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Recognizing the Limited Applicability of Job Plots in Studying Host−Guest Interactions in Supramolecular Chemistry

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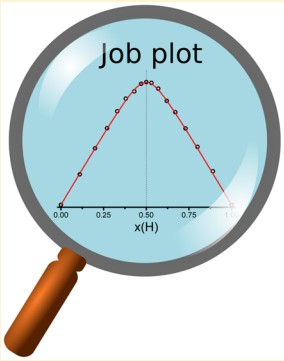
Supporting Information

ABSTRACT: Continuous variation method, known as Job plot, is the most commonly applied method for the determination of stoichiometry of complex chemical entities for over 100 years. Although, the method was proven successful in the analysis of very stable metal−ligand complexes, we demonstrate that its use in supramolecular chemistry often provides false results. We support this statement with multiple simulations as well as cases studies of several real host−guest systems. We propose an alternative, general method relying on the analysis of residual distribution in titration data fitting. The latter method is more convenient compared to the Job plot and unlike it gives correct results in all real cases studied.



Found not working in supramolecular systems

Misleading about stoichiometry of assemblies

Incorrect K_a determination

1. INTRODUCTION

Ever since Chemistry got rid of the prefix "al-" (alchemy), equations and numbers have become an integral part of the science. This revolution took place in the 19th century and is exemplified with the ground-breaking works of Lavoisier, Dalton, Clapeyron, Avogadro, and many others who established the principle of mass conservation, the relations between volume, pressure and temperature in gases, the law of definite proportions, etc. Later, the theories of thermodynamics, kinetics, and many others evolved. All of these laws and theories are defined in the language of mathematics and therefore require transformation of the properties of matter into numbers. Indeed, these numbers are very convenient for making various comparisons of substituents in physical organic chemistry and make it possible to predict some properties of new compounds based on previous measurements.

There is, however, an underestimated responsibility in publishing the numerical results of experiments. It takes a lot of effort to identify the incorrectly determined value and publish the correct one. The invalid numbers and false conclusions circulate in the consciousness of the scientific community for a very long time even after the correction is announced.

An example of a numerical measurement is determination of stoichiometry of supramolecular complexes. From the very beginning of host−guest chemistry studies, a continuous variation method, also referred as a Job plot, has been routinely employed for such analyses. Although there were some reports on the limited applicability of this method,[1−4] it was never fully validated in the range of low to moderate association constants which are often encountered in supramolecular assemblies. In this paper, we critically evaluate the continuous variation method by performing multiple theoretical simulations and analysis of several case studies.

2. RESULTS AND DISCUSSION

2.1. Theory and Simulations. *2.1.1. Consequences of Choosing a Model.* Although the vast majority of supramolecular systems form only simple 1:1 host (H) to guest (G) complexes, there are also numerous examples of supermolecules with more composite stoichiometry, the most common among them being 1:2 and 2:1 (HG_2 and H_2G). Such stoichiometry is usually a desired and planned property, but in some cases it may be discovered unexpectedly in novel systems.

The principles of titration have quite recently been discussed in detail by Thordarson[5] and Hirose,[6] so we shall only recapitulate the key issues here. In a solution containing both host and guest, supramolecular complexes may form according to eqs 1-3), with stabilities defined by the association constants[7] shown in eqs 4−6).

$$H + G \rightleftharpoons HG \quad (1)$$

$$HG + G \rightleftharpoons HG_2 \quad (2)$$

$$HG + H \rightleftharpoons H_2G \quad (3)$$

$$K_a^{1:1} = \frac{[HG]}{[H]\cdot[G]} \quad (4)$$

$$K_a^{1:2} = \frac{[HG_2]}{[HG]\cdot[G]} \quad (5)$$

$$K_a^{2:1} = \frac{[H_2G]}{[HG]\cdot[H]} \quad (6)$$

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During the course of a typical titration, aliquots of the guest are added to the solution of the host and an analytical signal (Y) is monitored throughout the process. The origin of the Y signal depends on the analytical method used:

- In UV−vis spectrometry, the absorbance ($Y = A$) is the sum of the absorbances of all components:

$$A = l\varepsilon_{\mathrm{H}}[\mathrm{H}] + l\varepsilon_{\mathrm{G}}[\mathrm{G}] + \sum_{n,m} (l\varepsilon_{\mathrm{H}_n\mathrm{G}_m}[\mathrm{H}_n\mathrm{G}_m])$$

  the same applies to fluorometry, with emission intensity ($Y = F$) as the analytical signal (relative absorption cross-section must be taken into account)
- In NMR spectroscopy, under the fast exchange regime,[8] the chemical shift ($Y = \delta$) of a host's nucleus depends on the mole fractions of pure host and its complexes:

$$\delta = \delta_{\mathrm{H}} x_{\mathrm{H}} + \sum_{n,m} (\delta_{\mathrm{H}_n\mathrm{G}_m} x_{\mathrm{H}_n\mathrm{G}_m})$$

- In calorimetry, the heat ($Y = \Delta H$) evolved after each portion of guest added is measured and ΔH arises from the formation of complexes as well as the heat of dilution of the guest ($\Delta H^0_{\mathrm{dil}}$ can be determined in a separate experiment):

$$\Delta H = \sum_{n,m} (\Delta H^0_{\mathrm{H}_n\mathrm{G}_m} \Delta n_{\mathrm{H}_n\mathrm{G}_m}) + \Delta H^0_{\mathrm{dil}}$$

Although the specific equations differ in each case, the main problem remains the same. A set of Y values obtained gives a titration curve that is a plot of Y (or $\Delta Y = Y - Y^0$) versus $[\mathrm{G}]_0$[9] (or equivalents of G). Based on the assumed model of binding, one then numerically fits the calculated set of Y values to the experimental ones by varying the values of association constants $K^{n:m}$ and the complexes' analytical coefficients $Y_{\mathrm{H}_n\mathrm{G}_m}$. In the simplest case, where only 1:1 complex is formed, $K^{1:1}$ and Y_{HG} are varied until the best match of Y values is reached.

It is of prime importance to know the stoichiometry of the complexes; otherwise, fitting the titration data with an inappropriate model will yield values that have no physical meaning, as is illustrated in Table 1. Here, theoretically simulated titration curves for a HG + HG_2 system with various $K^{1:1}/K^{1:2}$ ratios at several concentrations were fitted with a 1:1 model. Quite expectedly, in a case with low $K^{1:2}$ (such as 1% of $K^{1:1}$ value) and low concentration of the host, the second binding event turns out to exert little impact on the titration curve. The fitted association constant (K^{fit}) is therefore close to the real $K^{1:1}$ of the system; however, the error already reaches approximately 17%. When the concentration of the host and/or the ratio $K^{1:2}/K^{1:1}$ is higher, the K^{fit} is no longer close to $K^{1:1}$. It becomes a misleading value with no physical meaning, which does not correspond to any combination of the two real constants. Note that K^{fit} reaches a value of ~$0.3K^{1:1}$ for the midrange values of $K^{1:2}$ and $[\mathrm{H}]_0$ in Table 1, while in the last row K^{fit} values are already 1 order of magnitude lower than the actual $K^{1:1}$. These data clearly indicate that correctness of the chosen model is not just important to make the fitting more accurate, it is in fact *crucial for performing any association constant determination* in the first place.

Table 1. Association Constants (K^{fit}) Obtained by Fitting a 1:1 + 1:2 System by a Simple 1:1 Model[a]

$[\mathrm{H}]_0$	$K^{1:2} = 10$	$K^{1:2} = 50$	$K^{1:2} = 100$	$K^{1:2} = 250$
0.0001	833	518	407	474
0.001	521	317	293	350
0.01	129	105	118	104

[a] $K^{1:1} = 1000$, $Y_{\mathrm{HG}} = 1$, and $Y_{\mathrm{HG_2}} = 1.5$ were assumed.

