The Relation Between Molecular Structure and Biological Activity Among Mononitrophenols Containing Halogens

Substituted Nitrosalicylanilides: A New Class of Selectively Toxic Sea Lamprey Larvicides

Great Lakes Fishery Commission

TECHNICAL REPORT No. 11

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DECEMBER, 1966

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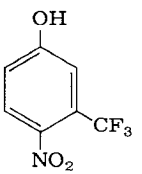
ABSTRACT

The results of tests of the biological activity of certain nitrophenols containing halogens are reported. Some of these are shown to be significantly more toxic to larvae of the sea lamprey *(Petromyzon marinus* L.) than to fishes. It is proposed that the death of lamprey larvae exposed to these compounds results from an acute hypotension (shock) with concomitant circulatory and respiratory failure. Rainbow trout *(Salmo gairdneri),* on the other hand, appear to die, at higher concentrations of the toxin, due to a chemically-caused mechanical interference with respiration through the gills. A systematic series of studies of mononitrophenols containing halogens disclosed that those phenols having the nitro group in the para-position and a halogen atom or group in the meta-position are generally more toxic to lampreys than to fish. The halogens or halogen groups used in this study were fluorine, chlorine, bromine, and trifluormethyl. The same substituents in other positions only occasionally gave rise to selectively toxic compounds. The relationship between the selectively active class of nitrophenols containing halogens and other related structures is discussed.

Introduction

Efforts to control the parasitic sea lamprey *(Petromyzon marinus* L.) in the Great Lakes are directed at present to the applications of a selective toxicant (called variously a lampricide or lamprey larvicide) to streams inhabited by lamprey larvae. Since 1961, the larvicide that has been used almost exclusively in the control program has been 3-trifluor(o)methyl-4-nitrophenol (TFM)' (Fig. 1A). The biological activity of this chemical has

1 Chemical Abstracts name: α, α, α -trifluoro-4-nitro-m-cresol.



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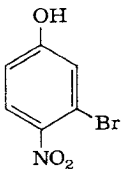

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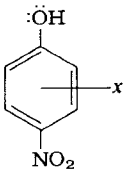
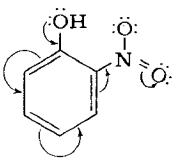


x = F, Cl, Br, CF_3

(C)



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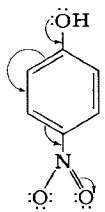
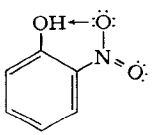


(E)



(F)

Figure 1. Halo-nitrophenols and nitrophenols: (A) 3-trifluormethyl-4-nitrophenol (TFM); (B) 3-bromo-4-nitrophenol; (C) 4-nitrophenols containing halogen(s); (D) 2-nitrophenol acidity enhancement by resonance interaction; (E) 4-nitrophenol acidity enhancement by resonance interaction; (F) 2-nitrophenol intramolecular hydrogen bonding.

been described by Applegate, Howell, and Smith (1958); Applegate, Howell, Moffett, Johnson, and Smith (1961); and, by Applegate and King (1962). Physical and chemical properties of the substance have been described by Smith, Applegate, and Johnson (1961) and methods of analysis for the purpose of controlling its application in natural waters have been described by Smith, Applegate, and Johnson (1960) and Kanayama (1963). This compound is, however, only one of about 15 closely related structures, all mononitrophenols containing halogens, that display, to a greater or lesser degree, a selectively toxic action upon lampreys. Applegate *et al.* (1958) have described the larvicidal properties of some of these compounds. Although not all of the mononitrophenols containing halogens (in fact, less than half of those tested) are selectively toxic to lampreys, no other "family" or group of related compounds has, to our knowledge, displayed any useful larvicidal activity except for the substituted nitro-salicylanilides which are reported upon by Starkey and Howell (1966) in another paper in this Report.

Between 1953 and 1957 a broad range of chemical substances was screened initially in an attempt to find a substance that would destroy larval sea lampreys without causing significant harm to any other animal life inhabiting the same waters. The results of these screening tests, presented by Applegate *et al.* (1957), included descriptions of the toxicity of a variety of phenolic compounds and their derivatives. It now appears not only possible, but also desirable, to review the findings of this and subsequent testing programs to demonstrate the relationship we found between the structure of phenolic compounds and their biological effects on lampreys and other fishes.

Although the phenols and their derivatives include some well-known herbicides and pesticides (e.g., "2,4-D", "2,4,5-T", "2,4,5-TP", etc.), there appears to be a scarcity, if not total absence, of published reports describing any selectively toxic action of this group of compounds among the different classes of chordates except for the following disclosures: Applegate and Howell, United States Patents No. 2,821,499 (Jan. 28, 1958), No. 3,219,521 (Nov. 23, 1965) and Dominion of Canada Patents No. 600,326 (June 21, 1960), No. 665,469 (June 25, 1963), and No. 683,165 (Mar. 31, 1964); Howell and King, United States Patents No. 3,238,098 (Mar. 1, 1966) and No. 3,271,246 (Sept. 6, 1966); Pyne, United States Patent No. 3,052,601 (Sept. 4, 1962); and, Sherer, Frensch, and Stähler, United States Patent No. 3,157,571 (Nov. 17, 1964)[2]. The importance of molecular dimensions in the

[2]All of these patents are related, directly or indirectly, to the subject matter discussed in this report.

case of the herbicidal activities of certain anilides has been reported by Huffman and Allen (1960). The type and position of substituent groups among the salicylic anilides have been shown by Schraufstätter (1962) to have a marked effect on their toxicity to molluscs. Either size or structure or some combination of these or other properties of the molecule may be responsible for the selectively toxic action of the mononitrophenols containing halogens upon lampreys.

