Sequence Specificity of Hydrogen-Bonded Molecular Duplexes

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Hydrogen-bonded molecular duplexes, **1·3** and **1·4**, each of which contains a mismatched binding site (acceptor-to-acceptor in **1·3**, and donor-to-donor in **1·4**), were designed and synthesized based on duplex **1·2**. One- and two-dimensional NMR studies demonstrated that, despite their single mismatched binding sites, the backbones of duplexes **1·3** and **1·4** still stayed in register through the formation of the remaining five H-bonds. The backbones of **1·3** and **1·4** adjusted to the presence of the mismatched binding sites by slightly twisting around these sites, which alleviate any head-on repulsive interactions between two H-bond donors (amide O) or between two acceptors (amide H). After 1 equiv of single strand **2**, which forms a perfectly matched duplex **1·2** with single strand **1**, was added into the solution of either **1·3** or **1·4**, only **1·2** and single strand **3** or **4**, were detected. Isothermal titration calorimetry (ITC, in chloroform containing 5% DMSO) indicated that duplexes **1·3** and **1·4** were significantly (>40 times) less stable than the corresponding perfectly hydrogen-bonded duplex **1·2**. These NMR and ITC results indicate that the pairing of two complementary single strands is not affected by another very similar single strand that contains only one wrong H-bond donor or acceptor, which demonstrates that the self-assembly of this class of H-bonded duplexes is a highly sequence-specific process. The role of these H-bonded duplexes as predictable and programmable molecular recognition units for directing intermolecular interactions has thus been established.

Introduction

High specificity of intermolecular interaction, which leads to the precise assembly of multicomponent architecture, is one of the most prominent features of natural supramolecular systems. Such high specificity results from the cooperative action of numerous noncovalent interactions. The formation of DNA or RNA double helix represents one of the most well-known and intensively studied examples of molecular recognition.[1] The association of nucleic acid strands is realized by nucleobase complementarity, which results in highly specific intermolecular interactions. Sequence-specific pairing of DNA and RNA strands is essential for the storage, transmission, and expression of genetic information, and forms the basis for techniques such as PCR,[2] hybridization techniques,[3] and DNA chip arrays.[4] To develop DNA-like, information-rich molecules that may lead to the specification of intermolecular interactions, efforts in designing complexes based on arrays (sequences) of hydrogen bond donor (D) and acceptor (A) sites have recently intensified.[5] The laboratories of Meijer,[6] Nowick,[7] Sessler,[8] Zimmerman,[9] and others[10] reported high affinity H-bonded systems based on heterocycles, nucleobases, and peptide strands containing unnatural amino acids.

We have also developed highly stable H-bonded duplexes based on linear oligoamide strands bearing arrays of hydrogen bond donors and acceptors on one edge.[11] These molecular duplexes are formed by pairing two strands of complementary H-bonding sequences. Due to the absence of secondary electrostatic interactions[12] in this system, the stability of a duplex is sequence-independent and is proportional to the number of H-bonds found in that duplex. By incorporating mismatched

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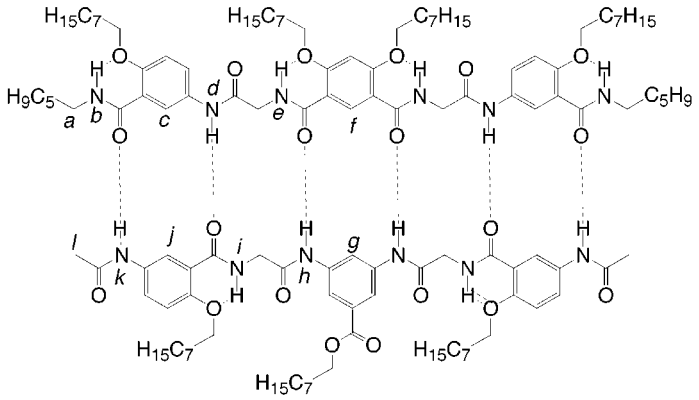
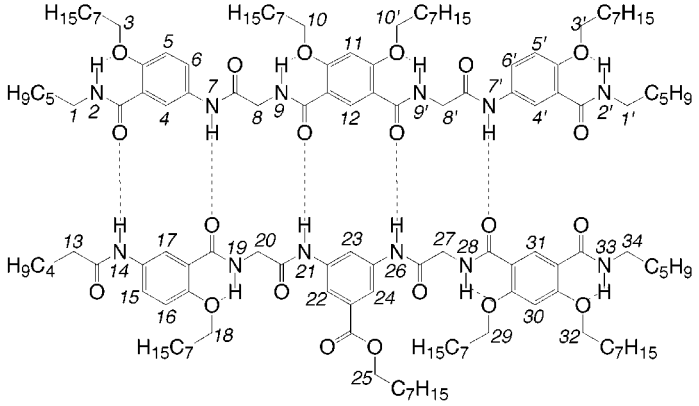
Chart 1



