Synthesis of Hydroxy- and Amino-Substituted Benzohydroxamic Acids: Inhibition of Ribonucleotide Reductase and Antitumor Activity

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Benzohydroxamic acids inhibit mammalian ribonucleotide reductase and exhibit antineoplastic activity in L1210 leukemic mice. Five new hydroxy- and amino-substituted benzohydroxamic acids (3,4- and 3,5-OH, 3,4-NH_2, 2,3,4-, and 3,4,5-OH) were prepared and tested along with 12 previously reported benzohydroxamic acids (BHA) for enzyme inhibition and antitumor activity. The most potent enzyme inhibitor in this series was 2,3,4-OH-BHA (ID_{50} = 3.5 μM), which is 140 times more potent than hydroxyurea, but its toxicity limited the antitumor activity to a 30% increase in life span of L1210 bearing mice at 125 (mg/kg)/day ip for 8 days. The most effective antitumor agent in this series was 3,4-OH-BHA which prolonged the life span of L1210 bearing mice 103% at 600 (mg/kg)/day ip for 8 days.

The reductive conversion of ribonucleotides to deoxyribonucleotides is a rate-controlling reaction in the biosynthetic pathway leading to DNA formation and cell replication. The level of enzyme that catalyzes this reduction, ribonucleotide reductase, is closely correlated with cellular replication.[1] This enzyme would therefore seem to be an excellent target for the development of cancer chemotherapeutic agents. The only inhibitor of ribonucleotide reductase presently in general clinical use is hydroxyurea.[2] The disadvantage of hydroxyurea is that frequent and high doses are required in order to maintain an effective concentration for antineoplastic activity. A number of hydroxamic acids, in which the amino group of hydroxyurea was replaced by various acyl and aryl groups, had been synthesized and tested for their effect on ribonucleotide reductase and L1210 tumor bearing mice in this laboratory with the results that some hydroxy-substituted benzohydroxamic acids were the most potent inhibitors of enzyme and tumor growth.[3] In addition, a number of arylhydroxamic acids had been reported by Gale et al. to inhibit DNA synthesis.[4,5]

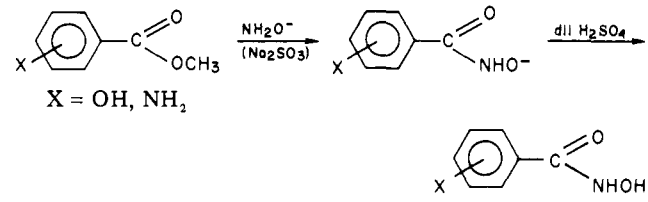
Based on these results, all mono-, di- and two of the trihydroxybenzohydroxamic acids were synthesized and tested for ribonucleotide reductase inhibition and antitumor activity in order to obtain more effective antineoplastic agents whose mode of action is inhibition of ribonucleotide reductase. Some of the corresponding aminobenzohydroxamic acids were prepared and tested as well.

Results and Discussion

Chemistry. The general method of preparing arylhydroxamic acids is the reaction of alkyl benzoates with NH_2OH solutions containing an excess of NaOH.[6] The synthesis of methyl 2,3,4-trihydroxybenzoate required the preparation of the acid from pyrogallol and sodium bicarbonate. Methylation of 2,6-dihydroxybenzoic acid with MeOH and H_2SO_4 led to different products, but methylation of the silver salt of 2,6-dihydroxybenzoic acid with methyl iodide[7] gave the desired ester. All other unavailable esters were prepared by refluxing the corresponding acids with MeOH–H_2SO_4.

The preparation of poly(hydroxy)benzohydroxamic acids was complicated by oxidation of the alkyl benzoate in alkaline medium. Rather than purging with N_2[4] which causes a loss of NH_2OH, the procedure was modified by adding Na_2SO_3 to the reaction mixture and by keeping the reaction flask covered prior to acidification (Scheme I). A summary of the properties of tested hydroxamic acids is given in Table I.

