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Synthesis and Biological Evaluation of Clitocine Analogues as Adenosine Kinase Inhibitors

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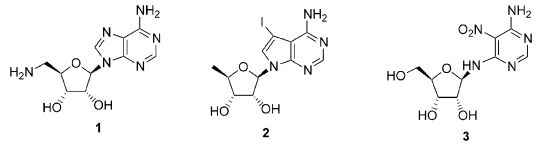
Abstract—Adenosine kinase (AK) is the primary enzyme responsible for adenosine metabolism. Inhibition of AK effectively increases extracellular adenosine concentrations and represents an alternative approach to enhance the beneficial actions of adenosine as compared to direct-acting receptor agonists. Clitocine (**3**), isolated from the mushroom *Clitocybe inversa*, has been found to be a weak inhibitor of AK. We have prepared a number of analogues of clitocine in order to improve its potency and demonstrated that 5′-deoxy-5′-amino-clitocine (**7**) improved AK inhibitory potency by 50-fold.

Extracellular concentrations of the endogenous neuromodulator, adenosine (ADO), are increased under conditions of metabolic stress and trauma (e.g., pain, inflammation, tissue damage, ischemia, seizure activity, etc.) and act to limit tissue damage and restore normal function[1] by activating members of the P1 receptor family, A_1, A_{2A}, A_{2B}, and A_3 receptors.[2] Increased extracellular ADO concentrations can result in the inhibition of excitatory amino acid (glutamate) release, suppression of free radical formation, and neutrophil adhesion, depending on the phenotype of the tissue involved.[3] Since ADO has a half-life of the order of seconds in physiological fluids,[4] its extracellular actions are restricted to the tissue and cellular sites where it is released.[5] The effects of extracellular ADO are terminated by its re-uptake and phosphorylation by ADO kinase (AK; ATP: adenosine 5′-phosphotransferase, EC 2.7.1.20) and via deamination by adenosine deaminase (ADA).[6] By preventing ADO phosphorylation, AK inhibition increases intracellular ADO concentrations, altering the equilibrium of the bi-directional transport systems responsible for ADO reuptake with the net effect of increasing the local concentration of ADO in the extracellular compartment.[7] AK inhibition is a more effective mechanism for increasing extracellular ADO levels than inhibition of the catabolic enzyme ADA,[7] and is more effective than ADA inhibition in reducing seizure and nociceptive activity in vivo.[8,9] Therefore, we initiated an effort to identify suitable AK inhibitors for exploring their potential as therapeutically useful CNS agents.

A number of agents such as 5′-deoxy-5′-amino-adenosine (**1**),[10] 5′-deoxy-5-iodotubercidin (**2**),[11] and clitocine (**3**)[12] have been found to inhibit AK (Scheme 1). Although clitocine is a weak AK inhibitor ($IC_{50} = 2\ \mu M$), we decided to investigate it as a lead structure because of its structural simplicity.

Clitocine was prepared by the procedure of Kamikawa et al.[13] The 5′-modified analogues of clitocine were prepared by 5′-hydroxy functional group modification of the intermediate[13] (**5**) prepared during its synthesis. Treatment of **5** with the PPh_3Br_2 gave the 5′-bromo compound **6**. Displacement of the 5′-bromo group with potassium phthalimide in DMF followed by sequential deprotection of the amine and the acetonide groups by



Scheme 1. Structures of known adenosine kinase inhibitors.

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using hydrazine in refluxing methanol and trifluoroacetic acid, respectively, gave 5′-deoxy-5′-aminoclitocine (**7**) (Scheme 2).

Heating **4** with aniline followed by acetonide deprotection gave N^6-phenyl-clitocine (**4a**). Deprotection of **6** using trifluoroacetic acid gave **6a**. The 5′-deoxy analogue of clitocine (**3**) could not be prepared by the general dehalogenation of **6** or **6a**. Therefore, an alternate synthetic route for preparation of compound **13** was designed (Scheme 3). Treatment of D-ribono-1,4-lactone (**8**) with PPh_3/NIS followed by hydrogenation with Pd/C gave 5-deoxy lactone. Reduction of the lactone to lactol with DIBAL provided **9** as a mixture of anomers.

The conversion of **9** to aminal (**10**) was accomplished by treating **9** with $AlCl_3$ followed by NaN_3 giving 2-azido intermediate. Hydrogenation of the resulting 2-azido intermediate with PtO_2 yielded aminal (**10**). The aminal (**10**) was then coupled with the aminochloropyrimidine (**11**) in DMF/Et_3N and deprotected with trifluoroacetic acid to give 5′-deoxyclitocine (**12**).