1

2

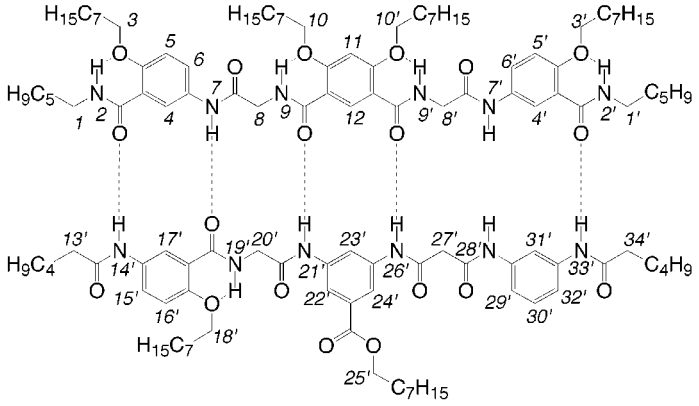
1·2



1

3

1·3



1

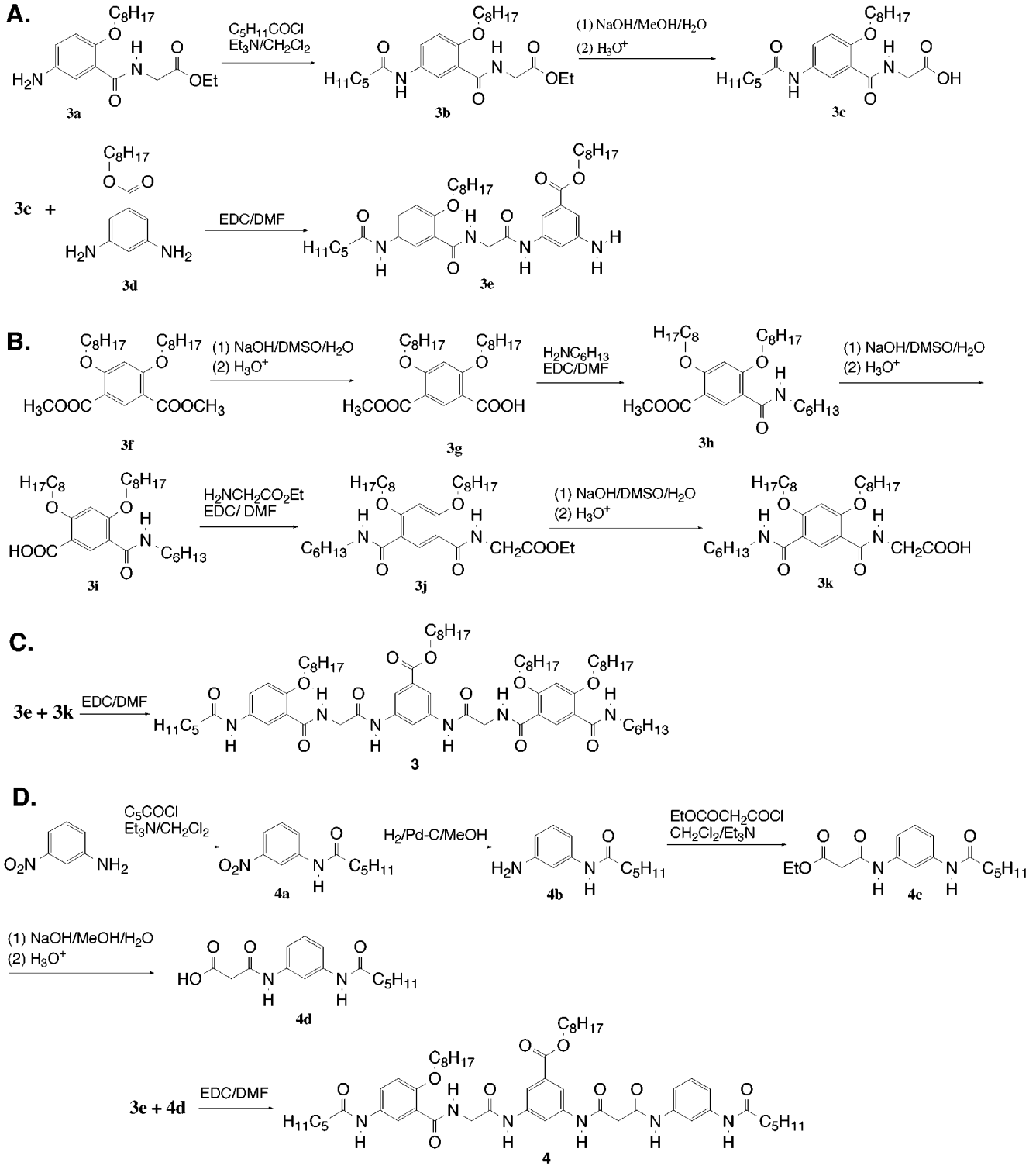
4

1·4

binding sites, such a molecular duplex system should provide an ideal platform for systematically probing the effect of sequence-specificity on unnatural self-assembling processes, which have so far been difficult to investigate due to the lack of appropriate model systems. Addressing the problem of sequence-specificity is critical to achieving the final objective of this research, which involves developing these molecular duplexes into a whole set of information-rich molecular recognition units that can lead to predictable and programmable intermolecular interactions. Such molecular recognition units will serve as powerful and versatile "sticky ends" for assembling multicomponent architecture.

Single strands **1** and **2** were found to pair into a highly stable, six-hydrogen-bonded duplex **1·2** (Chart 1).[11b] Single strands **3** and **4**, with the hydrogen-bonding sequences of DADDAA and DADDDD, were designed and synthesized to pair with **1**. Each of the resultant duplexes, **1·3** or **1·4**, should contain a mismatched binding site. Can duplexes **1·3** and **1·4** still form? Will the single strands in duplexes **1·3** and **1·4** be partially aligned to avoid the mismatched binding sites? What are the

Scheme 1



stabilities of **1·3** and **1·4** compared to that of **1·2**? How does the placement of the mismatch within each strand influence the thermodynamics of duplex formation?

We report here that, similar to duplex DNA, the self-assembly of these unnatural H-bonded duplexes is a highly sequence-specific process. Comparing duplexes **1·3** and **1·4** with **1·2**, which contains a perfectly matched H-bonding sequence, revealed the much higher stability of the latter. When three different single strands coexisted in solution, only the two with perfectly matched H-bonding sequences paired into the corresponding duplexes.

Results and Discussion

Synthesis. Single oligoamide strands **3** and **4** were synthesized by iterative coupling steps using either acid chloride or EDC (Scheme 1). Compound **3a**[11b] was acylated into **3b** which was then hydrolyzed to give acid **3c**. Diamine **3d**[11b] was then monoacylated by **3c** using EDC coupling, leading to **3e**.

Dimethyl 4,6-dioctyloxy-1,3-benzoate **3f**[11b] was partially hydrolyzed by using 1 equiv of NaOH in DMSO and water to give the monoacid **3g**. The coupling between **3g** and hexylamine using EDC in DMF gave **3h**. Hydrolysis of **3h** gave **3i**, which was first coupled with glycine ethyl ester to give **3j**. Subsequent hydrolysis of **3j** lead to **3k**. Coupling between **3e** and **3k** using EDC in DMF lead to **3**.

Amide **4a** was obtained by acylating 3-nitroaniline using hexanoyl chloride. Hydrogenation of **4a** and subsequent acylation lead to ester **4c** which was hydrolyzed to give acid **4d**. Coupling between **3e** and **4d** using EDC in DMF lead to **4**.

^{1}H NMR Spectroscopy. The ^{1}H NMR (400 MHz, 298 K in $CDCl_3$) spectra of single strand **3** or **4** showed broad, poorly defined resonances, indicating a slowly equilibrating mixture of many conformations. Upon mixing 1 equiv of **3** or **4** with 1 equiv of **1**, both **3** and **4** showed sharp sets of signals that can only be assigned to a single conformer, suggesting the association of **3** or **4** with **1.** Interestingly, protons *7* and *7′*, which are equivalent in single strand **1** or in duplex **1·2** as proton *d*, became nonequivalent upon mixing **1** with **3** or **4** (Figure 1). At 25 mM in $CDCl_3$, proton *7* of **1·3** appeared at 10.31 ppm, and that of **1·4** at 9.98 ppm; proton *7′* of **1·3** appeared at 10.04 ppm, and that of **1·4** at 9.73 ppm.[13] These data suggest the formation of duplexes **1·3** and **1·4** whose unsymmetrical structures placed protons *7* and *7′* in different chemical environment. Obviously, the exchange of single strands **1**, **3**, and **4** with the corresponding duplex **1·3** or **1·4** was slow on the NMR time scale.

To provide more detailed evidence for the formation of duplexes **1·3** or **1·4** in solution, two-dimensional NMR (NOESY) spectra were recorded (400 MHz, 293 K, 25 mM in $CDCl_3$). In each case, numerous interstrand contacts were observed, which confirms the dimeric structures of **1·3** and **1·4**. Figure 2 shows the regions of the NOESY spectrum of duplex **1·3**, which indicate strong NOE contacts between protons *13* and *1*, and *20* and *8*, as well as *27* and *8′* (Figure 2a). In addition, Figure 2b shows contacts between protons *14* and *4*, *21* and *11*, *23* and *11*, and *26* and *1*. Weak contact between protons *31* and *4′*, and *31* and *7′*, also exist. No NOE contact between protons *34* and *1′* is found. These results suggest that one end of **1·3** is locked by H-bonds while the other is open due to the presence of the mismatched binding site consisting of the two amide carbonyl groups. NOESY spectrum of **1·4**, as shown in Figure 3a, indicates strong NOE contacts between protons *13′* and *1*, and between *34′* and *1′*, along with contacts between protons *20′* and *8*, and *27′* and *8′*, suggesting that the two strands align in an end-to-end fashion. The NOESY results indicate that both ends of **1·4** are locked despite the presence of mismatched binding site consisting of the two amide NH groups. Figure 2b shows additional contacts between protons *21′* and *11*, *23′* and *11*, *26′* and *11*, and between *33′* and *4′*. The fact that no cross-strand contacts are observed between protons *28′* and *7′*, and only extremely weak contacts between protons *31′* and *7′*, and between *28′* and *4′* are detected, indicates that the backbones of **1·4** must be twisted to alleviate any head-on repulsive interaction between the two NH groups. Consistent with this explanation, in **1·4**, proton *28′* exhibits very strong NOE contact with *29′*, and strong NOE with *30′*; while proton *14′* exhibits no NOE contacts at all with *15′* and *16′*. However, this type of twist seems not to disrupt the formation of the other five attractive H-bonds, which is supported by the presence of cross-strand NOE contacts. Such a twist in backbone should also be true for **1·3**, which is supported by the lack of NOE contact at one end of this duplex as shown by NOESY studies. In **1·3** the five attractive H-bonds also formed as evidenced by cross-strand NOE contacts.