Scheme I



Biological Data. Enzyme Inhibition. A summary of the data obtained with substituted benzohydroxamic acids in tests for inhibition of ribonucleotide reductase and for antitumor acitivity is given in Table II. It should be noted that all of the compounds reported here were stronger inhibitors of ribonucleotide reductase than hydroxyurea, although a wide range of values was observed. Generally, the monosubstituted hydroxy- and aminobenzohydroxamic acids were more inhibitory than the unsubstituted benzohydroxamic acid, the substitution on the 2 or 4 position resulting in greater enzyme inhibition than the 3-hydroxy- or aminobenzohydroxamic acids. Enzyme inhibition was increased by the addition of a second hydroxy or amino group. Inhibition increased dramatically, on the order of 1 log unit, when the substituted groups were adjacent. Addition of a third adjacent hydroxyl group increased the enzyme inhibition still further, the most potent inhibitor being 2,3,4-trihydroxybenzohydroxamic acid, which was 140 times more inhibitory than hydroxyurea. The amino group may be acting in a manner similar to the hydroxy group because the amino-substituted benzohydroxamic acids follow the same pattern of inhibition as the hydroxybenzohydroxamic acids. The strongest enzyme inhibitions were obtained with 2,3,4-trihydroxy, 2,3-dihydroxy, 3,4,5-trihydroxy, and 3,4-dihydroxy substitutions in decreasing order of potency.

Antineoplastic Activity. Ribonucleotide reductase inhibition may lead to antineoplastic activity, although a direct correspondence between the two parameters would not necessarily occur due to differences in solubility, absorption, transport, metabolism, and toxicity which occur in vivo. Most of the hydroxybenzohydroxamic acids in this series prolonged the life span of mice bearing L1210

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Table I. Synthesis and Properties of Substituted Benzohydroxamic Acids

compd	subst	mp, °C	recrystn solvent	yield, %	formula	anal.
3	none	130[a]	H_2O		$C_7H_7NO_2$	
4	2-OH	177[b] dec	H_2O-MeOH		$C_7H_7NO_3$	
5	3-OH	110[c]	EtOAc-Et_2O	40	$C_7H_7NO_3 \cdot 0.25H_2O$	C, H, N
6	4-OH	179[d] dec	H_2O		$C_7H_7NO_3$	
7	2-NH_2	142[e] dec	H_2O		$C_7H_8N_2O_2$	
8	3-NH_2	151[f] dec	MeOH-pet. ether		$C_7H_8N_2O_2$	
9	4-NH_2	185[g] dec	H_2O		$C_7H_8N_2O_2$	
10	2,3-OH	218[h] dec	H_2O		$C_7H_7NO_4$	
11	2,4-OH	178[i] dec	H_2O	70	$C_7H_7NO_4$	C, H, N
12	2,5-OH	216[j] dec	H_2O		$C_7H_7NO_4$	
13	2,6-OH	215[k] dec	H_2O		$C_7H_7NO_4$	
14	3,4-OH	174 dec	EtOH-EtOAc	80	$C_7H_7NO_4$	C, H, N
15	3,5-OH	230 dec	H_2O	38	$C_7H_7NO_4$	C, H, N
16	2-OH, 4-NH_2	190[l] dec	H_2O	69	$C_7H_8N_2O_3$	C, H, N
17	3,4-NH_2	190 dec	H_2O-MeOH	51	$C_7H_9N_3O_2$	C, H, N
18	2,3,4-OH	201 dec	H_2O-MeOH	70	$C_7H_7NO_5 \cdot H_2O$	C, H, N
19	3,4,5-OH	230 dec	H_2O	66	$C_7H_7NO_5$	C, H, N

[a] E. Bamberger [*Chem. Ber.*, 33, 1786 (1900)] reported mp 126 °C. [b] A. Jeanrenaud [*Chem. Ber.*, 22, 1274 (1889)] reported mp 169 °C. [c] Reference 4 reported 43-45 °C for solvated sample. [d] Reference 4 reported mp 185-186 °C. [e] Reference 6 reported mp 149 °C. [f] Reference 4 reported mp 153-155 °C. [g] Reference 4 reported mp 197-200 °C. [h] Reference 4 reported mp 220-223 °C dec. [i] A. W. Scott and W. O. Kearse [*J. Org. Chem.*, 5, 598 (1940)] reported mp 171 °C dec. [j] Reference 5 reported mp 202 °C dec. [k] Reference 4 reported mp 221-223 °C. [l] H. Priewe and R. Rutkowski [German Patent 855 866 (1952); *Chem. Abstr.*, 52, P10184*b* (1958)] reported mp 187 °C dec.

