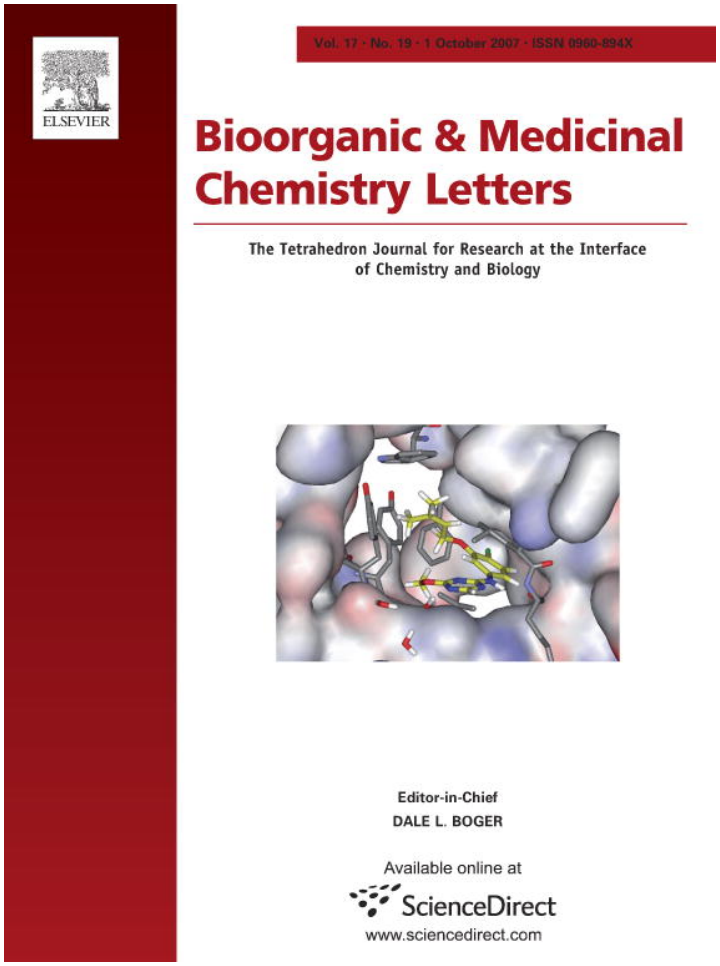

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Carbocyclic 3′-deoxyadenosine-based highly potent bisubstrate-analog inhibitor of basophilic protein kinases

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Abstract—Carbocyclic analogs of 3′-deoxyadenosine were synthesized as racemates and the resulting stereoisomers were separated by chromatography on a chiral column. The conjugation of obtained compounds with hexa-(D-arginine) via 6-aminohexanoic acid linker led to a highly potent inhibitor of several basophilic protein kinases with some selectivity towards cAMP-dependent protein kinase.

Protein kinases (PKs) play a key role in the regulation of cellular protein functions. More than 400 human diseases have been linked to aberrant PK signaling.[1] This fact and the recent success with PK inhibitor-based drugs have made PKs the second largest drug target after G protein-coupled receptors.[2] Due to apparent ease of development of high-affinity low molecular weight inhibitors targeted to the well-defined hydrophobic adenine-binding cleft, and despite serious selectivity problems (additionally to 500 coded PKs in human genome 1500 other proteins are able to bind adenine nucleotides)[1] in combination with high concentration of competing ATP in the cellular milieu, most of the developed and studied inhibitors of PKs are competitive with ATP. The high degree of 3D structural conservation of adenine nucleotide binding site of these proteins makes the development of specific inhibitors a challenging task.

The structure of protein substrate-binding domain of kinases is more variable and the development of highly selective peptide inhibitors has been described.[3] However, longer peptidic structures are needed for achieving nanomolar potency which leads to problems with cellular transport and stability of the compounds.