Knowledge of the precise effect of these compounds on lampreys and fishes is still incomplete. Both gross and microscopic pathological examinations of test lampreys and fishes exposed to 3-trifluormethyl-4-nitrophenol and observations of many thousands of specimens from the beginning of exposure to the chemical until their death indicate strongly that they die of a complex of circulatory and respiratory failures (Applegate *et al.,* 1961; Christie and Battle, 1963, Sawyer, 1959). Test lampreys exhibited first an excoriation of the respiratory epithelium with subsequent edema, hemorrhaging, and excessive mucus secretion in the gill pouches. Severe vasodilatation then followed (presumably due to an increase in the permeability of the vascular endothelium and/or relaxation of the vascular nerves) with much of the animal's blood volume accumulating in the now flacid post-cardinal veins and in the hepatic sinuses. Vasodilation by 3-trifluormethyl-4-nitrophenol is consistent with the facts that a nitro functional group is present and that nitrites have been found to be vasodilators DiPalma, 1964). Agris (1966) found no significant evidence of anoxia in lampreys exposed to the compound but the pathological symptoms observed infer that it must contribute to their death, at least in their final moments of life.

Rainbow trout *(Salmo* gairdneri) exposed to high concentrations of 3-trifluormethyl-4-nitrophenol exhibited the classic symptoms of anoxia in fishes up to the time of their death. Although edema of the gill lamellae and the coating of these with excessive amounts of mucus were evident, no hemorrhaging from the respiratory epithelium took place. Vasodilatation in the trout is apparently restricted to the gills; it was not evident elsewhere in the circulatory system. Christie and Battle (1963) postulate that 3-trifluormethyl-4-nitrophenol enters the body via the gills and that the differential susceptibility of the two species could be attributable to a greater permeability of the respiratory epithelium to the chemical in the larval lampreys than in the rainbow trout as well as to a more generalized toxic effect on the endothelium throughout the lamprey's whole vascular system.

In view of the severity of these symptoms, as observed by ourselves and others, we consider it very possible that the

lampreys die simply of acute hypotension (with concomitant circulatory and cardiac failure) and that the death of the trout results directly from an anoxia induced by a progressive mechanical interference with respiration from the accumulation of mucus on the gill epithelium.

Other more subtle pathological conditions of a metabolic nature may also exist and contribute to both the general and selectively toxic properties of various mononitrophenols containing halogens described in this report. The mononitrophenols are known to be methemoglobin-formers (Patty, 1962). Agris (1966), however, found no visual evidence in lampreys killed by 3-trifluormethyl-4-nitrophenol of the brown-colored blood that accompanies the production of methemoglobin. An irreversible block of the uptake of oxygen and inhibition of certain enzymes involved in respiration and carbohydrate metabolism may take place as with the molluscicide BAYLUSCIDE which is also a substituted nitrophenol as well as a salicylanilide[3]. It is also possible to speculate that 3-trifluormethyl-4-nitrophenol and its selectively toxic relatives, like 2,4-dinitrophenol and its related compounds, uncouple oxidative phosphorylation thus causing an increase in oxygen consumption and elevation of the basal metabolic rate (Harborne, 1964). In addition to the metabolic changes possible with exposure to nitrophenols, rates of biological degradation (metabolization) of aromatic structures may vary not only among different species of aquatic vertebrates but also with the nature and location of the substituent atoms or groups in a compound to which a single species is exposed. Maickel, Jondorf, and Brodie (1958; 1959), however, reported that "fish" are essentially unable to conjugate "foreign phenols." Maickel (personal communications) reported further that lampreys, like fishes, did not metabolize either certain drugs or phenolic compounds to which they were exposed. He noted, in fact, that the lampreys excreted in an unmetabolized form approximately 95 percent of the phenols taken up by the specimens from the surrounding treated medium.

We attempt in this report to integrate the results of the several diverse series of bioassays, conducted between 1953 and 1961, in which phenols and their related compounds or derivatives were tested to determine if a differential toxic action existed between lampreys and fishes. The results of some of

[3]See collected papers in Pflanzenschutz-Nachrichten "Bayer," Vol. 15, No. 1 (1962); published by Farbenfabriken Bayer AG, Leverkusen, West Germany.

these tests have already been published (loc. *cit.)* but it would be worthwhile at this juncture to summarize briefly the series of testing programs that took place in the order in which they evolved. A total of 4,346 chemicals, predominantly organic compounds, were subjected to rough screening as described by Applegate, Howell, Hall, and Smith (1957). Among all of these test compounds, one chemical, 3-bromo-4-nitrophenol, was found to have the desired biological effect as well as seemingly acceptable physical properties (Fig. 1B). This finding led to a more exhaustive series of tests in which a number of related mononitrophenols containing halogens were examined at closer intervals of concentration and over a wider range of concentrations. The results of these tests, as reported by Applegate *et al.* (1958) and by Applegate *et al.* (1961), brought to 10 the number of compounds that were significantly more toxic to larval lampreys than they were to certain native fishes. The present report increases this list of presumably useful larvicides by five additional substances. Finally, a number of other substituted phenols not tested in the initial screening program, nor in the subsequent "intensive test series" were examined. The results observed for the additional five selectively toxic agents as well as the other substituted phenols just mentioned have not been published or reported upon before. The fundamental purpose of this report is to present the results of all tests, regardless of character, made with the mononitrophenols containing halogens, with the other substituted phenols, and with the phenol derivatives to demonstrate that: (1) Those that are more toxic to lampreys than they are to fishes are associated with a particular molecular structure; and, (2) that very specific differences in the differential toxicities displayed by this group are associated with variations in the structure of this molecule.