Let us consider a different way of dealing with the stoichiometry problem. Namely, we might try to fit every set of titration data with a composite model assuming formation of HG, HG_2, H_2G, ... complexes. If any of the complexes do not occur, then the corresponding fitted $K^{n:m}$ should be negligibly low. This approach, however, fails to actually work in practice. Even if only three complexes are considered (HG, HG_2, H_2G), then there are six parameters (3 K and 3 ΔY) to be fitted, which requires approximately 30−40 data points. Moreover, in our experience, the application of such a composite model to a system that in fact forms only one or two complexes usually results in failure of the fitting procedure, generates multiple results depending on initial guess conditions, or gives false values with huge uncertainties.

Therefore, some measurements must be applied to determine the stoichiometry prior to performing titration and fitting. The first step should involve a rational analysis of the structure and properties of the receptors, i.e., two anchoring points (binding arms) in a receptor may act cooperatively but sometimes independently as well, large cavities may accommodate more than one guest molecule, multiply charged hosts may attract more than one singly charged guest, etc. Any binding model so assumed must then be evidenced by an appropriate experimental method. A comprehensive list of available methods was given by Thordarson[5] together with some critical comments. In this paper, we analyze the applicability of some of these methods in detail.

2.1.2. Job Plots. The continuous variation method, known as the Job plot, is the most popular way of determining the stoichiometry of complexes, although this method is based on assumptions that are in fact never actually met.[10] It is assumed that the H_nG_m complex is the only one formed and, therefore, the only one giving rise to ΔY. A Job plot should reach its maximum for a solution with $[\mathrm{H}]_0$:$[\mathrm{G}]_0 = n{:}m$. In a real situation, the HG complex and complexes with all possible intermediate stoichiometry will also be present in the solution, and each complex will influence the observed ΔY. This does not mean that the continuous variation method is useless, but it does mean that it requires careful interpretation. For example, if both HG and HG_2 complexes are formed, the maximum on the Job plot will not lie at molar fraction $x(\mathrm{H}) = 0.33$ but probably somewhere between 0.33 and 0.5. Depending on the system, a maximum shifted to 0.45 may already be an indication of 1:2 complex formation; however, more complicated cases are also possible.

To better illustrate this point, we performed a simulation of the Job plots for various $K^{1:1}$ and $K^{1:2}$ values as well as concentrations of the reactants for typical cases met in supramolecular systems. The results are presented in Table 2.

Note that at low concentrations all plots have a maximum at $x = 0.5$ and are symmetrical regardless of the $K^{1:2}$ values. At higher concentrations the maximum is shifted, but in the case of $K^{1:2} = K^{1:1}/20$ the shift of the maximum is visible only at the highest concentrations. Even in the model with the receptor bearing two independent binding sites ($K^{1:2} = K^{1:1}/4$, last column) a concentration corresponding to $K^{1:1}[\mathrm{H}]_0 = 10$ is essential to indicate the formation of 1:2 complex. If a classical interpretation were to be applied to these plots, only four of them would be treated as indicative of HG_2 complex formation.

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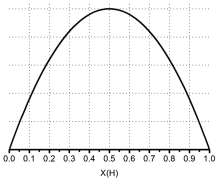
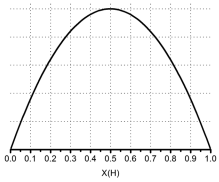
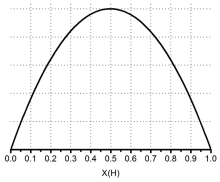
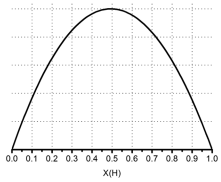
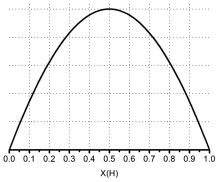
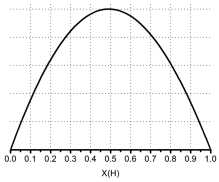
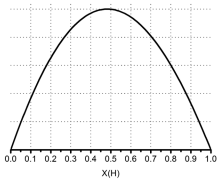
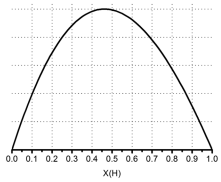
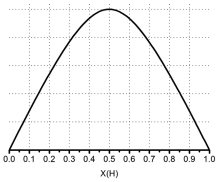
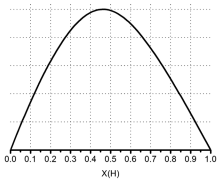
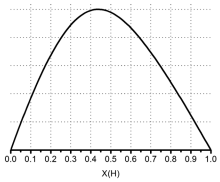
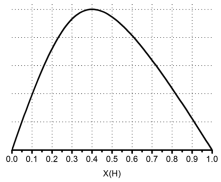
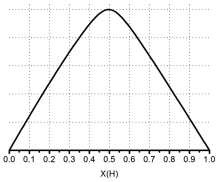
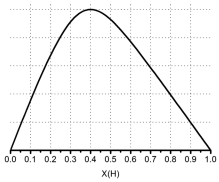
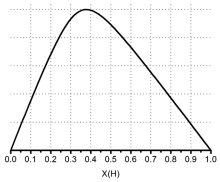
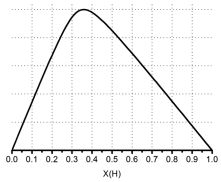
Table 2. Job Plot Shapes at Various Concentrations and $K^{1:1}$ to $K^{1:2}$ Ratios[a]

	Association constants			
concentration	$K^{1:2}$=0	$K^{1:2}$=50	$K^{1:2}$=100	$K^{1:2}$=250
0.0001	 x_{max}=0.50	 x_{max}=0.50	 x_{max}=0.50	 x_{max}=0.49
0.001	 x_{max}=0.50	 x_{max}=0.49	 x_{max}=0.48	 x_{max}=0.46
0.01	 x_{max}=0.50	 x_{max}=0.46	 x_{max}=0.44	 x_{max}=0.40
0.1	 x_{max}=0.50	 x_{max}=0.40	 x_{max}=0.38	 x_{max}=0.36

[a] $K^{1:1}$ = 1000, Y_{HG} = 1, and Y_{HG} = 2 were assumed.

The other plots would mislead the researcher and give rise to false results.

The relation between Y_H, Y_{HG}, and Y_{HG_2} is another factor that very strongly influences the shape of a Job plot. Four cases simulated for an HG + HG_2 system are presented in Table 3. This is a well-defined 1:1 + 1:2 system, for which, in a classical interpretation, the maximum is expected at $x = 1/3$. However, in none of the cases would the Job plot actually guide the researcher to the correct stoichiometry. In case A, the Y parameter of the two complexes is the same ($Y_{HG} = Y_{HG_2}$) and the maximum on the plot is very close to $x(H) = 0.5$. In plot B, the Y of the 1:2 complex is similar to the Y of the free host. This causes a shift in the maximum of the plot toward higher $x(H)$ values, which is indicative for H_2G complex formation, but not for HG_2, actually present in the solution. In the third simulation (case C), only the HG_2 complex has an impact on the Y parameter, resulting in the maximum being shifted to $x(H) = 0.29$. This value lies midway between $x = 0.33$ and $x = 0.25$, which typically indicates the formation of HG_2 and HG_3 complexes, respectively, thus leading to some confusion. Finally, if Y_{HG} and Y_{HG_2} have opposite signs, a wavelike plot is obtained with two extrema (maximum and minimum), neither of which correspond to the real stoichiometry. This set of examples shows how for some combinations of Y_{HG} and Y_{HG_2} the Job plot will be misleading, even if the formation of the HG_2 complex is significant and the measurement is conducted at high reactant concentrations. Moreover, it is possible for a combination of the A–D types of plots to be observed simultaneously in a single experiment for different nuclei (NMR) or different wavelengths (UV–vis, fluorometry).

