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Accounts

Threading Intercalators as a New DNA Structural Probe

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Nogalamycin is a threading intercalator and when it binds with double stranded DNA, one of the two sugar substituents goes between adjacent base pairs of DNA to form a stable complex in which the two substituents of nogalamycin are projecting out in each groove of DNA. Since the molecular volume of the substituents of nogalamycin is larger than that of the space created through the motion of DNA strands constituting the double helix, a cleavage of hydrogen bonds in the base pairs of DNA duplex is necessary to form such a complex. The structure of double-stranded DNA is dynamic, and the base pair cleavage (base pair opening) and re-formation (closing) occur frequently at ambient temperature. A wider variety of threading intercalators is obtainable synthetically, and they include several families such as 1,5-disubstituted anthraquinones, 9,10-disubstituted anthracenes, and naphthalene bis(dicarboximide) derivatives. Kinetic study of the complex formation and dissociation of naphthalene bis(dicarboximide) carrying substituents of different size have made one recognize the dynamic character of duplex DNA in intercalation reaction. A cyclic bis-intercalator was also able to form a threading complex of a catenane-like structure. A thorough study of the binding behavior of natural and synthetic threading intercalators with DNA revealed that a proper design of substituents on threading intercalators can produce ligands which specifically bind either to single- or double-stranded DNA. These ligands, synthetic ones in particular, will hopefully serve not only as new DNA structural probes but also as chemotherapeutic agents.

Nogalamycin, one of the anthracycline antibiotics, is furnished with two bulky substituents on the opposite sides of the rectangular, intercalating chromophore (Fig. 1).[1] Therefore, upon its intercalation to double-stranded DNA, one of the two sugar substituents is located in the major groove and the other in the minor groove when its anthraquinone chromophore intercalates between adjacent nucleic base pairs.[2,3] To form such a complex, a bulky sugar substituent has to pass through the space created between the adjacent base pairs of double-stranded DNA. An intercalator which forms such a type of complex is called a "threading intercalator". Many detailed studies have been made on the complexes of nogalamycin with duplex oligonucleotides to indicate the expected architecture for a threaded complex. A dynamic aspect of DNA-threading intercalators seems to open a new chemistry of DNA-intercalator complexes.

In this paper, we will review the binding chemistry of nogalamycin with DNA first and then of synthetic threading intercalators, which recently started to be actively studied. An application of the latter in probing the dynamics of duplex intercalation and single-/double-stranded DNA structure will be emphasized.

Nogalamycin

Nogalamycin (nog) is an antibiotic from *Streptomyces* and shows a huge spectrum of activity to several kinds of tumor cells. Aroa[1] reported the X-ray crystallographic analysis of nog itself, featuring a dumbbell-like structure where nogalose and amino sugar parts are attached to the opposite sides of the rectangular anthraquinone chromophore plane along its long axis and their molecular sizes are represented by their effective diameters, 12 and 6 Å respectively (Fig. 1).[1] Adjacent base pairs of double-stranded DNA can create a cavity of 3.4 Å in thickness under the classical intercalation process, while a maximum cavity size of 10 Å was reported.[4] Such information suggests that the hydrogen bonding of DNA base pairs has to cleave before the DNA can form an intercalated complex with nogalamycin. The fact that a threaded complex is readily formed for nogalamycin suggests that the structure of duplex DNA is dynamic in nature, where the hydrogen bonds in base pairs are rapidly cleaved and reformed at ambient temperature, providing temporary cavities great enough for the bulky substituents of nogalamycin to penetrate. A regeneration of the base pairs then completes the threading intercalation (Fig. 2). The complex thus obtained can be kinetically stabilized due to the two projecting bulky substituents, which can act as anchors. The kinetic data necessary for discussion here are summarized in Table 1. The complexation of nogalamycin with DNA is characterized by a very small dissociation rate constant (k_d = 0.001

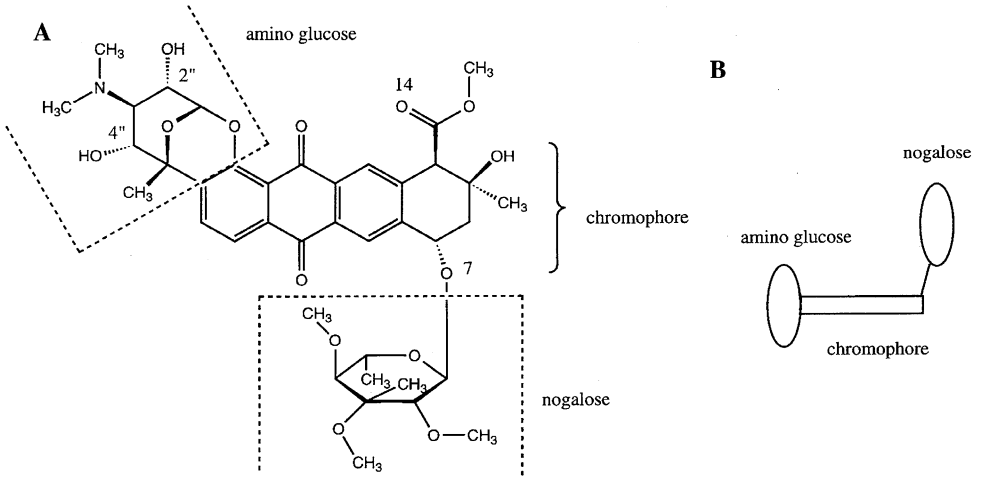


Fig. 1. Structure of nogalamycin (A) and carton of its side view (B).

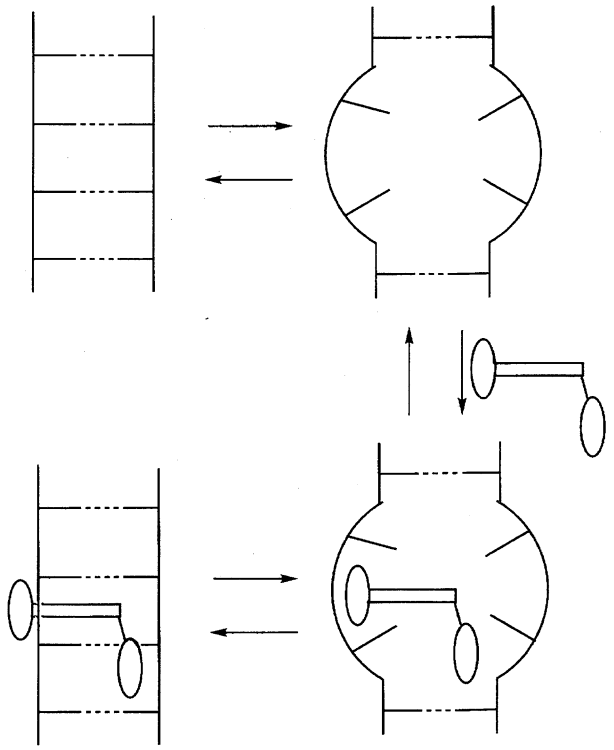


Fig. 2. Schematic model for the threading intercalation of nogalamycin molecules to double stranded DNA.