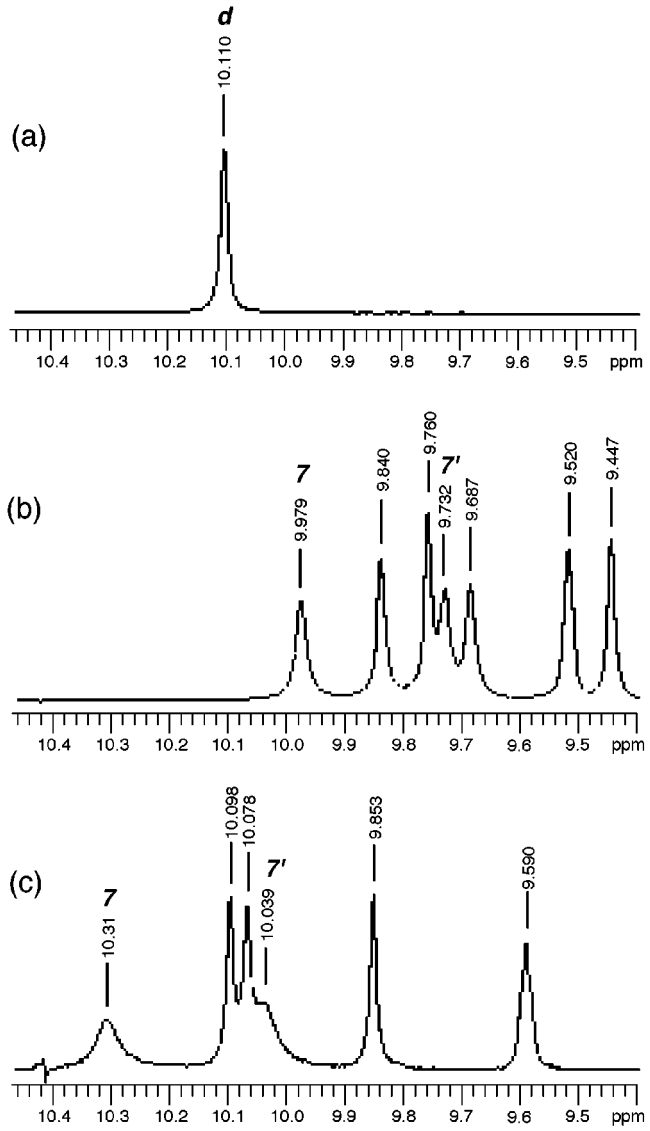


Figure 1. Amide NH resonances of (a) proton *d* of single strand **1**, (b) protons *7* and *7′* of **1·4**, and (c) protons *7* and *7′* of **1·3** at 25 mM.

^{1}H NMR competition titration experiments were carried out to examine the sequence specificity in forming **1·2**. The ^{1}H NMR signals within the 9.30−9.85 ppm region are good indicators for deriving the information of the sequence-specific pairing of **1·2** (Figure 4). The ^{1}H NMR spectra of **1·3** (Figure 4a) and **1·4** (Figure 4e) (at 2 mM in 5%DMSO-d_6 in $CDCl_3$) show two and three sharp peaks in this region, respectively. With the addition of 1 equiv of **2** into either **1·3** (Figure 4b) or **1·4** (Figure 4d), the only signal left in this region is the one corresponding to proton *i* of **1·2** (Figure 4d). Signal of proton *14* in **1·3**

(13) The assignment for *7* and *7′* was made based on the assumption that *7* is "locked" in the fully H-bonded side of **1·3** or **1·4** and should appear at a more downfield position than that of *7′*.

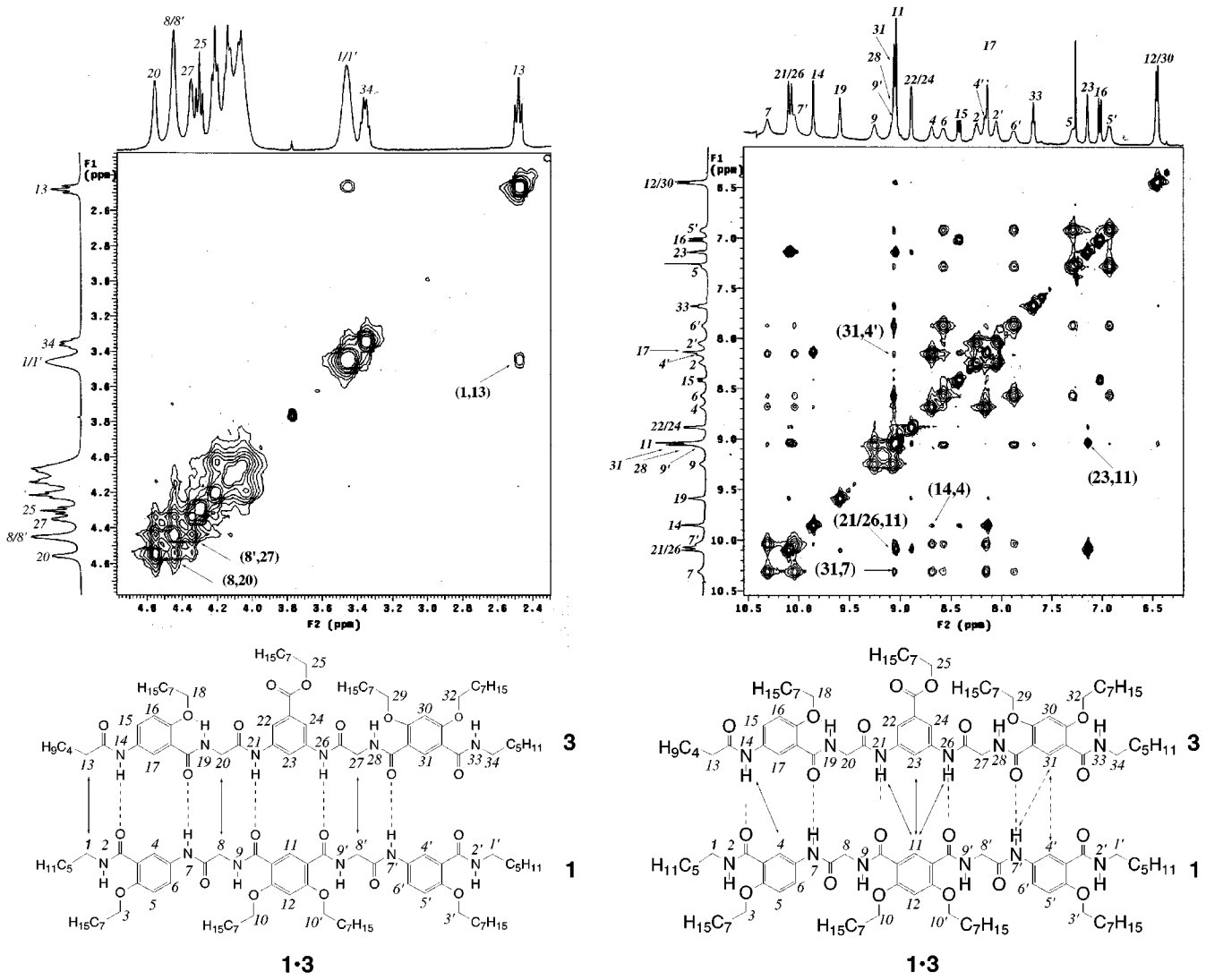


Figure 2. NOESY spectra of **1·3** (25 mM in $CDCl_3$, mixing time 0.5 s) showing cross-strand contacts: (a) between protons *1* and *13*, *8* and *20*, and *8′* and *27*, (b) between protons *14* and *4*, *21* and *11*, *23* and *11*, and *26* and *11*. Weak contacts (dashed arrows) exist between protons *31* and *4′*, and *31* and *7′*. No contacts can be detected between protons *34* and *1′*.

shifts upfield (δ < 9.00 ppm), and the two signals of the amide protons *26′* and *33′* in **1·4** also show significant upfield shifts (δ < 9.10 and 8.50 ppm, respectively). The signals of protons *19* and *19′* remain in this range by merging into the same position as the corresponding proton *i* (9.56 ppm) in **1·2**. It needs to be pointed out that signals in this region (9.30−9.85 ppm) are not from free single strands **1** and **2** which, if existed in solution, should show only one signal corresponding to protons *d* (9.67 ppm) and *h* (9.79 ppm), respectively. Free single strands **3** and **4** do not have any corresponding peaks within this region. These results have confirmed that single strand **2** specifically pair with **1** by displacing **3** or **4** when added into the solution of **1·3** or **1·4**.

