Synthesis of Some 1,4-Benzoxazine Derivatives and their Antimicrobial Activity

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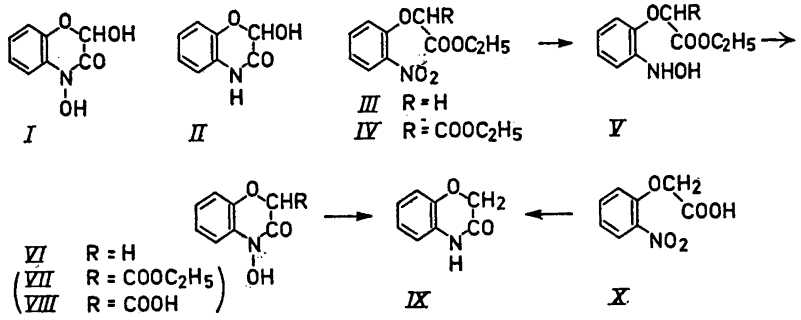
After the natural aglucone was isolated from crushed plants, and it was found to possess antimicrobial properties, the activity of different derivatives of this compound arose attention. In an earlier paper the synthesis of 2,4-dihydroxy-1,4-benzoxazin-3-one (I) and 2-hydroxy-1,4-benzoxazin-3-one (II) are described [1]. Another method for preparing 4-hydroxy-1,4-benzoxazine derivatives is also developed. When the reduction of *o*-nitrophenoxy acetic (or *o*-nitrophenoxy malonic) acid ester (III or IV) is performed in neutral medium with zinc dust, the hydroxylamino group in the first formed intermediate product (V) immediately reacts with the carboethoxy group, and ring closure to compounds VI and VII occurs. The compound VII can be readily hydrolyzed to the corresponding acid (VIII) with dilute sodium hydroxide solution.

4 - Hydroxy - 1,4 - benzoxazine - 2,3 - dione (XIII) was prepared by a similar method as 2,4-dihydroxy-1,4-benzoxazin-3-one [1]. The starting material was also now *o*-methoxymethoxyphenyl hydroxylamine (XI). By allowing this compound to react with oxalic acid ethyl ester chloride and hydrolyzing the intermediate product (XII) with methanolic hydrochloric acid, 4 - hydroxy - 1,4 - benzoxazine - 2,3 - dione (XIII) is formed. The rearrangement of 2,4 - dihydroxy - 1,4-benzoxazin - 3-one to benzoxazolinone [3] is obtained also by 4-hydroxy-1,4-benzoxazine-2,3-dione. Benzoxazolinone (XIV) could be detected in the water solution of XIII after heating for half an hour at 100°C.

The syntheses of 1,4-benzoxazine-2,3-dione [8] (XV) and 3,4-dihydro-2H-1,4-benzoxazine [9] (XVI) are described earlier in the literature.

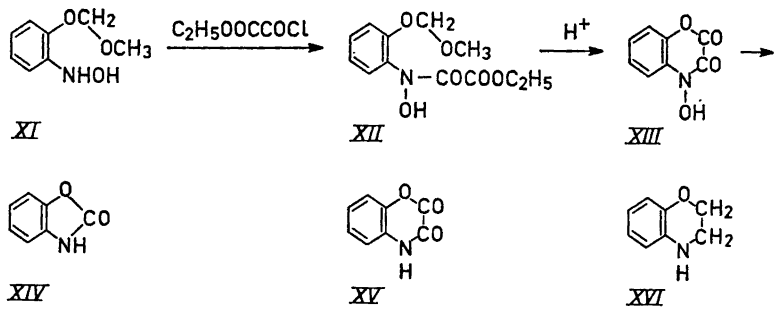
The growth-inhibiting effect of these compounds (I, II, VI, VIII, IX, XIII, and XVI) on *Fusarium nivale* was investigated. The results are given in Fig. 1. The antibacterial activity of the 4-hydroxy-1,4-benzoxazine derivatives (I, VI, VIII, and XIII) was determined with *St. aureus*, *Ps. fluorescens*, and *E. coli*. The results are shown in Fig. 2.

