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Accurate Method To Quantify Binding in Supramolecular Chemistry

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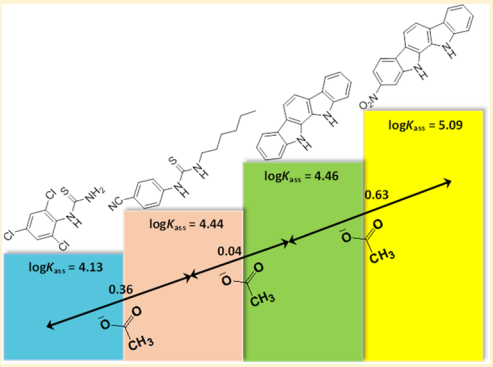
Supporting Information

ABSTRACT: An approach for accurate and comparable measurement of host–guest binding affinities is introduced whereby differences in binding strength ($\Delta\log K_{ass}$ values) are measured between two host molecules toward a particular guest under identical solvent conditions. Measuring differences instead of absolute values enables obtaining highly accurate results, because many of the uncertainty sources (the solvation/association state of the guest in solution, deviations in solvent composition, etc.) cancel out. As a proof of concept, this method was applied to the measurement of the binding strength of 28 synthetic anion receptors toward acetate in acetonitrile containing 0.5% water. The receptors included differently substituted indolocarbazoles, ureas, thioureas, and some others. Possible deprotonation of more acidic receptors of each compound class by acetate was checked by measuring their acidities (ΔpK_a values) relative to acetic acid in the same solvent. A self-consistent (consistency standard deviation 0.04 log units) binding affinity scale ranging for around 2.7 log units was constructed from the results. Absolute $\log K_{ass}$ values were found by anchoring the scale to the absolute $\log K_{ass}$ values of two receptor molecules, determined independently by direct measurements. This new approach is expected to find use in accurate quantification of a wide range of binding processes relevant to supramolecular chemistry.



INTRODUCTION

Noncovalent binding is the core concept in supramolecular chemistry and is the means by which supramolecular architectures, supermolecules, are formed.[1] The stability of a supermolecule is determined by the binding strength between the species involved (often termed the host and the guest) and is characterized by a binding (or association or stability) constant (K_{ass}, eq 1) of the supermolecule.[2] Binding constants are key characteristics of supermolecules, and when determined for a series of molecules they can reveal important trends and be useful for predicting the properties of new molecular assemblies. Differences in binding strength (ratios of K_{ass}), when binding different guests by the same host, characterize the selectivity of the interaction, which is very important in molecular recognition studies. Without question, accurate and reliable determination of binding constants is vital for supramolecular studies.

Binding of a guest G to a host H in 1:1 ratio with formation of the complex HG can be described by equilibrium eq 1. The equilibrium constant K_{ass} (or its logarithm $\log K_{ass}$) expresses the affinity of the host H toward the guest G. K_{ass} is expressed in eq 2, where a_{HG}, a_H, and a_G are the activities of the species in the solution.

$$H + G \overset{K_{ass}}{\rightleftarrows} HG \qquad (1)$$

$$K_{ass} = \frac{a_{HG}}{a_H a_G} \qquad (2)$$

Up until now binding constants have been generally measured directly according to eq 2 (or analogous equations corresponding to different stoichiometries). The reliability of the measurements is mostly evaluated by repeatability (standard deviation of measured values obtained on the same day or within the same series).[2] This leaves possible systematic effects (often highly influential sources of measurement uncertainty in equilibrium measurements[3]) out of consideration. Systematic effects introduce bias, by shifting all of the results in a series in the same direction, while at the same time the agreement between the individual results can be good. For example, in the case of anion binding by synthetic receptors such effects may be caused, e.g., by ion-pairing[4] and homoconjugation (association of acid and its anion).[5] Both of these can significantly decrease the activity of the free anion leading to biased results. As another example, even low levels of water in organic solvents (often used as media) will decrease the effective activity of both hydrogen bond donors and acceptors (by selective solvation) and consequently also the strength of hydrogen bonds, which are usually the main interactions involved in binding.

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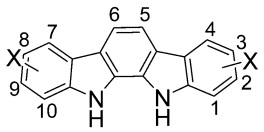
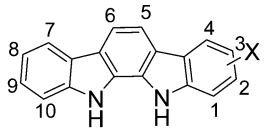
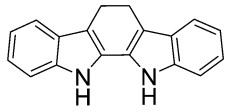
Scheme 1. Structures of the Molecular Receptors



1 X = H, H
6 X = 2,9-$(OCH_3)_2$
7 X = 2,7-$(OCH_3)_2$
8 X = 4,7-$(OCH_3)_2$
11 X = 1,10-$(CF_3)_2$
12 X = 1,10-Cl_2
14 X = 2,9-Cl_2
15 X = 2,7-Cl_2
16 X = 4,7-Cl_2



4 X = 2-NO_2
5 X = 4-NO_2
9 X = 2-OCH_3
10 X = 4-OCH_3
13 X = 1-Cl



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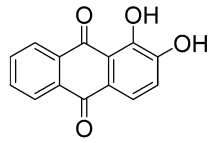

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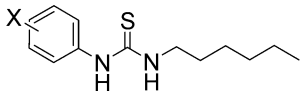
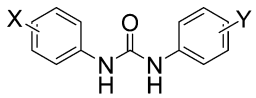
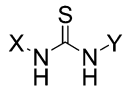


17 X = 4-NO_2
18 X = 4-CN
19 X = 4 -$COOCH_3$
20 X = 4-I



21 X = H Y = H
22 X = 4-NO_2 Y = 4-NO_2
23 X = 3,4-Cl_2 Y = 4-Cl



25 X = 4-CF_3-C_6H_4 Y = 4-CF_3-C_6H_4
26 X = 4-NO_2-C_6H_4 Y = H
27 X = 4-CF_3-C_6H_4 Y = H
28 X = 2,4,6 -Cl_3-C_6H_2 Y = H

Table 1. Self-Consistent Scale of Acetate Binding[a]

No	Receptor molecule	logK_{ass}	u_c[b]	u_c[c]	ΔlogK_{ass}
22	1,3-bis(4-nitrophenyl)urea	6.04	0.04	0.05	
5	4-NO_2-indolocarbazole	5.24	0.04	0.05	
23	3,4,4′-Cl_3-diphenylurea	5.20	0.02	0.04	
16	4,7-Cl_2-indolocarbazole	5.20	0.03	0.04	
4	2-NO_2-indolocarbazole	5.09	0.00	0.03	
15	2,7-Cl_2-indolocarbazole	5.05	0.01	0.04	
14	2,9-Cl_2-indolocarbazole	4.95	0.02	0.04	
17	1-(4-nitrophenyl)-3-hexylthiourea	4.70	0.02	0.04	
26	1-(4-nitrophenyl)-2-thiourea	4.69	0.02	0.04	
9	2-MeO-indolocarbazole	4.50	0.02	0.04	
8	4,7-$(MeO)_2$-indolocarbazole	4.48	0.01	0.03	
1	**Indolocarbazole**	4.46	0.01	0.03	
7	2,7-$(MeO)_2$-indolocarbazole	4.46	0.02	0.04	
18	1-(4-CN-phenyl)-3-hexylthiourea	4.44	0.02	0.04	
2	5,6-dihydroindolocarbazole	4.36	0.00	0.03	
21	1,3-diphenylurea	4.28	0.01	0.03	
13	1-Cl-indolocarbazole	4.24	0.01	0.03	
27	(4-CF_3-phenyl)thiourea	4.23	0.01	0.03	
28	N-(2,4,6-trichlorophenyl)thiourea	4.13	0.02	0.04	
19	1-(4-$MeOOCC_6H_4$)-3-hexylthiourea	4.09	0.01	0.03	
20	1-(4-I-phenyl)-3-hexylthiourea	3.87	0.01	0.03	
12	**1,10-Cl_2-indolocarbazole**	3.84	0.01	0.03	
11	1,10-$(CF_3)_2$-indolocarbazole	3.36	0.05	0.06	

[a]Solvent: acetonitrile with 0.5% water (m/m). In all cases 1:1 stoichiometry. Absolute logK_{ass} values are found by anchoring the scale to the logK_{ass} of compounds indicated in bold. [b]Standard uncertainties for comparing logK_{ass} values on the scale. [c]Standard uncertainties for comparing logK_{ass} values with those from other research groups.

