Studies on Anesthetics for Fish*

Miyoshi HIRATA**, Sumiro ISODA**, Munefumi KANAO**,
Hiroya SHIMIZU** and Shin-ichi INOUE***

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Recently anesthetics for fish, for example, urethane, amylalcohol, tricaine methane-sulfonate (MS-222)¹⁻³⁾, phenoxetol⁴⁾, propylene phenoxetol⁵⁾, and quinaldine⁶⁾, are widely used for transportation, tagging and research. Anesthetic efficacy of chlorpromazine for *Crustacea*⁷⁾ has also been reported. Of these compounds, urethane and MS-222 had especially been used for these purposes, but nowadays the former is hardly utilized because of possible carcinogenic properties⁸⁾.

In general, the following characteristics have been required for fish anesthetics: 1) intensive anesthetic activity 2) effectiveness to both marine and fresh-water fish 3) effectiveness at low water temperature 4) quick and complete recovery from anesthesia 5) high solubility in both marine and fresh water.

Novel anthranilic ester, DP-1166 synthesized by the authors, has been found to exhibit the above characteristics.

Materials and Methods

Syntheses of Anesthetics. All anesthetics used in this study were synthesized according to the procedures described in Fig. 1. Type A compounds tested are tabulated in Table 1 and type B compounds tested are in Table 2. The chemical structure and purity of these compounds were confirmed by elementary analyses and infrared absorption spectra.

Fig. 1. Synthetic procedures of anesthetics for fish. Type A compounds (upper) are shown in Table 1 and type B compounds (lower) are shown in Table 2.

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** Research Laboratories, Daiichi Seiyaku Co., Ltd., Edogawa-ku, Tokyo, Japan (平田三四司・磯田純郎・金尾宗史・清水弘也: 第一製薬株式会社総合研究所).

*** Product Development Department (Veterinaries and Chemicals), Daiichi Seiyaku Co., Ltd., Chuo-ku, Tokyo, Japan (井上進一: 第一製薬株式会社特薬開発部).
(印刷費負担)

Table 1. Anesthetic potencies of aminoalkyl anthranilate derivatives (type A) in goldfishes at 25°C (the first screening test).

No.	R	HX	Anesthetic potency (ppm)*				RR**	Sol***
			100	60	30	10		
1	CH ₃	HCl	+++	+	+	+	+	±
2	C ₂ H ₅	HCl	+++	+++	++	±	+	±
3	C ₂ H ₅	CH ₃ SO ₃ H	+++	+++	++	±	+	±
4	C ₃ H ₇ (n)	HCl	+++	+++	++	+	+	±
5	C ₃ H ₇ (i)	HCl	++	++	±	—	+	±
6	C ₄ H ₉ (n)	HCl	++	++	++	±	±	±
7	CH ₂ C ₆ H ₅	HCl	+++	++	++	±	+	—
8	CH ₂ CH ₂ OH	HCl	+++	++	++		+	±
9	CH ₂ CH ₂ OH	HBr	+	+	+	—	—	+
10	CH ₂ CH ₂ N(CH ₃) ₂	HCl	++	++	+	±	+	+
11	CH ₂ CH ₂ N(C ₂ H ₅) ₂	HCl	+++	++	++	±	+	+
12	CH ₂ CH ₂ N(C ₃ H ₇ (n)) ₂	HCl	++	++	±	±	+	±
13	CH ₂ CH ₂ N(C ₄ H ₉ (n)) ₂	HCl				+++	±	±
14	CH ₂ CH ₂ N	HCl	++	++	++	+	+	+
15	CH ₂ CH ₂ NH	HCl	+	±	—	—		+
16	CH ₂ CH ₂ N	HCl	—	—	—	—		+
17	CH ₂ CH ₂ CH ₂ N(C ₂ H ₅) ₂	HCl	++	++	++	+	±	+
18	CH ₂ CH ₂ CH ₂ N	HCl	++	++	++	++	+	±
19	CH ₂ CH ₂ CH ₂ N	HCl	+	±	—	—	+	+
20	CH ₂ CH ₂ CH ₂ CH ₂ N(C ₂ H ₅) ₂	HCl	+	+	+	±	+	+
21	NH ₂	2CH ₃ SO ₃ H		—	—			+
22	NH ₂	2p-TsOH		±	±			+
23	NH ₂	2CH ₃ SO ₃ H	++	+	±	—	+	+
24	NH ₂	2CH ₃ SO ₃ H	++	++	±	±		+
25	NH ₂	2p-TsOH		+	±			—

