Conjugation of Adenosine and Hexa-(D-arginine) Leads to a Nanomolar Bisubstrate-Analog Inhibitor of Basophilic Protein Kinases

Erki Enkvist, Darja Lavogina, Gerda Raidaru, Angela Vaasa, Indrek Viil, Marje Lust, Kaido Viht, and Asko Uri*

Institute of Organic and Bioorganic Chemistry, 2 Jakobi Street, 51014 Tartu, Estonia

Received May 18, 2006

Conjugates of oligoarginine peptides with adenine, adenosine, adenosine-5′-carboxylic acid, and 5-isoquinolinesulfonic acid were synthesized and characterized as bisubstrate-analog inhibitors of cAMP-dependent protein kinase. Adenosine and adenine derivatives were connected to the N- or C-terminus of peptides containing four to six L- or D-arginine residues via a linker with a length that had been optimized in structure−activity studies. The orientation of the peptide chain strongly affected the activity of compounds incorporating D-arginines. The biligand inhibitor containing Hidaka's H9 isoquinolinesulfonamide connected to the L-peptide had 65 times higher potency than the corresponding adenosine-containing conjugate, while both types of the conjugate comprising D-peptides had similar low nanomolar activity. Two of the most active adenosine- and H9-peptide conjugates were tested in the panel of 52 different kinases. At 1 μM concentration, both compounds showed strong (more than 95%) inhibition of several basophilic AGC kinases, including pharmaceutically important kinases ROCK II and PKB/Akt.

Introduction

Protein kinases (PKs[a]) play a key role in the regulation of protein functions in living cells. It has been estimated that the activity of one-third of proteins is regulated through phosphorylation of one or more of the serine, threonine, or tyrosine residues. More than 400 human diseases have been linked to aberrant PK signaling.[1] This has made PK the second largest drug target after G protein-coupled receptors.[2] Despite serious selectivity problems (all 500 protein kinases and more than 1500 other proteins are able to bind purine nucleotides[3]) and the high concentration of competing ATP in the cellular milieu, the main efforts of drug companies have been directed to the development of ATP competitive inhibitors.

The second type of active site targeted inhibitors of PKs that have been under investigation for a longer period of time are compounds that selectively interfere with protein−protein interactions and block the binding of the substrate protein to the PK.[4,5] Recent progress in this field has shown that such inhibitors could be invaluable as biological reagents and serve as therapeutically useful compounds for the treatment of a wide variety of disease states.[6] Usually, longer peptidic structures are needed for achieving nanomolar potency, which leads to problems with cellular transport and stability of the compounds.[7]

Logically, a combination of the aforementioned approaches and development of bisubstrate-analog (biligand) inhibitors that simultaneously associate with both ATP and protein binding domains of the dual substrate enzymes could give selective and potent inhibitors of PK. Several strategies of the design of bisubstrate-analog inhibitors have been described.[8] The most potent inhibitors of cAPK Cα with K_i values in the low nanomolar region were constructed by connecting a Hidaka isoquinolinesulfonamide-based ATP-competitive inhibitor H9 ($K_i = 2$ μM) to the N-terminus of the hexaarginine peptide via a linker incorporating *beta*-alanine and L-serine.[9]

Phosphoryl transfer mechanism-based inhibitor design with connection of an adenine nucleotide via its phosphate groups with a substrate peptide at the phosphorylatable serine residue has given compounds with micromolar potency.[10,11] The application of oligophosphate-containing motifs was more successful in the case of the first introduction of this principle into the design of bisubstrate inhibitors for (adenylate) kinase,[12] giving a compound, P1,P5-di(adenosine-5′)pentaphosphate, with nanomolar potency. By virtue of low bioavailability of substances comprising polar peptide moieties and highly charged oligophosphate fragments, the pharmacological potential of these compounds has been limited.

We have previously developed inhibitors for basophilic protein serine/threonine kinases with activities in the submicromolar region. These inhibitors, adenosine−arginine conjugates, comprise moieties of analogs of both substrates of PKs: ATP binding site targeted adenosine-5′-carboxylic acid and the protein substrate domain directed oligo-(L-arginine). The design of the latter fragment was based on the knowledge that phosphorylation sites of the substrates of basophilic PKs (cAPK, PKC, Akt/PKB, PKG, etc.) are flanked by regions rich in arginine and/or lysine residues.[13] Two active fragments of our inhibitors were connected via a linker chain with a length that was optimized in structure−activity studies.[14] By the incorporation of adenosine or other native nucleosides as building blocks, it was hoped to afford the application of existing structure−activity data concerning the affinity of structurally diverse nucleotides, constituents of bisubstrate inhibitors, for the construction of selective inhibitors of PKs.[15]

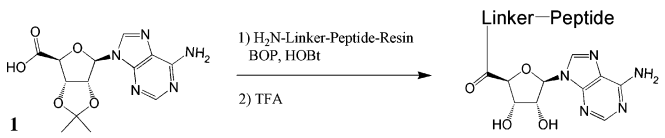
Later we showed that fragments of the conjugates may be chosen in a way that they coincidently increase the inhibitory potency and cell plasma membrane penetration ability of the inhibitors.[16] Moreover, it was established that structures of such biligand inhibitors could afford the attachment of voluminous

* Corresponding author. Phone: +3725175593. Fax: +3727375275. E-mail: asko.uri@ut.ee.

[a] Abbreviations: Adc, adenosine-5′-carboxylic acid; Adn, 5′-amino-5′-deoxyadenosine; Ado, adenosine; AGC, group of protein kinases containing cAMP-dependent protein kinase A, cGMP-dependent protein kinase G, and phospholipid-dependent protein kinase C; Ahx, 6-aminohexanoic acid; ARC, adenosine-arginine conjugate; BAEE, *N*-benzoyl-L-arginine ethyl ester; CAMK, calmodulin-dependent protein kinase; cAPK, cAMP-dependent protein kinase; Cα, catalytic subunit type α; DCE, 1,2-dichloroethane; DIEA, diisopropylethylamine; HOBt, *N*-hydroxybenzotriazole; HTS, high-throughput screening; IQS, 5-isoquinolinesulfonic acid; MBHA, 4-methylbenzhydrylamine; PK, protein kinases; PKI, heat-stable protein kinase inhibitor; Suc, succinic acid; TBTU, *O*-(1*H*-benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate; TFA, trifluoroacetic acid.

10.1021/jm0605942 CCC: $33.50 © 2006 American Chemical Society
Published on Web 11/09/2006

Scheme 1. Synthesis of Adenosine-5′-carboxylic Acid-Based Inhibitors



	Linker	Peptide
2	NH-$(CH_2)_5$CO	(L-Arg)$_4$-NH_2
3	NH-$(CH_2)_5$CO	(L-Arg)$_6$-NH_2
4	NH-$(CH_2)_5$CO	(D-Arg)$_4$-NH_2
5	NH-$(CH_2)_5$CO	(D-Arg)$_6$-NH_2
6	N(CH_3)-$(CH_2)_5$CO	(L-Arg)$_4$-NH_2
7	NH-CH_2-Ph-4-CO	(L-Arg)$_4$-NH_2
8	NH-Phe-*beta*Ala-CO	(L-Arg)$_4$-NH_2

tags carrying some physical (e.g., fluorescent marker) or biological (e.g., biotin for binding avidin or streptavidin) properties.[17]

In this paper, we report on the development of new synthetic procedures for the connection of oligoarginine peptides with adenine, adenosine, adenosine-5′-carboxylic acid, and 5-isoquinolinesulfonic acid and the biological characterization of the compounds as bisubstrate-analog inhibitors of cAPK. An examination of the selectivity of two of the most potent inhibitors, conjugates of Adc and IQS with hexa-(D-arginine) peptide, against a panel of 52 PKs revealed high affinity of the compounds toward basophilic kinases.

Results and Discussion

Chemistry. On the basis of the results of previous structure−activity studies,[14] it was supposed that the optimal length of the oligoarginine moiety was six arginine residues: the removal of two arginines from the C-terminal of the conjugate decreased the inhibitory potency by an order of magnitude (IC_{50} increased from 120 nM to 1.2 μM toward cAPK).[14] On the other hand, the majority of basophilic serine/threonine kinases preferentially phosphorylate substrates with Arg at the P-3 position but vary greatly in additional preference for Arg at P-2 or P-5 (P-designates amino acid residues N-terminal to the phosphorylation site).[18] Arginines at P-2, P-3, and P-6 are essential for cAPK. Hence, six sequential arginine residues in the peptide chain are enough to enable the interaction of three important pharmacophore elements with the target basophilic kinase. Introduction of more than six arginine residues into the ARC-type conjugate could lead to substantial nonspecific interaction of the compound with other basophilic proteins and nucleic acids (e.g., furins, RNA, and DNA). If compared to lysine, another basic amino acid residue, arginine is usually the preferred residue in high-affinity substrates and inhibitors of basophilic PK.[19,20]

However, as the biological characterization of inhibitors with micromolar activity was easier to perform, compounds comprising four arginine residues were utilized for the optimization of structures of the linker and nucleoside moieties of the conjugates. Compounds containing six arginines were later synthesized to achieve higher inhibitory potency. For the elimination of the negative charge from the C-terminal carboxylate group, the conjugates were synthesized in the form of C-terminal amides on Rink amide MBHA resin. Previous results[17,21] had pointed to the possibility that the interaction of the carboxylate group with a positively charged arginine residue could lead to the reduction of inhibitory activity of the conjugates toward PKs.

