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Synthesis and Structure-Activity Relationships of Benzothienothiazepinone Inhibitors of Protein Kinase D

Karla Bravo-Altamirano[†], Kara M. George[†], Marie-Céline Frantz[†], Courtney R. LaValle[‡], Manuj Tandon[‡], Stephanie Leimgruber^{‡,§}, Elizabeth R. Sharlow^{‡,§}, John S. Lazo^{‡,§}, Q. Jane Wang^{‡,§}, and Peter Wipf^{†,§,*}

[†]Department of Chemistry, University of Pittsburgh, Pittsburgh PA 15260, United States of America

[‡]Department of Pharmacology and Chemical Biology, University of Pittsburgh, Pittsburgh PA 15260, United States of America

[§]Drug Discovery Institute, University of Pittsburgh, Pittsburgh PA 15260, United States of America

Abstract

Protein kinase D (PKD) is a member of a novel family of serine/threonine kinases that regulate fundamental cellular processes. PKD is implicated in the pathogenesis of several diseases, including cancer. Progress in understanding the biological functions and therapeutic potential of PKD has been hampered by the lack of specific inhibitors. The benzoxoloazepinone CID755673 was recently identified as the first potent and selective PKD inhibitor. The study of structure-activity relationships (SAR) of this lead structure led to further improvements in PKD1 potency. We describe herein the synthesis and biological evaluation of novel benzothienothiazepinone analogs. We achieved a ten-fold increase in the *in vitro* PKD1 inhibitory potency for the second generation lead kb-NB142-70 and accomplished a transition to an almost equally potent novel pyrimidine scaffold, while maintaining excellent target selectivity. These promising results will guide the design of pharmacological tools to dissect PKD function and pave the way for the development of potential anti-cancer agents.

Keywords

Protein kinase D; small molecule inhibitor; benzothienothiazepinone; pyrimidines; CID755673

Diacylglycerol (DAG) is a key lipid secondary messenger that primarily targets protein kinase C (PKC) in cells.¹ In addition to PKC, an increasing number of DAG receptors have been discovered: protein kinase D (PKD), the chimerin Rac GTPase-activating proteins, the Ras guanyl-nucleotide-releasing proteins (RasGRPs), Munc13s, and the DAG kinases (DGKs).² All of these receptors are thought to be partially responsible for the diverse biological actions of DAG signaling. The PKD family has attracted particular attention because it is not only a target of DAG, but also a direct substrate of PKC,³ thus positioning itself downstream of DAG and PKC at a central position in the signal transduction pathway.

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*To whom correspondence should be addressed. Department of Chemistry, Parkman Ave. CSC 1301, University of Pittsburgh, Pittsburgh, PA 15260. Tel: 412-624-8606. Fax: 412-624-0787. pwipf@pitt.edu.

SUPPORTING INFORMATION AVAILABLE

Experimental procedures, assay results and spectral data for compounds. This information is available free of charge via the Internet at <http://pubs.acs.org>.

PKDs are serine/threonine kinases that are now classified as a subfamily of the Ca^{2+} /calmodulin-dependent kinase (CaMK) superfamily.⁶ To date, three PKD isoforms have been identified: PKD1 (formerly PKC μ),^{7,8} PKD2,⁹ and PKD3 (formerly PKC ν).¹⁰ They share a highly homologous sequence that comprises a catalytic domain and a regulatory domain. The regulatory domain of each PKD isoform is mainly constituted by a C1 domain, which binds DAG/phorbol esters, and a pleckstrin homology (PH) domain, which mediates protein-protein interactions and PKD autoinhibition.^{7–10}

DAG-responsive PKC is known to activate PKD by direct interaction with the PH domain of PKD and transphosphorylation of its activation loop at the conserved Ser⁷⁴⁴ and Ser⁷⁴⁸.^{3,11} Subsequent autophosphorylation occurs at multiple sites, including Ser⁹¹⁶.¹² Furthermore, DAG regulates the intracellular localization of PKD by binding to its C1 domain.^{4,5} Indeed, activated PKD seems to be mobile and able to shuttle between different subcellular compartments. Accordingly, the DAG-PKC-PKD signaling cascade represents a key mechanism that controls PKD function in cells. Recent evidence also indicates that PKD can be activated independently of PKC, adding to the complexity of the signaling network.^{4b} PKD contributes to a range of cellular responses, including cell proliferation, cell survival through oxidative stress-induced activation of nuclear factor-kappaB (NF- κ B) signaling, gene expression by regulation of class IIa histone deacetylases, protein trafficking from the Golgi to the plasma membrane, cell motility, and immune responses.^{4,5,13} This ubiquitous role of PKD at the cellular level accounts for its implication in pathological processes such as cardiac hypertrophy,¹⁴ angiogenesis,^{15,16} as well as tumor cell proliferation and metastasis,^{17–19} therefore constituting a potential therapeutic target for cardiovascular diseases and oncology.¹³

