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Fluorescent Substrates for Potential Use in Enzyme-Linked Immunosorbent Assay of Membrane-Bound Nucleic Acids

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A number of phosphorylated fluorochromes were synthesized and tested for the alkaline phosphatase-linked fluorescence assay of membrane-bound nucleic acids. 3-Hydroxy-*N*-2′-biphenyl-2-naphthalenecarboxamide phosphate ester (HBNP) was found to be the most suitable for the assay. Nonfluorescent HBNP is hydrolyzed by phospholipase bound to the probe DNA to the highly fluorescent 3-hydroxy-*N*,2′-biphenyl-2-naphthalenecarboxamide (HBN). HBN is insoluble enough in aqueous medium to precipitate on the nylon membrane. Spots containing as little as 5 fg of λ DNA can be successfully detected on the membrane with HBNP.

The DNA hybridization technique is a powerful tool in molecular biology and DNA diagnosis. Radioactively labeled DNA probes have been widely used for this purpose, but have been replaced recently by nonradioactive versions. Nonradioisotopic (non-RI) detection kits hitherto available are classified into two types: enzymatic and nonenzymatic methods. An example of the latter is a method using a fluorescent isothiocyanate-labeled probe.[1-3] It is widely used for in situ hybridization, but is not applicable to the detection of DNA hybrids on the nylon membrane because of the high fluorescent noise caused by the membrane. On the other hand, the enzymatic method is more suitable for the detection of membrane-bound nucleic acids. This owes much to the development of both the colorimetric method, utilizing 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium,[4-6] and the chemiluminescent method, utilizing a chemiluminescent substrate such as adamantyl-1,2-dioxetane phosphate (AMPPD)[7,8] for the detection of the phosphatase coupled to an antibody against haptenized probes. Although the isotopic detection is generally more sensitive than the non-RI enzymatic methods, it has been reported that the enzymatic detection shows higher sensitivity than the isotopic method in detection of membrane-bound nucleic acids under certain limited conditions. In search for a more sensitive enzymatic method, attempts have been made in our laboratory to synthesize fluorochromes which can be used as substrates for alkaline phosphatase, because it has been reported that a ~100-fold increase of sensitivity has been achieved by the enzyme-linked fluorescence assay (ELFA) as compared with an enzyme-linked immunosorbent assay using a colorimetric substrate.[9] The time-resolved fluorescence immunoassay using europium or terbium chelates[10,11] has been used to detect DNA hybrids in solutions, but has not been applied to membrane-bound nucleic acids because of (i) a high self-fluorescence of the nylon membrane, (ii) low substantivity (affinity to membranes) of fluorescent products, and (iii) quenching of their fluorescence when bound to the nylon membrane. In order to develop a highly sensitive enzymatic method, it is necessary to design fluorogenic compounds that should be good substrates for alkaline phosphatase and, after hydrolysis, should become water-insoluble substances with high fluorescence to precipitate on the membrane; such fluorochrome should have an emission wavelength longer than 450 nm, at which no inhibition by membrane fluoresence could be present. We report the synthesis of a number of aromatic carboxamide derivatives and their properties in solution and on the nylon membrane as fluorescent substrates for the enzyme-linked fluorescence assay of membrane-bound nucleic acids.

EXPERIMENTAL SECTION

Design of Fluorescent Substrates. Figure 1 shows schematically the ELFA detection method of nucleic acids on nylon membrane. Alkaline phosphatase having an anti-DIG

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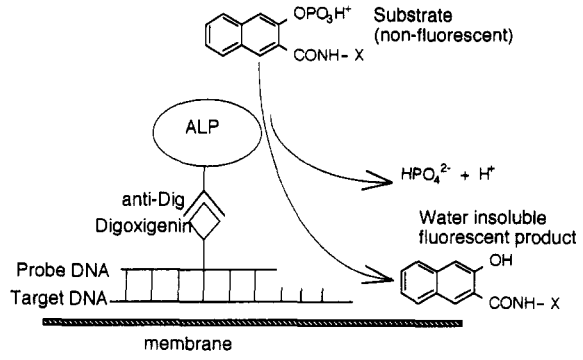

Figure 1. Detection of nucleic acids by enzyme-linked fluorescence assay on nylon membrane.