The synthesis of the first class of compounds containing Adc (**2**−**8**, Scheme 1) was performed as previously described,[14] with the application of Rink amide resin instead of Wang resin. Structures of the linker and peptide components were varied. It was decided to preserve the optimal length of the linker[14] corresponding to that of 6-aminohexanoic acid (equivalent to seven chemical bonds) between the nucleoside and the peptide moieties. Amides of Adc may form an intramolecular hydrogen bond between its amide group and the N3 atom of adenine.[22,23] This leads to the *syn*-conformation of the nucleoside moiety that contradicts the usual binding of ATP and other nucleotides to PKs in their *anti*-conformation. Methylated analog **6**, possessing no hydrogen bond donor characteristics, was synthesized to preclude the formation of an intramolecular hydrogen bond: the resin-bound peptide was first acylated with 6-bromohexanoic acid and then reacted with methylamine. The *N*-methylamino group of the resin-bound peptide was conjugated with **1** (2′,3′-*O*-isopropylidene adenosine-5′-carboxylic acid), according to the standard procedure, to give **6**. 4-(Aminomethyl)benzoic acid was used as an example of a rigid and aromatic linker in the compound **7**. Compound **8**, comprising the phenylalanine-(β)-alanine dipeptide linker, was synthesized to find out the effect of a bulky aromatic group in the linker region.

The diversity of the structure of the peptide part of the conjugates was increased by the variation of length and chirality of the oligoarginine chain, as represented by peptide moieties (L-Arg)$_4$-NH_2 (**2**), (L-Arg)$_6$-NH_2 (**3**), (D-Arg)$_4$-NH_2 (**4**), and (D-Arg)$_6$-NH_2 (**5**).

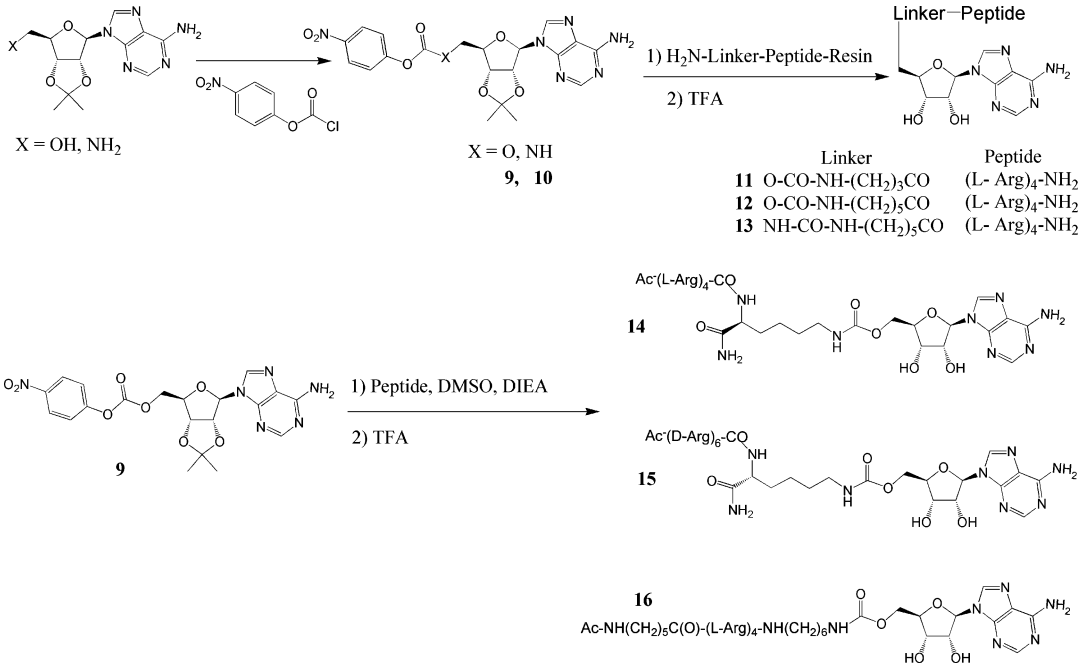
The second group of compounds contained adenosine and 5′-amino-5′-deoxyadenosine derivatives **11**−**13** (Scheme 2). The reaction of 4-nitrophenyl chloroformate with 2′,3′-isopropylidene adenosine[23] resulted in the activated carbonate **9**, which gave adenosine-5′-urethanes **11** and **12** in the reaction with amines. The length of the linker between nucleoside and peptide moieties was varied. Compound **9** turned out to be a suitable reagent for the synthesis of conjugates where adenosine was connected to the C-terminus of the peptide. Peptides containing a free amine group near the C-terminus (e.g., the side chain of lysine or a diamine linker) were prepared on solid phase, cleaved, and purified by HPLC. This was followed by the reaction with **9** and the removal of isopropylidene protection by TFA treatment, leading to compounds **14**−**16**. Activated 4-nitrophenyl urethane **10** was produced in the reaction of 5′-amino-5′-deoxy-2′,3′-*O*-isopropylidene adenosine with 4-nitrophenyl chloroformate. It was then used in the reaction with the N-terminal amino group of the peptide to give the conjugate with a carbamide group (**13**).

The reaction of 5′-amino-5′-deoxy-2′,3′-*O*-isopropylidene adenosine with glutaric anhydride and Boc-protected iminodiacetic acid anhydride yielded **17** and **18**, respectively. These compounds were coupled to resin-bound peptides with the aid of TBTU activation. Cleavage and deprotection of the conjugates in TFA solution gave compounds **19** and **20** that differed from each other by the presence of a CH_2 or NH group in the linker chain (Scheme 3).

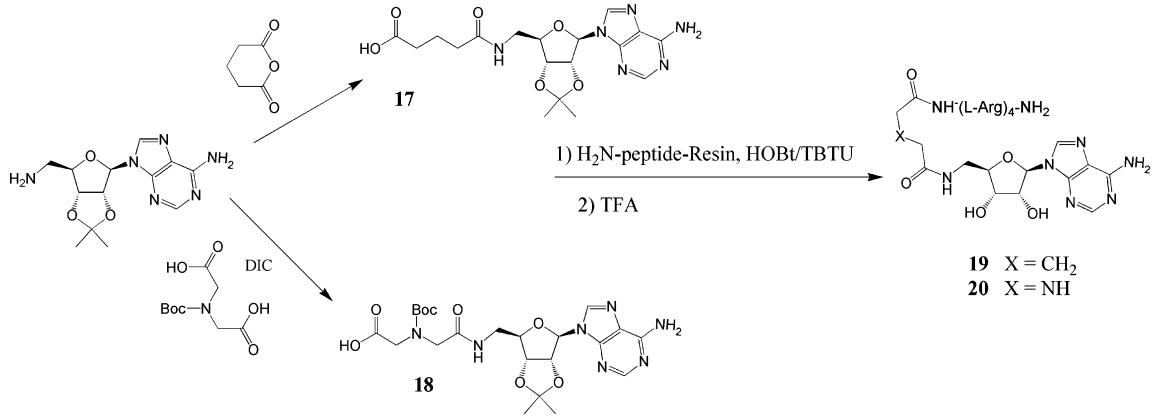
A special class of biligand inhibitors deprived of the ribose moiety, conjugates of adenine and oligoarginine, were synthesized by connecting adenine at the C8 or N9 position to a peptide via a linker chain. Precursors of these molecules containing a linker with a free carboxylate group (**21**, **22**) were synthesized in solution. Coupling of **21** and **22** with the resin-bound peptides resulted in conjugates **23** and **24** after cleavage and deprotection with TFA (Scheme 4).

Conjugates of isoquinolinesulfonamide and oligoarginine were synthesized to compare their activity and selectivity to those of analogous adenosine-containing compounds. If com-

Scheme 2. Synthesis of Compounds with Urethane and Urea Connections and Positioning of Adenosine to the C-Terminus of Peptides



Scheme 3. Synthesis of a Compound with a Secondary Amino Group in the Linker Region and Its CH_2-Containing Counterpart



pared to the previously synthesized isoquinolinesulfonyl peptides,[9] several modifications were introduced into the structure of the conjugate, and the synthesis was carried out on solid phase with the application of Fmoc-peptide and peptoid chemistry procedures. The use of Rink amide resin excluded the presence of the negatively charged C-terminal carboxylate group and resulted in products in the form of their C-terminal amides. The conventional Fmoc-peptide chemistry allowed the circumvention of the harsh HF treatment in the final step of Boc-chemistry procedures.[9] The (β-Ala)-Ser linker of previous conjugates[9] was replaced with 6-aminohexanoic acid, which eliminated potentially phosphorylatable serine residue, removed an unnecessary chiral center, and simplified the overall synthetic procedure. Resin-bound oligoarginine peptides were acylated at N-terminus with 6-bromohexanoic acid, and the following reaction with 5-isoquinolinesulfonyl ethylenediamine and cleavage with TFA lead to conjugates **25** and **26** (Scheme 5).

