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Molecular Recognition: Improved Binding of Biotin Derivatives with Synthetic Receptors

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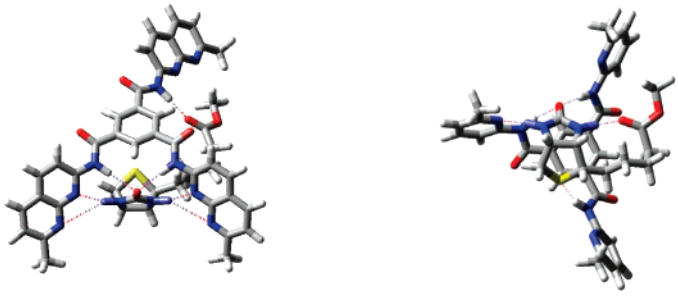
Received November 23, 2005



NMR titrations and Monte Carlo conformational searches have been used to study the molecular recognition features of five urea derivatives with two synthetic hosts. We have improved the binding constant (K_b) values for all the studied guests and measured the largest binding constant of a complex involving a biotin derivative (biotin methyl ester) bound to a synthetic host by means of several interaction points and not only through the urea moiety.

Introduction

Most papers on the field of host−guest chemistry dealing with the molecular recognition of urea derivatives focus on the synthesis of new hosts able to interact by hydrogen bond formation.[1−3] This is the natural way to obtain more stable complexes by use of all of the functional groups capable of interacting in urea derivatives. Unfortunately, when this strategy has been followed with biotin derivatives, this has led to the development of several hosts that interact with biotin through four hydrogen bonds with the urea moiety.[4−6] However, as long as these hosts do not use all of the functional groups that biotin possess, as happens in the biological recognition, no real improvement is obtained, and therefore, no new insights can be found. If the aim is to get stronger interactions with biotin derivatives, it is clear that the strategy should be the development of hosts capable of complexing with biotin by the use of more than one of the binding points in the structure of the guest (compound **1** in Figure 1).

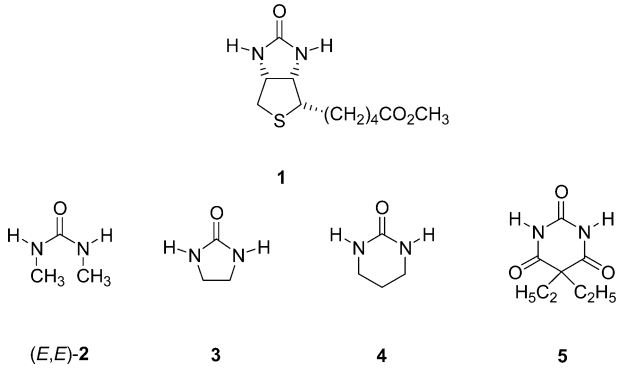


FIGURE 1. Structure of methylbiotin (**1**), *N*,*N*′-dimethylurea (**2**), 2-imidazolidone (**3**), *N*,*N*′-trimethyleneurea (**4**), and barbital (**5**).

Another feature frequently disregarded in this field is a careful revision of previously designed hosts. If the final purpose is to obtain better interactions with biotin and to reach a deeper understanding on the molecular recognition features of this molecule, the efforts should not be only on the synthesis of

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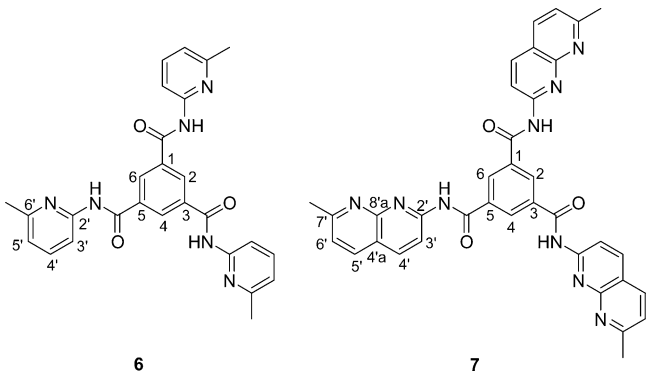
10.1021/jo052424n CCC: $33.50
Published on Web 03/14/2006



6 **7**

FIGURE 2. Hosts studied in this paper.

new compounds if they show nothing new in their properties toward biotin. If better candidates, not yet used with this guest, are in the literature they should be used to get more information on the host−guest properties of biotin.

By this approach, we have used two previously known hosts[7] (Figure 2), never used as complexing agents for biotin, closely related to previous compounds studied by us (Figure 1).[8,9] We have selected these compounds with the aim of obtaining a complex with methylbiotin through several interaction points and showing a great binding constant.

This paper reports the measurement and analysis of the binding constants, K_b, of five guests [methylbiotin (**1**), *N*,*N*′-dimethylurea (**2**), 2-imidazolidone (**3**), *N*,*N*′-trimethylenurea (**4**), and barbital (**5**)] (Figure 1) with two hosts, *N*,*N*′,*N*″-tris(6-methylpyridin-2-yl)-1,3,5-benzenetricarboxamide (**6**) and *N*,*N*′,*N*″-tris(7-methyl-1,8-naphthyridin-2-yl)-1,3,5-benzenetricarboxamide (**7**) (Figure 2). We have carried out the conformational search for all the complexes by the use of Monte Carlo method with AMBER and OPLS force fields, employing the GB/SA model for chloroform, obtaining the most probable structure and the associated energy.

Results and Discussion

Complex Stoichiometry. Before the quantification of the binding constants for these two hosts, the stoichiometry of the complexes must be determined to use the right equations on the titrations. We have used the method of continuous variation to generate Job plots by preparing different mixtures of host (H) and guest (G) covering the whole range of molar fractions of the host but keeping constant the total concentration of the solutions. The plot of the product between the increment in the chemical shift and the host concentration versus the molar fraction of the host affords a curve, from the value of the maximum (X) the stoichiometry of the complex, which can be obtained by means of eq 1.

$$mH + nG \rightleftharpoons H_mG_n$$

$$X = m/(m + n) \qquad (1)$$

We have obtained 1:1 stoichiometries for all of the complexes save for **6**:**5**. Figure 3a depicts the Job plot for the complex **7**:**1**, this plot is representative of the results obtained for all the complexes showing a 1:1 stoichiometry (**6**:**1**, **6**:**2**, **6**:**3**, **6**:**4**, **7**:**1**, **7**:**3**, and **7**:**4**). Figure 3b shows the Job plot for the complex **6**:**5**, the maximum appears at 0.66. By introducing this value in eq 1 a 2:1 stoichiometry is obtained, two hosts **6** for each guest **5**.

