Synthesis of Site-Directed Chelating Agents II: 2-Formyl-3-hydroxy-4,5-bis(hydroxymethyl)pyridine Thiosemicarbazone

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Abstract □ 2-Formyl-3-hydroxy-4,5-bis(hydroxymethyl)pyridine thiosemicarbazone was synthesized in an attempt to direct the chelating potential of this agent to the active site of the zinc-requiring enzyme pyridoxal phosphokinase. Evaluation of the antineoplastic activity of this agent in the sarcoma 180 and L-1210 leukemia systems in mice showed potent activity. The presence or absence of pyridoxine hydrochloride in the diet did not influence the degree of inhibition of the growth of sarcoma 180 ascites cells. In addition, 2-formyl-3-hydroxy-4,5-bis(hydroxymethyl)pyridine thiosemicarbazone was inactive against a subline of sarcoma 180 resistant to 1-formylisoquinoline thiosemicarbazone, suggesting that the former agent may have a biochemical mechanism of action similar to that of the latter. In keeping with this expectation, 2-formyl-3-hydroxy-4,5-bis(hydroxymethyl)pyridine thiosemicarbazone inhibited the synthesis of DNA but not of RNA or protein in sarcoma 180 ascites cells *in vitro*.

Keyphrases □ Chelating agents, site directed—synthesis of 2-formyl-3-hydroxy-4,5-bis(hydroxymethyl)pyridine thiosemicarbazone, potential antineoplastic agent □ 2-Formyl-3-hydroxy-4,5-bis(hydroxymethyl)pyridine thiosemicarbazone—synthesized, screened for antineoplastic activity □ Antineoplastic agents, potential—2-formyl-3-hydroxy-4,5-bis(hydroxymethyl)pyridine thiosemicarbazone synthesized and screened

Numerous heterocyclic carboxaldehyde thiosemicarbazones with the side chain alpha to a heteroaromatic ring nitrogen possess both antineoplastic and antiviral activities in various experimental systems (1). Furthermore, one agent of this series, 5-hydroxy-2-formylpyridine thiosemicarbazone, demonstrated weak carcinostatic potency in humans (2, 3). The antineoplastic activity of this class of agents, as elucidated with 5-hydroxy-2-formylpyridine thiosemicarbazone and the related compound, 1-formylisoquinoline thiosemicarbazone, requires metal-binding potential and the capacity to inhibit the synthesis of DNA (4). These related phenomena result from inhibition of the enzyme ribonucleoside diphosphate reductase, an iron-requiring biocatalyst.

BACKGROUND

It is conceivable that the chelating potential of the formyl thiosemicarbazone side chain, positioned alpha to a heteroaromatic ring nitrogen atom, might be directed toward other metal-containing enzymes by modification of the heterocyclic ring system such that it resembles the natural substrate of the targeted metalloenzyme. An earlier report (5), based in part upon this concept, involved the synthesis of pteridine carboxaldehyde thiosemicarbazones directed against the enzyme dihydrofolate reductase. Since heterocyclic carboxaldehyde thiosemicarbazones have been shown to form coordination complexes with various transition metals (4, 6), pyridoxal phosphokinase, a zinc-requiring enzyme (7) that catalyzes the phosphorylation of pyridoxine hydrochloride at position 5 to form the active coenzyme form, was chosen as a likely candidate for such direct metabolic assault.