* The anesthetic potency was expressed by the time required for immobilizing the fish after the addition of the test compound. +++: within 2 minutes, ++: 2 to 10 minutes, +: 10 to 60 minutes, +: depression of movement, —: ineffective.

** Recovery rate was expressed by the time at which fishes completely recovered from anesthesia in fresh water. +: within 10 minutes, ±: 10 to 60 minutes, —: over 60 minutes.

*** Solubility in water. +: easily soluble, ±: slightly soluble, —: insoluble.

Method of screening test. The first screening test: Two liters of water ($25 \pm 1^\circ\text{C}$) treated with ion exchange resin were poured into each of four 5 l. styrol vessels, and respective 5 gold-fish, *Carassius auratus*, weighing about 5 g each were kept in each vessel. Then test compounds were dissolved in the water to test their anesthetic effect. After administrating the anesthetics, the fish were transferred into fresh water to measure the recovery rate. The compounds which were proved to have the anesthetic effect by this test were subjected to the second screening test.

The second screening test: In order to examine the anesthetic effect at low temperatures, the second screening test was performed at 15 to 18°C . Other conditions were similar to those of the first screening test.

The third screening test: In this test, the puffer, *Fugu niphobles*, was used to select the compounds which were effective on the marine fish as well as the fresh-water fish. The

Table 2. Anesthetic potencies of aminoacyl anthranilate derivatives (type B) in gold-fish at 25°C (the first screening test).

No.	R	R'	R''	HX	Anesthetic potency (ppm)*				RR*	Sol*
					100	60	30	10		
26	CH ₃	C ₄ H ₉ (n)	H	HCl		±	—			+
27	CH ₃		H	HCl		+	±			+
28	C ₂ H ₅	CH ₃	H	HCl	—	—	—	—		+
29	C ₂ H ₅	C ₂ H ₅	H	HCl	±	±	—	—		+
30	C ₂ H ₅	CH ₃	CH ₃	HCl	—	—	—	—		+
31	C ₂ H ₅	C ₂ H ₅	C ₂ H ₅	1/2(COOH) ₂	+	+	+	±	+	±
32	C ₂ H ₅	C ₃ H ₇ (n)	H	HCl		±	±			+
33	C ₂ H ₅	C ₃ H ₇ (i)	H	HCl		±	—			+
34	C ₂ H ₅	C ₄ H ₉ (n)	H	HCl	±	±	—	—		+
35	C ₂ H ₅	C ₄ H ₉ (i)	H	HCl		++	++			+
36	C ₂ H ₅		H	HCl	++	++	++	±	+	+
37	C ₂ H ₅		H	CH ₃ SO ₃ H		+	+			+
38	C ₂ H ₅		H	HBr		++	±			+
39	C ₂ H ₅		CH ₃	HCl	++	+	+	+	+	+
40	C ₂ H ₅	—(CH ₂) ₄ —		HCl		+	±			+
41	C ₂ H ₅	—(CH ₂) ₅ —		HCl	+	+	+	—	+	+
42	C ₂ H ₅	—(CH ₂) ₂ O(CH ₂) ₂ —		HCl	++	++	++	+	+	+
43	C ₄ H ₉ (n)	C ₄ H ₉ (n)	H	HCl		++	+			+

* Abbreviations: see Table 1.