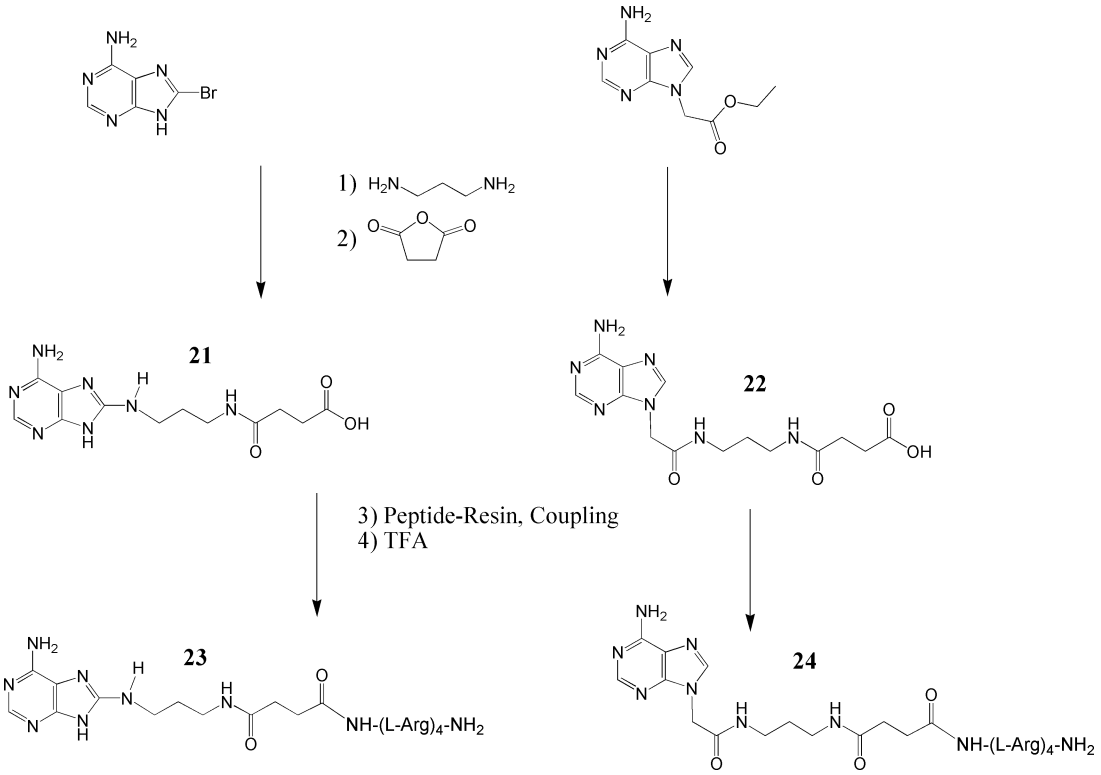
Peptide Ac-(D-Arg)$_6$-(D-Lys)-NH$_2$ (**27**) was synthesized to characterize the inhibitory potency of an all-D oligoarginine peptide.

Biological Evaluation of Compounds. The synthesized oligoarginine conjugates were evaluated as inhibitors of cAPK Cα with the application of the recently described fluorometric TLC kinase activity assay.[24] The inhibitory potency of compounds is expressed in the form of IC_{50} values (Table 1). The following substrate concentrations were used for the determination of IC_{50} values: 100 μM ATP (K_m = 20 μM) and 30 μM TAMRA-kemptide (5-carboxytetramethylrhodamine-LRRASLG, K_m = 3 μM).

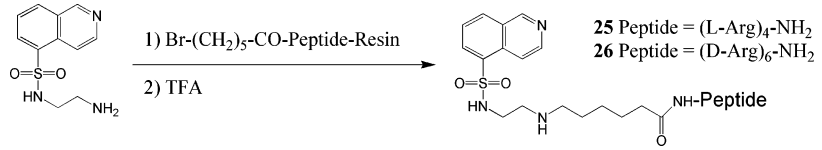
Amidation of the C-terminal carboxylate increased the activity of the conjugates by 4−6 times, leading to IC_{50} values of 2.0 and 0.17 μM for **2** and **3**, respectively. Their carboxylate counterparts showed IC_{50} values of 11.5 and 0.70 μM, respectively. This may be caused by the compensation of the positive charge of an arginine residue that participates in a favorable contact with the kinase by the negative charge of the carboxylate group. Substantial modifications in the structure of the tether between adenosine and peptide moieties of conjugates were well-tolerated by cAPK (Table 1, compounds **2**, **6**, **8**, **11**, and **12**), while some small structural modifications lead to a significant decrease of activity (**7**, **13**). The addition of a *N*-methyl group to the 5′-position of the adenosine moiety of the conjugate (**6** and **2**, respectively) left the activity unchanged, which pointed to the negligible impact of the intramolecular hydrogen bond and putative *syn*-conformation of Adc-amides to their binding to the kinase in *anti*-conformation.[22,23]

Incorporation of a phenylalanine residue into the linker chain (**8**) caused only a minor decrease of activity, which refers to

Scheme 4. Synthesis of Conjugates of Adenine and Oligoarginine



Scheme 5. Synthesis of Isoquinolinesulfonamide-Based Conjugates



the possibility of inclusion of bulky hydrophobic moieties into this part of the inhibitor. Adenosine-5′-urethanes **11** and **12** were synthesized to increase the structural diversity of adenosine-oligoarginine conjugates. Activities of these compounds were well comparable to those of Adc derivatives, while the corresponding 5′-urea-connected compound **13** showed 4-fold lower inhibitory potency. The compound with a 4-(aminomethyl)-benzoic acid linker (**7**) had about 10-fold lower activity than the compound with a 6-aminohexanoic acid linker (**2**). This effect may result from the steric hindrance of the rigid tether to the optimal positioning of interacting fragments, adenosine and tetraarginine, for kinase targeting. Exclusion of the ribose fragment from conjugates (**23** and **24**) lead to substantial reduction of inhibitory potency, which showed the importance of the interaction between the sugar-part of compounds and the kinase; still, both conjugates were better inhibitors of cAPK than the fragments, adenine and oligoarginine.

Variation of the structure of the conjugates also included the connection of adenosine to the C-terminus of the oligoarginine chain (**14**−**16**) and the incorporation of residues of D-arginine (**4**, **5**, and **15**) into the compounds. The latter means also served the aim of increasing the proteolytic stability of the compounds to make them applicable in cell experiments. Inhibitors with peptide moieties connected to the nucleoside part by C- and N-termini had similar affinities in the case of compounds containing peptides with L-amino acid residues (**2** and **14**), whereas the compound comprising the peptide with D-arginine residues connected via the C-terminus (**15**) had 500 times lower activity than the counterpart with N-terminal peptide (**5**). This great difference in activity may originate from the "wrong" spatial positioning of the oligo-(D-arginine) chain caused by D-lysine linker in the structure of **15**. A similar drastic effect occurs with the substitution of a D-alanine linker for a L-alanine in an inhibitor peptide, which lead to a 100-fold decrease of the inhibitor potency in the case of cAPK, which was previously reported.[25]

The compounds with adenosine connected to the N-terminus of the all-D-arginine peptide (**4**, **5**) had remarkably higher affinity than the conjugates with L-amino acids. The compound **4** with four D-arginines (IC_{50} of 0.33 μM) had six times higher potency than the conjugate **2** with four L-amino acid residues. An even greater increase of potency (20 times) was detected for compounds with six arginines: IC_{50} value of 0.17 μM was found for conjugate **3**, comprising l-arginines and 0.0083 μM for its all-D-arginine counterpart **5**. Extrapolated K_i value of 3.2 nM was found for **5** (Supporting Information). This result is surprising by virtue of the fact that cAPK has a strong preference for L-configuration of amino acids both at the phosphorylatable serine residue as well as at the N-terminal arginine residues of the substrate.[26] On the other hand, compounds **2**−**8**, depicted in Scheme 1, comprise oligoarginine peptides attached to Adc N-terminally, not C-terminally, which could be the reasonable positioning of the peptide originating from the consensus sequence of substrates of cAPK (arginines at positions −2, −3, and −6 from the phosphorylation site of the substrate). Introduction of arginine residues into ARC in the form of their D-configuration converts the peptide motif into retro-inverso counterpart of the C-terminally appended oligo(L-arginine)

Table 1. Inhibitory Potencies of Compounds toward cAPK Cα[a]

cmpd	structure	IC_{50} (μM)[b]
2	AdcAhx(L-Arg)$_4$-NH_2	2.0 ± 0.3
3	AdcAhx(L-Arg)$_6$-NH_2	0.17 ± 0.04
4	AdcAhx(D-Arg)$_4$-NH_2	0.33 ± 0.03
5	AdcAhx(D-Arg)$_6$-NH_2	0.0083 ± 0.0015 (0.109 ± 0.021)
6	AdcN(Me)Ahx(L-Arg)$_4$-NH_2	2.6 ± 0.6
7	AdcNHCH$_2$Ph-4-C(O)(L-Arg)$_4$-NH_2	23 ± 4
8	AdcPhe-*beta*Ala(L-Arg)$_4$-NH_2	3.7 ± 0.3
11	AdoC(O)NH(CH$_2$)$_3$C(O)(L-Arg)$_4$-NH_2	3.2 ± 0.3
12	AdoC(O)Ahx(L-Arg)$_4$-NH_2	3.0 ± 0.3
13	AdnC(O)Ahx(L-Arg)$_4$-NH_2	12.5 ± 3.7
14	Ac(L-Arg)$_4$-L-Lys[AdoC(O)]-NH_2	1.4 ± 0.2
15	Ac(D-Arg)$_6$-D-Lys[AdoC(O)]-NH_2	3.4 ± 0.6
16	AcAhx(L-Arg)$_4$-NH(CH$_2$)$_6$-NHC(O)Ado	0.77 ± 0.16
19	AdnC(O)(CH$_2$)$_3$C(O)(L-Arg)$_4$-NH_2	16.5 ± 4.7
20	AdnC(O)CH$_2$NHCH$_2$C(O)-(L-Arg)$_4$-NH_2	13 ± 6
23	Ade-8-NH(CH$_2$)$_3$NH-Suc-(L-Arg)$_4$-NH_2	75 ± 8
24	Ade-9-AcNH(CH$_2$)$_3$NH-Suc-(L-Arg)$_4$-NH_2	26 ± 10
25	H9-(CH$_2$)$_5$C(O)(L-Arg)$_4$-NH_2	0.030 ± 0.007 (0.22 ± 0.03)
26	H9-(CH$_2$)$_5$C(O)(D-Arg)$_6$-NH_2	0.0053 ± 0.0007 (0.067 ± 0.019)
27	Ac-(D-Arg)$_6$-D-Lys-NH_2	~3000
Adenosine		350 ± 40
H89		0.10 ± 0.02 (0.85 ± 0.09)
H9		3.7 ± 0.3
Ado + **27**[c]		57 ± 19

