# Potential Antileukemic and Immunosuppressive Drugs. II. Further Studies with Benzo-2,1,3-oxadiazoles (Benzofurazans) and Their N-Oxides (Benzofuroxans)

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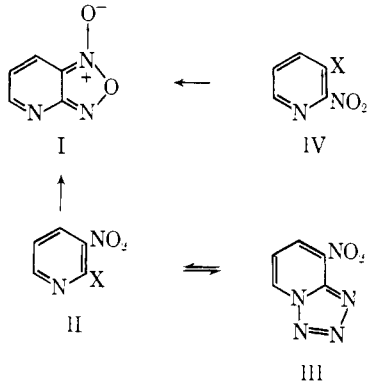
The synthesis of pyrido[2,3-*c*]furoxan and some novel amino- and hydroxybenzofurazans is described. The activity of these compounds as inhibitors of protein and nucleic acid biosynthesis *in vitro* in lymphocytes was determined.

An earlier paper[1] described the preparation and *in vitro* biological activity of some benzo-2,1,3-oxadiazoles (benzofurazans) and their N-oxides (benzofuroxans). Compounds with the 4-nitro substituent were the most potent of those investigated, acting on intracellular thiols,[2,3] and showed some selective action in preferentially inhibiting lymphocytes (potential immunocytes) when tested on a number of animal cells in culture and microorganisms.[3]

We have now prepared and tested *in vitro* pyrido[2,3-*c*]furoxan (I) to determine if the ring-bound nitrogen atom would simulate the effect of a nitro group in these novel drugs.

We also prepared and tested some hydroxybenzofurazans which, because of their structure and acidity,[4] might pharmacologically mimic the substituted phenols, some of which are powerful uncoupling agents[5] (*i.e.*, inhibitors of mitochondrial ATP biosynthesis) and therefore inhibitors of polymer biosynthesis. Some 7-substituted 4-nitrobenzofurazans (and their N-oxides) were also examined for their drug activity *in vitro*.

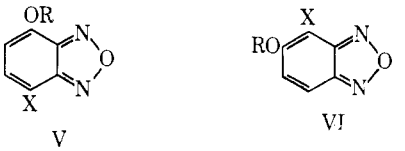
**Preparation of Compounds.**—Pyrido[2,3-*c*]furoxan was successfully prepared by pyrolysis of 8-nitropyridotetrazole (III) as described by Boyer and coworkers.[6]



I IV II III

However, the reaction is accompanied by extensive charring, and we therefore sought alternative routes to I. 3-Azido-2-nitropyridine (IV, X = $N_3$) merited attention since this intermediate could not form a tetrazole, unlike its isomer (II, X = $N_3$). However, attempts to prepare IV (X = $N_3$) have been unsuccessful to date mainly because of the lability of the 2-nitro group induced by the pyridine nitrogen. Thus, treatment of 3-methoxy-2-nitropyridine[7] (IV, X = $OCH_3$) with $NaN_3$ in DMSO afforded 8-methoxypyridotetrazole in place of the expected 3-azido-2-nitropyridine. With $NH_3$ in a sealed tube the methoxy group was replaced to yield 3-amino-2-nitropyridine[8] (IV, X = $NH_2$), while attempts to convert the amino group to azido by treating the diazonium solution with aqueous $NaN_3$ afforded 3-azido-2-pyridone.

