Synthesis and Biological Activity of 7,8-Disubstituted Isoalloxazines. Synthesis of Nitrogen Mustard Derivatives[1]

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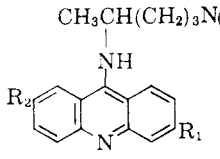
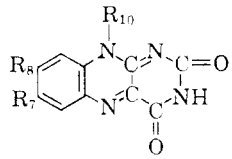
Two basically substituted isoalloxazines, 7,8-dimethyl- and 7,8-dichloro-10-{2-[bis(2-hydroxyethyl)amino]-ethyl}isoalloxazine hydrochloride, have been synthesized. The 2-[bis(2-hydroxyethyl)amino]ethyl substituent provides a terminal hydroxyl group at a distance from the isoalloxazine nucleus comparable to that of the 5′-hydroxyl group of riboflavin. Both compounds are biologically inert for *Lactobacillus casei* but the former is a reversible inhibitor of riboflavin in the rat. The 2-[bis(2-chloroethyl)amino]ethyl derivatives of the two basically substituted isoalloxazines were synthesized successfully only from the diol free bases. 2-Nitro-4,5-dimethylchlorobenzene or 4,5-dichloro-1,2-dinitrobenzene and 2-[bis(2-hydroxyethyl)amino]ethylamine were heated with pyridine at 150° for 10 hours to yield 2-nitro-4,5-dimethyl- (49%), and 2-nitro-4,5-dichloro-N-2-[bis(2-hydroxyethyl)amino]ethylaniline (79%), respectively. The nitro anilides were reduced to phenylenediamines which, as the dihydrochlorides, were condensed with alloxan monohydrate to yield 7,8-dimethyl- (43%), and 7,8-dichloro-10-{2-[bis(2-hydroxyethyl)amino]ethyl}isoalloxazine (13%), respectively. The free base of the 7,8-dimethyl- or the 7,8-dichloroisoalloxazine was refluxed with thionyl chloride to yield 7,8-dimethyl- (91%), and 7,8-dichloro-10-{2-[bis(2-chloroethyl)amino]ethyl}isoalloxazine hydrochloride (87%), respectively. Molar extinction coefficients are given for the two diol isoalloxazines. Routine procedures were used for the biological evaluation of these materials.

Quinacrine (I) and riboflavin (III) bear a structural[2] resemblance. This had prompted the synthesis of



I, R_1 = Cl, R_2 = OCH_3, R_3 = H
II, R_1 = Cl, R_2 = OCH_3, R_3 = Cl



III, R_7 = R_8 = CH_3, R_{10} = $-CH_2(CHOH)_3CH_2OH$[3]

several basically substituted isoalloxazines[2b,4] as potential antimalarial drugs. None possessed antimalarial activity nor did they show any antagonism toward riboflavin. Certain basically substituted 7,8-dichloroisoalloxazines[5] are weak inhibitors of D-amino acid oxidase,[5a] an enzyme which requires flavin adenine dinucleotide as a prosthetic group.

All the isoalloxazines, except one, which have a basic substituent at position 10 have a dialkylaminoalkyl side chain. The only exception is a derivative synthesized by Adams, *et al.*,[2b] which has a single hydroxyl group on the second carbon of the side chain. The absence of a hydroxyl group on the side chain at a distance comparable to the terminal hydroxyl group of riboflavin, which is required for the formation of the two biologically essential coenzymes, may be a reason why these basically substituted analogs of riboflavin are inactive.

Two basically substituted isoalloxazines, 7,8-dimethyl-10-{2-[bis(2-hydroxyethyl)amino]ethyl}-isoalloxazine hydrochloride (IV) and 7,8-dichloro-10-{2-[bis(2-hydroxyethyl)amino]ethyl}isoalloxazine hydrochloride (V), have been synthesized and tested for their biological activity for *L. casei* and IV has been tested in the rat. Both isoalloxazines have terminal hydroxyl groups as is the case for riboflavin. Further, Godfrey molecular models[6] of the D-ribityl and the 2-[bis(2-hydroxyethyl)amino]ethyl side chains show that they may assume comparable steric conformations.