Bioassay Methods

Laboratory facilities and equipment were similar to those described by Applegate et al. (1957). Bioassay methods for all of the mononitrophenols containing halogens were essentially the same as those described by Applegate and King (1962). Animals were placed in lo-liter glass battery jars (lo-inch diameter) each containing 6 liters of test solution. The test jars were aerated by means of standard stone aerators (vigorous aeration does not decrease the concentration of these nonvolatile chemicals). Oxygen levels in the jars during the tests were maintained

at near-saturation. Temperatures were held constant at 55°F. by immersion of the test jars in specially constructed water baths.

Test animals were larvae of the sea lamprey *(Petromyzon marinus* L.) and fingerling rainbow trout *(Salmo gairdneri).* Lamprey larvae ranged from 3.5 to 5.0 inches and rainbow trout from 4.5 to 5.5 inches in total length. All larvae were collected from a restricted area of the Ocqueoc River, Presque Isle County, Michigan. The rainbow trout were obtained from a hatchery of the Michigan Department of Conservation. In all experiments with this group of substances, two specimens of larval lampreys and two of rainbow trout were placed in each test container. After the animals were tempered and acclimated to the test temperature, appropriate amounts of a compound in aqueous solutions were added to produce the desired concentrations. All compounds were purchased or synthesized by us as C. P. grade materials. However, when any doubt as to purity existed, e.g., the melting point of a sample was significantly lower than that reported in the literature, the chemical was recrystallized from an appropriate solvent until a suitable substance was produced.

In the initial test of each chemical, two simultaneous replications were made to give a total of four individuals of each species exposed at each concentration up to a maximum of 40 ppm. No further tests were made with those mononitrophenols containing halogens that were biologically inactive at this maximum concentration nor with those that were unquestionably more toxic to rainbow trout than to larval lampreys at any concentration. All compounds in this group that were more toxic to lampreys than to fishes were tested again to determine their biological activity with more precision. Eight to 16 simultaneous replications were run with a total of 16 to 32 individuals of each species at each concentration. In these tests the intervals between concentrations varied from as little as 0.5 ppm to no more than 2 ppm and were so selected that accurate mortality curves could be devised. In the tabular material that is presented subsequently, the concentrations required to kill 100 percent of the larval lampreys and 25 percent of the rainbow trout are given only for those compounds that were more toxic to lampreys than to rainbow trout. The values given were derived from the mortality curves[4].

[4]Deviations of the $LD_{100}/24$ hrs. for lampreys and $LD_{25}/24$ hrs. for rainbow trout between those given in this report and those given in earlier publications are unquestionably due to the time of year when the several investigations were made. Seasonal changes in the biological

Bioassay methods for all other phenolic structures and their derivatives discussed in this report have been described by Applegate et al. (1957). The only variation in these methods took place in the rough screening of certain compounds in the period 1957-1960 when either fingerling brook trout *(Salvelinus fontinalis)* or fingerling brown trout *(Salmo trutta)* were substituted as test animals for the rainbow trout when the latter were not immediately available from hatcheries.

Dilution water used in all tests was taken from Hammond Bay of Lake Huron. During the period of years when the tests discussed in this report were conducted, the quality of this water obviously varied from season to season and from year to year. Our records show, however, that the physical and chemical characteristics of the water were usually within the following ranges : Oxygen, 8.5-13.7 ppm; carbon dioxide, 0.5-9.0 ppm; methyl-orange alkalinity, 90 - 118 ppm; phenolphthalein alkalinity, 0.0 ppm; pH, 7.5-8.2; and, conductivity, 176.3-182.3 micromhos/18°C.

Our tabular material for the phenolics and their derivatives, other than the mononitrophenols containing halogens, give only brief verbal descriptions of the biological activity of the compounds rather than specific concentrations at which they were toxic or nontoxic. We believe that this procedure is necessary because of the variety of test fishes used, variations in water quality over the many years when the tests were conducted, and because most, if not all, of these chemicals were tested at concentrations no higher than 5 ppm. We wish to make it very clear that, with other dilution waters, other test fishes, or at higher concentrations, these compounds might display substantial differences in toxicity other than those that we observed.

Relation of Molecular Structure to Biological Activity

The group of compounds that have the desired selectively toxic properties is a small class of closely related chemicals. These compounds are phenols containing (1) a single nitro group in the 4- position, and, (2) halogens or a trifluormethyl group substituted directly on the ring (Fig. 1C). The trifluormethyl group is more effective than halogen atoms. Among the halogens,

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activity of the halo-mononitrophenols have been reported by Applegate et al. (1961). Another study that may have some bearing on this phenomenon is that by Ivanova-Berg and Sokolova (1959).

bromine and chlorine are somewhat more effective than fluorine. The most desirable substances from a practical standpoint are those having a single halogen or trifluormethyl group in the 3-position.

Table 1 summarizes the data for 31 mononitrophenols containing either halogens or a trifluormethyl group. Thirteen of these compounds are derived from 4-nitrophenol and 17 are derived from 2-nitrophenol. Numerous attempts were made to secure compounds related to 3-nitrophenol but only one such compound was obtained. Many of the halogenated 3-nitrophenols are reported in the literature but facilities for preparing them were not available. Except for iodine, all of the halogens and trifluormethyl are well represented[5].

All of the trifluormethyl, monohalogen, and dihalogen derivatives of 4-nitrophenol studied were significantly more toxic to lamprey larvae than to fishes. This was not the case with the tri- and the tetra-halogen-4-nitrophenols tested. These were much more toxic to fish than to lamprey larvae. Furthermore, these compounds killed fish at much lower concentrations than did any of the mono- or disubstituted 4-nitrophenols. It appears that the addition of more than two halogen atoms results in an "overloading" of the molecule with such a pronounced increase in the toxic effect upon fishes that the compounds become more toxic to these than to lampreys. This is not the first time this effect has been observed. Schraufstatter (1962), commenting on the moluscicidal activity of chloronitroanilides of salicylic acid, observed the same thing in polychloro derivatives.