The 4-methoxy- (V, R = Me; X = H) and 5-methoxybenzofurazans (VI, R = Me; X = H) were prepared by published methods[9] and demethylated to obtain the corresponding hydroxy compounds.[9] Nitration and bromination of the hydroxy- and methoxybenzofurazans is described in detail in the Experimental Section. Substituted aminobenzofurazans were prepared as described.[10]



V VI

**Biological Activity.**—Table I shows that the most acidic hydroxybenzofurazan and analog of 2,4-dinitrophenol (a powerful uncoupling agent[5]) had no effect on polymer biosynthesis in lymphocytes, although these biosynthetic processes were sensitive to dinitrophenol. The simple methoxy derivatives were almost devoid of activity toward these lymphocytes (derived from two lymphoid tissues) even though the 5-methoxybenzofurazan is a fairly effective inhibitor of metabolism in circulating lymphocytes[2] (from sheep and rat lymph). 5-Hydroxybenzofurazan showed significant drug activity *in vitro*, but the 4-hydroxy isomer was consistently less potent. None of these phenolic derivatives displayed drug activity comparable with 4-nitrobenzofurazan[2] and showed negligible activity in reacting with thiols *in vitro*.

The pyridine analog (**1**) of benzofuroxan was only a feeble inhibitor of uridine incorporation into lymphocytes (25% inhibition at 0.25 m*M*) and therefore compared rather unfavorably with 4-nitrobenzofuroxan as a drug *in vitro*,[2,3] probably because of the greater hydrophilic character of I. It exhibited approximately the same activity as 4-nitrobenzofurazan and N-ethylmale-

(1) To whom inquiries should be addressed at the Department of Medicinal Chemistry, Riker Research Laboratories, Thornleigh, N.S.W. Australia.
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Table I

Drug Activity of Some Hydroxybenzofurazan Derivatives on Polymer Biosynthesis in Chicken Bursacytes (B) and Rabbit Thymocytes (T)

| | | | % inhib of incorp of radioactivity from | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Amino acids-$^{14}$C | | Uridine-5-$^3$H | | Thymidine-6-$^3$H | |
| Benzofurazan | p$K_a$ | Concn, $\mu M$ | B | T | B | T | B | T |
| 5-$CH_3O$- | | 200 | 25 | 5 | 15 | 0 | 25 | 25 |
| 4-Br-5-MeO- | | (200)[a] | 20 | 5 | 15 | 5 | 10 | 10 |
| 4-Br-5-MeO-, N-oxide | | (200)[a] | 0 | 0 | 30 | 10 | 40 | 25 |
| 4-MeO- | | 200 | 25 | 0 | 30 | 10 | 0 | 5 |
| 4-MeO-7-$NO_2$- | | 50 | | 65 | | 60 | | 80 |
| 4-OH- | 6.8 | 200 | 10 | 10 | 20 | 20 | 10 | 30 |
| 4,6-($NO_2$)$_2$-7-OH- | 1.4 | 100 | 0 | 10 | 20 | 10 | 0 | 10 |
| 5-OH- | 7.3 | 200 | 70 | 50 | 40 | 60 | 50 | 65 |
| 4-Br-5-OH- | 5.2 | 200 | 70 | 45 | 62 | 70 | 85 | 60 |
| 4-$NO_2$-7-(*p*-chloranilino)[b] | | 10 | 45 | | 55 | | 80 | |
| 2,4-Dinitrophenol | 4.0 | 100 | 90 | 95 | 80 | 75 | 85 | 80 |
| 4-Nitrobenzofurazan | | 5 | 70 | 35 | | | 50 | 50 |

[a] In saturated solution (<200 $\mu M$). [b] Also see Table II.

imide in reacting with aliphatic thiols (see Experimental Section). Benzofuroxan itself at 0.5 m$M$ had no effect (see also ref 1).

Some of the 7-amino derivatives of 4-nitrobenzofurazan and its N-oxide were very effective inhibitors of polymer biosynthesis, their activity being structure dependent (Table II). Thus, the isomeric 5-amino deriv-