Table II. Inhibition of Ribonucleotide Reductase and Antitumor Activity of Substituted Benzohydroxamic Acids

compd	substitut	enz inhibn.: ID_{50}, μM	T/C L1210 mice, % ± SD	dose to achieve max life span, (mg/kg)/day, for 8 days
hydroxyurea		500	162 ± 10	2500
3	none[a]	400	144 ± 16	500
4	2-OH[a]	150	147 ± 7	500
5	3-OH	350	177 ± 12	500
6	4-OH[a]	250	124 ± 2	400
7	2-NH_2	120	150 ± 11	500
8	3-NH_2	350	110 ± 18	500
9	4-NH_2	150	162 ± 18	500
10	2,3-OH	8	136 ± 11	200
11	2,4-OH	250	142 ± 7	500
12	2,5-OH	200	130 ± 14	300
13	2,6-OH	100	NT[b]	
14	3,4-OH	30	203 ± 15	600
15	3,5-OH	400	152 ± 22	1000
16	2-OH, 4-NH_2[a]	200	115 ± 5	1000
17	3,4-NH_2	40	140 ± 5	300
18	2,3,4-OH	3.5	130 ± 7	125
19	3,4,5-OH	10	153 ± 19	400

[a] Compounds 3, 4, 6, and 16 have been assigned National Cancer Screening numbers NCS 3136, 5088, 111680 and 18350, respectively. [b] NT, not tested.

leukemia. A majority of the compounds were as effective as hydroxyurea in increasing the life span but at lower concentrations than hydroxyurea. One of the newly synthesized compounds, **14**, appears to be the best antineoplastic agent of this series, with a T/C of 203%. In some cases, such as some of the 2-substituted benzohydroxamic acids, compounds **10**, **12**, and **18**, the compounds were too toxic to administer in a dose equivalent to that at which **14** was most potent. The ranges of toxicity can be seen in Table III. In the case of compound **5**, a higher in vivo response than would be expected from its in vitro data may be due in part to the greater solubility of the 3-substituted compounds. Despite these variations, however, it is significant that of all the hydroxamic acids tested in this laboratory all of those that were active in vivo were also active in vitro.

The schedule followed for administration of the drugs for purposes of comparison in this study apparently does not give an optimum increase of life span. For instance, when **19** was administered twice daily at an ip dose of 139 mg/kg per dose for 8 days, the T/C was found to depend on the interval between the two injections. At intervals of 1, 3, 5, and 7 h, the T/C values were 151 ± 17, 168 ± 13, 189 ± 13, and 172 ± 8, respectively. This is in accord with previous reports that S-phase specific drugs, including hydroxyurea, are more effective when administered repeatedly at shorter time intervals[8-10] than once a day.

Of the five new benzohydroxamic acids reported here, 3,4- and 3,5-dihydroxy, 3,4-diamino-, and 2,3,4- and 3,4,5-trihydroxybenzohydroxamic acid all inhibit ribonucleotide reductase to a greater extent than does hydroxyurea. The trihydroxybenzohydroxamic acids, which are the most potent ribonucleotide reductase inhibitors, represent a group of compounds which has never been synthesized before. Four of the newly synthesized compounds were among the five strongest inhibitors in this series. Of those compounds in this series which had been synthesized previously, only 2,3-dihydroxybenzohydroxamic acid exhibited the same range of enzyme inhibition. Several of the new drugs were also effective antineoplastic agents against L1210 leukemia. The life span of L1210 leukemic mice was increased by 3,4-dihydroxybenzo-