Among the monosubstituted derivatives of 4-nitrophenol, the monofluorine derivatives are the least toxic and show a narrower differential toxicity than other members of this group. There is little difference between the chlorine and the bromine derivatives. They are significantly more toxic than the fluorine compounds and show a more pronounced differential toxicity. There is no consistent change in toxicity among any of the pairs of halogen-4-nitrophenols when the halogen is moved from the 2- to the 3- position.

The two trifluormethyl-4-nitrophenols differ from the fluorine, chlorine, and bromine compounds discussed previously. First, there is a marked difference in effect associated with the position of the trifluormethyl group. When this group is in the

[5] No great effort to obtain the iodonitrophenols was made since these compounds are notoriously unstable and presumably would not have been suitable for either critical bioassays or practical application.

Table 1. Toxic effects of 31 mononitrophenols containing halogens or a trifluormethyl group on larval lampreys and fingerling rainbow trout.

[All tests conducted for a 24-hour period at a water temperature of 55°F. No tests of compounds conducted at concentrations greater than 40 ppm. Abbreviations: Lampreys = larval lampreys; RBT = rainbow trout]

Compounds	Biological activity				Properties of compounds selectively toxic to lampreys		
	No toxic effect on either lampreys or RBT	Equally toxic to lampreys and RBT	More toxic to RBT than to lampreys	More toxic to lampreys than to RBT	LD_{100}/24 hrs. for lampreys (Ppm)	LD_{25}/ 24 hrs. for RBT (Ppm)	Differential toxicity expressed as ratio of concentrations
Derived from 2-nitrophenol:							
3-fluoro-			X				
4-fluoro-	X						
5-fluoro-	X						
6-fluoro-	X						
4,5-difluoro-			X				
4,6-difluoro-			X				
3-chloro-			X				
4-chloro-			X				
5-chloro-				X	3	5	1.7
4,5-dichloro-			X				
4,6-dichloro-			X				
3,4,6-trichloro-				X	5	17	3.4
4-bromo-			X				
5-bromo-		X					
4,6-dibromo-			X				
3-trifluormethyl-				X	<15	>40	2.7+
4-trifluormethyl-			X				

Table 1 (Continued).

Derived from 3 -nitrophenol:					
2-iodo-		X	>3	4	1.0^{-4}
Derived from 4-nitrophenol:					
2-fluoro-		X	17	23	1.4
3-fluoro -		X	12	17	1.4
2-chloro-		X	7	12	1.7
3-chloro-		X	4	10	2.5
2,5 -dichloro-		X	3	13	4.3
2,6 -dichloro-		X	?+	?+	?+
2,3,6-trichloro-	X				
2,3,5,6-tetrachloro-	X				
2-bromo-		X	5	12	2.4
3-bromo-		X	5	11	2.2
2,6 -dibromo -		X	19	25	1.3
2-trifluormethyl-		X	13	19	1.5
3-trifluormethyl-		X	2	9	4.5

+ Tolerances poorly defined due to low solubility of compound.

2- position, the compound is intermediate between the fluoro and the chloro analogues, both in general toxicity and differential toxicity between lampreys and fishes. When the trifluormethyl group is in the 3- position, however, the LD_{100} for lampreys drops to 2 ppm and the differential toxicity rises to 4.5 (see Table 1). This combination of properties is responsible for the widespread use of 3-trifluormethyl-4-nitrophenol (TFM) in sea lamprey control (Applegate et al., 1961; Great Lakes Fishery Commission, 1961, 1962, 1963, 1964).

Of the 17 substituted 2-nitrophenols, three are selectively toxic to lampreys, ten are selectively toxic to fish, and three are biologically inactive. One compound is equally toxic to both fish and lampreys. Any close comparison of the monosubstituted 2-nitrophenols with similar 4-nitrophenols is out of the question. In both cases, the nitro group is in a position to enhance the acidity of the phenolic group by means of resonance interaction with the benzene ring (Figs. 1D and 1E)[6]. On the other hand, only in the 2-nitrophenols is there a strong intramolecular hydrogen bonding between the nitro and the hydroxyl groups (Fig. 1F). The 4- and 5- substituted derivatives of 2-nitrophenols invite comparison with both 2- and 3- substituted 4-nitrophenols for they all are 1,2,4-trisubstituted benzenes. It is clear enough, however, that the derivatives of 2-nitrophenol are generally less toxic and less selective than those of 4-nitrophenol.

In no instance did any phenol that varied from a structure containing one nitro group and one or more halogens possess any indication of the desired selective toxicity (Tables 2 and 3). Phenol itself was more toxic to fish than to larval lampreys. Twenty of 22 mono-halo-, poly-halo-, and mixed poly-halo-phenols were similar in this regard; pentachlorophenol was equally toxic to all test animals and pentabromophenol had no toxic effect on any species (Applegate *et* al., 1957). The three

[6]In his study of nitrohaloanilides of salicylic acid, Schraufstätter observed that p-nitroanilides were more toxic and more selective. A similar but less striking toxic effect was present in the o-nitroanilides. However, nitro groups in the meta position did not have this effect. The m-nitroanilides were actually less toxic than when the nitro group was absent. It is tempting to speculate that this behavior of the nitro group in the 3- position is associated with its inability to transmit a resonance effect to the amido nitrogen. The only halogenated 3-nitrophenol tested was 2-iodo-3-nitrophenol. It proved to be quite toxic, the lampreys being slightly more susceptible than the fish. The inadvisability of comparing the toxicity of this single substance with that of the other substances is obvious, particularly when it is noted that there are no other iodo compounds with which to compare it.

