Isolation, Characterization, and Properties of a Labile Hydrolysis Product of the Antitumor Nucleoside, 5-Azacytidine

John A. Beisler

Drug Design and Chemistry Section, Laboratory of Medicinal Chemistry and Biology, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20014.
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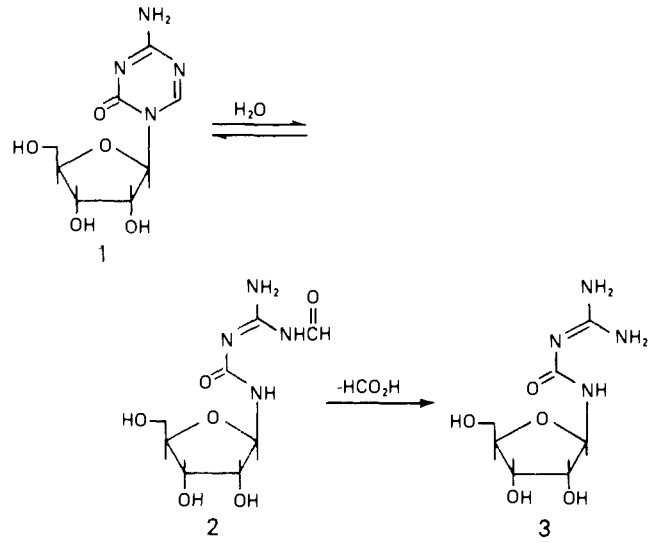
The antitumor nucleoside, 5-azacytidine (5-AC), is best administered clinically by prolonged intravenous infusion to minimize toxic effects. In opposition to this administration technique is facile drug decomposition in aqueous formulations giving products of unknown toxicity. Analysis of 24-h-old water solutions of 5-AC with high-pressure liquid chromatography (HPLC) indicated a threefold mixture of 5-AC, *N*-(formylamidino)-*N'*-β-D-ribofuranosylurea (RGU-CHO), and 1-β-D-ribofuranosyl-3-guanylurea (RGU). Preparative HPLC allowed the isolation and subsequent identification of each component in the mixture, including RGU-CHO which, until now, has not been available for chemical and biological study. It was shown that RGU-CHO in water solution readily equilibrates to 5-AC and more slowly deformylates to give RGU irreversibly. The latter hydrolysis product exhibited no pronounced toxicity when tested either in vitro or in vivo. Although RGU-CHO showed considerable antitumor activity against murine L1210 leukemia, hydrolysis studies indicated that all of the observed activity could be attributed to 5-AC formed by in vivo equilibration from RGU-CHO. Moreover, RGU-CHO seemed to impart to test animals a toxicity which was no greater than that anticipated from its ability to generate 5-AC.

5-Azacytidine (1, 5-AC) is a nucleoside antimetabolite[1] which has a clinical specificity for acute myelogenous leukemia.[2] When administered by rapid intravenous (iv) injection,[3] the drug causes severe, often dose limiting,[2] gastrointestinal toxicity which can be greatly reduced[4] or virtually eliminated[5] by slow, continuous iv infusion of the drug in lactated Ringer's solution over a 5-day period. However, the latter technique is thwarted by the facile hydrolysis of 5-AC in aqueous formulations,[6] leading not only to solutions of decreasing 5-AC potency but also to hydrolysis products having toxicological or therapeutic effects which have not been determined. Microbiological assay of 24-h-old aqueous solutions of 5-AC indicates twice the cytotoxicity as would be anticipated from the results of chemical stability data.[5,7] The discrepancy between the chemical and biological analysis of "aged" 5-AC solutions suggests that one or more hydrolysis products, in addition to 5-AC, contribute to the observed cytotoxic effect. Because of implications relevant to the clinical usage of 5-AC, it was of interest to examine partially hydrolyzed 5-AC solutions for the presence of biologically active hydrolysis products. Scheme I shows the predominant hydrolytic pathway of 5-AC (1) at room temperature and neutral pH such as would occur in a clinical formulation. The presence of the initial hydrolysis product, 2 (RGU-CHO), formed as a consequence of nucleophilic attack by water at C-6 of 5-AC followed by ring opening, was inferred by spectroscopic observations.[6] However, all attempts to isolate or synthesize RGU-CHO were unsuccessful.[6] The ribosylguanylurea (3, RGU), formed by an irreversible loss of the *N*-formyl group from RGU-CHO, was sufficiently stable to allow isolation but was found to be weakly cytotoxic against *Escherichia coli*[6] and inactive in vivo against murine L1210 leukemia while exhibiting no pronounced toxicity in the test animals.[8]

The present report describes the isolation [by high-pressure liquid chromatography (HPLC) in a preparative mode], characterization, and some chemical and biological properties of the labile *N*-formyl intermediate 2 from "aged" water solutions of 5-AC.