Compounds IV and V were suitable precursors for the synthesis of bis(2-chloroethyl)amino (nitrogen mustard) analogs of riboflavin. The isoalloxazine mustards may be considered analogous to the quinacrine mustards[7] (*e.g.*, II) which were synthesized to take advantage of the favorable distribution of the antimalarial drugs to the nucleus of cells.[8]

In view of these structural similarities and also because nitrogen mustard analogs related to only one vitamin have been synthesized,[9] we undertook the synthesis of two nitrogen mustard analogs; 7,8-dimethyl-10-{2-[bis(2-chloroethyl)amino]ethyl}isoalloxazine hydrochloride (VI) and 7,8-dichloro-10-{2-[bis(2-chloroethyl)amino]ethyl}isoalloxazine hydrochloride (VII).

The synthesis of compounds IV and V as the hydrochlorides was achieved *via* the nucleophilic substitution reaction of 2-nitro-4,5-dimethylchlorobenzene (VIII) or 4,5-dichloro-1,2-dinitrobenzene (IX) with 2-[bis(2-hydroxyethyl)amino]ethylamine (X) to give the intermediate nitro anilides XI and XII which were isolated and characterized. The nitro anilides were reduced to the phenylenediamines, which were not isolated, but were converted to their hydrochloride salts and con-

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(2) (a) J. Madinaveitia, *Biochem. J.*, **40**, 373 (1946); (b) R. R. Adams, C. A. Weisel, and H. S. Mosher, *J. Am. Chem. Soc.*, **68**, 883 (1946).

(3) The numbers used in structure III are the same as the numbers used for the systematic nomenclature of isoalloxazines by *Chem. Abstracts*.

(4) (a) H. Burkett, *J. Am. Chem. Soc.*, **69**, 2555 (1947); (b) F. Kipnis, N. Weiner, and P. E. Spoerri, *ibid.*, **69**, 799 (1947); (c) F. E. King and R. M. Acheson, *J. Chem. Soc.*, 681 (1946).

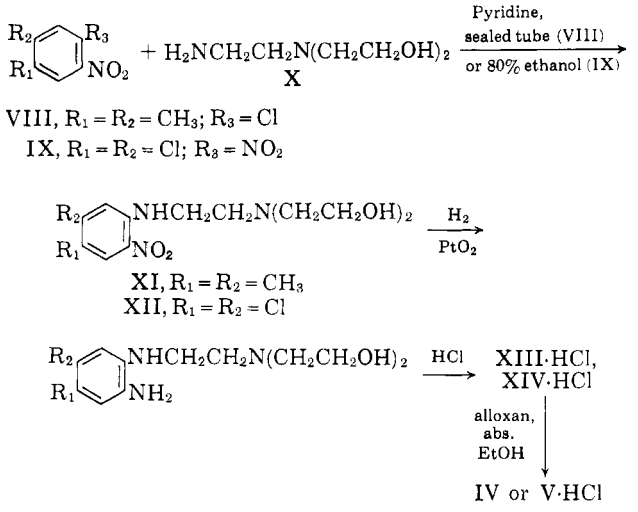
(5) (a) R. B. Barlow, *ibid.*, 2225 (1951); (b) R. B. Barlow and H. R. Ing, *ibid.*, 713 (1950).

(6) J. C. Godfrey, *J. Chem. Educ.*, **36**, 140 (1959).

(7) (a) R. M. Peck, R. K. Preston, and H. J. Creech, *J. Am. Chem. Soc.*, **81**, 3984 (1959); (b) R. M. Peck, R. K. Preston, and H. J. Creech, *J. Org. Chem.*, **26**, 3409 (1961).

(8) R. Jones, Jr., U. Jonsson, M. Browning, H. Lessner, C. C. Price, and A. K. Sen, *Ann. N. Y. Acad. Sci.*, **68**, 1133 (1958).