The lack of specificity of early PKD inhibitors, *i.e.* the isoquinoline sulfonamide H89, staurosporine analogs, and resveratrol, has significantly impeded the further analysis of the regulation and biology of this kinase, but, more recently, several more selective inhibitors have been reported.^{13,14b,19b} A significant accomplishment in this area was recently achieved by the identification and characterization of CID755673, the first potent and selective PKD inhibitor (Table 1).²⁰ CID755673 inhibits PKDs with an *in vitro* IC₅₀ of 182 nM for PKD1, 280 nM for PKD2, and 227 nM for PKD3. In addition to its potency, CID755673 was unique because it was not an ATP-competitive inhibitor, implying that it may bind to an alternative site on PKD, and, consequently, offer a greater selectivity for PKD *vs* other protein kinases. CID755673 effectively blocked PKD-mediated cellular functions and revealed novel tumor-promoting functions of PKD isoforms in prostate cancer cells.²⁰

Herein, we report our structure-activity relationship (SAR) efforts based on CID755673. The search for further information of the binding interaction and the identification of more potent analogs was guided by the dissection of the parent compound CID755673 into four major structural zones (Table 1): zone I (aryl moiety), zone II (furan), zone III (azepine) and zone IV (amide function).²¹ The compounds resulting from the structural modifications of each of these zones were evaluated *in vitro* and in cells for their PKD1 inhibitory activity. Among the *ca.* 50 analogs that were prepared, data for the most representative in each series are summarized in Table 1.

All modifications to zone IV, which included functional group interconversions and replacement of the amide moiety, failed to enhance the inhibitory activity (data not shown).

In zone II, replacement of the oxygen in the parent compound CID755673 with a nitrogen atom resulted in equivalent or slightly enhanced potency, as exemplified by pyrrole kb-NB123-57, which showed an IC₅₀ of 130 nM. Interestingly, replacement of the oxygen with

a sulfur atom significantly improved the activity and led to the discovery of the potent benzothienothiazepinone kb-NB142-70, exhibiting an *in vitro* IC₅₀ of 28.3 nM.

Zone I modifications included functionalization across the aryl moiety and substitutions on the phenolic hydroxyl group. Most of these zone I derivatives were less active than CID755673. Specifically, carbon substituents *ortho* to the phenol and *O*-benzylations were detrimental, whereas *ortho*-halogenation²¹ had a minor effect on the *in vitro* activity (data not shown). Furthermore, phenol *O*-methylation was well tolerated, as illustrated by an IC₅₀ of 82.5 nM for the methoxy analog, kb-NB165-09. This remarkable potency of the methyl ether compared to the phenol kb-NB142-70 may indicate that a hydrogen bond donor at this position is not crucial for activity. In contrast, the dramatic loss in activity of the benzyl analog suggests that zone I pocket in the protein is sterically limiting. Notably, the azide analog mcf292-08 maintained a high inhibitory activity with an IC₅₀ of 74.9 nM. This result confirms the limited significance of a hydrogen bond donor at this position and also suggests that an azide group can fit quite well inside the sterically restricted binding pocket.

In zone III, the ring size was altered by addition or removal of methylene groups and substitution at the benzylic position. Concomitant to zone II modification by a thiophene, thioether insertion *exo* to the five-membered heterocycle was not detrimental to the activity, as shown for kb-NB142-70 and kb-NB165-09, although no conclusion could be drawn at this point regarding the relative contribution of each modification. Moreover, increasing the ring size from 7 to 8 atoms by inserting an additional methylene group in zone III was also well tolerated, as suggested by the potency of the benzothienothiazocinones kb-NB184-02 (IC₅₀ 193 nM) and kb-NB165-92 (IC₅₀ 111 nM). It is also worth noting that in the case of the 8-membered ring, the same trend applies regarding the slightly decreased activity of the methoxy *vs* the free phenol substituent in zone I.

The synthesis of representative analogs in each series required the development of flexible synthetic routes. β -Carboline kb-NB123-57 was prepared from phenylhydrazine **2** *via* a Fischer-like indole synthesis with the corresponding 7-membered α -ketolactam, obtained *in situ* by acid-catalyzed hydrolysis of the enamine **1** (Scheme 1).²² Debenzylation by transfer hydrogenation led to the phenol kb-NB123-57.²³

Benzothienothiazepinones kb-NB142-70 and kb-NB165-09 were synthesized according to a literature protocol^{24,25} beginning with benzyl protection of commercially available 3-hydroxycinnamic acid (Scheme 2). Thionyl chloride-mediated Higa cyclization of acid **4** provided the benzo[*b*]thiophene acid chloride, which was subsequently converted to methyl ester **5**. Treatment of **5** with cysteamine hydrochloride in the presence of DBU resulted in formation of the desired benzothienothiazepinone core.²⁵ Deprotection of the aryl benzyl ether with boron tribromide provided kb-NB142-70 in good yield, and *O*-methylation of kb-NB142-70 with methyl iodide led to kb-NB165-09.