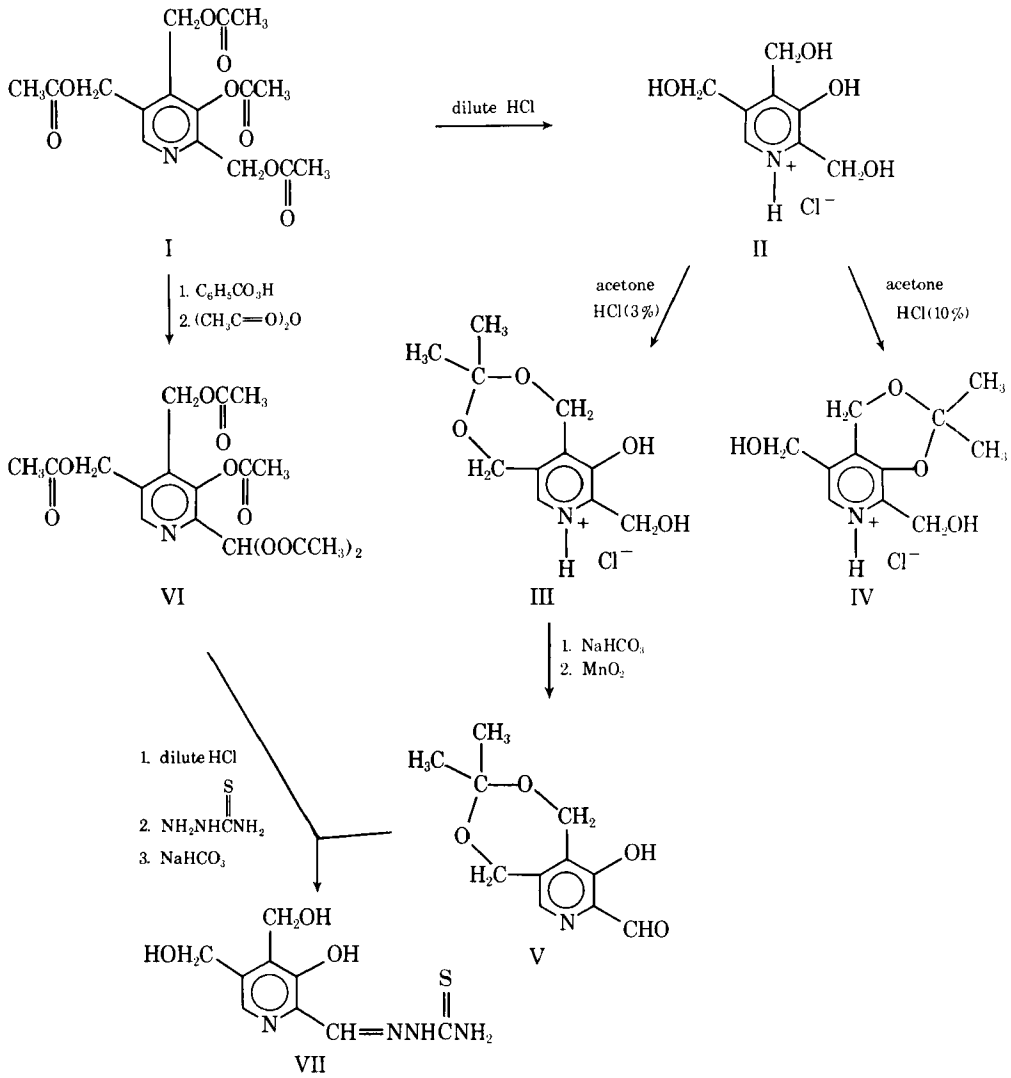
The idea that interference with the function of pyridoxine hydrochloride can lead to impairment of neoplastic growth gains support from the findings (8) that the incidence of complete regression of sarcoma 180 brought about by administration of methylglyoxal bis(N^4-methylthiosemicarbazone) was markedly enhanced in animals fed a pyridoxine-deficient diet from the day of initial tumor transplantation, although this relatively mild degree of pyridoxine hydrochloride deficiency alone did not impair the growth of the tumor significantly. Although clinical studies in patients on diets low in pyridoxine and as well as on pyridoxine antimetabolites did not show definite antitumor effects (9, 10), observations were reported in which tumor growth in model systems was depressed by creation of a pyridoxine hydrochloride deficiency (11, 12). Thus, blockade of pyridoxal phosphokinase activity by chelation of the zinc cofactor could conceivably yield a useful cancer chemotherapeutic agent.