Table III. Antitumor Activity of Some Substituted Benzohydroxamic Acids at Various Dosages

compd	substitut	T/C L1210 mice, % ± SD	(mg/kg)/day for 8 days
5	3-OH	123 ± 6.4	200
		177 ± 12	500
		156 ± 17	600
		148 ± 15	700
		68 ± 18	1000
10	2,3-OH	136 ± 11	200
		107 ± 8	300
		103 ± 5	450
		100 ± 16[a]	500
14	3,4-OH	125 ± 9	100
		156 ± 9	300
		154 ± 8	400
		184 ± 13	500
		203 ± 15	600
		28 ± 2[b]	800
18	2,3,4-OH	111 ± 8	63
		109 ± 15	75
		130 ± 7	125
		108 ± 14	200
		92 ± 18	300
		46 ± 6	320
19	3,4,5-OH	125 ± 10	100
		147 ± 4	200
		153 ± 4	400
		56 ± 10	800

[a] Administration of drug was discontinued after 3 days because of noted toxicity. [b] Administration of drug was discontinued after 2 days because of noted toxicity.

hydroxamic acid nearly twice as much as by hydroxyurea at a concentration that was one-tenth that of hydroxyurea on a molar basis.

Experimental Section

The uncorrected melting points were determined in open capillary tubes with a Thomas-Hoover melting point apparatus. The elemental analyses were performed by Atlanta Microlabs, Inc., Atlanta, Ga., and by Micro-Tech Laboratories, Skokie, Ill. All results were within ±0.4% of the theoretical values. Confirmation of structures was obtained by ^{1}H NMR spectra on a Hitachi Perkin-Elmer R24 spectrometer. Previously reported hydroxamic acids were identified by melting or decomposition points and by equivalent weight determined by titration with 0.10 M NaOH, using a recording pH meter (Heath, Model EU 301V).

Chemical. 2,3,4-Trihydroxybenzoic Acid. The only commercially unavailable substituted benzoic acid, 2,3,4-trihydroxybenzoic acid, was prepared by heating pyrogallol (20 g) with $NaHCO_3$ (30 g) in a mixture of 30 mL of H_2O and 20 mL of mesitylene to boiling in a covered flask for 1 h. The suspension was acidified slowly with HCl. The precipitated acid weighed 6.9 g after drying: equiv wt 171; mp 220 °C dec (lit.[11] 221 °C).

Methyl Benzoate Esters. The Me ester of 2,3,4-trihydroxybenzoic acid was prepared by heating the acid with MeOH–H_2SO_4, mp 151 °C (lit.[12] 153 °C). The Me ester of 2,6-dihydroxybenzoic acid was synthesized by first preparing the Ag salt of the acid and then treating with MeI, mp 67 °C (lit.[13] 67–68 °C).

Methyl benzoates with substitutions, 2-, 3-, 2,3-, 2,5-, 3,4-, and 3,4,5-OH and 3-, 4-, and 3,4-NH_2, were prepared in more than 60% yield by refluxing 0.10 mol of the corresponding acid for 24 h with 100 mL of MeOH containing 2% (v/v) H_2SO_4. Extra H_2SO_4 was added to the aminobenzoic acids to form their bisulfate salts. After evaporation of MeOH under reduced pressure, the residues were suspended in water, and the esters were isolated according to published procedures. Melting points of the substituted methyl benzoates: 3-OH 68 °C (lit.[14] 69 °C); 2,3-OH 80–83 °C (lit.[15] 80–81 °C); 2,5-OH 83–84 °C (lit.[16] 85–86 °C); 3,4-OH 135–136 °C (lit.[17] 134.5–135 °C); 3,4,5-OH 201 °C (lit.[18] 201 °C); 3-NH_2·HCl salt 207 °C (lit.[19] 202 °C); 3,4-NH_2 106–107 °C (lit.[20] 108–109 °C); 2-OH, 4-NH_2 121 °C (lit.[21] 121 °C).

