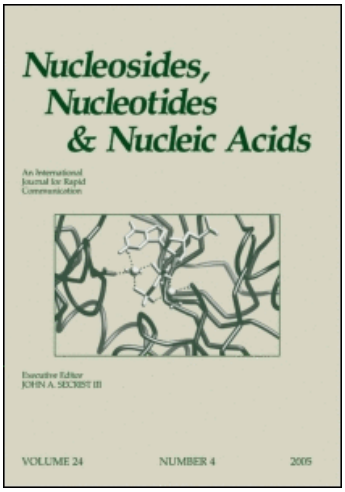

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

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To cite this Article Shirai, Ryuichi , Ito, Taro , Iwasaki, Shigeo and Hashimoto, Yuichi(1998) 'Sequence Selective Interaction Between Nucleotides and Intercalators Bound to Water Soluble Dextran: An Application to the Affinity Chromatography of Dinucleotides', Nucleosides, Nucleotides and Nucleic Acids, 17: 1, 593 — 601
To link to this Article: DOI: 10.1080/07328319808005202
URL: http://dx.doi.org/10.1080/07328319808005202

NUCLEOSIDES & NUCLEOTIDES, 17(1-3), 593-601 (1998)

SEQUENCE SELECTIVE INTERACTION BETWEEN NUCLEOTIDES AND INTERCALATORS BOUND TO WATER SOLUBLE DEXTRAN: AN APPLICATION TO THE AFFINITY CHROMATOGRAPHY OF DINUCLEOTIDES†

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ABSTRACT: The analysis of association constant between dextran coupled intercalators and nucleotides revealed the base- and sequence-selective affinity to mono- and dinucleotides in aqueous solution. Acridine bound CH-Sepharose 4B, designed as the affinity stationary phase for nucleotides, also showed base- and sequence-selective affinity.

Intercalation has been recognized as the major interaction between DNA and so-called intercalator such as acridine, pyrene and ethidium bromide.[1] Intercalator generally possesses a planar fused polycyclic aromatic skeleton. In addition to the intercalative interaction to doubly stranded DNA, these ligands also exhibit some affinity to nuclear bases by π-π interaction. However, hydrophobic nature of them makes it difficult to analyze such interactions between nuclear bases and intercalative ligands.

Polyaromatic hydrophobic compounds generally stack spontaneously to form dimers and/or higher aggregates in aqueous solutions. For the observation of real interactions between hydrophobic intercalative ligands and hydrophilic nucleic acids in water, aggregation of hydrophobic ligands should be dissociated. We reported the dextran-coupling method to solubilize extremely hydrophobic molecules such as tetraphenylporphyrins and Zimmerman's molecular tweezers into water as monomerized state (FIG. 1). Based on the success of dextran-coupling method to solubilize hydrophobic ligands in water, we turned our interest to the non-intercalative interaction between dinucleotides and intercalators.

†Dedicated to the memory of Professor Tsujiaki Hata

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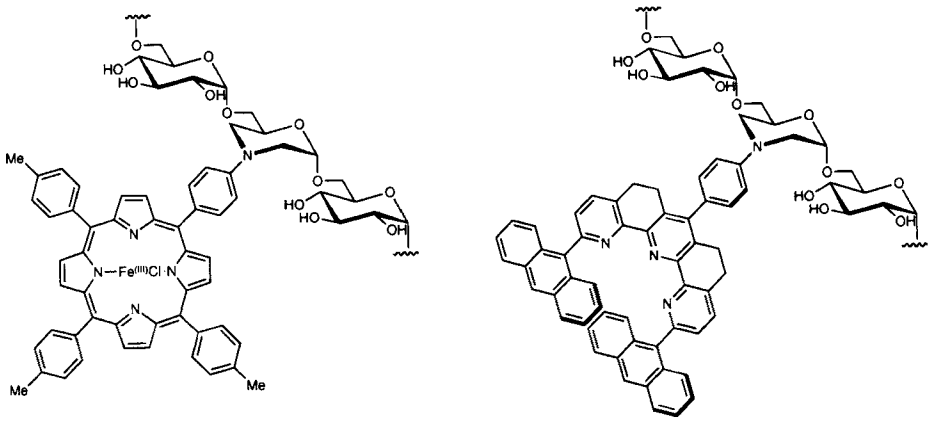


FIG. 1. Dextran-coupled Tetraphenylporphirin and Molecular Tweezers

In this paper, we describe the base- and sequence-selective interaction between diribonucleotides and intercalative ligands using intercalators covalently bound to water soluble dextran (dextran-coupled intercalators, **DEX-IC**), and its application to the affinity chromatography for diribonucleotides analysis.