Table 2. Synopsis of the biological activity among lampreys and fishes characterizing certain phenolic groups.

[Biological activity described in more detail in Table 3.]

Phenol	More toxic to certain fish species than to lampreys (1 compound tested).
Mono- and poly-halo-phenols	More toxic to fish than to lampreys or toxic to fish and not toxic to lampreys except pentachlorophenol which was equally toxic to all species and pentabromophenol which had no toxic effect on any species, at least at 5 ppm (20 compounds tested).
Mixed poly-halo-phenols	Toxic to fish; not toxic to lampreys (2 compounds tested).
Mononitrophenols	No toxic effect on any test species (4 compounds tested).
Dinitrophenols	No toxic effect on any test species (1 compound tested).
Halo-dinitrophenols	No toxic effect on any species except 2-chloro-4,6-dinitrophenol which was toxic to fish but not to lampreys (6 compounds tested).
Halo-trinitrophenols	No toxic effect on any species (1 compound tested).
Poly-halo-mononitrophenols	More toxic to fish than to lampreys (1 compound tested).

mononitrophenols and 2,4-dinitrophenol were biologically inactive at a concentration of 5 ppm. Six of the seven mono-halo-di- and trinitrophenols subjected to the same screening tests were inactive; the exception was 2-chloro-4,6-dinitrophenol which was toxic to fish but not to l amp r e y s. The only mixed halonitrophenol examined was 4-bromo-2-chloro-6-nitrophenol; it was more toxic to fish than larval lampreys[7].

In view of the widespread occurrence of oxidation-reduction processes in living systems, it would have been interesting to

[7]The biological activity of 24 of these phenolic compounds have been reported by Applegate et *al*. (1957). The toxicity of 12 additional compounds of this type has been determined since this report was issued.

Table 3. Biological activity of phenol and some substituted pheonlic compounds other than mononitrophenols containing halogens.

[“Report No.” refers to numerical designation of compound in Table 1 in report by Applegate et al. (1957); “FWS No.” is Accession number of compounds tested, but not yet reported or published anywhere† . Where both “Report No.” and “FWS No.” are given for a chemical, it indicates that early tests with the compound were reported (op. cit.), while results of later tests with other samples of the same substance have not been published until now. (Maximum concentration tested, 5 ppm, except where otherwise noted after name of chemical. Abbreviations: RBT = rainbow trout; BrT = brook trout; BT = brown trout; BG = bluegills; and L = larval lampreys].

Report or Accession number	Compound	Biological activity
	Phenol	
Report No. 2704	Phenol (liquified USP XIV)	Toxic to RBT; not toxic to BG & L
	Mono- and *poly-halo-phenols*	
Report No. 2761	2-chlorophenol	Toxic to RBT; not toxic to BG &L
Report No. 2762	3-chlorophenol	Toxic to RBT & BG; not toxic to L
Report No. 2763	4-chlorophenol	More toxic to RBT &BG than to L
Report No. 2801	2, 4-dichlorophenol	More toxic to RBT than to BG &L
FWS No. 5072; 5230	2, 5-dichlorophenol	More toxic to RBT &BG than to L
Report No. 2605	2, 6-dichlorophenol	ditto
Report No. 2803; FWS No. 5228	3, 4-dichlorophenol	ditto
Report No. 2878	2, 4, 5-trichlorophenol	ditto
Report No. 2879	2, 4, 6-trichlorophenol	ditto
Report No. 2871	2, 3, 4, 6-tetrachlorophenol	ditto
Report No. 2872	2, 3, 5, 6-tetrachlorophenol	ditto
Report No. 2857	pentachlorophenol	Equally toxic to RBT, BG &L
FWS No. 5074	3-bromophenol	More toxic to RBT than to L
FWS No. 5087	4-bromophenol	ditto
Report No. 2790	2, 4-dibromophenol	Toxic to RBT &BG; not toxic to L
FWS No. 5139	2, 4, 6-tribromophenol	More toxic to RBT &BG than to L
Report No. 2856	pentabromophenol	No toxic effect on any species
FWS No. 5146	2-iodophenol	More toxic to RBT than to L
Report No. 2829	4-iodophenol	Toxic to RBT &BG; not toxic to L
FWS No. 5145	2, 4, 6-triiodophenol	More toxic to RBT &BG than to L

Table 3 (Continued)

	Mixed poly-halo-phenols	
Report No. 2721	4-bromo -2, 6 dichlorophenol	Toxic to RBT & BG; not toxic to L
Report No. 2792	2, 6-dibromo-4-chlorophenol	ditto
	Mononitrophenols	
Report No. 2844	x-nitrophenol	No toxic effect on any species
Report No. 2845	2-nitrophenol	ditto
FWS No. 5149	3-nitrophenol	ditto
Report No. 2846	4-nitrophenol	ditto
	Dinitrophenols	
Report No. 2814	2, 4-dinitrophenol	Not toxic to L
	Halo-dinitrophenols	
FWS No. 5265	4-fluoro-2, 6-dinitrophenol (40 ppm)	Not toxic to RBT &L
Report No. 2767; FWS No. 5088	4-chloro-2, 6-dinitrophenol (40 ppm)	ditto
Report No. 2766	2-chloro-4, 6-dinitrophenol	Toxic to RBT &BG; not toxic to L
FWS No. 5235	2, 5-dichloro-4, 6-dinitrophenol	Not toxic to RBT, BG, or L
FWS No. 5241	3-bromo-2, 4-dinitrophenol	Not toxic to RBT or L
FWS No. 5239	3-bromo-4, 6-dinitrophenol	ditto
	Halo-trinitrophenols	
FWS No. 5240	3-bromo-2, 4, 6-trinitrophenol	Not toxic to RBT or L
	Poly-halo-mononitrophenols	
FWS No. 5227	4-bromo-2-chloro-6-nitrophenol	More toxic to RBT & BrT than to L