In order to synthesize the thiazocinone annulated benzothiophenes kb-NB165-92 and kb-NB184-02, the aminopropanethiol hydrochloride **8** was obtained through ring opening of thiazinanethione **7**, obtained in 2 steps from commercially available 3-amino-1-propanol (Scheme 3).²⁶ Aminothiol **8** was isolated as an approximately 1:1.3 thiol/disulfide mixture and used directly in the cyclocondensation-deprotection sequence to yield the desired benzothienothiazocinones.

Replacement of the phenol group by an azide on the benzothiophene scaffold required the development of an alternative route (Scheme 4). Aromatic nucleophilic substitution of methyl 2-chloro-5-nitrobenzoate by the methyl thioglycolate anion followed by immediate Dieckman cyclization^{27,28} afforded the benzothiophene precursor **10**. Cyclization of the corresponding triflate **11** with cysteamine hydrochloride provided the desired tricyclic core

12 in 50% yield (67% based on recovered starting material **10**). After reduction of the nitro group, treatment of aniline **13** with *tert*-butyl nitrite and TMS-azide using Moses' method²⁹ yielded the aryl azide mcf292-08.

Our SAR had established that in general benzothienothiazepinones were superior for PKD inhibition compared to benzofuroazepinones. The benzothienothiazepinone kb-NB142-70 was the most potent analog with an *in vitro* IC₅₀ of 28 nM for PKD1, achieving a nearly 7-fold improvement in potency over the earlier lead structure CID755673. This trend was further confirmed in cell-based assays, for which the cellular IC₅₀ was lowered 5-fold from 11.8 μM for the inhibition of PMA-induced activation of endogenous PKD1 by CID755673 to single digit 2.2 μM by kb-NB142-70. While this result boded well for the ability of kb-NB142-70 to inhibit PKD1 in intact cells, *in vivo* studies revealed a short plasma half-life for the benzothienothiazepinone.³⁰ Accordingly, we further explored a zone I modification of kb-NB142-70 to install a more electron-deficient pyrimidine moiety in place of the phenol ether, a known site of active phase I and II metabolism.

The synthetic route to arrive at this new thiazepinothiophenopyrimidinone scaffold is summarized in Scheme 5. Starting with commercially available methyl 3-aminothiophene-2-carboxylate, formation of the pyrimidine moiety using potassium cyanate, followed by chlorination with POCl₃ gave dichloride **15**.³¹ Palladium-catalyzed regioselective dechlorination of **15** in the presence of Na₂CO₃ occurred exclusively at the C-4 position,³² and substitution of the remaining C-2 chloride with methoxide provided **17** in 79% yield over the two steps. Electrophilic bromination of **17** using bromine in AcOH³³ gave the desired C-7 brominated compound **18**. Functionalization at C-6 under Knochel's conditions³⁴ gave the required cyclization precursor **19**. Formation of the thiazepinone moiety was then achieved by one-pot nucleophilic displacement-condensation of **19** with cysteamine hydrochloride, providing the desired methoxypyrimidine kmg-NB4-23 in good yield.

Gratifyingly, pyrimidine kmg-NB4-23 exhibited an IC₅₀ of 124 nM, which is only a slight decrease in activity compared to the parent lead kb-NB165-09. This encouraging result thus confirms the validity of our design and also suggests that decreased π-electron density is well tolerated in the aryl region.

In order to establish the selectivity profile of our analog series against other kinases, we also performed a variety of counterscreens using AKT, CAK (CDK7), PLK1, PLK2, several PKCs and CaMKs. In contrast to the low nanomolar activity of our analogs against PKD, none of our inhibitors were found to be significantly active at concentrations <10 μM in these screens. Furthermore, we tested the selectivity of our leads kmg-NB4-23, mcf292-08 and kb-NB123-57 against PKCα, PKCβI, and PKCδ, and we did not detect significant activities in these assays. For CaMKIIα, we found weak (<40%) inhibition at 10 μM for mcf292-08 and kb-NB123-57. These counterscreens clearly establish a high selectivity profile for PKD inhibition in our analog series.³⁵

We have also started to probe the cellular effects of kmg-NB4-23, mcf292-08 and kb-NB123-57.³⁵ The azidobenzothienothiazepinone mcf292-08 inhibited PMA-induced PKD1 S⁹¹⁶ autophosphorylation in LNCaP prostate cancer cells with an IC₅₀ of 2.2 μM. For the thiazepinothiophenopyrimidinone kmg-NB4-23 an IC₅₀ of 6.8 μM was found in this assay. In contrast, kb-NB123-57 did not significantly inhibit the PMA-induced PKD1 autophosphorylation at S⁹¹⁶ (IC₅₀ >50 μM), possibly due to limited membrane permeability, adverse protein binding, metabolic degradation, or other compensatory pathways in cells.