Designed **DEX-IC** (**DEX-Acr**, **DEX-Pyr**, **DEX-Flu** and **DEX-Ant**) are depicted in FIG. 2. Ligands (**1**, **2**) for **DEX-Acr** and **DEX-Pyr** with amino group spacer were prepared from 6,9-dichloro-2-methoxyacridine and 1-aminopyrene as described previously.[2] Fluorene and anthracene ligands (**3**, **4**) for **DEX-Flu** and **DEX-Ant** were synthesized from 2-aminofluorene and 9-anthracenecarboxylic acid[3] as shown in SCHEME 1.

These intercalative ligands were covalently supported on dextran (MW=2,000,000) by the King's method with minor modifications as follows (SCHEME 2).[4,5] Partial glycol cleavage of dextran **7** by $NaIO_4$ (0.06 eq.) in acetate buffer (pH 5) gave dialdehyde **8**, which was reductively aminated by sodium cyanoborohydride in DMSO with the amino spacer of intercalative ligand. The adduct was precipitated by the addition of EtOH, which was redissolved in water and chromatographed on Sephadex G-50 (eluted with H_2O) to give **DEX-IC**. The content of covalently bound ligand in dextran polymer could be controlled by varying the reaction conditions, and was determined by the UV absorption at 285 nm for **DEX-Acr**, 287 nm for **DEX-Pyr**, 291 nm for **DEX-Flu** and 258 nm for **DEX-Ant**.

Then, the interactions of **DEX-IC** in clear aqueous solution with various nucleotides, i.e., ribonucleoside 5'-monophosphates (pG, pA, pC and pU) and diribonucleotides (GpG, GpC, CpG, CpC, ApA, ApU, UpA and UpU) were analyzed. For the binding experiments

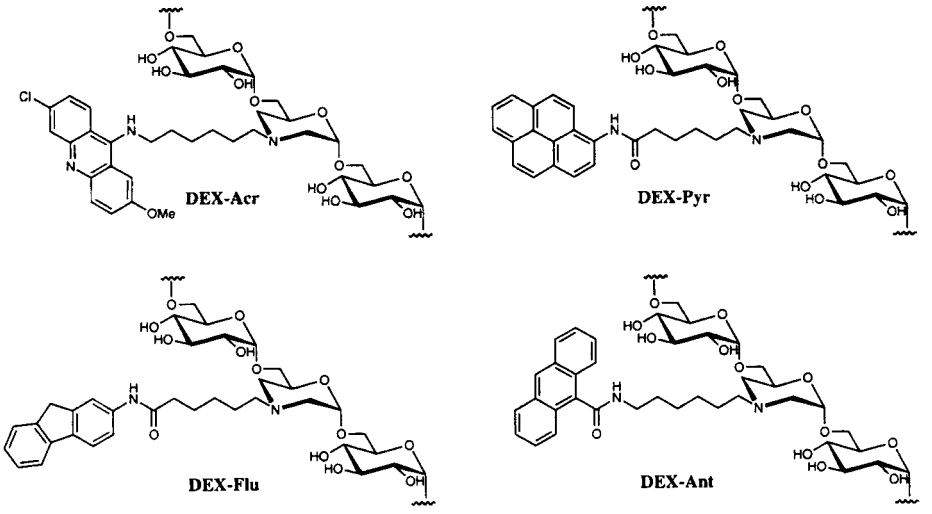
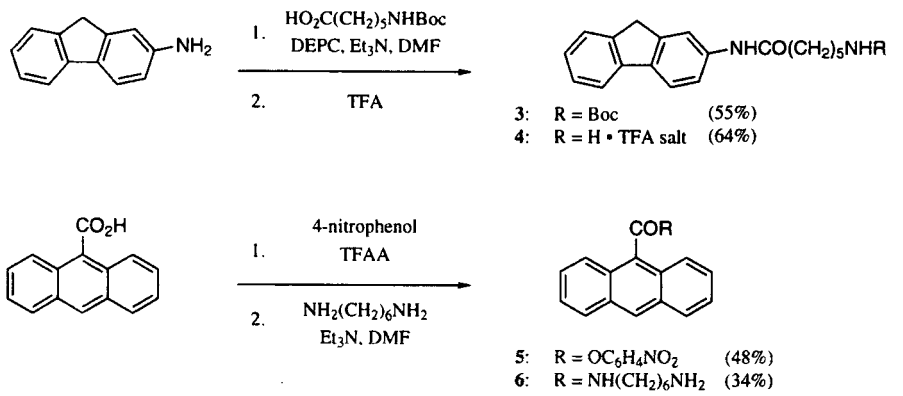
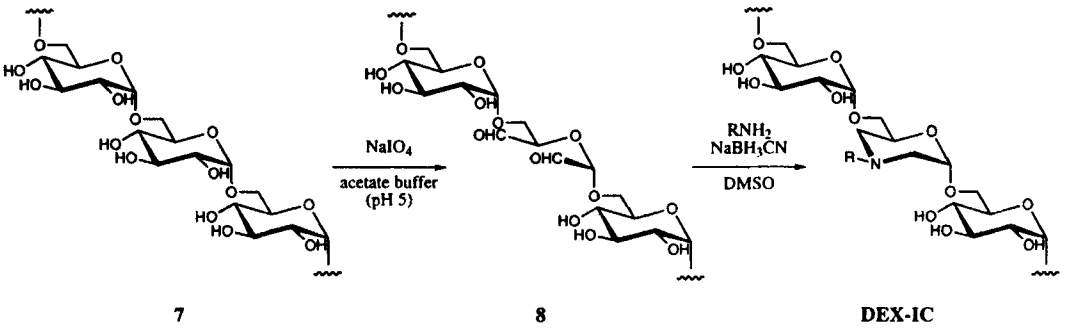