The simulated Job plots presented here serve to illustrate the limitations on the method's applicability in supramolecular chemistry, limitations which we feel crucially need to become more widely recognized. How can these limitations be counteracted? First of all, it is beneficial for experiments to be carried out at the highest possible concentration; otherwise, the resulting plot may be misleadingly centered at $x = 0.5$. More generally, we propose that a cautious method for Job plot interpretation should proceed as follows:

- a maximum shifted to at least 0.45 or 0.55 already indicates a composite binding stoichiometry

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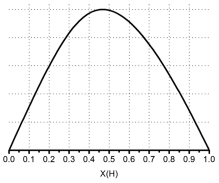
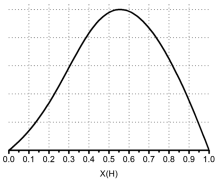
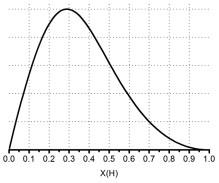
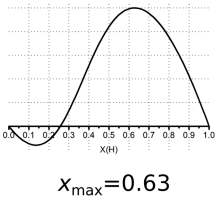
- a maximum near 0.5 *does not* provide proof of a simple 1:1 model, unless the plot is very sharp ($K_a[H]_0 > 100$)
- a slight shift in the maximum (approximately 0.45−0.55) may arise from experimental errors
- if possible, more than one nucleus or absorption/emission band should be analyzed

In order to reduce the experimental effort, a simplified procedure for obtaining Job plots was suggested. In this method, the results of typical titration are transformed into a Job plot; however, in this case even the basic assumptions of the method are no longer valid and the procedure was experimentally proved to provide false results.[11]

Table 3. Simulated Shapes of Job Plots for 1:1 + 1:2 Stoichiometry[a]

A: Y_{HG}=1; Y_{HG_2}=1	**B**: Y_{HG}=1; Y_{HG_2}=0
	
x_{max}=0.47	x_{max}=0.56
C: Y_{HG}=0; Y_{HG_2}=1	**D**: Y_{HG}=1; Y_{HG_2}= -1
	
x_{max}=0.29	x_{max}=0.63 x_{min}=0.14

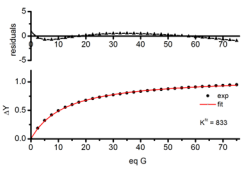
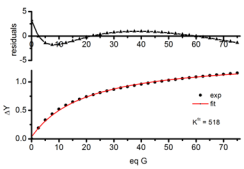
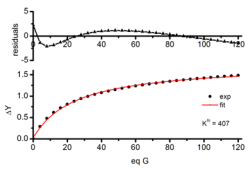
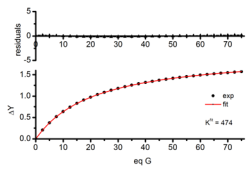
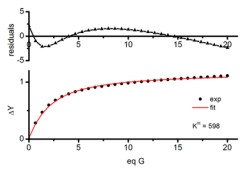
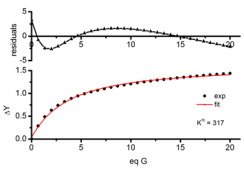
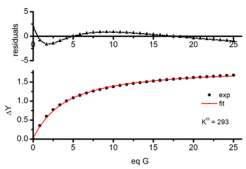
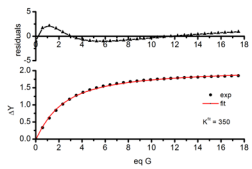
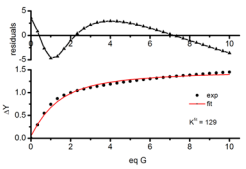
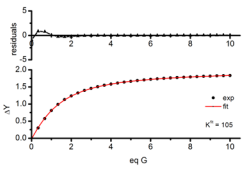
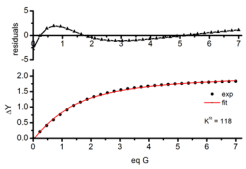
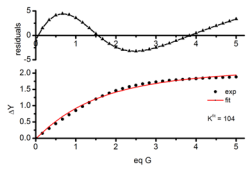
[a] $K^{1:1}$ = 1000, $K^{1:2}$ = 250, $[H]_0$= 0.01.

2.1.3. Quality of Fitting and Residual Distribution. The authors of some papers in the literature claim that their assumed model of binding is proved by a high correlation coefficient between the experimental and fitted data.[12] However, it should be recognized that obtaining a good correlation coefficient (R^2 close to unity) with a simple model does not necessarily mean that the fitting might not be even better with a more composite model, and so such argumentation is ultimately unconvincing. Moreover, R^2 is a particularly poor indicator of quality of fitting.[13] On the other hand, as we already mentioned, the application of a model with overly high complexity may give false results even if the fitting seems quite good. A very nice example of examination of multiple possible binding modes with χ^2 used to assess the quality of the fit was presented for a receptor with two anion-binding and two cation-binding sites.[14]

In fact, it is not the correlation coefficient or the sum of residual squares but rather the residuals distribution that may be used as an indicator of model correctness, as already suggested in Thordarson's review.[5] If the data are fitted with a proper model, the residuals will be distributed randomly, since they arise from random errors. On the contrary, when data are fitted with an improper model, a regular, sinusoidal distribution of the residuals will be observed. This characteristic is one of the most sensitive indicators that an assumed model may be incorrect; unfortunately, however, it does not suggest what the actual stoichiometry might be.

To illustrate this, in Table 4 we plotted several graphs of a simulated HG + HG_2 system fitted with a simple 1:1 model. The values and distributions of the residuals differ depending on the association constants and the host concentrations, yet they maintain a sinusoidal shape.

Table 4. Titration Data Simulated for a HG + HG_2 System Fitted with a Simple 1:1 Model: Titration Curves and Residual Distributions[a]

	Association constants			
concentration	$K^{1:2}$=10	$K^{1:2}$=50	$K^{1:2}$=100	$K^{1:2}$=250
0.0001				
0.001				
0.01				

[a] Y_{HG} = 1 and Y_{HG} = 2 were assumed. $K^{1:1}$ = 1000.

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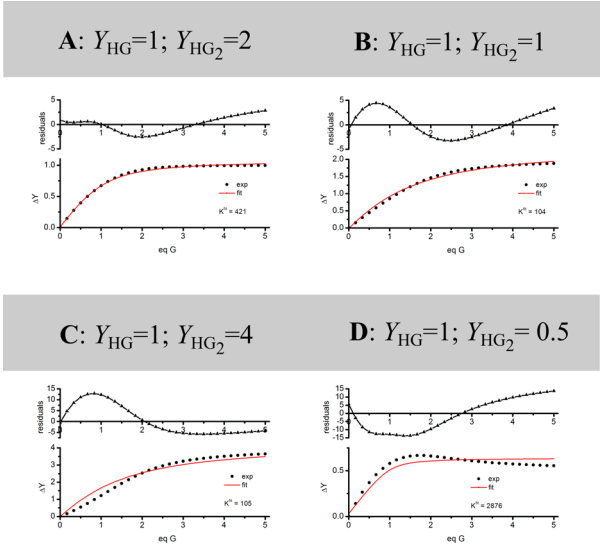
In a case where both the ratio $K^{1:2}/K^{1:1}$ and the product $[H]_0K^{1:1}$ are low (e.g., <0.1 and <1, respectively) the sinusoidal distribution of errors has a very low amplitude and may be hidden in the noise of random errors in a real situation. The amplitude of this residual distribution increases with increasing $[H]_0$ and $K^{1:2}$; however, for certain combinations of association constants and concentration, the residuals may diminish coincidentally. Therefore, as likewise concluded above for the continuous variation method, it is beneficial for a titration to be performed at the highest possible host concentration, intended not for K determination, but only for model validation.

