[Chem. Pharm. Bull. 31(1) 162—167 (1983)]

New Chromogenic Substrates for the Assy of Esterases——Acetates and Butyrates of Phenolic Naphthylazo Compounds with Sulfonic Acid Group

SHINICHI KAMACHI,[a] KIYOSHIGE WAKABAYASHI,[a] KIYOSHI ZAITSU,[b] and YOSUKE OHKURA*,[b]

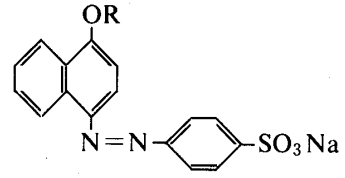
New Drug Research Laboratories, Chugai Pharmaceutical Co., Ltd.[a] Takada, Toshima-ku, Tokyo 171, Japan and Faculty of Pharmaceutical Sciences, Kyushu University 62,[b] Maidashi, Higashi-ku, Fukuoka 812, Japan

(Received July 17, 1982)

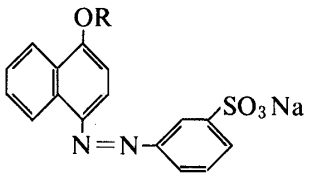
Eight esters (acetates and butyrates) of four phenolic naphthylazo compounds with sulfonic acid group(s) were synthesized and assessed as water-soluble chromogenic substrates for the assay of esterases. The absorption maxima of azo compounds produced enzymatically from these esters occur at wavelengths about 100 nm longer than those of the esters and their molar absorptivities are higher than those of the esters. Investigations on the substrate specificities of these esters revealed that all the esters are hydrolyzed by carboxyl esterase- and aryl esterase-mediated reactions, and butyrates of *m*-(4-hydroxy-1-naphthylazo)benzenesulfonic acid and Azorubin should be particularly useful as substrates for cholinesterase and aryl esterase, respectively.

Keywords——ester of phenolic naphthylazo compound; chromogenic substrate; water-soluble substrate; carboxyl esterase; aryl esterase; cholinesterase; esterase activity in serum

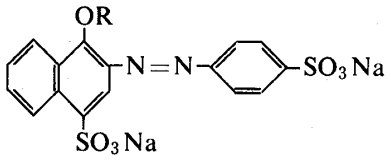
Carboxyl esterase (carboxylic ester hydrolase, EC 3.1.1.1), aryl esterase (aryl ester hydrolase, EC 3.1.1.2) and cholinesterase (acylcholine acyl-hydrolase, EC 3.1.1.8; pseudocholinesterase) catalyze hydrolyses of esters of aliphatic alcohols, esters of phenols and esters of choline



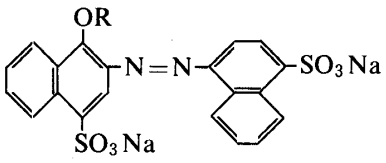
Orange I: R=H
Orange I acetate:[a] R=$COCH_3$
Orange I butyrate: R=$COCH_2CH_2CH_3$



HNB: R=H
HNB acetate: R=$COCH_3$
HNB butyrate: R=$COCH_2CH_2CH_3$



SHNB: R=H
SHNB acetate: R=$COCH_3$
SHNB butyrate: R=$COCH_2CH_2CH_3$



Azorubin S: R=H
Azorubin S acetate: R=$COCH_3$
Azorubin S butyrate: R=$COCH_2CH_2CH_3$

Chart 1. Structures of Phenolic Azo Compounds and Their Esters

a) Known compound.[12]

or its analogs, respectively. The clinical significance of carboxyl esterase *in vivo* has not been elucidated. It has been reported that aryl esterase is useful in diagnosing leukemia, since the enzyme is present on the leukocyte membrane.[3-9] In liver diseases, a decrease in activity of cholinesterase in serum has been observed.[2] Synthetic chromogenic substrates used for measuring the activities of enzymes, except for cholinesterase, are poorly soluble in water.[3-9] Therefore the enzymatic reactions are run with suspended substrates, but these must be removed before measurement of the absorbance.