(9) C. C. Stock, S. M. Buckley, K. Sugiura, and C. P. Rhoads, *Cancer Res.*, **11**, 432 (1951), report that three "one-arm" mustards and two "two-arm" mustards related to the structure of pyridoxine were synthesized for them by Drs. M. Tishler and Evelyn H. Wilson of the Merck Company.



densed with alloxan monohydrate to form the flavins.[10]

Basically substituted isoalloxazines have been synthesized previously[2b,4,5] by two different procedures: (A) by the reaction of a substituted phenylenediamine with alloxan monohydrate in glacial acetic acid using boric acid as a catalyst and (B) by the reaction of the phenylenediamine hydrochlorides with alloxan monohydrate in alcoholic solution. When procedure A was used for the synthesis of IV, a mixture of products was obtained from which IV could be obtained in poor yield by a chromatographic separation. No V could be obtained by the use of procedure A. Procedure B gave a good yield of IV and a small yield of V as the hydrochlorides. In contrast to the observation of Kipnis, *et al.*,[4b] on the solubility of some basically substituted isoalloxazines, IV was insoluble in chloroform.

The conversion of IV and V to the nitrogen mustard derivatives VI and VII did not take place when the hydrochlorides were refluxed with thionyl chloride, although Lin and Price[11] were able to convert the hydrochlorides of similarly substituted adenine diols to their bis(2-chloroethyl)derivatives. By using the free base of the flavin and a large excess of thionyl chloride, the conversion to the nitrogen mustards was achieved readily.

Biological Activity. A. For *L. casei*—Compounds IV and V were tested with *L. casei* for their biological activity by methods described previously.[12] They were inactive as growth inhibitors at a ratio of up to 500 μg. of analog to 0.1 μg. of riboflavin per tube. In addition IV was tested for any vitamin-like properties in tubes containing no additional riboflavin and it was found to be inactive as a vitamin for *L. casei*.

B. For the Rat.—Male weanling Wistar rats were maintained on a riboflavin deficient diet[12] until they were deficient.[13] They were then assigned at random to experimental groups. Animals in the different groups received a supplement by stomach tube, daily, just prior to feeding. The results are given in Table I.

TABLE I

GROWTH OF RATS RECEIVING ANALOG, RIBOFLAVIN, OR ANALOG PLUS RIBOFLAVIN

Group	Supplement[a]	Weight change,[b] g.	Survivors
1	H_2O	3 ± 1.6	15/16
2	2 mg. A	−12 ± 1.6	10/25
3	2 mg. A + 25 μg. R	41 ± 4.3	9/9
4	25 μg. R	119 ± 3.8	8/8
5	10 μg. R	57 ± 3.8	7/7
6	2 mg. A + 100 μg. R	106 ± 7.0	9/9
7	100 μg. R	143 ± 6.6	8/8

[a] 0.5 ml. solutions or suspensions, in 6% gum acacia, of compound IV (Analog or A), riboflavin (R) or compound IV plus riboflavin were administered. [b] Net weight change of survivors for the 4-week test period. An estimate of the standard error is given. *P* values (null hypothesis) for 1 *vs.* 2, 1 *vs.* 3, 1 *vs.* 4, 1 *vs.* 6, 1 *vs.* 7, 3 *vs.* 4 and 6 *vs.* 7 are much less than 0.01. *P* for 3 *vs.* 5 is 0.015. Net weight change for survivors of groups of animals receiving 2 mg. A plus smaller quantities of riboflavin were for: 2 mg. A + 1γR, −11 g. (3/9), 2 mg. A + 5γR, −6 g. (6/10) and 2 mg. A + 10γR, + 5 g. (8/10).

Compound IV is a reversible inhibitor[14] of riboflavin for the riboflavin deficient rat. The quantity or riboflavin required to obtain a 50% reversal of the growth inhibition from a dose of 2 mg. of IV per day can be estimated as about 40 μg. The inhibition index would be approximately 50. In view of the lack of activity of V for *L. casei* and the small supply of this compound available for study, it was not tested in the rat.

The nitrogen mustards VI and VII have been submitted to the Cancer Chemotherapy National Service Center for screening.