Some special cases can also be considered for different relations between Y_{HG} and Y_{HG_2}, as presented in Table 5. Case A corresponds to a receptor equipped with two equal and independent binding sites. In case B, the observed nucleus/wavelength is insensitive to the formation of HG_2 complex, yet the residuals are significant and regularly distributed. The next possibility, case C, describes a nucleus/wavelength which is more sensitive to formation of HG_2 complex. A sigmoidal shape of the binding isotherm is observed, which cannot be accurately fitted, and again large residuals are observed. The last case, D, depicts a situation where $Y_{HG_2} < Y_{HG}$, which results in the presence of an extremum on a binding isotherm. The latter case is the easiest to spot and the most convincing indication of a nontrivial stoichiometry of complexes.

Table 5. Titration Data Simulated for Special Cases within a HG + HG_2 System Fitted with a Simple 1:1 Model[a]



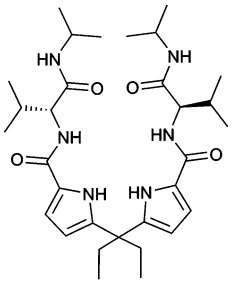
[a] $K^{1:1}$ = 1000, $K^{1:2}$ = 250.

The residual analysis method does not prove that an applied model is correct; it can in fact only reveal a model's incorrectness. However, one can try to treat the collected data with several (physically acceptable) models and compare the residual distributions. The model with the lowest residuals and most random distribution of residuals is the most likely to be correct.

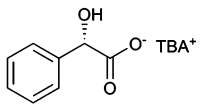
Unfortunately, residual analysis is very rarely applied in the literature. Moreover, we found quite a large number of titration plots with residuals, large enough to be spotted by the naked eye, that follow regular-sinusoidal distributions.[15] Residual distribution analysis is a method of model validation that is quite sensitive and does not require additional experimental work. It is trivial to calculate the residual distribution, and most of the common software packages do so automatically (i.e., WinEqNMR,[16] HYPERQUAD, HYPNMR,[17] Open Data Fit,[18] *etc.*) Such calculation should therefore be done routinely for every titration performed, and such residual distribution plots provided in the supporting information would increase the reliability of the presented data.

2.1.4. Comparing Varying Concentrations. Another possible way to check the correctness of an applied model follows as a direct consequence of Table 1. Namely, a strong and regular dependence of fitted K^{fit} on the $[H]_0$ in several experiments indicates a model mismatch, whereas good consistency of results across concentrations of 2 orders of magnitude offers evidence of the model's correctness. The $[H]_0$ in the experiments being compared should differ by at least 5× (although the greater the difference, the better). This method introduced previously by Thordarson[5] is likewise not widely applied in the literature, even though it is also quite simple and very sensitive.

2.2. Case Studies. In what follows, we illustrate the theoretical considerations presented above with some real experimental examples, starting with cases taken from our own recent work, where a composite stoichiometry was found quite unexpectedly. In the first example, a bipyrrolic receptor **1** equipped with chiral amino acid arms was being investigated for its chiral recognition properties. We assumed that the six hydrogen bond donors (two pyrrolic and four amidic) would act cooperatively to bind a single carboxylate in the binding pocket.



1



(*S*)-ManOTBA

The Job plot for **1** with (*S*)-mandelate (tetrabutylammonium salt) (Figure 1a) had a maximum at $x(H) \approx 0.43$, which may already indicate a more composite stoichiometry of binding. Fitting of the titration data with a simple 1:1 model at first sight seems to provide a good match between the calculated and experimental data. However, a closer look reveals systematic distribution of residuals, thus judging the incorrectness of this model (Figure 1b). When the data were fitted with a HG + HG_2 model, a perfect match was obtained with stochastic residuals of very low values (Figure 1c). Unfortunately, the obtained association constants $K^{1:1}$ and $K^{1:2}$ are subject to huge uncertainties, as is quite common for composite systems. We noticed, within the error boundaries, the ratio $\frac{K^{1:1}}{K^{1:2}} \approx 4$, indicating the presence of two, equal and quite independent, binding sites within our receptors. We concluded that binding of carboxylate is achieved by one half of the receptor and that the two halves cannot act cooperatively (Scheme 1).

Another case of unpredicted composite stoichiometry arose within our series of thioureidic receptors of type **2** and **3**.[19] Receptor **2** was proved by residual distribution analysis to form exclusively 1:1 complexes. However, host **3** incorporating tryptophane moieties is equipped with additional heterocyclic NH hydrogen bond donors, which may interfere with the

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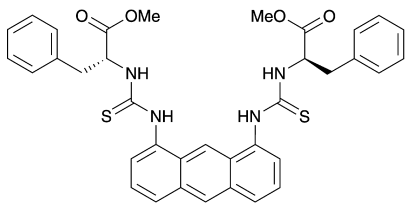
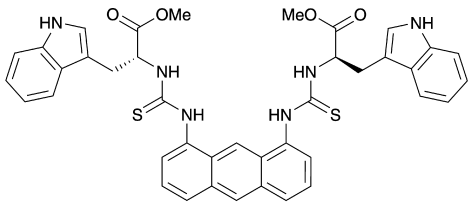


2



3

simple binding model. The Job plot of **3** with (*S*)-mandelic acid ((*S*)-ManOTBA) TBA salt plotted for three different protons is presented in Figure 2a. Two protons, thioureidic and C9, form symmetrical plots with maxima at $x(H) = 0.5$, whereas the plot of the indole NH proton is slightly asymmetrical and has a maximum shifted to $x(H) \approx 0.47$. These results could be indicative of a simple binding model. The titration curves plotted for these protons, however, are much more informative (Figure 2b). Namely, the thioureidic protons seem to reach a plateau after 1 equiv of guest is added, but the indolic proton continues to move downfield; furthermore, the shift for the C9 aromatic proton moves slightly backward after 1 equiv of guest added. Such behavior cannot be explained and fitted by a simple model; it provides clear evidence of a composite stoichiometry. The data were indeed nicely fitted with a HG + HG_2 model, and the following values were obtained: $K^{1:1} = 8400 \pm 25\%$, $K^{1:2} = 11 \pm 80\%$ [M^{-1}]. The large Y_{HG_2} value for indolic protons indicates that they are engaged in the second binding event. Although the second binding constant $K^{1:2}$ is only 0.1% of $K^{1:1}$, it has a very pronounced effect on the titration curves and the final results. If the ureidic proton shifts are fitted with an inappropriate 1:1 model, a value of $K^{fit} \approx 6700$ is obtained.