[a] A table with graphical structures and IC_{50} values has been added to the Supporting Information. [b] IC_{50} values for inhibition of cAPK Cα at standard substrate concentrations (ATP, 100 μM; TAMRA-kemptide, 30 μM). Values in parentheses are determined at 1.0 mM ATP concentration. [c] The IC_{50} value for an equimolar mixture of the two compounds.

peptide. A retro-inverso peptide can be regarded as a derivative of a normal peptide in which the relative amino acid side chain topology is maintained, while the backbone termini and direction of the peptide bonds are reversed.[27] Compound **5** is, to the best of our knowledge, the most effective adenosine-based PK inhibitor ever described.

The conjugate of 5-isoquinolinesulfonyl ethylenediamine with the peptide of four L-arginine residues (**25**) had an IC_{50} value of 0.03 μM, which was 65-fold lower than that of the adenosine counterpart **2**. The same difference in potency was measured for the fragments, adenosine and H9 (IC_{50} values of 350 μM and 3.7 μM, respectively, Table 1). This result indicates that combining the peptide of four L-arginine residues via a suitable linker to two structurally different ATP binding site-targeted inhibitors leads to an equal increase in potency for both types of compounds. These results are in good agreement with the results from previous studies.[9,14]

The isoquinolinesulfonylamide-based compound **26**, incorporating six D-arginines, is a highly effective inhibitor of cAPK, exhibiting an IC_{50} value of 5.3 nM in the described assay conditions. The obtained IC_{50} value is similar to that of the most potent adenosine derivative **5**. Still, accurate determination of inhibitory constants for inhibitors with low nanomolar and higher potency is problematic because of comparable initial concentrations of the enzyme (0.5 − 1.0 nM) and inhibitors in the assay solution. To circumvent these problems, to find out the kinetic mechanism of inhibition and to establish the inhibitory potency of these active inhibitors at physiological (millimolar) concentrations of ATP, IC_{50} values of highly active

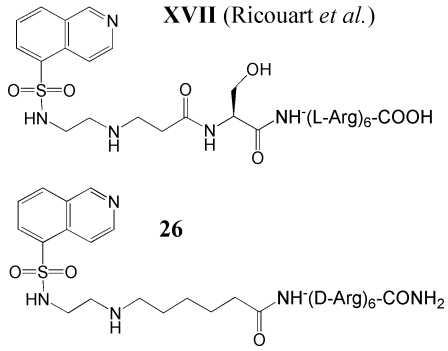


Figure 1. Structures of 5-isoquinolinesulfonamide-oligoarginine conjugates **XVII**[9] and **26**.

compounds were also measured in the presence of higher ATP concentrations. IC_{50} values of all measured compounds were from 7 to 13 times (0.85−1.11 in logarithmic scale) higher in the presence of 1.0 mM ATP if compared to 0.1 mM substrate concentration. These results indicate that all these inhibitors are competitive toward ATP. Apparent K_m of ATP increased continuously when higher concentrations of **5** were applied, while V_{max} maintained its initial value. The increase of concentration of the peptide substrate (TAMRA-kemptide) from 10 μM to 100 μM caused a slight effect on the IC_{50} values of **5**. K_m of TAMRA-kemptide was independent of concentration of **5**, but V_{max} decreased when higher concentrations of the inhibitor were applied (details of competitiveness studies are appended in Supporting Information). These results are in accord with previous studies.[9] The usual explanation to such behavior of bisubstrate analog-type inhibitors arises from the catalytic mechanism of phosphoryl transfer for the catalytic subunit of cAPK that is ordered with the nucleotide binding first.[28,29] Competitive inhibition with respect to ATP and noncompetitive inhibition toward the peptide substrate for the inhibitors targeting simultaneously both substrate binding sites of the kinases has been reported previously and was confirmed in the present study. On the other hand, high inhibitory potency of biligand inhibitors (much higher than that of the constituent single-substrate-targeted inhibitors) points to the simultaneous binding of biligand inhibitors to the ATP-binding pocket and the protein/peptide-binding domain of the kinase. The biligand character of the inhibitors can be monitored directly in binding assays with its competitive displacement from the complex with the kinase by both ATP- and protein/peptide site targeted monofunctional inhibitors (our unpublished data).

The modification of the structure of isoquinolinesulfonamide-based bisubstrate inhibitor, the compound **XVII** (K_i = 4 nM toward cAPK), which was disclosed by Ricouart et al. 15 years ago,[9] lead us to the compound **26** (estimated K_i = 1−2 nM, cAPK) in the present study (Figure 1). In the course of elaboration of the new structure, we relied on the knowledge that was accumulated in the process of the development of ARC-type compounds and followed speculations and recommendations that were recently expressed by David Lawrence.[5]

First, phosphorylatable and chiral serine residue was removed from the linker chain. Second, subsequent β-alanine and L-serine residues were replaced with a flexible and hydrophobic α,ω-hexanoic acid linker, while the length of the tether (seven bonds) between the amino group of isoquinolinesulfonyl ethylenediamine (Hidaka's inhibitor H9) and the N-terminus of the oligoarginine chain was retained.

In **26**, all-D-arginine peptide was substituted for unstable in biological systems all-L-arginine peptide (**XVII**). The C-terminal negatively charged carboxylate group of **XVII** was replaced with

neutral amide group in **26**. All these structural and synthetic modifications and simplifications did not reduce the inhibitory potency of the conjugate but made the structure of **26** more promising for application in biological experiments.

The analysis of inhibition data measured at 1 mM ATP shows that adenosine and isoquinolinesulfonamide derivatives with six D-arginines (**5** and **26**) have similar inhibition potencies, whereas analogous compounds with L-arginines differ by 65-fold in activity. The reason for this different effect of D-arginines in the adenosine and isoquinlinesulfonamide conjugates is unknown. Compounds **25** and **26** have a 3- to 5-fold difference in activity, while in the case of other conjugates the addition of two extra arginines increases the potency by 10-fold. The high affinity of the conjugate of adenosine and D-arginine-peptide for the kinase indicates that an excellent structural fit is induced during the process of complex formation between the partners in the active site of cAPK Cα. This may lead to a tight and closed conformation of the kinase like what happens when PKI and MgATP synergistically bind to the catalytic subunit in the ternary complex.[30,31] The constituents of bisubstrate inhibitor **5** [adenosine and hexa-(D-arginine)-containing peptide **27**] were weak inhibitors. The testing of the inhibitory potency of these fragments in an equimolar mixture (Table 1, Ado + **27**) lead to slightly stronger inhibition than that of the fragments alone, the potency is still incomparably weaker that of the bisubstrate inhibitor **5**.

In earlier experiments, ARC-type compounds were shown[16] to enter cells of different origins and localize in the cytoplasm and nucleus. To be usable in cellular experiments, an inhibitor has to retain its initial structure during the membrane penetration and in intracellular milieu. The introduction of D-arginine residues into cell-penetrating peptides leads to a substantial increase in the stability of peptides to enzymatic degradation of these structures.[32] To test the effect of incorporation of D-Arg residues into ARC-type compounds, the resistance to enzymatic degradation by trypsin and fetal bovine serum of all-D-Arg conjugates **4** and **5** was compared to that of all-L-Arg conjugates **2** and **3**. All these compounds were stable in fetal bovine serum within at least 10 min (data not shown). The trypsin treatment of L-amino acid-containing compounds gave a mixture of all possible deletion analogs in a ratio that shifted in time toward the formation of less-arginine-containing ARCs that could be separated by ion-exchange chromatography with a Mono S column. The half-lives of the compounds **2** and **3** in trypsin solution (50 BAEE-units/mL) were 68 and 37 s, respectively, while at higher concentration (500 BAEE-units/mL) these compounds were completely consumed in less than a minute. In contrast, their D-analogs **4** and **5** remained intact for at least 20 min at even 2-fold higher activity of the protease (1000 BAEE units/mL).