Thus, the determination of exact activities of the species can be difficult and can introduce a large measurement uncertainty. In order to avoid these problems we propose an alternative method, which is based on measuring the *relative* binding affinity of two hosts H_1 and H_2 toward the same guest as described by eq 3, whereby all the species are dissolved in the same solvent. The relative binding affinity is expressed by $\Delta\log K_{ass}$ defined in eq 4.

$$H_1 + H_2G \overset{\Delta\log K_{ass}}{\rightleftharpoons} H_1G + H_2 \qquad (3)$$

$$\Delta\log K_{ass} = \log K_{ass}(H_1G) - \log K_{ass}(H_2G) = \log \frac{a_{H_1G}a_{H_2}}{a_{H_2G}a_{H_1}} \qquad (4)$$

From eqs 3 and 4 it can be seen that the need for determining the activity of the guest is eliminated. This means that the possible side processes involving the guest such as ion-pairing and homoconjugation, which influence binding to both hosts simultaneously, cancel out and thus do not affect the

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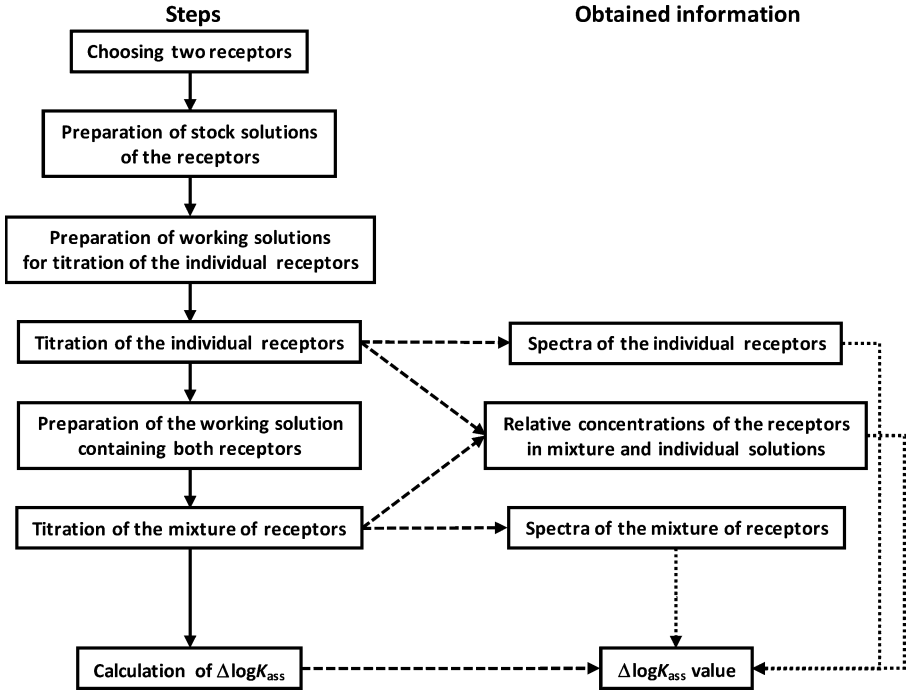

Figure 1. Flowchart of $\Delta\log K_{ass}$ value measurement.

measurement result. The activities of the free and bound hosts enter eq 4 as ratios. Thus, possible effects affecting the hosts also largely cancel, e.g., the composition of the solvent is automatically identical for both hosts. A reasonable assumption to make is that the ratios of activity coefficients $\gamma(H_x)/\gamma(H_xG)$ are similar for both host molecules.[3,5] Consequently the activities in eq 4 can be replaced with equilibrium concentrations:

$$\Delta\log K_{ass} = \log K_{ass}(H_1G) - \log K_{ass}(H_2G) = \log \frac{[H_1G][H_2]}{[H_2G][H_1]} \quad (5)$$

Because many sources of error cancel with relative binding affinity measurements, it is possible to obtain highly accurate results. The proposed method is by its nature analogous to relative acidity and basicity measurement methods, which have been used for determination of pK_a values in nonaqueous media,[3,5] and to competition experiments.[6]

As a proof of concept the proposed method was applied to the measurement of binding constants between chelating anion-binding receptors (thioureas, ureas, indolocarbazoles, and anthraquinones; see Scheme 1) as hydrogen bond donors and acetate anions as hydrogen bond acceptors. Molecular recognition of anions by synthetic receptors has become a topic of much interest.[7,8] In biological systems there are macromolecular receptors, which are highly selective toward specific ions.[9] Designing artificial receptors that have both high binding affinity toward a specific anion of interest and low binding affinity toward other anions has proven to be quite a challenge.[8,10,11] However, emerging applications for anion receptor systems in extraction[12] and transport[13] continues to drive the exploration of new selective anion receptor systems forward.[14−22]

If multiple measurements with different hosts (or guests) are carried out, then this approach enables the construction of scales of guest binding to numerous hosts (or a host binding numerous guests). Such scales can be anchored to known $\log K_{ass}$ values, and as a result the absolute $\log K_{ass}$ values for all host−guest complexes in the scale can be calculated.

Such scales would be excellent tools for the accurate comparison of binding efficiencies within compound series under the same experimental conditions. Currently, because of the above-mentioned bias effects, it is often almost impossible to reliably compare the binding constant data from different groups, even if formally obtained in a comparable way (the same species and solvent).

RESULTS

Relative Binding Measurements. The newly proposed method was tested on 28 different receptor molecules. The binding constants of 23 compounds, which showed measurable binding affinity and did not deprotonate, were successfully measured with acetate. The resulting binding affinity scale (ladder) ranging for ca. 2.7 orders of magnitude is presented in Table 1. Each arrow in the ladder represents difference between the absolute binding strengths of two receptor molecules in logarithmic scale expressed as the $\Delta\log K_{ass}$ value. Figure 1 presents a schematic of the determination of the $\Delta\log K_{ass}$ value (see Experimental Section for details).

All compounds are linked to the scale by at least two (mostly three or more) relative binding measurements against different partners. Each additional measurement contributes to circular validation[23] of the whole scale.

The absolute $\log K_{ass}$ values of the compounds on the scale were found by minimizing the sum of squares of the differences between the directly measured $\Delta\log K_{ass}$ values and the assigned $\log K_{ass}$ values, which is denoted as SS in the following equation:[5]

$$SS = \sum_{i=1}^{n_m} \{\Delta\log K^i_{ass} - [\log K_{ass}(R_yHA^-) - \log K_{ass}(R_xHA^-)]\}^2 \quad (6)$$

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Table 2. Results of Absolute logK_{ass} Value Measurements

receptor molecule	abs logK_{ass} [a]	s [b]	n [b]	CI (95%) [b]	abs logK_{ass} from scale [c]	difference [c]
3,4,4′-trichlorodiphenylurea [d]	5.22	0.02	3	0.04	5.20	−0.021
indolocarbazole	4.49	0.06	9	0.04	4.46	−0.024
1,10-dichloroindolocarbazole	3.82	0.05	9	0.04	3.84	0.024

[a]logK_{ass} values were obtained as averages of the independent measurement runs. [b]Standard deviations, numbers of measurement runs, and confidence intervals of the mean values at 95% probability, [c]logK_{ass} values obtained for the same receptor molecules using the scale method through least-squares procedure and the differences between the logK_{ass} values obtained directly and using the scale-method. [d]Included for comparison only, not used for assigning the absolute values (see text).

Every $\Delta\log K^{i}_{ass}$ value is the directly measured relative acetate binding strength of the receptors R_yH and R_xH. The absolute logK_{ass} values for all the compounds in the ladder were calculated by the least-squares procedure with the logK_{ass} values of indolocarbazole (**1**) and 1,10-dichloroindolocarbazole (**12**) (Table 2) taken as the anchor points of the scale. The consistency of the results, the goodness of match between the assigned absolute logK_{ass} values and the measured $\Delta\log K_{ass}$ values, can be assessed by the consistency standard deviation s of the scale,[3] which is found according to the following equation:

$$s = \sqrt{\frac{SS}{n_m - n_c}} \quad (7)$$

where $n_m = 46$ is the number of $\Delta\log K_{ass}$ measurements and $n_c = 23$ is the number of absolute logK_{ass} values that were determined. For the current data $s = 0.04$ log units.

For each compound on the scale standard uncertainties of the logK_{ass} value were estimated using two approaches (see Supporting Information for details). The uncertainties obtained using the first approach describe the accuracy of the logK_{ass} values as values on the scale and are the appropriate uncertainty estimates to use when comparing the logK_{ass} values of different compounds on the scale. These uncertainty estimates do not take into account uncertainty due to anchoring of the scale. The uncertainties obtained via the second approach estimate how accurately it is possible to obtain the absolute binding constant values as thermodynamic equilibrium constant values in the used solvent. These uncertainties are appropriate to use when comparing the absolute logK_{ass} values from this work with those from other research groups.

The following compounds showed no measurable binding toward acetate in 0.5% H_2O/AN (m/m): 1,2-diaminoanthraquinone, nitrate ionophore V, 1,2-diamino-4-nitrobenzene.