In summary, we have identified a series of novel benzothienothiazepinones as PKD inhibitors. A first round of optimization from the initial lead CID755673 led to the discovery

of kb-NB142-70 and its methoxy analog kb-NB165-09. These new lead compounds inhibited PKD1 *in vitro* in the low nanomolar range, achieving a nearly one-log IC₅₀ enhancement compared to the HTS hit CID755673. Additionally, the latter compounds inhibited PKD1 autophosphorylation at Ser⁹¹⁶ in LNCaP prostate cancer cells in the low micromolar range, a 10-fold improvement compared to CID755673. In order to circumvent metabolism issues, further core modification with a pyrimidine moiety led to the promising new lead structure kmg-NB4-23. SAR studies are currently in progress to improve the activity of kmg-NB4-23 as well as its selectivity towards PKD1 vs PKD2 or PKD3, for which the IC₅₀ values were found in the 50–500 nM range for all derivatives tested (data not shown). In conclusion, our results to date demonstrate that modifications of CID755673 significantly modulate its potency, but have only limited effect on isoform selectivity.²¹ This suggests that the binding domain may be highly conserved among the three isoforms. Finally, the azide analog mcf292-08, also active at nanomolar (*in vitro*) or micromolar (in cells) concentrations, represents a valuable tool for future photoaffinity labeling studies that may provide key structural information for binding site identification.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS

CAK	CDK activating kinase
CaMK	Ca ²⁺ /calmodulin-dependent kinase
CDK	cyclin-dependent kinase
DAG	diacylglycerol
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
DGK	DAG kinase
DMAP	<i>N,N</i> -dimethylaminopyridine
HDAC	histone deacetylase
NF-κB	nuclear factor-kappaB
PH	pleckstrin homology
PKC	protein kinase C
PKD	protein kinase D
PLK	polo-like kinase
PMA	phorbol 12-myristate 13-acetate
RasGRP	Ras guanyl-nucleotide-releasing protein
SAR	structure-activity relationship