Table 1. Dissociation Rate Constants of the Several Threading Intercalators

Compound	Na^+/M	k_d[a]/s^{-1}	Ref.
Nogalamycin	0.5	0.001	5
Ethidium bromide	0.5	13.4	6
2	0.2	1.2	27
3	0.2	19.0	27
9	0.035	0.02	34, 37
1	0.2	0.63	25
1	0.035	0.42	25
15	0.035	0.06	44
15[b]	0.035	4.2	44

a) All data were obtained from stopped-flow kinetic traces for the SDS-driven dissociation of intercalators from calf thymus DNA and k_d values are the average defined as $(k_1A_1+k_2A_2)/(A_1+A_2)$, where the k and A values are the amplitudes and the first-order rate constants, respectively, for a double exponential fit to the traces. b) Denaturation of calf thymus DNA, prepared by heating at 100 °C for 10 min and then immediately cooling on ice, was used in this experiment.

s^{-1}, Table 1),[5] which was in sharp contrast with a classic intercalator such as ethidium bromide which assumes a rate constant k_d = 13.4 s^{-1} (Table 1).[6] Such a small dissociation rate constant is considered to be linked to its biological activity.[7]

X-Ray crystallographic analyses of nog–DNA complexes have been reported for four hexanucleotides: d(^{5m}CGT($_p$S)-A^{5m}CG)$_2$,[8–11] d(CGT($_p$S)ACG)$_2$,[8] d(TGATCA)$_2$,[12,13] and d(TGTACA)$_2$,[14] (where ^{5m}C is methylated cytosine at C5 position and $_p$S is an internucleotidic phosphorothioate linkage in the *R* configuration). For all of them, two nog molecules bind to one duplex hexamer.

In the complexes with d(^{5m}CGT($_p$S)A^{5m}CG)$_2$ and d(CGT-($_p$S)ACG)$_2$, nog molecules intercalate at the CG steps, where the amino sugar is located in the major groove and the nogalose moiety in the minor groove of the hexamer duplex. This intercalation did not cause any unwinding of the hexamer DNA helix. In the case of d(^{5m}C$_1$G$_2$T$_3$($_p$S)A$_4$^{5m}C$_5$G$_6$)·d-(^{5m}C$_7$G$_8$T$_9$($_p$S)A$_{10}$^{5m}C$_{11}$G$_{12}$), a specific hydrogen bonding was observed in the minor groove (one between the carbonyl oxygen at position 14 of nog and N2 of G$_{12}$ and another between the ether oxygen at position 7 of nog and N2 of G$_{12}$) and in the major groove (one between the hydroxy group at 2″ position and N7 of G$_2$ and another between the hydroxy group at 4″ position and N4 of ^{m5}C$_{11}$). 2D NMR study of d(GCATGC)$_2$ complex also gave a similar conclusion.[15] However, 2D NMR study of d(AGCATGCT)$_2$ complex revealed the intercalation of nog at a TG step.[16] An enzymatic study using DNase I footprinting indicated the preference of nog for CA and TG steps.[17] To solve this discrepancy, Smith and co-workers[12,13] studied the crystal structure of nog complex of d(TGATCA)$_2$ complex. They confirmed the intercalation of nog molecules at two TG steps. They also

confirmed that a strong hydrogen bonding between nog and DNA took place in the major groove which comprised 2″ hydroxy group of the amino sugar and N7 of guanine.

There is another structural problem in the nog–hexamer DNA interaction described above. In the complex, there can be two different orientations for nogalose and amino sugar substituents on nog molecule anchored on DNA. The situation is schematically illustrated in Fig. 3, where nogα is directed to the center and nogβ is directed toward the end of the duplex DNA. In the reality, 2D NMR study indicated that nog_2–d$(CGTACG)_2$[18] complex contained only center-directed nog molecules, while nog_2–d$(GCATGC)_2$ complex involved only end-directed nog molecules.[15] To solve this discrepancy, Petal and co-workers[19] conducted 2D NMR studies on the complexes of nog with d(GCGT) duplex (the complementary strand, d(ACGC)) and d(GCAT) (the complementary strand, d(ATGC)) duplex. For nog–d(GCGT) duplex complex, the substituents of nog are directed toward the 5′-end of d(GCGT), whereas the substituents in nog–d(GCAT) duplex complex are in an equilibrium directed either to the 5′-end or the 3′-end of d(GCAT). They concluded from these results that nog has an orientation selectivity toward 3′ side of C. The 2D NMR study at different temperatures suggested that a GC step is better than an AT step for intercalation, but a neighboring AT site is necessary to make the "partial melting" easy enough so that one of the substituents of nog can readily penetrate the base pair stacks.

Recently, the complex of arugomycin with d$(GCATGC)_2$ was analyzed by 2D NMR (Fig. 4).[20] Arugomycin, whose basic structure is similar to that of nogalamycin but features much bulkier substituents, was found to be intercalated to a CA step with specific hydrogen bonding between its hydroxy

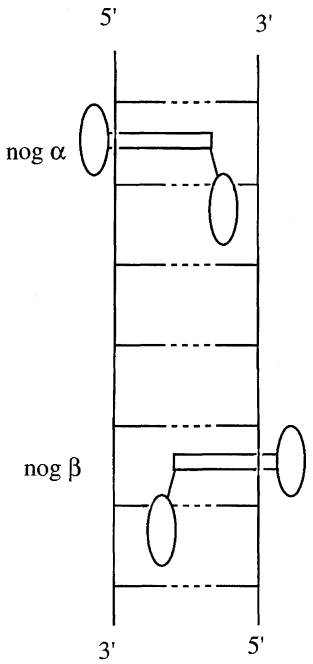


Fig. 3. Possible orientation of nogalamycin substituents on DNA. Nogα is directed to the center and nogβ is directed toward the end.