Experimental[15]

4,5-Dimethyl-2-nitro-N-2-[bis(2-hydroxyethyl)amino]ethylaniline (XI).—2-Nitro-4,5-dimethylchlorobenzene[2b] (VIII) (5 g., 0.027 mole) (prepared from 2-nitro-4,5-dimethylaniline[16]), the free base from 11.1 g. (0.056 mole) of 2-[bis(2-hydroxyethyl)-amino]ethylamine dihydrochloride[7a,17] (X) and 25 ml. of pyridine were heated in a sealed tube at 140–150° for 10 hr. When cold, the contents of the tube were concentrated to dryness, made acid with 60 ml. of 6 *N* hydrochloric acid and the unreacted VIII was extracted with ether. The aqueous layer was neutralized with ammonium hydroxide. The crystalline product was filtered and recrystallized from 50 ml. of 50% alcohol to give 3.9 g. (49%) of XI, m.p. 107–108°. An analytical sample had m.p. 109–110°.

Anal. Calcd. for $C_{14}H_{23}N_3O_4$: C, 56.6; H, 7.8; N, 14.1. Found: C, 56.2; H, 7.7; N, 13.9.

4,5-Dichloro-2-nitro-N-2-[bis(2-hydroxyethyl)amino]ethylaniline (XII).—To a cold solution of 320 ml. of concd. sulfuric acid and 212 ml. of concd. nitric acid was added 108 g. (0.56 mole) of 3,4-dichloronitrobenzene,[16] and the mixture was stirred and heated at 110–115° for 6 hr. The reaction mixture was poured onto ice, the product collected on a filter and washed with cold water. The crude product was recrystallized twice from ethanol, twice from glacial acetic acid and finally from ethanol to give

(10) Flavin is a general term referring to any isoalloxazine.

(11) H. H. Lin and C. C. Price, *J. Org. Chem.*, **26**, 265 (1961).

(12) J. P. Lambooy and H. V. Aposhian, *J. Nutrition*, **71**, 132 (1960).

(13) The rats were deficient after 3–4 weeks on the riboflavin deficient diet.

(14) Quinacrine was found to be an irreversible inhibitor of cytochrome reductase by E. Haas, *J. Biol. Chem.*, **155**, 321 (1944), and D-amino acid oxidase by L. Hellerman, A. Lindsay, and M. R. Bovarnick, *J. Biol. Chem.*, **163**, 553 (1946).

(15) All melting points are corrected and were taken in an electrically heated bath. Decomposition points were obtained by immersing the capillary into a rapidly heated bath at 150° with rapid heating to the decomposition point. This procedure permitted a high degree of reproducibility in the determination of decomposition points. Ultraviolet spectra were obtained on a Beckman DU with water as solvent. R_f values were obtained in 1-butanol, water, acetic acid (4:5:1) on Whatman number one paper by the ascending technique.

(16) Now available from the Aldrich Chemical Co.

(17) S. R. Aspinall, *J. Am. Chem. Soc.*, **63**, 852 (1941).

24.7 g. (18%) of 4,5-dichloro-1,2-dinitrobenzene[18] (IX), m.p. 107–108°. The free base from 46.4 g. (0.21 mole) of X and 24.7 g. (0.104 mole) of IX were dissolved in 400 ml. of 80% aqueous ethanol and refluxed for 46 hr. A negative test for evolved nitric oxide was obtained with starch–potassium iodide paper at this time. The reaction mixture was diluted with 400 ml. of water and after cooling, the product was filtered. Recrystallization from 50% ethanol gave 28.7 g. (79%) of XII, m.p. 114–115°.

Anal. Calcd. for $C_{12}H_{17}Cl_2N_3O_4$: C, 42.6; H, 5.1; Cl, 21.0; N, 12.4. Found: C, 42.9; H, 5.1; Cl, 21.3; N, 12.1.