Since the 2-formyl thiosemicarbazone derivative of pyridoxine

Table I—Antitumor Effect of 2-Formyl-3-hydroxy-4,5-bis(hydroxymethyl)pyridine Thiosemicarbazone against Murine Neoplasms Sarcoma 180, Leukemia L-1210, and Sarcoma 180/IQ-1

	Sarcoma 180							
	Complete Diet		Pyridoxine-Deficient Diet		L-1210 Leukemia		Sarcoma 180/IQ-1	
Daily Dose, mg/kg[a]	Average Weight Change, %[b]	Average Survival Time, days	Average Weight Change, %[b]	Average Survival Time, days	Average Weight Change, %[b]	Average Survival Time, days	Average Weight Change, %[b]	Average Survival Time, days
Control	+11.0	12.2	+6.0	12.0	+7.1	8.0	+18.5	13.0
20	+7.0	16.2	+6.9	19.3	—	—	+3.0	16.2
40	+5.6	21.8	+8.1	17.7	—	—	+3.5	16.2
60	+6.2	16.0	+4.1	19.8	—	—	—	—
80	+0.0	16.4	+2.3	15.4	—	—	—	—
20 × 2	−4.6	19.8	—	—	−2.8	12.7	—	—
40 × 2	−5.7	29.8	—	—	−2.7	14.4	—	—
60 × 2	—	—	—	—	+1.5	16.2	—	—
IQ-1[c] (40)	−10.8	39.7	—	—	+2.4	16.6	−0.6	13.8

[a] Administered once or twice daily at 12-hr intervals, as indicated, for 6 consecutive days beginning 24 hr after tumor implantation. [b] Average change in body weight from onset to termination of drug therapy. [c] IQ-1 = 1-formylisoquinoline thiosemicarbazone.



Scheme I

has structural features similar to those deemed necessary for inhibition of the ribonucleoside diphosphate reductase enzyme (13), it is probable that this compound would exert blockade of two metabolic processes *in vivo*. This paper describes the synthesis of 2-formyl-3-hydroxy-4,5-bis(hydroxymethyl)pyridine thiosemicarbazone (VII) as well as several biological and biochemical effects of this compound; a preliminary report of this work was presented earlier in abstract form (14).

RESULTS AND DISCUSSION

The synthesis of VII was accomplished by two different procedures (Scheme I). The common intermediate, 3-acetoxy-2,4,5-tris(acetoxymethyl)pyridine (I), was synthesized according to the procedure of Bedford *et al.* (15). Acid hydrolysis of I afforded 3-hydroxy-2,4,5-tris(hydroxymethyl)pyridine hydrochloride (II), which was treated with hydrogen chloride as a suspension in acetone according to the procedure of Korytnyk (16). The amount of hydrogen chloride taken up by the suspension is a critical factor, and, accordingly, yields are variable.

If the quantity of hydrogen chloride utilized was about 10%, only a six-membered ring ketal (IV) was formed; 3% hydrogen chloride afforded the seven-membered ketal (III) and unreacted compound II. Recently, Korytnyk *et al.* (17) reported that acetonation of II in the presence of *p*-toluenesulfonic acid yielded primarily III in 65% yield. Compound III as the free base was then oxidized with manganese dioxide to yield the corresponding aldehyde (V). Compound V, after hydrolysis with dilute hydrochloric acid and subsequent reaction with thiosemicarbazide and neutralization with sodium bicarbonate, produced the desired compound, VII.

An alternative route produced a better yield of VII. This procedure required the initial formation of the *N*-oxide of I which, upon rearrangement with acetic anhydride, yielded the pentaacetate VI (15). After acid hydrolysis of the pentaacetate, the solution was reacted with thiosemicarbazide, which yielded the desired compound VII upon neutralization with sodium bicarbonate.