We synthesized as water-soluble substrates lower fatty acid esters of phenolic naphthylazo compounds with sulfonic acid group(s), acetates and butyrates of *p*-(4-hydroxy-1-naphthylazo)-benzenesulfonic acid sodium salt (Orange I), *m*-(4-hydroxy-1-naphthylazo)-benzenesulfonic acid disodium salt(HNB), *p*-(1-sulfo-4-hydroxy-2-naphthylazo)-benzenesulfonic acid disodium salt (SHNB) and 4-(1-sulfo-4-hydroxy-2-naphthylazo)-naphthalenesulfonic acid disodium salt (Azorubin S) (Chart 1), and studied their substrate specificities with such esterases as carboxyl esterase, aryl esterase and cholinesterase using human serum and purified enzyme preparations.

Experimental

Materials———All chemicals were of reagent-grade, unless otherwise noted. Orange I, Azorubin S, physostigmine sulfate, and parathion (standard) were obtained from Wako Pure Chemical Industries Ltd. (Japan), and HNB and SHNB were synthesized according to the literature.[10] Cholinesterase (from human serum, 3—9 U/mg protein, lyophilized powder), carboxyl esterase (from porcine liver, approximately 100 U/mg protein, suspension in 3.2 M $(NH_4)_2SO_4$), cholesterol esterase (from bovine pancreas, 2000—4000 U/g protein, lyophilized powder) and lipase (from hog pancreas, 51100 U/mg protein, lyophilized powder) were obtained from Sigma Chemical Co. (U.S.A.). Aryl esterase was separated from human serum and purified according to the method of Kurooka *et al.* (approximately 30 U/mg protein).[11] Normal human sera were obtained from Kyushu University Hospital.

Syntheses of Esters of Azo Compounds———Azo compounds (25 mmol) was dissolved in 100 ml of dimethylformamide, then 10 ml of pyridine and 10 ml of acetic anhydride or butyric anhydride were added and the mixture was heated at 70—90°C for 30 min.

The precipitate formed on cooling was filtered off and recrystallized from a dimethylformamide–acetone mixture.

All the synthesized esters were pale yellow crystalline powders; yield 70—75%; mp over 250°C (dec.).

Apparatus———Infrared (IR) spectra were measured in KBr pellets, with a Hitachi 260-30 grating infrared spectrophotometer and ultraviolet and visible spectra were taken with a Hitachi 124 spectrophotometer in 10-mm quartz cells. pH was measured with a Hitachi-Horiba M-7 pH meter at 25°C. Melting points are uncorrected.

Standard Procedure for Testing of Esters———The synthetic esters were dissolved in 0.1 M phosphate-borate buffer (pH 7.5) at the concentration of 0.5 mM, except for Orange I butyrate, which was dissolved in 0.1 M Tris-HCl buffer (pH 7.5). To 3 ml of this solution, 10 μl of serum or enzyme solution was added. The mixture was incubated at 37°C for about 20 min, and then the increment of absorbance per minute (ΔA/min) at the absorption maximum wavelength (see Table I) was measured in the range of linear change in the absorbance. For the blank, the substrate solution without addition of the serum or enzyme solution was treated in the same way.

When enzyme inhibitor was used, 10 μl of serum was added to 100 μl of the inhibitor solution which was prepared so as to give the indicated concentration in the enzyme reaction mixture (see Tables V and VI), then the solution of the ester was added after preincubation for 10 min, and the same procedure as above was carried out.

Results and Discussion

All the synthesized substances show IR absorption caused by stretching vibration of the carbonyl group around 1750 cm^{-1}, absorptions ascribable to stretching vibrations of methylene and methyl groups of the fatty acid moiety around 2950 and 2850 cm^{-1} and absorptions due to deformation vibrations of methylene and methyl groups of the fatty acid moiety around 1460 and 1380 cm^{-1}. These results and the elemental analytical data (Table I) suggest that the synthesized substances are the esters of the corresponding azo compounds.