Isothermal Titration Calorimetry. To quantitatively evaluate the effect of a single mismatched binding site on the stabilities of the resultant duplexes, a solution of 8 mM **1** was titrated into 1 mM of **3** or **4** in 5% DMSO/$CHCl_3$ by isothermal titration calorimetry (ITC). Analysis of the resulting binding isotherm (Figure 5) gave the dimerization constants and the thermodynamic data as shown in Table 1. With just one mismatched hydrogen bonding site, the two duplexes were found to be at least 40 times less stable ($K_a = (7.5 \pm 0.4) \times 10^4$ M^{-1} for **1·3**, and $K_a = (8.2 \pm 0.6) \times 10^4$ M^{-1} for **1·4**) than duplex **1·2** ($K_a = (3.5 \pm 1.3) \times 10^6$ M^{-1}) whose two single strands are perfectly matched. Like the binding of **2** to **1**, the binding of both **3** or **4** to **1** is also largely enthalpically driven. Unlike the formation of **1·2** whose entropy contributed negatively to the duplex stability ($T\Delta S$ = −0.8 kcal/mol),[11b] the mismatched duplex **1·4** has an almost negligible entropy contribution ($T\Delta S$ = −0.2 kcal/mol), while in **1·3**, it becomes even positive ($T\Delta S$ = +0.9 kcal/mol). In combination with 2D NOESY data, these results indicate that, due to its one open end, duplex **1·3** has the most flexible backbones; the backbones of **1·4** are less flexible; in **1·2**, the backbones are relatively rigid. These results also suggest that, although the positions and the types of the mismatched bonding sites have a considerable effect on the mismatched duplexes in terms of enthalpy and entropy, the overall stability of these mismatched duplexes are similar within experimental error. There seems to be a mechanism for duplexes **1·3** and **1·4** to compensate their loss in binding energy caused by mismatched sites to achieve similar stabilities that are consistent with the number of H-bonds in each complex.

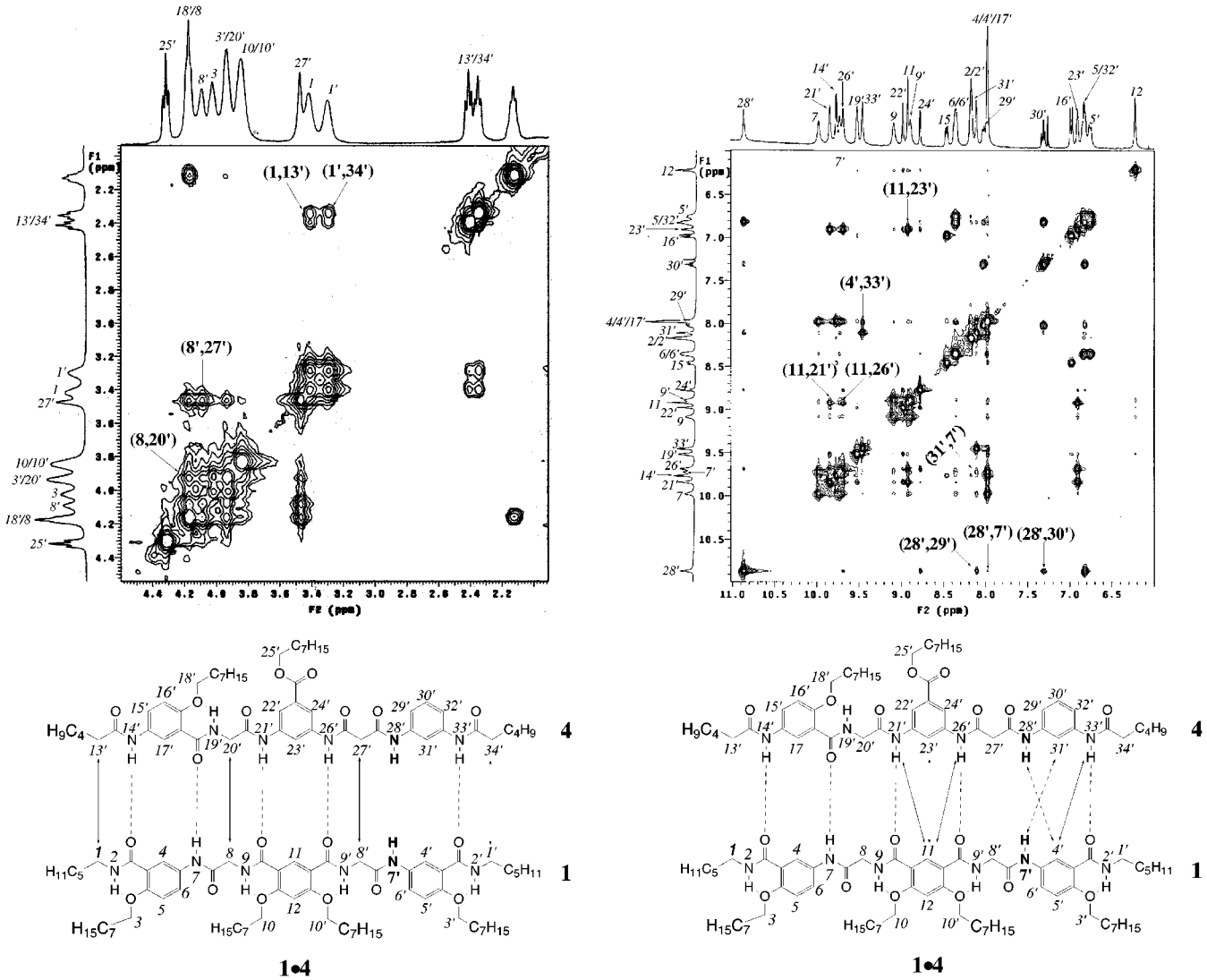


Figure 3. NOESY spectra of **1·4** (25 mM in $CDCl_3$, mixing time 0.5 s) showing cross-strand contact between (a) protons *1* and *13′*, *8* and *20′*, *8′* and *27′*, and *1′* and *34′*, (b) between protons *21′* and *11*, *23′* and *11*, *26′* and *11*, and between *33′* and *4′*. Very weak contacts (dashed arrows) exist between protons *31′* and *7′*, and between *28′* and *4′*. No contacts can be detected between protons *28′* and *7′*.

Conclusions

The above data have demonstrated that replacement of a hydrogen bond by single mismatched binding sites in a six-hydrogen-bonded duplex still lead to complexes with registered backbones. However, the mismatched sites resulted in duplexes with significantly reduced stabilities. Such a reduction in stabilities indicates that a single strand will indeed only pair with another single strand with a complementary sequence, forming a duplex with perfectly matched single strands. Other strands with imperfect sequences are not likely to interfere with the formation of the "correct" duplex, which is confirmed by NMR competition studies. The high precision as demonstrated by the complementary molecular strands in their pairing has thus confirmed this class of compounds as unnatural information-rich molecular strands. Combined with results previously reported by us,[11] the role of this class of H-bonded duplexes as versatile unnatural molecular recognition units have now been established. With their adjustable stabilities and programmable sequence specificities that parallel the molecular recognition characteristics of duplex DNA, these duplexes can be applied to direct the assembly of a wide variety of structural fragments. The design and construction of self-assembling, multicomponent architectures can be envisioned.

Experimental Section

General Methods. All chemicals were purchased from Aldrich, Fluka, and Sigma and used as received unless otherwise noted. The organic phase from all liquid extractions was dried over Na_2SO_4 unless specified otherwise. All products were detected as single spots by thin-layer chromatography (precoated 0.25 mm silica plates from Aldrich). All samples were purified either by recrystallization or by flash column chromatography and dried completely under high vacuum before characterized by ^1H NMR (400 MHz), ^{13}C NMR (100 MHz), and elemental analysis. NMR chemical shifts were reported in ppm relative to TMS. For the ^1H NMR dilution experiments, $CDCl_3$ (99.8% D) and DMSO-d_6 (99.8% D) were purchased from Cambridge Isotope Laboratory and used without further purification. T1 measurements were carried out with degassed samples (freeze−thaw cycle with nitrogen). NOE measurements were performed with the steady-state NOEDIF protocol on degassed samples.