In addition to excellent inhibitory activity of the compound **5**, it could possess even better cellular uptake characteristics than its counterpart with L-arginine residues.[16] This makes **5** a good candidate for its application for the regulation of protein phosphorylation balance in live cells.[33,27]

Selectivity Study. Selectivity testing was performed (on the commercial basis at the Division of Signal Transduction Therapy, University of Dundee) for two compounds, **5** and **26**, that had revealed the highest inhibitory potency toward cAPK Cα. The panel of kinases included all 52 kinases available for testing. To the best of our knowledge, the selectivity profiling has not been carried out for bisubstrate-type inhibitors with a wide panel of PK before. To make the inhibitory potencies comparable, assays were run at ATP concentrations that were close to the K_m value of the kinase, essentially as it has been described previously.[34,35] For comparison, the selectivity of the compound **3** incorporating L-arginine residues was assayed in the PK selectivity panel (KinaseProfiler, Upstate Biotech Inc.) toward 10 different PK in the presence of 100 μM ATP. The data presented in Tables 2 and 3 characterize the activity of the compounds at their 1 μM concentration. They are expressed as the percent of residual activity of the kinase relative to that in control incubations where the inhibitor was omitted.

The activity profiles of the two compounds incorporating D-arginines were mostly similar, but the isoquinolinesulfonamide derivative **26** was generally more effective than the adenosine

Table 2. Residual Activities of PKs in the Presence of Inhibitors **5** and **26**[a]

PK	kinase group[39]	ATP (μM)	residual activity[b] (%) cmpd **5** (1 μM)	cmpd **26** (1 μM)
ROCK-II	AGC	20	0 (±0)	0 (±0)
MAPKAP-K1a/rsk-1	AGC	50	28 (±7)	0 (±1)
SGK	AGC	20	16 (±0)	1 (±0)
MSK1	AGC	20	2 (±0)	1 (±1)
PKBα-Δph	AGC	5	2 (±0)	2 (±0)
P70 S6K	AGC	20	7 (±2)	2 (±0)
MAPKAP-K1b/rsk-2	AGC	50	12 (±0)	2 (±0)
PRK2	AGC	5	2 (±1)	2 (±0)
PKBβ	AGC	50	3 (±0)	3 (±0)
CAMK-1	CAMK	50	15 (±2)	6 (±0)
PKA/cAPK	AGC	20	11 (±7)	6 (±2)
PIM2	CAMK	5	38 (±3)	9 (±0)
PKD1	CAMK	50	54 (±8)	9 (±1)
PKCα	AGC	20	31 (±7)	15 (±5)
CHK2	CAMK	20	14 (±2)	22 (±5)
MST2	STE	20	77 (±3)	24 (±0)
CHK1	CAMK	20	16 (±4)	31 (±1)
MNK2	CAMK	50	99 (±9)	31 (±3)
AMPK	CAMK	50	48 (±6)	39 (±4)
ERK8	CMGC	5	50 (±9)	52 (±0)
Aurora B	other	20	103 (±5)	53 (±1)
MARK3	CAMK	5	40 (±3)	53 (±4)
PDK1	AGC	20	62 (±4)	56 (±1)
NEK7	other	20	58 (±1)	56 (±9)
PBK	TK	50	88 (±5)	67 (±4)
MNK1	CAMK	50	102 (±1)	68 (±9)
DYRK1a	CMGC	50	72 (±6)	69 (±1)
MAPKAP-K3	CAMK	20	67 (±0)	69 (±8)
CSK	TK	20	48 (±9)	70 (±1)
CDK2/cyclin A	CMGC	20	88 (±7)	73 (±3)
JNK/SAPK1c	CMGC	20	70 (±7)	75 (±4)
PLK1	other	5	81 (±10)	76 (±2)
SAPK2a/p38	CMGC	50	79 (±2)	76 (±7)
SAPK2b/p38ss2	CMGC	20	85 (±0)	80 (±2)
MAPKAP-K2	CAMK	20	85 (±6)	80 (±2)
MKK1	STE	5	77 (±9)	84 (±6)
IKKβ	other	5	94 (±11)	87 (±1)
CK2	other	5	104 (±9)	87 (±7)
Src	TK	50	84 (±9)	88 (±4)
Lck	TK	50	70 (±3)	88 (±3)
smMLCK	CAMK	50	72 (±0)	90 (±5)
EF2K	atypical	5	92 (±9)	91 (±8)
JNK3	CMGC	50	91 (±6)	92 (±9)
SAPK4/p38δ	CMGC	5	107 (±1)	93 (±1)
GSK3β	CMGC	5	56 (±4)	94 (±9)
MAPK2/ERK2	CMGC	50	100 (±3)	95 (±7)
CK1	CK1	20	106 (±8)	97 (±4)
PRAK	CAMK	20	89 (±4)	97 (±8)
NEK2a	other	50	82 (±2)	98 (±6)
SAPK3/p38γ	CMGC	5	111 (±4)	99 (±1)
SRPK1	CMGC	50	100 (±3)	99 (±2)
NEK6	other	50	94 (±8)	100 (±8)

[a] The experiments were performed essentially as it has been described previously.[34,35] [b] Percent of residual activity of the kinase (at 1 μM concentration of the inhibitor) as relative to that in control incubations where the inhibitor was omitted (means of duplicate determinations).

Table 3. Residual Activities of PKs in the Presence of the Inhibitor **3**[a]

PK	residual activity[b] (%)	PK	residual activity[b] (%)
Aurora-A	62 (±1)	PKBα	30 (±3)
CHK1	49 (±6)	PKCβII	76 (±4)
CK2	86 (±1)	PKCη	17 (±2)
MSK1	14 (±3)	ROCK-II	4 (±2)
PKA	11 (±2)	SGK	75 (±0)

[a] Assayed in a PK selectivity panel (KinaseProfiler, Upstate Biotech, Inc.) in the presence of 100 μM ATP. [b] Percent of residual activity of kinases (at 1 μM concentration of the compound **3**) as relative to that in control incubations where the inhibitor was omitted (means of duplicate determinations).

derivative **5**. Both conjugates inhibited strongly ROCK-II, MSK1, PKBβ, PKBΔph, and PRK2 (residual activity ≤3%). Compound **26** inhibited almost completely MAPKAP-K1a/rsk1, p70S6K, and MAPKAP-K1b, while residual activity in the presence of **5** was 7−28% for these kinases. The biggest difference between the two compounds was observed for the inhibition of MAPKAP-K1a/rsk1, where the corresponding values were 0 and 28% for **26** and **5**, respectively.

CAMK-1, PKA, PIM2, and PKD1 retained less than 10% of activity in the presence of **26**. The significant difference between the two compounds was also found for PKD1 (the residual activities were 54% for **5** and 9% for **26**). Weaker inhibition (residual activities were 11 and 6% for **5** and **26**, respectively) was established for PKA in this assay than it could be expected, based on inhibitory potency determined in this work (Table 1); actually, this special measurement was subjected to large errors (Table 2). Checkpoint kinases (CHK1 and CHK2) were both inhibited more strongly by **5** than by **26**. Several kinases were weakly inhibited by biligand inhibitors, while some of them were not inhibited at all, for example, acidophilic kinases represented by casein kinases CK1 and CK2 retained full activity in the presence of **5** as well as **26**. As illustrated by data in Table 2, oligo-(D-arginine)-containing biligand inhibitors were not specific to any particular PK but slowed down the reactions catalyzed by basophilic kinases, especially of the AGC and CAMK groups. Of the 14 PK inhibited by more than 85% by compound **26** at 1 μM concentration, 11 kinases belong to the AGC group and 3 kinases belong to the CAMK group. All these 14 kinases have been reported[18,36] to be of basophilic type, which points to the biligand character of the inhibitors, where the inhibitory potency of the conjugates is influenced by binding to sites of both substrates of the kinase. The only AGC group kinase that falls out of this selection is PDK, an AGC kinase that expresses badly the defined consensus sequences and lacks strong Arg preference at any position.[18]

The selectivity of L-arginine-containing compound **3** was tested toward 10 different PKs (Table 3). The results point to the similarity of the activity profile of the compound **3** to those of D-arginine-containing conjugates **5** and **26** (Table 2): ROCK-II was the most strongly inhibited kinase, followed by other basophilic kinases PKA, MSK1, and PKCη. A remarkable difference in the inhibitory potency of **3** toward PKCβII and PKCη (residual activities of 76 and 17%, respectively) was observed. Although the lower potency of **3** if compared to that of its D-arginine-containing counterpart **5** is apparent toward all basophilic kinases tested, the different assay formats (e.g., dissimilar ATP concentration) do not afford a direct comparison of the activities of the compounds.

The further modification of the structure of the conjugates with the aim of increasing the specificity could be supported by the established structural factors important for the selectivity of the inhibitors targeting the ATP-binding pocket[15,37] and the protein/peptide binding domain.[38] Such an approach makes it possible to retain high affinity of inhibitors toward special kinases but suppresses the activity toward others. Group-selective inhibitors might be valuable tools for several biological applications, for example, as probes in bioanalytical methods for the determination of the active concentration of kinases and the evaluation of new inhibitors in HTS assays.

Conclusions

Flexible and productive solid-phase synthetic methods were developed for the preparation of conjugates of oligoarginine peptides with adenine, adenosine, adenosine-5′-carboxylic acid, and 5-isoquinolinesulfonic acid. Amidation of peptide C-terminus increased the potency of bisubstrate inhibitors 4- to 6-fold. Surprisingly, high affinity (low nanomolar in the case of Adc-based conjugate **5**) was found for the D-arginine-containing compound toward cAPK Cα; this compound was also resistant to trypsin degradation. The bisubstrate character of the inhibitor is supported by the high inhibitory potency of the compound if compared to the submillimolar potencies of the single site-targeted constituents of the conjugate, adenosine, and oligo-(D-arginine). On the other hand, profiling with the 52-kinase selectivity panel reveals a strong tendency of the arginine-rich conjugates to inhibit specifically basophilic PKs, which points to the active participation of both functionary moieties in the formation of the binary complex with the kinase.