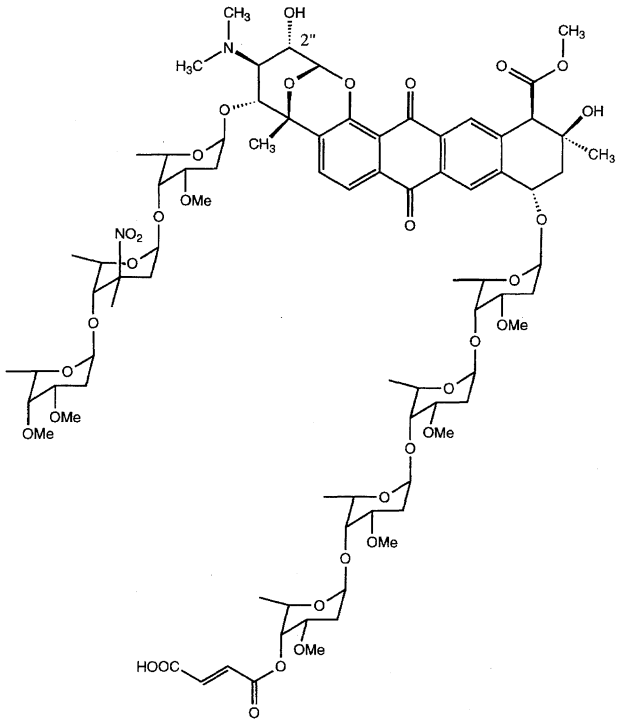


Fig. 4. Structure of arugomycin.

group at C2″ and N7 of G. This further demonstrates that DNA can accept such huge substituents for intercalation in the threading mode.

Synthetic Threading Intercalators

Figure 5 shows some typical synthetic threading intercalators, **1**, **2**, **4**—**6**. Naphthalene bis(dicarboximide) (hereafter referred to as naphthalene diimide) derivative **1**, which in fact includes a series of homologues characterized by different alkyl chains and amine groups, was studied by Gabby and Wilson in detail.[21,22] Threading intercalation of naphthalene diimide has not yet been proved by direct evidence such as X-ray crystallography, but they provided a lot of indirect evidence which supports a threading intercalation.[23] Kinetic studies are among them.

The absorption spectra of naphthalene diimide part in **1** show a strong hypochromic shift when the intercalator is bound to DNA along with a negative Cotton effect in the region of the chromophore in circular dichroism (CD) spectra. DNA becomes unwound when **1** binds to DNA; this is a typical behavior for intercalation. The salt effect on the association rate constant (k_a) of the naphthalene diimide with double stranded DNA is somewhat different from that of the classic intercalator; the slope of the plot of $\log k_a$ against $\log [Na^+]$ was one in the case of **1** (dicationic in the neutral pH range), whereas a classical dicationic intercalator, e.g., quinacrine,[24] gives a slope of two. This suggests that only one of the two cationic substituents in the threading intercalator is important in the association process. The dipositive charge on the conventional intercalator is placed exclusively in one of the grooves (minor groove), while that of **1** is placed evenly in the two grooves. A theoretical basis of the

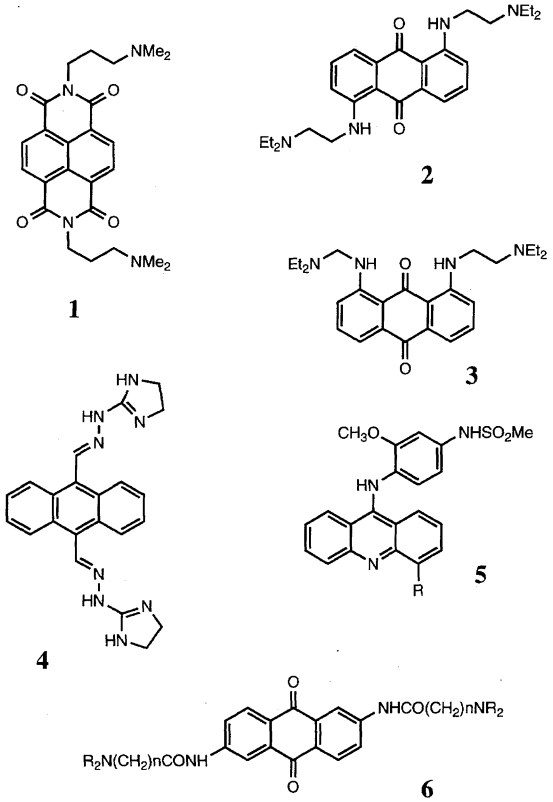


Fig. 5. Structures of synthetic threading intercalators **1**, **2**, **4**, **5**, and **6**. **3** is a classical intercalator.

observed interesting salt effect is yet to be studied.

Associaton kinetics of **1** with double-stranded DNA is strongly dependent on its substituent size. This implies that naphthalene diimide-type intercalators can be used as a dynamic probe for studying the base pair opening process in double-stranded DNA.[25] Dissociation of **1** from double-stranded DNA is also slower than that of the classical intercalating compounds as shown in Table 1. Comparison of the DNA binding behavior between 1,5-(**2**) and 1,8-disubstituted anthraquinone (**3**) exemplifies some characteristic features of threading intercalation.[26,27] Geometrically, 1, 5-disubstituted anthraquinone **2** will bind to DNA only by the threading mode as long as the anthraquinone group is fully intercalated. Experimentally, both anthraquinone derivatives **2** and **3** showed a similar hypochromic shift in their absorption spectra and indicated similar unwinding angles upon intercalation. However, the DNA double helix was more stabilized by 1,5-derivative **2** (ΔT_m of **2** was higher than that of the 1,8-isomer **3**, where ΔT_m represents the increment in the melting point of DNA on intercalation), and 1,5-derivative binds to DNA more strongly (larger binding constant) than does 1,8-isomer. In addition, 1,5-isomer **2** dissociates from DNA more slowly than 1,8-isomer **3** (Table 1). These results are in accord with the idea of threading intercalation for 1,5-isomer **2**. Yao and Wilson[28] established the threading intercalation of bisantrene **4**, a 9,10-disubstituted anthracene derivative, by 1D and 2D NMR. Anthraquinone derivative **6** also intercalates to double-stranded DNA by threading.[26,29] Recently, **6** was found to stabilize a triple helix DNA through a threading intercalation mode, where the positively charged substituents were regarded to act as molecular anchors.[30]

Tris-Intercalators Containing Threading Intercalating Units

Large series of poly-intercalators have been studied from the viewpoint of improved DNA binding affinity and the associated biological activities.[31] However, most of the poly-intercalators presented up to now are featured by "classical" intercalation. We synthesized a linear tris-intercalator **7** (Fig. 6) carrying a threading intercalator part (1,5-disubstituted anthraquinone) in the center of the linear skeleton.[32—34] We also synthesized a 1,8-disubstituted anthraquinone **8** carrying two acridine units at the molecular termini. Figure 6 includes also other tris-intercalators carrying a naphthalene diimide group as a threading intercalator part in the middle of their molecular skeleton.