***N*-Ethoxycarbonylmethyl-5-hexanoamido-2-octyloxybenzamide (3b).** To a solution of amine **3a** (2.00 g, 5.70 mmol)

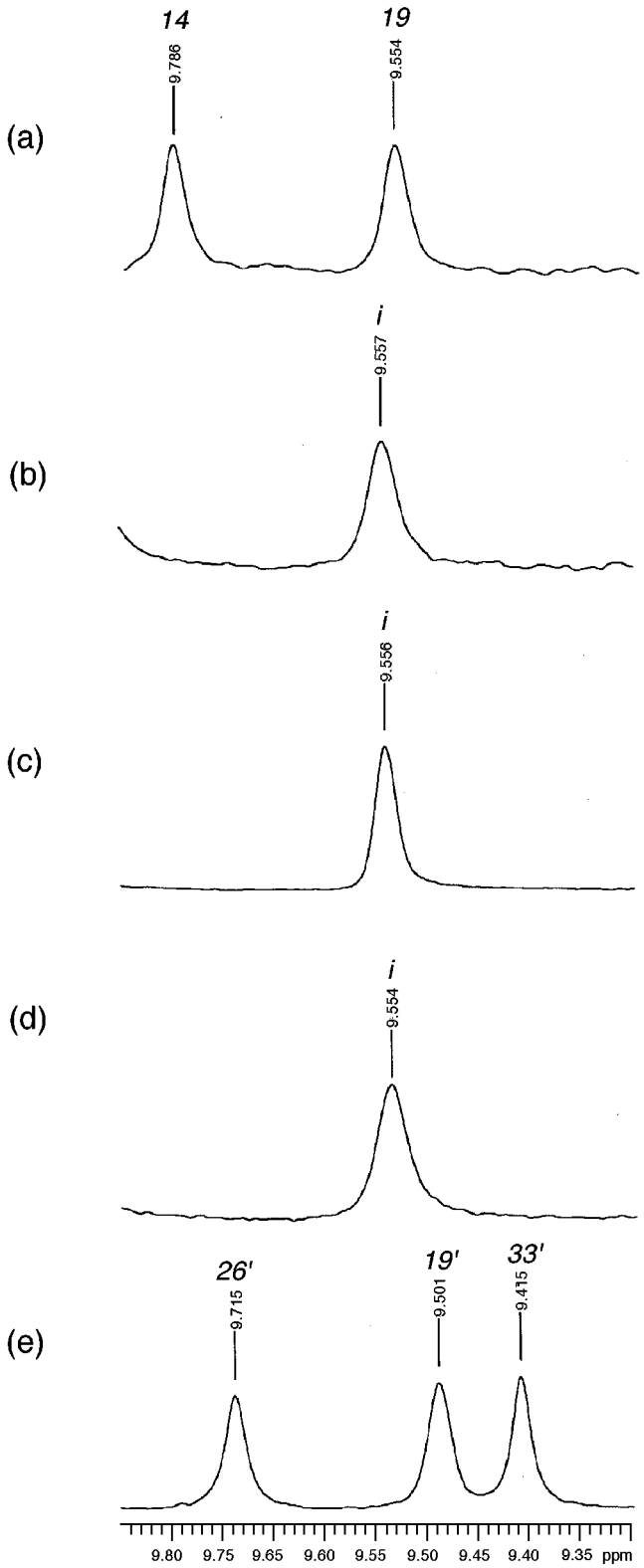


Figure 4. Amide NH resonances of (a) **1·3**, (b) **1·3** + **2**, (c) **1·2**, (d) **1·4** + **2**, and (e) **1·4** (2 mM) in the region of 9.30−9.85 ppm in $CDCl_3$ containing 5% DMSO-d_6 at 400 MHz. There are no peaks corresponding to single strands **3** and **4** exist within this region.

and triethylamine (0.57 g, 5.70 mmol) in 60 mL of methylene chloride was added dropwise hexanoyl chloride (0.79 g, 5.70 mmol) over 5 min. at 0 °C. The ice−water bath was removed. After 6 h the solvent was evaporated, and the residue was dissolved in ethyl acetate and washed with diluted aqueous HCl and NaOH solutions alternatively. Drying and evaporation of the solvent gave the crude product which was recrystallized from MeOH, giving the pure product as a white solid (2.28 g, 89%). ^{1}H NMR ($CDCl_3$) δ 8.714 (s, 1H), 8.192 (d, 1H; J = 8.8 Hz), 7.849 (s, 1H), 7.607 (br, 1H), 6.941 (d, 1H; J = 8.8 Hz), 4.273−4.220 (m, 4H), 4.124 (t, 2H; J = 6.4 Hz), 2.353 (t, 2H; J = 7.2 Hz), 1.932 (m, 2H), 1.716 (m, 4H), 1.477−1.285 (m, 15H), 0.898−0.863 (m, 6H). ^{13}C NMR ($CHCl_3$)δ 171.91, 169.80, 164.87, 153.85, 131.76, 125.40, 123.08, 120.72, 113.11, 69.78, 61.37, 42.16, 37.58, 31.77, 31.41, 29.26, 29.18, 20.07, 26.14, 25.31, 22.61, 22.41, 14.18, 14.04, 13.89. Anal. Calcd for $C_{25}H_{40}N_2O_5$: C, 66.93; H, 8.99; N, 6.25. Found: C, 67.07; H, 9.06; N, 6.27.

***N*-Carboxymethyl-5-hexanoamido-2-octyloxybenzamide (3c).** The ester **3b** (2.24 g, 5.00 mmol) was dissolved in hot MeOH (30 mL), to which 1 N NaOH (5.50 mL, 5.50 mmol) and 10 mL of H_2O was added. The mixture was heated under reflux for 30 min, to which more water (100 mL) was added. The aqueous layer was neutralized by addition of concentrated HCl to pH 3.0. The precipitated crude product was collected. Recrystallization of the crude product from MeOH gave a white solid (2.01 g, 96%). ^{1}H NMR ($CDCl_3$) δ 8.799 (s, 1H), 8.425 (s, 1H), 8.143 (q, 1H; J = 2.3 Hz, 9.2 Hz), 7.829 (d, 1H, J = 2.4 Hz), 7.462 (br, 1H), 6.856 (d, 1H, J = 9.2 Hz), 4.243 (d, 2H, J = 4.0 Hz), 4.045 (t, 2H; J = 6.4 Hz), 2.348 (t, 2H; J = 7.2 Hz), 1. 698 (m, 2H), 1.668 (m, 2H), 1.410−1.235 (m, 14H), 0.845 (t, 6H; J = 6.4 Hz). ^{13}C NMR ($CHCl_3$) δ 172.51, 172.12, 165.64, 153.82, 131.87, 125.82, 123.24, 119.79, 112.85, 69.64, 42.37, 37.31, 31.72, 31.37, 29.193, 28.94, 25.99, 25.34, 22.60, 22.38, 14.05, 13.88. Anal. Calcd for $C_{23}H_{36}N_2O_5$: C, 65.69; H, 8.63; N, 6.66. Found: C, 65.49; H, 8.67; N, 6.59.

Octyl 3-Amino-5-[(5′-hexanoamido-2′-octyloxy)phenylcarbonylaminomethyl-carbonylamino]benzoate (3e). To a solution of acid **3c** (1.96 g, 4.66 mmol) in 30 mL of methylene chloride were added EDC [1-ethyl-3-(3-dimethyl aminopropyl)-carbodiimide hydrochloride] (0.90 g, 4.67 mmol) and 1-hydoxybenzotriazole (0.71 g, 4.67 mmol). After stirring for 30 min, the solution was added dropwise within 10 min to octyl 3,5-diaminobenzoate **3d** (3.38 g, 12.8 mmol) in 40 mL of methylene chloride. The reaction was allowed to proceed for 6 h. The precipitated solid was filtered and dried in the air to give the pure product as a white solid (2.37 g, 76%). ^{1}H NMR (DMSO-d_6) δ 10.000 (s, 1H), 9.868 (s, 1H), 8.646 (s, 1H), 8.051 (d, 2H; J = 2.8 Hz), 7.783 (q, 1H; J = 2.8 Hz, J = 8.8 Hz), 7.405 (s, 1H), 7.102 (d, 1H; J = 8.8 Hz), 7.066 (s, 1H), 6.896 (s, 1H), 5.413 (s, 2H), 4.187 (t, 2H; J = 6.0 Hz), 4.135 (d, 2H; J = 4.8 Hz), 4.110 (t, 2H; J = 6.4 Hz), 2.251 (t, 2H; J = 6.0 Hz), 1.818 (m, 2H), 1.648 (m, 2H), 1.564 (m, 2H), 1.380−1.157 (m, 22H), 0.859−0.829 (m, 6H), 0.767 (t, 3H; J = 6.8 Hz). ^{13}C NMR (DMSO) δ 171.04, 167.09, 166.17, 164.23, 152.57, 149.35, 139.54, 132.67, 130.78, 123.69, 121.87, 121.22, 113.55, 109.83, 108.56, 107.77, 69.28, 64.35, 43.60, 36.26, 31.26, 30.92, 28.79, 28.76, 28,68, 28.65, 28.58, 28.28, 25.80, 25.50, 24.87, 22.10, 21.92, 13.95, 13.93, 13.90. Anal. Calcd for $C_{38}H_{58}N_4O_6$: C, 68.43; H, 8.77; N, 8.40. Found: C, 68.13; H, 8.62; N, 8.35.