†Test records on file at U.S. Bureau of Commercial Fisheries, Hammond Bay Biological Station, Millersburg, Michigan, 49759.

discover the biological activity of oxidation or reduction products of the selectively active 4-nitrophenols. Unfortunately, the original screening program included few such compounds. The polyhydric phenols such as catechol might be considered as oxidation products. None of the nitro-halo derivatives of these compounds was tested. The three halogenated resorcinols tested were either indiscriminately toxic or more toxic to fish. A second class of oxidation products would be the quinones. No benzoquinones were tested (quinhydrone was biologically inactive). Derivatives of 1,4- and 1,2-napthoquinone, however, were very active biologically, particularly when halogens were present, but were generally more toxic to fish than to larval lampreys. Further examination of this class might prove to be interesting.

In considering reduction products of the nitrophenols, a chemist would first think of azo and azoxy benzenes. Seven compounds which belonged to this class were tested, but none was toxic at 5 ppm except for two instances when a phenolic and a nitro group were also present [2-nitro-4-phenylazophenol and 4- (4-nitrophenylazo)phenol]. These two compounds were more toxic to fish than to larval lampreys.

The end reduction product of a nitro group is an amino group. No phenols containing halogen and an amino group were tested. The unsubstituted aminophenols are inactive. In this behavior, they resemble the mononitrophenols although 4-aminophenol exhibited indiscriminate toxicity. Anilines can be considered as nitrogen analogues of phenols rather than reduction products of aromatic nitro compounds. Anilines and nitroanilines are generally nontoxic unless a halogen is present. When a halogen, with or without a nitro group, is present, the compounds are either equally toxic to lampreys and fishes or they are more toxic to fish.

The original screening program included also a wide variety of compounds related to phenol that might be expected to have biological properties similar to the halo-nitrophenols. Most of these chemicals were alkylated phenols like the cresols but a fair number of naphthols were also included. When these compounds contained neither halogen nor nitro groups, they exhibited no biological activity. A single nitro group did not make these related compounds toxic, but two nitro groups did. Seven dinitro-alkylphenols (e.g., 4,6-dinitro-2-cresol) were tested; all of them were toxic but none was significantly more toxic to lampreys than to fish. A single halogen atom often sufficed to produce a toxic substance (e.g., 6-bromo-2-naphthol) and additional halogens usually intensified the effect, but among

more than 30 mono- and polyhalogenated substances, none was more toxic to lamprey larvae than to fish. Eight alkylated phenols containing halogen and nitro groups were examined (e.g., 4-bromo-6-nitro-2-cresol) but all of them were either indiscriminately toxic or more toxic to the fish.

Acknowledgments

We wish to thank Dr. John D. Drumheller, Maumee Chemical Company, Mr. John F. LesVeaux, Niagara Chemical Division, FMC Corp., Dr. Clarence L. Moyle, Biochemical Research Laboratory, The Dow Chemical Company, and, Dr. Bennett R. Willeford, Department of Chemistry, Bucknell University, for their review and constructive criticism of the manuscript.

The investigations summarized in this report were supported by the U. S. Fish and Wildlife Service from 1953 to 1956; thereafter, they were supported by the Great Lakes Fishery Commission.

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SUBSTITUTED NITROSALICYLANILIDES: A NEW CLASS OF SELECTIVELY TOXIC SEA LAMPREY LARVICIDES

Roland J. Starkey and John H. Howell

ABSTRACT

Structure activity relationships of substituted 3-nitro- and 5-nitrosalicylanilides and related compounds have been evaluated to elucidate their activity as potent selectively toxic sea lamprey larvicides.

Introduction

A substantial reduction of the sea lamprey in Lake Superior has been accomplished by the widespread treatment of larval habitat in streams with 3-trifluormethyl-4-nitrophenol (TFM) (Applegate, Howell, Moffett, Johnson and Smith, 1961). The larvicidal LD_{100} for this compound varies from 3-10 ppm depending upon the physical and chemical characteristics of the water in which it is used. The LD_{25} for 11 species of fish has been reported between 5 and 42 ppm (Applegate and King, 1962). During a continuing screening program to detect more potent, selectively toxic sea lamprey larvicides, substituted 3-nitro- and 5-nitrosalicylanilides were found to be more toxic to sea lamprey than TFM.

Salicylanilide was first used as a textile fungistat (Fargher, Galloway, and Probert, 1930), and later clinically in the topical treatment of dermatophytoses (Schwartz, Peck, Botvinick, and Leibovitz, 1946; Sullivan and Bereston, 1952). Derivatives of salicylanilide have exhibited a diverse bioactivity spectrum, including antimicrobial activity (Taborsky, Darker, and Kaye, 1959; Taborsky and Starkey, 1962; Taborsky and Starkey, 1963); antitumor activity (Taborsky and Starkey, 1962); molluscicidal function (Gönnert and Strufe, 1962); and as a taeniacide (Forbes, 1963). One of these compounds, 2',5-dichloro-4'-nitrosalicylanilide (Bayer 73), has already been used successfully as a synergist of TFM in control of sea lampreys (Howell, King, Smith, and Hanson, 1964).

Preliminary mammalian oral toxicity studies with substituted 3-nitro- and 5-nitrosalicylanilides indicate lack of side effects in dosages greater than 2.5 gm/kg. Halo-3-nitrosalicylanilides, introduced by the intraperitoneal route in the rate, produce an LD_{50} of 35 mg/kg and halo-5-nitrosalicylanilides an LD,, of 125 mg/kg (Taborsky and Starkey, 1962).