Absolute Binding Measurements. The absolute logK_{ass} values were found for three receptor molecules: 3,4,4′-trichlorodiphenylurea (**23**), indolocarbazole (**1**), and 1,10-dichloroindolocarbazole (**12**). These compounds were selected for anchoring the scale as they are in the top, middle, and bottom of the scale. Such anchoring enabled us to check the newly developed method and reveal possible artificial expansion or contraction of the scale. For each of these compounds the absolute logK_{ass} value was measured on at least three different days. Several independent data sets were obtained on each day, and for each of the data sets three independent calculation procedures were applied (see the Experimental Section). Absolute logK_{ass} value for 3,4,4′-trichlorodiphenylurea (**23**) could be calculated only from the data obtained immediately after preparation of the stock solutions. This was due to the slow decrease of its concentration in the stock solutions, possibly caused by its adsorption on the walls of the vial or aggregation resulting in precipitation. No such problems were found at the low concentrations used for measurements with the two indolocarbazoles. The results of the measurements are presented in Table 2.

In order to obtain absolute binding constant values for all of the studied receptors, the scale of acetate binding was anchored to these independently measured logK_{ass} values. Two of the three values were used. Receptor **23** (3,4,4′-trichlorodiphenylurea) was not used for assigning the absolute values because its behavior was not fully understood. Anchoring was done by minimizing the sum of squares of the differences between directly measured absolute logK_{ass} values and values obtained from the scale. The span of the scale was not altered by anchoring. This allowed independent testing of the span of the scale for possible expansion or contraction[24] by comparing the differences of logK_{ass} values of the selected anchor compounds obtained from the scale and from direct measurements. Differences are presented in Table 2 and show good agreement between direct measurement and relative measurement, which offers evidence for absence of artificial expansion or contraction of the scale. Also, the small discrepancy between the directly measured absolute value and the one obtained from the scale for 3,4,4′-trichlorodiphenylurea (**23**) indirectly validates the absolute measurement of this compound.

DISCUSSION

Addition of electron-withdrawing groups generally increases the binding strength of the receptor toward anions. This was also observed in the current study. As expected the chloro- and nitro-compounds display binding strength higher than that of the respective compounds having electron-donating groups such as methoxy. Studies of differently substituted indolocarbazoles show that changing the position of the substituent groups does not change the binding affinity significantly. For example, changing the positions of two chloro- substituents varies the absolute logK_{ass} value within ca. 0.3 units, unless positions 1 and 10 are involved. Comparison between electron-donating (methoxy-) and electron-withdrawing groups (chloro-, nitro-) shows that two chloro- groups or one nitro- group increase the binding strength of the unsubstituted indolocarbazole by around 0.5 logK_{ass} units. The results also demonstrate that methoxy-substituted indolocarbazoles have higher binding strengths than nonsubstituted indolocarbazole. The methoxy group is electron-donating by the resonance mechanism, which decreases the positive polarization of the hydrogen atom of the N−H group and decreases its HBD ability. However, it is also an electron-withdrawing group by the field-inductive mechanism increasing the HBD ability. The combination of these effects leads to an almost complete canceling out, and the measured differences in binding strength between the methoxy substituted compounds and the unsubstituted indolocarbazole are very small.

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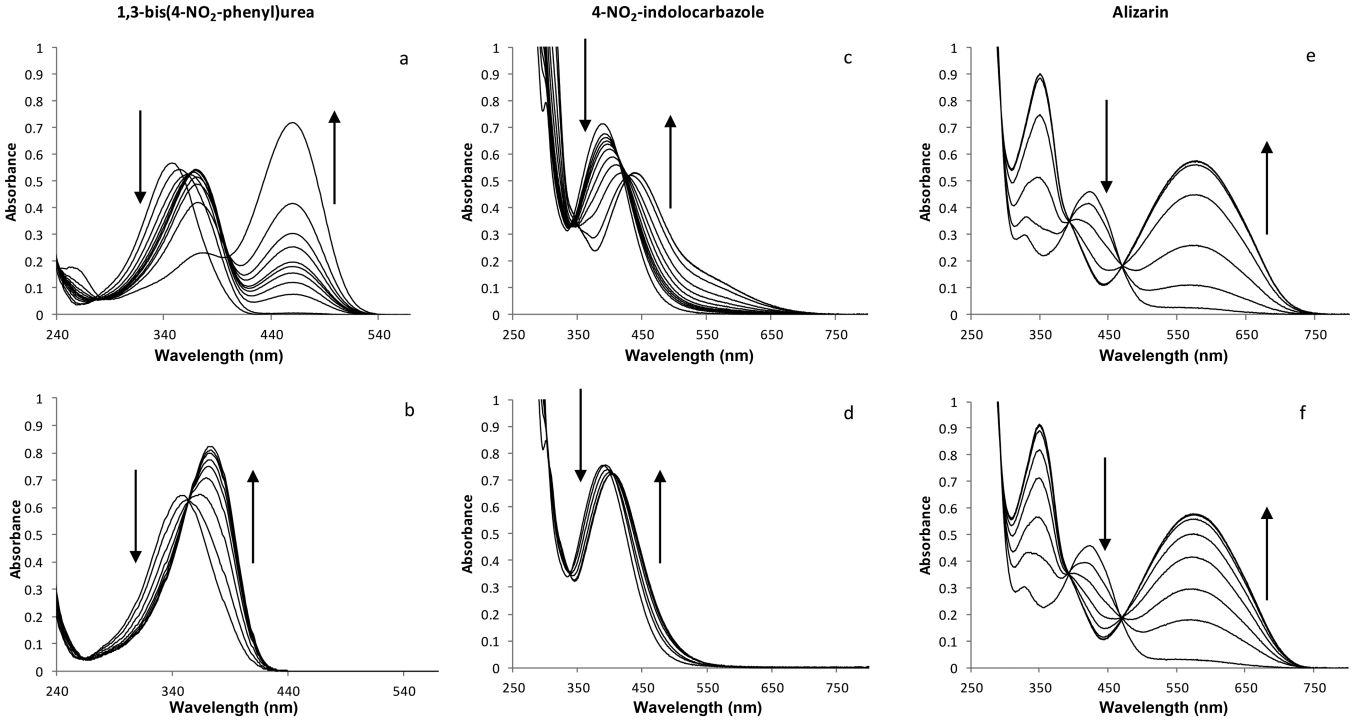
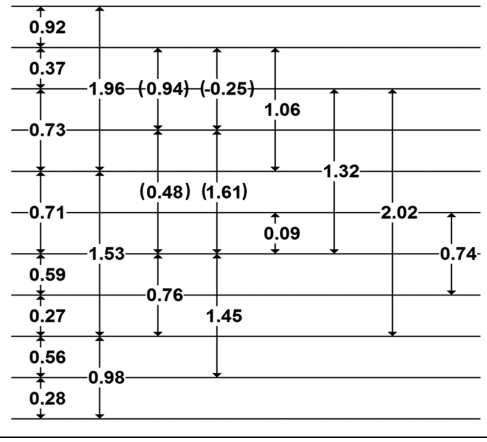


Figure 2. Absorbance spectra collected from titration of 1,3-bis(4-NO_2-phenyl)urea, 4-NO_2-indolocarbazole, and alizarin with OH^- and AcO^- . Upper spectra were recorded using OH^-, and lower ones with AcO^-.

Table 3. Differences of Acidities of the Most Acidic Receptor Molecules and Acetic Acid[a]

Acid	pK_a	ΔpK_a
4-NO_2-Indolocarbazole	**3.16**	
2,3,4,5,6-$(CF_3)_5$-aniline	2.22	
1-(4-nitrophenyl)-3-hexylthiourea	**1.87**	
1,3-bis(4-nitrophenyl)urea	**1.1 .. 2.5**	
9-COOMe-fluorene	1.22	
$(C_6F_5)CH(COOEt)_2$	0.71	
$(4\text{-}CH_3\text{-}C_6F_4)_2CHCN$	0.58	
Acetic Acid	**0.00**	
$(4\text{-}CH_3\text{-}C_6F_4)(C_6F_5)CHCN$	-0.23	
1,3-bis(4-CF_3-phenyl)thiourea	**-0.85**	
Alizarin	**-1.17**	



[a]Solvent: acetonitrile with 0.5% water (m/m). Acidity increases downward. The pK_a values are given relative to acetic acid. The acidity of 1,3-bis(4-NO_2-phenyl)urea is uncertain; see the text for details. The values in parentheses are based on different assumptions and are not consistent with the remaining measurements.