Table 1. Crystallographic Experiments of HBN

formula	$C_{23}H_{17}NO_2$
formula weight	339.39
crystal system	monoclinic
space group	$P2_{1/c}$
a (Å)	17.925(4)
b (Å)	6.716(2)
c (Å)	15.772(4)
α (deg)	90
β (deg)	114.70(2)
γ (deg)	90
Z	4
V (Å^3)	1724.9(7)
ρ_c (g/cm^3)	1.307
crystal size (mm)	0.12 × 0.14 × 0.3
μ (cm)	0.778
$2\theta_{max}$ (deg)	60.0
no. of unique reflections	5609
no. of observed reflections with $F_o > 3\sigma(F_o)$	2780
R (%)	7.27
R_w (%)	6.68
S (goodness of fit)	1.076
no. of reflections used at the last stage of least squares fitting	2780

antibody is first linked to the digoxigenin-labeled DNA bound to a target DNA and then hydrolyzes fluorogenic substrates to water-insoluble fluorochromes. Thus, target nucleic acids can be visualized as fluorescent signals of the fluorochromes precipitated on the nylon membrane. Before enzymatic reaction, the substrate is not fluorescent and soluble in water, but after the reaction, the products become highly fluorescent and precipitate on the nylon membrane. In order to design such fluorescent substrates, naphthalene and anthracene rings were used as rigid molecular skeletons, and the carboxyamide group was linked at the 3-position to other aromatic rings.

Synthesis of N-Substituted 3-Hydroxy-2-naphthalenecarboxamide. A suspension of 3-hydroxy-2-naphthoic acid (0.053 mol) and an aniline derivative (0.048 mol) in dry xylene were stirred at 80 °C for 10 min. To the mixture was added PCl_3 (0.018 mol), and the mixture was heated under reflux for 2 h to form precipitates. After the mixture was cooled, the solubent was removed by decantation. To the resulting solid was added 3% HCl, and the mixture was heated for 10 min at 80 °C. Precipitates were filtered and washed with hot water to give the 3-hydroxy-2-naphthalenecarboxamide, which was recrystallized from dimethylformamide–methanol (1:1).

Table 1 shows the results of X-ray crystal structure analysis of 3-hydroxy-*N*-2′-biphenyl-2-naphthalenecarboxamide (HBN). Three-dimensional intensity data were obtained by graphite-monochromatized Mo Kα radiation (λ = 0.71073 Å) with a Rigaku AFC-5 diffractometer. The $\omega - 2\theta$ scan technique was employed at a scan rate of 2 deg min^{-1}. The crystal structure, solved by a direct method (MULTAN78), is shown in Figure 2. The dihedral angles between the naphthalene ring and the benzene ring bonded to the amide group, between the *a* naphthalene ring and another benzene ring, and between two benzene rings are 35.8°, 77.2°, and 59.5°, respectively. The OH group is situated near the oxygen of the carbonyl group of an adjacent molecule by intermolecular hydrogen bonding.

Synthesis of the Phosphate Esters of N-Substituted 3-Hydroxy-2-naphthalenecarboxamides. The N-substituted 3-hydroxy-2-naphthalenecarboxamide (3 g) was heated at 50 °C for 1 h with 5 mol equiv of PCl_5 in dioxane (120 mL). The reaction mixture was poured into cold water (300 ml) and stirred for 5 min. The precipitate was filtered, dried, and dissolved in dimethylformamide (4 mL). To the solution was added 0.1 N sodium carbonate (300 mL), and the mixture was stirred at 0 °C for 2 h, to give precipitates which were filtered through a 0.2-μm nylon membrane filter. To the filtrate was added 30% HCl to give a precipitate which was filtered, washed with dilute hydrochloric acid, and dried in vacuo, affording the phosphate ester as a colorless solid.

Synthesis of the Phosphate Esters of N-Substituted 3-Hydroxy-2-anthracenecarboxamides. 2-Hydroxy-3-anthranoic acid was prepared according to the method of Joshi et al.[12] Both the carboxamides and the phosphate esters were prepared in the same manner as above.

Screening of Substrates. Fluorescent intensities of fluorochromes in solution (3 μM in DMF) were measured with a spectrofluorophotometer (RF-5000, Shimadzu). Detection limits were determined by spot tests as follows. λ DNA was labeled with digoxigenin (DIG) using a DNA labeling and detection kit (Boehringer Mannheim). The labeled DNA was diluted with 10 mM tris (pH 8), 1 mM EDTA, and 100 ng/μL carrier DNA to make a dilution series of 2.5 fg–10 ng and 100 ng/μL. A 1-μL aliquot of each dilution was then spotted on nylon membrane (Biodyne A, 0.45 μM, Pall) and baked at 80 °C for 30 min in vacuo. The membrane was then soaked in 0.1% nonfat dry milk solution for 30 min and treated with phosphatase-labeled anti-DIG-Fab fragment for 30 min, followed by washing with buffer (0.1 M tris, pH 7.5, 0.15 M NaCl) three times for 10 min each. Enzymatic reaction was performed with a substrate solution (0.3 mM synthesized fluorescent substrate, 100 mM tris, pH 8, 100 mM NaCl, 50 mM $MgCl_2$) for 2 h at 37 °C, and the membrane was treated with 0.5 N NaOH. Fluorescent signals were detected under UV light (302 nm, 2 mW/cm^2).