FIG. 2. Dextran-coupled Intercalators (DEX-Acr, DEX-Pyr, DEX-Flu, DEX-Ant)



SCHEME 1. Introduction of Amino Spacer into Fluorene and Anthracene

described below, **DEX-IC** with ligand content of 31.6 μmol (**Acr**), 77.6 μmol (**Pyr**), 112 μmol (**Flu**) and 126 μmol (**Ant**) per 1 g of dextran, respectively, were used. Association constants (*Ka*) between **DEX-IC** and nucleotides were determined by the ultrafiltration method. **DEX-IC** (corresponding to 5.0 mM as ligand unit, 35 μl) was incubated with nucleotides (2.5 mM, 35 μl) in TE [10 mM Tris-HCl (pH 7.5) - 1 mM EDTA] at 4 °C for 24 h. The mixture and the corresponding nucleotide solution as a control were ultrafiltrated using a Centricut W-50 UF tube (Kurabou Co.). The content of nucleotides in the filtrate was determined by the measurement of UV absorption, and the value was taken as the



SCHEME 2. Synthesis of Dextran-coupled Intercalative Ligands

concentration of free nucleotides. The results of binding experiments are shown in TABLE 1. **DEX-Acr** possesses higher affinity to mononucleotides/dinucleotides than **DEX-Pyr**, **DEX-Flu** and **DEX-Ant**. The highest affinity was observed between **DEX-Acr** and GpG. Since dextran itself did not show any interaction with nucleotides, the binding of mononucleotides with **DEX-IC** observed here should be due to some interaction between nucleobase of mononucleotides and polycyclic aromatic skeleton. Affinity of mononucleotides to the most potent **DEX-Acr** was decreased in the order of pG > pA > pC > pU, while **DEX-Pyr**, **DEX-Flu** and **DEX-Ant** showed relatively weak or no affinity to all mononucleotides. The order of affinity of dinucleotides to **DEX-Acr** was "GpG >> CpG > ApA > GpC > UpU > ApU >> CpC > UpA", while that to **Dex-Pyr**, **DEX-Flu** and **DEX-Ant** was "GpG >>> CpG > ApA > GpC > UpU > ApU >>> CpC,UpA", "GpG >>> GpC > ApA > UpA >>> CpG,CpC,ApU,UpU" and GpG >>> ApA > GpC > UpA > CpG > ApU > UpU >>> CpC. It should be noted that GpG showed strongest affinity to all **DEX-IC** investigated. Concering hetero-dinucleotides, **DEX-Acr** and **DEX-Pyr** possess higher affinity toward CpG and ApU than GpC and UpA, respectively. In contrast, **DEX-Flu** and **DEX-Ant** showed reversed affinity. We cannot interpret this sequense-selectivity at this stage, however, encouraged by the results of binding experiment using **DEX-IC**, we introduced the acridine (**Acr**) and anthracene (**Ant**) into activated CH-Sepharose 4B (Pharmacia Biotech) as the promising affinity ligands for the sequence-selective analysis of dinucleotides. TE buffer solution of mononucleotides (pG, pA, pC, pU), homo-dinucleotides (GpG, ApA, CpC, UpU) and hetero-dinucleotides (GpC, CpG, ApU, UpA) were independently chromatographed through above affinity gels with TE as eluent. Elution patterns of nucleotides monitored by UV absorption are shown in FIG. 4. As predicted by the binding experiment of **DEX-IC** with nucleotides, base- and sequence-selective retention

TABLE 1. Association Constant (*Ka*) of DEX-IC with Ribonucleotides

Nucleotide	Association Constant (M^{-1})			
	DEX-Acr	DEX-Pyr	DEX-Flu	DEX-Ant
pG	441	57	53	38
pA	279	48	30	89
pC	74	38	n.o.*	38
pU	118	n.o.*	5	75
GpG	834	375	257	429
GpC	207	84	60	99
CpG	438	143	n.o.*	71
CpC	33	n.o.*	n.o.*	n.o.*
ApA	281	102	33	153
ApU	85	25	n.o.*	47
UpA	17	n.o.*	11	87
UpU	108	50	n.o.*	38

*Interaction was not observed.

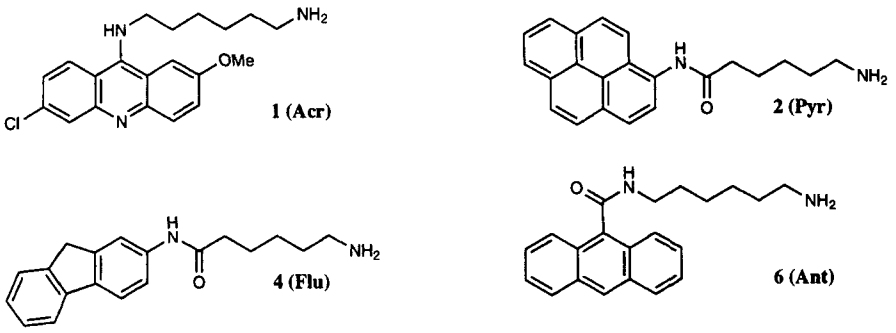


FIG. 3. Intercalative Ligands with Amino Group Spacer

of certain nucleotides was observed. An excellent differentiation of mononucleotides (pG, pA from pC, pU); homo-dinucleotides (GpG, ApA from CpC, UpU); hetero-dinucleotides (CpG from GpC, ApU, UpA) was achieved by **Sepharose-Acr gel**, however, could not be done by **Sepharose-Ant gel**.

In conclusion, hydrophobic intercalators were converted to be water-soluble by coupling with dextran polymer (**DEX-Acr**, **DEX-Pyr**, **DEX-Flu** and **DEX-Ant**). It has become possible to observe the interactions of hydrophobic polyaromatic skeletons with