TABLE 1. Experimental Binding Constants K_b (M^{-1}) and Free Energy Changes ΔG (kJ mol^{-1}) at 300 K for the Complexes of Hosts 6 and 8

guest	K_b(**6**)	ΔG(**6**)	K_b(**8**)	K_b(**6**)/K_b(**8**)
1	4000 ± 900	−20.7	975 ± 53	4.0 ± 1.1
2	34 ± 5	−8.8	<10	
3	4800 ± 400	−21.1	1450 ± 62	3.3 ± 0.4
4	5700 ± 750	−21.6	2300 ± 271	2.5 ± 0.6
5	6100 ± 800[a]	−21.7	2375 ± 242	2.6 ± 0.6
	1400 ± 280[b]	−18.1		

[a] K_b for the 1:1 complex. [b] K_b for the 2:1 complex.

TABLE 2. Experimental Binding Constants K_b (M^{-1}) and Free Energy Changes ΔG (kJ mol^{-1}) at 300 K for the Complexes of Hosts 7 and 9

guest	K_b(**7**)	ΔG(**7**)	K_b(**9**)	K_b(**7**)/K_b(**9**)
1	148000 ± 20000	−29.7	35000 ± 5250	4.2 ± 1.2
3	33000 ± 2900	−26.0	9500 ± 1900	3.5 ± 1.0
4	21000 ± 900	−24.8	6000 ± 1000	3.5 ± 1.2

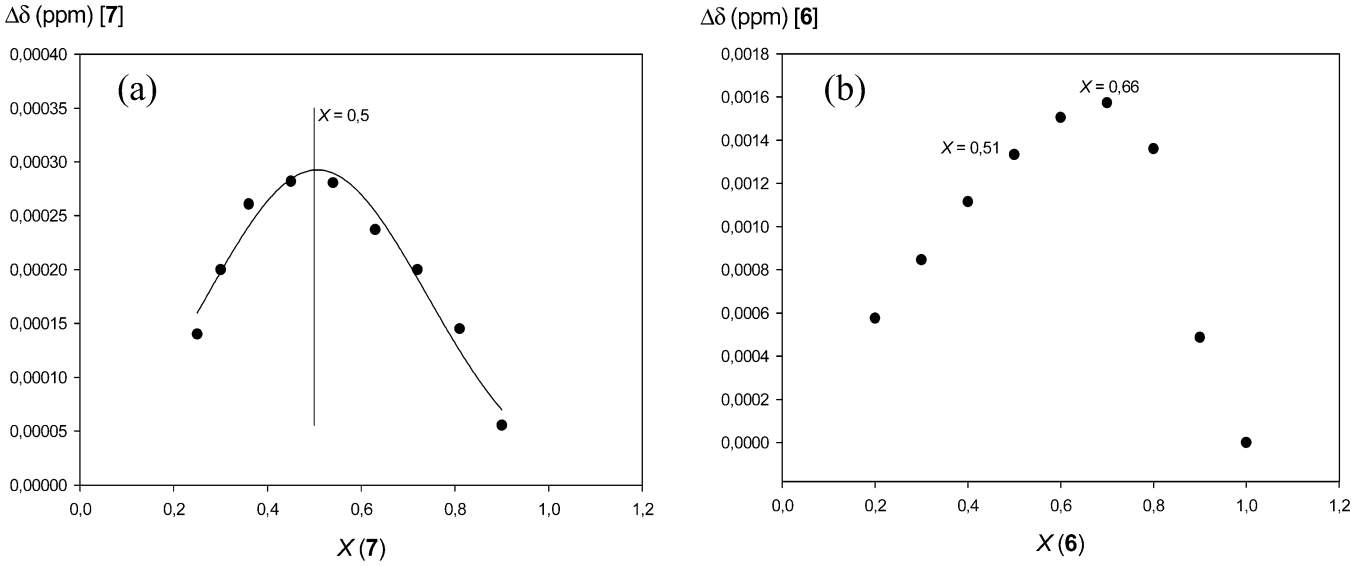
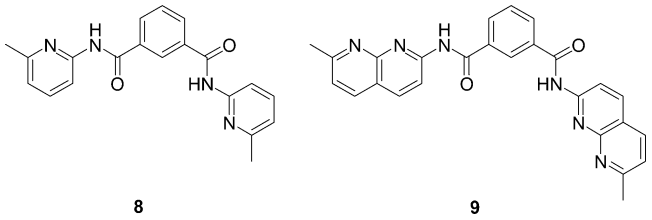


FIGURE 3. Job plots for complexes (a) **7**:**1** and (b) **6**:**5**.



8 **9**

FIGURE 4. Two hosts previously studied.

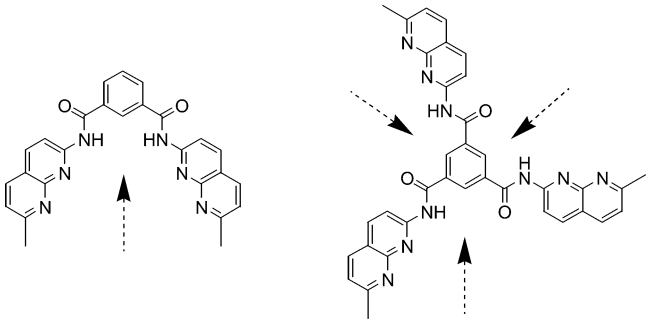


FIGURE 5. Hosts **9** and **7**.