The development of bisubstrate-analog (biligand) inhibitors that simultaneously associate with both ATP and protein-binding domains of PK could give selective and potent inhibitors of these dual substrate enzymes.[4] A linker enabling optimal positioning of the active fragments comprising the bisubstrate inhibitor could lead to a conjugate with kinase-binding energy substantially exceeding the sum of binding energies of the fragments.[5]

Recently[6] we described novel bisubstrate-analog-type inhibitors of PK that consisted of adenosine and oligo-(D-arginine) moieties connected via 6-aminohexanoic acid linker (ARCs, adenosine–oligoarginine conjugates). The most active of the compounds, **ARC-902** (number **5** in Table 1 of Ref. 6), showed low nanomolar inhibitory potency toward the catalytic subunit (type α) of cAMP-dependent protein kinase (cAPK Cα, also known as PKA Cα), and high proteolytic stability.[6] Later we demonstrated that such highly potent ARCs really behave as bisubstrate-analog inhibitors and can be displaced from their complex with cAPK Cα by both ATP- and protein substrate-competitive inhibitors.[7]

The further increase of inhibitory potency of ARC-type inhibitors could be achieved by optimization of structures of the moieties and their spatial positioning. The earlier structure–activity studies with adenosine and adenine nucleotide analogs have shown that contrary to other modifications of the structure that decrease the affinity of adenosine analogs, the removal of the hydroxyl group from the 3′-carbon of the ribose moiety

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Table 1. Inhibition of cAPK Cα

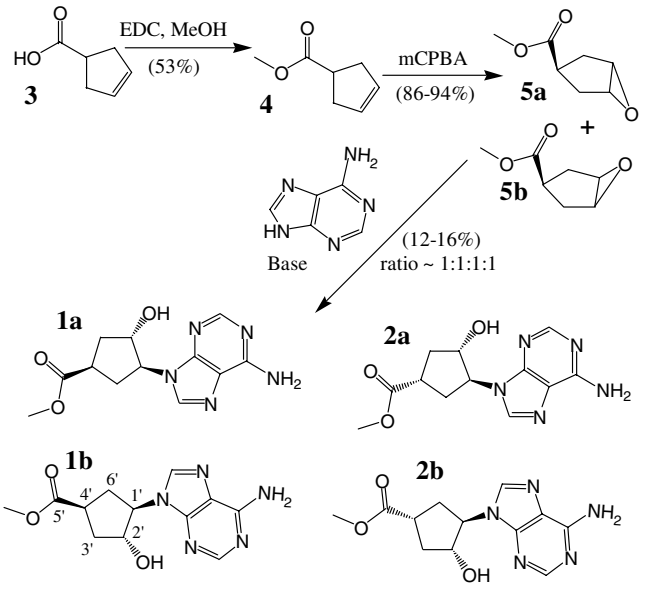
Compound	IC_{50} (0.1 mM ATP)[a]	IC_{50} (1 mM ATP)[a]
1a/1b	328 μM (3.48 ± 0.20)[b]	—
2a/2b	93 μM (4.03 ± 0.16)	—
1a	>1000 μM	—
1b	168 μM (3.77 ± 0.18)	—
ARC-659-1a/1b/2a/2b[c]	3.45 nM (8.46 ± 0.22)	24.5 nM (7.61 ± 0.19)
ARC-659-1a/1b	—	16.8 nM (7.77 ± 0.23)
ARC-659-2a/2b	—	571 nM (6.24 ± 0.31)
ARC-659-1a	—	97.3 nM (7.01 ± 0.22)
ARC-659-1b	2.41 nM (8.62 ± 0.12)	12.9 nM (7.89 ± 0.25)

[a] Concentration of the substrate (ATP) in the kinetic assay.
[b] Values in brackets express pIC_{50}-s with 95% confidence intervals.
[c] **ARC-659-1a/1b/2a/2b** was synthesized from the mixture of racemic diastereomers **1a/1b** and **2a/2b**.

of adenosine may increase the affinity of compounds towards several PKs up to 5-fold.[8] Additionally, crystal structures of cAPK Cα with adenosine derivatives reveal the absence of polar interactions between the 4′-oxygen of the ribose and the kinase. These facts point to the possibility of the use of chemically and enzymatically more stable[9] carbocyclic (cyclopentane) adenosine mimics for the design of potent ARC-type inhibitors.