Hydroxamic Acids. The hydroxamic acids were prepared from the corresponding esters under the following conditions. Sodium hydroxide (0.5 mol) in 25% aqueous solution was added slowly to a mixture of $(NH_2OH)_2$·H_2SO_4 (0.1 mol) and 100 g of ice. Then, 2 g of Na_2SO_3 and 0.10 mol of the ester were added. The mixture was stirred at room temperature in the covered flask until the ester had dissolved and then left overnight at 45 °C or for 2 days at room temperature. The solution was acidified with 25% H_2SO_4 to pH 6.0 while being cooled. Some of the hydroxamic acids precipitated at this point. In all cases, the aqueous solvent was evaporated under reduced pressure, the residue was extracted with hot MeOH and filtered, and the MeOH was then evaporated. Recrystallization of both the initial precipitate and the residue of the MeOH extract was generally done from hot H_2O after treatment with carbon, except for **4**, **5**, **8**, **14**, **17**, and **18** as indicated in Table I.

Biological. In Vitro. Ribonucleotide reductase was partially purified from Novikoff hepatoma by a procedure similar to that outlined previously.[22] The activity of the enzyme was assayed by measuring the conversion of CDP to dCDP by the use of a slightly modified assay procedure originally developed by Reichard et al.[23] The assay mixture (0.34 mL) contained 3 μCi of [^{3}H]CDP (specific activity 14–19 Ci/mmol), 3.3 mM ATP, 5.9 mM magnesium chloride, 8.8 mM Hepes buffer (pH 7.5), 15 mM dithiothreitol, and enzyme protein between 0.4 and 1.3 mg. Incubation occurred for 40 min at 30 °C. The inhibitors were dissolved in water or in a mixture of water and up to 1% ethanol or 2% dimethyl sulfoxide, which were not inhibitory at these concentrations.

Each inhibitor was tested at a minimum of three concentrations. The active compounds were reassayed at least one additional time. ID_{50} (μM), that is, the concentration of drug which reduces by 50% the observed activity of the enzyme in controls, was estimated from graphs summarizing the results for each compound.

In Vivo. L1210 leukemia cells were propagated in DBA/2 mice. Diluted ascitic fluid (0.10 mL, 10^5 cells) was administered ip to female C6D2F_1 mice, weighing about 20 g at day 0. The life span of eight control mice varied from 7 to 9 days. Test agents were given ip once a day for 8 days, starting on day 1. Compounds were generally tested at three doses in the range of 100–1000 mg/kg, each dose in 8 mice. Other dose levels were given dependent upon toxicity in screening tests and in follow-up experiments.

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Synthesis and Biological Activity of 5-Fluoro- and 5-Methyl-1,3-oxazine-2,6(3*H*)-dione

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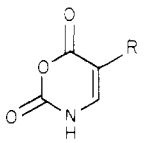
5-Fluoro-1,3-oxazine-2,6(3*H*)-dione (3-oxa-FU) was synthesized by reacting 3-oxauracil with fluoroxytrifluoromethane and decomposing the adduct in the presence of a catalytic amount of Et_3N. 5-Methyl-1,3-oxazine-2,6(3*H*)-dione (3-oxathymine) was prepared by polyphosphoric acid catalyzed ring closure of β-(*N*-ethoxycarbonylamino)-2-methacrylic acid and by treatment of citraconimide with sodium hypochlorite. As determined in vitro, 3-oxa-FU was markedly inhibitory to *S. faecium* ($ID_{50} = 9 \times 10^{-8}$ M) and *E. coli* ($ID_{50} = 1 \times 10^{-7}$ M) but was less active against leukemia L-1210 cells ($ID_{50} = 1 \times 10^{-5}$ M). At 1×10^{-4} M, 3-oxathymine was inactive in these cell systems. Inhibition of the growth of *S. faecium* by 3-oxa-FU was reversed competitively by the natural pyrimidines. The relatively rapid hydrolysis of the compounds in the growth media is a major factor in determining their biological effectiveness.