TABLE 3. Corrected Interaction Energies for Complexes of Hosts 6 and 7 and the Energies for Hosts 8 and 9, $-E_{min}$ (kJ mol^{-1}), Obtained with AMBER

guest	**8**	**9**	**6**	**7**
1	60.7	78.6	72.9	89.4
2	29.0		42.8	
3	51.7	71.1	60.9	80.8
4	53.0	72.3	59.3	82.7
5	65.0		73.0	

Binding Constant Quantification. We have used NMR titrations to quantify the interactions between hosts and guests. The K_b values measured in $CDCl_3$ at 300 K for complexes of the five guests **1**−**5** with host **6** are gathered in Table 1 and in Table 2 for the complexes of host **7** with **1**, **3**, and **4** as guests. As a consequence of the structure of hosts **6** and **7** we have been able to use two independent signals, benzene and amide protons, for K_b determination. Together with the results for hosts **6** and **7**, we present as well the K_b values for the two hosts **8** and **9** (Figure 4) previously studied by us with these guests.[8,9] These compounds are the equivalents of hosts **6** and **7**, with only two amide groups, and we have introduced them for comparative purposes.

The quantification of the binding constants with host **7** has been carried out with guests **1**, **3**, and **4** but not with guests **2** and **5**, due to the low solubility of this host in most solvents used in NMR. It is only possible to solubilize **7** when a small quantity of the guest is added to the solution with two conditions: the guest must be soluble and show a good K_b with the host. Because with *N*,*N*′-dimethylurea (**2**) the K_b value is small and barbital (**5**) itself shows a low solubility in chloroform it was not possible to carry out the quantification with these two guests. However, the quantification could be achieved with the most interesting compounds.

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TABLE 4. Corrected Interaction Energies for Hosts 6 and 7 and the Energies for Hosts 8 and 9, $-E_{min}$ (kJ mol^{-1}), Obtained with OPLS

guest	**8**	**9**	**6**	**7**
1	92.4	106.3	111.0	120.9
2	56.0		82.7	
3	80.3	99.2	94.7	112.8
4	81.7	98.6	91.4	108.9
5	92.5		103.9	

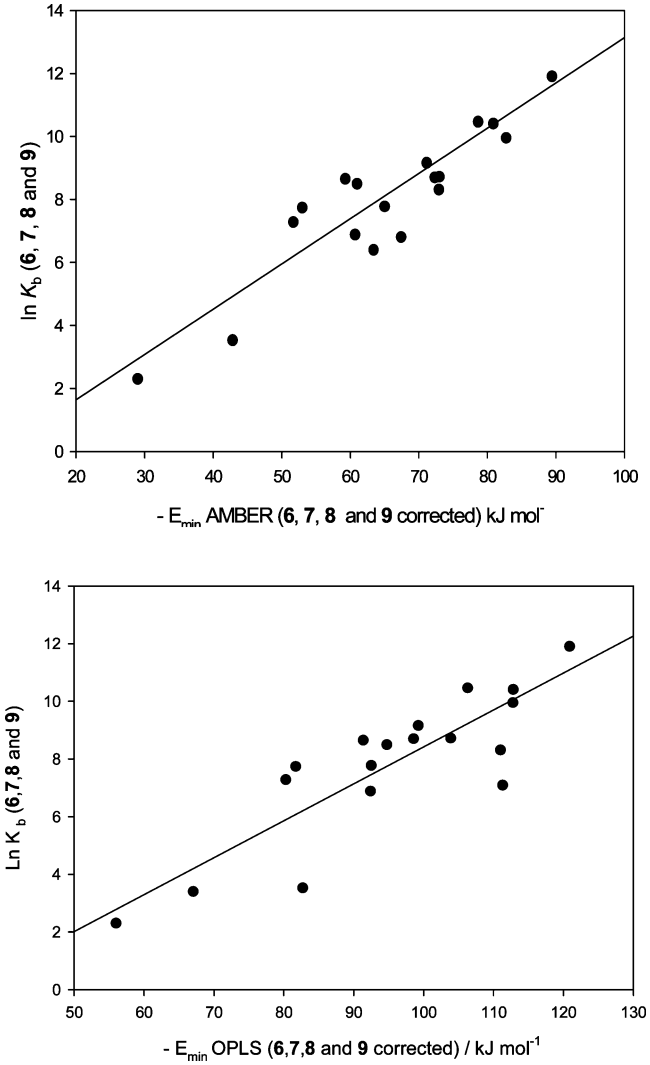

FIGURE 6. Plot of the experimental results (Tables 1 and 2) vs the calculated interaction energies for the complexes of **6**−**9** with (a) AMBER (Table 3) and (b) OPLS (Table 4) force fields.

Binding constant values for the complexes of these two hosts are clearly much better than those for the compounds with two amide substituents. In fact, the average improvement factor (*a*) is 3.1 times for host **6** and 3.7 times for host **7** compared to hosts **8** and **9,** respectively. The reason for this becomes clear if we look at the structures of hosts **7** and **9** (Figure 5).

In host **9** (and **8**), as in most of the known hosts, the interaction can only take place when the guest approximates to the host in the right direction toward the cleft. However, due to the structure of host **7** (and **6**), showing a C_3 symmetry, there are three ways that the guest can be used to bind to the host.

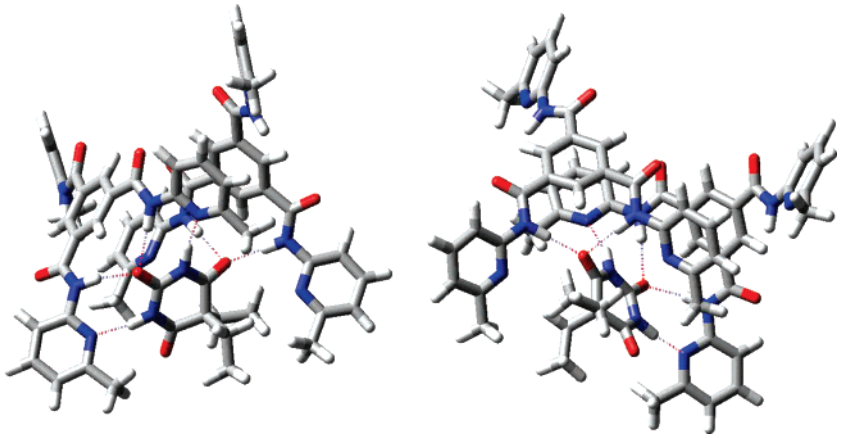

FIGURE 7. Two views of the structure of complex **6**:**5** as predicted using the AMBER force field.