TABLE I. Elemental Analytical Data for Esters of Azo Compounds

	Formula	C (%)		H (%)		N (%)	
		Calcd	Found	Calcd	Found	Calcd	Found
Orange I butyrate	$C_{20}H_{17}N_2NaO_5S$	57.14	57.33	4.08	3.95	6.66	6.42
HNB acetate	$C_{18}H_{13}N_2NaO_5S$	55.10	54.92	3.34	3.30	7.14	7.33
HNB butyrate	$C_{20}H_{17}N_2NaO_5S$	57.14	57.41	4.08	4.24	6.66	6.82
SHNB acetate	$C_{18}H_{12}N_2Na_2O_8S_2$	43.73	43.52	2.45	2.73	5.67	5.61
SHNB butyrate	$C_{20}H_{16}N_2Na_2O_8S_2$	45.98	45.73	3.09	3.32	5.36	5.24
Azorubin S acetate	$C_{22}H_{14}N_2Na_2O_8S_2$	48.53	48.23	2.59	2.43	5.14	5.31
Azorubin S butyrate	$C_{24}H_{18}N_2Na_2O_8S_2$	50.34	50.32	3.17	3.52	4.89	4.81

As shown in Table II, all the esters in aqueous solution show absorption maxima at wavelengths about 100 nm shorter than those of the azo compounds. Molar absorptivities of the azo compounds are high, being greater than those of the esters. As an example, the absorption spectra of solutions of HNB and its acetate and butyrate are shown in Fig. 1. This provides a basis for a convenient, sensitive colorimetric assay of esterase activity by using these esters as substrates. The esters of Orange I and HNB can be dissolved in water or the buffers used in the standard procedure to give concentrations of by more than 2 mM at room temperature (25°C) and those of SHNB and Azorubin S can give concentrations of more than 5 mM. This solubility is sufficient for the standard procedure.

These esters were scarcely decomposed in 0.1 M phosphate-borate buffer (pH 7.5) and 0.1 M phosphate buffer (pH 7.5) at 37°C (decomposition rate, ΔA/min, 0.000—0.002), but decomposed slightly in 0.1 M Tris-HCl buffer (pH 7.5) (decomposition rate, ΔA/min, 0.004—0.021). When Orange I butyrate was dissolved in buffers containing phosphate, a precipitate was formed for an unknown reason.

The potential of these esters as substrates for esterases was preliminarily examined using normal human serum. It was found that all the esters were hydrolyzed by enzymes in the

TABLE II. Ultraviolet Absorption Maxima and Molar Absorptivities (ε) of Azo Compounds and Their Esters

	Absorption maximum (nm)	log ε
Orange I[a,b]	475	4.43
Orange I acetate[a]	377	4.08
Orange I butyrate[b]	378	4.12
HNB[a]	475	4.45
HNB acetate[a]	374	4.01
HNB butyrate[a]	374	4.04
SHNB[a]	495	4.35
SHNB acetate[a]	333	4.21
SHNB butyrate[a]	333	4.31
Azorubin S[a]	507	4.22
Azorubin S acetate[a]	394	4.06
Azorubin S butyrate[a]	393	4.08

a) Measured in 0.1 M phosphate-borate buffer (pH 7.5).
b) Measured in 0.1 M Tris-HCl buffer (pH 7.5).

serum, and in general the highest hydrolysis rate was attained in the 0.1 M phosphate-borate buffer. The optimal pH was in the range of 7.2—8.4 (Table III). Apparent K_m values for the esters were low in general (Table III). This suggests that the affinity of each ester for esterases in the serum is high.

On the basis of the above observations, the enzyme reactions with the esters were carried out in 0.1 M phosphate borate buffer (0.1 M Tris-HCl buffer for the reaction with Orange I butyrate) of pH 7.5 at an ester concentration of 0.5 mM.

Degrees of hydrolysis of the esters by enzyme preparations and normal human serum are shown in Table IV. The esters of SHNB and Azorubin S are selective substrates for carboxyl esterase and aryl esterase, respectively. The esters of Orange I and HNB are hydrolyzed by the reactions mediated by carboxyl esterase, aryl esterase and cholinesterase. Orange I butyrate is hydrolyzed in the presence of cholesterol esterase. Lipase slightly catalyzes the hydrolyses of the esters of SHNB and the butyrate of Azorubin S. Serum hydrolyzes all the synthetic esters (Table IV).