In view of established physical and biological characteristics of developed inhibitors **5** and **26** (high inhibitory potency, resistance proteolytic degradation, good water solubility, penetration of cell plasma membrane), these compounds first of all show potential for successful development of tools for bioanalysis, including in vitro assays of functional kinomics and inhibitor testing.

Materials and Methods

All chemicals were obtained commercially unless otherwise noted. Solvents were from Rathburn and Fluka. Solid-phase resins and other peptide synthesis chemicals were from Neosystem, Novabiochem, Advanced ChemTech, and AnaSpec. Other chemicals were from Sigma-Aldrich. Compound **9**[23], *N*-(*tert*-butyloxycarbonyl)-iminodiacetic acid,[40] H9,[41] and 5′-amino-5′-deoxy-2′,3′-*O*-isopropylidene adenosine[42] were synthesized according to previously described methods. ^{1}H and ^{13}C NMR spectra were taken on Bruker AC 200P spectrometer. Mass spectra of all synthesized compounds were measured with MALDI-TOF mass spectrometer Voyager DE-Pro (Applied Biosystems). Unicam UV 300 (ThermoSpectronic) spectrometer was used for measuring UV-vis spectra and quantification of the products. The solution-phase reactions were monitored by thin-layer chromatography (TLC) on Polygram Sil G/UV$_{254}$ plates (Macherey−Nagel), and a UV-lamp was used for the visualization of the products. Fluorescence imaging was performed by Molecular Imager FX Pro Plus (Bio-Rad Laboratories; excitation at 532 nm, 555 nm LP emission filter, 100 μm resolution), and scanned images were processed with Quantity One software (Bio-Rad). Langford Sonomatic 375H ultrasonic bath was used for sonication. Column chromatography was performed on silica gel 60 (0.04−0.063 mm), purchased from Fluka. The final products were purified with Gilson HPLC system using C18 reversed-phase column (GL Sciences) Inertsil ODS-3 (5 μm, 25 × 0.46 cm), with monitoring at 260 nm or 220 nm (peptides). Elution was performed with water−acetonitrile gradient (0.1% TFA), with a flow rate of 1 mL/min. The separated products were freeze-dried. The purity of the products was characterized by cation exchange chromatography (Mono S HR 5/5, Pharmacia Biotech) by using the eluent systems of A (20 mM phosphate buffer, pH = 7.6, 18% isopropanol) and B (40 mM phosphate buffer, pH = 7.6, 1.2 M NaCl, 18% isopropanol) with a gradient of 15−100% B for 10 min,

followed by 100% B for 10 min, with a flow rate of 1 mL/min. All compounds used in biological tests were >95% pure by HPLC (detected at 260 nm, Table 1). Concentrations of compounds for biological testing were measured by UV spectroscopy [molar extinction coefficient of 15 000 $M^{-1}cm^{-1}$ at 259 nm was used for adenosine derivatives and **24**, ~20 000 $M^{-1}cm^{-1}$ at 245 nM for **7**, 4400 $M^{-1}cm^{-1}$ at 323 nm for isoquinolinesulfonamide derivatives, and 17 000 $M^{-1}cm^{-1}$ at 295 nm (pH 2) for **23**].

5′-*p*-Nitrophenyloxycarbonylimino-5′-deoxy-2′,3′-*O*-isopropylidene Adenosine (10). *p*-Nitrophenyl chloroformate (40.5 mg, 0.20 mmol) was dissolved in DCE (250 μL). Pyridine (20 μL), DIEA (50 μL), and DCE (750 μL) were added to the solution. The formed suspension was transferred to solid 5′-amino-5′-deoxy-2′,3′-*O*-isopropylidene adenosine (45.9 mg, 0.15 mmol) and stirred. A clear solution formed within 15 min. The solvents were removed in vacuo, and the product was separated by column chromatography (EtOAc/acetone 2/1, $R_f = 0.47$; 41 mg, 58% yield). MALDI-TOF MS (*m*/*z*): $C_{20}H_{21}N_7O_7$, 472 $[M + H]^+$. ^1H NMR (200 MHz, $CDCl_3$): δ 1.37 and 1.64 (6H, s, CH_3 of isopropylidene (Ip)), 3.56 (1H, m, $J = 14.3$, 2.8 and 1.6 Hz, 5′), 3.88 (1H, m, $J = 14.3$, 8.5 and 2.6 Hz, 5′), 4.56 (1H, m, $J = 2.8$, 2.7 and 2.5 Hz, 4′), 5.02 (1H, dd, $J = 6.5$ and 2.7 Hz, 3′), 5.30 (1H, dd, $J = 6.5$ and 4.3 Hz, 2′), 5.89 (1H, d, $J = 4.3$ Hz, 1′), 6.01 (2H, br, NH_2), 7.37 (2H, d, $J = 9.2$ Hz, *o*-PNP (4-nitrophenyl)), 7.87 (1H, s, A), 8.25 (2H, d, $J = 9.2$ Hz, *m*-PNP), 8.39 (1H, s, A), 8.89 (1H, br m, $J = 8.5$ and 1.6 Hz, NH).

5′-Glutarylamido-5′-deoxy-2′, 3′-*O*-isopropylidene Adenosine (17). 5′-Amino-5′-deoxy-2′,3′-*O*-isopropylidene adenosine (130 mg, 0.425 mmol) and glutaric acid anhydride (60 mg, 0.526 mmol) were stirred in DMF (1.3 mL) for 1.5 h. The solvent was removed, and the residue was purified by column chromatography ($CHCl_3$/MeOH 10/3). The product was dissolved in EtOH and precipitated by adding *t*-BuOMe (106 mg, 60%). UV_{max} 259 nm (methanol). MALDI-TOF MS (*m*/*z*): $C_{18}H_{24}N_6O_6$, 421 $[M + H]^+$. ^1H NMR (200 MHz, DMSO-d_6): δ 1.31 and 1.53 (2 × 3H, s, Ip), 1.70 (2H, m, CH_2), 2.15 (4H, m, CH_2CO), 3.32 (2H, t, $J = 6.0$ Hz, 5′), 4.16 (1H, dt, $J = 6.0$ and 3.3 Hz, 4′), 4.90 (1H, dd, $J = 6.5$ and 3.3 Hz, 3′), 5.42 (1H, dd, $J = 6.5$ and 3.0 Hz, 2′), 6.12 (1H, d, $J = 3.0$ Hz, 1′), 3.37 (2H, br, NH_2), 8.11 (1H, t, $J = 6.0$ Hz, amide), 8.18 and 8.33 (2H, s, A2 and A8).

5′-[*N*-Carboxymethyl-(*N*-*tert*-butyloxycarbonyl)-acetamido]-5′-deoxy-2′,3′-*O*-isopropylidene Adenosine (18). *N*-(*tert*-Butyloxycarbonyl)-iminodiacetic acid (101 mg, 0.43 mmol), HOBt (65 mg, 0.425 mmol), and *N*,*N*′-diisopropylcarbodiimide (65 μL, 0.42 mmol) were stirred at 0 °C for 15 min in DMF (0.75 mL). The mixture was then poured into 5′-amino-5′-deoxy-2′,3′-*O*-isopropylidene adenosine (105 mg, 0.34 mmol) in DMF (0.75 mL). The solution was stirred for 2 h, and the solvent was removed. The residue was purified by column chromatography ($CHCl_3$/MeOH 10/3). The product was precipitated form EtOH with *t*-BuOMe (126 mg, 70%). UV_{max} 259 nm (methanol). MALDI-TOF MS (*m*/*z*): $C_{22}H_{31}N_7O_8$, 522 $[M + H]^+$. ^1H NMR (200 MHz, DMSO-d_6): δ (signals of the major rotamer are marked with asterisk*) 1.27* and 1.32 (9H, s, *t*-Bu), 1.29 and 1.51 (2 × 3H, s, Ip), 3.5−3.9 (6H, m, 5′ and CH_2 of iminodiacetic acid (Ida)), 4.20 (1H, m, 4′), 4.92 (1H, m, 3′), 5.37 and 5.47* (1H, dd, $J = 6.2$ and 3.2 Hz, $J^* = 6.3$ and 2.9 Hz, 2′), 6.08 and 6.12* (1H, d, $J = 3.2$ Hz, $J^* = 2.9$ Hz, 1′), 7.36 (2H, br, NH_2), 8.16*, 8.17, 8.38*, and 8.41 (2H, s, A2 and A8), 8.70 (1H, br, CONH).

8-(Succinylamido-3-propylamino)-adenine (21). 8-Bromoadenine (54.6 mg, 0.255 mmol) and diaminopropane (1 mL, 11.9 mmol) were heated 24 h under argon at 135−140 °C. Volatiles were removed under reduced pressure, and the residue was triturated with chloroform (5 mL). The precipitate was separated and dried (73 mg). The crude 8-(amino-3-propylamino)-adenine was suspended in DMF (1 mL) and DIEA (0.1 mL). Succinic anhydride (35 mg, 0.35 mol) was added, the suspension was sonicated for 25 min, and the solvents were removed under reduced pressure. The residue was dissolved in K_2CO_3 solution (0.6 mL), and after 1 h, the product was precipitated by adding 10% $KHSO_4$ solution until pH was about 4. The residue was separated, washed with water (3×), and dried to give **21** (49.3 mg, 63%). UV_{max} 295 nm (pH 2). MALDI-TOF MS (*m*/*z*): $C_{12}H_{17}N_7O_3$, 308 $[M + H]^+$. ^1H NMR (200 MHz, DMSO-d_6): δ 1.66 (2H, qn, $J = 6.8$ Hz, CH_2), 2.31 (2H, t, $J = 5.8$ Hz, CH_2), 2.43 (2H, t, $J = 5.8$ Hz), 3.11 (2H, dt, $J = 6.8$ and 6.0 Hz, CH_2), 3.26 (2H, dt, $J = 6.8$ and 6.0 Hz, CH_2), 6.33 (2H, br, NH_2), 6.94 (1H, br, NH), 7.91 (1H, s, A2), 7.94 (1H, t, $J = 6.0$ Hz, NHCO).