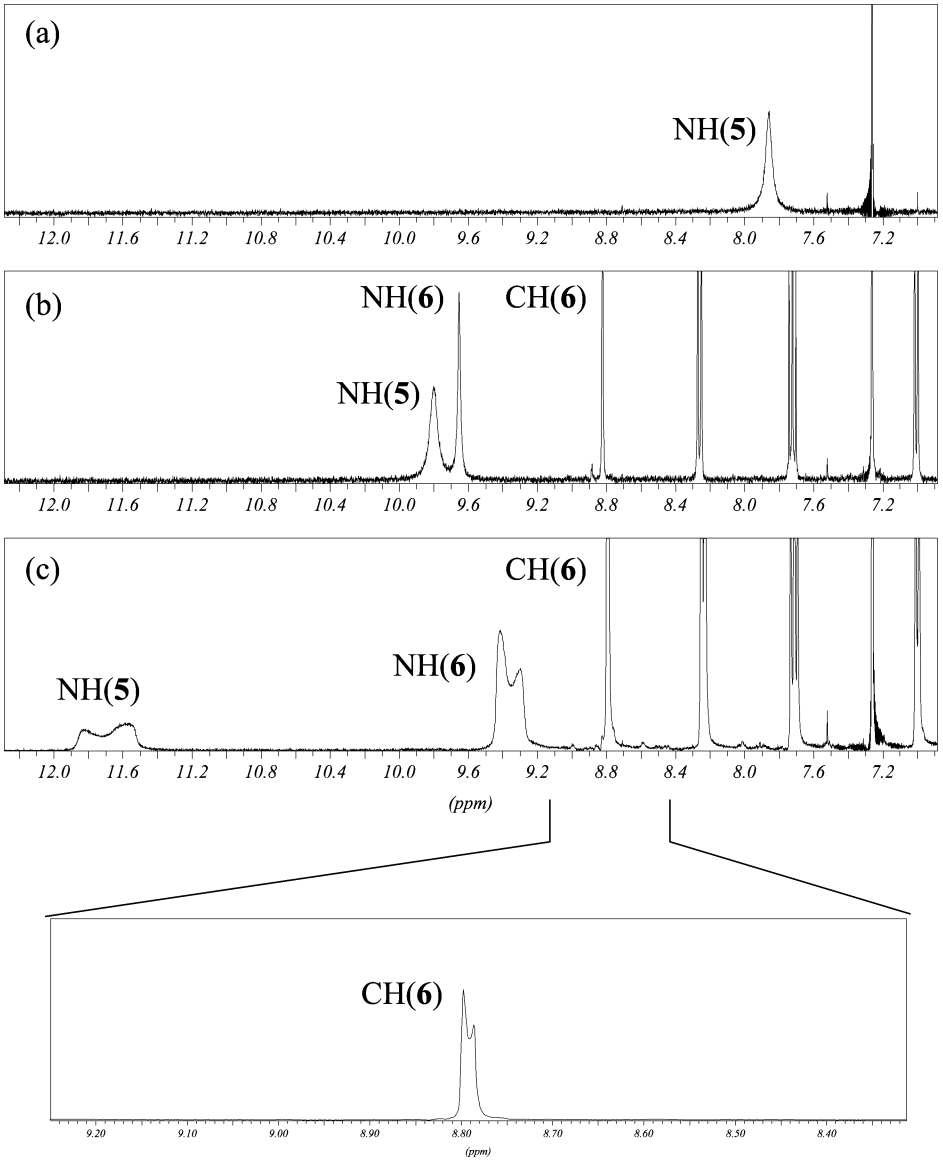

FIGURE 8. Expanded region (6.9−12.3 ppm) of ^{1}H NMR spectra in the titration of complex **6**:**5**: (a) initial solution of the guest, (b) for a 1:1 ratio, (c) for a 2:1 ratio.

Then, there are three degenerate complexes with an equal probability of existing; therefore, the entropy of the system is bigger than in those cases where there is only one possible complex, according to Boltzmann's entropy definition (eq 2)

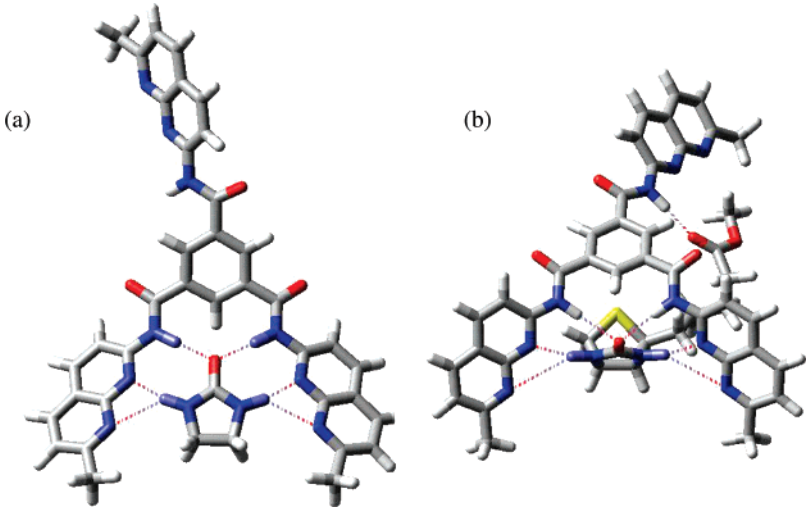

FIGURE 9. Structures for complexes (a) **7**:**3** and (b) **7**:**1**.