Figure 7 illustrates the geometry and topology of binding expected for tris-intercalators. The binding mode IV is for classical poly-intercalators, where intercalating units are bound to a flexible aliphatic chain so that each intercalating unit behaves rather independently from others. Compounds **11**[35] and **12**[36] in Fig. 6 are typical examples. In other words, the DNA binding process of this type of tris-intercalators could be simply a repetition or an accumulation of the intercalation reaction of a classical mono-intercalator, although, in practice, the stereochemistry imposed by the connecting linker chain can introduce quite a complexity in the actual binding behavior of the total tris-intercalator molecule and the exact binding stereochemistry cannot be explained simply. The tris-intercalator **8** is also expected to bind to DNA with a classical intercalation mode like mode IV in Fig. 7. However, an emphasis is placed on its special molecular design which is not involved in the classical poly-intercalators like **11** and **12**. All the intercalating units of **8** are disposed to cover fully or to be properly sewed into the ladder of the base pairs of DNA, so that the whole molecule **8**, which is made up of three individual intercalating units, behaves as an integrated entity so as to produce a maximally stabilized DNA–ligand complex. This makes a critical difference between the binding modes III and IV.

In other words, complexes III and IV are similar in that tris-intercalators bind to DNA from one side of the groove. However, if one examines the linker effect more closely, binding modes III and IV are quite different. In binding mode III, both linker chains connecting the terminal intercalating units are separated by the central intercalating units and hence the binding of one terminal site of intercalator and central intercalator does not affect the interaction of the other terminal intercalating unit. On the other hand, there is a steric restriction in the binding mode IV because of the bifurcated linker arms connecting the terminal intercalating units.

Tris-intercalator **7** carrying a threading intercalator unit in the middle position is expected to assume unique binding modes such as I and II in Fig. 7. Binding model I may be called the penetrating mode, where one of the terminal in-

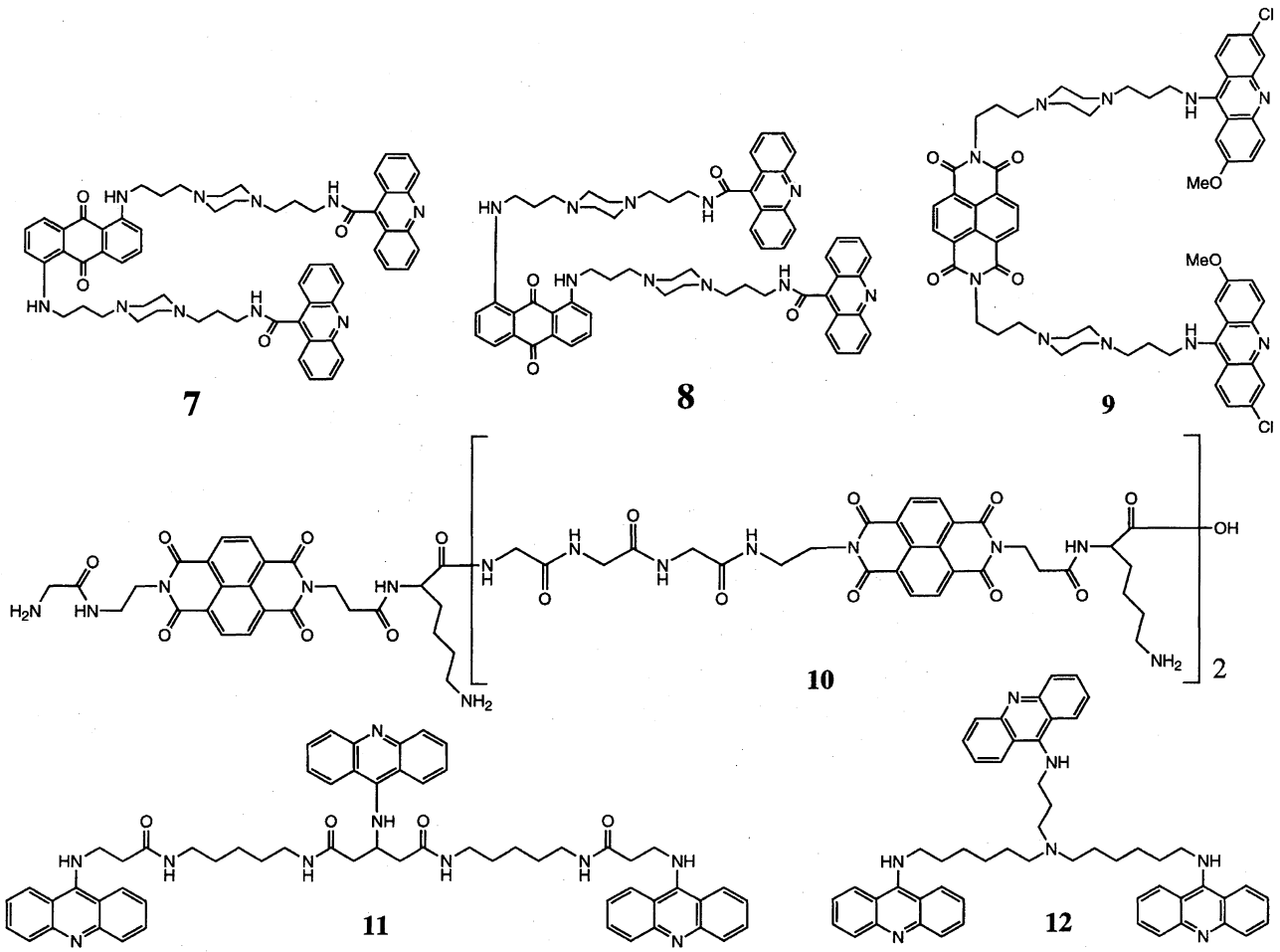


Fig. 6. Structure of tris-intercalators carrying classical or threading intercalating units.

tercalating parts need to go through between base pairs to complete the DNA–ligand binding. In the resultant complex, the two arms on the central intercalator protrude in the major and minor grooves of DNA and the two terminal intercalating parts then become bound back to the base pairs. In binding model II, the molecular skeleton of the ligand stays exclusively in one of the grooves of DNA. Only the two terminal intercalating units become fully stacked with base pairs, while the intercalating unit in the middle remains unbound or groove-bound. A molecular model consideration for **7** suggests that all the molecular parts can be well accommodated on the double-stranded DNA according to the penetration mode I. Each of the piperazine rings is at least singly protonated in the pH range of our study, and should be stabilized in the groove through external binding to the stacked base pairs. On molecular modeling, one does not see any positive reason for **7** to prefer binding model II.

A similar consideration on **8** by molecular modeling suggests that it should prefer binding model III. **7** and **8** are the geometrical isomers of each other, and the resultant DNA complexes are a kind of topological isomers. Then, what kind of physicochemical similarities and differences can we expect between the two complexes?