9-(Succinylamido-3-propylaminocarbonylmethyl)-adenine (22). Ethyl adenyl-9-acetate (172 mg, 0.777 mmol) was dissolved in diaminopropane (1.4 mL, 16.7 mmol) and stirred for 1 h until the precipitation was complete. The excess of diaminopropane was removed, and 9-aminopropylaminoacetyladenine was used in the next step without further purification. UV_{max} 259 (water pH 7). MALDI-TOF MS (*m*/*z*): $C_{10}H_{15}N_7O$, 250 $[M + H]^+$. ^1H NMR (200 MHz, DMSO-d_6): δ 1.52 (2H, qn, $J = 6.8$ Hz, CH_2), 2.57 (2H, t, $J = 6.8$ Hz, CH_2), 3.13 (2H, dt, $J = 6.8$ and 5.6 Hz, CH_2), 4.81 (2H, s, CH_2 Ac), 7.22 (2H, br, NH_2), 8.06 and 8.11 (2H, s, A2 and A8), 8.35 (1H, t, $J = 5.6$ Hz, NHCO).

9-Aminopropylaminoacetyladenine was suspended in water (1.5 mL), and succinic anhydride (158 mg, 1.58 mmol) was added and stirred for 10 min. K_2CO_3 (250 mg) was added, and the reaction mixture was stirred for 2 h and acidified with $KHSO_4$ solution until the pH was about 4. The precipitate was washed with water (3 × 10 mL) and dried under reduced pressure to give **22** (184 mg, 68%). UV_{max} 259 (water, pH 7). MALDI-TOF MS (*m*/*z*): $C_{14}H_{19}N_7O_4$, 350 $[M + H]^+$. ^1H NMR (200 MHz, DMSO-d_6): δ 1.54 (2H, qn, $J = 7.0$ Hz, CH_2), 2.29 (2H, t, $J = 6.0$ Hz, CH_2), 2.41 (2H, t, $J = 6.0$ Hz, CH_2), 3.0−3.15 (4H, m, 2 × CH_2), 4.81 (2H, s, CH_2), 7.21 (2H, br, NH_2), 7.85 (1H, t, $J = 5.3$ Hz, NH), 8.06 and 8.11 (2H, s, A2 and A8), 8.27 (1H, t, $J = 5.3$ Hz, NH).

Solid-Phase Synthesis. Peptide fragments were prepared by using traditional Fmoc solid-phase peptide synthesis methods on Rink amide MBHA resin. Protected amino acids (3 equiv) were dissolved in DMF and activated with BOP/HOBt (2.94 equiv each) in DMF/*N*-methylmorpholine. Coupling solutions were added to the resin and shaken for 40−60 min. The completeness of each step was monitored with the Kaiser test, which was followed by deprotection of the Fmoc group with 20% piperidine solution in DMF (20 min). Fmoc-protected linkers were attached to the peptide part following the same protocol.

Carboxylate containing adenosine analog (1.5 equiv) was used for coupling to the resin-bound peptide. HOBt/BOP (1.47 equiv each) activation was used for **1** and HOBt/TBTU (1.47 equiv each) was used for **17**, **18**, **21**, and **22**. One-hour reaction times in DMF/DIEA were used. Coupling of **22** was repeated several times due to incomplete reaction.

4-Nitrophenyl-containing adenosine derivatives (1.2−1.3 equiv; **9**, **10**) were dissolved in DMF/DIEA and added to the resin, and the reaction mixture was agitated overnight.

6-Bromohexanoic acid (10 equiv) was activated with *N*,*N*′-diisopropylcarbodiimide (5 equiv) at 0 °C in DMF and added to the resin. After a 45-min agitation, the resin was washed. The resulting alkyl bromide was reacted with primary amine (4 equiv for H9 and 10 equiv for methylamine) in DMSO/DIEA for 15 h to give resin-bound secondary amine.

Finally, the resins were washed five times with each solvent (DMF, isopropanol, and DCE) and dried. Treatment with TFA/H_2O/triisopropylsilane (90/5/5 by volume) for 2−3 h was used as the standard cleavage procedure. The products were purified by reversed-phase HPLC and lyophilized.

Precursor peptides for the synthesis of compounds **14**−**16** were prepared on solid phase, cleaved, and finally purified by HPLC. Lysine-containing precursors for the synthesis of **14** and **15** were prepared on Rink amide resin, while the precursor of **16** was prepared on 1,6-diaminohexane trityl resin. Ac-(L-Arg)$_4$-Lys-NH_2, HPLC: $R_t = 11.2$ min. MALDI-TOF MS (*m*/*z*): 812 $[M + H]^+$. Ac-(D-Arg)$_6$-Lys-NH_2, HPLC: $R_t = 6.4$ min. MALDI-TOF MS (*m*/*z*): 1125 $[M + H]^+$. Ac-NH-$(CH_2)_5$-C(O)-(L-Arg)$_4$-C(O)-NH-$(CH_2)_6$-NH_2, HPLC: $R_t = 15.8$ min. MALDI-TOF MS (*m*/*z*): 897 $[M + H]^+$. Amine-containing peptide and **9** (1.3 equiv) were dissolved in DMSO and DIEA. The solution was stirred overnight

and evaporated to dryness. The removal of the isopropylidene group by TFA/H_2O treatment (1 h) and purification by HPLC gave products **14**−**16**.

Proteolytic Degradation. The proteolytic degradation experiments were carried out by incubating the inhibitors (400 μM) at 30 °C in fetal bovine serum or a phosphate buffered (40 mM, pH = 7.2) solution of trypsin at various concentrations in a final volume of 20 μL. The degradation was monitored by following the change in the concentration of the starting material and Adc-bearing degradation products by cation exchange chromatography with NaCl gradient (0.0−1.0 M for10 min, with a flow rate of 1 mL/min) in 18% isopropanol/40 mM phosphate buffer, pH = 7.2. The absorbance was detected at 258 nM, and the collected fractions were characterized by MALDI MS. Tryptic activity was measured spectrophotometrically using BAEE as substrate.

Fluorometric TLC Kinase Assay. The IC_{50} values of the inhibitors were measured as previously described.[24] The inhibitors in various concentrations were incubated at 30 °C in Hepes buffer (50 mM, pH = 7.5) containing cAPK Cα (about 1 nM), TAMRA-kemptide (10, 30 or 100 μM), ATP (100 μM or 1 mM), magnesium acetate (10 mM), and bovine serum albumin (0.2 mg/mL). ATP was added last to initiate the phosphorylation reaction. At fixed time points, the reaction was stopped by a 20-fold dilution with 75 mM phosphoric acid, and obtained solutions were analyzed by normal-phase TLC (without fluorescence indicator, eluted with 1-butanol/pyridine/acetic acid/water, 15/10/12/12 by volume). The visualization and quantification of the fluorescent spots were carried out by fluorescence imaging. Data were processed with Graphpad Prism software (version 4, GraphPad).

Acknowledgment. The authors thank Prof. Sir Philip Cohen for his support with the use of kinase selectivity panel, Ursel Soomets (Department of Biochemistry, University of Tartu) for access to the MALDI TOF mass spectrometer, and Sulev Kõks (Department of Physiology, University of Tartu) for access to the Molecular Imager. The work was supported by grants from the Estonian Science Foundation (6710) and the Estonian Ministry of Education and Sciences (0182611s03).