Southern Blot Hybridization. A dilution series of *Hin*dIII-digested λ DNA was electrophoresed on 0.8% agarose gels (5 × 6 × 0.6 cm; sample well 0.5 × 0.1 × 0.5 cm) with a tris–acetate buffer (pH 8.3). After electrophoresis, depurination, and denaturation, the DNA was transferred onto nylon membrane (Biodyne A, 0.2 μm, Pall), using a VacuGene transfer apparatus (LKB), and fixed by UV irradiation. Hybridization was performed in 5 × SSC (1 × SSC: 150 mM sodium citrate, pH 7, 150 mM NaCl) –5% nonfat dry milk

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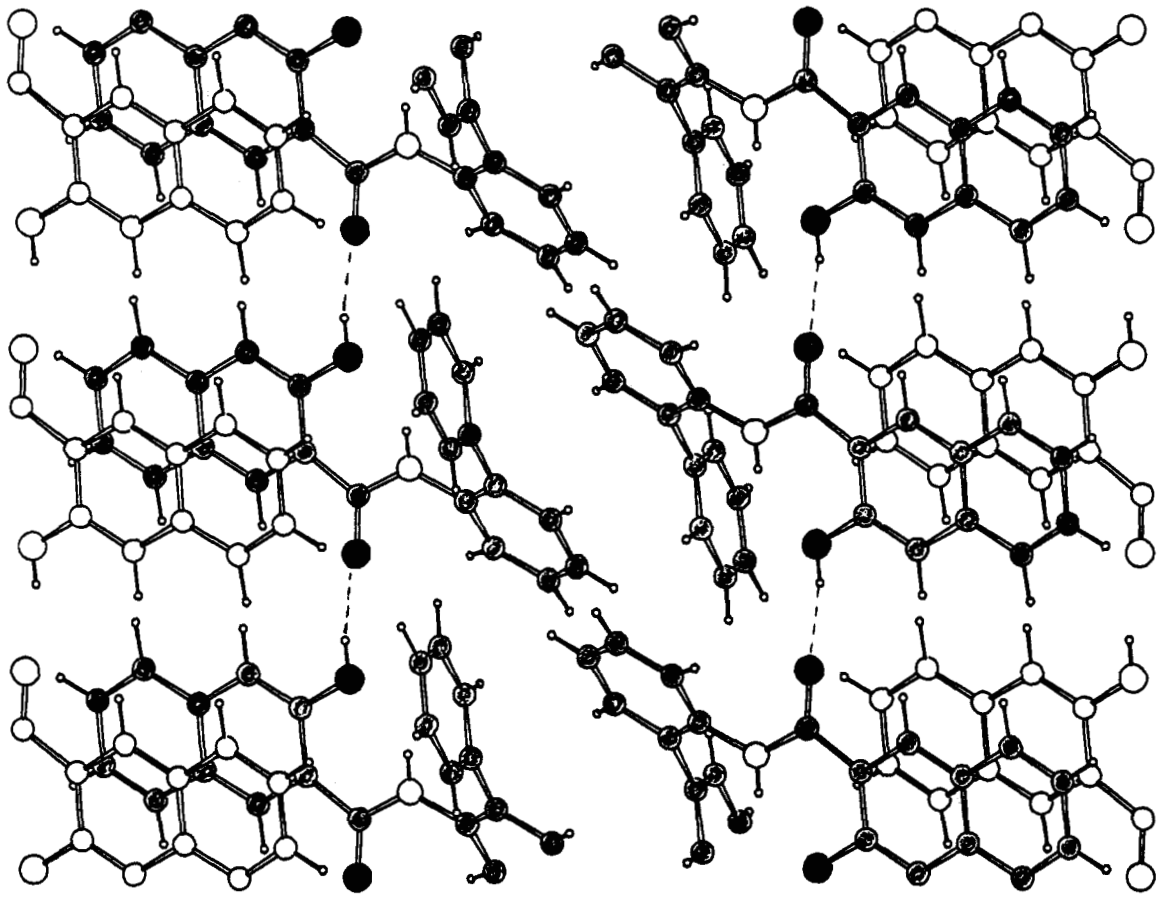


Figure 2. Crystal structure of HBN.

–0.1% *N*-lauroylsarcosine sodium salt–0.02% sodium dodecyl sulfate (SDS) –50% formamide–denatured digoxigenin-labeled *Hin*dIII-digested λ DNA (50 ng/mL) for 16 h at 42 °C. After washing in 0.1 × SSC–0.05% SDS first at room temperature and then at 55 °C, signals were detected with HBNP or AMPPD as a substrate. Fluorometric detection was carried out under UV light (302 nm, 2 mW/cm^2), and the results were recorded with a Polaroid camera. Chemiluminescent signals were detected by exposure to X-ray films (Eastman Kodak) for 1 h after 30 min of preincubation.