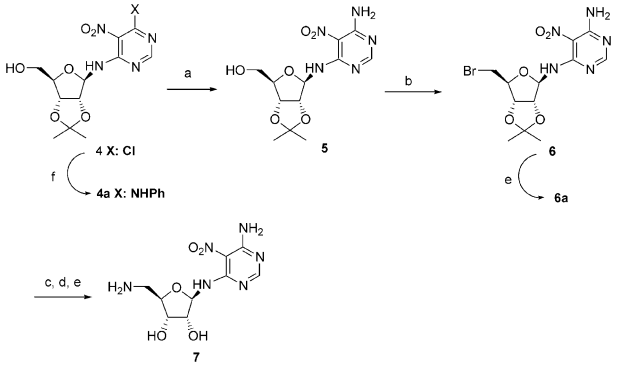
In an attempt to find a carboxylate replacement for the 5-nitro group of clitocine, 4,6-dihydroxy-pyrimidine (**13**) was formylated and chlorinated in one pot using Bredereck's procedure[14] and the resulting formylated product was oxidized to the corresponding carboxylic acid by $KMnO_4$ in acetone. Esterification of the carboxylic acid with diazomethane gave **14**. Coupling of **14** with **15** gave a mixture of anomers, which were purified by column chromatography to give the pure β-anomer (**16**) which is shown in Scheme 4. Amination of **16** followed by deprotection gave **18**.

Mitsunobu reaction of **17** with diphenyl phosphorylazide gave the corresponding azido compound (**19**). Reduction of the azide to amine followed by deprotection of the acetonide gave the 5′-amino compound (**20**) (Scheme 5).

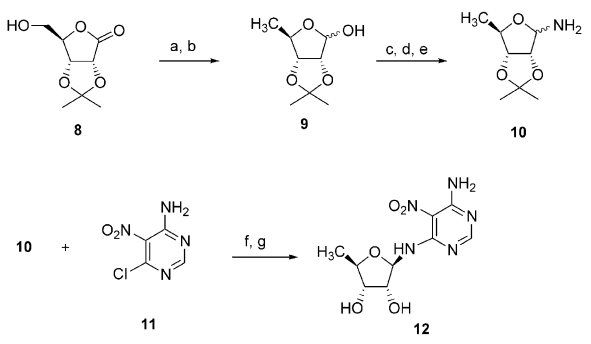
The corresponding 5′-deoxy compound (**22**) was prepared by the coupling of **10** with **14** followed by amination at the 4-position of **21** and deprotection of the acetonide (Scheme 6). The intermediate **16** was converted to **16a**, **16b**, and **16c** as shown in Scheme 7.

Compounds **26** and **27** were prepared as shown in Scheme 8. Bromination of 4,6-dihydroxy-pyrimidine (**13**) yielded 5-bromo-4,6-dihydroxy-pyrimidine followed by 5-bromo displacement with sodium thiophenol to give 5-mercaptophenyl (**23**). Treatment of **23** with $POCl_3$ gave the corresponding dichloride and by sequential oxidation with *m*-CPBA yielded **24** and **25**. Ribosylation of **24** and **25** followed by aminolysis and deprotection gave **26** and **27**.

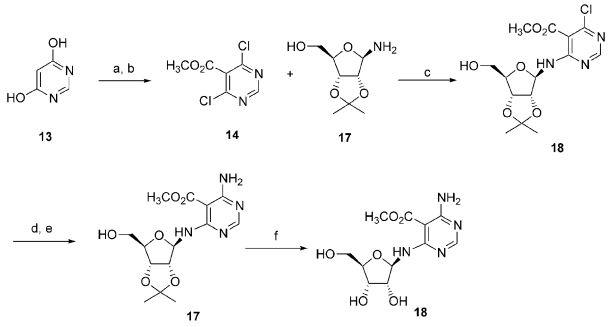
Compound **30** with an oxazoline at the 5-position of pyrimidine was prepared as shown in Scheme 9. 4,6-Dichloro-pyrimidine-5-carboxylic acid (prepared as



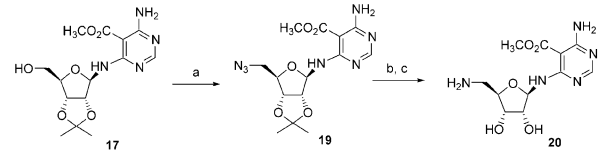
Scheme 2. Reagents: (a) NH_3/MeOH; (b) PPh_3Br_2; (c) potassium phthalimide; (d) N_2H_4; (e) TFA/H_2O, 12% overall; (f) $PhNH_2$/DMF.