Fractionation. As shown in Figure 1, a water solution of 5-AC (1) exhibits a single peak in the chromatogram (trace A) when analyzed with HPLC immediately after the solution is formed. After the solution is stored at room temperature for 2 h a second peak due to RGU-CHO (2) can be observed (trace B). A third peak emerges from baseline noise after 6 h (trace C) which is primarily due to RGU (3). After 24 h (trace D), 2 is approximately at its maximum concentration in the mixture, and thereafter both 1 and 2 decrease in concentration while the ratio of their peak areas remains essentially constant. From its first appearance in the chromatogram, the concentration of RGU increases continuously until it is the only detectable material in solution after about 10 days.

Scheme I



The three peaks of the 24-h-old solution were sufficiently separated to encourage an attempt to isolate and identify the individual components of the mixture by preparative HPLC. It was found that 1 (32 mg) in water solution (2 mL), after 24-h storage at room temperature to give the threefold mixture, could be chromatographed without significant peak overlap. Multiple collection of the first eluted peak (t_R = 2.0 min), due primarily to 3, gave a glass on lyophilization from which the picrate of 3 was isolated in 52% yield. The somewhat low yield of picrate and the presence of a small shoulder on the peak in the chromatogram suggest the presence of other materials, although triazine products can be eliminated from consideration since absorptivity in the UV was absent.

The second peak (t_R = 5.9 min) due to 2 was collected, frozen immediately, and lyophilized to give a white solid. Typically, fractions from five or six runs were combined in water solution (2 mL) and rechromatographed to provide pure samples of 2 which were used for charac-

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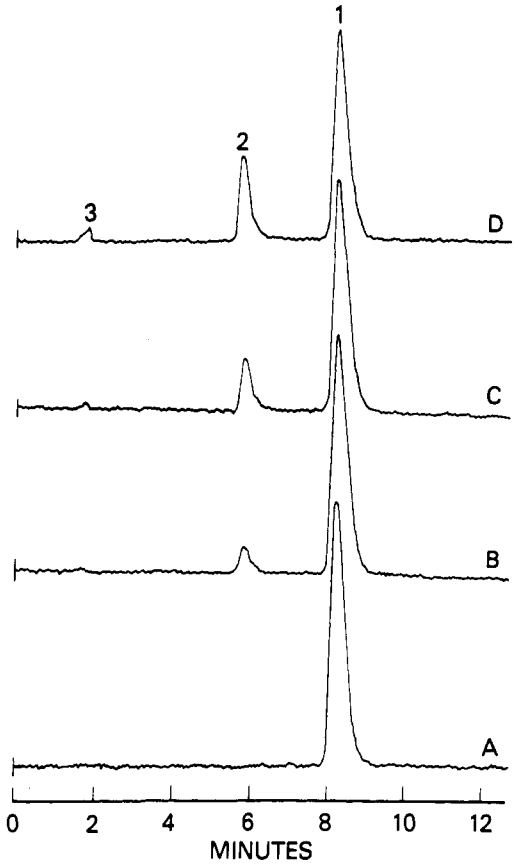

Figure 1. HPLC traces of 5-AC in aqueous solution (10 mg/mL, 25 °C) at increasing time intervals showing the response from a refractive index detector (×8) produced by 20-μL injections. The column and conditions are described in the Experimental Section. Traces A, B, C, and D were produced at time = 0, 2, 6, and 24 h, respectively. Peak 3 = RGU (**3**), peak 2 = RGU-CHO (**2**), and peak 1 = 5-AC (**1**).