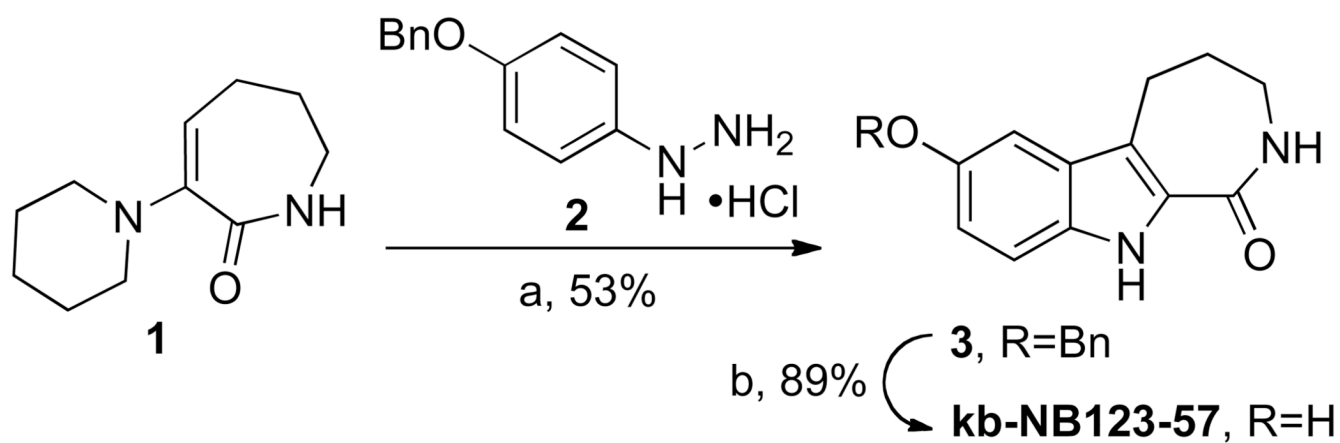
TMP 2,2,6,6-tetramethylpiperidine

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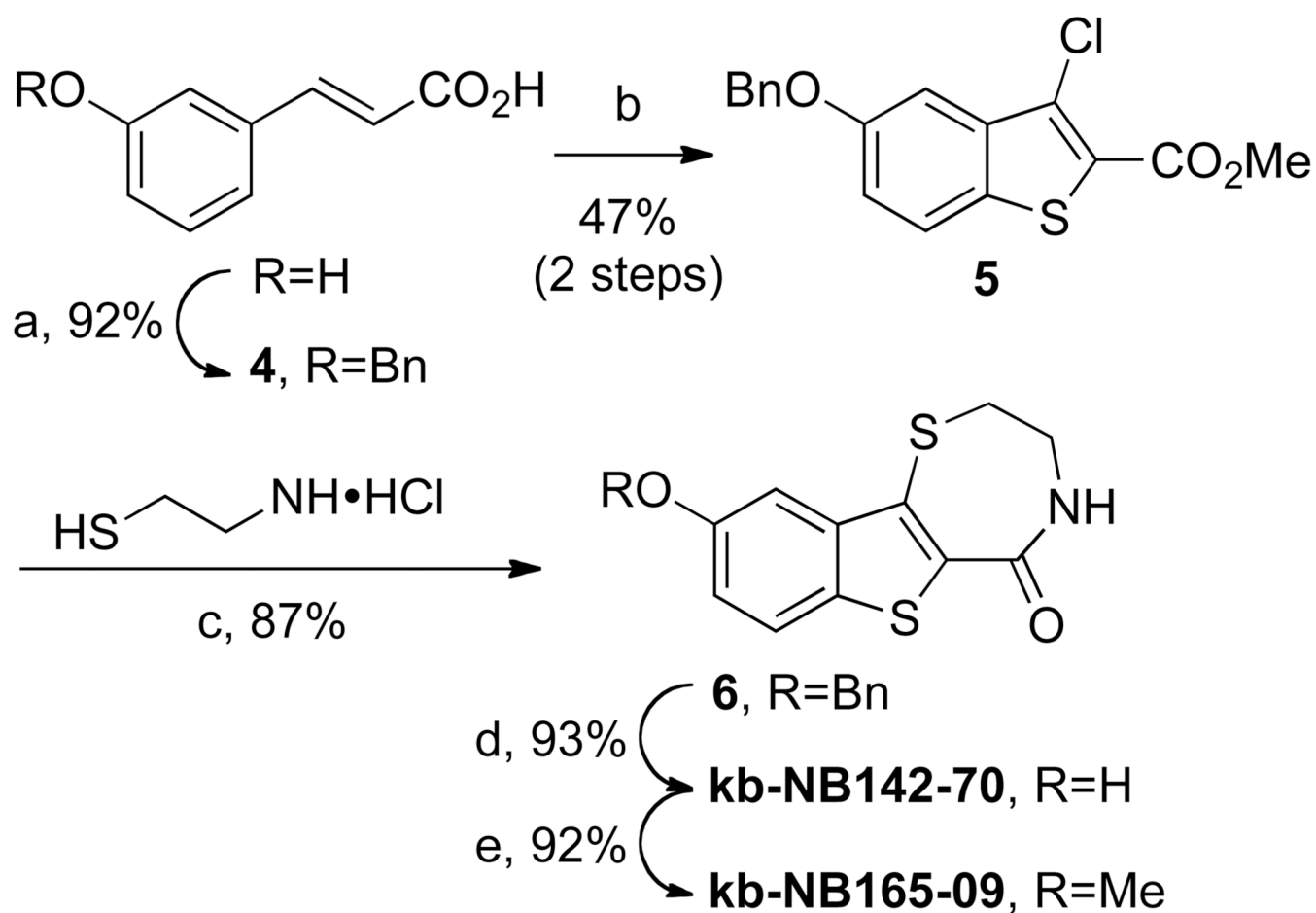
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35. See Supporting Information for assay details.