The antitumor effects of VII in three different murine neoplastic test systems are shown in Table I. Although preliminary experiments showed that relatively high levels of VII (about 10^{-4} *M*) caused slight inhibition of pyridoxal phosphokinase from *Escherichia coli*, the effects of this agent on sarcoma 180, as measured by the average survival time of mice bearing this ascites cell neoplasm, were similar in tumor-bearing animals fed a complete diet or a pyridoxine hydrochloride-deficient diet. Thus, in each group, a single daily dose of VII exhibited slight, but significant, antitumor activity as compared to a dose of 1-formylisoquinoline thiosemicarbazone in the same range, which was employed as a standard control. However, when VII was administered twice daily at 12-hr intervals, a level of 40 mg/kg/dose increased the average survival time of tumor-bearing mice to 29.8 days as compared to 12.2 days for untreated control animals bearing this tumor.

In the L-1210 lymphoma system, however, VII showed antineoplastic activity equivalent to 1-formylisoquinoline thiosemicarbazone, which essentially doubled the lifespan of lymphoma-bearing animals. In a strain of sarcoma 180 ascites tumor, selected for resistance to 1-formylisoquinoline thiosemicarbazone, VII was essentially inactive, suggesting a similar biochemical mechanism of action for these two compounds. To gain evidence for this possibility, the effect of VII on the synthesis of DNA, RNA, and protein was measured *in vitro*.

The results indicated that the synthesis of DNA, as measured by the incorporation of ^{3}H-thymidine into acid-insoluble material, was significantly inhibited by this compound at a concentration of 4×10^{-5} *M* (Table II). RNA synthesis, as measured by ^{3}H-uridine incorporation, was unaffected by VII, while a 33% inhibition of protein synthesis was obtained at the relatively high concentration of 4×10^{-4} *M*. The inhibition of the formation of DNA, with little or no effect on the syntheses of RNA and protein, is similar to that found for 1-formylisoquinoline thiosemicarbazone (18) and 5-hydroxy-2-formylpyridine thiosemicarbazone (19) under these conditions. Thus, the findings suggest that the antineoplastic activity of VII in these systems is primarily due to its inhibition of the replication of DNA in a manner similar to that of other α-*N*-heterocyclic carboxaldehyde thiosemicarbazones.

Table II—Effect of 2-Formyl-3-hydroxy-4,5-bis(hydroxymethyl)pyridine Thiosemicarbazone on Incorporation of ^{3}H-Thymidine, ^{3}H-Uridine, and ^{14}C-Leucine into DNA, RNA, and Protein, Respectively[a]

Concentration, *M*	Inhibition, %		
	DNA	RNA	Protein
4×10^{-6}	12	0	0
4×10^{-5}	49	5	0
4×10^{-4}	66	0	33

[a] All data are average values of duplicate flasks from each of two experiments. Control specific activities were: ^{3}H-thymidine into DNA = 2.9×10^5 cpm/μmole, ^{3}H-uridine into RNA = 4.0×10^6 cpm/μmole, and ^{14}C-leucine into protein = 2.1×10^5 cpm/μmole. Results are expressed as the percent change in radioactivity present in nucleic acids or protein from controls containing 2% dimethyl sulfoxide.

EXPERIMENTAL[1]

Antitumor Activity—Antitumor activity was determined in mice bearing the ascites cell forms of either sarcoma 180, sarcoma 180 resistant to 1-formylisoquinoline thiosemicarbazone (sarcoma 180/IQ-1), or L-1210 leukemia. The experimental details of such tests were described previously (20).

Transplantation of the neoplasms was accomplished by inoculating mice intraperitoneally with approximately 6×10^6 ascites cells. The drug was administered by intraperitoneal injection beginning 24 hr later, and such therapy was continued once or twice daily at 12-hr intervals for 6 consecutive days in mice bearing sarcoma 180 or sarcoma 180/IQ-1 ascites cells. Mice bearing the L-1210 leukemia were treated twice daily at 12-hr intervals for 4 consecutive days.

Animals were weighed throughout the experiments, and the percent change in body weight from onset to termination of therapy was used as an indication of drug toxicity. Determination of the sensitivity of the tumors toward this agent was based upon the prolongation of survival time afforded by the drug treatments.