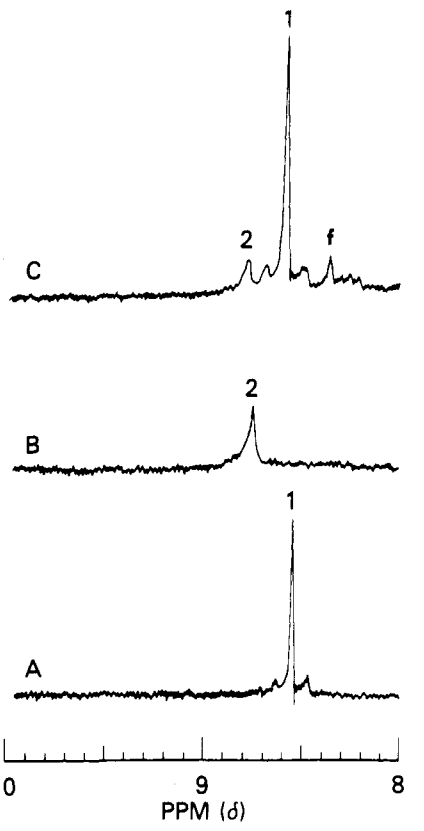

Figure 2. The low-field portions of the proton NMR spectra (Me_2SO-d_6) are shown for pure 5-AC (spectrum A), pure RGU-CHO (spectrum B), and a hydrolysis mixture obtained by lyophilization of a 5-AC solution in distilled water after storage at 25 °C for 24 h (spectrum C). Peak 1 is the C-6 aromatic proton of 5-AC (flanked by spinning side bands). Peak 2 is the formyl proton of RGU-CHO. Peak f is due to the formyl proton of formate anion.

terization and biological testing described in this report.

Examination of the δ 8–10 region of the NMR spectra of compounds **1** and **2** showed characteristic differences (Figure 2). The C-6 aromatic proton of 5-AC (**1**) produces a sharp singlet at δ 8.60 (Figure 2, spectrum A) which is absent from the spectrum of **2** (spectrum B). Compound **2**, however, shows the formyl proton as a broadened singlet (δ 8.80). For purposes of comparison, the analogous proton of formylguanidine[9] in the same solvent (Me_2SO-d_6) was found to exhibit a singlet at δ 8.45.

NMR analysis presented a method to qualitatively corroborate our HPLC analysis of 24-h-old aqueous solutions of 5-AC. For that purpose, a water solution of **1** was stored at room temperature for 24 h, lyophilized, and dissolved in Me_2SO-d_6. The NMR spectrum of the hydrolysis mixture (Figure 2, spectrum C) showed singlets assignable to the formyl proton of **2** and the C-6 proton of **1**. The formyl proton of formate anion, which indirectly indicates the presence of the ribosylguanylurea (**3**), appeared at δ 8.39 as a singlet. Incremental addition of formic acid caused a corresponding stepwise strengthening of the singlet with concomitant stepwise high-field shifts. The formyl proton of ammonium formate (δ 8.43, Me_2SO-d_6) exhibited the same behavior in the NMR with incrementally added formic acid. Using ^{13}C NMR, Israili et al.[10] also found evidence for formate in a similar preparation of 5-AC.

Chromatographically pure **2**, run as soon as possible after making a water solution, gave a maximum in the UV at 238 nm (ε 18700). After 1 h the extinction coefficient decreased to 15200 with no change in the maximum position. It was reported[6] that **1** in aqueous solution showed an increase in UV absorbance with time from its initial value. The increase continues for several hours at room temperature before a gradual decrease is noted. With UV data for **2** in hand, these observations can now be interpreted. The early hydrolysis product, **2**, because of its high extinction coefficient relative to **1** [λ_{max} 241 nm (ε 6800)] augments UV absorbance as the hydrolysis of **1** proceeds. Since the difference in the maxima of pure samples of **1** and **2** is small (3 nm), no apparent shift in maximum is observed during the hydrolysis course. A point in time is reached when **2** in turn is hydrolyzed to **3** (nonchromophoric) at a rate which exceeds the production of **2** from **1**. An observed net decrease in UV absorbance then results.