The measurement results confirm that when bulky groups such as chloro- or trifluoromethyl- are located next to the binding sites as in the case of 1-substituted **13** or 1,10-disubstituted indolocarbazoles **11** and **12**, then the anion complexation is hindered. This hindrance effect is further enhanced by the negative partial charge of the substituents leading to charge−charge repulsion. In the case of acetate binding the introduction of a chloro- substituent in the 1-position **13** decreases the binding strength toward acetate by 0.2 logK_{ass} units. Addition of the second chloro substituent in the 10-position **12** decreases binding strength by further 0.4 logK_{ass} units. Steric hindrance in the case of 1,10-bis-(trifluoromethyl)indolocarbazole (**11**) is even more pronounced, making this compound the weakest acetate binder.

Table 1 reveals high consistency of the results and serves as a demonstration of the usefulness of this approach. On the basis of the consistency of the results, we estimate that the method allows differentiation between compounds with logK_{ass} values differing by 0.04−0.08 logK_{ass} units. This is significantly lower than is possible to differentiate in the case of absolute measurements, especially if made in different laboratories.

Investigating Possible Deprotonation. As outlined above the high acidity of the hydrogen bond donor sites of the receptor molecule can present a problem as Brønsted acid−

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base reaction (deprotonation of the receptor molecule by the anion) may occur according to eqs 8 and 9[25] instead of formation of the hydrogen-bonded complex according to eq 1.

$$RHA^- \rightleftarrows R^- + HA \quad (8)$$

$$RHA^- + A^- \rightleftarrows R^- + AHA^- \quad (9)$$

Deprotonation is a completely different process and is not welcome because any geometry-based selectivity is lost (any sufficiently basic anion can deprotonate the receptor). The probability of deprotonation increases at the end of the titration experiment where the anion concentration is high and homoconjugation of the anion with its protonated form according to eq 9 can significantly facilitate the deprotonation.

In order to be confident that the right process is studied, the most acidic receptor molecules from each substance class under study were tested using two different approaches.

First, the compounds were titrated with tetrabutylammonium hydroxide in the same solvent that was used for binding measurements, and the recorded UV−vis spectra were compared with the spectra obtained during the titration with acetate (Figure 2). Hydroxide is both an anion and a strong base, which means that with receptors of lower acidity hydroxide at first forms a hydrogen-bonded complex and by further addition of the anion deprotonation occurs. If the receptor has high acidity then deprotonation takes place immediately. Such comparison of spectra conveniently gives information whether deprotonation occurs.

Second, the relative acidities with respect to acetic acid (ΔpK_a values)[5] of the most acidic receptor molecules in the solvent used for binding studies were determined (Table 3). In order to act as a receptor molecule toward acetate, its pK_a value in the same medium should be higher than that of acetic acid by at least 1 unit. This tentative criterion is based on our experience that in such cases the formation of the anion is not detectable in the UV−vis spectrum. This means that there most probably is some small proportion of the receptor-anion complex where intracomplex proton transfer has occurred but the complex is still intact.

Figure 2a shows that during the titration of 1,3-bis(4-NO_2-phenyl)urea (**22**) with hydroxide the formation of the hydrogen-bonded complex and the deprotonation process overlap, although during the first stage of titration deprotonation occurs only to a small extent. Characteristic changes in the absorbance can be observed in the maxima at 342 and 366 nm, which correspond to the binding of the anion. The rise of an intense maximum at 455 nm is related to the deprotonation of the compound. Titration with acetate (Figure 2b) does not involve formation of the maximum at 455 nm, which suggests that 1,3-bis(4-NO_2-phenyl)urea (**22**) does not undergo deprotonation with acetate even when adding a large excess of the anion. These spectra agree well with those from previous studies,[22,26] where deprotonation of ureas was studied thoroughly using UV−vis spectrophotometric and NMR methods. Because 1,3-bis(4-NO_2-phenyl)urea (**22**) has the highest acidity of the ureas studied in this work (also demonstrated by the determined pK_a values), it is reasonable to assume that less acidic ureas also do not deprotonate upon addition of acetate.

The most acidic indolocarbazole studied was 4-NO_2-indolocarbazole (**5**), and the spectra obtained through titration with hydroxide are presented in Figure 2c and with acetate in Figure 2d. Again, the spectral changes on titration with OH^- are much more extensive than with AcO^-.

Initially a number of binding measurements were carried out with alizarin (**24**) as a receptor. However, the spectra obtained from titration with OH^- and AcO^- (Figure 2e and f) are identical, which suggests that alizarin (**24**) undergoes immediate deprotonation and is not usable as a receptor molecule for acetate. This is supported by the fact that alizarin (**24**) is by ∼1 pK_a unit a stronger acid in the used solvent than acetic acid (see below).

The relative acidity data in Table 3 reveal that the most acidic thiourea 1,3-bis(4-CF_3-phenyl)thiourea (**25**) is also a markedly stronger acid than acetic acid. However, 1-(4-nitrophenyl)-3-hexylthiourea (**17**) is by more than 1.5 pK_a units a weaker acid than acetic acid, meaning that the complex essentially remains intact. Out of the indolocarbazoles, 4-NO_2-indolocarbazole (**5**) is a weaker acid than acetic acid by more than 3 pK_a units. The relative acidity of 1,3-bis(4-NO_2-phenyl)urea (**22**) against acetic acid could not be reliably determined because of the overlap between the binding of the OH^- (see the Experimental Section) and deprotonation. Different assumptions and approximations led to ΔpK_a values for this compound ranging from 1.1 to 2.5 relative to acetic acid. The spectra in Figure 2 imply that the ΔpK_a is significantly above 1 because if the acidities were indeed different by around 1 pK_a unit, then in the spectra of the titration with acetate at high acetate ion concentrations at least a weak maximum at 455 nm corresponding to the anion of 1,3-bis(4-NO_2-phenyl)urea (**22**) should be visible.

Hydrogen Bond Donicity and Acidity. The acidity of a molecule refers to the difference in the stabilities of the neutral acid and the anion formed after deprotonation. Hydrogen bond donicity on the other hand refers to the extent of polarization of the relevant hydrogen atom in the molecule and its steric accessibility. Acidity and HB donicity are correlated but not strongly.[27] Thioureas are stronger acids than ureas[28] but show significantly lower binding affinity toward acetate due to lower hydrogen bond donicity. This is further demonstrated by the fact that although nitro groups should have larger effect on the acidity in the *para* position in the phenyl ring than trifluoromethyl groups, 1,3-bis(4-CF_3-phenyl)thiourea (**25**) is by at least 2 pK_a units a stronger acid than 1,3-bis(4-NO_2-phenyl)urea (**22**) in acetonitrile with 0.5% water (m/m) as shown in Table 3. The compounds most suitable for use as anion receptors should have strong positive partial charges on the HBD hydrogen atoms, while the stability of the anion formed on deprotonation (i.e., the acidity of the receptors) should be low.

■ CONCLUSIONS

The construction of a ladder of binding affinities allows for the possibility of mutual comparability of binding efficiencies of receptor molecules synthesized by different groups. This can be achieved by linking the receptor molecules of interest to the scale presented here under the same experimental conditions. The applicability of this approach is not limited to anion-receptor binding constants but can be used for any binding/association reactions. This approach is also not limited to UV−vis spectrophotometry as experimental technique: it can be used with any technique enabling simultaneous determination of concentration ratios of free and bound receptor for two receptors in the same solution, such as NMR or fluorescence.