It can be seen in Fig. 1 that the strongest antifungal effect of the 1,4-benzoxazine derivatives was exerted by compound XVI in which the heterocyclic ring is completely hydrogenated. The difference in



If the reduction of *o*-nitrophenoxy acetic acid (X) is carried out with sodium hydrogen sulphite the lactam, 2H-1,4-benzoxazin-3(4H)-one, is obtained [2]. This compound (IX) is also formed when 4-hydroxy-1,4-benzoxazin-3-one (VI) is reduced with zinc dust in boiling acetic acid.

effectivity between compounds VI and IX on the one hand and XIII and XV on the other shows that the compounds with the NOH group are about twice as effective as the corresponding compounds with the NH group. On this basis the NOH compound corresponding to com-



XI XII XIII XIV XV XVI

pound XVI would be the most effective one of the substances in this group. The compound has not, however, been synthesized. The oxidation of 2 as well as 3 carbon atoms lowers the effectivity. The effect of 4-hydroxy-1,4-benzoxazine on bacteria is almost the same as its effect on *Fusarium*. In a concentration of 1 mg/ml compound VI inhibited the growth of *St. aureus*, *Ps. fluorescens*, and *E. coli* (Fig. 2).

All melting points are corrected.

o-*Nitrophenoxy acetic acid ethyl ester* was prepared according to the method of Duparc [4].

4-Hydroxy-1,4-benzoxazin-3-one. A mixture of 225 mg of o-nitrophenoxy acetic acid ethyl ester, 200 mg of ammonium chloride and 200 mg of zinc dust in 10 ml of 60 % ethanol was shaken for 2 h at room temperature. The solution was filtered, and the filter washed first quickly with 2 % acetic acid (to decompose the sparingly soluble zinc salt of the formed hydroxamic acid) and then with alcohol. Water was added to the filtrate, and the solution was extracted with ether. The ether solution was washed with dilute sodium hydroxide. Upon neutralisation of the aqueous layer with hydrochloric acid, the hydroxamic acid was extracted with ether. After drying with sodium sulphate the solvent was evaporated and the residue crystallized from water. Yield 100 mg (60 %); m. p. 168–169°C. (Found: N 8.28. Calc. for $C_8H_7NO_3$: N 8.48.) The compound gave an intensely blue-violet colour with aqueous ferric chloride. UV-Spectrum (in ethanol): max. 258 mμ, ε = 6 300; max. 286

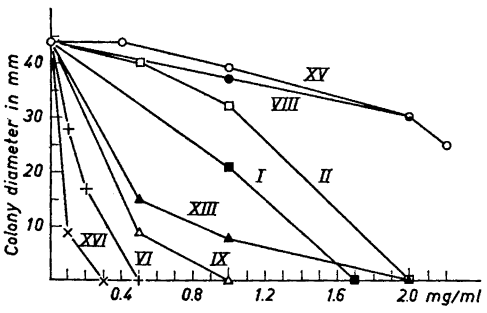


Fig. 1. Growth-inhibiting effect of 1,4-benzoxazine derivatives on *Fusarium nivale*. Oat-glycerol-agar nutrient solution, pH 6.4.

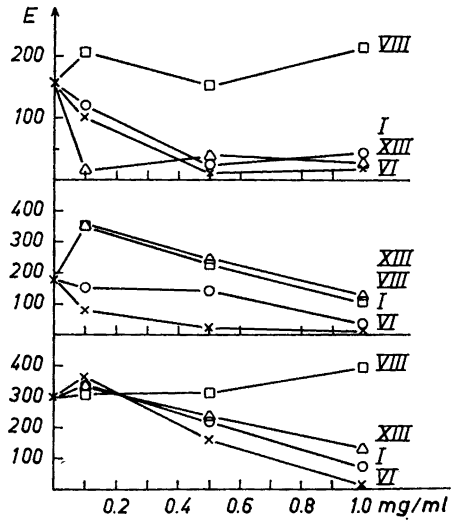


Fig. 2. Growth-inhibiting effect of 4-hydroxy-1,4-benzoxazine derivatives on *St. aureus* (topmost), *Ps. fluorescens* (middlemost), and *E. coli* (undermost). Broth nutrient solution, pH 6.5. Extinction (E) read at 622 mμ.

mμ, ε = 5 100. R_F-Value (Whatman No. 4 paper, solvent *i*-PrOH:NH_4OH:H_2O, 8:1:1) 0.39 (desc.).