Table II

Drug Activity of Some Amino-4-nitrobenzofurazans Assayed Using Chicken Bursacytes (*in Vitro*)

| 4-Nitro- benzofurazan | Concn, $\mu M$ | % inhib of thymidine-$^3$H incorpn |
|---|---|---|
| 7-Piperidino- | (<200)[a] | 15 |
| 7-Piperidino-, N-oxide | 200 | 45 |
| 7-Morpholino- | (<200)[a] | 10 |
| 7-Morpholino-, N-oxide | 200 | 75 |
| 7-(*p*-Methoxyanilino)- | 50 | 80 |
| 5-(*p*-Chloranilino)- | 50 | 5 |
| 7-(*p*-Chloranilino)-[b] | 5 | 70 |
| 7-(*p*-Chloranilino)-, N-oxide | 5 | 60 |

[a] In saturated solution. [b] Also see Table I.

atives were virtually inactive and compounds with aliphatic amino substituents (*e.g.*, NHMe, $NMe_2$) at C-7 were less potent than the 7-anilino compounds.[2] The drug activity of these potent aniline derivatives was not abolished by preincubation with aliphatic thiols (twentyfold excess of 2-mercaptoethanol, cysteine, and its ethyl ester or glutathione) which distinguishes them from the parent compound 4-nitrobenzofurazan.[2] These compounds merit further investigation.

## Experimental Section

Analyses were performed by Dr. Joyce Fildes and her staff at the Australian National University, Canberra, Australia.

**8-Methoxypyridotetrazole.**—3-Methoxy-2-nitropyridine[6] (IV, X = OMe) (1.54 g) and $NaN_3$ (0.7 g) in 20 ml of DMSO were heated together at 165–170° for 5 min. The clear red solution was cooled to room temperature, diluted with 100 ml of $H_2O$, and adjusted to pH 3.0 with 5 $N$ HCl. Extraction with AcOEt, drying ($MgSO_4$), and solvent removal afforded the tetrazole (0.6 g, 40%) as light tan prisms from $H_2O$. Sublimation (100°, 0.2 mm) gave white prisms, mp 179–181° dec. *Anal.* ($C_6H_6N_4O$) C, H, N.

**3-Azido-2-pyridone.**—3-Amino-2-nitropyridine[8] (3.0 g) in 30 ml of glacial AcOH was stirred into nitrosylsulfuric acid (made from 3 g of $NaNO_2$ and 20 ml of concentrated $H_2SO_4$) at 0°. After stirring 3 min the solution was poured into 150 g of crushed ice and added with shaking to a solution of $NaN_3$ (5 g) in 100 ml of $H_2O$. The resulting yellow solution was extracted (AcOEt) and dried ($MgSO_4$), and the solvent was evaporated under reduced pressure (200 mm) to yield after two recrystallizations from AcOEt (charcoal) the pyridone (0.5 g, 17%) as light-sensitive plates, mp 130° dec. *Anal.* ($C_5H_4N_4O$) C, H, N.

**Dinitration of 4-Hydroxy- and 4-Methoxybenzofurazans.**—The benzofurazan (V, X = H; R = H or Me) (0.001 mole) in 3 ml of 98% $H_2SO_4$ was cooled to 0° and treated with 0.21 g (0.0021 mole) of $KNO_3$ in 2 ml of 98% $H_2SO_4$. After standing at 20° for 15 min, the mixture was heated to 50° for 5 min, cooled, and poured into 35 g of crushed ice. The precipitate was collected (water-soluble hydroxy derivative extracted with AcOEt), washed ($H_2O$), and crystallized. **5,7-Dinitro-4-hydroxybenzofurazan** (60%) separated from AcOEt–petroleum ether (bp 40–60°) (1:2) as tan prisms, mp 150° dec. *Anal.* ($C_6H_2N_4O_6$) C, H, N.

**5,7-Dinitro-4-methoxybenzofurazan** (53%) crystallized from EtOH as pale yellow prisms, mp 147–148°. *Anal.* ($C_7H_4N_4O_6$) C, H, N.

