Institute of Chemistry[1], Agricultural University, Department of Drug Chemistry[2], Medical University of Warsaw, Laboratory of Experimental Pharmacology[3], Polish Academy of Sciences, Medical Research Center, Warsaw, Poland

Alkaline hydrolysis of cladribine

M. Andrzejewska[1], A. Dzierzgowska-Szmidt[2], Z. Kazimierczuk[1,3]

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Prof. Dr. habil. Zygmunt Kazimierczuk, Institute of Chemistry, Agricultural University, 159C Nowoursynowska st., PL-02-787 Warsaw, Poland
kazimierczuk@delta.sggw.waw.pl

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The kinetics of hydrolysis of 2-chloro-2′-deoxyadenosine (cladribine) was studied at various sodium hydroxide concentrations and temperatures. HPLC analysis of reaction mixtures showed that the main products were 2′-deoxyisoguanosine and 2′-deoxyguanosine. The first one was the result of the hydroxyl anion attack, whereas the presence of the other nucleoside has evidenced the existence of hitherto undescribed rearrangement reaction in purine derivatives.

1. Introduction

Cladribine (2-chloro-2′-deoxyadenosine, 2-CdA, **3**) is an analog of the naturally occurring nucleoside 2′-deoxyadenosine. Cladribine is resistant to deamination by adenosine deaminase, and is phosphorylated intracellularly to monophosphate by deoxycytidine kinase. The monophosphate is further phosphorylated to diphosphate and triphosphate, the latter being thought to be the active metabolite. Incorporation of 2-CdA triphosphate into a growing DNA strand results in DNA breaks. Cladribine also activates poly(ADP)ribosylation and enhances the consumption of NAD, and therefore results in ATP depletion that may cause cell death due to exhaustion of energy stores. Cladribine shows also immunosuppressive activity because of its toxicity to lymphoid cells, and particularly to lymphocytes T that contain ten-fold higher adenosine deaminase concentration than most other cells and are therefore most sensitive to this drug. As an antileukaemic drug, cladribine is registered under a number of trade names, e.g., Leustatine®, Leustat®, and Biodribin®. Because of its lymphotoxicity, the drug has also shown some promise of being useful for the treatment of a number of autoimmunoaggressive diseases, e.g., rheumatoid arthritis, psoriasis and multiple sclerosis. The molecular basis of activity and therapeutic applications of cladribine were discussed in several recent review articles [1–5].

Whereas 2-CdA is stable in neutral media even at elevated temperatures, it has, being a 2′-deoxynucleoside, a limited stability in acidic solution. At pH 1, which corresponds to that of the human gastric fluid, cladribine decomposes to 2-chloroadenine and deoxyribose [6]. Therefore the reported oral bioavailability of the drug is only about 50% [7]. The other pathway of degradation of 2-CdA in the gastrointestinal tract is its phosphorolysis, e.g., by purine nucleoside phosphorylase present in *Escherichia coli*, a common Enterobacterium [8]. In this study, we report the results of alkaline degradation of cladribine that revealed a new type of rearrangement taking place in purine nucleosides.

2. Investigations, results and discussion

Of the two naturally occurring purine 2′-deoxynucleosides, 2′-deoxyadenosine is less stable in alkaline medium than 2′-deoxyguanosine that is able to form an anion. Under such conditions, 2′-deoxyadenosine undergoes imidazole ring opening with subsequent rearrangement and glycosyl bond cleavage [9]. It was quite a surprise for us to find that its 2-halogeno analog behaves completely different under these conditions. Showing a very low pK value of protonation (1.8), cladribine is practically a neutral molecule at pH values above 4. We have found that 2-chloro-2′-deoxyadenosine was completely stable at pH 12 at 37–60 °C. At 80 °C, some decomposition products were detected after 6 h. Still, over 90% of cladribine remained unchanged after 6 h of heating at temperatures 37–60 °C and at pH 13. Increasing temperature to 80 °C resulted in almost 40% decomposition of 2-chloro-2′-deoxyadenosine, whereas practically complete decomposition was achieved using the same hydrolysis time at pH 14 and 80 °C. Fig. 1 presents the time-course of decomposition of 2′-chloro-2′-deoxyadenosine at 80 °C at various pH values. Kinetic analysis showed a pseudo-first order rate of the reaction with activation energy 90.11 kJ/mol at pH 14 (Fig. 2). The other energetic parameters of the reaction – enthalpy (88.17 kJ/mol) and entropy (-66.55 J/K · mol) were determined using the ln k/T vs. 1/T relationship (Fig. 3). A simple TLC analysis showed a single hydrolysis product. However, HPLC analysis revealed two products with similar retention times (4.458 min and 5.030 min), whereas the starting 2-chloro-2′-deoxyadenosine eluted at 21.023 min (Fig. 4). For identification, both main products were isolated by preparative HPLC. The slower-eluting compound was spectrally (UV, NMR) and chromatographically identical with the expected 2′-deoxyisoguanosine (**1**). ESI MS of the other product showed a molecular peak (MH^+ 268) identical with that of 2′-deoxyisoguanosine. However, the UV spectrum of this product was different from that of authentic 2′-deoxyisoguanosine and its dependence on pH has indicated two pK values, one in acidic and one in