Reduction of 4-hydroxy-1,4-benzoxazin-3-one. The reduction was carried out with zinc dust by a similar procedure as in the case of 2,4-dihydroxy-1,4-benzoxazin-3-one [1]. The reduction product melts at 171—172°C, mixed m.p. 171—172°C with 2H-1,4-benzoxazin-3(4H)-one [2].

o-*Nitrophenoxy malonic acid diethyl ester* has been prepared previously from the potassium salt of *o*-nitrophenol and bromomalonic acid diethyl ester by Bischoff [5] who obtained this compound as a crystalline solid (m. p. 116—118°C). When this procedure was repeated, a small amount of crystals were obtained which melted at 115—118°C. On the basis of the molecular weight determination and analysis, this compound should be the bis-(*o*-nitrophenoxy)-malonic acid diethyl ester, for which Bischoff [6] reports the m. p. 119°C. To obtain the *o*-nitrophenoxy malonic acid the uncrystallized part of the reaction mixture was hydrolyzed with aqueous sodium hydroxide at room temperature. Hydrochloric acid was then added to pH 6, and the unreacted *o*-nitrophenol was extracted with ether. The water solution was then made strongly acid and extracted several times with ether. The ether solution was dried, and the solvent evaporated. The residue was dissolved in a few ml of conc. ammonium hydroxide solution, and ethanol was added until separation of crystals occurred. The ammonium salt was filtered off and washed with cold ethanol. After recrystallization from aqueous alcohol the crystals were dissolved in water. The solution was made acid by hydrochloric acid and extracted with ether. After drying the solvent was evaporated, and the residue crystallized from a mixture of ether-benzene. M. p. 128—130°C. (Found: N 5.87. Calc. for $C_9H_7NO_7$: N 5.81.)

The *o*-nitrophenoxy malonic acid was then esterified by refluxing for 4 h with 1 % hydrogen chloride in absolute ethanol. The solvent was evaporated, and the residue dissolved in ether and washed with sodium hydrogen carbonate solution. After drying the ether was evaporated and the residue distilled *in vacuo*. The *o*-nitrophenoxy malonic acid diethyl ester was obtained as a pale yellow oil, which did not crystallize on standing for several weeks at room temperature or in a refrigerator. B. p. 145—150°/0.05 mm. (Found: N 4.77. Calc. for $C_{13}H_{15}NO_7$: N 4.71.)

4-Hydroxy-2-carbethoxy-1,4-benzoxazin-3-one. A mixture of 297 mg of *o*-nitrophenoxy malonic acid diethyl ester, 200 mg of ammonium chloride, and 200 mg of zinc dust in 10 ml of 60 % alcohol was shaken for 2 h at room temperature. The solution was filtered, and water was added to the filtrate. After extraction with ether and drying, the solvent was evaporated, and the residue crystallized from benzene. Yield 120 mg (50 %); m. p. 133—134°C. (Found: N 5.98. Calc. for $C_{11}H_{11}NO_5$: N 5.90.) Violet colour reaction with ferric chloride in alcohol. UV-Spectrum (in ethanol) max. 266 mμ, ε = 5 100; max. 288 mμ, ε = 5 100.

4-Hydroxy-2-carboxy-1,4-benzoxazin - 3 - one. 24 mg of 4-hydroxy-2-carbethoxy-1,4-benzoxazin-3-one were dissolved in 1 ml of 1 N sodium hydroxide solution and allowed to stand overnight. The solution was made slightly acid by hydrochloric acid and extracted several times with ether. After drying the solvent was evaporated and the residue crystallized from an ether-benzene mixture. Yield 15 mg (71 %); m. p. 144—146°C. (Found: N 6.70. Calc. for $C_9H_7NO_5$: N 6.65.) Blue colour reaction with ferric chloride. UV-Spectrum (in ethanol) max. 265 mμ, ε = 5 350; max. 289 mμ, ε = 5 300. R_F-Value (Whatman No. 4 paper, solvent *i*-PrOH:NH_4OH:H_2O, 8:1:1) 0.11 (desc.).