RESULTS AND DISCUSSION

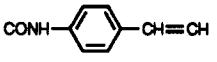
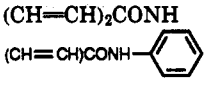
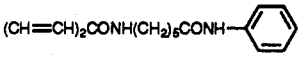
Properties of Fluorescent Substrates. Table 2 shows the detection limits of the ELFA using naphthol derivatives and lists the properties of the derivatives. The fluorescent intensities are shown as relative values to that of 3-hydroxy-*N*-2′,4′-(dimethylphenyl)-2-naphthalenecarboxamide (**13**), which is commercially available as naphthol AS-MX (Sigma).

As little as 10 fg of spotted DNA could be detected with the 3-hydroxy-*N*-phenyl-2-naphthalenecarboxamide phosphate ester (**1**), but a slight diffusion of the hydrolyzed product from the spot was observed. Among the monosubstituted derivatives, *N*-biphenyl-substituted naphthalenecarboxamide derivative **11** (HBNP) showed the lowest detection limit of 5 fg without diffusion of the reaction product, as well as the lowest background fluorescence. Though the *N*-2-methylphenyl derivative **2** showed a detection limit as low as HBN, a slight diffusion of the fluorescent signal was seen. The effect of a number of other substituents on the phenyl was examined. Isopropyl, methoxy, and chloro derivatives **3–5** showed higher detection limits. Cyano and acetoamide derivatives **6**, **8**, **9** were inadequate for ELFA on the nylon membrane because of the weak substantivities of their OH forms. The 3-nitro derivative **7** was nonfluorescent. The 3-phenyl and 2-phenyl-2′-phenyl substitutions (**10**, **12**) deteriorated the detection limit. Dimethyl derivatives **13** and **14** showed a higher detection limit than **2** because of nonspecific adsorption to the membrane. Isopropyl substitutions (**15**, **16**) also suffered from significant diffusion of the OH forms and fluorescent noise of the phosphates. Chloro and bromo substitutions (**19–21**) did not improve the detection limit. Dimethoxy and chloromethoxy derivatives (**17**, **23**) deteriorated the sensitivity. The detection limit of the 2-phenyl derivative (**25**) increased slightly with phenyl substitution and was significantly improved by methoxy substitution (**24**). Significant diffusion was observed in all the trisubstituted derivatives (**26–30**). As a result, di- and trisubstituted derivatives showed lower sensitivities than monosubstituted compounds. The 8-bromo- and 7-hydroxynaphthalene derivatives did not have improved sensitivity (**31** compared with **13**, **32** compared with **4**, and **49** compared with **11**).

We next examined 3-hydroxy-2-naphthalenecarboxamides having other substituents. The *N*-naphthyl derivatives **33** and **34** (Chart 1) showed weaker fluorescence, and that with dibenzofuran or anthracene (**35**, **36**) had high backgrounds. Fluorescein substitution (**46–48**) increased the solubility in water, thereby worsening the sensitivity drastically.

Table 2. Comparison of Fluorescent Properties of Naphthalenecarboxamide Derivatives

no.	R	detectn limit (fg)	fluor. properties in soln: λ_{ex}	λ_{em}	rel fluor[a]	fluor. properties on membrane: substantivity of a OH form	nonspecific adsorptn of a phosphate ester[b]
1	none	10	348	498	0.41	+[c]	low
2	2-methyl	5	430	493	0.74	±	low
3	2-isopropyl	20	405	480	0.39	+	low
4	2-methoxy	10	437	494	0.56	+	low
5	4-chloro	20	400	515	0.3	+	low
6	4-cyano	5000	356	522	1.2	−	high
7	3-nitro		no fluorescence			+	
8	3-acetoamide	5000	437	512	1.0	−	high
9	4-acetoamide	none[d]	362	480	2.5	none[d]	high
10	3-phenyl	200	437	496	1.3	●	moderate
11	2-phenyl	5	290	510	0.3	+	low
12	2-phenyl-2′-phenyl	40	315	510	1.7	+	moderate
13	2,4-dimethyl	40	436	490	1.0	+	moderate
14	3,5-dimethyl	20	316	505	0.4	+	moderate
15	2-isopropyl-6-methyl	200	428	484	0.4	●	moderate
16	2,6-diisopropyl	200	341	388	0.9	±	moderate
17	2,4-dimethoxy	20	436	495	0.5	+	low
18	3,5-dimethoxy	20	316	506	0.2	+	low
19	2-methyl-4-chloro	20	335	500	0.3	+	low
20	2-methyl-5-chloro	10	266	509	0.3	+	low
21	2-methyl-4-bromo	20	441	501	2.3	+	low
22	2-bromo-4-methyl	40	435	508	0.8	+	moderate
23	2-methoxy-5-chloro	40	400	515	0.3	+	moderate
24	2-phenyl-5-methoxy	200	420	490	1.8	±	moderate
25	2,6-diphenyl	20	285	347	0.7	+	low
26	2,4,6-trimethyl	250	344	405	0.7	±	moderate
27	3,4,5-trimethoxy	125	398	508	1.1	●	moderate
28	5-chloro-2,4-dimethoxy	2000	337	506	1.1	−	high
29	4-chloro-2,5-dimethoxy	400	420	491	1.0	−	moderate
30	4-chloro-2-methoxy-5-methyl	400	395	494	0.2	−	moderate
31	2,4-dimethyl	40	440	501	0.4	+	moderate
32	2-methoxy	20	450	508	0.6	+	low