It should be mentioned that as a consequence of nucleophilic attack by water at the 6 position of **1**, ring opening could conceivably occur in a manner which would locate the formyl group on the nitrogen atom bearing the ribosyl moiety (N-1) and *not* as indicated in structure **2**. However, it can be reasoned that **3**, which has no UV absorptivity at wavelengths greater than 225 nm, if substituted at the terminal amidino residue with a formyl group, as in **2**, would have the ability to extend the conjugation of the chromophore through enolization of the formyl group. Therefore, the maximum observed at 238 nm supports structure **2**. On the other hand, if the formyl group is situated on N-1, enolization is not possible, and the observed bathochromic shift would not be anticipated.

The third peak eluted with water (t_R = 8.35 min) from

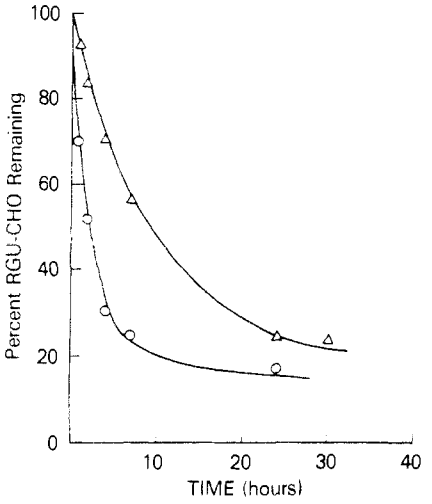

Figure 3. Time course of the disappearance of RGU-CHO in distilled water (2.6 mg/mL) at 37 °C (O-O) and 25 °C (Δ-Δ) as determined by HPLC using a UV detector (linear response in the 2.0 AUFS range used) and the conditions described in the Experimental Section.

the HPLC column was collected, lyophilized, and identified as starting material 1 by melting point, mixture melting point, UV, and NMR.

Hydrolysis Studies. The rate of disappearance of **2** at 25 and 37 °C in water solution was conveniently followed by HPLC using a UV detector having a fixed wavelength of 254 nm. Immediately after dissolving **2** in water a single peak was observed in the chromatogram. After storing the solution in a 37 °C thermostatic bath for 1 h and repeating the HPLC analysis, it was found that the peak area due to **2** had decreased to 70% of the initial value and a peak due to 1 (formed by equilibration from **2**) appeared in the chromatogram. Further aliquots were withdrawn and analyzed at intervals of 2, 4, 7, and 24 h to provide, in part, the data for Figure 3 from which it can be seen that the half-life of **2** in water at 37 °C is about 2 h. At t = 4 h, 31% of **2** remained. Almost all of the loss of **2** up to t = 4 h can be accounted for by its equilibration to 1. In addition to loss to equilibration, **2** can also react irreversibly with water to form **3**, but very little **3** was evident in the t = 4 h chromatogram (refractive index detector, ×8). Moreover, the sum of the peak areas of 1 and **2** (UV detector), after application of a factor to account for the difference in extinction coefficients at 254 nm (ϵ_{254} RGU-CHO/ϵ_{254} 5-AC = 1.77), was nearly equal to the area of **2** at t = 0. Another interesting property of this hydrolysis system was observed at t = 7 h when 25% of the initial concentration of **2** remained. The peak area ratio of 1 and **2** became essentially constant, and, thereafter, 1 and **2** decreased in a fixed concentration ratio of 1 part of RGU-CHO to 2.43 parts of 5-AC.

In the same way, the hydrolysis of **2** was studied at 25 °C (Figure 3). The half-life occurred at t = 9 h and a constant ratio of 1 and **2** (2.42:1) was reached at t = 24 h when 24% of **2** remained.

For comparison, the hydrolysis of 1[11] (5 mg/mL) was studied at 25 °C. Samples were withdrawn and analyzed with HPLC at 1, 3, 6, 24, 30, and 48 h. Over these intervals the percent of 1 remaining as determined by peak area comparisons was 96, 89, 82, 63, 58, and 48%, respectively. The half-life was reached after 47 h and a constant ratio of 1 and **2** (2.67:1) was established at t = 24 h when 63% of 1 remained and 24% of **2** had formed by hydrolysis.