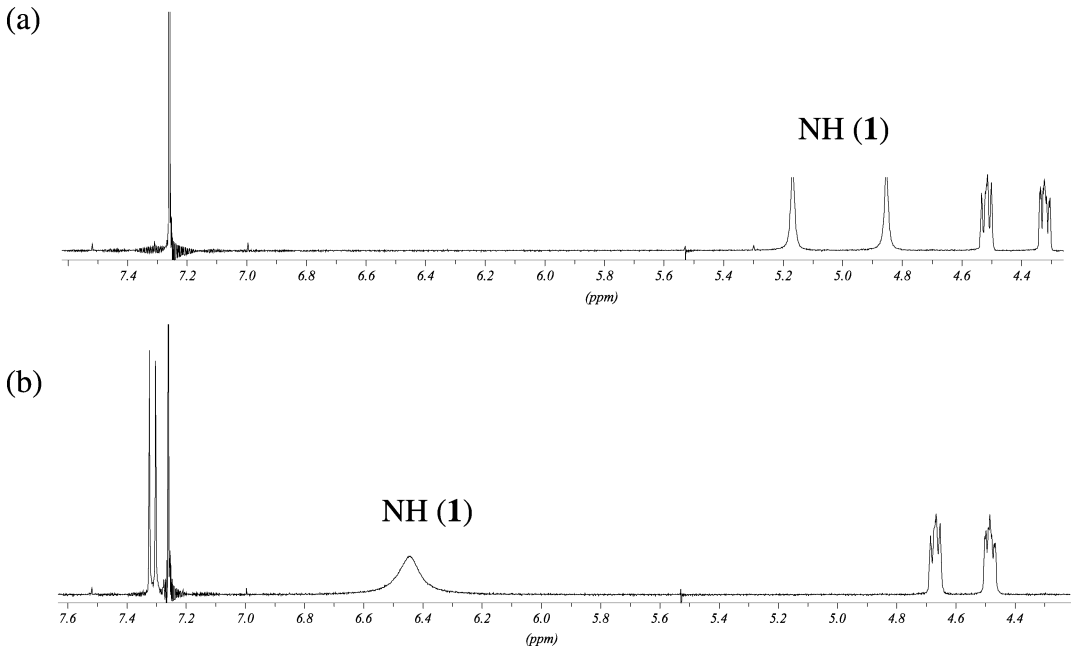

FIGURE 10. Expanded region (4.2−7.6 ppm) of the ^{1}H NMR spectra of (a) methylbiotin (**1**) and (b) **7**:**1** complex.

where W is the number of states accessible to the system.

$$S = k \ln W \qquad (2)$$

The K_b values are especially significant for host **7**; mainly with methylbiotin (**1**) the increase of the interaction affords the largest binding constant ever measured for a complex between a biotin derivative and a synthetic host with a K_b value of 1.48 10^5 M^{-1} for the complex **7**:**1**.

In this situation with an average improvement of 3 times (3.1 for **6** and 3.7 for **7**), there are some differences between the guests. With methylbiotin (**1**), the improvement is larger than the average in both hosts; for instance, it is better than that of the closely related 2-imidazolinone (**3**) by a small but significant factor of 1.2. To explain this different behavior, we have to use the molecular modeling results.

Molecular Modeling: Experimental versus Theoretical Data. In our previous papers, we found a quite good correlation between experimental binding constants and predicted interaction energies; because of that, we are confident that this is a correct approximation for the study of these systems.[8,9] We have performed this theoretical study with the aim of obtaining the most probable structure of the complexes and the associated energies. But as long as the interaction strength for these hosts is due to the probabilistic factor, it has an entropic cause; this is not taken into account in the modeling, affording wrong interaction energies. To be able to correlate experimental and theoretical data, the interaction energies must be corrected with an improvement factor (a), obtained from the average ratio of the NMR binding constants, and using the interaction energies for the complexes of hosts **8** and **9**. We are aware that this does not increase the ability of modeling these complexes, where the entropic factor is important, but it proves that the enthalpic contribution is still well modeled for this kind of similar compounds.

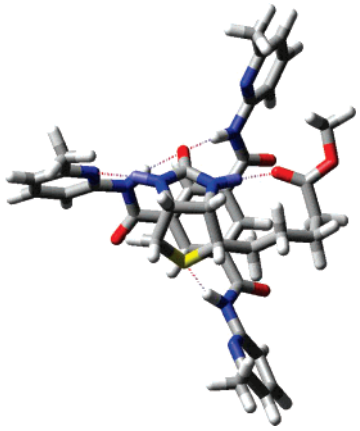

FIGURE 11. Structure for complex **6**:**1**.

If we consider two types of energies, E_1 without the probabilistic factor and E_2 with it, E_1 would be the energy for hosts **8** and **9**, while E_2 would be the one for hosts **6** and **7**. As we proved that the energy predictions for hosts such as **8** and **9** are quite well correlated with the experimental values, we can use them to obtain the corrected values for **6** and **7**. By introducing the improvement factor (a), in the energy term E_1 we obtain eq 3 and then the corrected energies E_2 as a function of nonprobabilistic energies.

$$E_2 \approx E_1/(\ln K_{b1}/(\ln a + \ln K_{b1})) \qquad (3)$$

The corrected energies for the probabilistic factor, with AMBER and OPLS force fields, are depicted in Tables 3 and 4. If the regression is performed with the original values, poor r^2 of 0.61, with AMBER data, and 0.50, with OPLS, are obtained, while with the corrected data the correlation is improved to 0.88 and 0.74, respectively. The regressions with both force fields are shown in Figure 6.

Molecular Modeling: Structure of the Complexes. The most probable structure for the complex **6**:**5** is shown in Figure 7. Looking at the complex with barbital, it is clear why the stoichiometry is 2:1 and not 1:1, as it was with host **8**, where titrations with enough concentration of host were made to eliminate the possibility of higher order complexes.[9] While the guest is interacting with the host, through one of the carbonyl groups, the probability, for the urea moiety, of finding a cleft ready to interact is three times bigger than with host **8**.

Therefore, again, there is a probability three times higher to form the ternary complex. This situation is not possible with the other guests due to the functional groups and the binding mode, showing a 1:1 stoichiometry.

The formation of this ternary complex **6**:**5** is clearly observed in the titration spectra (Figure 8). The first spectrum is for the initial solution of the guest with the NH signals of barbital (**5**). The second spectrum corresponds to the titration when a 1:1 ratio is obtained; the shift of the signals for the host and the guest can be observed. Finally, the third spectrum is the one observed when a 2:1 ratio is reached and shows how the guest and the host NH signals and the host CH signals are split.