no.	X	detectn limit (fg)	fluor. properties in soln: λ_{ex}	λ_{em}	rel fluor.[a]	fluor. properties on membrane: substantivity of a OH form	nonspecific adsorpn of phosphate ester[b]
33	1-naphthyl	200	428	493	0.4	+[c]	moderate
34	2-naphthyl	100	436	498	0.9	+	moderate
35	2-anthryl	10^5	337	490	0.2	+	high
36	2-fluorene	1000	330	480	0.2	+	high
37	2-(6-methylbenzothiazolyl)	5000	382	509	1.4	−	high
38	2-perylenyl	no[e]	412	436	22.7	no	high
39	1-perylenyl	no	438	472	52.7	no	high
40	3-perylenyl	no	417	449	102.3	no	high
41	$CONH(CH_2)_{11}$	1000	412	442	46.5	±	high
42	$(CH{=}CH)_2$	80	442	468	71.3	±	moderate
43	$(CH{=}CH)_4$	none[d]	438	469	36.1	none	high
44		none	437	469	71.3	none	high
45	$CH{=}CHCH{=}CH$	400	368	470	0.07	−	moderate
46	$(CH{=}CH)_2CONH$	2000	345	475	0.25	−	high
47		2000	484	512	1.5	−	high
48		10^4	438	510	2.0	−	high
49	2-biphenyl	10^4	266	462	0.4	+	high
50	3-perylenyl	no	360	512	0.04	no	low
51	3,5-dimethylphenyl	5000	370	450	0.05	−	high
52	3,5-dimethylphenyl	2500	336	472	0.6	−	high

[a] Measured for 3×10^{-6} mol/L DMF solution. [b] Fluorescence intensity of phosphate form as a blank. [c] Key: (+) high substantivity; (±) weak substantivity; (−) no substantivity. [d] Nonspecific adsorption of the phosphate form on nylon. [e] No enzymatic reaction.

Fluorescent intensities of naphthoic acid directly bonded with aminoperylene (**38**–**40**) became 20–100 times higher than **13**, although they could not be good substrates for alkaline phosphatase. Since the above fact may be attributed to the steric bulkiness of perylene, we coprepared the naphthol derivatives **41** and **42** bound to perylene through an alkyl straight chain or an olefin spacer. Indeed, **42** could be hydrolyzed by phospholipase to show the detection limit of 80 fg although with relatively high background. **43** and **44**, having longer spacers, showed higher background due to nonspecific

Chart 1

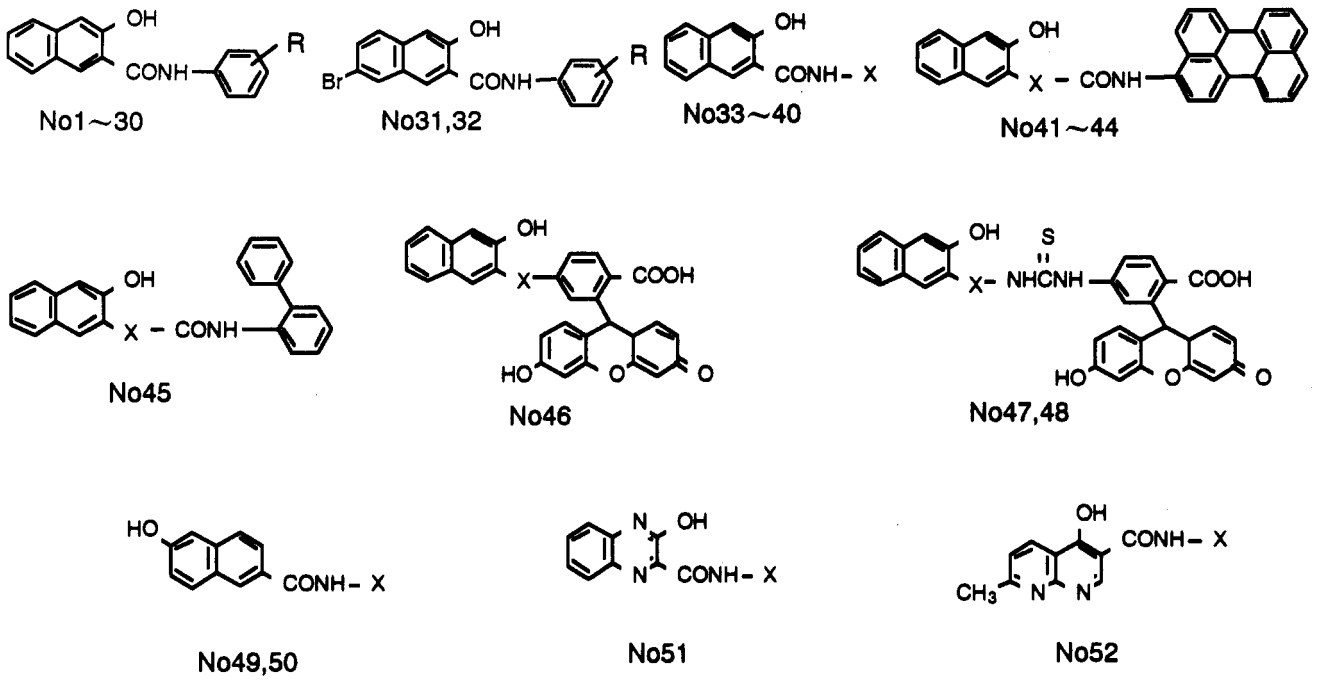
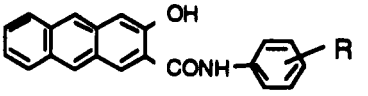