Spectrophotometric titration of DNA with tris-intercalator **7** or **8** showed hypochromic and bathochromic shifts of a similar magnitude for **7** and **8** in the region of acridine (360 nm for both **7** and **8**) and anthraquinone chromophores (539 nm for **7** and 555 nm for **8**). The CD spectra of calf thymus DNA showed an additional negative Cotton effect at both acridine and anthraquinone absorptions in the presence of tris-intercalator **7** or **8**. The negative induced CD in this region indicates the intercalation of these chromophores to DNA duplex. We also measured an unwinding angle of base pairs in double-stranded DNA caused by intercalation through viscometric titration with supercoiled plasmid DNA as 48° and 49° for the tris-intercalators **7** and **8**, respectively. The unwinding angle of classical mono-intercalators is known to be around 17°, and the values obtained above were nearly three times that for mono-intercalators. This is another line of evidence of the participation of all the intercalating units of **7** and **8** in intercalation.

Penetration of the substituent in tris-intercalation I is expected to stabilize the DNA duplex against melting or dissociation. We compared the effect of tris-intercalators **7** and **8** on DNA melting temperature. Tris-intercalator **7** stabilized the DNA double-strand by 28 °C (ΔT_m) which was larger than 24 °C for **8**. All these experimental results seem to support the penetrating intercalation mode for **7**.

On forming the DNA complex of **7** by the penetration mode, one of the acridine chromophores has to go through

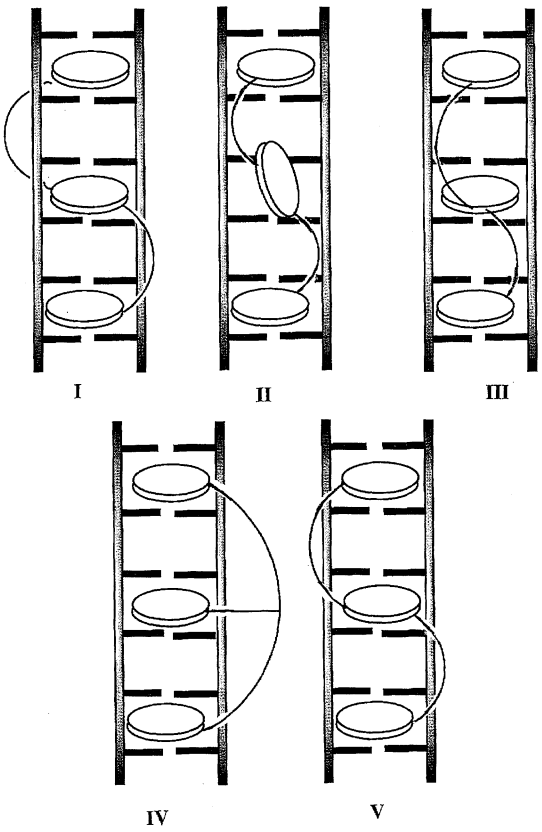


Fig. 7. Schematic representation of the binding of a tris-intercalator to double stranded DNA. In modes I, III, IV, and V, all the intercalating units of tris-intercalator were intercalated between adjacent base pairs of double stranded DNA. However, if one examines the complex structure of the central intercalating unit more closely, they intercalate in quite a different manner. In mode I, two linker chains are projecting out in each groove. In binding mode III, both linker chains connecting the terminal intercalating units are separated by the central intercalating units and hence the binding of one terminal site of intercalator and central intercalator does not affect the interaction of the other terminal intercalating unit. On the other hand, there is a steric restriction in binding mode IV because of the bifurcated linker arms connected with the terminal intercalating units.

the stack of base pairs. Kinetically, this process should occur in AT-rich regions more easily than in GC-rich regions. This association step is not necessarily reflected in the equilibrium binding behavior. Nevertheless, it is interesting to note that most of the classical poly-intercalators hitherto reported showed a preference for AT-rich regions on double-helical DNA. In this connection, we estimated the DNA base sequence selectivity of **7** by C_{50} measurement. The C_{50} values represent the tris-intercalator concentration necessary to displace 50% of DNA-bound ethidium bromide. The smaller the C_{50} values, the stronger the binding for DNA. The C_{50} values for DNA from several sources with different AT (adenine and thymine) content shows that both tris-intercalators **7** and **8** intercalated preferably with AT-rich DNAs but **8** had a higher AT-selectivity than **7**. The reason for this difference is not clear, but it is known that when a poly-intercalator binds to DNA in one side of the groove only because of a structural restraint of the linker chain, it tends to prefer AT-rich DNAs where the structure is more flexible. DNase I footprinting showed that **7** and **8** covered or bound to AT-contiguous sequences with high selectivity. Since AT-contiguous sequences of DNA are known to be very important for the regulation of gene expression, this type of tris-intercalators is expected to be useful in arificial gene regulation in the future.

We designed tris-intercalator **9** (Fig. 6) for extending the scope of this class of threading tris-intercalators.[34,37] Tris-intercalator **9** is furnished with a naphthalene diimide group as a threading intercalating unit in the middle of the molecular skeleton and two 9-aminoacridine moieties are connected through alkylamino chains at the ends. Absorption and fluorescence intensity of **9** in aqueous solution decreased as the buffer and salt concentration of the medium was increased. This was interpreted to indicate that **9** is in equilibrium between an elongated and an intramolecularly stacked conformation. Higher concentrations of buffer or supporting electrolyte shift the equilibrium toward the stacked conformation, where the three aromatic chromophores are associated together, resulting in a decrease in the apparent molar absorptivity and a quenching of monomeric acridine fluorescence. This made it difficult to analyze the interaction of **9** with DNA in detail. However, we managed to determine the binding constant in pure water to be 10^7 M^{-1} (1 M = 1 mol dm^{-3}) with calf thymus DNA using absorption and fluorescence photometry.

A kinetics study was made for the dissociation of **9**–DNA complex in a buffered solution according to the method of Wilson.[38] The absorption of the naphthalene diimide and acridine chromophores and the fluorescence of acridine chromophore were monitored. The two methods gave the same dissociation rate constant of 0.02 s^{-1} (Table 1). Since the naphthalene diimde threading intercalator **1** has a much larger dissociation constant of 0.40 s^{-1}, the threading tris-intercalator **9** dissociates at least 20 times more slowly than the threading mono-intercalator **1**. This supports the penetrating mode I for the binding of **9** with double-stranded DNA.

