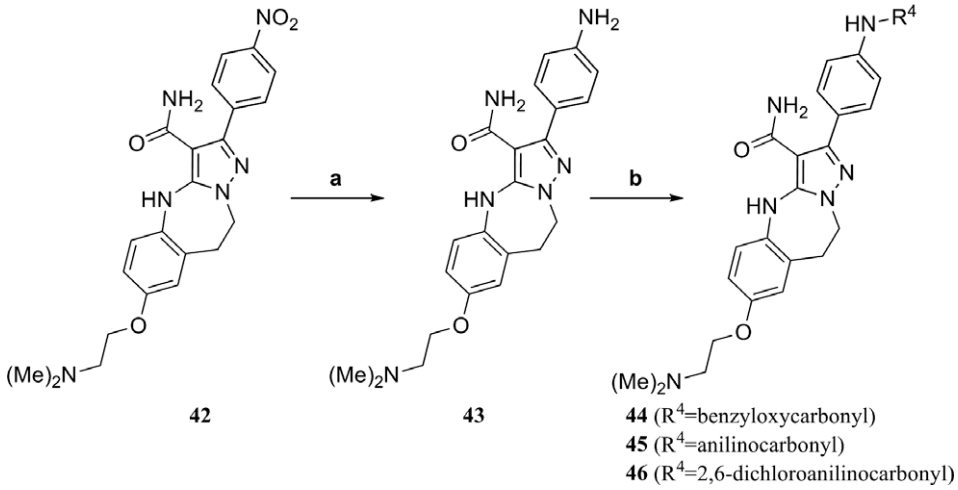


34 35 36

Scheme 2. Reagents and conditions: (a) H_2 gas, Pd–C, MeOH–THF, 60 °C; (b) TsCl, Et_3N, $CHCl_3$, rt; (c) BBr_3, $CHCl_3$, 0 °C–rt; (d) $ClCH_2CH_2N(CH_3)_2$, K_2CO_3, DMF, 90 °C; (e) KOH, THF–EtOH–H_2O, 60 °C.

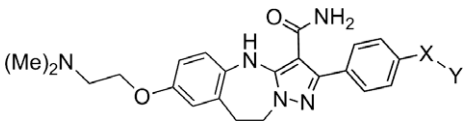


Scheme 3. Reagents and conditions: (a) H_2 gas, Pd–C, MeOH–THF, rt; (b) preparation of **38**: triphenylbismuth, $PhI(OAc)_2$, $Cu(OAc)_2$, $CHCl_3$, rt–60 °C; preparation of **39**: 2,6-dichlorobenzylbromide, Et_3N, DMF, 80 °C; (c) BBr_3, $CHCl_3$, −78 °C–rt; (d) $ClCH_2CH_2N(CH_3)_2$, K_2CO_3, DMF, 90 °C.



Scheme 4. Reagents and conditions: (a) H_2 gas, Pd–C, MeOH–THF, rt; (b) preparation of **44**: benzyl chloroformate, Et_3N, $CHCl_3$, 50 °C; preparation of **45**, **46**: corresponding isocyanate, Et_3N, THF–DMF, rt.

Table 2



Compound	X	Y	IC_{50} (nM)	
			Lck[a]	MLR[b]
25	O	Ph	0.72	0.74
36	O	H	0.44	38
43	NH	H	3.9	180
40	NH	Ph	0.80	8.2
41	$NHCH_2$	2,6-Dichlorophenyl	1.0	1.3
44	$NHCO_2CH_2$	Ph	2.0	23
45	NHCONH	Ph	2.3	50
46	NHCONH	2,6-Dichlorophenyl	0.94	21

[a] Mean values from at least two independent experiments. IC_{50} values were determined from full eight-point, half-log concentration–response curves.

[b] Mean values from at least two independent experiments. IC_{50} values were determined from full nine-point, half-log concentration–response curves.

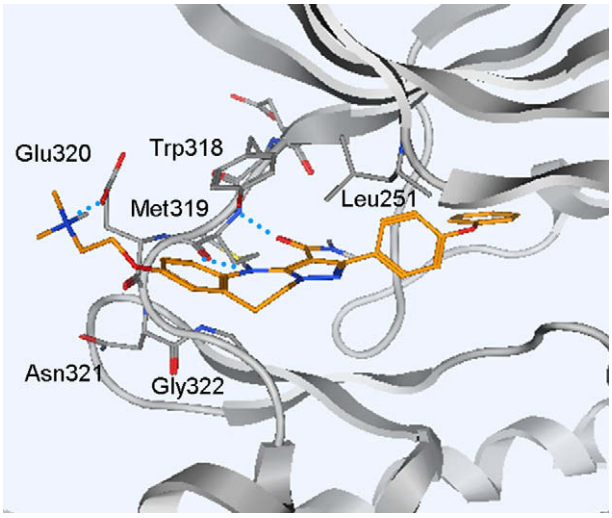


Figure 2. Model of **25** (orange) bound to the ATP-binding site of Lck. Oxygen atoms are shown in red and nitrogen atoms in blue. Hydrogen bonds are shown as cyan dotted lines.