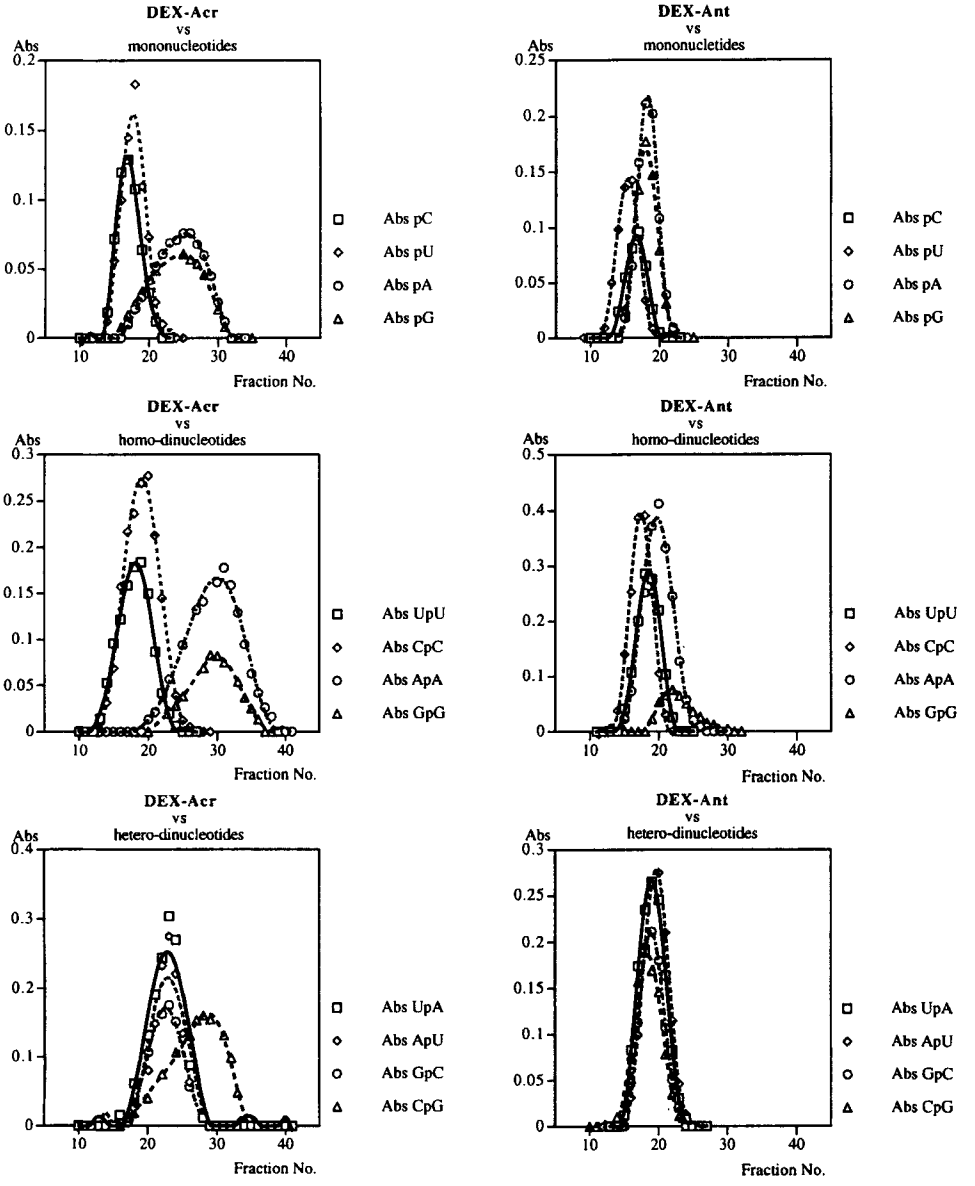


FIG. 4. Affinity Chromatography by CH-Sepharose 4B Bound Intercalator

mono- and dinucleotides in aqueous solution by the development of dextran-coupled intercalator. The analysis of association constant between **DEX-IC** and nucleotides revealed the base- and sequence-selective affinity of these hydrophobic intercalative skeletons to mono- and dinucleotides. On the basis of these findings, effective **Sepharose-Acr gel** was also developed for dinucleotides analysis. Although the nature of these interactions between intercalators and nucleotides still remains unknown, these results should provide new aspects of molecular recognition in the chemistry of nucleic acids.

EXPERIMENTAL

All ^{1}H-NMR spectra were measured on a JEOL JMN-A500 spectrometer with tetramethylsilane as internal standard. IR spectra were recorded on a JASCO A-102 infrared spectrophotometer. Mass spectra (MS) were obtained on a JEOL JMS-HX110 spectrometer. All reactions were carried out in an atmosphere of dry argon at room temperature unless otherwise stated. Analytical thin-layer chromatography (TLC) was performed on Merck precoated silica gel 60 F254 plates.