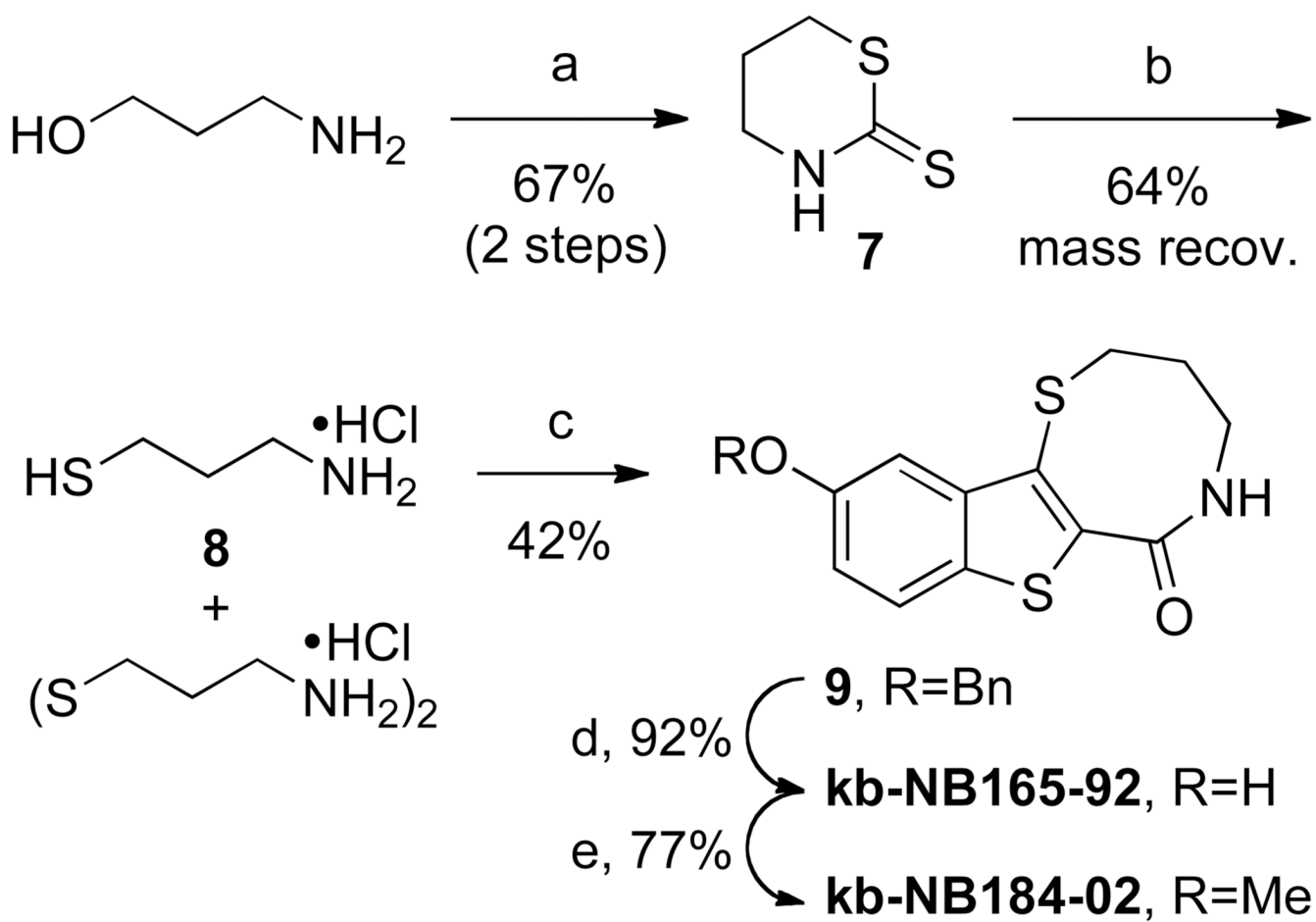


Scheme 1. Synthesis of the β -carboline analog kb-NB123-57.a

^aReagents and conditions: (a) H_2SO_4 , EtOH, reflux, 5 h. (b) Pd/C, NH_4HCO_3 , MeOH, reflux, 2 h.