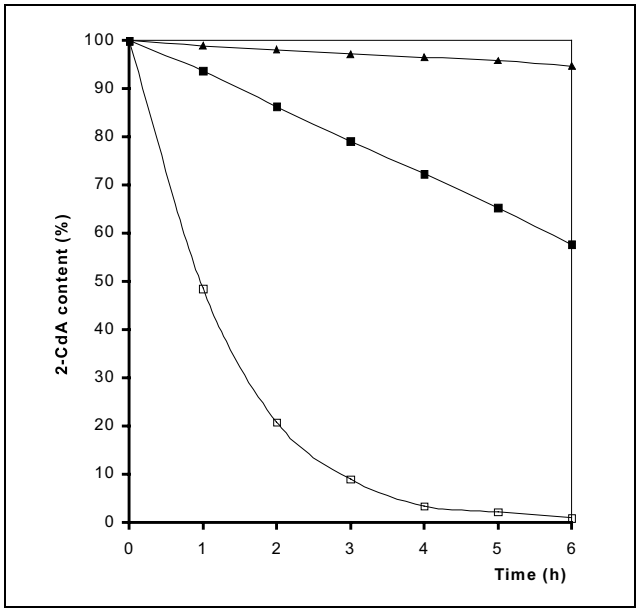


Fig. 1: Decomposition of cladribine at pH 12 (▲), pH 13 (■) and pH 14 (□) at 80 °C

alkaline medium. Values of λ_{max} were identical to those given for 2′-deoxyguanosine (**2**). Comparison of both ^{1}H NMR spectra (ppm in D_6(DMSO)): 2.21 and 2.50, 2m, H-2′ and 2″; 3.52, m, H-5′ and 5″; 3.80, m, H-4′; 4.32, q, H-3′, 4.31, t, HO-5′; 5.23, d, HO-3′, 6.12, t, H-1′, 6.45, s, H_2N; 7.91, s, H-8; 10.6, bs, H−N) and ^{13}C NMR spectra (ppm in D_6(DMSO)): C-2′ (superimposed DMSO), 61.6, C-5′; 70.7, C-3′; 82.6, C-1′; 87.6, C-4′; 116.6, C-5; 135.2, C-8; 150.8, C-4; 153.6, C-2; 156.7, C-6) with those of authentic 2′-deoxyguanosine suggested identity with this nucleoside (within experimental error). The HPLC analysis revealed that the two daughter 2′-deoxynucleosides formed with similar yields (approximately 1 : 1, after correction for the difference in the respective extinction coefficients), independently of the reaction pH and temperature employed, thus excluding the possibility of a sequential formation of the main products. The comparison of authentic deoxyisoguanosine (retention time 4.451 min) and deoxyguanosine (retention time 5.028 min) confirmed also the identity of the compounds isolated. This indicated that the two compounds were formed through independent