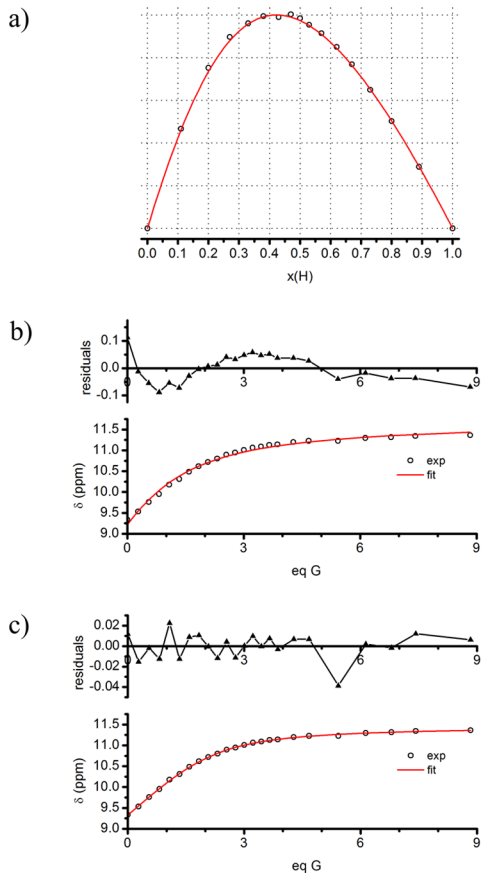
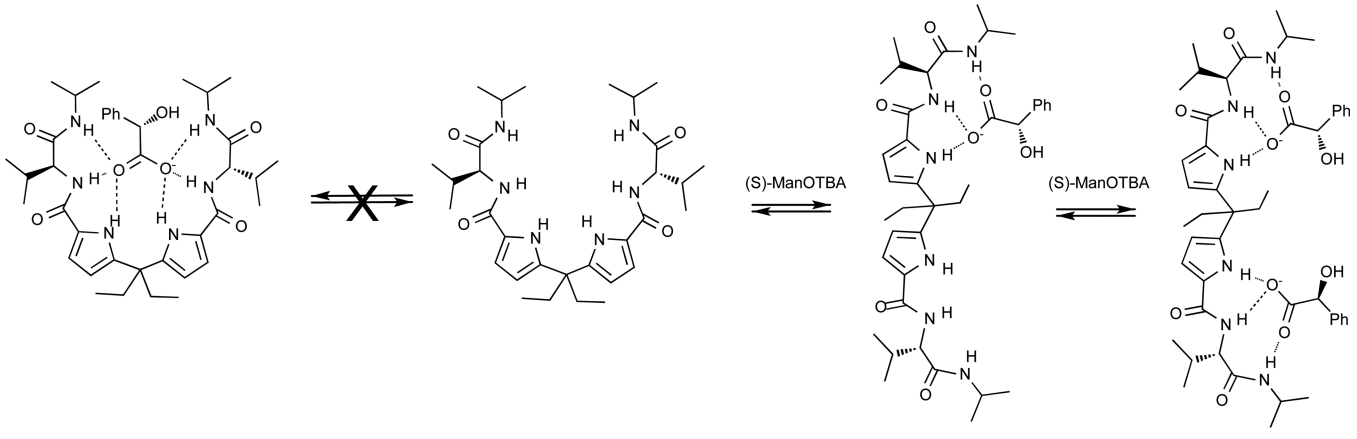


Figure 1. (a) Job plot of **1** with (*S*)-ManOTBA in $CDCl_3$; (b) titration data fitted with 1:1 model; (c) same titration data fitted with 1:1 + 1:2 model.

Let us now consider one of the cases from the literature. The photoswitchable chloride receptor/transporter **4**, recently described by Jeong,[20] attracted our attention because of its similarity to our receptors based on an azobenzene core[21] (**5**). Host **4** is equipped with two urea moieties capable of anion binding. For the receptor in the *E*-form, however, these groups are very distant (about 16 Å), and their cooperative action in binding a single anionic guest such as chloride is rather impossible. Indeed, in our recent work we showed that a similar host **5** forms HG and HG_2 complexes with the urea moieties acting independently. After analyzing the structure of host **4**, the researchers from Jeong's group could likely have concluded that their host forms complexes of composite stoichiometry, but the Job plot experiment they conducted misled them to a conclusion that complexes with composite stoichiometry even if formed may

Scheme 1. Independent Binding of Two Guest Molecules by Single Halves of Receptor 1



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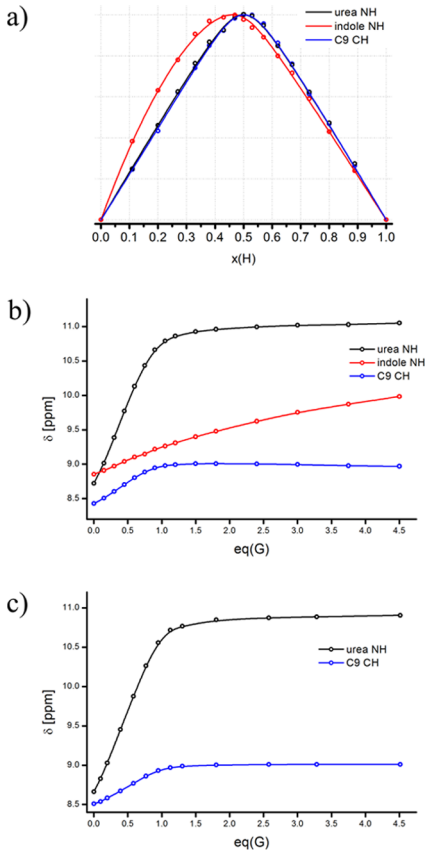
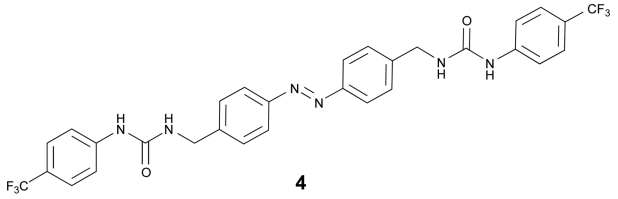
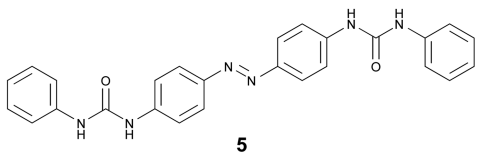


Figure 2. (a) ^{1}H NMR Job plots for receptor **3** with (*S*)-ManOTBA in MeCN-d_3 (all plots were scaled to the same range); (b) NMR titration curves of **3** with (*S*)-ManOTBA in MeCN-d_3; (c) NMR titration curves of **2** with (*S*)-ManOTBA in MeCN-d_3 shown for comparison.

be disregarded. To prove that HG_2 complexes are formed and influence titrations, and to demonstrate what we suggest is a proper route of analysis, we synthesized receptor **4** and performed the necessary measurements.