Dimethyl 4,6-Dioctyloxy-1,3-benzenedicarboxylate (3f). A mixture of **3l** (6.79 g, 30 mmol), K_2CO_3 (24.86 g, 180 mmol), and 1-bromooctane (23.2 g, 120 mmol) in 150 mL of DMF containing 30 mL of methanol was heated at 100 °C for 2 days. The solid was filtered off and the solvent was removed in vacuo at 130 °C. The residue was dissolved in ethyl acetate and washed with diluted HCl and NaOH solutions alternatively. Evaporation of the solvent gave pure **3f** (11.9 g, 88%). ^{1}H NMR ($CDCl_3$) δ 8.458 (s, 1H), 6.423 (s, H), 4.057 (t, 4H; J = 6.4 Hz), 3.853 (s, 6H), 1.867 (m, 2H), 1.507 (m, 4H), 1.348−1.284 (m, 16H), 0.884 (t, 3H; J = 6.8 Hz). ^{13}C NMR ($CDCl_3$) δ 165.43, 163.63, 137.01, 112.05, 97.70, 69.28, 51.62, 31.80, 29.28, 29.21, 29.06, 25.53, 22.64, 14.02. Anal. Calcd for $C_{26}H_{42}O_6$: C, 69.30; H, 9.40. Found: C, 69.46; H, 9.68.

2,4-Dioctyloxy-5-methoxycabonylbenzoic Acid (3g). To a solution of Dimethyl 4,6-dioctyloxy-1,3-benzenedicarboxylate **3f** (13.52 g, 30 mmol) in 50 mL of DMSO at 130 °C was added KOH (1.68 g, 30 mmol) in 15 mL of MeOH drop by drop. The reaction was kept at 130 °C for 3 h. After cooling the solution, 5 mL of concentrated HCl and 150 mL of distilled water was added. The aqueous layer was extracted with 2 × 200 mL ethyl

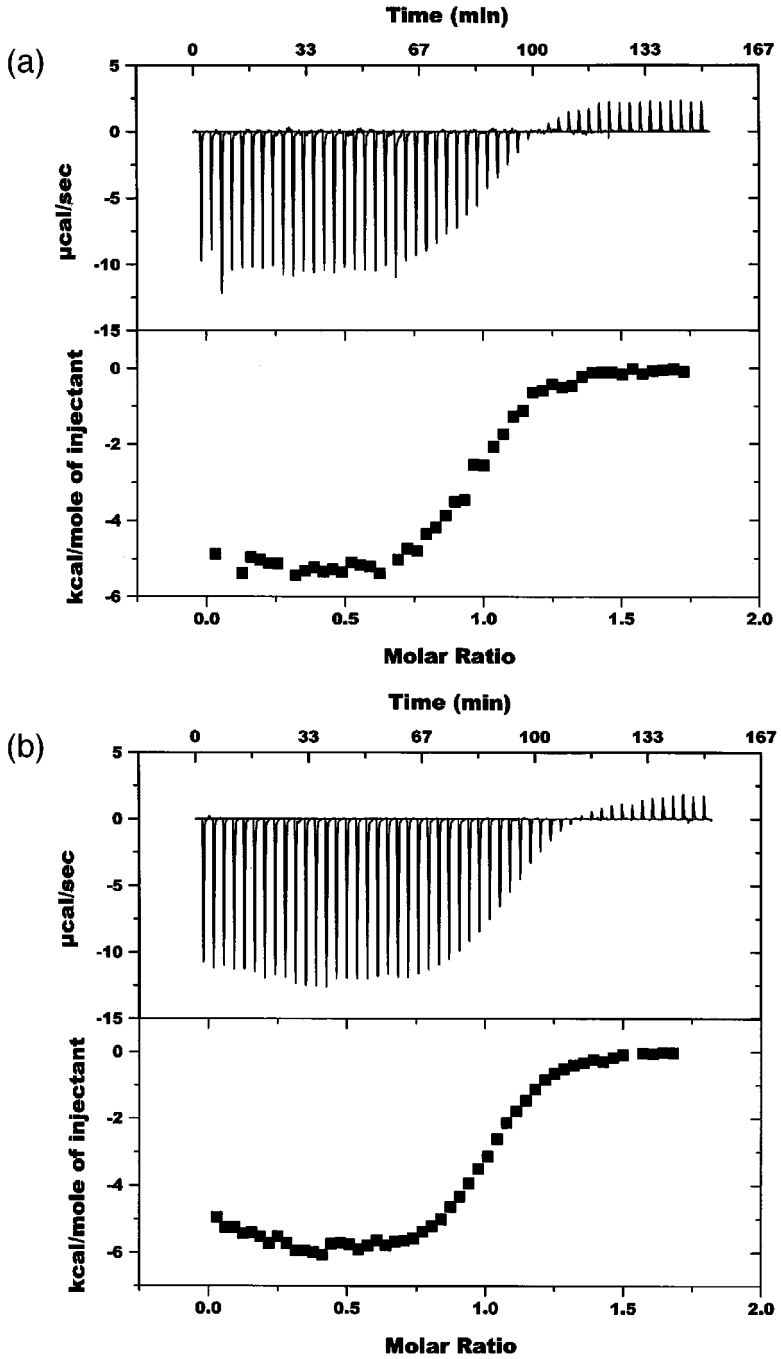


Figure 5. Calorimetry binding isotherms for the titration of (a) **3** with **1**, and (b) **4** with **1** in 5% DMSO/chloroform.

Table 1. Association Constants (*KA*) and Thermodynamic Data for the Binding of 2−4 to 1 in $CHCl_3$ Containing 5% DMSO.

	1·2	**1·3**	**1·4**
K_a (M^{-1})	$(3.5 \pm 1.3) \times 10^6$	$(8.2 \pm 0.4) \times 10^4$	$(7.5 \pm 0.6) \times 10^4$
ΔG (kcal/mol)	-8.9 ± 0.2	-6.7 ± 0.4	-6.6 ± 0.4
ΔH (kcal/mol)	-9.7 ± 0.2	-5.8 ± 0.2	-6.8 ± 0.2
$T\Delta S$ (kcal/mol)	-0.8 ± 0.4	0.9 ± 0.5	-0.2 ± 0.4

acetate. After drying and evaporation of ethyl acetate the residue was recrystallized twice from 100 mL hexane to give pure **3g** as a white solid (7.99 g, 61%). ^{1}H NMR ($CDCl_3$) δ: 0.870−0.904 (6H, m), 1.293−1.361 (16H, m), 1.462−1.538 (4H, m), 1.847−1.972 (4H, m), 3.859 (3H, s), 4.081 (2H, t, J = 6.4 Hz), 4.260 (2H, t, J = 6.8 Hz), 6.488 (1H, s), 8.694 (1H, s). ^{13}CNMR ($CDCl_3$) δ: 13.99, 14.02, 22.58, 22.63, 25.86, 25.88, 28.89, 28.97, 29.05, 29.15, 29.18, 29.23, 31.69, 31.79, 51.80, 69.64, 70.65, 97.07, 109.86, 114.70, 138.40, 161.49, 164.23, 164.78. Anal. Calcd for $C_{25}H_{40}O_6$: C, 68.78; H, 9.24; Found: C, 68.53; H, 9.33.

***N*-Hexyl-2,4-dioctyloxy-5-methoxycabonylbenzamide (3h).** To a solution of acid **3g** (5.72 g,13.1 mmol), EDC (2.52 g, 13.1 mmol), and 1-hydoxybenzotriazole (1.77 g, 13.1 mmol) in 40 mL of DMF was added hexylamine (1.33 g, 13.1 mmol) in 10 mL of DMF. The reaction mixture was kept heating for 1 h to dissolve any precipitate and was allowed to proceed for additional 5 h at room temperature. Distilled water (150 mL) and ethyl acetate (150 mL) were added. The ethyl acetate layer was washed with distilled water (100 mL) at least four times to ensure complete removal of DMSO. Drying and evaporation of ethyl acetate gave pure **3h** as a white solid (5.58 g, 82%).^{1}H NMR ($CDCl_3$) δ: 0.885 (9H, d, J = 4.4 Hz), 1.300−1.327 (22H, m), 1.501 (4H, m), 1.580 (2H, m), 1.882 (4H, m), 3.429 (2H, q, J = 6.8 Hz, J = 12.4 Hz), 3.838 (3H, s), 4.043

(2H, t, J = 6.4 Hz), 4.135 (2H, t, J = 6.0 Hz), 6.425 (1H, s), 7.709 (1H, s), 8.741 (1H, s). ^{13}C NMR ($CDCl_3$) δ: 13.99, 14.02, 22.59, 22.63, 25.91, 26.26, 26.88, 29.09, 29.19, 29.20, 29.23, 29.28, 29.33, 29.66, 31.59, 31.77, 31.80, 39.80, 51.55, 69.43, 97.13, 113.44, 114.21, 136.94, 160.85, 162.60, 164.22, 165.40. Anal. Calcd for $C_{31}H_{53}NO_5$: C, 71.64; H, 10.28; N, 2.69. Found: C, 71.50; H, 10.48; N, 2.98.