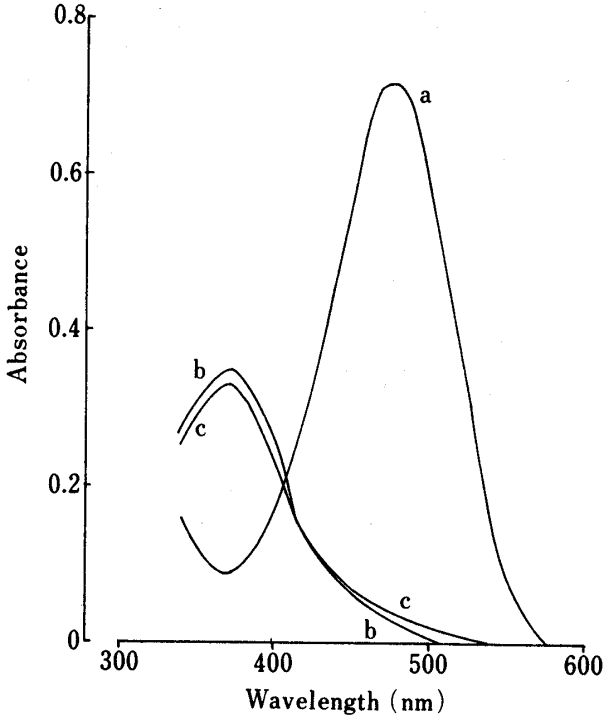


Fig. 1. Absorption Spectra of HNB, HNB Acetate and HNB Butyrate (28.5 μM Phosphate-Borate Buffer (pH 7.5)

a, HNB; b, HNB acetate; c, HNB butyrate.

TABLE III. Optimal pHs for the Reactions of Esterase present in Normal Human Serum and Apparent K_m Values for the Esters of Azo Compounds[a)]

Orange I acetate	7.2—7.8	0.04
Orange I butyrate	7.5—8.1	0.05
HNB acetate	7.5—7.8	0.16
HNB butyrate	7.5—8.1	0.08
SHNB acetate	7.5—7.8	0.04
SHNB butyrate	7.2—7.8	0.04
Azorubin S acetate	7.3—8.1	0.06
Azorubin S butyrate	7.5—8.4	0.04

a) Portions (10 μl) of serum were treated as in the standard procedure.

In order to clarify esterase(s) in serum which acted on the individual esters, an investigation was carried out by using enzyme inhibitors, parathion (inhibitor of both carboxyl esterase and cholinesterase),[13,14] ethylenediaminetetraacetic acid (EDTA) (inhibitor of aryl esterase,[13] physostigmine sulfate (inhibitor of cholinesterase),[14] quinine sulfate and sodium fluoride (inhibitors of lipase)[15] and sodium cholate (inhibitor of carboxyl esterase, aryl esterase and cholinesterase).[13,14] The results, shown in Table V suggest that the esters of Orange I are hydrolyzed by aryl esterase, those of HNB by cholinesterase, aryl esterase and carboxyl esterase, and those of both SHNB and Azorubin S by aryl esterase. In particular, physostigmine sulfate and EDTA strongly inhibit the enzyme reactions with butyrates of HNB and

TABLE IV. Hydrolysis of the Esters by Enzyme Preparations and Normal Human Serum[a]

	Hydrolysis rate[b] in the presence of enzyme preparation or serum					
	Carboxyl esterase (2 mU)	Aryl esterase (32.5 mU)	Cholin-esterase (3.45 mU)	Cholesterol esterase (51.1 U)	Lipase (300 mU)	Serum (10 μl)
Orange I acetate	++	++	++	−	−	++
Orange I butyrate	++	+	++	++	−	++
HNB acetate	++	+	++	−	−	++
HNB butyrate	++	+	++	−	−	++
SHNB acetate	++	++	−	−	−	++
SHNB butyrate	++	+	−	−	−	++
Azorubin S acetate	++	++	−	−	−	++
Azorubin S butyrate	+	++	−	−	−	++

a) Portions (10 μl) of enzyme solutions of serum were treated according to the standard procedure.
b) Hydrolysis rate (ΔA/min): ++, > 0.01; +, 0.001 − 0.01; −, < 0.001.