The structures for complexes **7**:**3** and **7**:**1** are depicted in Figure 9. The structure for the complex with 2-imidazolidone (**3**) can be taken as the usual way of binding for all the guests with both hosts except for barbital and methylbiotin. That conformation is the reason guests **2**, **3**, and **4** show a 1:1 stoichiometry because upon complex formation the guest does not have more functional groups able to interact with a second host, as happens with barbital (**5**). The theoretical prediction explains as well the high improvement observed in the complexes with methylbiotin (**1**): Two of the three binding sites of the guest are used, this happens with both hosts, so for the first time the recognition of a biotin derivative by the use of several interaction points is described; this, together with the probabilistic factor, affords the largest binding constant in a

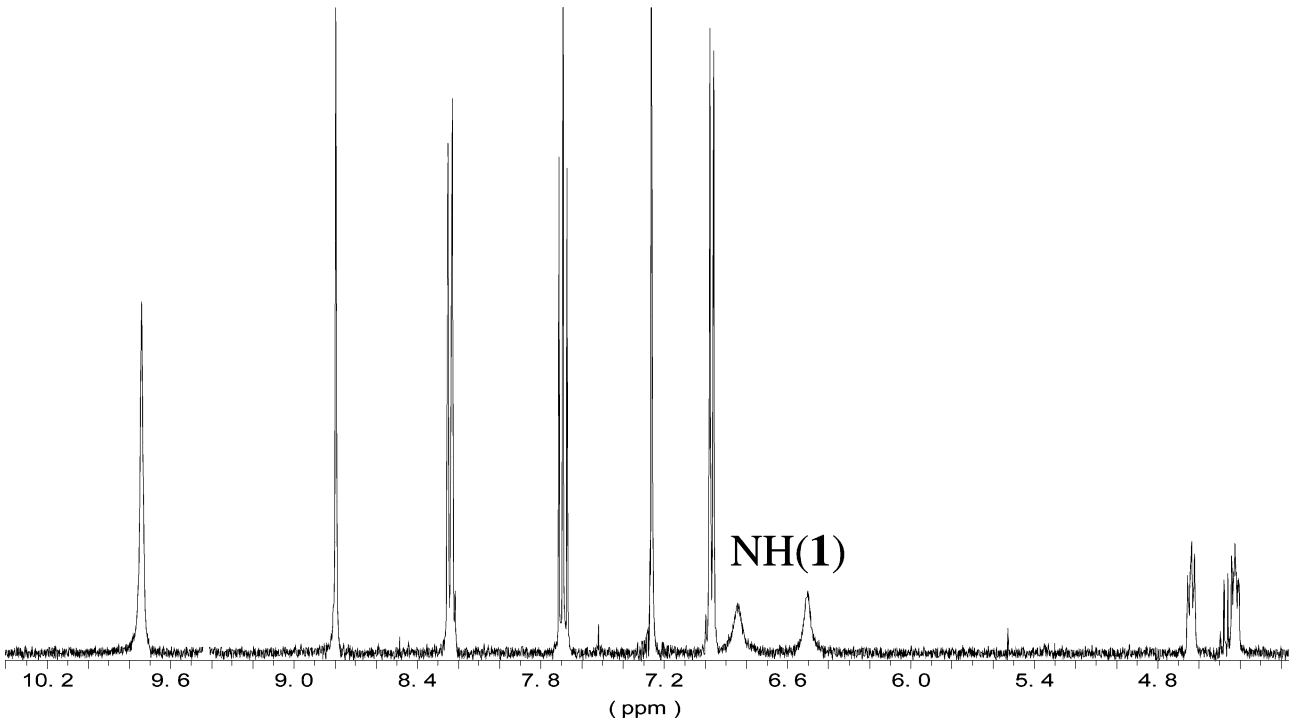

FIGURE 12. Expanded region (4.5−10.3 ppm) of the ^{1}H NMR spectrum of **6**:**1** complex.

complex between methylbiotin and a synthetic receptor. The hydrogen bonds represented in the structure of complex **7**:**1** are all in the default limits for hydrogen bonds in Macromodel software (maximun distance 2.5 Å, minimum donor angle 90° and maximum acceptor angle 60°).

The predicted conformation of the biotin side chain is confirmed by analysis of the NMR spectra. If the side chain in the complex is spread out, it should be reflected in the loss of the split for the NH signals of methylbiotin, as we have shown previously. In Figure 10 two spectra are presented: Figure 10a shows an expanded region of the spectrum for free methylbiotin (**1**) where the two signals for the NH are observed, and the spectrum in Figure 10b corresponds to the complex with host **7** and for the same concentration of methylbiotin the split is lost. Thus, in the equilibrium, most of the methylbiotin has lost the intramolecular hydrogen bond according to the predicted structure.

The utility of these experiments to identify the conformation of biotin side chain is clear if we look at the spectrum of the complex **6**:**1**; in this case the intramolecular hydrogen bond remains unchanged upon complex formation (Figure 11), and this is reflected in the split of biotin NH signals in the ^{1}H NMR spectrum of complex **6**:**1** (Figure 12). For this complex the improvement in the K_b value is explained due to the extra hydrogen bond with the sulfur atom in biotin structure.

Conclusions

To obtain better interactions with biotin derivatives it could be more important to carry out a careful revision of the literature than to synthesize new hosts that do not lead necessarily to new insights. Good correlations between experimental and theoretical data have been obtained when the probabilistic factor is taken into account.

The use of two hosts *N*,*N*′,*N*″-tris(6-methylpyridin-2-yl)-1,3,5-benzenetricarboxamide (**6**) and *N*,*N*′,*N*″-tris-(7-methyl-1,8-naphthyridin-2-yl)-1,3,5-benzenetricarboxamide (**7**) allowed us to improve the binding constants values of the complexes formed with urea and biotin derivatives. We have determined the largest binding constant between a biotin derivative, methylbiotin (**1**), and a synthetic host, compound **7**.