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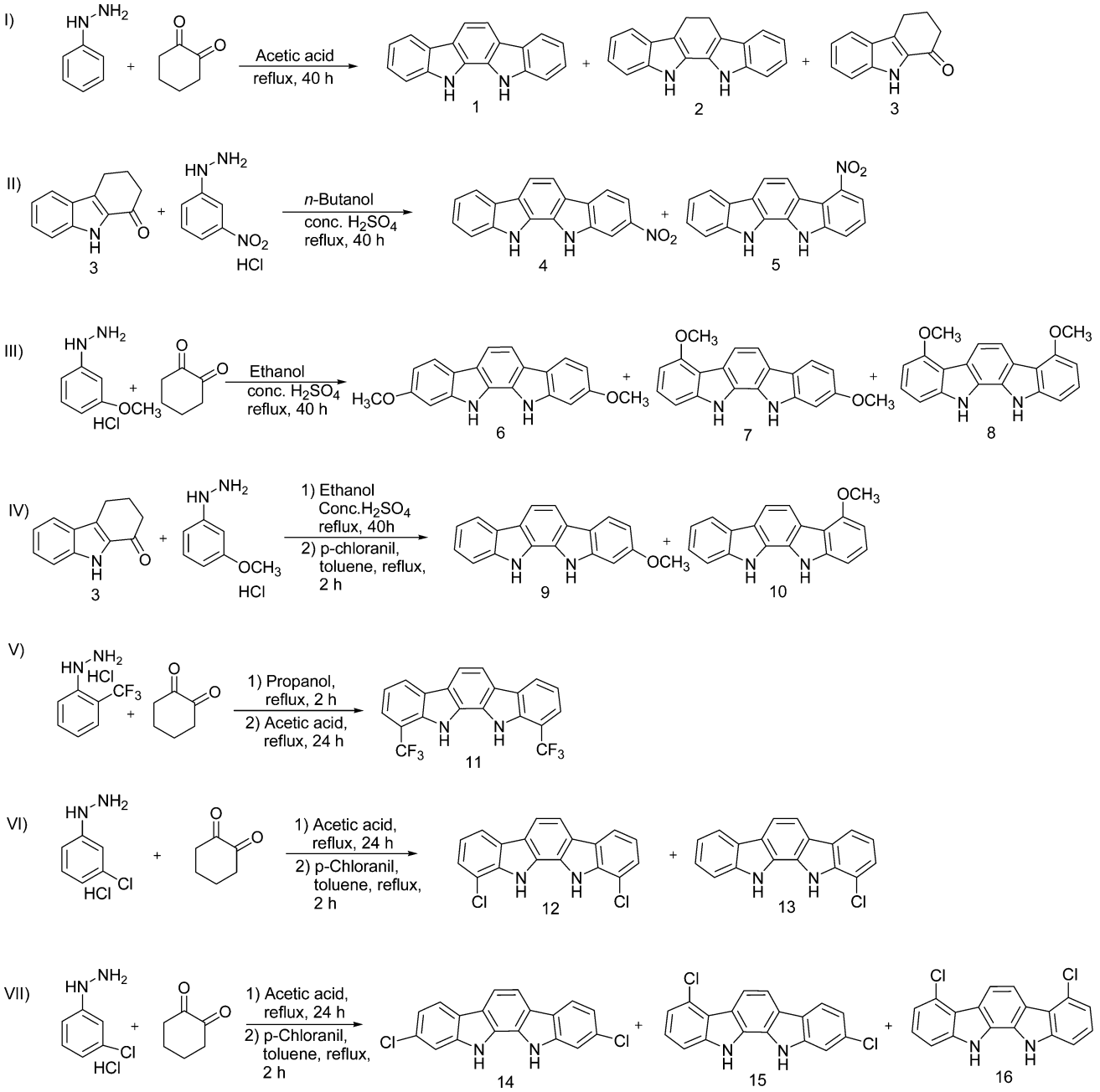
Scheme 2. Synthesis of Different Substituted Indolocarbazoles



EXPERIMENTAL SECTION

Instruments. UV−vis spectrophotometric measurements were carried out using a spectrophotometer. The water content of the solvent was checked with coulometric Karl Fischer titrator. NMR spectra of synthesized compounds were obtained on a 200 MHz NMR and on a 400 MHz NMR. High-resolution mass spectrometric analyses were done using a hybrid mass spectrometer. The ions from the ion source are first directed (for possible preselection, fragmentation) into a triple-quadrupole MS (which can also be used for low-resolution MS analysis) and then into the FT-ICR mass spectrometer for high-resolution mass spectral analysis. For ionization an APCI source was used with the following parameters: spray chamber temperature, 40 °C; nebulizing gas (N_2) pressure, 50 psi (1 psi = 6894 Pa) at 400 °C; auxiliary gas (N_2) pressure, 25 psi; API-drying gas (N_2), 10 psi at 100 °C; corona needle current, 2 mA; shield voltage, 600 V; and capillary voltage, 80 V. Solvents used for different indolocarbazoles were 80% ACN/20% H_2O (0.1% formic acid), MeOH, and 50% MeOH/50% DCM.

Solvents and Chemicals. The solvent for binding measurements, acetonitrile with 0.5% water (m/m), was prepared gravimetrically using acetonitrile with water content max 0.02% and water corresponding to the ASTM Type I (or better). Final water content of solvent was checked with Karl Fischer titration. Titrant solutions for binding measurements were prepared from different batches of tetrabutylammonium acetate (TBAA; 97% and 99%). For deprotonation studies the titrant was prepared from tetrabutylammonium hydroxide (0.1 M in 2-propanol/methanol (10:1 v/v)) in the same solvent.

Receptor Molecules. The following commercially available receptor molecules were used: 1,3-bis(4-NO_2-phenyl)urea (**22**), 3,4,4′-trichlorodiphenylurea (**23**), 1-(4-NO_2-phenyl)thiourea (**26**), 1,3-diphenylurea (**21**), 1-(4-CF_3-phenyl)thiourea (**27**), 1-(2,4,6-trichlorophenyl)thiourea (**28**), alizarin (**24**), 1,2-diaminoanthraquinone, 1,2-diamino-4-nitrobenzene, nitrate ionophore V. The following

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compounds were synthesized by Gale's group, and their synthesis has been described elsewhere: 1,3-bis(4-CF_3-phenyl)thiourea (**25**),[29] 1-(4-CN-phenyl)-3-hexylthiourea (**18**),[30] 1-(4-I-phenyl)-3-hexylthiourea (**20**),[30] 1-(4-NO_2-phenyl)-3-hexylthiourea (**17**),[30] and 1-(4-methoxycarbonylphenyl)-3-hexylthiourea (**19**).[30] All indolocarbazoles under study were synthesized in our laboratory.

Reference Acids for pK_a Studies. The reference acids used in pK_a measurements (Table 3) were the same as in refs 31 (2,3,4,5,6-$(CF_3)_5$-aniline) and 5 (all other reference acids).

Synthesis of Indolocarbazoles. Indolocarbazole derivatives have recently found application in a range of anion receptor systems.[32] In this work, the main focus was on synthesis and characterization of indolocarbazoles containing a range of functional groups such as -NO_2, -OCH_3, -CF_3, and -Cl attached to the indolocarbazole framework. The aim was 2-fold: to supply a set of molecules with gradually changing binding strength for building the continuous ladder, and on the other hand to understand the influence of the substituents on anion binding. The general approach to the synthesis of the desired indolocarbazole involves reaction of substituted phenylhydrazine and cyclohexane-1,2-dione refluxed in acetic acid (see Scheme 2).[33]

We describe the use of acetic acid and concentrated sulfuric acid to perform the efficient double Fisher indolization. Compounds **1**−**16** having a range of different functional groups on indolocarbazole were synthesized using cyclohexane-1,2-dione with improved yield (23−78%). The reaction of phenylhydrazine and cyclohexane-1,2-dione under reflux conditions in acetic acid results in a mixture of compounds (**1**−**3**) (Scheme 2). Compound **3** reacted with 3-nitrophenylhydrazine hydrochloride and 3-methoxyphenylhydrazine hydrochloride reacted with conc H_2SO_4 in *n*-butanol and ethanol to form the structural isomers **4**, **5**, **9**, and **10**.[34] Compounds **6**, **7**, and **8** were obtained from the reaction of 3-methoxyphenylhydrazine hydrochloride and cyclohexane-1,2-dione heated at reflux in ethanol for 40 h (Scheme 2). Compound **11** was obtained from 1-trifluoromethylphenylhydrazine hydrochloride and cyclohexane-1,2-dione reflux in acetic acid (Scheme 2). We attempted the reaction of cyclohexane-1,2-dione with 2-chlorophenylhydrazine hydrochloride followed by dehydrogenation using *p*-chloranil to obtain a mixture of compounds **12** and **13**; one chlorine is removed in acidic medium (Scheme 2). A similar procedure for the reaction between 3-chlorophenylhydrazine and cyclohexane-1,2-dione, as shown in Scheme 6 in Supporting Information, was used to obtain the following mixture of isomers (**14**−**16**) (see Scheme 2).[35]

Preparation of Compound 1−3. Phenylhydrazine (3.00 g, 27.27 mmol) and cyclohexane-1,2-dione (1.00 g, 8.92 mmol) were dissolved in glacial acetic acid (50 mL), and the reaction mixture was stirred at reflux temperature for 40 h. The disappearance of the starting materials was monitored by TLC during the heating. The reaction mixture was cooled to room temperature, quenched with saturated aqueous $NaHCO_3$ solution (100 mL), and extracted with ethyl acetate (2 × 75 mL). The combined organic layers were washed with water (300 mL), dried over anhydrous $MgSO_4$ for 5 min, and filtered. The filtrate was evaporated under reduced pressure to obtain crude product (3.00 g). The crude product was purified by column chromatography (silica 230−400 mesh) eluting with 2−8% ethyl acetate in hexane to obtain pure compounds with combined yield 97.9%. Indolo[2,3-*a*]carbazole **1** (0.90 g, 3.51 mmol, 39.4%) was obtained as a white solid, 5,6,11,12-tetrahydroindolo[2,3-*a*]carbazole **2** (0.10 g, 0.38 mmol, 4.3%) was obtained as a light yellow solid, and 2,3,4,9-tetrahydrocarbazol-1-one **3** (0.90 g, 4.83 mmol, 54.2%) was obtained as a brown solid.