Table 2. (Continued)

No.	R	R'	R''	HX	Anesthetic potency (ppm)*				RR*	Sol*
					100	60	30	10		
44	CH ₃	C ₂ H ₅	C ₂ H ₅	HCl	—	—	—	—		+
45	CH ₃		H	HCl	+++	++	++	++	+	±
46	CH ₃		H	CH ₃ SO ₃ H	++	++	++	—	+	+
47	CH ₃	—(CH ₂) ₅ —		HCl	++	++	+	—	+	+
48	C ₂ H ₅	CH ₃	H	HCl	+	+	±	—	+	±
49	C ₂ H ₅	CH ₃	CH ₃	HCl	—	—	—	—		+
50	C ₂ H ₅	C ₂ H ₅	H	HCl	+	±	—	—	±	±
51	C ₂ H ₅	C ₂ H ₅	C ₂ H ₅	HCl	++	++	+	—	+	+
52	C ₂ H ₅	C ₂ H ₅	C ₂ H ₅	COOH (CHOH) ₂ COOH		±	—		+	+
53	C ₂ H ₅	C ₃ H ₇ (n)	H	HCl		—	—			+
54	C ₂ H ₅	C ₃ H ₇ (n)	C ₃ H ₇ (n)	HCl	+	+	+	—	+	±
55	C ₂ H ₅	C ₃ H ₇ (i)	H	HCl		—	—			+
56	C ₂ H ₅	C ₄ H ₉ (n)	H	HCl	++	++	+	+	+	+
57	C ₂ H ₅	C ₄ H ₉ (n)	C ₄ H ₉ (n)	HCl	+	±	±	—	+	±
58	C ₂ H ₅	C ₄ H ₉ (i)	H	HCl		++	+			+
59	C ₂ H ₅		H	HCl	+++	+++	+++	++	±	±
60	C ₂ H ₅		H	CH ₃ SO ₃ H	+++	+++	++	+	±	+
61	C ₂ H ₅		H	p-TsOH	++	++	±	—	+	±
62	C ₂ H ₅		CH ₃	HCl	++	++	++	±	+	+
63	C ₂ H ₅	—(CH ₂) ₄ —		HCl		—	—			+
64	C ₂ H ₅	—(CH ₂) ₅ —		HCl	+	+	±	—	+	±
65	C ₂ H ₅	—(CH ₂) ₂ O(CH ₂) ₂ —		HCl	—	—	—	—		+
66	C ₂ H ₅	CH ₂ — CH ₂ OH	H	HCl	—	—	—	—		+
67	C ₂ H ₅	CH ₂ — CH ₂ OH	CH ₂ — CH ₂ OH	HCl	—	—	—	—		+
68	C ₃ H ₇ (i)		H	HCl		+	+			±
69	C ₃ H ₇ (i)	—(CH ₂) ₅ —		HCl		++	+			+

* Abbreviations: see Table 1.

Table 2. (Continued)

No.	R	R'	R''	HX	Anesthetic potency (ppm)*				RR*	Sol*
					100	60	30	10		
70	C ₂ H ₅	CH ₃	CH ₃	HCl		±	—			+
71	C ₂ H ₅	C ₂ H ₅	H	HCl		±	—			+
72	C ₂ H ₅	C ₂ H ₅	C ₂ H ₅	HCl		+	+		+	+
73	C ₂ H ₅	C ₃ H ₇ (i)	H	HCl		—	—			+
74	C ₂ H ₅	C ₄ H ₉ (i)	H	HCl		+	+		+	+
75	C ₂ H ₅		H	HCl		+	+		+	+
76	C ₂ H ₅	—(CH ₂) ₄ —		HCl		—	—			+
77	C ₂ H ₅	—(CH ₂) ₅ —		HCl		±	—			+
78	C ₂ H ₅	—(CH ₂) ₂ O(CH ₂) ₂ —		HCl		±	±			+

* Abbreviations: see Table 1.