Biochemical Studies—The effects of VII on the incorporation of ^{3}H-thymidine, ^{3}H-uridine, and ^{14}C-leucine into DNA, RNA, and protein, respectively, were determined by previously described methodology (18, 19) (Table II). The experiments were carried out in duplicate on pooled 6-day growths of sarcoma 180 ascites cells. Approximately 1.5×10^8 cells were incubated for 30 min at 37° in 10 ml of Fischer's medium minus horse serum, with the inhibitor dissolved in dimethyl sulfoxide and diluted to yield a final concentration of dimethyl sulfoxide no greater than 2%.

^{3}H-Thymidine (200 μg/flask, 2.1×10^4 cpm/μg) and ^{3}H-uridine (200 μg/flask, 1.3×10^4 cpm/μg) were utilized to monitor nucleic acid biosynthetic pathways. 1-^{14}C-D,L-Leucine (125 μg/flask, 5.3×10^4 cpm/μg) was used to measure the rate of protein synthesis.

Acetonation of 3-Hydroxy-2,4,5-tris(hydroxymethyl)pyridine Hydrochloride (II)—Compound II (1.11 g, 0.005 mole) was suspended in 50 ml of dry acetone and cooled to −10°. Hydrogen chloride was bubbled through the suspension slowly until 1.2 g (3% w/v) was utilized. The mixture was then stirred at 0° for 2 hr and kept at −15° for an additional 48 hr. The product was filtered and washed with acetone and then with ether. The yield was 1.1 g, consisting of unreacted II and the cyclic ketal with a seven-membered ring (III).

The mixture was dissolved in 5 ml of water and neutralized with 5% $NaHCO_3$. Compound III, as the free base, was isolated by extraction with ether. The ether extracts were dried (magnesium sulfate), the solvent was removed, and the residue was crystallized from benzene to yield 0.27 g (24%) of white crystals, mp 145–147° [lit. (13) mp 149–151°].

Anal.—Calc. for $C_{11}H_{15}NO_4$: C, 58.66; H, 6.66; N, 6.22. Found: C, 58.41; H, 6.72; N, 6.02.

In another experiment, hydrogen chloride was passed into a suspension of 1.11 g of II in 50 ml of dry acetone at −10° until 5.0 g (10% w/v) was gained. The mixture was stirred at room temperature for 30 min and kept in a freezer overnight. Ether was added to the acetone solution, and the obtained precipitate was filtered and washed with ether to give 1.0 g (76%) of IV. The hydrochloride salt was dissolved in 5 ml of water, neutralized with 10% Na_2CO_3, and evaporated under vacuum to dryness. Then IV, as the free base, was extracted with hot benzene, which, upon cooling, gave 0.7 g of white crystals, mp 116° [lit. (13) mp 110°].

Anal.—Calc. for $C_{11}H_{15}NO_4$: C, 58.66; H, 6.66; N, 6.22. Found: C, 58.54; H, 6.80; N, 6.08.

Oxidation—Compound III (225 mg, 0.001 mole) as the free base was dissolved in 50 ml of chloroform, and the solution was stirred with 500 mg of active manganese dioxide[2] at room temperature for 48 hr. The mixture was then filtered through diatomaceous earth[3], the filtrate was evaporated under vacuum, and the residue was crystallized from petroleum ether to yield 100 mg (45%) of V as white needles, mp 93–94°; NMR (deuterochloroform): δ 1.53 [$(CH_3)_2C$], 4.9, 4.97 (two CH_2), 8.07 (C_6-H), and 10.03 (CHO) ppm.

Anal.—Calc. for $C_{11}H_{13}NO_4$: C, 59.19; H, 5.83; N, 6.28. Found: C, 58.91; H, 5.74; N, 6.24.

2-Formyl-3-hydroxy-4,5-bis(hydroxymethyl)pyridine Thiosemicarbazone (VII)—Compound V (111.5 mg, 0.5 mmole) or VI (11) (206 mg, 0.5 mmole) was heated at 100° for 1 hr in 5 ml of 10% HCl. Thiosemicarbazide (46 mg) was then added, and heating was continued for 5 min. The solution was neutralized with 10% $NaHCO_3$, and the resulting precipitate of VII was filtered, washed with water, and dried to yield 95 mg of yellow solid (74%), mp 200–202°; IR: λ_{max} (potassium bromide) 3170 (NH), 1615 (CH=N), and 1117 (C=S) cm^{-1}.