7,8-Dimethyl-10-{2-[bis(2-hydroxyethyl)amino]ethyl}isoalloxazine (IV). Procedure A.—Five grams (0.017 mole) of XI was reduced as described under Procedure B. The catalyst and alcohol were removed and the residue of XIII condensed with 3.2 g. (0.02 mole) of alloxan monohydrate by the procedure of Lambooy.[19] One gram (16%) of IV was obtained, m.p. 214–216° dec.

Anal. Calcd. for $C_{18}H_{23}N_5O_4$: C, 57.9; H, 6.2; N, 18.8; Found: C, 57.6; H, 6.3; N, 18.6. ϵ_{max}[20] (λ) 265 (30,500), 370 (10,500), 455 (12,200); ϵ_{min} (λ) 240 (11,300), 300 (1,100), 400-(7,800). $R_f = 0.32$.

Procedure B is a modification of the procedure of Adams, *et al.*[2b] Five grams (0.017 mole) of XI in 150 ml. of absolute ethanol was reduced over platinum oxide at 3.5 kg./cm.^{2} for 17 hr. The catalyst was filtered and dry hydrogen chloride gas passed into the filtrate for a few min. The solution was concentrated to an oil and 50 ml. of absolute ethanol was added to dissolve the residue. To this solution of XIII hydrochloride was added a hot solution of 4 g. (0.02 mole) of alloxan monohydrate in 100 ml. of absolute ethanol. The resulting dark solution was heated for 10 min. on the steam bath and kept at room temperature overnight. The crude flavin hydrochloride was filtered and recrystallized from 775 ml. of 60% ethanol. After cooling, the product was collected and recrystallized from 150 ml. of 50% ethanol to give 3 g. (43%) of IV hydrochloride, m.p. 264–265° dec.

Anal. Calcd. for $C_{18}H_{24}ClN_5O_4$: Cl, 8.6. Found: Cl, 8.7.

7,8-Dichloro-10-{2-[bis(2-hydroxyethyl)amino]ethyl}isoalloxazine Hydrochloride (V).—Five grams (0.015 mole) of XII in 150 ml. of absolute ethanol was reduced over platinum oxide at 4.2 kg./cm.^{2} for 11 hr. The catalyst was filtered and dry hydrogen chloride gas passed into the filtrate. The alcohol was distilled and 50 ml. of absolute ethanol added to dissolve the residue. A hot solution of 4 g. (0.02 mole) of alloxan monohydrate in 150 ml. of absolute ethanol was added and the solution heated for 10 min. on the steam bath and let stand for 2 days in the dark at room temperature. The crude product was filtered and recrystallized from a mixture of 155 ml. of water, 10 ml. of concd. hydrochloric acid and 175 ml. of ethanol. The product was filtered and recrystallized from a mixture of 20 ml. of water, 5 ml. of concd. hydrochloric acid and 25 ml. of ethanol to give 0.87 g., m.p. 255–256° dec. A second crop of 0.23 g. was isolated from the filtrate, m.p. 253–255° dec.; total yield of V hydrochloride, 1.1 g. (13%). An analytical sample was obtained from 50% ethanol, m.p. 259–260° dec.

Anal. Calcd. for $C_{18}H_{18}Cl_3N_5O_4$: C, 42.6; H, 4.0; Cl, 23.6; N, 15.5. Found: C, 42.3; H, 4.0; Cl, 23.6; N, 15.4. ϵ_{max} (λ) 225 (32,400), 270 (34,900), 350 (8,500), 440 (12,000); ϵ_{min} (λ) 240 (22,400), 300 (2,900), 380 (4,700). $R_f = 0.44$.

7,8-Dimethyl-10-{2-[bis(2-chloroethyl)amino]ethyl}isoalloxazine Hydrochloride (VI).—The free base of IV hydrochloride was prepared by the neutralization of 3.33 g. (0.008 mole) of the hydrochloride with *N* sodium hydroxide to give 3.02 g. of IV, m.p. 210–212° dec. IV and 200 ml. of thionyl chloride were kept at room temperature for 12 hr. and refluxed for 2 hr. The thionyl chloride was distilled and 100 ml. of absolute ethanol added to the residue and distilled. The crystalline residue was transferred to a filter with the aid of 75 ml. of absolute ethanol. There was obtained 3.4 g. (91%) of VI hydrochloride, m.p. 252–253° dec. A 1.1 g. sample was recrystallized for analysis from a mixture of 20 ml. of concd. hydrochloric acid, 40 ml. of water and 80 ml. of ethanol. The product, m.p. 252–253°, was filtered and dried at 3 mm./100°/5 hr., m.p. 250–252° dec.