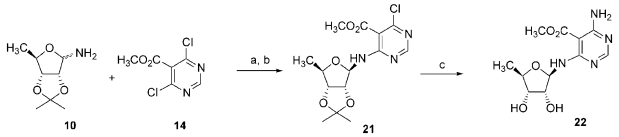
Scheme 3. Reagents: (a) PPh_3/NIS; (b) DIBAL; (c) $AlCl_3$; (d) NaN_3; (e) PtO_2/H_2, 44% overall yield; (f) Et_3N/DMF/rt; (g) TFA/H_2O, 11% overall yield.



Scheme 4. Reagents: (a) $POCl_3$/DMF; (b) $KMnO_4$/acetone; (c) CH_2N_2; (d) Et_3N/DMF; (e) NH_3/MeOH; (f) TFA/H_2O.



Scheme 5. Reagents: (a) $(PhO)_2PON_3$/DEAD; (b) PPh_3/Py; (c) TFA/H_2O.



Scheme 6. Reagents: (a) Et_3N/DMF; (b) NH_3/MeOH; (c) TFA/H_2O.

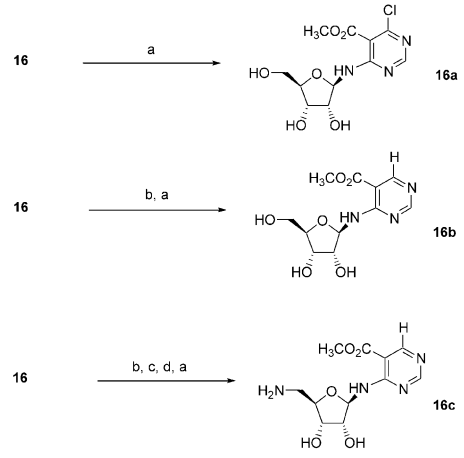
shown in Scheme 4) was amidated via the acid chloride to give **28**. Conversion of the β-hydroxy amide to the β-chloro amide followed by cyclization in NaH gave the oxazoline (**29**).

Ribosylation of **29**, followed by aminolysis and deprotection, gave the desired oxazoline **30** and the ring opened β-hydroxy amide (**31**).

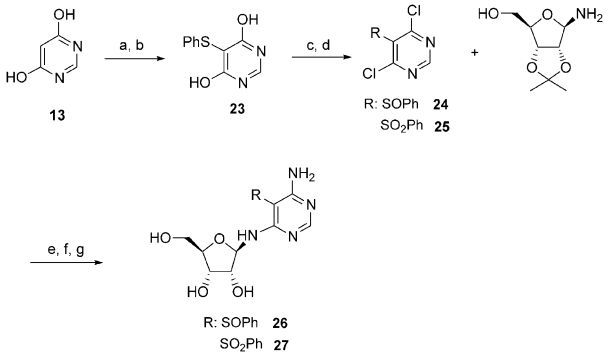
Adenosine kinase activity of rat brain cytosol was assayed radiometrically. Assays were carried out at ambient temperature in a final volume of 100 μL. The standard reaction mixture contained 64 mM Tris–HCl, pH 7.5, 40 mM KCl, 0.2 mM $MgCl_2$, 1 mM ATP, 0.2 μM U-[^{14}C]-adenosine (2×10^{10} Bq/mmol, Amersham), an appropriate concentration of the AK inhibitor, and an aliquot of rat brain cytosol. The reaction was terminated after 15 min by spotting 40 μL of the reaction mixture onto disks of Whatman DE-81 anion exchange paper. DE-81 disks were then air-dried, washed for 10 min in 2 mM ammonium formate, rinsed successively with distilled water, methanol and acetone, and dried. Disks were then soaked for 5 min in 0.1 N HCl/0.4 M KCl prior to liquid scintillation.

The structural variation of clitocine was focused initially at the 5′- and 4-positions (Table 1). Replacing the 5′-hydroxy group of clitocine by a 5′-bromo or 5′-deoxy group, respectively, led to a slight decrease of in vitro activity. Replacement by an amino group, on the other hand, led to a 50-fold improvement in the enzyme inhibitory activity ($IC_{50}=35$ nM for **7** vs 2 μM for **3**). Replacing the 4-amino group of clitocine by a chloro or aniline group led to a significant decrease in activity.