Data for **1**: Mp 372−376 °C. R_f = 0.31 (8% ethyl acetate in hexane). ^{1}H NMR (400.1 MHz, DMSO-d_6, 25 °C) δ 7.20 (ddd, $^3J_{HH}$ = 7.8 Hz, $^3J_{HH}$ = 7.1 Hz, $^4J_{HH}$ = 1.0 Hz, 2H, C*H*-3,8); 7.39 (ddd, $^3J_{HH}$ = 8.1 Hz, $^3J_{HH}$ = 7.1 Hz, $^4J_{HH}$ = 1.2 Hz, 2H, C*H*-2,9); 7.69 (ddd, $^3J_{HH}$ = 8.1 Hz, $^4J_{HH}$ = 1.0 Hz, $^5J_{HH}$ = 0.8 Hz, 2H, C*H*-1,10); 7.91 (bs, 2H, C*H*-5,6); 8.15 (dddd, $^3J_{HH}$ = 7.8 Hz, $^4J_{HH}$ = 1.2 Hz, $^5J_{HH}$ = 0.8 Hz, $^5J_{HH}$ = 0.7 Hz, 2H, C*H*-4,7); 11.03 (bs, 2H, N*H*-11,12). ^{13}C NMR (100.6 MHz, DMSO-d_6, 25 °C) δ 111.52 (*C*H-5,6); 111.53 (*C*H-1,10); 118.8 (*C*H-3,8); 119.6 (*C*H-4,7); 120.0 (*C*-5′,6′); 123.7 (*C*-4′,7′); 124.4 (*C*H-2,9); 125.6 (*C*-11′,11″); 138.9 (*C*-10′,12′). IR (ATR-FT-IRS) $\tilde{\nu}$: 3307 cm^{-1}. APCI-ICR (*m*/*z*): solvent 80% ACN/20% H_2O (0.1% formic acid), [M + H]$^+$ calcd for $C_{18}H_{13}N_2$ 257.10732, found 257.10726.

Data for **2**: Mp 330 °C. R_f = 0.36 (5% ethyl acetate in hexane). ^{1}H NMR (400.1 MHz, DMSO-d_6, 25 °C) δ 3.04 (bs, 4H, CH_2-5,6); 7.01 (m, $^3J_{HH}$ = 8.0 Hz, $^3J_{HH}$ = 7.0 Hz, $^4J_{HH}$ = 1.0 Hz, 2H, C*H*-3,8); 7.05 (m, $^3J_{HH}$ = 8.1 Hz, $^3J_{HH}$ = 7.0 Hz, $^4J_{HH}$ = 1.1 Hz, 2H, C*H*-2,9); 7.45 (m, $^3J_{HH}$ = 8.1 Hz, $^4J_{HH}$ = 1.0 Hz, $^5J_{HH}$ = 0.8 Hz, 2H, C*H*-1,10); 7.47 (m, $^3J_{HH}$ = 8.0 Hz, $^4J_{HH}$ = 1.1 Hz, $^5J_{HH}$ = 0.8 Hz, $^5J_{HH}$ = 0.7 Hz, 2H, C*H*-4,7); 10.81 (bs, 2H N*H*-11,12). ^{13}C NMR (100.6 MHz, DMSO-d_6, 25 °C) δ 20.3 (*C*H_2-5,6); 109.8 (*C*-5′,6′); 111.6 (*C*H-1,10); 117.8 (*C*H-4,7); 119.2 (*C*H-3,8); 120.7 (*C*H-2,9); 127.0 (*C*-4′,7′); 128.0 (*C*-11′,11″); 136.3(*C*-10′,12′). IR (ATR-FT-IRS) $\tilde{\nu}$: 3376, 3396 cm^{-1}. APCI-ICR (*m*/*z*): solvent 80% ACN/20% H_2O (0.1% formic acid), [M + H]$^+$ calcd for $C_{18}H_{15}N_2$ 259.12297, found 259.12302.

Data for **3**: Mp 170 °C. R_f = 0.49 (2% ethyl acetate in hexane). ^{1}H NMR (400.1 MHz, DMSO-d_6, 25 °C) δ 2.55 (m, 2H, CH_2-2); 2.15 (m, 2H, CH_2-3); 2.94 (dd, $^3J_{HH}$ = 6.5 Hz, $^3J_{HH}$ = 5.7 Hz, 2H, CH_2-4); 7.07 (ddd, $^3J_{HH}$ = 8.1 Hz, $^3J_{HH}$ = 6.9 Hz, $^4J_{HH}$ = 1.0 Hz, 1H, C*H*-6); 7.30 (ddd, $^3J_{HH}$ = 8.3 Hz, $^3J_{HH}$ = 6.9 Hz, $^4J_{HH}$ = 1.2 Hz, 1H, C*H*-7); 7.40 (ddd, $^3J_{HH}$ = 8.3 Hz, $^4J_{HH}$ = 1.0 Hz, $^5J_{HH}$ = 0.9 Hz, 1H, C*H*-8); 7.66 (dddd, $^3J_{HH}$ = 8.1 Hz, $^4J_{HH}$ = 1.2 Hz, $^5J_{HH}$ = 0.9 Hz, $^5J_{HH}$ = 0.7 Hz, 1H, C*H*-5); 11.57 (bs, 1H, N*H*-9). ^{13}C NMR (100.6 MHz, DMSO-d_6, 25 °C) δ 20.8 (*C*H_2-4); 24.7 (*C*H_2-3); 38.1 (*C*H_2-2); 112.7 (*C*H-8); 119.6 (*C*H-6); 121.1 (*C*H-5); 125.2 (*C*-5′); 126.1 (*C*H-7); 127.9 (*C*-4′); 137.9 (*C*-8′); 131.2 (*C*-9′); 190.3 (*C*=O). IR (ATR-FT-IRS) $\tilde{\nu}$: 1636, 3277 cm^{-1}. APCI-ICR (*m*/*z*): solvent 80% ACN/20% H_2O (0.1% formic acid), [M + H]$^+$ calcd for $C_{12}H_{12}NO$ 186.09134, found 186.09132.

Preparation of Compounds 4 and 5. Compound **3** (0.20 g, 1.06 mmol) and 3-nitrophenylhydrazine hydrochloride (0.32 g, 1.7 mmol) were dissolved in *n*-butanol (25 mL) and stirred at room temperature for 15 min, then conc H_2SO_4 (0.01 mL) was added dropwise, and the mixture was stirred at reflux temperature for 40 h until the disappearance of the starting material (monitored by TLC). The reaction mixture was then cooled to room temperature and concentrated under reduced pressure, after which the residue was dissolved in ethyl acetate (50 mL). The ethyl acetate solution was washed with water (200 mL), dried over anhydrous $MgSO_4$ for 5 min, and filtered. The filtrate was evaporated under reduced pressure to obtain a crude product. The crude product was purified by column chromatography (silica 230−400 mesh) eluting with 10−15% ethyl acetate in hexane to afford **4** and **5** with combined yield 23.4%. 2-Nitroindolo[2,3-*a*]carbazole **4** was obtained as a brown solid (35 mg, 0.11 mmol, yield 10.9%), and 4-nitroindolo[2,3-*a*]carbazole **5** was obtained as a brown solid (40 mg, 0.13 mmol, yield 12.5%).