Table 3. Comparison of Fluorescence Properties of Anthracenecarboxamide Derivatives



			fluor. properties in soln			fluor. properties on membrane	
no.	R	detectn limit (fg)	λ_{ex}	λ_{em}	rel fluor.[a]	substantivity OH form	nonspecific adsorptn of a phosphate ester[b]
53	2-methyl	20	415	480	1.1	+[c]	low
54	2-methoxy	20	417	462	1.5	+	low
55	2-phenyl	20	318	406	9.0	+	low
56	2-phenyl-2′-phenyl	2000	434	472	5.3	-	high
57	2,4-dimethyl	40	435	465	2.0	+	moderate
58	3,5-dimethyl	2000	360	477	0.75	-	high
59	2,4-dimethoxy	80	436	466	0.4	±	moderate
60	2-methyl-4-bromo	none[d]	409	467	1.1	none	high
61	2-methyl-4-chloro	400	412	465	1.4	-	moderate
62	3,4,5-trimethoxy	2000	435	465	1.1	-	high

[a] Measured for 3×10^6 mol/L DMF solution. [b] Fluorescence intensity of phosphate form as a blank. [c] Key: (+) high substantivity; (±) weak substantivity; (-) no substantivity. [d] Nonspecific adsorption of the phosphate form on nylon.

adsorption on the membrane of the phosphate form. Heterocyclic derivatives **51** and **52** showed a weak substantivity of the OH form and nonspecific adsorption of the phosphate.

The fluorescent properties of many *N*-aryl 3-hydroxy-2-anthracenecarboxamides were next examined, and the results are summarized in Table 3. The sensitivities obtained with di- and trisubstituted derivatives **56-62** were lower than those obtained with monosubstituted derivatives **53–55**, as in the case of naphthalenecarboxamide compounds.

Several different aspects of the substituent effects between naphthalenecarboxamide derivatives and anthracenecarboxamide derivatives were observed. Monosubstituted forms of naphthalenecarboxamide derivatives showed sensitivities higher than anthracenecarboxamide derivatives (**53**, **54**, and **55** compared with **2**, **4**, and **11** respectively). Dimethyl substitution of both naphthalenecarboxamide and anthracenecarboxamide derivatives at the 2,4-position showed almost equal sensitivities (**13** compared with **57**), whereas the dimethoxy-substituted naphthalenecarboxamide derivative had higher sensitivity than the corresponding anthracenecarboxamide derivative (**17** compared with **59**). Dimethoxy substitution (**14**) at the 3,5-position of the anthracenecarboxamide derivative resulted in lower sensitivity than that of the naphthol compound (**14**). All other substitutions on the *N*-phenyl group in the anthracenecarboxamide derivatives lowered the sensitivities (**60**, **61**, and **62** compared with **21**, **19**, and **27**, respectively). Stokes shifts of most napthalenecarboxamide derivatives were found to be larger than those of anthracenecarboxamide derivatives; i.e., the Stokes shifts of naphthalenecarboxamide derivatives are ~200 nm, whereas those of anthracenecarboxamide derivatives are smaller than 120 nm. Most anthracenecarboxamide derivatives showed slightly lower sensitivities than the naphthalenecarboxamide derivatives, probably because of significant fluorescent backgrounds