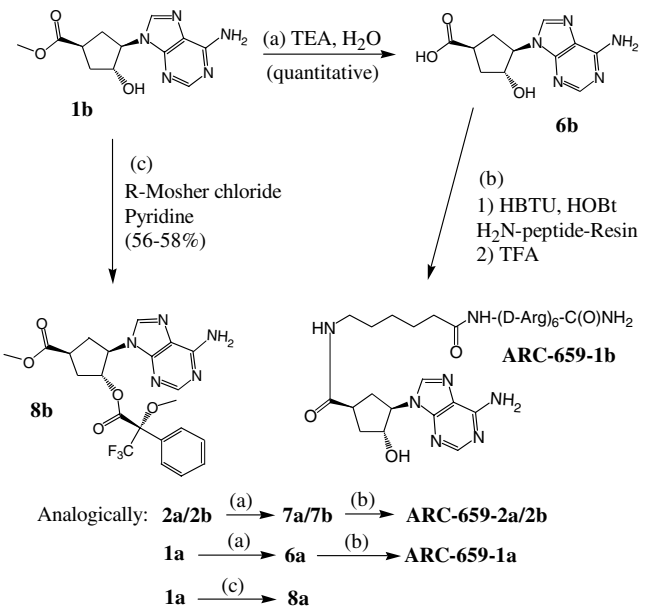
Here we present the synthesis of cyclopentane-based carbocyclic analogs of 3′-deoxyadenosine (3′-deoxyaristeromycin), chromatographic separation of the formed stereoisomers, their conjugation with oligo-(D-arginine)-containing peptides, and characterization of the latter compounds as highly potent inhibitors of basophilic protein kinases.

The shortest synthetic scheme was selected to prepare the derivatives of carbocyclic 3′-deoxyadenosine **1a**, **1b**, **2a**, and **2b** that are applicable for easy conjugation with peptides. 3-Cyclopentene-1-carboxylic acid (**3**) was converted to the methyl ester **4** and the obtained ester was oxidized to the appropriate epoxide (**5a/5b** ratio 3:1) with mCPBA (Scheme 1). Cis and trans isomers (**5a** and **5b**) of the epoxide were separated by column chromatography as described before.[10] The products were treated with the excess of adenine in the presence of NaH or a phosphazene base.[11] Nucleophilic ring opening of epoxide required increased temperatures (90–110 °C).[12] The reaction led to a mixture of four stereoisomers (two diastereomers as racemates—**1a/1b**[13] and **2a/2b**) in almost equal amounts. The molar ratio of formed stereoisomers was the same irrespective of whether the starting epoxide existed as a pure single isomer (**5a** or **5b**) or a mixture of isomers (**5a/5b**). This was apparently caused by the racemization occurring at the most acidic 4′-carbon adjacent to the ester group in basic medium at higher temperatures (>100 °C). **1a/1b** and **2a/2b** were obtained as racemates due to the symmetric structure of the starting epoxides **5a** and **5b**. The application of both NaH and the phosphazene base led to similar yields and relative proportions of the products. NaH appeared to be the preferred base due to easier purification of the products. Adenine alkylation reaction producing a mixture of **1a/1b** and **2a/2b** was the most critical step due to low overall yield (12–16%). Apparently, the low yield of the synthesis resulted from instability of the starting epoxides (**5a** and **5b**) in the reaction medium. The pairs of diastereomers (**1a/1b** and **2a/2b**) were separated by column chromatography on silica gel. The overlapping fractions were purified repeatedly. Structural elucidation of the diastereomers was based on different NOE signals between 1′- or 2′-protons and 4′-proton of the isomers. The diastereomer **1a/1b** eluted faster than **2a/2b** in case of normal phase chromatography, which is consistent with the previous data concerning ribose counterparts of the compounds.[14] Enantiomers **1a** and **1b** were separated by HPLC on a chiral chromatography column (Chiralpak AD-H).



Scheme 1. Synthesis of the carbocyclic nucleoside analogs.

Reactions of the enantiomers (**1a** and **1b**) with (*R*)-α-methoxy-α-trifluoromethylphenylacetyl chloride in pyridine gave Mosher esters **8a** and **8b** (Scheme 2),



Scheme 2. Synthesis of the peptide conjugates and Mosher esters.

respectively, which were used for the determination of the absolute configuration of the compounds by NMR.[15] Methyl esters (**1a**, **1b** and racemates **1a/1b** and **2a/2b**) were hydrolyzed in the presence of triethylamine (TEA). The obtained carboxylic acids (**6a/6b**, **6a**, **6b**, and **7a/7b**[16]) were coupled to the peptides with *O*-(1*H*-benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU) and *N*-hydroxybenzotriazole (HOBt) activation. Cleavage and deprotection of the resin-bound compounds with trifluoroacetic acid (TFA) led to the target conjugates of carbocyclic 3′-deoxyadenosine and hexa-(D-arginine) (**ARC-659**). The peptide conjugates made from different isomers are designated by adding the number of the isomer (**1a**, **1b**, etc.) to the common code of the compounds **ARC-659**.