4-Hydroxy-1,4-benzoxazine-2,3-dione. 1.5 g of oxalic acid ethyl ester chloride [7] in 20 ml of ether were gradually added to a solution of 3.3 g of crude *o*-(methoxymethoxy)phenyl hydroxylamine [1] in 50 ml of dry ether under cooling (0°C). The ether solution was decanted from the dark brown oil and evaporated to dryness. The residue was dissolved in 20 ml of methanol and 2 ml of 2 N hydrochloric acid and refluxed for 15 min. The solvent was evaporated under reduced pressure. Water was added, and the mixture was extracted several times with ether. The solvent was evaporated after drying, and the residue crystallized from a mixture of alcohol-benzene. Yield 380 mg (21 %); m. p. 230—233°C (decomp.). (Found: N 7.98. Calc. for $C_8H_5NO_4$: N 7.82.) Red-violet colour reaction with ferric chloride. UV-Spectrum in ethanol max. 303 mμ, ε = 5 900; in water max. 280 mμ, ε = 2 900. R_F-Value (Whatman No. 4 paper, solvent *i*-PrOH:NH_4OH:H_2O, 8:1:1) 0.08 (desc.).

Conversion of 4-hydroxy-1,4-benzoxazine-2,3-dione to benzoxazolinone. A dilute water solution (20 μg/ml) of 4-hydroxy-1,4-benzoxazine-2,3-dione was heated for half an hour on a water bath. The UV-spectrum was then measured. The maximum by 280 mμ was shifted to 270 mμ (ε = 4 200) which is characteristic for benzoxazolinone (max. 270 mμ, ε = 4 200).

1,4-Benzoxazine-2,3-dione was prepared according to the method of Puxeddu and Sanna [8]. UV-Spectrum (in ethanol) max. 303 mμ, ε = 5 800.

3,4-Dihydro-2H-1,4-benzoxazine was prepared from 2H-1,4-benzoxazin-3(4H)-one by reduction with lithium aluminium hydride [2]. UV-Spectrum (in ethanol) max. 274 mμ, $\varepsilon = 5\,350$, max. 296, $\varepsilon = 3\,150$.

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Amino Acid Composition of Seal Myoglobin I

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The isolation and purification of seal myoglobin I has been described recently [1]. The quantitative composition of amino acids has been determined by the method of Moore and Stein [3] using Amberlite IR-120 columns. For each analysis, 20 mg of salt-free myoglobin I was dissolved in 6 N HCl, the solution was cooled in an ice bath and the tubes were evacuated with a water pump and sealed. The hydrolyses were conducted in pairs in an oven at 110°C for 20 and 70 h. The cooled tubes were opened and the contents centrifuged and evaporated to dryness over NaOH pellets in a vacuum desiccator at room temperature. The dry material was dissolved in a small amount of water and dried. Each, nearly colorless, residue was dissolved in 0.2 N sodium citrate buffer, pH 3.25 just before putting it on the column. A sample containing 0.0949 μmole of amino acids, calculated on the basis of protein, was used in each run.

The calculations are based on Moore and Stein's [3] values for color yields, and residues/mole are based on a molecular weight of 18 600 deduced from the iron content [1]. It has been possible to detect small peaks for methionine sulfoxide in 20 and 70 h hydrolysates. Table 1 shows the values obtained.

The amide ammonia values are not included in the summation of amino acid residues.

In order to provide a check on the amide NH_3 values calculated from the chromatographic results, the amide nitrogen was determined by two different methods, micro Kjeldahl and Nessler. For the Nessler nitrogen a solution of 3 mg of protein in 2 ml N H_2SO_4 was heated for 4, 6 and 8 h in a sealed tube at 105°C[4]. An ammonium sulfate solution was used as a standard. In the micro-Kjeldahl technique 22 mg of protein was heated with 0.9 ml of 6 N HCl for 20 h in a sealed tube at 110°C. The resulting ammonia was titrated with 0.5 N H_2SO_4 using an "Agla" micrometer

Table 1. Amino acid composition of hydrolysates of seal myoglobin I.

Amino acid	Time of hydrolysis 20 h	70 h	Number of residues to nearest integer
	Residues/mole		
Aspartic acid	10.5	10.4	11
Threonine	4.7	4.7	5
Serine	6.8	5.8	7
Glutamic acid	15.8	15.7	16
Proline	4.2	3.9	4
Glycine	11.8	11.5	12
Alanine	13.9	13.8	14
Valine	5.3	5.9	6
Methionine	1.6	1.3	2
*Iso*leucine	6.3	7.2	7
Leucine	17.9	18.0	18
Tyrosine	1.8	1.6	2
Phenylalanine	6.7	6.6	7
Lysine	17.9	17.9	18
Histidine	12.3	12.0	12
Arginine	4.6	4.9	5
Amide NH_2	6.7	8.1	7
Total			146

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