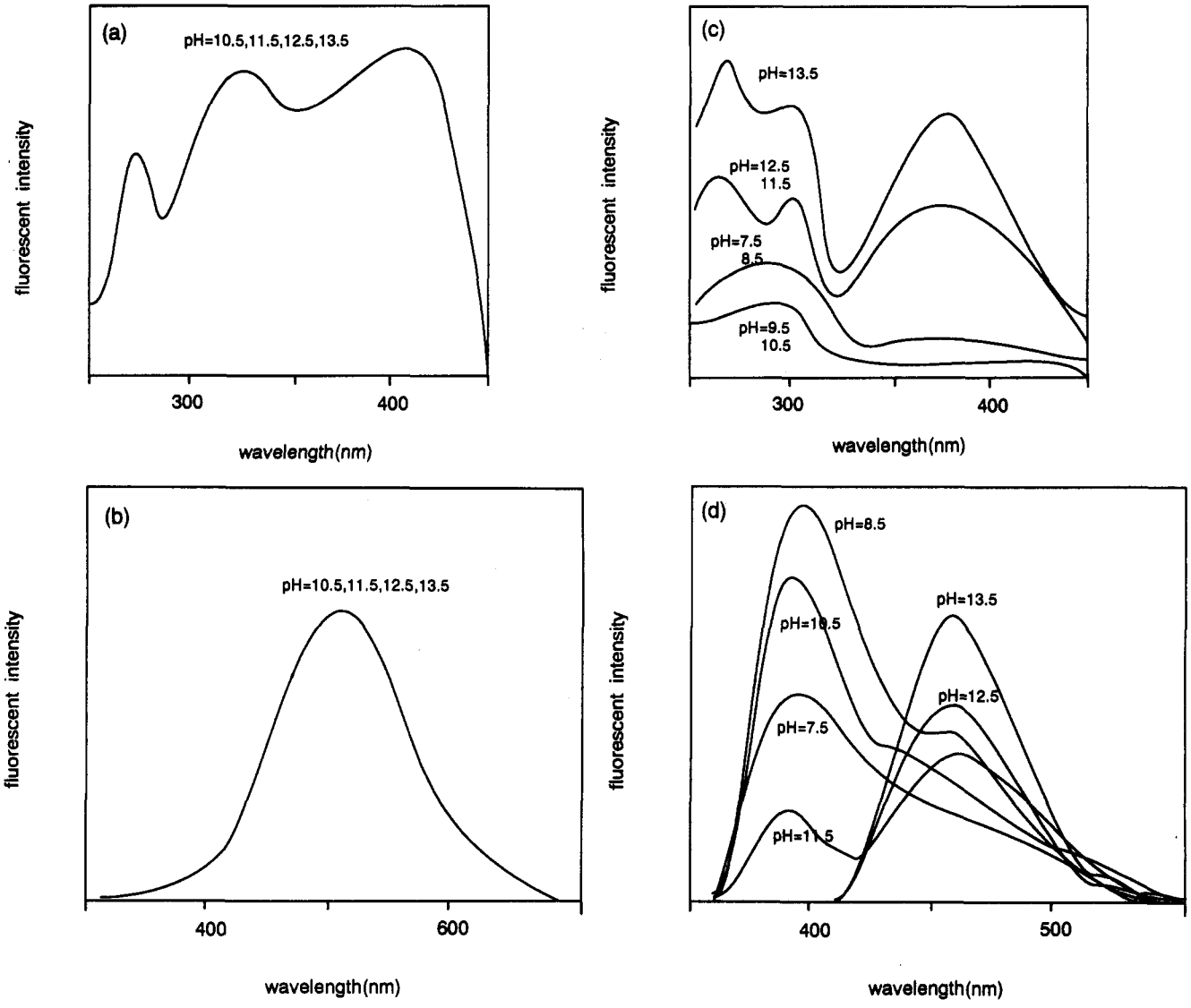


Figure 3. Effect of pH on excitation and emission spectra of HBN in solution and on membrane: excitation spectra (a) and (c) with λ_{em} = 500 nm; emission spectra (b) and (d) with λ_{ex} = 300 nm.

derived from nonspecific adsorption of the anthracene phosphates.

Further Studies on HBN. The properties of HBN (dephosphorylated forms of HBNP) were studied in further detail. The acid dissociation constant of both forms of HBN (0.3×10^{-4} M) was pK_a ~11.5, which was estimated by pH dependence of their ultraviolet spectra. Since the pK_a values of 2-naphthol derivatives are generally in a range between 8 and 10, neutral species of HBN are assumed to have intramolecular hydrogen bondings.

Panels a and b of Figure 3 show the effect of pH on the excitation and emission spectra in solution. HBN has an excitation maximum (λ_{ex}) at 410 nm and an emission maximum (λ_{em}) at 510 nm in a pH range between 10.5 and 13.5. No effects of pH on the excitation and emission spectra were observed in solution.