The inhibitory potencies of the synthesized compounds were evaluated against cAPK Cα with the application of fluorometric TLC kinase activity assay,[17] and the results are expressed as the IC_{50} values in Table 1. TAMRA-kempide (30 μM) and ATP (0.1 or 1.0 mM) were used as substrates. The application of higher concentration of ATP (1.0 mM) in the test system increased the IC_{50} value and enabled to avoid tight-binding conditions of the assay ($IC_{50} \leqslant C_{kinase}$).[18] The racemic nucleoside analogs **1a/1b** and **2a/2b** showed expectedly weak inhibitory potencies. The IC_{50} values for these compounds were comparable to that of adenosine (IC_{50} = 350 μM). The diastereomer **2a/2b**, being structurally less similar to the native adenosine than **1a/1b**, showed slightly stronger inhibition. The inhibitory potency of the conjugates of these racemates with hexa-(D-arginine) differed by more than 30-fold, and **ARC-659-1a/1b** (structurally more similar to adenosine) revealed much higher potency. This difference in activity may originate from the more effective positioning of the 6-aminohexanoic acid linker and the peptide in complex with the enzyme.

The separated enantiomer **1b** was ca. 10-fold more potent than **1a**. This result indicates the importance of correct positioning of 2′-hydroxy group for inhibitor binding to the enzyme. The conjugates with peptides revealed similar one-magnitude difference in activity. To the best of our knowledge **ARC-659-1b** is the most potent bisubstrate-analog inhibitor of protein kinases described so far.

The selectivity of **ARC-659-1b** was tested (on the commercial basis at the Division of Signal Transduction Therapy, University of Dundee) against the panel of protein kinases. Inhibition efficiency was determined at two concentrations of **ARC-659-1b** (1 and 0.01 μM).

The inhibition data for 34 kinases are presented as the percent of residual activity of the kinase in the presence of the inhibitor relative to control incubations where the inhibitor was omitted (Table 2). The analysis of the data reveals that **ARC-659-1b** most potently inhibits cAPK. The K_i value of ~0.5 nM was estimated for this PK (Table 1; calculated according to the Cheng–Prusoff equation[21] with K_m of 20 μM for ATP). The compound also showed strong inhibition (ca. 50% at 10 nM concentration) of ROCK-II, MSK1, and PRK2. Similar inhibition potency was determined toward Akt/PKB, SGK1, and CAMK1. The moderately inhibited kinases (>90% of inhibition at 1 μM concentration of the inhibitor) were PIM1, S6K1, PAK5, MELK, and AMPK. Some other kinases retained higher activity in the presence of **ARC-659-1b** and several tested kinases were not inhibited at all. Generally, the inhibition profile of **ARC-659-1b** was similar to that of the previously reported conjugate of adenosine and oligo-(D-arginine), **ARC-902**.[6] However, **ARC-659-1b** has remarkable cAPK selectivity while its adenosine counterpart **ARC-902** revealed preference for ROCK-II. Several kinases of the CAMK group like AMPK, PIM-2, and CAMK1 were inhibited more strongly by **ARC-659-1b**, whereas **ARC-902** was more potent towards the kinases of the RSK group. PAK5 and MELK kinases (both known as basophilic kinases) were also strongly inhibited by **ARC-659-1b**.