Supporting Information Available: Tables listing structures and analytical data of the evaluated inhibitors and their IC_{50} values, results of competitiveness studies of **5**, full names of the kinases used in the selectivity panels, and ^{13}C spectra of synthesized compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Pelech, S. Tracking cell signaling protein expression and phosphorylation by innovative proteomic solutions. *Curr. Pharm. Biotechnol.* **2004**, *5*, 69−77.
(2) Cohen, P. Protein kinases: The major drug targets of the twenty-first century? *Nat. Rev. Drug Discovery* **2002**, *1*, 309−315.
(3) Fischer, P. M. The design of drug candidate molecules as selective inhibitors of therapeutically relevant protein kinases. *Curr. Med. Chem.* **2004**, *11*, 1563−1583.
(4) Bogoyevitch, M. A; Barr, R. K.; Ketterman, A. J. Peptide inhibitors of protein kinases−discovery, characterisation and use. *Biochim. Biophys. Acta* **2005**, *1754*, 79−99.
(5) Lawrence, D. S. New design strategies for ligands that target protein kinase-mediated protein−protein interactions. In *Handbook of Experimental Pharmacology*, Inhibitors of protein kinases and protein phosphatases; Pinna, A. L., Cohen, P. T. W., Eds.; Springer-Verlag: Berlin, Heidelberg GmbH & Co. K, 2005; Vol 167, pp 11−44.
(6) Luo, Y.; Smith, R. A.; Guan, R.; Liu, X.; Klinghofer, V.; Shen, J.; Hutchins, C.; Richardson, P.; Holzman, T.; Rosenberg, S. H.; Giranda, V. L. Pseudosubstrate peptides inhibit Akt and induce cell growth inhibition. *Biochemistry* **2004**, *43*, 1254−1263.
(7) Gumireddy, K.; Reddy, M. V.; Cosenza, S. C.; Boominathan, R.; Baker, S. J.; Papathi, N.; Jiang, J.; Holland, J.; Reddy, E. P. ON01910, a non-ATP-competitive small molecule inhibitor of Plk1, is a potent anticancer agent. *Cancer Cell* **2005**, *7*, 275−286.
(8) Parang, K.; Cole, P. A. Designing bisubstrate analog inhibitors for protein kinases. *Pharmacol. Ther.* **2002**, *93*, 145−157.
(9) Ricouart, A.; Gesquiere, J. C.; Tartar, A.; Sergheraert, C. Design of potent kinase inhibitors using bisubstrate approach. *J. Med. Chem.* **1991**, *34*, 73−78.
(10) Medzihradszky, D.; Chen, S. L.; Kenyon, G. L.; Gibson, B. Solid-phase synthesis of adenosine phosphopeptides as potential bisubstrate inhibitors of protein kinases. *J. Am. Chem. Soc.* **1994**, *116*, 9413−9419.
(11) Shen, K.; Hines, A. C.; Schwarzer, D.; Pickin, K. A.; Cole, P. A. Protein kinase structure and function analysis with chemical tools. *Biochim. Biophys. Acta* **2005**, *1754*, 65−78.
(12) Lienhard, G. E.; Secemski, I. I. P1,P5-Di(adenosine-5′)pentaphosphate, a potent multisubstrate inhibitor of adenylate kinase. *J. Biol. Chem.* **1973**, *248*, 1121−1123.
(13) Kreegipuu, A.; Blom, N.; Brunak, S.; Jarv, J. Statistical analysis of protein kinase specificity determinants. *FEBS Lett.* **1998**, *430*, 45−50.
(14) Loog, M.; Uri, A.; Raidaru, G.; Jarv, J.; Ek, P. Adenosine-5′-carboxylic acid peptidyl derivatives as inhibitors of protein kinases. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 1447−1452.
(15) Shugar, D. The NTP phosphate donor in kinase reactions: is ATP a monopolist? *Acta Biochim. Pol.* **1996**, *43*, 9−23.
(16) Uri, A.; Raidaru, G.; Subbi, J.; Padari, K.; Pooga, M. Identification of the ability of highly charged nanomolar inhibitors of protein kinases to cross plasma membranes and carry a protein into cells. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 2117−2120.
(17) Viht, K.; Padari, K.; Raidaru, G.; Subbi, J.; Tammiste, I.; Pooga, M.; Uri, A. Liquid-phase synthesis of a pegylated adenosine-oligoarginine conjugate, cell-permeable inhibitor of cAMP-dependent protein kinase. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 3035−3039.
(18) Zhu, G.; Fujii, K.; Liu, Y.; Codrea, V.; Herrero, J.; Shaw, S. A single pair of acidic residues in the kinase major groove mediates strong substrate preference for P-2 or P-5 arginine in the AGC, CAMK, and STE kinase families. *J. Biol. Chem.* **2005**, *280*, 36372−36379.
(19) Kemp, B. E.; Graves, D. J.; Benjamini, E.; Krebs, E. G. Role of multiple basic residues in determining the substrate specificity of cyclic AMP-dependent protein kinase. *J. Biol. Chem.* **1977**, *252*, 4888−4894.
(20) Loog, M.; Oskolkov, N.; O'Farell, F.; Ek, P.; Jarv, J. Comparison of cAMP-dependent protein kinase substrate specificity in reaction with proteins and synthetic peptides. *Biochim. Biophys. Acta* **2005**, *1747*, 261−266.
(21) Loog, M.; Uri, A.; Järv, J.; Ek, P. Bi-substrate analogue ligands for affinity chromatography of protein kinases. *FEBS Lett.* **2000**, *480*, 244−248.
(22) Prasad, R. N.; Bariana, D. S.; Fung, A.; Savic, M.; Tietje, K.; Stein, H. H.; Brondyk, H.; Egan, R. S. Modification of the 5′ position of purine nucleosides. 2. Synthesis and some cardiovascular properties of adenosine-5′-(N-substituted)carboxamides. *J. Med. Chem.* **1980**, *23*, 313−319.
(23) Pehk, T.; Uri, A. Synthesis and structural characterization of conjugates of adenosine and tetra-aspartate, novel analogs of ATP. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 2159−2164.
(24) Viht, K.; Vaasa, A.; Raidaru, G.; Enkvist, E.; Uri, A. Fluorometric TLC assay for evaluation of protein kinase inhibitors. *Anal. Biochem.* **2005**, *340*, 165−170.
(25) Yan, X.; Lawrence, D. S.; Corbin, J. D.; Francis, S. H. Distinguishing between closely related protein kinases: a variation on the bisubstrate inhibitor theme. *J. Am. Chem. Soc.* **1996**, *118*, 6321−6322.
(26) Eller, M.; Sepp, A.; Toomik, R.; Ekman, P.; Järv, J.; Ragnarsson, U.; Engstrom, L. Studies on the substrate specificity of cAMP-dependent protein kinase using diastereomeric peptides. *Biochem. Int.* **1991**, *25*, 453−460.
(27) Fischer, P. M. The design, synthesis and application of stereochemical and directional peptide isomers: a critical review. *Curr. Protein Pept. Sci.* **2003**, *4*, 339−356.
(28) Whitehouse, S.; Feramisco, J. R.; Casnellie, J. E.; Krebs, E. G.; Walsh, D. A. Studies on the kinetic mechanism of the catalytic subunit of the cAMP-dependent protein kinase. *J. Biol. Chem.* **1983**, *258*, 3693−3701.
(29) Adams, J. A. Kinetic and catalytic mechanisms of protein kinases. *Chem. Rev.* **2001**, *101*, 2271−2290.
(30) Whitehouse, S.; Walsh, D. A. Mg•ATP^{2-}-dependent interaction of the inhibitor protein of the cAMP-dependent protein kinase with the catalytic subunit. *J. Biol. Chem.* **1983**, *258*, 3682−3692.
(31) Lew, J.; Coruh, N.; Tsigelny, I.; Garrod, S.; Taylor, S. S. Synergistic binding of nucleotides and inhibitors to cAMP-dependent protein kinase examined by acrylodan fluorescence spectroscopy. *J. Biol. Chem.* **1997**, *272*, 1507−1513.
(32) Elmquist, A.; Langel, Ü. In vitro uptake and stability study of pVEC and its all-D analog. *Biol. Chem.* **2003**, *384*, 387−393.
(33) Borghouts, C.; Kunz, C.; Groner, B. J. Current strategies for the development of peptide-based anti-cancer therapeutics. *J. Pept. Sci.* **2005**, *11*, 713−726.

(34) Kuma, Y.; Sabio, G.; Bain, J.; Shpiro, N.; Marquez, R.; Cuenda, A. BIRB796 inhibits all p38 MAPK isoforms in vitro and in vivo. *J. Biol. Chem.* **2005**, *280*, 19472−19479.
(35) Murray, J. T.; Campbell, D. G.; Morrice, N.; Auld, G. C.; Shpiro, N.; Marquez, R.; Peggie, M.; Bain, J.; Bloomberg, G. B.; Grahammer, F.; Lang, F.; Wulff, P.; Kuhl, D.; Cohen, P. Exploitation of KESTREL to identify NDRG family members as physiological substrates for SGK1 and GSK3. *Biochem. J.* **2004**, *384*, 477−488.
(36) Pinna, L. A.; Ruzzene, M. How do protein kinases recognize their substrates? *Biochim. Biophys. Acta* **1996**, *1314*, 191−225.
(37) Zachary, A.; Knight, Z. A.; Shokat, K. M. Review features of selective kinase inhibitors. *Chem. Biol.* **2005**, *12*, 621−637.
(38) Livnah, N.; Yechezkel, T.; Salitra, Y.; Perlmutter, B.; Ohne, O.; Cohen, I.; Litman, P.; Senderowitz, H. Protein kinase inhibitors comprising ATP mimetics conjugated to peptides or peptidomimetics. Eur. Pat. Appl. EP1416934, 2004.
(39) Manning, G.; Whyte, D. B.; Martinez, R.; Hunter, T.; Sudarsanam, S. The protein kinase complement of the human genome. *Science* **2002**, *298*, 1912−1934.
(40) Ni, Z.-J.; Maclean, D.; Holmes, C. P.; Murphy, M. M.; Ruhland, B.; Jacobs, J. W.; Gordon, E. M.; Gallop, M. A. Versatile approach to encoding combinatorial organic synthesis using chemically robust secondary amine tags. *J. Med. Chem.* **1996**, *39*, 1601−1608.
(41) Hidaka, H.; Inagaki, M.; Kawamoto, S.; Sasaki, Y. Isoquinolinesulfonamides, novel and potent inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C. *Biochem. J.* **1984**, *23*, 5036−5041.
(42) Liu, F.; Austin, D. J. Synthesis of 5′-functionalized adenosine: suppression of cyclonucleoside formation. *Tetrahedron Lett.* **2001**, *42*, 3153−3154.

JM0605942