Table 2. (Continued)

No.	R	R'	R''	HX	Anesthetic potency (ppm)*				RR*	Sol*
					100	60	30	10		
79	C ₂ H ₅	CH ₃	H	1/2(COOH) ₂		—	—			±
80	C ₂ H ₅	CH ₃	CH ₃	1/2(COOH) ₂		+	—			±
81	C ₂ H ₅	C ₂ H ₅	C ₂ H ₅	(COOH) ₂		±	±			±
82	C ₂ H ₅	C ₃ H ₇ (n)	H	(COOH) ₂			—			±
83	C ₂ H ₅	C ₃ H ₇ (i)	H	HCl		++	++		+	+
84	C ₂ H ₅	C ₄ H ₉ (n)	H	HCl		+	+		+	+
85	C ₂ H ₅	C ₄ H ₉ (i)	H	HCl		++	+		+	+
86	C ₂ H ₅		H	HCl		+	+		+	±
87	C ₂ H ₅		CH ₃	HCl	+	+	+	±	—	+
88	C ₂ H ₅	—(CH ₂) ₄ —				++	++		+	±
89	C ₂ H ₅	—(CH ₂) ₅ —		HCl		++	++		+	+
90	C ₃ H ₇ (i)	—(CH ₂) ₅ —		HCl		+	+		+	+
91	C ₄ H ₉ (n)	—(CH ₂) ₅ —		HCl		++	++		+	±
92	C ₂ H ₅	—(CH ₂) ₂ O(CH ₂) ₂ —		HCl		++	+		—	+

* Abbreviations: see Table 1.

experimental procedures were the same as those of the first screening test except for the use of artificial marine water instead of fresh water.

In addition, favourable compounds were subjected to the anesthetic test in various kinds of fish, i.e., carp, *Cyprinus carpio*, rainbow trout, *Salmo gairdnerii* f. *irideus*, yellow tail, *Seriola macrocephalus*, pearl eye, *Girella punctata*, and the parrot bass, *Oplegnathus fasciatus*.

Acute toxicity. The acute toxicity was expressed in TLm in gold-fish weighing 2 to 3 g and LD₅₀ in male mice of ddYF strain, 19–23 g in body weight.

TLm (24-hours). TLm was calculated from the mortalities which were obtained after 24-hours of keeping the fish in 6 l. of tap water solution of the test compound. In this case, the fish treated with the solution were transferred into 6 l. of fresh water for 1 hour to ascertain whether or not they survived.

LD₅₀ (72-hours). LD₅₀ in mice was determined by intravenous and subcutaneous injections.

TLm (24-hours) and LD₅₀ (72-hours) were calculated according to the method of LITCHFIELD and WILCOXON⁹⁾.

Results and Discussion

The results of the first screening test are presented in Tables 1 and 2. The anesthetic potencies of some compounds of type A were fairly high in gold-fish at 25°C. However, since these compounds, except slightly soluble compounds i.e., Nos. 1, 4, and 6, exhibited the low potency by the pre-examination in rainbow trout at a low temperature of about 5°C, the compounds of another type (type B compounds) were synthesized to test the anesthetic effect. As shown in Table 2, effective compounds were also found in this group. In view of the relationship between the chemical structure and potency of the compounds in this group, the compounds with $n=1$ and $n=2$ in $\text{NHCO}(\text{CH}_2)_n\text{NR}'\text{R}''$ moiety were found to be more effective in anesthetizing gold-fish than the compounds with $n=3$. However, as described above, the high solubility of anesthetics and easy recoverability of the fish from anesthesia in fresh water are required for their practical use. In consideration of these respects, 8 compounds (Nos. 36, 39, 45, 47, 51, 56, 60, and 62) that seemed to be favourable for fish anesthetics were selected and subjected to the test at low temperature (the second screening test). The results showed that a compound No. 36, DP-1166, exhibited the highest potency as presented in Table 3. Further, the results of the third screening test (Table 4) indicate that this compound possesses more desirable properties than the other compounds tested in view of the effectiveness on puffer. And as shown in Table 5, the effectiveness of DP-1166 on various kinds of marine and fresh-water fish, i.e., carp, rainbow trout, yellow tail, pearl eye, and parrot bass, was superior to that of MS-222.