Prompted by the results of preliminary larvicidal screening studies and the relatively low mammalian toxicity, it was decided to evaluate structure-activity relationships of a series of 3-nitro- and 5-nitrosalicylanilides and related benzanilides in an attempt to design more effective and selective sea lamprey larvicides. A similar study, undertaken during the development of halo-nitrophenols as sea lamprey larvicides (Applegate, Johnson, and Smith, 1966), is described in the preceding paper of this report.

This paper presents the general activity-relationships for selected compounds from this series. We believe this information, although preliminary, merits presentation on the following grounds: (a) agreement of structure-activity relationships with prior studies with salicylanilides (Baichwal, Baxter, Kandel, and Walker, 1960); (b) consistency of structure-activity relationships within our own data and, (c) correlation of preliminary screening data with compounds chosen for comprehensive evaluation.

Methods

Substituted 3-nitro- and 5-nitrosalicylanilides were prepared according to previously reported techniques (Taborsky *et al.,* 1959; Taborsky and Starkey, 1963). Included in the study were 3-nitrosalicylotoluidides, 3-nitro-salicyloxylidides, a 3-nitro-salicylanisidide, and miscellaneous benzanilides[1].

The facilities, methods, and various techniques used in evaluating sea lamprey larvicides were reported by Applegate et al. (1957; 1953). Minimal requirements for larvicidal selectivity have been documented (Howell and Marquette, 1962).

Results

Of one hundred salicylanilides and benzanilides, 80 demonstrated larvicidal activity at the maximum screening level of 10

[1]Method of preparation by Starkey unpublished.

ppm. Among these active compounds were 56 that exhibited selective toxicity for larval lamprey over fingerling rainbow trout, Salmo *gairdneri,* 13 which were equally toxic to both species and 11 more toxic to rainbow trout.

Table 1 illustrates the obligatory molecular requirements for the ortho phenolic hydroxyl substituent in the carboxylic acid moiety of salicylanilide as depicted by the complete lack of activity in benzanilides. This finding conforms to the observations of Baichwal *et al.* (1960) in regard to the *ortho* hydroxy requirements for salicylanilide and derivatives for antifungal activity.

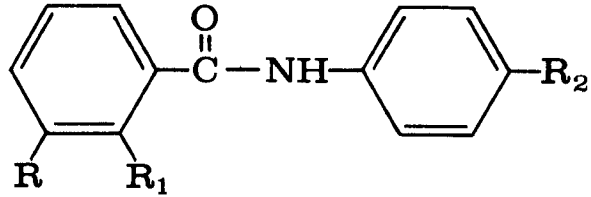
In addition, maximum toxicity and selectivity require the optimum combination of a halogen and a nitro group in the molecule. For example, salicylanilide and 3-nitrosalicylanilide, although active in concentrations less than 10 ppm, produce minimal selectivity. The addition of a halogen, as in 4'-chloro-3-nitrosalicylanilide, further increased toxicity and enhanced selectivity. Additional proof of the requirement for a nitro group in regard to selectivity is based on the reduction of this to an acetamido configuration (Taborsky and Starkey, 1963). The resulting 4'-chloro-3-acetamidosalicylanilide retains some of its toxicity but loses selectivity.

Mono-halonitrosalicylanilides, except 3 -chloro-5-nitrosalicylanilide, produced an LD_{100} for lamprey larvae at concentrations less than 10 ppm. Failure of this compound to exhibit an LD_{100} in concentrations not exceeding 15 ppm has been verified by repeated assays. Because this is the only 5-nitrosalicylanilide substituted in the *meta* position of the aniline moiety, studies are being undertaken with additional 5-nitrosalicylanilides substituted in the same position with other halogens to determine if the relatively low toxicity is position dependent.

Discussion

Larval toxicity of the mono-halonitrosalicylanilides generally falls into three levels at 0.3, 0.5, and 0.9 to 3.0 ppm, respectively. The importance of the halogen locus in relation to the toxicity of 3-nitro-and 5-nitrosalicylanilides is depicted in Table 2. *Meta* and *para* substituents in the aniline moiety of 3-nitrosalicylanilides produce a toxic effect at 0.3 ppm. In 5-nitrosalicylanilides substitution of a halogen in the *para* position results in a group of compounds consistently active at 0.5 ppm. Compounds larvicidal in the 0.9 to 3.0 ppm range are

Table 1. Comparison of molecular requirements for substituted mono-halo-nitrosalicylanilides exhibiting selective toxicity to larval sea lamprey and fingerling rainbow trout



Compound	Substituents			Lamprey	Trout
	R	R_1	R_2	Woo (Ppm)	LD_{25} (ppm)
Benzanilide	.	. .	. .	210.0	>10.0
4'-chlorobenzanilide	. .	. .	-Cl	>10.0	>10.0
Salicylanide	. .	-OH	. .	9.5	9.51
3-nitrosalicylanilide	$-NO_2$	. .	. .	3.0	3.0
4'-chloro-3-nitrobenzanilide	$-NO_2$	. .	-Cl	>10.0	>10.0
4'-chloro-3-nitrosalicylanilide	$-NO_2$	-OH	-Cl	0.3	0.7
4'-chloro-3-acetamidosalicylanilide	-NH $COCH_3$	-OH	-Cl	3.0	3.02

[1] $LD_{,,}$ at 9.5 ppm

[2] LD_{100}

Table 2. Comparative toxicity of halonitrosalicylanilides to larval sea lamprey and fingerling rainbow trout as a function of substituent loci.