Table 3
Kinase selectivity IC_{50} values (nM)

	LcK	Src	Csk	ZAP-70	MEK1	PKA	PKBα	PKCα	CDK1	CaMKIV	MAPK1
25	0.72	0.6	1.7	>1000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000

Met319 main-chain NH. Furthermore, the phenyl ring of the pyrazolobenzodiazepine skeleton is presumed to be in contact with a hydrophobic site consisting of Trp318, Leu251, and Gly322. In addition, we think that the terminal N atom at the 7-position of the pyrazolobenzodiazepine skeleton could be in H-bond contact with the Glu320 side chain.

Compound **25**, the most potent analog, was tested for selectivity against several kinases, and these results are summarized in Table 3.[19] Compound **25** exhibited >2-fold selectivity versus Csk, however, it exhibited non-selectivity versus the Src family member Src. Compound **25** was inactive for other kinases, including ZAP-70, MEK-1, PKA, PKBα, PKCα, CDK1, CaMKIV, and MAPK1.

In summary, we have described the synthesis, enzyme inhibitory activity, cellular activity, SAR, and proposed binding mode of a novel class of ring-fused pyrazole derivatives for Lck, as represented by compound **1**. Optimization of compound **1** led to compound **25** with excellent activity against Lck (IC_{50} = 0.72 nM) and MLR (IC_{50} = 0.74 nM). Further work for an in vivo analysis of compound **25** will be disclosed in due course.

References and notes

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13. Inhibition of Lck activity was measured using a recombinant human Lck kinase domain, expressed as a GST fusion in insect cells. Lck (5 ng/well) was incubated in kinase buffer (50 mM Hepes, 50 mM KCl, 25 mM $MgCl_2$, 5 mM $MnCl_2$, 100 μM Na_3VO_4, 0.01% CHAPS,1 mM DTT) with test compound. The final concentration of 5 μM ATP, 0.1 μCi/well γ^{33}P-ATP, and 10 μM poly (L-glutamic acid-L-tyrosine, 4:1) were added and incubated at 25 °C for 60 min and stopped with 100 μL of 100 mM phosphoric acid. The mixture was transferred to a MultiScreen-HA mixed cellulose ester membrane plate and harvested by filtration. Scintillation cocktail was added, and radioactivity was measured on a Packard Topcount instrument.
14. To isolate naïve T cells from C57BL/6 mice (Charles River Japan), spleen and lymph node cell suspensions were passed through nylon wool columns. Nonadherent T cells (3×10^5 cells/well) were cocultured with mitomycin-C treated spleen cells from BALB/c mice (Charles River Japan) (2×10^5 cells/well). These cultures were incubated at 37 °C in 5% CO_2 for 72 h. Cell proliferation was assayed by pulsing the cells with ^{3}H-thymidine for the last 4 h. ^{3}H-Thymidine incorporation into DNA was measured by Topcount. Compounds were added at the start of the culture.
15. The two regioisomers were successfully separated by column purification.
16. General procedure for preparation of ring-fused pyrazole derivative (**12**). To a mixture of **6** (1 mmol) and K_2CO_3 (1.5 mmol) in DMF (3.5 ml) was added 1-bromo-2-(2-bromoethyl)-4-methoxybenzene (1 mmol), stirred at 80 °C for 3 h, then cooled to room temperature. The mixture was diluted with AcOEt, and washed with brine. The organic layer was dried over $MgSO_4$, filtered, and concentrated in vacuo. The residue was purified by column chromatography to give **10**. Compound **10** (1 mmol) was treated with diglyme (20 ml). Cs_2CO_3 (2 mmol), $Pd_2(dba)_3$ (6 mol %) and *rac*-BINAP (6 mol %) were added to the mixture, heated at 160 °C for 1 h, cooled to room temperature. The mixture was diluted with AcOEt, and washed with brine. The organic layer was dried over $MgSO_4$, filtered, and concentrated in vacuo. The residue was purified by column chromatography to give the cyclization derivative. This compound (1 mmol) was treated with DMSO (10 ml). K_2CO_3 (3 mmol) and H_2O_2 (30% solution, 2.8 g) were added to the mixture, stirred at 40 °C for 2 h, then cooled to room temperature. The mixture was diluted with AcOEt, and washed with brine. The organic layer was dried over $MgSO_4$, filtered, and concentrated in vacuo. The residue was purified by column chromatography to give **12**.
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18. *The binding model was examined and visualized using MOE™ (Molecular Operating Environment) Version* 2007.09, Chemical Computing Group: Montreal, Canada.
19. Lck, Src, and Csk were purchased from Upstate (NY, USA). Kinase assays for ZAP-70, MEK1, PKA, PKBα, PKCα, CDK1, CAMKIV and MAPK1 were run by Upstate (NY, USA) using Kinase profiler service in according to the manufacturer's procedures.