By combination of theoretical and experimental studies we have reached a deeper understanding on the molecular recognition features of these types of guests. These results open a new way to a continuous improvement of the binding in biotin derivatives.

Experimental Section

General Methods. The six guests are commercially available: biotin methyl ester (methylbiotin, **1**) (> 99%, dried under vacuum), *N*,*N*′-dimethylurea (**2**) (99%, recrystallized from ethyl acetate), 2-imidazolidone (**3**) (96%, recrystallized from ethyl acetate), *N*,*N*′-trimethyleneurea (**4**) (> 98%, recrystallized from ethyl acetate), and barbital (**5**) (>99%). Melting points were determined in a hot-stage microscope and are uncorrected.

NMR Spectroscopy. NMR spectra were recorded at 300 K (9.4 T, 400.13 MHz for ^{1}H, 100.62 MHz for ^{13}C, and 40.56 MHz for ^{15}N). Chemical shifts (δ in ppm) are given from internal solvent $CDCl_3$ (7.26 for ^{1}H and 77.0 for ^{13}C) and DMSO-d_6 (2.49 for ^{1}H and 39.5 for ^{13}C), and for ^{15}N NMR nitromethane was used as external standard. gs-HMQC (^{1}H−^{13}C), gs-HMBC (^{1}H−^{13}C), and gs-HMBC (^{1}H−^{15}N) were carried out with the standard pulse sequences[10] to assign the ^{1}H, ^{13}C, and ^{15}N signals.

Synthesis of *N*,*N*′,*N*″-Tris(6-methylpyridin-2-yl)-1,3,5-benzenetricarboxamide (6). 1,3,5-Benzenetricarbonyl trichloride (2 g, 7.53 mmol) was dissolved under Ar in 90 mL of dry CH_2Cl_2 and then added for 2 h, from a pressure-equalizing addition funnel, over a solution of 2-amino-6-methylpyridine (5.0 g, 46.0 mmol) and freshly distilled Et_3N (3.2 mL) in 80 mL of dry CH_2Cl_2. The resulting solution was stirred for 4 h and then washed with saturated solution of $NaHCO_3$ and water, dried (Na_2SO_4), and concentrated to yield a white solid which is purified by chromatography (silica, ethyl acetate/hexane/dichloromethane 15:10:1.5, $R_f = 0.46$) affording 1.4 g (39%) of **6**: mp 236 °C; ^{1}H NMR ($CDCl_3$) δ (ppm) 8.93 (s, 3H, NH), 8.72 (s, 3H, H-2), 8.17 (d, 3H, $J_{3',4'} = 8.2$ Hz, H-3′), 7.66 (t, 3H, H-4′), 6.96 (d, 3H, $J_{5',4'} = 7.5$ Hz, H-5′), 2.48 (s, 9H, CH_3); ^{13}C NMR ($CDCl_3$) δ (ppm) 163.4 (CO), 157.9 (C6′), 150.4 (C2′, $^3J = 9.3$ Hz), 138.9 (C4′, $^1J = 161.0$ Hz), 135.7 (C1/C3/C5), 129.3 (C2/C4/C6, $^1J = 162.6$ Hz, $^3J = 6.1$ Hz), 119.8 (C5′, $^1J = 162.6$ Hz, $^3J = 6.2$ Hz, $^2J = 3.1$ Hz), 111.1 (C3′, $^1J = 171.8$ Hz, $^3J = 6.1$ Hz), 23.9 (CMe, $^1J = 127.3$ Hz); ^{15}N NMR ($CDCl_3$) δ (ppm) −242.6 (NH), −98.4 (N1′).

Synthesis of *N*,*N*′,*N*″-Tris(7-methyl-1,8-naphthyridin-2-yl)-1,3,5-benzenetricarboxamide (7). 2,6-Diaminopyridine (3,00 g, 27.5 mmol) was dissolved in 35 mL of H_3PO_4 at 90 °C under Ar, 3-ketobutanal dimethyl acetal (3.70 g, 28.2 mmol) was slowly added from a pressure-equalizing addition funnel, and the mixture was heated at 115 °C for 3 h. After cooling, NH_4OH (15%) was added until pH 8, extracted with $CHCl_3$, washed with brine, dried ($MgSO_4$), and concentrated to yield a dark-red solid which was recrystallized from toluene to afford 1.90 g of 2-amino-7-methyl-1,8-naphthyridine (**10**) (45%): mp 215 °C; ^{1}H NMR($CDCl_3$) δ (ppm) 7.80 (d, 1H, $J_{5,6} = 7.97$ Hz, H-5), 7.78 (d, 1H, $J_{4,3} = 8.62$ Hz, H-4), 7.05 (d, 1H, H-6), 6.70 (d, 1H, H-3), 2.66 (s, CH_3).

1,3,5-benzenetricarbonyl trichloride (1.3 g, 4.90 mmol) was dissolved under Ar in 20 mL of dry CH_2Cl_2 and then added for 2 h, from a pressure-equalizing addition funnel, over a solution of 2-amino-7-methylnaphthyridine (**10**, 2.3 g, 14.5 mmol) and freshly distilled Et_3N (2.1 mL) in 100 mL of dry CH_2Cl_2. The resulting solution was stirred for 24 h, and then 100 mL of water was added and the mixture stirred for an additional 20 min. The dichloromethane was evaporated and the precipitate filtered and washed several times with water. The compound was purified by chromatography (silica, chloroform/methanol 7:1, $R_f = 0.62$) affording 2.4 g (78%) of **7** as a pale yellow solid: mp 220 °C; ^{1}H NMR (DMSO-d_6) δ (ppm) 11.63 (s, 3H, NH), 8.91 (s, 3H, H-2), 8.53 (d, 3H, $J_{3',4'} = 9.0$, H-3′), 8.50 (d, 3H, H-4′), 8.33 (d, 3H, $J_{5',6'} = 8.2$, H-5′), 7.47 (d, 3H, H-6′), 2.71 (s, 9H, CH_3); ^{13}C NMR (DMSO-d_6) δ (ppm) 165.4 (CO), 162.7 (C7′), 154.4 (C8′a), 154.0 (C2′), 139.5 (C4′, $^1J = 165.6$ Hz, $^2J = 3.8$ Hz), 136.9 (C5′, $^1J = 164.1$ Hz, $^2J = 3.4$ Hz), 134.3 (C1/C3/C5), 131.5 (C2/C4/C6, $^1J = 165.6$ Hz, $^3J = 6.1$ Hz), 121.7 (C6′, $^1J = 165.6$ Hz, $^2J = 4.6$ Hz), 118.3 (C4′a) 114.4 (C3′, $^1J = 171.8$ Hz, $^2J = 3.1$ Hz), 25.4 (CMe, $^1J = 126.8$ Hz); ^{15}N NMR (DMSO-d_6) δ (ppm) −237.7 (NH).