Anal.—Calc. for $C_9H_{12}N_4O_3S$: C, 42.19; H, 4.69; N, 21.87. Found: C, 42.31; H, 4.52; N, 21.62.

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N-Bromosuccinimide Assay of Penicillins and Cephalosporins

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Abstract □ All penicillins and cephalosporins known to possess biological activity respond to an *N*-bromosuccinimide assay. The developed method is not yet usable for determining stability, but it is useful as a bulk or batching assay.

Keyphrases □ *N*-Bromosuccinimide—reagent for iodometric analysis of penicillins and cephalosporins □ Penicillins—analysis, using *N*-bromosuccinimide, iodometric titration □ Cephalosporins—analysis, using *N*-bromosuccinimide, iodometric titration □ Titrimetry, iodometric—analysis of penicillins and cephalosporins

The iodometric assay for penicillin has been used successfully for almost 30 years (1), and it also has been used for cephalosporins (2). Recently, the results of some iodometric assays of synthetic cephalosporins proved difficult to interpret because of the complexity of the side chains. For such cases, another simple and rapid method was sought that might permit easier interpretation of the assays of complicated structures and that might supplement the iodometric assay.

A bromometric method, reported for penicillin O (3), differed from the iodometric method in that no inactivation of penicillin with alkali was required prior to the measurement. In this laboratory, the reaction of *N*-bromosuccinimide with penicillin was found to require no inactivation of the antibiotic with alkali or penicillinase. An attempt to use a direct assay procedure with this reagent, which had been used for several years by a host of investigators (4–12), led to the realization that the reaction between the penicillins and *N*-bromosuccinimide was time dependent. Moreover, the reagent was found to be somewhat light sensitive and subject to oxidation in air when used as a direct titrant for penicillins, in spite of its prior use in the determination of such simple compounds as sulfides, thiocyanates, cysteine, thiourea, mercaptans, phenols, and sulfur-containing amino acids (4–12). Technical grade *N*-bromosuccinimide deteriorated rapidly in solution.

To offset these difficulties, a different approach was used. In experiments involving the reaction of penicillin with an excess of *N*-bromosuccinimide, a rapid rise in the consumption of *N*-bromosuccinimide was evident from time zero to 1 hr, after which consumption increased at a slower rate. The most reproducible results were obtained when the reaction time was limited to 2.0 hr; it is suggested that this period be used. Results on samples shielded from light or assayed at a lower temperature did not differ significantly from those obtained under the described assay conditions.

EXPERIMENTAL

Reagents—*0.02* N *N-Bromosuccinimide*[1]—Dissolve 1.9 g of *N*-bromosuccinimide in about 5 ml of reagent grade dimethylformamide. Pour into a 1-liter volumetric flask containing about 500 ml of distilled water. Stir or shake and then dilute to volume with distilled water. Transfer to a dark, amber bottle and store immediately in the refrigerator.

Despite reports of the instability of *N*-bromosuccinimide, this solution will maintain its titer for several weeks under proper storage conditions, with a loss of less than 5% of its original value.

0.01 N *Sodium Thiosulfate*—Dissolve 2.5 g of sodium thiosulfate pentahydrate in cooled, freshly boiled distilled water and add 2–3 ml of toluene (preservative). Then dilute to 1 liter with water.

Buffer Solution—To 40 g of anhydrous sodium acetate dissolved in about 500 ml of distilled water, add 1.93 ml of concentrated sulfuric acid. Dilute to 1 liter with distilled water.

Starch Solution—Prepare a 1% solution of starch in saturated saline.

Dimethylformamide (reagent grade), potassium iodide (reagent grade), and acetic acid were used also.

[1] Aldrich analyzed reagent grade, Aldrich Co., Milwaukee, WI 53233