Table 2. Residual activities of PKs in the presence of the inhibitor **ARC-659-1b**

Kinase	Kinase group[19]	Residual activity (%)	
		0.01 μM	1 μM
PKA/cAPK	AGC	7 ± 1	0 ± 0
ROCK 2	AGC	41 ± 2	1 ± 0
PKBβ	AGC	74 ± 1	2 ± 0
MSK1	AGC	37 ± 8	3 ± 0
PRK2	AGC	59 ± 7	3 ± 1
PIM1	CAMK	92 ± 1	6 ± 1
S6K1	AGC	81 ± 7	7 ± 0
PAK5	STE	71 ± 7	10 ± 1
MELK	CAMK	86 ± 11	10 ± 3
AMPK	CAMK	70 ± 6	12 ± 1
SGK1	AGC	40 ± 4	19 ± 3
CAMK1	CAMK	62 ± 1	19 ± 1
PIM2	CAMK	103 ± 2	20 ± 1
CHK1	CAMK	84 ± 7	27 ± 4
PDK1	AGC	76 ± 1	28 ± 1
RSK2	AGC	91 ± 2	28 ± 8
PKCα	AGC	96 ± 1	30 ± 2
PKBα	AGC	25 ± 4	32 ± 1
PKCζ	AGC	83 ± 7	33 ± 4
DYRK3	CMGC	81 ± 4	51 ± 3
Lck	TK	86 ± 7	63 ± 6
AURORA B	Other	76 ± 6	72 ± 10
PKD1	CAMK	81 ± 5	73 ± 15
CSK	TK	97 ± 3	77 ± 4
ERK1	CMGC	82 ± 5	84 ± 5
Src	TK	85 ± 9	84 ± 5
GSK3β	CMGC	82 ± 0	88 ± 7
CK2	Other	86 ± 5	88 ± 5
MAPKAP-K3	CAMK	97 ± 4	91 ± 13
PRAK	CAMK	88 ± 1	96 ± 8
RSK1	AGC	98 ± 3	98 ± 4
JNK3	CMGC	103 ± 9	104 ± 10
NEK6	Other	101 ± 11	111 ± 8
CDK2-Cyclin A	CMGC	103 ± 4	114 ± 5

To make the inhibitory potencies comparable, assays were run at ATP concentration which was close to the K_m value for the given kinase.[6,20]

In conclusion, we have accomplished synthesis of the carbocyclic analog of 3′-deoxyadenosine along with separation and characterization of its stereoisomers. The conjugation of these compounds with hexa-(D-arginine)

peptide led to potent inhibitors of basophilic PKs. The stereochemistry of the nucleoside part of most active conjugate **ARC-659-1b** is similar to that of adenosine, but the novel conjugate does not incorporate a native nucleoside with a chemically and enzymatically degradable glycosidic bond. The selectivity study revealed that **ARC-659-1b** strongly inhibited basophilic kinases and was almost inactive toward a number of PKs that do not contain multiple basic amino acids (arginine and/or lysine) residues in their consensus phosphorylation sequences.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2007.08.016.

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13. Analytical data of the key compound (+/−)-(1*S**,3*R**,4*R**)-3-(6-amino-purin-9-yl)-4-hydroxy-cyclopentanecarboxylic acid methyl ester (**1a/1b**). UV_{max} 261 nm (MeOH). HRMS: *m/z* calcd for $C_{12}H_{16}N_5O_3$ ($M+H^+$): 278.1243. Found: 278.1237. ^1H NMR (500 MHz, CD_3SOCD_3) δ 1.88 (1H, ddd, J = 13.6, 10.0 and 6.8 Hz, 3′h), 2.30 (1H, ddd, J = 13.6, 7.5 and 6.5 Hz, 3′l), 2.35 (1H, ddd, J = 13.1, 10.4, 9.4 Hz, 6′h), 2.46 (1H, ddd, J = 13.1, 8.1 and 7.4 Hz, 6′l), 3.17 (1H, m, J = 10, 9.4, 8.1 and 6.5 Hz, 4′), 3.64 (3H, s, CH_3), 4.49 (1H, td, $J = 2 \times 7.5$ and 6.8 Hz, 2′), 4.63 (1H, dt, J = 10.4 and 2×7.4 Hz, 1′), 8.39 and 8.49 (2H, s, 2 and 8), 8.67 (2H, br, NH_2). ^{13}C NMR (125 MHz, CD_3SOCD_3) δ 32.2 (6′), 34.6 (3′), 37.8 (4′), 51.8 (CH_3), 62.6 (1′), 73.8 (2′), 118.7 (5), 142.0 (8), 146.9 (2), 149.0 (4), 152.0 (6), 174.8 (COO).
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