**5-Hydroxy-4-nitrobenzofurazan (VI, X = $NO_2$; R = H).**—5-Methoxy-4-nitrobenzofurazan[11] (VI, X = $NO_2$; R = Me) (0.1 g) was refluxed for 10 min with 5 ml of HBr (48%). Dilution with $H_2O$ (10 ml) and extraction with AcOEt yielded, after recrystallization from 95% EtOH, the hydroxy compound (65%) as white needles, mp 187–188°. *Anal.* ($C_6H_3N_3O_4$) C, H, N.

**4-Bromo-5-methoxybenzofuroxan.**—5-Methoxybenzofuroxan[11] (0.83 g, 0.005 mole) in 20 ml of AcOH was treated dropwise at 15° with 1 g of $Br_2$ in 10 ml of AcOH. After standing 30 min at 15°, the yellow solution was diluted with 100 ml of ice water, and the yellow precipitate was collected and twice crystallized (EtOH) to yield the bromo compound (90%), mp 157–158° (lit.[12] 157.4–157.8°). Identical procedures were used to prepare the 4-bromo-5-methoxy-[13] and 4-bromo-5-hydroxybenzofurazans.[13]

**Biological Testing.**—Lymphocytes were drawn from the bursa of Fabricius (10–14-week-old chickens) or from thymus tissue (8–12-week-old rabbits, 150-g rats, or 2-month-old puppies) in Hank's medium, isolated by slow centrifugation, and washed once again with Hank's medium. These cells (3–6 × $10^7$/ml) were incubated with radioactive precursors and the compounds to be tested were added from solutions in DMF or THF (final concentrations of these solvents, 1% v/v) in Hank's medium–0.1 $M$ sodium phosphate, pH 7.4 (4:1 v/v), for 40 min at 37°. Radioactivity incorporated into polymers from 0.1 $\mu$Ci of uridine-5-$^3$H or thymidine-6-$^3$H/ml or from 0.01 $\mu$Ci of amino acids-$^{14}$C (algal protein hydrolysate)/ml was determined as described.[2]

Data for percentage inhibition given in Table I was obtained

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from (at least) duplicate incubations at a given drug concentration.

**Thiol-Neutralization Assay.**—Solutions of drugs in DMF (0.2 ml) were mixed with 0.5 ml of 0.4 mM solutions of 2-mercaptoethanol, cysteine hydrochloride, or glutathione in 0.1 *M* sodium phosphate, pH 7.4. After standing for 2 min at 25°, residual thiol was measured by the coloration produced (and read immediately at 412 mμ) on adding excess Ellman's reagent [5,5′-dithio(2-nitrobenzoic acid)] in 0.1 *M* sodium phosphate, pH 7.4. Appropriate blanks were established with drugs and thiol and drugs and Ellman's reagent. Relative thiol-blocking activity was determined as the molar ratio (drug:thiol) to neutralize 50% of the thiol, using N-ethylmaleimide as reference.

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# [3-(2-Mercaptoethylamino)propyl]oxamide and Related Compounds as Potential Antiradiation Agents[1]

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Thiols and the corresponding hydrogen thiosulfate esters were prepared as potential radioprotective agents from [ω-(1-aziridinyl)alkyl]oxamides by ring-opening reactions. Of 18 such compounds prepared, only [3-(2-mercaptoethylamino)propyl]oxamide (**6a**) showed considerable radioprotective activity in mice.

In the course of a continuing search for superior antiradiation agents through modifications of 2-aminoethanethiol, appropriate ring openings of the known[2] N,N′-bis[3-(1-aziridinyl)propyl]oxamide (**2b**) were effected as an entry into the area of 2-(ω-acylaminoalkylamino)ethanethiols and related compounds. The terminal substituent in this case is an oxamoyl group, and the resulting products were N,N′-bis[3-(2-mercaptoethylamino)propyl]oxamide (**3b**) dihydrochloride and the corresponding bis(hydrogen thiosulfate) (**3c**). As shown in Scheme I and described in the Experimental Section, variations of the general reaction sequence led to other oxamide derivatives (**3a**, **b**, **e** and **6a–p**). Such compounds are, in effect, oxamoylated analogs of the recently described S-2-(ω-aminoalkylamino)ethyl dihydrogen phosphorothioates, which showed an exceptionally high level of radioprotective activity.[3] Ring-opened products were limited, however, to thiols and the corresponding hydrogen thiosulfate esters, since, as an example, the treatment of [3-(1-aziridinyl)propyl]oxamide (**5a**) with $Na_3SPO_3$ in $H_2O$ in the presence of 2 molar equiv of AcOH resulted in the isolation of an impure dihydrogen phosphorothioate ester.