***N*-Hexyl-2,4-dioctyloxy-5-hydroxycabonylbenzamide (3i).** Hydrolysis of **3h** based on the similar procedures as described for **3c** gave the crude product, which was crystallized from MeOH to give the pure product as a white solid (94%). ^{1}H NMR ($CDCl_3$) δ: 0.868−0.893 (9H, m), 1.301−1.596 (26H, m), 1.913 (4H, m), 3.428 (2H, q, J = 6.8 Hz, J = 12.8 Hz), 4.155 (2H, t, J = 6.4 Hz), 4.228 (2H, t, J = 6.4 Hz), 6.466 (1H, s), 7.561 (1H, t, J = 3.2 Hz), 8.957 (1H, s). ^{13}C NMR ($CDCl_3$) δ: 13.99, 22.59, 25.86, 26.23, 26.87, 28.93, 29.06, 29.17, 29.20, 29.32, 29.63, 31.59, 31.70, 31.78, 39.91, 69.85, 70.66, 96.60, 111.21, 138.70, 160.59, 161.53, 163.58, 164.06, 194.62. Anal. Calcd for $C_{30}H_{51}NO_5$: C, 71.25; H, 10.16; N, 2.77. Found: C, 71.10; H, 10.39; N, 3.00.

***N*-Ethoxycarbonylmethyl-*N*′-hexyl-4,6-dioctyloxy-1,3-benzenedicarboxamide (3j).** To a solution of acid **3i** (0.384 g, 0.76 mmol) and EDC (0.146 g, 0.76 mmol) and HOBt (1-hydoxybenzotriazole) (0.103 g, 0.76 mmol) in 30 mL of DMF were added glycine ethyl ester hydrochloride (0.420 g, 3.0 mmol) and triethylamine (0.203 g, 2 mmol) in 10 mL DMSO. The reaction mixture was allowed to proceed for 6 h at room temperature. Distilled water and ethyl acetate were added. Cooling the solution gave pure **3j** as a white solid (0.40 g, 89%). ^{1}H NMR ($CDCl_3$) δ: 0.875−0.900 (9H, m), 1.299−1.612 (31H, m), 1.894 (2H, m), 1.974 (2H, m), 3.440 (2H, q, J = 6.8 Hz, J = 12.4 Hz), 4.138 (4H, q, J = 6.4 Hz, J = 12.4 Hz), 4.245 (4H, m), 6.442 (1H, s), 7.614 (1H, t, J = 4.2 Hz), 8.272 (1H, t, J = 4.8 Hz), 9.016 (1H, s). ^{13}C NMR ($CDCl_3$) δ: 14.03, 14.06, 14.20, 22.62, 26.16, 26.26, 26.89, 28.97, 29.18, 29.23, 29.26, 29.34, 29.63, 31.60, 31.78, 39.78, 42.08, 61.27, 69.42, 69.74, 96.20, 114.42, 115.37, 137.19, 160.23, 160.28, 164.21, 164.25, 170.23. Anal. Calcd for $C_{34}H_{58}N_2O_6$: C, 69.12; H, 9.89; N, 4.74. Found: C, 68.89; H, 9.75; N, 4.77.

***N*-Hydroxycarbonylmethyl-*N*′-hexyl-4,6-dioctyloxy-1,3-benzenedicarboxamide (3k).** To a solution of ester **5h (**0.591 g, 1 mmol) in 10 mL of DMSO under reflux was added NaOH (0.4401 g, 1.1 mmol) in 3 mL of H_2O. The reaction was kept refluxing for 20 min. After cooling the solution, 0.3 mL of concentrated HCl and 150 mL of distilled water were added. The precipitate was filtered off and recrystallized from MeOH to give pure **5i** as a white solid (0.512 g, 91%). ^{1}H NMR ($CDCl_3$) δ: 0.858−0.907 (9H, m), 1.301−1.596 (28H, m), 1.921 (4H, m), 3.432 (2H, q, J = 6.8 Hz, J = 12.4 Hz), 4.131 (4H, t, J = 6.0 Hz), 4.273 (2H, d, J = 4.4 Hz), 6.430 (1H, s), 7.681 (1H, t, J = 4.8 Hz), 8.310 (1H, t, J = 4.4 Hz), 8.947 (1H, s). ^{13}C NMR ($CDCl_3$) δ 13.99, 14.02, 22.60, 25.74, 26.28, 26.96, 28.52, 29.12, 29.26, 29.32, 29.50, 31.66, 31.73, 31.80, 39.79, 43.86, 69.38, 69.59, 96.06, 113.77, 114.17, 136.28, 160.00, 160.46, 164.24, 164.46, 174.67. Anal. Calcd for $C_{32}H_{54}N_2O_6$: C, 68.29; H, 9.67; N, 4.98; Found: C, 68.46; H, 9.95; N, 5.17.

Octyl 3-[(5′-Hexanoamido-2′-octyloxy)phenylcarbonylaminomethyl carbonyl-amino]-5-[(5″-hexylaminocarbonyl-2″,4″-dioctyloxy)phenylcarbonylaminomethyl-carbonylamino]benzoate (3). To a solution of **3k** (0.348 g, 0.618 mmol), EDC (0.119 g, 0.618 mmol) and HOBt (0.095 g, 0.618 mmol) in 40 mL of CH_2Cl_2 was added amine **3e** (0.412 g, 0.618 mmol) in 5 mL of DMF. The reaction mixture was stirred for 6 h. The solvent was removed *in vacuo*. The residue was trituated with hot MeOH, which was filtered off and washed with acetone to give pure white solid **3** (0.370 g, 50%). ^{1}H NMR (DMSO) δ 10.399 (1H, s), 10.382 (1H, s), 9.879 (1H, s), 8.676 (2H, t, J = 4.0 Hz), 8.470 (1H, s), 8.086 (1H, s), 8.061 (1H, d, J = 2.8 Hz), 8.040 (1H, s), 8.012 (1H, s), 7.854 (2H, t, J = 4.4 Hz), 7.788 (1H, q; J = 2.4 Hz, J = 8.8 Hz), 7.087 (1H, d, J = 9.2 Hz), 6.752 (1H, s), 4.234−4.194 (8H, m), 4.093 (2H, t, J = 6.0 Hz), 3.252 (2H, q, J = 6.4 Hz, J = 12.4 Hz), 2.245 (2H, t, J = 7.2 Hz), 1.865−1.784 (5H, m), 1.671 (2H, m), 1.563 (2H, m), 1.477−1.126 (50H, m), 0.850−0.799 (9H, m), 0.736−0.707 (6H, m). ^{13}C NMR (10% DMSO in $CDCl_3$) δ 172.05, 167.50, 167.41, 166.10, 165.15, 164.45, 164.10, 160.47, 160.32, 153.29, 139.11, 136.45, 132.64, 131.36, 124.88, 123.02, 120.81, 116.08, 114.86, 114.65, 114.12, 112.82, 96.40, 69.77, 69.56, 69.46, 65.05, 44.33, 33.87, 31.64, 31.60, 31.45, 31.34, 29.44, 29.21, 29.11, 29.05, 28.93, 28.80, 28.60, 26.14, 26.08, 25.78, 25.30, 24.88, 22.48, 22.35, 14.02, 13.95. Anal. Calcd for $C_{70}H_{110}N_6O_{11}$: C, 69.39; H, 9.15; N, 6.94. Found: C, 69.54; H,9.27; N, 7.02.