4



5

Chloride anion guest was used as tetrabutylammonium salt (TBACl). Due to low solubility of the receptor in the examined solvent mixture, the measurements were run at quite low concentration (c = 0.5 mM), which as we have noted is disadvantageous for stoichiometry determination. The Job plot performed by authors exhibits a maximum at $x(H)$ = 0.5, but its rounded shape indicates that this experiment is rather uninformative. We carried out titration measurements until a plateau was reached, and the data were fitted with simple (HG) and composite ($HG + HG_2$) binding model. A suggestive wave-like distribution of residuals was obtained when the simple model

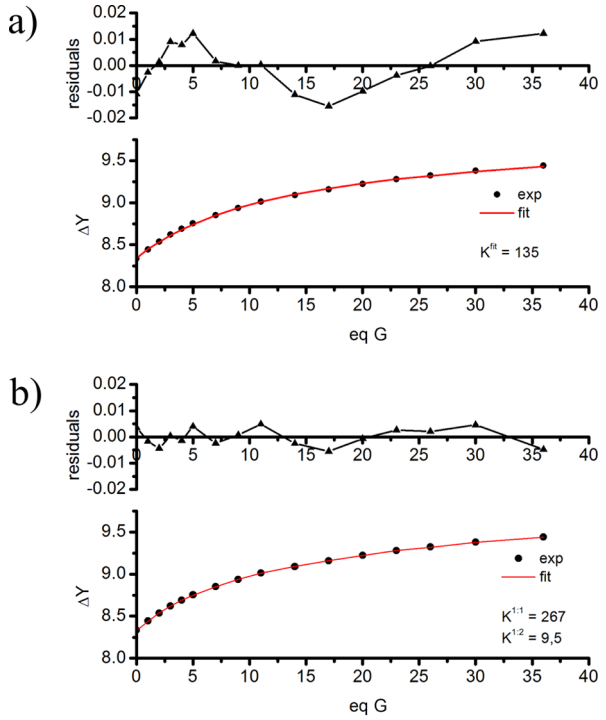
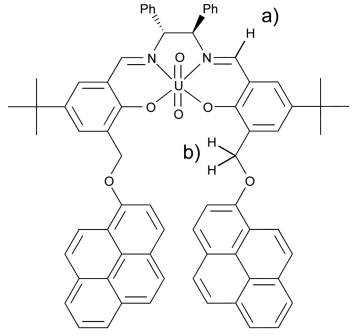


Figure 3. ^{1}H NMR titration of receptor **4** with TBACl fitted with 1:1 model (a) and composite 1:1 + 1:2 model (b). Changes in a shift of urea protons are plotted.



6

Figure 4. Structure of receptor **6** and labeling of selected hydrogen atoms.

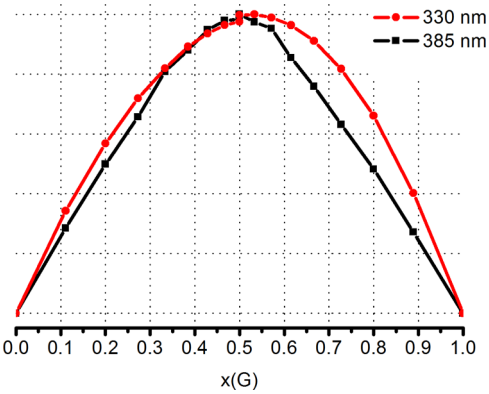


Figure 5. Job plot for receptor **6** with D-PheOTBA under UV−vis control.

was applied with K^{fit} = 135 (Figure 3). On the contrary, the composite model, consistent with rational analysis of the receptor, gave a stochastic distribution of residuals of far lower

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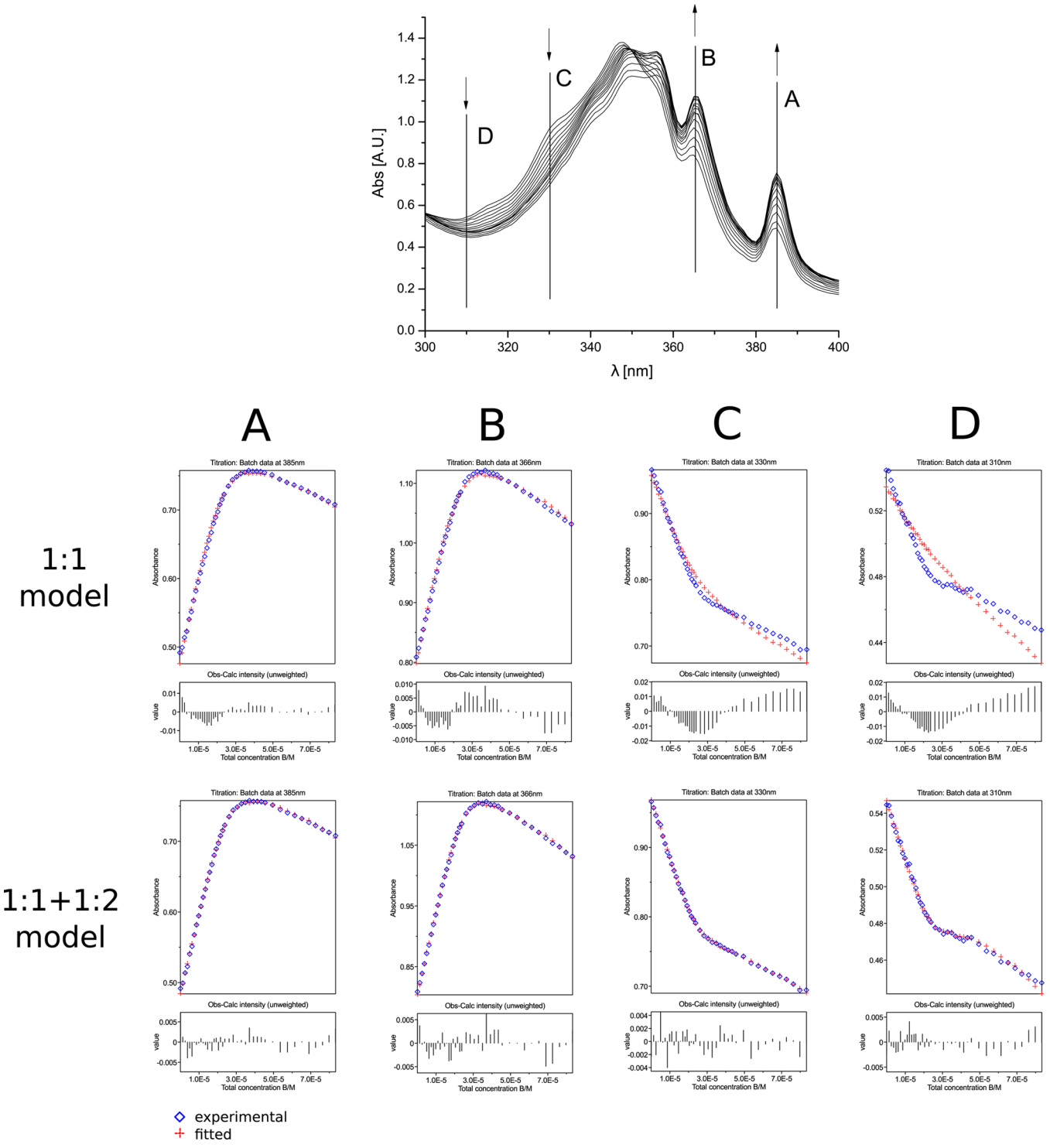

Figure 6. UV−vis titration data for receptor **6** with D-PheOTBA fitted by simple and composite binding models.

values. This latter result proves that the proper binding model differs from what cited authors claimed, as do the association constants. For receptor **4**, the association constants we determined[22] are $K^{1:1}$ = 267 ± 15, $K^{1:2}$ = 9.5 ± 0.5. By some experimental coincidences Jeong obtained similar value of K_a,[23] although the binding mode was incorrectly reported. These results prove that the possibility of formation of complexes with composite stoichiometry should never be disregarded.