The preparation of N-[3-(1-aziridinyl)propyl]-N′-methyloxamide (**5c**) from ethyl [3-(1-aziridinyl)propyl]-oxamate (**4**) is an exception to the general route and was followed after difficulties had been encountered in the separation of the required intermediate, ethyl methyloxamate, from N,N′-dimethyloxamide following the reaction of diethyl oxalate with $MeNH_2$. Analytically pure N-[3-(1-aziridinyl)propyl]-N′-cyclohexyloxamide (**5d**) was obtained by the alternative route, *i.e.*, the reaction of **4** with cyclohexylamine, although the general route was also effective. Hydrogen thiosulfate esters were prepared by aziridine-ring openings with either $Na_2S_2O_3$ and AcOH[4] or $(NH_4)_2S_2O_3$.[3,5] The thiol **6p** hydrochloride was not obtained pure but was converted into pure [3-(2-phenyl-3-thiazolidinyl)propyl]-oxamic acid 2-phenylhydrazide (**7**) with benzaldehyde.

[3-(2-Mercaptoethylamino)propyl]oxamide (**6a**) hydrochloride was the only end product among those described here that showed appreciable radioprotective activity in mice in tests carried out at the Walter Reed Army Institute of Research, Washington, D. C.[6] The approximate $LD_{50}$ dose of **6a** was 700 mg/kg; a dose of 400 mg/kg of **6a** administered intraperitoneally 30 min prior to irradiation (1000 R, γ rays) gave 53% survival as compared to 0% among untreated control mice, and a dose of 200 mg/kg gave 40% survival. All the other thiols and thiosulfates tested were nonprotective with the exception that the thiosulfate **6b** and the thiol **6c** gave slight protection at a high dose level relative to the respective $LD_{50}$ dose.

## Experimental Section[7]

**1-(2-Aminoethyl)aziridine** (**1a**), bp 126°, was prepared from 2-(2-aminoethylamino)ethanol (1.0 mole) in 17% yield by a published procedure[8] (lit.[8] bp 126–127.5°). On a larger scale (4.8 moles of the alcohol) rearrangement of **1a** to piperazine was predominant, and the yield of **1a** was only 1%.

**N,N′-Bis[ω-(1-aziridinyl)alkyl]oxamides** (**2**) were prepared by the method reported by Bestian[2] for the preparation of **2b**. A solution of diethyl oxalate (7.30 g, 50.0 mmoles) in EtOAc (10 ml) was added slowly to a stirred solution of 100 mmoles of the appropriate aziridine (**1a**, **1b**,[2,3] **or 1c**[3]) in EtOAc (50 ml). The mixture was allowed to stand at 25° for 3 hr and was then refrigerated. The crystalline product was collected and washed with EtOAc: **2a** (mp 159–160°) was obtained in 84% yield; **2b** (mp 142°, lit.[2]

(1) This investigation was supported by the U. S. Army Medical Research and Development Command under Contract No. DA-49-193-MD-2028.

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(7) All analytical samples were dried *in vacuo* over $P_2O_5$ at room temperature unless another temperature is specified. Melting points were determined with a Kofler Heizbank unless noted otherwise. Where analyses are indicated only by symbols of the elements, analytical results obtained for those elements were within 0.4% of the theoretical values.

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