***N*-(3-Nitrophenyl)hexanoamide (4a)**. Hexanoyl chloride (1.34 g) in CH_2Cl_2 was added to a solution of 3-nitroaniline (1.38 g) and triethylamine (1.01 g) in CH_2Cl_2 (50 mL) at 0−5 °C. After stirring for overnight, the solvent was removed and the residue was dissolved in ether and washed with acidic and basic solutions. Evaporation of ether gave the pure product as a yellow oil, which solidified upon standing (1.98 g, 84%). ^{1}H NMR ($CDCl_3$) δ 9.351 (s, 1H), 9.932 (m, 2H), 7.649 (s, 1H), 7.458 (t, 1H, J = 8.4 Hz), 2.388 (t, 2H, J = 7.6 Hz), 1.715 (m, 2H), 1.324−1.350 (m, 4H), 0.905 (t, 3H, J = 7.2 Hz). ^{13}C NMR ($CDCl_3$) δ 171.85, 148.48, 139.02, 129.83, 125.40, 118.73, 114.39, 37.66, 31.34, 25.08, 22.39, 13.90. Anal. Calcd for $C_{12}H_{16}N_2O_3$: C, 61.00; H, 6.83; N, 11.86. Found: C, 61.27; H, 6.95; N, 12.01.

***N*-(3-Aminophenyl)hexanoamide (4b)**. This compound was prepared from **4a** based on the same procedure for preparing **3a**. Yield: 89%. ^{1}H NMR (DMSO) δ 7.581 (s, 1H), 7.137 (s, 1H), 7.033 (t, 1H, J = 8.0 Hz), 6.702 (d, 1H, J = 7.6 Hz), 6.393 (d, 1H, J = 7.2 Hz), 3.190 (s, 2H), 2.293 (t, 2H, J = 7.2 Hz), 1.689 (m, 2H), 1.295 (m, 4H), 0.881 (t, 3H, J = 6.4 Hz). ^{13}C NMR ($CDCl_3$) δ 171.68, 147.13, 139.03, 129.55, 110.90, 109.76, 106.67, 37.72, 31.35, 25.28, 22.35, 13.84.

***N*-[3-(Ethoxycarbonylmethylcarbonylamino)phenyl]-hexanoamide (4c).** To a solution of **4b** (0.80 g, 3.88 mmol) and triethylamine (0.39 g, 3.88 mmol) in 40 mL of methylene chloride was added dropwise ethyl 3-chloro-3-oxo-propionate (1.05 g, 6.98 mmol) over 5 min. at 0 °C. The ice−water bath was removed. After 6 h the solvent was evaporated, and the residue was dissolved in ethyl acetate and washed with diluted HCl and NaOH solutions alternatively. Drying and evaporation of the solvent gave the crude product which was recrystallized from MeOH, giving the pure product as a white solid (1.03 g, 83%). ^{1}H NMR ($CDCl_3$) δ 9.263 (s, 1H), 7.777 (s, 1H), 7.423 (s, 1H), 7.245 (d, 2H; J = 5.6 Hz), 7.218 (q, 1H; J = 8.0 Hz, J = 10.8 Hz), 4.234 (q, 2H; J = 6.8 Hz, J = 14.0 Hz), 3.456 (s, 3H), 2.326 (t, 2H; J = 7.2 Hz), 1.704 (m, 2H), 1.349−1.312 (m, 6H), 0.896 (t, 3H; J = 7.2 Hz). ^{13}C NMR ($CDCl_3$) δ 171.63, 169.71, 163.20, 138.58, 137.90, 129.50, 115.85, 115.54, 111.20, 61.94, 41.64, 37.74, 31.37, 25.23, 22.41, 14.02, 13.92. Anal. Calcd for $C_{17}H_{24}N_2O_4$: C, 63.73; H, 7.55; N, 8.74. Found: C, 63.63; H, 7.39; N, 8.64.

***N*-[3-(Hydroxycarbonylmethylcarbonylamino)phenyl]-hexanoamide** (**4d**). This compound was prepared from **4c** based on the same procedure for preparing **3c**. Yield: 0.82 g, 94%. ^{1}H NMR (DMSO): δ 10.116 (s, 1H), 9.880 (s, 1H), 7.899 (s, 1H), 7.243 (d, 2H; J = 7.2 Hz), 7.197 (t, 1H; J = 7.2 Hz), 3.322 (s, 2H), 2.266 (t, 2H; J = 7.2 Hz), 1.564 (m, 2H), 1.295−1.261 (m, 4H), 0.855 (t, 3H; J = 6.8 Hz). ^{13}C NMR (DMSO) δ 171.48, 169.44, 164.62, 139.71, 139.22, 128.95, 114.35, 113.90, 110.05, 44.03, 36.45, 30.98, 24.94, 22.00, 13.97. Anal. Calcd for $C_{15}H_{20}N_2O_4$: C, 61.63; H, 6.90; N, 9.58. Found: C, 61.69; H, 7.94; N, 9.64.

Octyl 3-[(5′-Hexanoamido-2′-octyloxy)phenylcarbonylaminomethylcarbonyl-amino]-5-[(3″-hexanoamidophenyl)aminocarbonylmethylcarbonylamino]benzoate (**4**). To a solution of acid **4d** (0.61 g, 2.10 mmol) and EDC (0.41 g, 2.13 mmol) and HOBt (0.33 g, 2.16 mmol) in 40 mL of DMF was added amine **3e** (1.32 g, 5 mmol) in 10 mL of DMF. The reaction was allowed to proceed overnight. Distilled water (100 mL) was then added to the reaction mixture. The precipitated solid was filtered and stirred with acetone under reflux for 30 min. The solution was cooled to room temperature and filtered, giving the pure product as a white solid (1.01 g, 51%). ^{1}H NMR (DMSO) δ 10.399 (s, 1H), 10.368 (s, 1H), 10.142 (s, 1H), 9.860 (s, 2H), 8.653 (t, 1H; J = 4.8 Hz), 8.153 (s, 1H), 8.057 (t, 2H; J = 6.4 Hz), 7.927 (d, 2H; J = 1.6 Hz), 7.794 (q, 1H; J = 2.8 Hz, 8.8 Hz), 7.307−7.251 (m, 2H), 7.187 (t, 1H, J = 8.0 Hz),

7.102 (d, 1H; $J = 8.8$ Hz), 4.246 (t, 2H; $J = 6.4$ Hz), 4.183 (d, 2H; $J = 5.2$ Hz), 4.110 (t, 4H; $J = 6.4$ Hz), 3.474 (s, 2H), 2.252 (q, 4H; $J = 7.6$ Hz, 11.2 Hz), 1.824 (m, 2H), 1.683 (m, 2H), 1.570 (m, 4H), 1.419−1.139 (m, 24H), 0.870−0.803 (m, 9H), 0.750 (t, 3H; $J = 6.4$ Hz). ^{13}C NMR (DMSO) 171.26, 170.94, 167.55, 165.75, 165.40, 165.16, 164.26, 152.50, 139.63, 139.53, 139.42, 139.09, 132.65, 130.70, 128.78, 123.60, 121.80, 121.26, 114.71, 114.63, 114.23, 113.84, 113.53, 110.04, 69.24, 64.74, 45.89, 43.54, 36.33, 36.19, 31.16, 30.85, 28.72, 28.66, 28.57, 25.54, 28.15, 25.72, 25.38, 24.79, 22.01, 21.99, 21.84, 13.86, 13.82. Anal. Calcd For $C_{53}H_{76}N_6O_{10}$: C, 66.50; H, 8.00; N, 8.78. Found: C, 66.12; H, 8.12; N, 8.79.

Isothermal Titration Calorimetry. The thermodynamic binding parameters were determined by titrating 1 mM solutions of **3** or **4** with 8 mM **1** in 5% DMSO/$CHCl_3$ in an Omega isothermal titration calorimeter (MicroCal, Northampton, MA). The cell was thermostated to ±0.1 °C using a circulating bath. All experiments were performed at 25 °C. The enthalpy of binding between strands were determined from multiple single injections. Injection volumes were 5 μL, with 3 min of equilibration time allowed between injections. The heat of dilution of **1** into 5% DMSO/$CHCl_3$ was determined and the **1**−**3** and **1**−**4** heats were adjusted via subtraction of the heat of dilution from the two data sets. The equilibrium binding constants K were extracted from the calorimetric data by employing the Origin data analysis software supplied with the calorimeter. A complete description of the data analysis has been published by Brandts and co-workers.[14]

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