Recently, Iverson et al.[39] synthesized tris-naphthalene diimide-type linear ligand **10** (Fig. 6) which made use of a tripeptide linkage containing glycine and lysine residues. A high DNA binding affinity was confirmed, and the DNA unwinding demonstrated the intercalation. However, they concluded that the intercalating unit actually did not thread in and proposed the binding mode V. The reason why **10** prefers this binding mode is yet to be studied.

Naphthalene Diimide-Based Intercalators Carrying Thymine Bases

Dumbbell-shaped synthetic intercalators seem to give a variety of DNA ligands with potentially new functions in DNA chemistry. In fact, the interest is not simply limited to the topology of intercalation. From the view point of molecular design, tris-intercalators of the type **1**—**9** may be seen as follows: The central aromatic ring (anthraquinone, naphtha-

lene diimide, etc.) is a universal affinity ligand for DNA and the two arms represent functional groups with which one can exert a particular chemical action on the DNA strand. The target nucleic acid with which the designed ligands interact is not limited to double-stranded DNA or RNA. A single-stranded DNA or RNA could well be studied with such ligands designed for specific purposes. The interaction of dumbbell-shaped synthetic intercalators with RNA is dealt with below as an example.

We measured the binding constant of **1** (Fig. 5) with polyA as a single-stranded nucleic acid and with polyApolyU as a double-stranded nucleic acid.[40] The binding constant is slightly larger for polyApolyU (2.8×10^5 M^{-1}) than for polyA (1.1×10^5 M^{-1}) in 10 mM MES and 1 mM EDTA (pH 6.24) at 25 °C. It was rather unexpected that the binding affinity of **1** is in the same order of magnitude for single-stranded and double-stranded RNAs. On the other hand, the binding constant for polyU was nearly ten times smaller than that for polyA (1.3×10^4 M^{-1}) under the same conditions. This suggested that the stacking interaction between the nucleic base plane and the naphthalene diimide ring is important for the binding with single stranded nucleic acid.

Naphthalene diimide **13** carrying two thymine moieties at the termini of the substituents on the imide nitrogens (Fig. 8) was synthesized.[40] The binding affinity of **13** for polyApolyU double-strand was not much different from that of **1** (binding constant, 1.1×10^5 M^{-1} for **13** and 2.8×10^5 M^{-1} for **1**). However, the binding of **13** to single-stranded polyA was much stronger (1.6×10^6 M^{-1} for **13** and 1.1×10^5 M^{-1} for **1**). As a result, the binding affinity of **13** for single-stranded polyA is about 15 times greater than that for double-stranded polyApolyU. On the other hand, the affinity of **13** for polyU is much lower with a binding constant equal to that of **1** with polyU as described above. These results indicate that the complex of **13** with single-stranded polyA is stabilized by hydrogen bond formation between the thymine bases of **13** and adenine bases of polyA, as schematically shown in Fig. 8(a). This rationalizes the molecular design outlined above. The naphthalene diimide moiety of **13** makes a hydrophobic, stacked complex with adenine bases on the flexible chain of polyA. This allows the thymine bases on **13** to gain an easy access to neighboring adenine bases on polyA to produce specific nucleic base pairing (two AT base pairs). All the processes are considered to help the molecule of **13** to assume a binding preference to polyA single-strand. **13** is the first example of a simple compound showing specificity for a flexible single-stranded A-contiguous sequence. In passing, we tested polydA as a DNA polymer as well, but **13** showed a decreased binding affinity (binding constant, 3.1×10^4 M^{-1}), due presumably to the conformational rigidity of polydA.

As an extension of this type of study, we tested the binding of **13** to d(GCGAAACGC) oligonucleotide.[41] This oligonucleotide assumes a hairpin structure in solution at ambient temperature which is formed by folding of the molecular chain. Therefore, the naphthalene diimide **13** was at first expected to bind to the AAA region of the hairpin oligonucleotide. However, **13** was found to be bound to and stabilized in the AAA region of the bulged dimer, and the content of this unique structure increased as the amount of **13** in solution was increased (Fig. 8(b)). The bulged dimer is an alternative structure of the hairpin for d(GCGAAACGC) oligonucleotide, but the dimer does not exist under ordinary conditions (10 mM MES, 1 mM EDTA, pH 6.25). That the dimer formation was induced by the presence of **13** is not surprising in light of the fact that **13** prefers a flexible single-stranded A-contiguous sequence for stacking interaction. The single-stranded AAA region of the bulged structure is more flexible than the AAA region of the hairpin structure. The AAA region of the bulged structure is like the pleats of an accordion: It can adjust its own fine conformation to accommodate most suitably the incoming naphthalene diimide moiety of **13**. It should be pointed out that the mixture of **1** and thymine base did not exert any stabilizing effect on the bulged dimer formation. Moreover, **13** did not cause any effect on d(GCGTTTCGC). These observations again emphasize the importance of specific hydrogen bonding between thymine bases in **13** and adenine bases in the bulged oligonucleotide. In order to obtain further proof on the complexing site of **13** with bulged d(GCGAAACGC), we titrated a solution of d(GCGAAACGC) with ethidium bromide in the presence of **1** or **13**. Since ethidium bromide mainly binds to double-stranded regions of a DNA duplex, there should

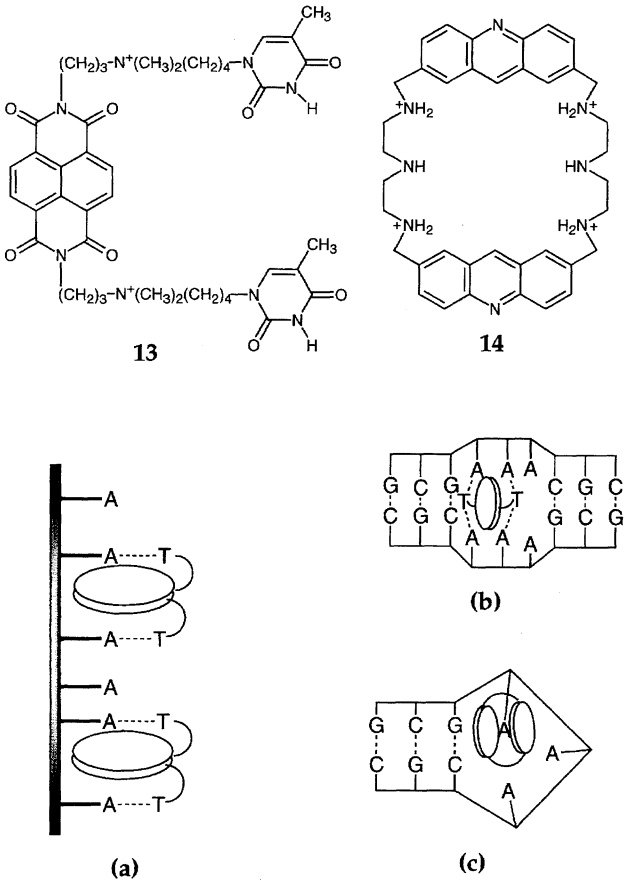


Fig. 8. Structure of intercalators, **13** and **14**. Examples of recognition patterns of high-order single stranded nucleic acids by **13** (**a**, **b**) and **14** (**c**).

not be much competition between ethidium and **13**. On the contrary, a considerable competition between ethidium and **1** may be expected since both ligands become bound to the duplex region. The experiment indicated that the fluorescence of ethidium bromide was suppressed more effectively by **1** than by **13**. This is in accord with the bulge site binding of **13**.