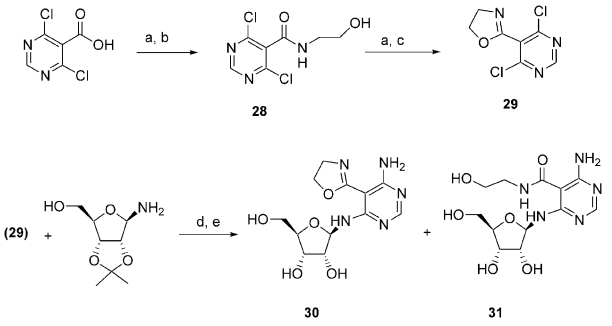
An attempt was made to find a replacement for the 5-nitro group of clitocine. It has been proposed that a crucial H-bond exists between the oxygen of the nitro group and the NH-group at the 6-position. As a result, a desirable orientation will form between the ribose and pyrimidine rings.[15] Electron withdrawing functional groups were chosen keeping this postulate in mind. The 5-methyl carboxylate analogue **18** was found to be equipotent to clitocine. Increasing the size of the ester group did not increase the activity (results not shown). Changing the 5′-substituent to an amino group again led to a large improvement in activity ($IC_{50}=2$ nM for **20** vs 1 μM for **18**). Interestingly, the analogue of this



Scheme 7. Reagents: (a) TFA/H_2O; (b) $Pd/C/H_2$; (c) $(PhO)_2PON_3$/DEAD; (d) PPh_3/py.

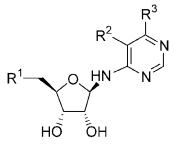


Scheme 8. Reagents: (a) Br_2/MeOH; (b) NaSPh; (c) $POCl_3$; (d) *m*-CPBA; (e) Et_3N/DMF; (f) NH_3/MeOH; (g) TFA/H_2O.



Scheme 9. Reagents: (a) $SOCl_2$; (b) $NH_2CH_2CH_2OH$; (c) NaH; (d) NH_3/MeOH; (e) TFA/H_2O.

Table 1. In vitro biological activity of ADO kinase inhibitors



Compd	R^1	R^2	R^3	Adenosine kinase inhibition IC_{50} (nM)[a]
1				15 (±14)
2				1.63 (±1.09)
3	OH	NO_2	NH_2	2000 (±1000)
4a	OH	NO_2	NHPh	>10,000
6a	Br	NO_2	NH_2	>10,000
7	NH_2	NO_2	NH_2	35 (±12)
12	H	NO_2	NH_2	4000 (±1400)
16a	OH	CO_2CH_3	Cl	>10,000
16b	OH	CO_2CH_3	H	600 (±323)
16c	NH_2	CO_2CH_3	H	4 (±1)
18	OH	CO_2CH_3	NH_2	1000 (±857)
20	NH_2	CO_2CH_3	NH_2	2 (±1)
22	H	CO_2CH_3	NH_2	6100 (±5200)
26	OH	SOPh	NH_2	>10,000
27	OH	SO_2Ph	NH_2	>10,000
30	OH	2-Oxazoline	NH_2	4000 (±3420)
31	OH	$CONHC_2H_4OH$	NH_2	>10,000

[a]All values are the mean±SEM of at least three separate observations run in triplicate.

compound with no substituent at the 4-position, **16c**, was equally active ($IC_{50}=4$ nM). Furthermore, the analogue of **18** with no substituent at the 4-position, **16b**, also had activity similar to that of **18** ($IC_{50}=0.6$ μM vs 1 μM).

Replacing the 5-nitro group of clitocine by a phenylsulfoxide or phenylsulfone led to a loss of activity. This result was disappointing since it was anticipated that the phenyl group of these compounds would fit in the site occupied by the iodo-group of **2**. The nitro group of clitocine lacks the bulky hydrophobic nature of the iodo-group of **2**. An oxazoline group at the 5-position would be an electron withdrawing group with two heteroatoms. Additionally, it would provide a handle to put a hydrophobic group at the 5-position. The oxazoline analogue **30** was found to be equipotent to clitocine. More analogues need to be prepared for evaluating this substitution further. The hydroxyethyl amide prepared as a side product was found to be inactive.

In conclusion, we have prepared analogues of clitocine and found that a 5′-amino group enhanced the potency of AK inhibition by more than 50-fold. The ester and oxazole functionalities were found to replace the 5-nitro group without loss of activity. The scope of the latter substitution needs to be explored further.

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