On the other hand, the fluorescent characteristics of HBN were strongly dependent on pH on the nylon membrane (Figure 3c,d). These pH-dependent changes of the fluorescent spectra were reversible. The λ_{em} was observed at 390 nm for neutral species and at 460 nm for anionic species, and the existence of the nylon membrane was essential for the pH-dependent shifts of the spectra. This suggests a specific interaction (for example, intermolecular hydrogen bonding) of anionic species with the nylon membrane. It is also suggested that the extremely strong substantivity of HBN is attributed to this kind of interaction.

Detection Limit of ELFA Using HBNP. Since 5 fg of λ DNA (48 502 base pairs) is equivalent to 3.1×10^{-22} mol and DIG is said to be incorporated every 36th base under optimum labeling conditions,[13] then 5 fg of λ DNA corresponds to ~4.2 $\times 10^{-19}$ mol of DIG. In addition, equal quantities of anti-DIG antibody and DIG are attached to each other, and the conjugation ratio between anti-DIG antibody and ALP is 1:3[14] therefore, it is concluded that 5 fg of λ DNA is equivalent to ~4.2 $\times 10^{-19}$ mol of ALP.

The results of Southern hybridizations are illustrated in Figure 4. The amounts of DNA applied on lanes were 20, 4,

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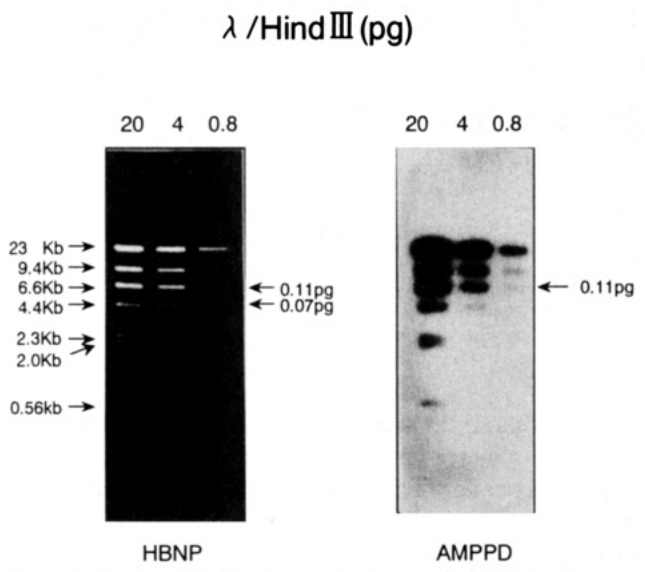

Figure 4. Southern hybridization. A dilution series of *Hind*III-digested λ DNA were electrophoresed, transferred onto nylon membrane, and hybridized with DIG-labeled *Hind*III-digested λ DNA. Fluorescent and chemiluminescent detections were carried out with HBNP and AMPPD, respectively.

and 0.8 pg, respectively. The resolution of each bands seems to be higher with HBNP than with AMPPD. In the case of HBNP, ~70 fg of DNA (2.5×10^{-20} mol) was detected, but this was not the case with AMPPD. It is estimated that 70 fg of DNA is equivalent to $\sim 5.9 \times 10^{-18}$ mol of ALP. The efficiencies of transfer of target DNA to membrane and hybridization of probe to target may reduce the sensitivity of Southern hybridization.

Several investigators have reported the usefulness of chemiluminescent methods for DNA detection[7,8,15,16] and stated that the chemiluminescent technique is more sensitive than colorimetric or fluorometric methods. However, as described above, the HBNP method has been proved to be as sensitive as the AMPPD method in the present study. The HBNP method has advantages over the AMPPD method. One is that no remarkable fluorescent decay is observed under UV light and fluorescent products can be stored for at least 1 year in the dark without losing high S/N ratios. Another advantage is that mixtures of varying amounts of differently sized DNA can be resolved at high fidelity; this is not possible with the AMPPD method, since bands containing larger amounts of DNA become diffuse, due to the low affinity of AMPPD product for the nylon membrane.

CONCLUSION

(1) The sensitivity is not always related to the fluorescent intensity. Occasionally, an increase of the fluorescent intensity of the OH form causes the increase of that of the phosphate form, lowering the S/N ratio. Actually, HBNP is the most suitable fluorochrome for the alkaline phosphatase-linked fluorescence assay of membrane-bound nucleic acids, although the relative fluorescent intensity of HBN is as low as 0.3.

(2) Both the affinity for the membrane of the OH form and the water solubility of the phosphate form are important for high sensitivity and fidelity. The low water solubility of the phosphate form causes nonspecific adsorption of the phosphate as well as increase of the fluorescent noise on the membrane.

(3) Stokes shifts of naphthalenecarboxamide derivatives are larger than those of anthracenecarboxamide derivatives, and HBNP has the largest Stokes shift of 220 nm among the synthesized fluorochromes.

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