Lehn et al.[42] studied the interaction of cyclic bis-acridine **14** with d(GCGAAACGC). This ligand **14** was proved to bind to the adenine base region of hairpin structure (Fig. 8(c)). The binding site preferences are similar between **13** and **14**, but the binding topology preferences are totally opposite.

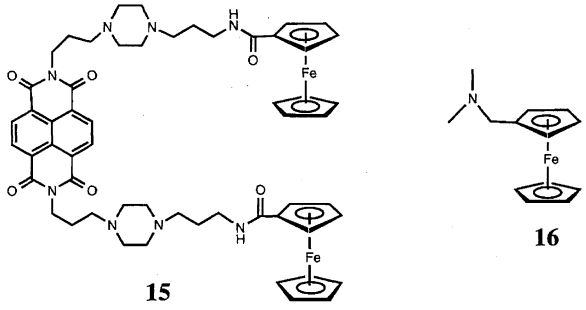
A-contiguous sequences, when present in natural nucleic acid, are known to assume a variety of higher-order structures such as base bulge or hairpin. These structures are associated with many biological functions of nucleic acid. Thus, it is very important to develop ligands that are able to recognize these special structures of nucleic acids. For example, molecules capable of binding to TAR or REE RNA sequences could be good candidates as anti-HIV agents because they should inhibit the interaction of Tat and Rev protein with TAR and REE RNA, respectively.[43] These sequences include many single-stranded nucleic acid regions, which may be a good target of our ligand of the type **13** or Lehn's cyclic-bisintercalator.

Naphthalene Diimide-Based Intercalator Carrying Ferrocenyl Moieties

Threading intercalators often show an unusually slow dissociation rate from double-stranded DNA. However, this does not hold for their complexes with single-stranded DNA but the dissociation rate from single-stranded DNA is greater. This can be used for the discrimination between double- and single-stranded DNA, which can find a unique application in DNA sensing.

We synthesized naphthalene diimide **15** (Fig. 9) which carries ferrocenyl moieties at the termini of its two substituents.[44,45] The ferrocene group offers two functions: (i) Improvement of discrimination ability between single- and double-stranded DNA in the dissociation of a ligand–DNA complex. The molecular thickness of ferrocene is about 4 Å. This value is slightly larger than the thickness of a base pair in double helix DNA, 3.4 Å. This suggests that the complex of **15** with double-stranded DNA is kinetically stable, because of the steric hindrance involved when the ferrocene part slides away between the adjacent base pairs. On the contrary, the complex with single-stranded DNA is not expected to be stabilized by such an effect and (ii) Electrical signal transducer. The presence of **15** is readily detected electrochemically through a characteristic redox reaction of a ferrocene group.

Our strategy of DNA sensing is as follows. A DNA probe, which carries a complementary sequence of the target gene, is chemically immobilized on the surface of an electrode. This electrode is soaked in the sample DNA solution containing the target DNA (hybridization reaction), and then treated with **15** for intercalation or complex formation. After washing the electrode briefly with a buffer solution, the amount of redox active species (**15**) on the electrode is measured by conventional cyclic voltammetry. The procedure is similar in principle to that described previously,[46,47] except that a maximum advantage was taken of the kinetically stable, double-stranded DNA–selective ligand, **15**. If a target gene is present in the sample, it forms a double-stranded DNA on the probe electrode, which then collects electroactive ligand **15**. The **15**–double helix complex is kinetically stable and thus should stay intact even after washing.



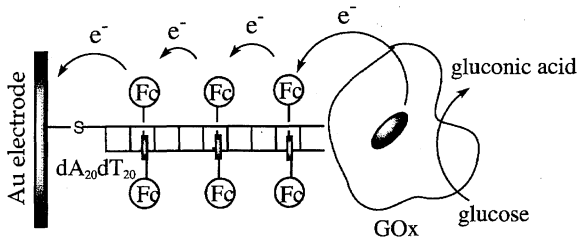


Fig. 9. Construction of a pseudo-polyferrocene array with **15** intercalated to a $dA_{20}dT_{20}$-immobilized gold electrode. Mediation of electron transfer between GO_x^{red} and the electrode.

The dissociation rate was measured for the complex formed from the ligand **15** and DNA. Native calf thymus DNA was used as a model of double-stranded DNA, and a heat-denatured calfthymus DNA was adopted as a model for single-stranded DNA. A heat-denatured calf thymus DNA still includes an appreciable fraction of double-stranded regions, but even with this reference **15** dissociates from double-stranded DNA about 100 times more slowly than from single-stranded DNA (Table 1). When compared with the dissocation rate constant of naphthalene diimide **1**, **15** has about 10 times smaller dissocation rate constant than **1** (Table 1). By following the procedure outlined above, yeast choline transfer gene was successfully detected at a femtomole level. Thus, the proposed method has a detection limit comparable to that of the conventional radioisotope-based technique.

Development of a gene detection method with high sensitivity becomes more and more important from the viewpoint of gene diagnosis and therapy. The DNA probe method is commonly in use for such purposes even now. In this method, one has to make a proper chemical modification (attachment of a label) to a DNA fragment, where a large number of chemical labels is necessary to improve the de-

tection limit. However, this causes a decrease in the stability of the complex between the labeled DNA probe and sample gene, resulting in a decrease in selectivity. In the proposed methodology, the chemical modification of DNA probe is unnecessary and thus DNA probe can bind to the target gene without disturbing the hybridized duplex stability. The method should be feasible for practical use in DNA analysis and gene diagnosis.

DNA-Strand Coating with Ferrocenyl-Modified, Naphthalene Diimide-Base Intercalators

As a dipositive intercalator, **15** shows a high binding affinity for double-stranded DNA. In fact, spectrophotometric titration of **15** with calf thymus DNA gave a binding constant of 1.3×10^5 M^{-1} with a site size of 2 and a cooperative parameter of 0.4 in 10 mM MES and 1 mM EDTA at pH 6.25 and 25 °C. The site size of 2 implies that at binding saturation, **15** is placed on double-stranded DNA every two base pairs. This means that the placement of the ferrocene group around the duplex strand is considerably dense, and the structure of the ferrocenyl ligand-DNA complex is like a pseudo-polyferrocenyl polymer coiled around the DNA strand along the two grooves. Such an arrangement of redox active ferrocene may effectively mediate an electron transfer from one end to the other along the double-stranded DNA.