As a final example, let us consider the chiral salen-uranyl complex **6**, used by Pappalardo[24] in chiral recognition studies of carboxylates. Pappalardo's paper attracted our attention because of the exceptionally high enantioselectivty values reported there and the significant residuals in the titration data fitting procedures found in the supplementary data. We therefore synthesized receptor **6** and analyzed its binding properties in depth. The first experiments, with TBA salt of D-phenylalanine (D-PheOTBA), were carried out with UV−vis monitoring, as in the cited paper. We conducted Job plot measurements at concentration of 5 × 10^{-5} M. The two plots presented in Figure 5, prepared for different wavelengths, are slightly asymmetrical, but the maxima

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are around x = 0.50 and x = 0.53 for 385 and 330 nm, respectively. The obtained curves are not very sharp, which means that they are uninformative. Next, we performed a complete UV−vis titration with $[H]_0 = 2.5 \times 10^{-5}$. The data obtained were fitted globally (all wavelengths simultaneously) with HypSpec software; the results depicted by four selected wavelengths are presented in Figure 6. Please note that the decrease in absorption in the final stage of titration is mainly due to the dilution of the sample with the guest solution. The titration curves obtained for the peaks at 385 and 366 nm (plots A and B) were quite nicely fitted with a simple 1:1 binding model, but for both curves the residuals show a regular, sinusoidal distribution, which as we have noted is already a strong indication of a model mismatch. The data obtained for 310−340 nm turned out to be much more informative. The shapes of curves at 330 and 310 nm (C,D in Figure 6) cannot be reproduced in the simple model and fitting of this data failed. When we applied a composite binding model ($HG + HG_2$), a perfect match between the experimental and fitted values was obtained for all wavelengths. The residuals for curves A and B became lower and took on a stochastic distribution. Curves C and D with nontrivial shape are properly reproduced, again with very low and irregularly distributed residuals. Unlike the Job plot, these results provide a strong indication of the formation of HG_2 complex.

For further verification, we performed another Job plot, this time under ^{1}H NMR control, and of course with a much higher concentration $c = 5 \times 10^{-3}$ M. As the graph in Figure 7 shows,

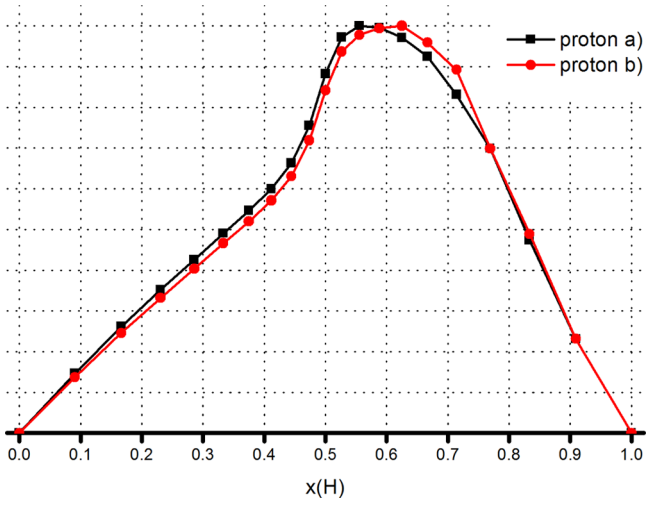

Figure 7. ^{1}H NMR Job plot for host **6** with D-PheOTBA plotted for protons (a) and (b) (labeled in Figure 4).

this curve might also misguide the researcher, as its maximum is close to x = 0.5. The asymmetrical shape of the obtained curve matches case B presented in Table 3, providing additional confirmation of the composite stoichiometry of complexes of **6** and D-PheOTBA. Finally, we performed an NMR titration, which strongly validates the assumed model. The titration curves plotted for two protons (Figure 8) exhibit a nontrivial shape which cannot be fitted with a simple model. This shape is in accordance with case D presented in Table 5.

Although the formation of HG_2 complex was difficult to predict in the case of receptor **6** and the mechanism by which two guest molecules are bound still remains unclear, the collected data nevertheless point in a consistent and indisputable way to the formation of two complexes. However, this means that the

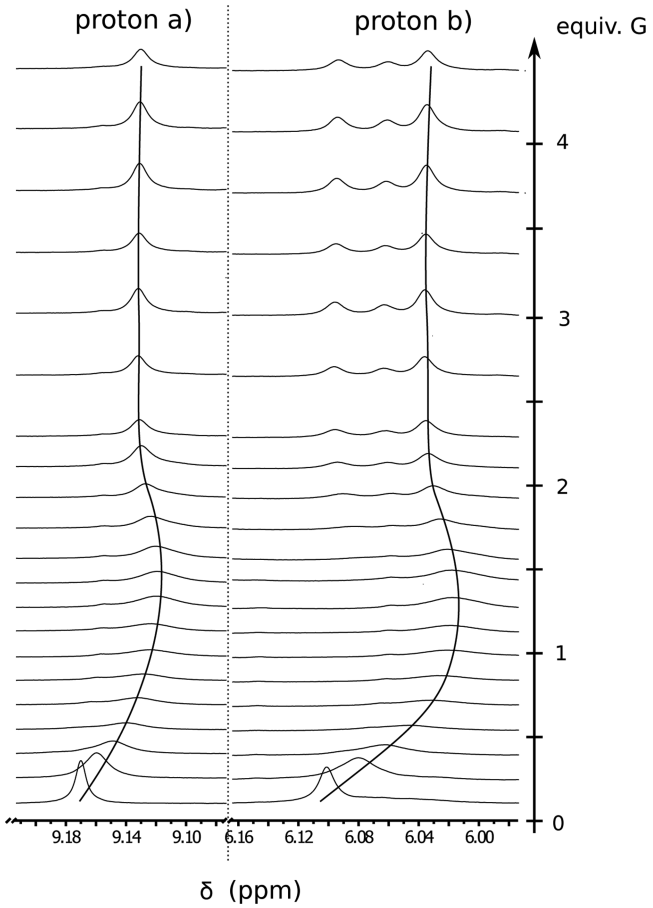

Figure 8. Titration course of **6** with D-PheOTBA indicating changes in chemical shifts of signals of protons (a) and (b) (labeled in Figure 4).

association constants reported in the original paper, which were determined using an incorrect model, have no physical meaning. Thus, all enantioselectivity values ($\alpha = K_S/K_R$) reported in that paper are also not valid.

3. CONCLUSIONS

By considering some theoretical simulations and case studies, we have shown herein that determining the stoichiometry of particular supramolecular complexes is no trivial task and that it must be reliably performed prior to any other analyses. We have demonstrated that even if supramolecular complexes of nontrivial stoichiometry have low abundances, taking them into account is important for proper determination of association constants. Although the Job plot method is successful in inorganic chemistry studies, it turns out to be misleading in some typical cases encountered in supramolecular chemistry. Indeed, as our analysis of several case studies has illustrated, a Job plot may often be misleading about the actual stoichiometry. As an alternative to the continuous variation method, therefore, we suggest analysis of the distribution of residuals. This method is much more sensitive to the occurrence of supermolecules with composite stoichiometry, and is quite simply a natural accompaniment to any titration experiment, a procedure which would be run anyway. All common titration data analysis software packages plot the residuals automatically. We have shown herein that in cases were the Job plot method failed to indicate the actual stoichiometry, a residuals distribution analysis successfully guided us to the proper binding model, paving the way

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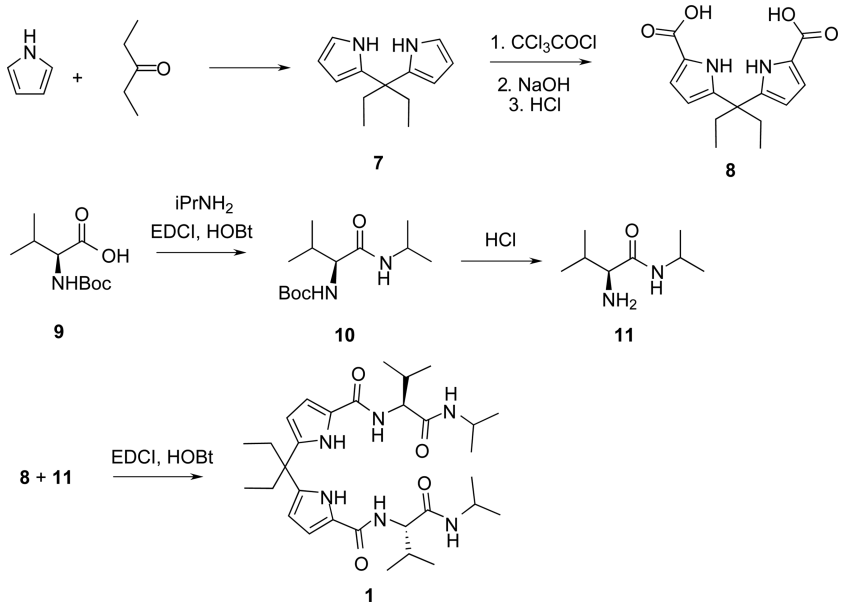
Scheme 2. Synthesis of Receptor 1



for accurate determination of the association constants. We therefore propose that Job plots should no longer be treated as a golden standard in the analysis of supramolecular systems, and that residual distributions should instead be analyzed to confirm the validity of an assumed model.