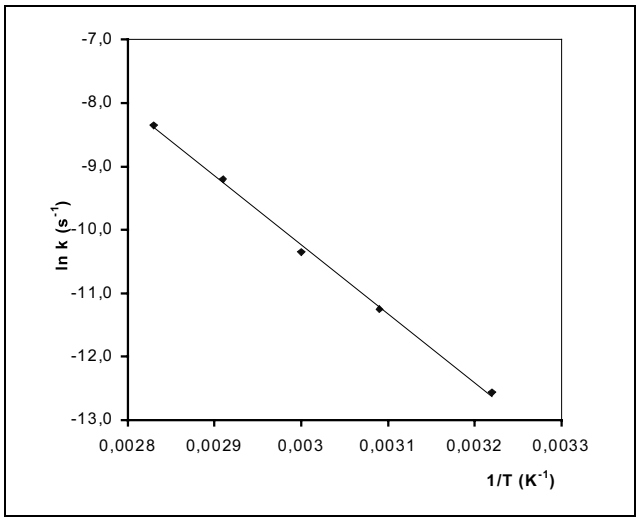


Fig. 2: Arrhenius-plot of hydrolysis of cladribine at pH 14 at various temperatures. Activation energy 90.11 kJ/mol

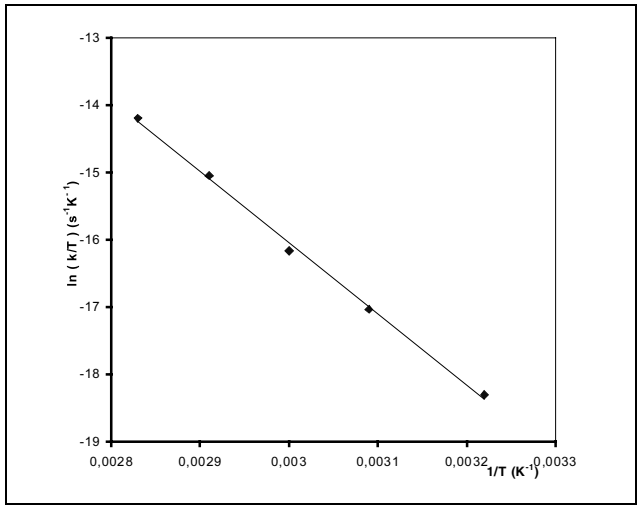


Fig. 3: The ln k/T vs. 1/T plot of alkaline hydrolysis of cladribine. Enthalpy 88.17 kJ/mol and entropy −66.55 J/K · mol

processes. The sum of the yields of 2′-deoxyisoguanosine and 2′-deoxyguanosine at pH 14 was about 90% at 80 °C. The relatively low amount of byproducts is particularly noteworthy considering the harsh reaction conditions.

Whereas the formation of 2′-deoxyisoguanosine can be explained by a nucleophilic attack of hydroxyl anion and subsequent elimination of the chlorine anion from position 2 of 2-chloro-2′-deoxyadenosine, the presence of 2′-deoxyguanosine in the hydrolysis products needs another explanation (Scheme). The purported mechanism of this reaction pathway includes hydrolytic opening of the purine nucleus and subsequent elimination of a hydrochloride molecule from the intermediate, followed by recyclization resulting in the formation of a new purine molecule. Such rearrangement with (N)1−C(6) bond-breaking in the purine series was never reported previously. A similar reaction, the so-called Dimroth rearrangement, was described for 1,9-disubstituted adenines. In that case, the hydrolytic bond breaking was between N(1) and C(2) purine ring atoms [10] and produced N^6, 9-disubstituted adenines after consecutive cyclization.