The isosteric replacement of the nitrogen atoms of the pyrimidine ring has been one of the approaches employed for obtaining potentially useful antitumor agents. One such modification, the replacement of nitrogen with oxygen, has been actively explored in recent years. Although 1,3-oxazine-2,6(3*H*)-dione (3-oxauracil) was first prepared by Rinkes, in 1927, by sodium hypochlorite oxidation of maleimide,[1] information on its biological activity became available only in 1972, when we reported the synthesis of 3-oxathymine and the in vitro inhibitory activity of these compounds against various microbial and tumor cell lines.[2] In the same year, Washburne et al. published a new synthesis of the agent, involving the reaction of maleic anhydride with trimethylsilyl azide.[3] In 1973, Skoda et al. demonstrated the inhibition of *E. coli* growth by 3-oxauracil,[4] and we reported the preparation of 3-oxa-2′-deoxyuridine, which is a much more potent inhibitor of cell growth than is the aglycon.[5] The synthesis of the ribonucleoside derivative of 3-oxauracil was published in 1974 by Chwang and Heidelberger,[6] and in a preliminary communication we presented the synthesis of the 5-fluoro derivative of 3-oxauracil.[7] Subsequently, the synthesis of 3-oxauracil variously substituted with halo, alkyl, and aryl groups was reported by Warren et al.,[8] McMillan and Washburne,[9] Farkas et al.,[10] and by Washburne and Park.[11]

Chemistry. The synthesis of 5-fluoro-1,3-oxazine-2,6(3*H*)-dione (I) was carried out by reacting 1,3-oxazine-2,6(3*H*)-dione dissolved in anhydrous acetone with fluoroxytrifluoromethane at −55 to −60 °C. The rapid disappearance of UV absorption at 260 nm indicated the formation of an adduct, as was previously reported to occur upon the interaction of this reagent with pyrimidine bases and nucleosides.[12]



I, R = F
II, R = CH_3

The attempted decomposition of the intermediate by procedures used in the synthesis of 5-fluoropyrimidines[13,14] led to the formation of decomposition products. This difficulty was overcome by adding catalytic amounts of Et_3N to the reaction mixture. When markedly greater amounts of catalyst were used, even under exclusion of protic solvents, the formation of I could not be detected by TLC.

Table I. Comparative Biological Effects of Some 1,3-Oxazines

compd	molar concn for 50% inhibn of the growth of		
	leuk L-1210	*S. faecium*	*E. coli*
5-fluoro-3-oxauracil	1×10^{-5}	9×10^{-8}	1×10^{-7}
3-oxauracil	4×10^{-5}	2×10^{-5}	4×10^{-7}
3-oxathymine	$>10^{-4}$	$>10^{-4}$	3×10^{-4}

Table II. Relative Effectiveness of Natural Pyrimidines in Preventing the Inhibition of *S. faecium* Growth Caused by 3-Oxa-5-fluorouracil

metabolite	inhibn index $([I]/[S]_{50})^a$
thymine	120
thymidine	110
2′-deoxyuridine	100
uracil	90
uridine	90
2′-deoxycytidine	10
cytidine	8
cytosine	7

[a] [I]/[S] for 50% growth inhibition at substrate concentrations ranging from 10^{-3} to 10^{-6} M.

The thymine analogue, 5-methyl-1,3-oxazine-2,6(3*H*)-dione (II) was prepared by polyphosphoric acid catalyzed ring closure of β-(*N*-ethoxycarbonylamino)-α-methacrylic acid at 75–80 °C or by treatment of the citraconimide with sodium hypochlorite at 0–5 °C.

Biology. To eliminate biologically active degradation products, e.g., halogenoacetaldehyde,[15] that may have formed upon storage of the relatively labile 3-oxa-FU, the compound was recrystallized from ethyl acetate–methylene chloride each time before its use. Under these conditions, 3-oxa-FU was found to be an effective inhibitor of bacterial cell growth, but it was only moderately active against leukemia L-1210 cells (Table I). In contrast, 3-oxauracil

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