Biological Results and Discussion. It was of interest to determine if RGU-CHO has intrinsic antitumor properties, and is the cytotoxic hydrolysis product suggested by tissue culture studies,[5,7] and if RGU-CHO imparts a host toxicity. In order to explore these two areas of enquiry, the in vivo murine L1210 leukemia model was selected to evaluate RGU-CHO biologically in a parallel assay with 5-AC. The inherent difficulty in this biological test is the interconvertibility of 5-AC and RGU-CHO by equilibration in aqueous environments, making the separation of their respective biological effects difficult since pure samples of both compounds in aqueous solution form nearly identical threefold mixtures of 1, **2**, and **3** after the requisite period of time. Accordingly, samples of 5-AC and RGU-CHO for biological studies were purified by preparative HPLC and were administered to the test mice within 15 min after solution in physiological saline. The dose–response results of the comparative evaluation in the L1210 test system are recorded in Table I. It can be seen that both compounds have considerable antitumor activity with the optimum dose for 5-AC occurring at 20 mg/kg and that for RGU-CHO apparently is >60 mg/kg. However, the activity shown by RGU-CHO could be accounted for by its equilibration to 5-AC if the assumption is made that the findings of the above hydrolysis studies at 37 °C are approximately applicable to the behavior of RGU-CHO in a mouse. A dose of 40 mg/kg of RGU-CHO (% ILS 85) corresponds to a 10 mg/kg dose of 5-AC (% ILS 86), and 20 mg/kg of RGU-CHO (% ILS 56) corresponds to 5 mg/kg of 5-AC (% ILS 63). Therefore, RGU-CHO has one-fourth the potency of 5-AC which was confirmed by a second, identical L1210 experiment. Since it was shown that 30% of RGU-CHO is converted to 5-AC after 1 h at 37 °C, it is reasonable to conclude that RGU-CHO exhibits antitumor activity only by virtue of its ability to generate 5-AC in aqueous systems. This conclusion is strengthened if one considers that 50% of RGU-CHO equilibrates to 5-AC in 2 h at 37 °C, suggesting a potential for 5-AC to exert an even greater influence on the antitumor response following RGU-CHO administration. The observed one-fourth potency actually shown by RGU-CHO might be explained by the interplay of the following possible departures from its behavior in aqueous solution: (a) the rate of 5-AC formation is retarded in vivo, (b) RGU-CHO is more rapidly deformylated in vivo, and (c) RGU-CHO is rapidly excreted relative to 5-AC. In any event, it seems unlikely that RGU-CHO itself has antitumor properties.

Although neither drug at the dose levels tested caused acute toxicity leading to early death, the highest 5-AC dose (40 mg/kg) showed significant indications of drug-related

Table I. Comparison of 5-AC and RGU-CHO against L1210 Leukemia[a]

5-AC				RGU-CHO			
Dose, mg/kg	% ILS[b]	T − C[c] (1-5)	T − C[d] (1-9)	Dose, mg/kg	% ILS[b]	T − C[c] (1-5)	T − C[d] (1-9)
40	84	−2.3	−6.5	60	93	−1.9	−4.3
20	130	−1.6	−5.1	40	85	−0.8	−4.7
10	86	−1.8	−4.5	20	56	−1.9	−3.8
5	63	−1.0	−2.9	10	33	−1.6	−3.4
2.5	33	−1.5	−5.7	5	17	−1.2	−2.6

[a] Drug treatment was on days 1, 5, and 9 after tumor cell implantation. The untreated control group had a mean survival time of 10.1 days. See the Experimental Section for details. [b] Mean percentage increase in life span. A duplicate test gave similar results. [c] The difference of the average body weight change in grams of the test group (T) and the control group (C) as measured on days 1 and 5. [d] The same as in footnote c except the weight differences of T and C are those recorded on days 1 and 9.

toxicity in terms of % ILS and T - C values. On the other hand, the highest RGU-CHO dose tested (60 mg/kg) was not accompanied by the same toxic indications. Moreover, weight losses (T - C) due to RGU-CHO treatment were generally less than those recorded for the corresponding 5-AC treated group at the same dose level. Therefore, it can be concluded that RGU-CHO is apparently neither toxic nor antitumor active beyond its ability to equilibrate to 5-AC.