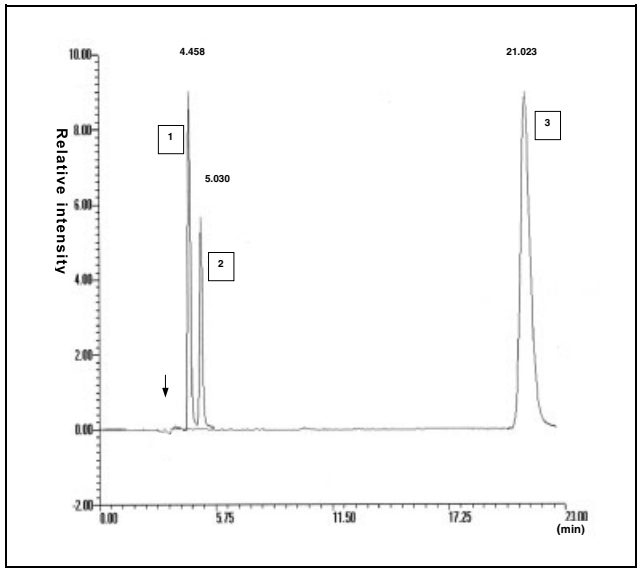
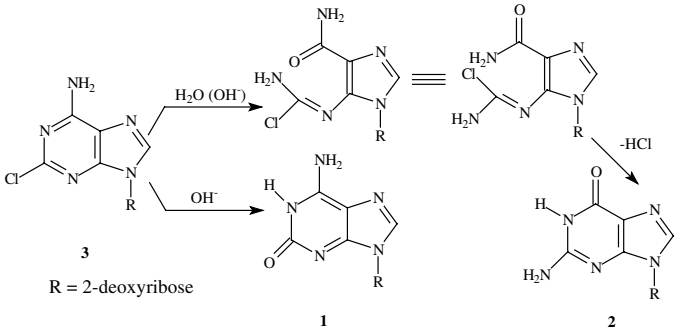


Fig. 4: HPLC-profile of alkaline hydrolysis of cladribine. Peaks: 2′-deoxyisoguanosine (**1**), 2′-deoxyguanosine (**2**), and cladribine (**3**). The reference substance (thiourea, retention time 3.29 min) is indicated as arrow

Scheme



3. Experimental

3.1. Chemistry

2-Chloro-2′-deoxyadenosine (**3**) was prepared according to a previously described procedure [11]. 2′-Deoxyisoguanosine (**1**) was obtained from cladribine using a photochemical approach [12]. 2′-Deoxyguanosine (**2**), other chemicals and solvents were from Sigma-Aldrich. HPLC analytical measurements were performed using a Shimadzu LC 6A instrument with UV detection ($\lambda = 270$ nm) and a Beckman Ultrasphere C18 column (250×4.6 mm). Mobile phase consisted of 10 mM phosphate buffer pH 3.0-MeOH−CH_3CN (84 : 13 : 3, v/v). The flow rate of elution was 1.5 ml/min and column temperature 20 °C. The samples were taken for analysis at 1-h intervals and were diluted with phosphate buffer (pH 3.0, 10 mM) to a final concentration of 5 μg/ml. Preparative separation of the hydrolysis products was performed on a Beckman 350 HPLC instrument using a column Ultraprep C18 (2.12×15 cm). The injections volume was 5 ml and column temperature 20 °C. The elution was performed with flow rate 25 ml/min and column temperature was 20 °C. UV spectra were measured on a Techcomp UV 8500 spectrophotometer. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Varian UNITYplus 500 MHz spectrometer. MS (electrospray) were obtained with a model AMD-604 (Inectra) spectrometer.

3.2. Hydrolysis procedure

One-ml 2-chloro-2′-deoxyadenosine solution (1 mg/ml) samples at pH 12, 13 or 14 were heated at 37–80 °C for up to 6 h. The resulting reaction mixtures were diluted to a final nucleoside concentration of about 5 μg/ml, neutralized and injected into the HPLC analytical system. The injection volume was 20 μl. The elution was performed with the aforementioned solvent.

3.3. Preparative hydrolysis

2-Chloro-2′-deoxyadenosine (285 mg, 1 mmol) was dissolved in 50 ml of 2 M NaOH and the solution was stirred at 80 °C for 4 h. A pale yellow solution formed was deposited on a Dowex 50W (H^+) 4×18 cm column and eluted with H_2O. UV absorbing fractions (TLC) were collected and evaporated to dryness. The residue was dissolved in H_2O (50 ml) and 5 ml volume portions were injected to the column. Separation of the components was performed using a Beckmann preparative HPLC apparatus and an unbuffered H_2O−MeOH (95 : 5, v/v) mixture as the eluent with rate flow 25 ml/min. The nucleoside-containing fractions were collected and evaporated to dryness. The isolated compounds were crystallized from EtOH-water (1 : 1,v/v) giving **1** (85 mg, 32%, m.p. dec. >230 °C) and **2** (105 mg, 39%, m.p. dec. >300 °C). The obtained deoxynucleosides were analyzed by spectroscopic and chromatographic methods.

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