***N*-(2-Fluorenyl)-6-*N*-*tert*-butoxycarbonylamino-1-hexamide (3).** 2-Amino-fluorene (597.3 mg, 3.3 mmol) in DMF (5 ml) and DEPC (541 mg, 3.3 mmol) were added to 6-*N*-*tert*-butoxycarbonyl-1-hexanoic acid (693.0 mg, 3.0 mmol) at 0°C. After stirring for a while, Et_3N (0.8 ml, 6.3 mmol) in DMF (5 ml) was added to the mixture. After stirring for 30 min at 0°C and at room temperature overnight, the reaction mixture was diluted with AcOEt, washed with 10% citric acid, sat. $NaHCO_3$, brine, dried over $MgSO_4$ and evaporated to give crude product. Recrystalization from benzene gave pure product (651 mg, 55 %). mp 151 °C. Anal. calcd. for $C_{24}H_{30}N_2O_3$: C, 73.09; H, 7.61; N, 7.11. Found: C, 72.98; H, 7.56; N, 7.17. ^{1}H-NMR ($CDCl_3$): δ 1.47 (s, 9H), 1.90 (m, 6H), 2.40 (m, 2H), 3.16 (m, 2H), 3.91 (s, 2H), 4.61 (b, 1H), 7.3-7.5 (m, 4H), 7.7-7.9 (m, 3H), 7.97 (b, 1H). FAB-MS (*m/z*, *m*NBA, Gly): 395 (M^++H), 394 (M^+), 295, 181. IR ($CHCl_3$): 3450, 3322, 2944, 2871, 1690, 1620, 1593, 1460, 1420, 1370, 1164, 1093, 1042, 1005, 953, 862, 824 cm^{-1}.

6-Amino-*N*-(2-fluorenyl)-1-hexanamide (4). *N*-(2-fluorenyl)-6-*N*-*tert*-butoxycarbonylamino-1-hexanoic amide (118.2 mg, 0.3 mmol) was dissolved in TFA (5 ml) and stirred at room temperature for 40 min. After evaporation of TFA, ether (10 ml) was poured into the residue and precipitates were collected by filtration. Recrystallization from EtOH/Ether gave **3** as TFA salt (77.8 mg, 64 %). mp 207.1-207.8 °C. Anal. calcd. for $C_{21}H_{23}F_3N_2O_3$: C, 61.76; H, 5.64; N, 6.86. Found: C, 61.44; H, 5.93; N, 6.94. IR (KBr): 3039, 2942, 2871, 1679, 1660, 1613, 1549, 1529, 1489, 1466, 1416, 1395, 1205, 1181, 1144, 836, 825, 801, 774, 722cm^{-1}. ^{1}H-NMR (CD_3OD): δ 1.64 (m, 6H), 2.49 (m,

2H), 3.02 (m, 2H), 3.93 (s, 2H), 7.4-8.0 (m, 7H). FAB-MS (*m/z*, *m*NBA, Gly): 295, 181, 114.

9-Anthracenecarboxylic acid 4-nitrophenylester (5). To a stirred suspension of 9-anthracenecarboxylic acid (400 mg, 1.8 mmol) in benzene was added trifluoroacetic anhydride (1 ml, 7.2 mmol). After 10 minutes, 4-nitrophenol (400 mg, 3.0 mmol) was added and stirred for 30min. Organic layer was washed with 10% aq. NaOH, brine, dried over $MgSO_4$. Evaporation of the solvent gave crude product, which was recrystallized from hexane/AcOEt to give **5** (300 mg, 48 %). mp 181.6-181.8 °C. Anal. calcd. for $C_{21}H_{13}NO_4$: C, 73.47; H, 3.79; N, 4.08. Found: C, 73.07; H, 4.18; N, 3.94. ^{1}H-NMR(CD_3OD): δ 7.54 (d, 2H, J=8.5Hz), 7.59 (d, 4H, J=8.0Hz), 8.01 (d, 4H, J=8.0Hz), 8.34 (d, 2H, J=8.5Hz), 8.56 (s, 1H). IR ($CHCl_3$): 1748, 1616, 1594, 1527, 1487, 1350, 1289, 1182, 1164, 1131, 1015, 972, 886, 864, 847cm^{-1}. FAB-MS (*m/z*, *m*NBA): 343(M^+), 205.