NMR Titrations. Each NMR titration was carried out at least three times at 300 K in $CDCl_3$ as a solvent (deuterium content >99.8%, water content <0.01%). Syringes: 5 μL (divisions 0.05 μL), 10 μL (divisions 0.1 μL), 250 μL (divisions 5 μL). ^{1}H NMR titrations are used in order to quantify K_b values, these titrations are carried out following the chemical induced shift (CIS) in one or several protons of host or guest while the concentration of the complex is changed by the addition of one of the components. For both hosts we performed a double-independent quantification following the CIS for amide proton and the benzene proton, while guest solution aliquots are added. There are a large number of ways to fit the data from a titration, but that consisting in nonlinear curve fitting is generally accepted as the method with the lowest error in the determination of K_b values, in comparison to others that employ approximations to reach a linear relationship between δ and K_b.

(10) Braun, S.; Kalinowski, H.-O.; Berger, S. *150 and More Basic NMR Experiments*; Wiley-VCH: New York, 1998.

To fit the experimental data the Sigmaplot 8.1 program was employed.[11] The basic equation used in this kind of titrations is represented by eq 4, showing the relationship between chemical shifts (δ), concentrations of host H, guest G, and complex C, and the binding constant K_b, this equation is valid only for a 1:1 stoichiometry. For complex **6**:**5** of stoichiometry 2:1 a more complex equation is necessary and the program HHG Tit. was used.[12]

$$\delta_{OBS} = (\delta_C - \delta_H)(\{(1 + [G]/[H] + 1/K_b[H])/2\} - \{(1 + [G]/[H] + 1/K_b[H])^2/4 - [G]/[H]\}^{1/2}) + \delta_H \quad (4)$$

To obtain K_b values with the lowest error the titrations are carried out in the 20−80% saturation range for the compound which CIS is being followed. This condition determines the concentrations to be used in the titrations for both host and guest and a calculation has to be done to find those concentrations that best cover the whole range of p in order to get the maximum information from the titration curve. The accuracy in the concentration range to be used in titrations is usually disregarded in most publications of the host−guest field, affording K_b values totally different from those obtained following this procedure. The error determined by this magnitude is intrinsic to the measurement method and it is not reflected by the standard deviation (S_d) which is a measure of the fit goodness of the data employed.

For the competitive titration, a unique solution of equal concentration in each of the two hosts is prepared in a volumetric flask. Aliquots of the guest solution are then added to 500 μL of both hosts solution. On this way the CIS in both hosts are measured, the relationship between this two CIS being the fundamental task to be considered.

Afterward, the fit of the data to the eq 5 gives the value of a relative binding constant. As the K_b for the complexes of the corresponding two amide substituted hosts (**8** and **9**) have been previously measured by us[8,9] we can calculate the value for the complexes of hosts **6** and **7** with the same guests.

$$K_{b(hostA)}/K_{b(hostB)} = [(1/F_{hostB}) - 1]/[(1/F_{hostA}) - 1] \quad (5)$$

F_{hostA} and F_{hostB} are the molar fractions of both hosts that are bound to the guest, if no another equilibria arise (which it has been proved with self-association titrations), then $F_i = (\delta_{i,free} - \delta_{i,observed})/(\delta_{i,free} - \delta_{i,complexed})$.

(11) Sigmaplot 8.1 from SPSS Science Software Gmbh.
(12) Hunter, C. *Chem. Eur. J.* **1998**, *4*, 845−851.

Job Plots. A series of solutions covering the whole range of molar fractions for host or guest, keeping the total concentration constant, are prepared. The chemical shifts for each solution are measured, plotting the molar fraction of the host versus the product between the increment in chemical shift and host concentration (Job's plot); a curve is generated where the maximum point indicates the stoichiometry of the complex by the use of eq 1.

MM Calculations. MacroModel v.8.1, with the GB/SA model for chloroform was used in order to perform the molecular simulations of hosts, guests, and complexes in all cases.[13] All calculations were achieved with Monte Carlo (MC) conformational analyses.[14] Previous to MC, all hosts, guests, and complexes were minimized; in all cases, the complexes were prepared from the minimized structure of host and guest, situating this one inside host cleft. Minimization is carried out using Polak-Ribiere conjugate gradient optimizer.[15] In a typical MC run, a MCMM is performed with, at least, 1000 steps for each degree of freedom, to carry out the search both torsional rotations in host and guest and translation/rotation (10 Å/360°) of the guest is performed, to be sure that the structure with the guest inside host cleft is the most stable one, for all the MC a cutoff is applied to van der Waals, electrostatic and H-bond interactions with 7, 12, and 4 Å, respectively. These calculations were carried out with two different force fields, AMBER,[16] and OPLS,[17] as implemented in the version of the program.

Acknowledgment. We deeply acknowledge Professor José Elguero (Instituto de Química Médica, CSIC, Madrid, Spain) for helpful discussions. The financial support was provided by the Spanish DGI/McyT (Project No. BQU2003-00976).

Supporting Information Available: ^{1}H and ^{13}C NMR spectra of compounds **6** and **7**; representative table for a continuous variation experiment (JOB curve) for complex **6**:**5**; representative table for a direct titration experiment for complex **6**:**1**; representative table for a competitive titration experiment for complex **7**:**1**. This material is available free of charge via the Internet at http://pubs.acs.org.

JO052424N

(13) MacroModel, Schrödinger LLC, 2004. http://www.schrodinger.com/Products/ macromodel.html.
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