A study was made whether the ferrocenyl threading intercalator **15** could be arranged on DNA to form a pseudo-polyferrocene array and thus effectively mediate the electron transfer between reduced glucose oxidase (GO_x^{red}) and DNA-immobilized gold electrode. Figure 9[48,49] illustrates the concept.

Cyclic voltammetry (CV) was run in a solution containing GO_x and glucose using a $dT_{20}dA_{20}$ (double-stranded DNA)-immobilized electrode treated with **15** in advance, followed by electrocatalytic anodic current determination.

The peak current was twice as large as that obtained with a reference compound **16**. The current was also twice as large as that for the case where dT_{20} (single-stranded DNA)-immobilized electrode was used along with the ligand **15**. Based on these observations, it was concluded that **15** bound to the double-stranded DNA facilitated the electron transfer between GO_x^{red} and the electrode as illustrated in Fig. 9. This is the first case of electron transfer along the DNA strand mediated by intercalated molecules.

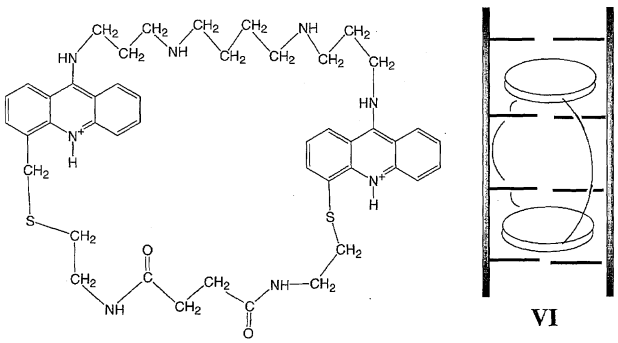
Development of nano-scale electronic devices is a real challenge for chemists. DNA is a good candidate for the construction of such nano-size assemblies.[50—52] However, one has to arrange functional molecules capable of conducting electrons through the strand of DNA, which possesses only a poor electron conducting ability. Recently, Braun and co-workers[48] succeeded in the preparation of a DNA wire by coating a DNA strand with metallic silver. Our system just described could be one alternative to this approach in utilizing a DNA matrix to construct an electron conduction network which takes advantage of all the peculiar supramolecular architecture of DNA.

Cyclic Bis-threading Intercalators

We already described the example of cyclic-bis-acridine **14**. However, this compound did not bind to double-stranded DNA, possibly because of the short linker chain length which connects the two acridine units. Zimmerman and co-workers[54] were the first to synthesize the cyclic bis-threading type compound **17** (Fig. 10), where 4- and 9-positions of acridine parts were connected by 13—14 atom-long chains (similar to **5** in Fig. 5). Since 4,9-disubstituted acridine unit offers a sound molecular structure for threading intercalation, the formation of a stable complex between **17** and duplex DNA strongly suggests the realization of a unique binding mode VI (Fig. 10). From a mechanistic point of view, this topology is to be obtained only through breakage of at least one or two base pairs of the DNA duplex; the details could be no more than a conjecture. Zimmerman and Wilson[55] studied the complex of **17** with $d(CGCG)_2$ by NOESY and COSY NMR spectroscopy. The aromatic proton of **17** showed an upfield shift when bound to the DNA oligomer, and the proton NOE cross peaks were observed between the aliphatic linker chain of **17** and the nucleic bases projecting out into the major and minor grooves of $d(CGCG)_2$. This is in agreement with the complex structure schematically shown by VI in Fig. 10. However, there is not any assurance that the basic structure of the duplex DNA remains the same, i.e., as B-structure DNA, after suffering a catenane type intercalation as in IV. It is noted in this connection that triostin, a natural bis-intercalator, makes the configuration of DNA change from the Watson–Crick base pairing type to the Hoogsteen base pairs when bound to DNA.[56] This shows that intercalators could affect the structure of DNA dramatically.

Summary

Threading intercalation was found for the first time in the interaction of nogalamycin with double-stranded DNA. This type of interaction does not seem to be special but occurs rather universally, reflecting the structural flexibility of double-stranded DNA in accepting a variety of intercalating



17

VI

Fig. 10. Chemical structures of cyclic bis-threading intercalators **17** and the proposed model of the catenated complex (VI).

ligands. The DNA complexes with a threading intercalating ligand can take varying geometrical and/or topological configurations depending on the steric requirement of the ligand and generally feature a slow rate of dissociation. Synthetic threading intercalators, typically *N*,*N*′-disubstituted naphthalene diimide derivatives, are of special interest, because they can be provided with added chemical functions other than simple threading intercalation. Some of them can be used to probe the dynamics (breathing) of double-stranded DNA at ambient temperature. An electrochemically labeled naphthalene diimide derivative (ferrocene-modification at *N*,*N*′-substituents) can label a duplex DNA strand. The labeled duplex DNA complex is kinetically differentiated from the complex of single-stranded DNA, and the principle can be applied to hybridization-based gene sensing. The naphthalene diimide derivative carrying thymine pendant arms indicates a binding selectivity to polyA due to AT base pair formation on the chain. This ligand also alters a hairpin-bulge duplex equilibrium of d(GCGAAACGC). A cyclic bis-thread intercalating ligand can give a catenated complex with duplex DNA, whose detailed physicochemical characterization, especially the kinetics of formation and dissociation, is yet to be finished. Inclosing, it is emphasized that threading intercalation has raised the importance of dynamic aspect of DNA complexation with small molecules, for the first time in an explicit way. Threading intercalation gives a vivid, working idea about the mechanism of base-pair cleavage and restoration on duplex DNA. It also stimulates synthetic chemists to design molecules for probing and/or regulating the dynamics of duplex DNA under physiological conditions.

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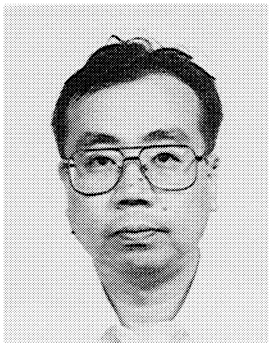

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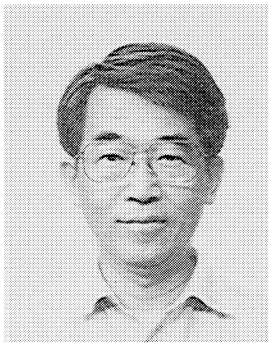

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