Anal. Calcd. for $C_{18}H_{22}Cl_3N_5O_2$: C, 48.4; H, 5.0; Cl, 23.8; N, 15.7. Found: C, 48.2; H, 5.1; Cl, 23.2, 23.4; N, 15.0, 15.1, 15.6.

7,8-Dichloro-10-{2-[bis(2-chloroethyl)amino]ethyl}isoalloxazine Hydrochloride (VII).—The free base of V hydrochloride was prepared by the neutralization of 1.23 g. (0.0027 mole) with *N* sodium hydroxide to give 1.1 g. of V, m.p. 202–204° dec. It was suspended in 200 ml. of thionyl, chloride, let stand at room temperature for 15 hr. and then refluxed for 3 hr. After removal of the thionyl chloride, 100 ml. of absolute ethanol was distilled from the residue and the crystalline product was transferred to a filter with the aid of 60 ml. of absolute ethanol. The yield of VII hydrochloride was 1.16 g. (87%), m.p. 229–230° dec. A 0.54 g. sample was recrystallized from a mixture of 30 ml. of concd. hydrochloric acid and 45 ml. of ethanol. The product, m.p. 234–235° dec., was dried for analysis at room temperature and 3 mm. for 12 hr., m.p. 234–235° dec. Heating the sample during drying caused loss of hydrogen chloride.

Anal. Calcd. for $C_{18}H_{16}Cl_5N_5O_2$: C, 39.4; H, 3.3; Cl, 36.3; N, 14.3. Found: C, 39.3; H, 3.2; Cl, 36.6; N, 14.1.

(18) R. Kuhn, F. Weygand, and E. Möller, *Ber.*, **76**, 1044 (1943).

(19) J. P. Lambooy, *J. Am. Chem. Soc.*, **72**, 5225 (1950).

(20) ϵ Has units of l./mole-cm.

Vitamin B_6 Analogs. I. 5-Hydroxy-6-methyl-4-trifluoromethyl-3-pyridinemethanol Hydrochloride[1]

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A total synthesis of 5-hydroxy-6-methyl-4-trifluoromethyl-3-pyridinemethanol hydrochloride is described. The condensation of trifluoroacetylacetone with 2-cyanoacetamide leads to 2-hydroxy-6-methyl-4-trifluoromethylnicotinonitrile. This pyridine is then converted to the final product by a five-step reaction sequence.

Although Stoerk[2] observed in 1947 that 4-desoxypyridoxine administered to hybrid mice maintained on a vitamin B_6-deficient diet inhibited the growth of lymphosarcoma 6C3H-ED, relatively little work has been done on the syntheses of other analogs of the B_6 vitamins for screening against animal neoplasms. Other workers have investigated the effect of 4-desoxypyridoxine and acid hydrazides, separately and in combination, on the growth of other rodent tumors.[3] In all cases the treatments were significantly more effective on a B_6-deficient diet.

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(2) H. C. Stoerk, *J. Biol. Chem.*, **171**, 437 (1947).

(3) D. M. Shapiro and A. Gellhow, *Cancer Research*, **11**, 35 (1951); R. R. Cerecedo, M. E. Lombardo, D. V. M. Reddy, and J. J. Travers, *Proc. Soc. Exper. Biol. Med.*, **80**, 648 (1952); B. L. Freedlander, F. A. French, and A. Furst, *ibid.*, **86**, 788 (1954); J. B. Loefer, *Cancer Research*, **11**, 481 (1951); and R. W. Brockman, J. R. Thomson, F. M. Schabel, Jr., and H. E. Skipper, *ibid.*, **16**, 788 (1956).