***N*-(6-Aminohexyl)-anthracene-9-carboxyamide (6).** To a stirred solution of hexamethylenediamine (580 mg,5.0 mmol) and Et_3N (0.8 ml, 6.3 mmol) in DMF (5 ml) was added 9-anthracenecarboxylic acid 4-nitrophenylester (343 mg, 1.0 mmol) in DMF (10 ml) and the mixture was stirred overnight at room temperature. After DMF and Et_3N was evaporated, H_2O and AcOEt were poured into the residue. Aqueous layer was extracted with AcOEt and concentrated to give crude product (20 mg), which was chromatographed (eluted with CH_2Cl_2:MeOH=9:1 with small amount of ammonium hydroxide) to give amide **2** (110 mg, 34 %). mp 114.6-115.2 °C. Anal. calcd. for $C_{21}H_{24}N_2O$: C, 78.75; H, 7.50; N, 8.75, Found: C, 78.38; H, 7.55; N, 8.58. ^{1}H-NMR(CD_3OD): δ 1.49 (m, 8H), 2.63 (m, 2H), 3.55 (m, 2H), 7.3-7.5 (m, 4H), 7.8-8.1 (m, 4H), 8.49 (s, 1H). IR (KBr): 2926, 2852, 1640, 1570, 1444, 1392, 1360, 1294, 1267, 1258, 1165, 1012, 971, 926, 883, 843, 800, 790, 737cm^{-1}. FAB-MS (*m/z*, *m*NBA): 321 (M^++H), 205.

Preparation of Dextran-coupled Intercalator: General Procedure. Ligand **1** (**Ant**) (2.3 mg, 7.19 μmol) in DMSO (2 ml) and Et_3N (1 mg) in MeOH (0.5 ml) were added to the solution of partially cleaved dextran by sodium metaperiodate (30 mg, 477 nmol aldehyde/mg) in DMSO (1 ml). After stirring for 1h, $NaBH_3CN$ (7.5 mg) in MeOH (0.5 ml) was added and the mixture was stirred for 4 days. EtOH (10 ml) was added to the reaction mixture to precipitate **Dex-Ant**. The precipitates were dissolved in water (2 ml) and chromatographed on Sephadex G-50 with H_2O as eluent. Lyophilization of the eluent gave **Dex-Ant** (24.4 mg). Concentration of **Ant** in **Dex-Ant** was determined to be 157 μmol/g by the measurement of UV absorption.

Preparation of CH-Sepharose 4B Bound Intercalator: General Procedure. Activated CH-Sepharose 4B (Pharmacia Biotech, 1 g) was swelled and washed with 1 mM HCl (200 ml) over glass filter (G3). The ligand was dissolved in coupling buffer, 0.1

M $NaHCO_3$ (pH 8). The ligand solution was mixed with gel suspension and rotated end-over-end (not with a magnetic stirrer) at room temperature for 1 h. After excess ligand was washed away with coupling buffer, the gel was transferred to 0.1 M Tris-HCl buffer (pH 8.0) and allowed to stand for 1 h. The product was washed repeatedly at least three cycles (1. washed with 0.1 M acetate buffer (pH 4.0) containing 0.5 M NaCl; 2. washed with 0.1 M Tris-HCl buffer pH 8.0 containing 0.5 M NaCl).

Affinity Chromatography by CH-Sepharose 4B Bound Intercalator: General Procedure. Nucleotides (10 µl of 5 mM in TE) was passed through the column (diameter:5.5 mm, height: 60 mm) of **Sepharose-Acr gel** (**Acr** concentration: 5.07 µmol/ml of gel) or **Sepharose-Ant gel** (**Ant** concentration: 6.14 µmol/ml of gel) with elution buffer (10 mM Tris-HCl (pH 7.5) - 1 mM EDTA) and 50 fractions (100 µl/fraction) were collected at room temperature.

ACKNOWLEDGEMENT

This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture, Japan. The authors are grateful to Professor Koichi Shudo (Faculty of Pharmaceutical Sciences, University of Tokyo) for his helpful discussion.

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