Compound	Lamprey LD_{100} (ppm)	Trout LD_{25} (ppm)
3 ' -chloro-3 -nitrosalicylanilide	0.3	0.9
4'-chloro-3-nitrosalicylanilide	0.3	0.7
3'-iodo-3-nitrosalicylanilide	0.3	1.0
4'-iodo -3-nitrosalicylanilide	0.3	0.7
3'-bromo-3-nitrosalicylanilide	0.3	1.0
4'-bromo-3 -nitrosalicylanilide	0.3	1.0
4'-chloro-5-nitrosalicylanilide	0.5	1.0
3'-fluoro-3-nitrosalicylanilide	0.5	0.9
4'-iodo -5-nitrosalicylanilide	0.5	1.0
4'-bromo -5 -nitrosalicylanilide	0.5	1.0
2'-chloro-5-nitrosalicylanilide	0.9	3.0
2'-iodo-3-nitrosalicylanilide	1.0	3.0
2'-bromo-3-nitrosalicylanilide	1.0	1.0'
4'-fluoro-3-nitrosalicylanilide	1.0	3.0
2'-fluoro-3-nitrosalicylanilide	3.0	3.0
2'-chloro-3-nitrosalicylanilide	3.0	7.0
4'-fluoro-5-nitrosalicylanilide	3.0	
3'-chloro-5-nitrosalicylanilide	15.0	15.0

[1] LD_{100}

either ortho substituted 3-n i t r o- or 5-nitrosalicylanilides. Fluoro-3-nitro-and 5-nitrosalicylanilides deviated from this pattern and cannot be explained on the basis of our present data.

Toxicity to rainbow trout and the resulting selectivity is not as consistent as for larvicidal activity. Compounds that produce larval LD_{100} in the *0.3* to 0.5 range produced LD_{25}'s for trout in concentrations of 0.7 to 1.0 ppm. Again, the *ortho* substituted halo-3-nitro- or 5-nitrosalicylanilides and fluoro derivatives were less toxic to trout in the 3.0 to 15.0 ppm range. Although selectivity varied considerably among the compounds, those with the highest degree of larvicidal and trout toxicity were usually the most selective.

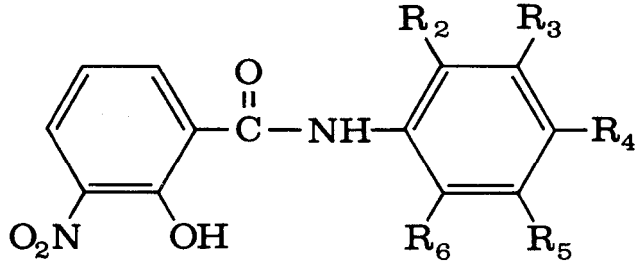
Comparative toxicity studies with substituted 3-nitrosalicylotoluidides, 3-nitrosalicyloxylidides, a 3-nitrosalicyanisidide, and a dichloro-3-nitrosalicylanilide are summarized in Table 3. These compounds have been arbitrarily subdivided into four groups based on substitutent patterns. The *ortho* hydroxy group in the acid moiety is common to all of the compounds while the *meta, para,* 5, and 6 position of the aniline moiety have been varied.

In all tests the 3-nitrosalicyloxylidides are less toxic to lamprey larvae than their corresponding substituted 3-nitrosalicylotoluidide. The only exception was with 3-nitrosalicyloxylidide but it was not tested at sufficiently high levels to determine if it is selective.

The loci of substituents in the aniline moiety of polysubstituted compounds appear to be correlated with toxicity to larval lamprey and rainbow trout. Also, dichloro substitutions evoke more potent activity than do chloro or methyl groups in the same positions. Activity becomes optimum when substitutions are made at the 2 and 5 positions in the aniline moiety as illustrated by 2',5'-dichloro-3-nitrosalicylanilide. Activity was minimal with compounds substituted at the 2 and 6 positions.

Structure-activity relationships seem to exist among other 3-nitro-and 5-nitrosalicylanilides screened. The data are of such a preliminary nature that we have mentioned only the more obvious correlations. For example, 4'-chloro-3-nitrosalicylanilide is more toxic to larval lamprey than to trout, but an inverse toxicity exists with 4'-phenylazo-3-nitrosalicylanilide. Certain dinitrosalicylanilides are moderately toxic to larval lampreys and demonstrate some selectivity. Substitution of more than two halogens in the aniline moiety produces a saturation effect and a loss of toxicity and selectivity. Many additional benzanilides have been tested in addition to those listed in the tables and in no instance were they active at the 10.0 ppm screening level.

Table 3. Selective toxicity of polysubstituted 3-nitrosalicylanilides to larval sea lamprey and fingerling rainbow trout as a function of atomic loci



Compound -3-nitrosalicylanilide	Substituents					Lamprey	Trout
	R_2	R_3	R_4	R_5	R_6	Woo (ppm)	LD_{25} (ppm)
2', 3'-dimethyl-	$-CH_3$	$-CH_3$		. .	. .	3.0	5.0
2'-methyl-3'-chloro-	$-CH_3$	-Cl		. .	. .	0.7	1.0
2', 4'-dimethyl-	$-CH_3$	.	$-CH_3$.	.	. .	3.0	7.0
2'-methyl-4'-chloro-	$-CH_3$.	.	-Cl .	.	. .	0.5	0.7
2', 5'-dimethyl-	$-CH_3$	.		$-CH_3$	. .	1.0	3.0
2'-methyl-5'-chloro-	$-CH_3$.	.		-Cl	.	0.5	0.9
2', 5'-dichloro-	-Cl .	.		-Cl	.	0.3	0.9
2'-methoxy-5'-chloro-	$-CH_3O$.	.		-Cl	. .	0.7	1.0
2', 6'-dimethyl-	$-CH_3$.	.		. .	$-CH_3$	>10.0	>10.0
2'-chloro-6'-methyl-	-Cl .	.		. .	$-CH_3$	0.7	1.0

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