Although traces of additional materials were detected by scrupulous HPLC analysis, the predominant 5-AC hydrolysis products at room temperature and neutral pH are RGU and RGU-CHO. Neither of these hydrolysis products seems to possess the intrinsic cytotoxicity necessary to explain the enhanced cytotoxicity found by microbiological assay[5,7] of "aged" 5-AC solutions. Excluding the presence, in trace amounts, of a very potent cytotoxic hydrolysis product, the microbiological results could be explained by the participation of RGU-CHO as a latent form of 5-AC as the latter is removed from equilibrium by cell uptake and anabolic reaction. In a recent report Presant et al.[13] demonstrated a prolonged residual activity of 5-AC in leukemic mice lasting 1–2 days after administration. Since the drug half-life in mice is relatively short (<6 h), it was suggested that the persistent antileukemic effect was perhaps mediated by a continued availability of a 5-AC metabolite but not by the intact drug. In view of the present findings, it is interesting to speculate that RGU-CHO might reversibly react with macromolecular sulfhydryl, amino, or hydroxyl groups, resulting in a depot form of 5-AC and slowing the irreversible conversion of RGU-CHO to the inactive RGU.

The technique of administering 5-AC by continuous iv infusion offers a clinical advantage, although strict control over 5-AC dosages is operationally difficult to achieve because of hydrolysis reactions. If fresh infusion solutions are prepared every 3–12 h as has been suggested,[4] drug loss to hydrolysis of about 10–25% would occur over that period as shown by the HPLC analyses herein reported. The terminal hydrolysis product, RGU, is slowly formed and is only detectable by HPLC after 6 h. Since RGU has only a moderate toxicity, and has a concentration of <5% in a 5-AC formulation at the end of 12 h, it seems unlikely that it would contribute significantly to clinical toxicity. The initial hydrolysis product, RGU-CHO, while present in substantial concentrations in formulated 5-AC, has no salient untoward biological effects but, rather, may play an important role in the pharmacodynamics of 5-AC.

Experimental Section

Chemical. HPLC was carried out with a Waters Associates, Inc., Model ALC/GPC-244 chromatograph which included the following modular components: a U6K septumless injector, a 440 fixed-wavelength (254 nm) UV detector, a R401 differential refractometer detector, and a 6000A solvent pump. Chromatograms were recorded with an OmniScribe Model A5211-1 two-pen recorder. Both analytical and preparative work was accomplished with a 7 mm (i.d.) × 300 mm μBondapak/C_{18} column (Waters Associates, Inc.) which was eluted with distilled water at 4 mL/min. HPLC fractions were lyophilized with a Virtis Unitrap Model 10-100-C apparatus. A Cary Model 15 spectrophotometer was used to obtain UV spectra and a Perkin-Elmer Model 621 was used to record infrared spectra. Proton NMR spectra were recorded with a Varian HA-100D spectrometer. Chemical shifts are reported in parts per million (δ) downfield from tetramethylsilane which was used as an internal standard. Melting points were determined with a Thomas-Hoover capillary apparatus and are uncorrected. Elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, Tenn. A generous quantity of 5-azacytidine was made available by Dr. H. B. Wood, Jr., Drug Synthesis and Chemistry Branch, NCI.

Hydrolysis Products Isolation. A solution of 1 (192 mg, 0.786 mmol) in distilled water (12 mL) was stored at 24–25 °C for approximately 24 h at which time a 2-mL sample was withdrawn and injected into the HPLC. As detected by the differential refractometer (attenuation ×64), the three eluted peaks (see Figure 1) were collected sequentially, immediately frozen, and lyophilized. The column loaded to this extent (ca. 32 mg) gave a complete separation of **3** from 1 and **2** and a near complete separation of 1 and **2** (valley = 1% of full scale) was achieved.

First Eluted Fraction: 1-β-D-Ribofuranosyl-3-guanylurea (3). The first peak eluted from the HPLC was collected, lyophilized, and pooled with the corresponding fractions from 11 earlier runs to provide a total of 32 mg (0.14 mmol) of **3** as a noncrystalline gum which showed only end absorbance in the UV. A solution of the gum in methanol (0.5 mL) was treated with picric acid (32 mg, 0.14 mmol) in absolute ethanol (0.5 mL). After allowing the solution to crystallize overnight, the picrate of **3** was removed by filtration, washed with cold absolute ethanol (three times), and dried to give 34 mg (52%) of yellow crystals, mp 172–174 °C dec (lit.[6] mp 172–174 °C dec). A mixture melting point with an authentic sample[6,8] was undepressed.