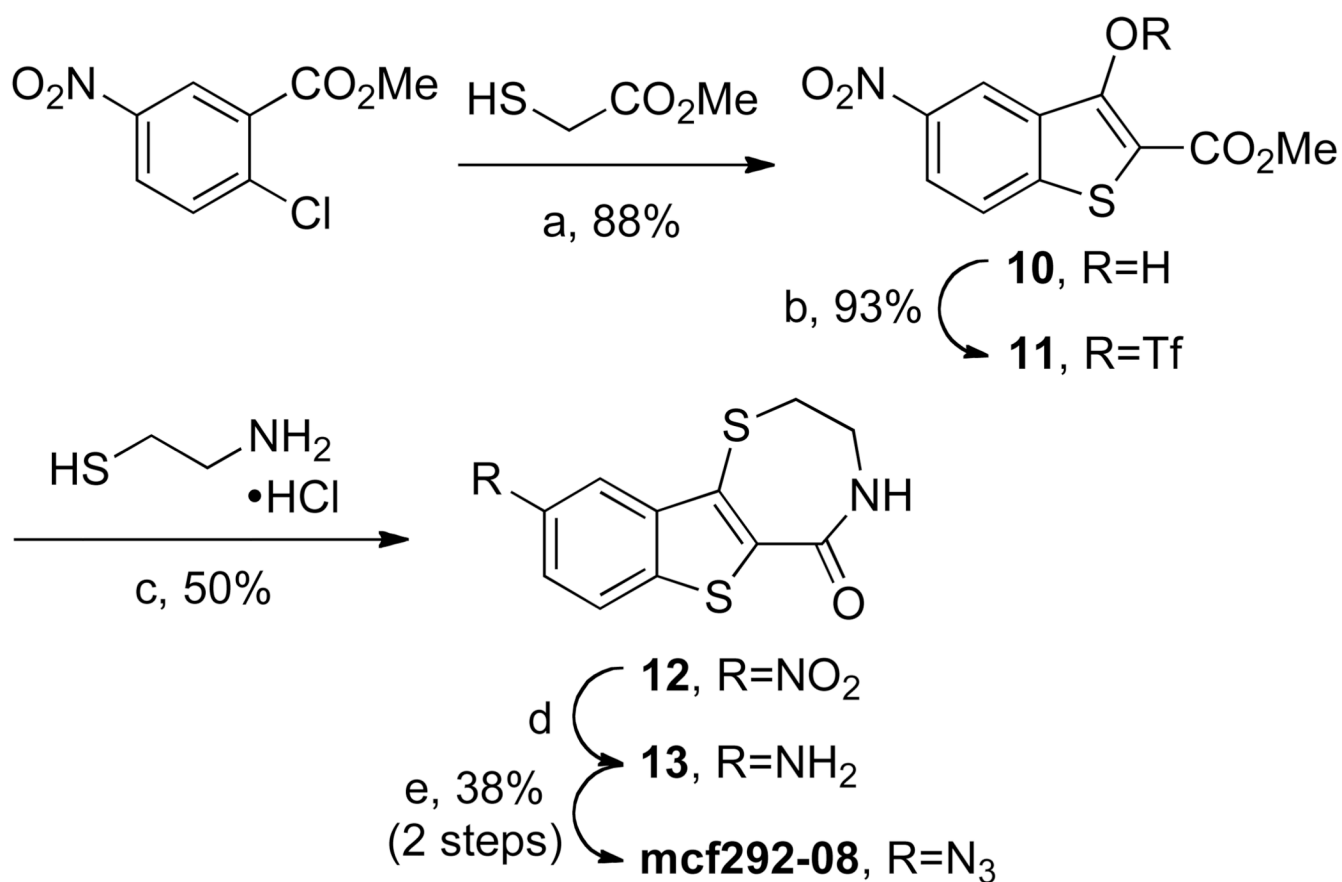


Scheme 2. Synthesis of the benzothienothiazepinone analogs kb-NB142-70 and kb-NB165-09. a ^aReagents and conditions: (a) BnBr, NaOH, EtOH, rt, 14 h. (b) (i) SOCl₂, pyr., DMF, PhCl, 120 °C, 22 h; (ii) Et₃N, MeOH, reflux, 12 h. (c) DBU, DMF, rt, 1.5 h; then 70 °C, 12 h. (d) BBr₃, DCM, -20 to 0 °C, 2.5 h. (e) MeI, K₂CO₃, DMF, rt, 12 h.



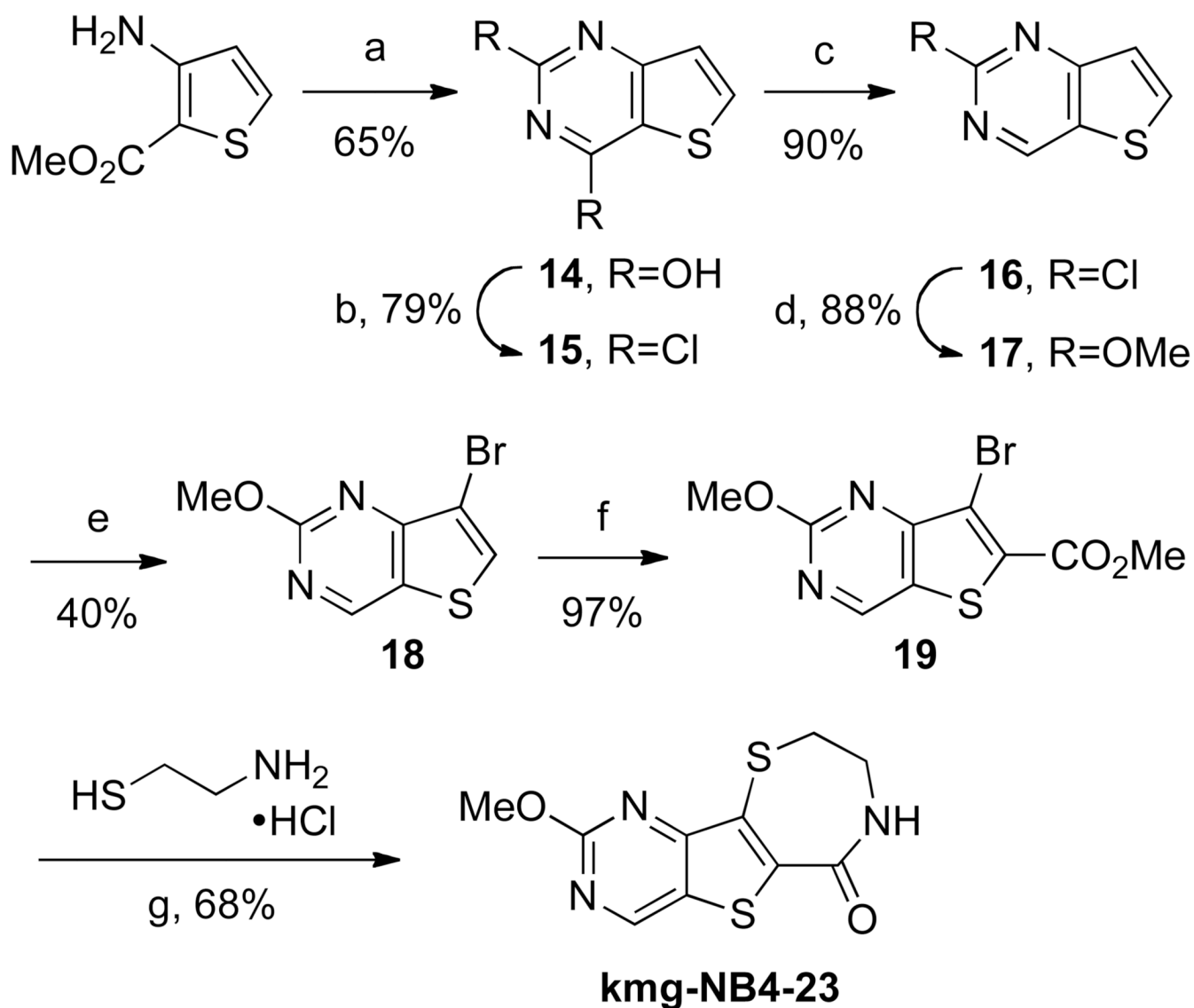
Scheme 3. Synthesis of the benzothienothiazocinones kb-NB165-92 and kb-NB184-02. a