4. EXPERIMENTAL SECTION

Synthesis of receptor **1** is outlined is Scheme 2. Dipyrromethane **7** was obtained according to a reported procedure.[25]

Diacid 8. In a three-neck, round-bottom flask were placed under argon diethyldipyrromethane **7** (5.05 g, 25 mmol) and dry THF (100 mL). The solution was warmed to 40 °C, and a solution of tricholoacetic acid chloride (8.7 mL, 75 mmol) in dry THF (30 mL) was added dropwise via an addition funnel over 30 min at 40 °C. After the addition was complete, the solution was stirred at 40 °C for another 4 h. Next, a 10% solution of NaOH (80 mL, 0.2 mol) was added, and the two-phase mixture was intensively stirred at 40 °C for 2 h. After the mixture was cooled to rt, water was added (100 mL), the phases were separated, and the water phase was washed with dichloromethane (2 × 50 mL). The water phase was concentrated to about 2/3 of the initial volume to remove the remaining THF. The water phase was then acidified with concentrated HCl to pH = 3. The brown precipitate was filtered off, washed with water, and dried under high vacuum. Brown powder. Yield 4.96 g (68%, two steps). Mp: 180 °C dec. ^{1}H NMR (200 MHz, acetone-d_6) δ: 9.78 (2H, bs); 7.35 (2H, dd, J = 2.4; 3.8 Hz); 6.27 (2H, dd, J = 2.4; 3.8 Hz); 2.10 (4H, q, J = 7.2 Hz). 0.78 (6H, t, J = 7.2 Hz). ^{13}C NMR (50 MHz, acetone-d_6) δ: 172.9; 145.7; 122.47; 122.09; 110.9; 45.2; 30.2; 8.5. Anal. Calcd for $C_{15}H_{18}N_2O_4$: C, 62.06; H, 6.25; N, 9.65. Found: C, 62.07; H, 6.22; N, 9.56.

BocValNHiPr (10). Boc-protected valine (**9**, 1.085 g, 5 mmol), isopropylamine (0,613 mL, 7.5 mmol), and dry dichloromethane (50 mL) were placed in a round-bottom flask, and the solution was cooled to 0 °C. EDCI (1.16 g, 6 mmol), HOBt (0.92 g, 6 mmol), and DIPEA (2.05 mL, 12 mmol) were added. The cooling bath was removed, and the solution was allowed to reach room temperature and stirred overnight. The solution was concentrated to about 1/4 of its volume, ethyl acetate was added (50 mL), and the organic phase was washed with 10% $NaHCO_3$ (2 × 50 mL), 10% $NaHSO_4$ (2 × 50 mL), and brine (20 mL). The organic phase was dried over anhydrous Na_2SO_4 and concentrated in vacuo. The amide was used without further purification. Colorless crystals. Yield: 572 mg (65%). Mp: 169−170 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ: 5.83 (1H, bs); 5.11 (1H, bs); 4.05 (1H, oct, J = 6.7 Hz); 3.78 (1H, dd, J = 6.5, 8.5 Hz); 2.01−2.12 (1H, m); 1.43 (9H, s); 1.142 (3H, d, J = 6.6 Hz); 1.138 (3H, d, J = 6.8); 0.93 (3H, d, J = 6.5 Hz); 0.90 (3H, d, J = 6.5 Hz). ^{13}C NMR (50 MHz,$CDCl_3$) δ: 170.7, 155.9, 79.50, 60.0, 41.2, 30.9, 28.2, 22.63, 22.46, 19.2, 18.0. Anal. Calcd for $C_{13}H_{26}N_2O_3$: C, 60.44; H, 10.14; N, 10.84. Found: C, 60.41; H,10.17; N, 10.86. [α]: −10.5 (c 1.0, CH_2Cl_2).

Receptor 1. N-Boc-protected amino acid S4 (1.03 g, 4 mmol) was dissolved in HCl in dioxane (4 M, 5 mL) and stirred at rt for 2 h. During the reaction time, some of the product precipitated. The mixture was concentrated on a rotary evaporator, and the obtained salt was used in the next step. The deprotected amino acid was dissolved in dry dichloromethane (50 mL), and DIPEA (2 mL) and diacid **8** (387 mg, 1.33 mmol) were added. The solution was cooled to 0 °C, and EDCI (580 mg, 3 mmol) and HOBt (459 mg, 3 mmol) were added. The reaction mixture was allowed to reach rt and was stirred overnight. Next, ethyl acetate (100 mL) was added, and the organic phase was washed with 10% $NaHCO_3$ (2 × 50 mL), 10% $NaHSO_4$ (2 × 50 mL), and brine (20 mL). The organic phase was dried over anhydrous Na_2SO_4 and concentrated in vacuo. The product was purified by flash chromatography on silica gel (20% acetone in dichloromethane). Yield: 318 mg (42%). Colorless powder. Mp: 172−175 °C. ^{1}H NMR (600 MHz, DMSO-d_6) δ: 10.86 (2H, s); 7.90 (2H,d, J = 7.5 Hz); 7.74 (2H, d, J = 9.0 Hz); 6.75 (2H, dd, J = 2.4; 3.6 Hz); 5.89 (2H, dd, J = 2.7; 3.6 Hz); 4.21 (2H, t. J = 8.4 Hz); 3.83 (2H, oct, J = 6.8 Hz.); 2.03−2.27 (4H, m); 1.94−2.00 (2H, m); 1.04 (6H, d, J = 6.8 Hz); 1.03 (6H, d, J = 6.8 Hz); 0.84−0.88 (12H, m); 0.65 (6H, t. J = 7.4 Hz). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 170.2; 160.0; 140.7; 128.2; 127.2; 125.36; 111.3; 106.6; 57.8; 42.8; 40.2; 30.6; 26.8; 22.4; 22.2; 19.2; 18.9; 8.2. HRMS: calcd for $[C_{31}H_{50}N_6O_4{\cdot}Na]^+$ 593.3786, found 593.3806. [α]: +66.6 (c = 1, acetone).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.joc.5b02909.

NMR spectra, raw titration data, and information about simulations (PDF)

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Notes

The authors declare no competing financial interest.

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(7) In all cases in this paper the concentrations are given in mol·dm^{-3}, and association constants in dm^3·mol^{-1}.
(8) Slow exchange equilibria (on the NMR time scale) are rare in supramolecular chemistry and will not be discussed here. The principles have been described by Hirose.[6]
(9) $[G]_0$ and $[H]_0$ correspond to total concentration of guest and host, respectively, in all forms.
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