Data for **4**: Mp 358 °C. R_f = 0.55 (10% ethyl acetate in hexane). ^{1}H NMR (400.1 MHz, DMSO-d_6, 25 °C) δ 7.24 (ddd, $^3J_{HH}$ = 7.8 Hz, $^3J_{HH}$ = 7.1 Hz, $^4J_{HH}$ = 1.0 Hz, 1H, C*H*-8); 7.44 (ddd, $^3J_{HH}$ = 8.1 Hz, $^3J_{HH}$ = 7.1 Hz, $^4J_{HH}$ = 1.2 Hz, 1H, C*H*-9); 7.72 (ddd, $^3J_{HH}$ = 8.1 Hz, $^4J_{HH}$ = 1.0 Hz, $^5J_{HH}$ = 0.7 Hz, 1H, C*H*-10); 8.02 (bs, 2H, C*H*-5,6); 8.08 (dd, $^3J_{HH}$ = 8.6 Hz, $^4J_{HH}$ = 2.2 Hz, 1H, C*H*-3); 8.19 (dddd, $^3J_{HH}$ = 7.8 Hz, $^4J_{HH}$ = 1.2 Hz, $^5J_{HH}$ = 0.7 Hz, $^5J_{HH}$ = 0.6 Hz, 1H, C*H*-7); 8.35 (ddd, $^3J_{HH}$ = 8.6 Hz, $^5J_{HH}$ = 0.6 Hz, $^5J_{HH}$ = 0.5 Hz, 1H, C*H*-4); 8.70 (dd, $^4J_{HH}$ = 2.2 Hz, $^5J_{HH}$ = 0.5 Hz, 1H, C*H*-1); 11.44 (bs, 1H, N*H*-11); 11.59 (bs, 1H, N*H*-12). ^{13}C NMR (100.6 MHz, DMSO-d_6, 25 °C) δ 108.0 (*C*H-1); 111.8 (*C*H-10); 112.4 (*C*H-5 or *C*H-6); 113.2 (*C*H-5 or *C*H-6); 114.1 (*C*H-3); 118.9 (*C*-5′); 119.3 (*C*H-8); 119.9 (*C*H-4); 120.1 (*C*H-7); 121.7 (*C*-6′); 123.4 (*C*-7′); 125.2 (*C*-11′); 125.3 (*C*H-9); 128.8 (*C*-11″); 128.9 (*C*-4′); 137.7 (*C*-12′); 139.3 (*C*-10′); 144.2 (*C*-2). IR (ATR-FT-IRS) $\tilde{\nu}$: 1296, 3349, 3443 cm^{-1}. APCI-ICR (*m*/*z*): solvent 80% ACN/20% H_2O (0.1% formic acid), [M + H]$^+$ calcd for $C_{18}H_{12}N_3O_2$ 302.09240, found 302.09251.

Data for **5**: Mp decomposed above 300 °C. R_f = 0.42 (15% ethyl acetate in hexane). ^{1}H NMR (400.1 MHz, DMSO-d_6, 25 °C) δ 7.24 (ddd, $^3J_{HH}$ = 7.9 Hz, $^3J_{HH}$ = 7.1 Hz, $^4J_{HH}$ = 1.0 Hz, 1H, C*H*-8); 7.44 (ddd, $^3J_{HH}$ = 8.1 Hz, $^3J_{HH}$ = 7.1 Hz, $^4J_{HH}$ = 1.2 Hz, 1H, C*H*-9); 7.60 (dd, $^3J_{HH}$ = 8.0 Hz, $^3J_{HH}$ = 8.0 Hz, 1H, C*H*-2); 7.74 (ddd, $^3J_{HH}$ = 8.1 Hz, $^4J_{HH}$ = 1.0 Hz, $^5J_{HH}$ = 0.7 Hz, 1H, C*H*-10); 7.98 (dd, $^3J_{HH}$ = 8.7 Hz, $^5J_{HH}$ = 0.5 Hz, 1H, C*H*-6); 8.02 (dd, $^3J_{HH}$ = 8.0 Hz, $^4J_{HH}$ = 0.9 Hz,

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1H, C*H*-3); 8.17 (dd, $^3J_{HH}$ = 8.0 Hz, $^4J_{HH}$ = 0.9 Hz, 1H, C*H*-1); 8.19 (dddd, $^3J_{HH}$ = 7.9 Hz, $^4J_{HH}$ = 1.2 Hz, $^5J_{HH}$ = 0.7 Hz, $^5J_{HH}$ = 0.6 Hz, 1H, C*H*-7); 8.19 (dd, $^3J_{HH}$ = 8.7 Hz, $^5J_{HH}$ = 0.5 Hz, 1H, C*H*-5); 11.21 (bs, 1H, N*H*-11); 11.95 (bs, 1H, N*H*-12). ^{13}C NMR (100.6 MHz, DMSO-d_6, 25 °C) δ 111.9 (*C*H-10); 112.5 (*C*H-6); 115.1 (*C*H-5); 115.7 (*C*-4′); 116.1 (*C*-5′); 116.4 (*C*H-3); 118.2 (*C*H-1); 119.3 (*C*H-8); 120.1 (*C*H-7); 121.3 (*C*-6′); 123.3 (*C*-7′); 123.8 (*C*H-2); 125.1 (*C*-11″); 125.3 (*C*H-9); 127.9 (*C*-11′); 139.4 (*C*-10′); 140.9 (*C*-12′); 142.7 (*C*-4). IR (ATR-FT-IRS) $\tilde{\nu}$: 1265, 3338, 3379 cm^{-1}. APCI-ICR (*m*/*z*): solvent 80% ACN/20% H_2O (0.1% formic acid), [M + H]$^+$ calcd for $C_{18}H_{12}N_3O_2$ 302.09240, found 302.09253

Preparation of Compounds 6, 7, and 8. Cyclohexane-1,2-dione (0.50 g, 4.46 mmol) and 3-methoxyphenylhydrazine hydrochloride (2.01 g, 11.6 mmol) were dissolved in ethanol (25 mL) and stirred at room temperature for 15 min, then conc H_2SO_4 (0.2 mL) was added dropwise, and the mixture was stirred at reflux temperature for 40 h, after which disappearance of the starting material was observed by TLC. The reaction mixture was cooled to room temperature and concentrated under reduced pressure. The residue was dissolved in ethyl acetate (50 mL), and the ethyl acetate solution washed with water (200 mL), dried over anhydrous $MgSO_4$ for 5 min, and filtered. The filtrate was evaporated under reduced pressure to obtain a crude product. The crude product was purified by column chromatography (silica 230−400 mesh) eluting with 2−10% ethyl acetate in hexane to obtain pure compounds with yield of 42.3%: 2,9-dimethoxyindolo[2,3-*a*]carbazole **6** (0.30 g, 0.94 mmol, yield 21.2%), 2,7-dimethoxyindolo-[2,3-*a*]carbazole **7** (0.25 g, 0.79 mmol, yield 17.6%), and 4,7-dimethoxyindolo[2,3-*a*]carbazole **8** (0.050 g, 0.15 mmol, yield 3.5%) all as brown solids.

Data **6**: Mp decomposed above 350 °C. R_f = 0.35 (10% ethyl acetate in hexane). ^{1}H NMR (400.1 MHz, DMSO-d_6, 25 °C) δ 3.86 (s, 6H, OC*H*$_3$-2,9); 6.80 (dd, $^3J_{HH}$ = 8.5 Hz, $^4J_{HH}$ = 2.3 Hz, 2H, C*H*-3,8); 7.19 (dd, $^4J_{HH}$ = 2.3 Hz, $^5J_{HH}$ = 0.5 Hz, 2H, C*H*-1,10); 7.75 (bs, 2H, C*H*-5,6); 7.97 (ddd, $^3J_{HH}$ = 8.5 Hz, $^5J_{HH}$ = 0.6 Hz, $^5J_{HH}$ = 0.5 Hz, 2H, C*H*-4,7); 10.91 (bs, 2H, N*H*-11,12). ^{13}C NMR (100.6 MHz, DMSO-d_6, 25 °C) δ 55.4 (O*C*H$_3$-2,9); 95.3 (*C*H-1,10); 107.9 (*C*H-3,8); 111.0 (*C*H-5,6); 117.8 (*C*-4′,7′); 119.6 (*C*-5′,6′); 120.2 (*C*H-4,7); 125.3 (*C*-11′,11″); 140.2 (*C*-10′,12′); 157.8 (*C*-2,9). IR (ATR-FT-IRS) $\tilde{\nu}$: 1155, 3423 cm^{-1}. APCI-ICR (*m*/*z*): solvent methanol, [M + H]$^+$ calcd for $C_{20}H_{17}N_2O_2$ 317.12845, found 317.12833.