Second Eluted Fraction: *N*-(Formylamidino)-*N'*-β-D-ribofuranosylurea (2). The second peak eluted from the HPLC was collected and lyophilized. Combining the lyophilized fractions from a total of six runs in distilled water (2 mL), and rechromatographing the solution, enabled the separation and collection of pure **2** (19 mg) as a white electrostatic solid after lyophilization: mp 112–114 °C (solidifies and remelts with effervescence at ~140 °C); UV (solutions maintained at 25 °C) λ_{max} (H_2O, t = 0) 238 nm (ε 19200); UV λ_{max} (H_2O, t = 1 h) 238 nm (ε 16100); UV λ_{max} (pH 7 buffer, t = 0) 238 nm (ε 18700); UV λ_{max} (pH 7 buffer, t = 1 h) 238 nm (ε 15200); IR (Nujol) 1723, 1652, 1602 cm^{-1}; NMR δ (Me_2SO-d_6) 8.80 (s, 1, CHO), 5.14 (d, J = 5 Hz, 1, C_1H). Anal. Calcd for $C_8H_{14}N_4O_6$ (262.2): C, 36.64; H, 5.38; N, 21.37. Found: C, 36.54; H, 5.40; N, 21.41.

Third Eluted Fraction: 4-Amino-1-β-D-ribofuranosyl-1,3,5-triazin-2(1*H*)-one (1). The third peak to elute from the HPLC was collected, frozen, and lyophilized to give 15 mg of a fluffy white solid. Crystallization from ethanol–water (2:1) gave 1 as white needles: mp and mmp 231–232 °C dec; UV (solutions maintained at 25 °C) λ_{max} (H_2O, t = 0) 241 nm (ε 6800); UV λ_{max} (H_2O, t = 1 h) 241 nm (ε 7700); UV λ_{max} (pH 7 buffer, t = 0) 241 nm (ε 6900); UV λ_{max} (pH 7 buffer, t = 1 h) 241 nm (ε 7800); NMR δ (Me_2SO-d_6) 8.60 (s, 1, C-6), 5.67 (d, J = 4 Hz, 1, C_1H).

Biological. CDF_1 female mice, 11–12 weeks old, were each inoculated ip (day 0) with 0.1 mL of a L1210 cell suspension (Lockes balanced salt) containing 1.43 × 10^6 cells/mL (Coulter counter). Beginning 24 h after tumor implantation (day 1) drugs (purified by preparative HPLC) were administered within 15 min after dissolution in sterile 0.85% saline by ip injection in volumes of 0.2–0.3 mL. Drug treatment was continued on days 5 and 9. Ten animals were used for testing each dose level of each drug and ten untreated control animals were maintained. The mice were weighed on days 1, 5, and 9 in order to determine the correct dosage for each animal as well as to provide comparative weight gain/loss data for drug toxicity evaluations. The increase in mean life span of the test animals beyond the mean survival time of the untreated control animals expressed as a percentage increase (% ILS) was used to evaluate antitumor activity. A % ILS value of ≥25% was considered indicative of antitumor activity.

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Synthesis and Antitumor Activity of Preactivated Isophosphamide Analogues Bearing Modified Alkylating Functionalities[1]

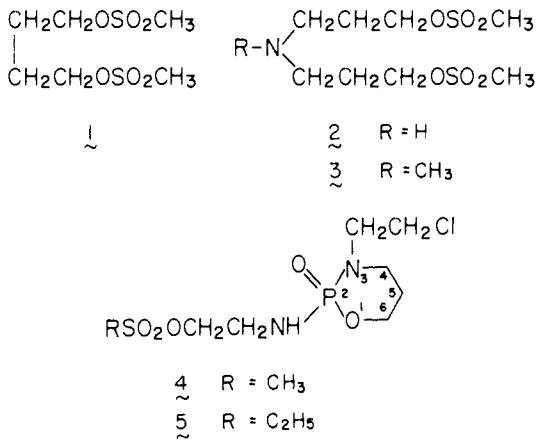
Akira Takamizawa,* Saichi Matsumoto, Tsuyoshi Iwata, Itsuo Makino, Kenji Yamaguchi, Naomi Uchida, Hisashi Kasai, Osamu Shiratori, and Shiro Takase