^aReagents and conditions: (a) (i) ClSO_3H , CCl_4 , rt, overnight; (ii) CS_2 , NaOH , $\text{EtOH}/\text{H}_2\text{O}$, $0\text{ }^\circ\text{C}$ to reflux, 40 min. (b) conc. HCl , reflux, N_2 , 14 d. (c) **5**, DBU, DMF, rt, 2 h; then $70\text{ }^\circ\text{C}$, 18 h. (d) BBr_3 , DCM, $-20\text{ }^\circ\text{C}$ to rt, 2 h. (e) MeI , K_2CO_3 , DMF, rt, overnight.



Scheme 4. Synthesis of the azidobenzothienothiazepinone analog **mcf292-08**. **a**

^aReagents and conditions: (a) Et_3N , MeOH, 40–50 °C, 4 h. (b) Tf_2O , Et_3N , DMAP, DCM, rt, 2 h. (c) DBU, DMF, rt, 1.5 h; then 70 °C, 13 h. (d) SnCl_2 , EtOH, reflux, 5 h. (e) *t*-BuONO, TMSN_3 , MeCN, rt, 1.5 h.

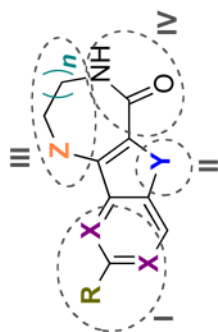


Scheme 5. Synthesis of the thiazepinothiophenopyrimidinone analog kmg-NB4-23.

^aReagents and conditions: (a) (i) KOCN, AcOH, H₂O, rt, 20 h; (ii) 2 N NaOH, rt, 2 h. (b) POCl₃, MeCN, reflux, 2 d. (c) H₂, Pd/C, EtOH, Na₂CO₃, rt, 24 h. (d) NaOMe, MeOH, reflux, 6 h. (e) AcOH, Br₂, 70 °C, 24 h. (f) (i) TMPMgCl•LiCl, −50 °C, 2 h; (ii) MeCO₂CN. (g) DBU, DMF, rt, 1.5 h; then 70 °C, 10 h.

Table 1

Chemical structure, SAR analysis and PKD inhibitory activity of CID755673 and selected synthetic analogs.



Zone	Compound	Structure				IC ₅₀	
		X	Y	Z	R	n	Cellular PKD1 (μM) ^b
-	CID755673	CH	O	CH ₂	OH	1	11.8 ± 4.0 (n=3)
II	kb-NB123-57	CH	NH	CH ₂	OH	1	>50 (n=3)
II, III	kb-NB142-70	CH	S	S	OH	1	2.2 ± 0.6 (n=3)
I	kb-NB165-09	CH	S	S	OMe	1	3.1 ± 0.5 (n=3)
I	mef292-08	CH	S	S	N ₃	1	2.2 ± 0.2 (n=3)
III	kb-NB165-92	CH	S	S	OH	2	2.6 ± 0.7 (n=2)
III	kb-NB184-02	CH	S	S	OMe	2	18.6 ± 2.0 (n=3)
I	kmg-NB4-23	N	S	S	OMe	1	6.8 ± 1.3 (n=3)

^aPKD1 IC₅₀ was determined using a radiometric kinase activity assay as previously described.²⁰ Each IC₅₀ was calculated as the mean ± S.E.M. of at least three independent experiments with triplicate determinations at each concentration in each experiment; n = number of independent experiments.

^b Cellular IC₅₀ was determined by densitometry analysis of Western blotting data for PKD1 autophosphorylation at S⁹¹⁶ in LNCaP cells as previously described.²¹ Each IC₅₀ was calculated as the mean ± S.E.M. of at least two independent experiments; n = number of independent experiments.