TABLE V. Effects of Various Inhibitors on Hydrolysis of the Esters by Serum Esterases[a]

	Activity[b] of serum esterases in the presence of inhibitor[c]					
	Parathion (0.1 mM)	Physostigmine sulfate (0.1 mM)	EDTA (5 mM)	Quinine sulfate (0.1 mM)	Sodium fluoride (10 mM)	Sodium cholate (2 w/v%)
Orange I acetate	100	100	55	100	100	0
Orange I	100	100	73	N.D.[d]	100	0
HNB	92	79	53	100	93	8
HNB	88	21	83	N.D.	87	0
SHNB	100	100	47	100	100	69
SHNB	100	100	73	100	100	75
Azorubin S acetate	100	100	44	100	100	23
Azorubin S butyrate	100	100	28	N.D.	100	0

a) Portions (10 μl) of serum were treated according to the standard procedure.
b) Activity in the absence of inhibitor was taken as 100.
c) The concentration of inhibitor was that in the enzyme reaction mixture.
d) Not determined successfully.

Azorubin S and so these esters should be good substrates for cholinesterase and aryl esterase, respectively.

The suitability of the esters for use as substrates for the colorimetric assay of esterase was examined under the conditions of the standard procedure using the acetate and butyrate of HNB as model esters and cholinesterase as an enzyme. The rates of the enzyme reactions with both esters increased in proportion to the concentration of the enzyme up to at least 17.2 mU/10 μl. The rates (ΔA/min) at the enzyme concentration of 17.2 mU/10 μl were 0.041 for HNB acetate and 0.061 for HNB butyrate. The enzyme activity was also checked in the presence of physostigmine sulfate at various concentrations (Table VI). Physostigmine sulfate inhibited almost all the activity at a concentration of 1×10^{-6} M or greater. Therefore, cholinesterase in serum may be assayed in terms of the difference between total esterase

TABLE VI. Effect of Physostigmine Sulfate Concentration on the Hydrolysis of HNB Acetate and HNB Butyrate by Cholinesterase[a]

	Hydrolysis rate[b] in the presence of physostigmine sulfate (μM)					
	0	0.1	1	10	100	500
HNB acetate	0.041	0.003	0.002	0.001	0.000	0.000
HNB butyrate	0.063	0.005	0.002	0.001	0.000	0.000

a) Portions (10 μl) of cholinesterase solution (17.2 mU) were treated according to the standard procedure.
b) ΔA/min.

activity obtained with HNB acetate or HNB butyrate as the substrate without addition of inhibitor and the esterase activity in the presence of physostigmine sulfate and EDTA.

The water-soluble chromogenic substrates newly found in this study should be useful for the assay of esterase activity in serum and other biological materials.

Acknowledgement We are grateful to the Central Clinical Laboratory of Kyushu University Hospital for the supply of sera.

References

1) W.K.W. Lam, J. Chen, E. Taft, and L.T. Yan, *Clin. Chem.*, **24**, 1177 (1978).
2) For example, H. Takahashi, *Rinsho Byori*, **5**, 94 (1957).
3) W. Pily, *Hoppe-Seyler's Z. Physiol. Chem.*, **328**, 1 (1962).
4) W.N. Aldridge, *Biochem. J.*, **53**, 110 (1953).
5) Y. Kobayashi, *Seikagaku*, **36**, 335 (1964).
6) A.C. Morrow and A.G. Motulsky, *J. Lab. and Clin. Med.*, **71**, 350 (1968).
7) G. Szaog, *Clin. Chem.*, **14**, 646 (1968).
8) J.A. Rider, H.C. Moeller, and K.P. DuBois, *Proc. Soc. Exp. Biol. Med.*, **76**, 427 (1951).
9) D.N. Kramer and R.H. Gamson, *Anal. Chem.*, **30**, 251 (1958).
10) J.B. Muller, L. Blangey, and H.E. Fierz-David, *Helv. Chim. Acta*, **35**, 2579 (1952).
11) S. Kurooka, S. Okamoto, and M. Hashimoto, *J. Biochem.* (Tokyo), **81**, 361 (1977).
12) R.E. Reeves, *J. Am. Chem. Soc.*, **97**, 6019 (1975).
13) K.B. Augustinsson, *Ann. N. Y. Acad. Sci.*, **94**, 844 (1961).
14) W. Kalow and R.O. Davies, *Biochem. Pharmacol.*, **1**, 183 (1958).
15) C. Hollet and H.C. Meng, *Am. J. Physiol.*, **184**, 428 (1956).
16) H.J. Raderecht, *Clin. Chim. Acta*, **4**, 221 (1959).
17) G.M. Nishart, *Biochem. J.*, **14**, 406 (1920).