Table 3. Anesthetic potencies of aminoacyl anthranilate derivatives (type B) in gold-fish at 15–18°C (the second screening test).

No.	R	n	R'	R''	HX	Anesthetic potency (ppm)*				RR*	Sol*
						100	60	30	10		
36	C ₂ H ₅	1		H	HCl	++	++	++	±	+	+
39	C ₂ H ₅	1		CH ₃	HCl	++	++	+	±	+	+
45	CH ₃	2		H	HCl	+	+	±	—	+	+
47	CH ₃	2	—(CH ₂) ₅ —		HCl	++	+	±	—	+	+
51	C ₂ H ₅	2	C ₂ H ₅	C ₂ H ₅	HCl	++	++			+	+
56	C ₂ H ₅	2	C ₄ H ₉ (n)	H	HCl	±	±	±	±	+	+
60	C ₂ H ₅	2		H	CH ₃ SO ₃ H	++	++	+	±	+	+
62	C ₂ H ₅	2		CH ₃	HCl	±	±	±	±	+	+

* Abbreviations: see Table 1.

Table 4. Anesthetic potencies of some compounds in puffer at 24°C.

No.	Compounds	Anesthetic potency (ppm)**				RR**	Sol*
		100	60	30	10		
36 (DP-1166)	HCl	+++	+++	++	++	+	+
39	HCl	++	++	++	++	+	+
51	HCl	+	—	—	—	—	+
60	CH ₃ SO ₃ H	++	+	+	—	+	+
MS-222	CH ₃ SO ₃ H	+++	++	+	+	+	+

* Sol: Solubility in artificial marine water. ** Other abbreviations: see Table 1.

Table 5. Anesthetic potencies of DP-1166 and MS-222 in various fish.

Species of fish	Compound	Anesthetic potency (ppm)*								RR*	Water temperature (°C)
		100	60	50	30	25	12.5	10	5		
Carp (7 g)**	DP-1166	++	+		+			+		+	23
	MS-222	+	+		+			-		+	
Rainbow trout	DP-1166	+++		+++		++		++	+	+	16
Yellow tail (100 g)**	DP-1166			+++		+++	+++		+	+	22.3
	MS-222			++		+				+	
Pearl eye (50-70 g)**	DP-1166			+++		+++	+++			+	22.3
	MS-222			++		++				+	
Parrot bass (85 g)**	DP-1166					++	++			+	20-21
	MS-222					+	+			+	

* Abbreviations: see Table 1.

** Figures in parentheses indicate the body weight.

Table 6. Toxicity of DP-1166.

1. TLm in gold-fish.

Waer used. Tap water allowed to stand overnight at room temperature.

Concentration of DP-1166 (ppm)	mortality
10	6/16
14.4	5/16
20.8	8/16
30	16/16

TLm (24-hours) = 20.2 (16.4-24.8) ppm*

2. LD₅₀ in mice.

Dose (mg/kg)	Administration route	mortality
40	i.v.**	1/10
45	i.v.**	4/10
50	i.v.**	5/10
60	i.v.**	8/10

LD₅₀ = 48.7 (44.2-53.5) mg/kg*

1,000	s.c.***	0/10
2,000	s.c.***	2/10

LD₅₀ > 2,000 mg/kg

* Figures in parentheses indicate 95% fiducial limits.

** Intravenous injection.

*** Subcutaneous injection.

In regard to the toxicity of DP-1166, 20.2 ppm of TLm (24-hours) was obtained by the test in gold-fish kept in tap water and then allowed to stand overnight at room temperature. On the other hand, its LD₅₀ values in mice were 48.7 mg (intravenous injection) and over 2000 mg (subcutaneous injection) per Kg of body weight, which suggests the poor absorption of this compound through the subcutaneous tissue in mammals.

Summary

Ninety-two anthranilate derivatives were synthesized and examined for the anesthetic effect of these compounds on gold-fish and puffer. From these results, it is clear that compound No. 36, DP-1166, is the most potent anesthetic for fish and this compound also showed an excellent effectiveness on other kinds of fish.

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