Shionogi Research Laboratory, Shionogi & Co., Ltd., Fukushima-ku, Osaka 553, Japan. *Received June 28, 1977*

In search of cancer chemotherapeutic agents with greater efficacy than cyclophosphamide, 4-hydroperoxyisophosphamide analogues bearing modified alkylating functionalities such as 2-bromoethyl, 2-iodoethyl, 2-methylsulfonyloxyethyl, and 2-ethylsulfonyloxyethyl groups were prepared by ozonolytic cyclization reaction of N,N'-substituted 3-butenyl phosphorodiamidates. Comparative cytotoxicity against L1210 cells and antileukemic life-span activity against L1210 implanted BDF_1 mice of the newly synthesized compounds were tabulated. The 4-hydroperoxyisophosphamide analogues which have different alkylating groups in a molecule showed slightly greater cytotoxicity in vitro than those with the same alkylating groups. Most of the compounds having different alkylating groups also showed high antileukemic activity in vivo. Among them, the highest efficacy was found for 2-[*N*-methyl-*N*-(2-chloroethyl)]amino-3-(2-methylsulfonyloxyethyl)-4-hydroperoxy-1,3,2-oxazaphosphorinane 2-oxide (NSC 280122D) whose life-span activity was also greater than that of 4-hydroperoxyisophosphamide, cyclophosphamide, and isophosphamide. The superiority of this compound was especially apparent by oral administration.

Busulfan (1)[2] is a representative antitumor alkylsulfonate having potential alkylating activity. However, in comparison with nitrogen mustard, which is representative of antitumor alkylating agents bearing 2-chloroethylamino groups and is effective against various kinds of experimental tumors, busulfan exerts only limited activity against Walker-256 carcinosarcoma and is practically ineffective against L1210 leukemia and other animal tumors. In 1964, Sakurai and El-Merzabani[3] synthesized nitrogen-containing methanesulfonates such as **2** and **3** which are structurally related to nitrogen mustard and found that these compounds showed increased activity against some tumors which were unaffected by busulfan.[4,5] In 1974, Brock and his co-workers[6,7] reported on the antitumor activities of a number of cyclophosphamide and isophosphamide analogues bearing alkylsulfonyloxyalkyl and chloroethyl groups. Most of the compounds reported by Brock et al. showed considerable activities against Yoshida ascitic sarcoma in rats, Walker-256 carcinosarcoma in rats, and L1210 leukemia in mice, and they concluded that isophosphamide analogues bearing mixed alkylating functions such as **4** and **5** were especially effective. Recently, we[8,9] synthesized C_4-oxidized cyclophosphamide and isophosphamide derivatives and found that C_4-hydroperoxylation was as effective as C_4-hydroxylation for activating these drugs and that the C_4-hydroperoxides showed greater stability than the corresponding C_4-hydroxy derivatives. The action mechanisms of the compounds reported by Brock et al. are thought to resemble those for isophosphamide (and cyclophosphamide), i.e., the antitumor effect might be exerted after in vivo C_4-oxidation of the 1,3,2-oxazaphosphorinane ring.[10] Therefore, the C_4-hydroperoxy derivative of **4** and **5** might also exert increased activity. We now wish to report on the synthesis and antileukemic activity of C_4-hydroperoxyisophosphamide analogues bearing mixed alkylating functions related to compounds **4** and **5** (Chart I).

Chart I



The ozonolysis reaction of 3-butenyl phosphorodiamidate, which is a general synthetic method for preparing C_4-functionalized 1,3,2-oxazaphosphorinane 2-oxides and related phosphorus-containing heterocyclics,[8,9,11,12] was also used for the present syntheses. A variety of 3-butenyl phosphorodiamidates bearing different alkylating substituents at the phosphorodiamidic nitrogen atoms were prepared via three routes (see Scheme I). Route a consisted of reaction of phosphoryl halide **6** (POX_3, X = Cl or Br) with 3-buten-1-ol followed by treatment with the corresponding N-substituted 2-haloethylamine salt ($XCH_2CH_2NHR \cdot HX$, X = Cl or Br) in the presence of

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