Data for **7**: Mp 251.4−253.3 °C. R_f = 0.50 (7% ethyl acetate in hexane). ^{1}H NMR (400.1 MHz, DMSO-d_6, 25 °C) δ 3.87 (s, 3H, OC*H*$_3$-2); 4.04 (s, 3H, OC*H*$_3$-7); 6.73 (dd, $^3J_{HH}$ = 7.4 Hz, $^4J_{HH}$ = 1.2 Hz, 1H, C*H*-8); 6.82 (dd, $^3J_{HH}$ = 8.5 Hz, $^4J_{HH}$ = 2.3 Hz, 1H, C*H*-3); 7.23 (dd, $^4J_{HH}$ = 2.3 Hz, $^5J_{HH}$ = 0.5 Hz, 1H, C*H*-1); 7.26 (dd, $^3J_{HH}$ = 8.1 Hz, $^4J_{HH}$ = 1.2 Hz, 1H, C*H*-10); 7.30 (dd, $^3J_{HH}$ = 8.1 Hz, $^3J_{HH}$ = 7.4 Hz, 1H, C*H*-9); 7.77 (dd, $^3J_{HH}$ = 8.2 Hz, $^5J_{HH}$ = 0.5 Hz, 1H, C*H*-5); 7.97 (dd, $^3J_{HH}$ = 8.2 Hz, $^5J_{HH}$ = 0.5 Hz, 1H, C*H*-6); 7.97 (ddd, $^3J_{HH}$ = 8.5 Hz, $^5J_{HH}$ = 0.6 Hz, $^5J_{HH}$ = 0.5 Hz, 1H, C*H*-4); 10.84 (bs, 1H, N*H*-12); 11.08 (bs, 1H, N*H*-11). ^{13}C NMR (100.6 MHz, DMSO-d_6, 25 °C) δ 55.3 (O*C*H$_3$-2); 55.4 (O*C*H$_3$-7); 95.3 (*C*H-1); 100.1 (*C*H-8); 104.5 (*C*H-10); 108.0 (*C*H-3); 111.0 (*C*H-5); 113.0 (*C*-7′); 114.0 (*C*H-6); 117.7 (*C*-4′); 118.7 (*C*-6′); 119.7 (*C*-5′); 120.3 (*C*H-4); 124.7 (*C*-11′); 125.0 (*C*-11″); 125.3 (*C*H-9); 140.3 (*C*-12′); 140.3 (*C*-10′); 155.2 (*C*-7); 157.9 (*C*-2). IR (ATR-FT-IRS) $\tilde{\nu}$: 1123, 1386, 3374 cm^{-1}. APCI-ICR (*m*/*z*): solvent methanol, [M + H]$^+$ calcd for $C_{20}H_{17}N_2O_2$ 317.12845, found 317.12862.

Data for **8**: Mp 143.2 °C. R_f = 0.62 (2% ethyl acetate in hexane). ^{1}H NMR (400.1 MHz, DMSO-d_6, 25 °C) δ 4.04 (s, 6H, OC*H*$_3$-4,7); 6.74 (dd, $^3J_{HH}$ = 7.5 Hz, $^4J_{HH}$ = 1.2 Hz, 2H, C*H*-3,8); 7.26 (dd, $^3J_{HH}$ = 8.1 Hz, $^4J_{HH}$ = 1.2 Hz, 2H, C*H*-1,10); 7.98 (bs, 2H, C*H*-5,6); 7.31 (dd, $^3J_{HH}$ = 8.1 Hz, $^3J_{HH}$ = 7.5 Hz, 2H, C*H*-2,9); 11.19 (bs, 2H, N*H*-11,12). ^{13}C NMR (100.6 MHz, DMSO-d_6, 25 °C) δ 55.3 (O*C*H$_3$-4,7); 100.0 (*C*H-3,8); 104.5 (*C*H-1,10); 112.9 (*C*-4′,7′); 113.9 (*C*H$_2$-5,6); 118.7 (*C*-5′,6′); 124.4 (*C*-11′,11″); 125.3 (*C*H-2,9); 140.3 (*C*-10′,12′); 155.2 (*C*-4,7). IR (ATR-FT-IRS) $\tilde{\nu}$: 1102, 3384 cm^{-1}. APCI-ICR (*m*/*z*): solvent methanol, [M + H]$^+$ calcd for $C_{20}H_{17}N_2O_2$ 317.12845, found 317.12838.

Preparation of Compounds 9 and 10. Compound **3** (0.70 g, 3.76 mmol) and 3-methoxyphenylhydrazine hydrochloride (0.72 g, 4.13 mmol) were dissolved in ethanol (25 mL) and stirred at the room temperature for 15 min, then conc H_2SO_4 (0.2 mL) was added dropwise, and the mixture was stirred at reflux temperature for 40 h, after which disappearance of the starting material observed by TLC. The reaction mixture was cooled to room temperature and concentrated under reduced pressure. The residue was dissolved in ethyl acetate (50 mL), and the ethyl acetate solution was washed with water (200 mL), dried over anhydrous $MgSO_4$ for 5 min, and filtered. The filtrate was evaporated under reduced pressure to obtain 1.3 g of crude product. The crude product was dissolved in toluene (25 mL), and *p*-chloranil (0.44 g, 2.05 mmol) was added. The reaction mixture was stirred for 2 h at reflux temperature. After disappearance of the intermediate as monitored by TLC, the reaction mixture was concentrated under reduced pressure. The obtained residue was dissolved with ethyl acetate (50 mL), and the solution was washed with saturated $NaHSO_4$ (100 mL) and with water (2 × 100 mL). The ethyl acetate solution was dried over anhydrous $MgSO_4$ for 5 min and filtered. The filtrate was evaporated under reduced pressure to obtain a crude product. The crude product was purified by column chromatography (silica 230−400 mesh) eluting with 4−8% ethyl acetate in hexane to obtain pure compounds with combined yield 47.4%: 2-methoxyindolo[2,3-*a*]carbazole **9** (0.41 g, 1.43 mmol, yield 38.1%) and 4-methoxyindolo[2,3-*a*]carbazole **10** (0.10 g, 0.34 mmol, yield 9.3%) as a brown solid.

Data for **9**: Mp decomposed above 350 °C. R_f = 0.52 (8% ethyl acetate in hexane). ^{1}H NMR (400.1 MHz, DMSO-d_6, 25 °C) δ 3.88 (s, 3H, OC*H*$_3$-2); 6.82 (dd, $^3J_{HH}$ = 8.5 Hz, $^4J_{HH}$ = 2.3 Hz, 1H, C*H*-3); 7.18 (ddd, $^3J_{HH}$ = 7.8 Hz, $^3J_{HH}$ = 7.0 Hz, $^4J_{HH}$ = 1.0 Hz, 1H, C*H*-8); 7.23 (dd, $^4J_{HH}$ = 2.3 Hz, $^5J_{HH}$ = 0.5 Hz, 1H, C*H*-1); 7.36 (ddd, $^3J_{HH}$ = 8.1 Hz, $^3J_{HH}$ = 7.0 Hz, $^4J_{HH}$ = 1.2 Hz, 1H, C*H*-9); 7.65 (ddd, $^3J_{HH}$ = 8.1 Hz, $^4J_{HH}$ = 1.0 Hz, $^5J_{HH}$ = 0.8 Hz, 1H, C*H*-10); 7.80 (dd, $^3J_{HH}$ = 8.2 Hz, $^5J_{HH}$ = 0.4 Hz, 1H, C*H*-5); 7.86 (dd, $^3J_{HH}$ = 8.2 Hz, $^5J_{HH}$ = 0.4 Hz, 1H, C*H*-6); 8.00 (ddd, $^3J_{HH}$ = 8.5 Hz, $^5J_{HH}$ = 0.6 Hz, $^5J_{HH}$ = 0.5 Hz, 1H, C*H*-4); 8.12 (dddd, $^3J_{HH}$ = 7.8 Hz, $^4J_{HH}$ = 1.2 Hz, $^5J_{HH}$ = 0.8 Hz, $^5J_{HH}$ = 0.6 Hz, 1H, C*H*-7); 10.88 (bs, 1H, N*H*-12); 11.01 (bs, 1H, N*H*-11). ^{13}C NMR (100.6 MHz, DMSO-d_6, 25 °C) δ 55.3 (O*C*H$_3$-2); 95.3 (*C*H-1); 108.1 (*C*H-3); 111.0 (*C*H-5); 111.4 (*C*H-10); 111.5 (*C*H-6); 117.7 (*C*-4′); 118.8 (*C*H-8); 119.3 (*C*-6′); 119.5 (*C*H-7); 120.3 (*C*H-4); 120.4 (*C*-5′); 123.8 (*C*-7′); 124.3 (*C*H-9); 125.2 (*C*-11″); 125.7 (*C*-11′); 138.9 (*C*-10′); 140.2 (*C*-12′); 157.9 (*C*-2). IR (ATR-FT-IRS) $\tilde{\nu}$: 1317, 3408 cm^{-1}. APCI-ICR (*m*/*z*): solvent methanol, [M + H]$^+$ calcd for $C_{19}H_{15}N_2O$ 287.11789, found 287.11778.

Data for **10**: Mp 231.7−232.3 °C. R_f = 0.6 (4% ethyl acetate in hexane). ^{1}H NMR (400.1 MHz, DMSO-d_6, 25 °C) δ 4.05 (s, 3H, OC*H*$_3$-4); 6.75 (dd, $^3J_{HH}$ = 7.2 Hz, $^4J_{HH}$ = 1.4 Hz, 1H, C*H*-3); 7.20 (ddd, $^3J_{HH}$ = 7.8 Hz, $^3J_{HH}$ = 7.1 Hz, $^4J_{HH}$ = 1.0 Hz, 1H, C*H*-8); 7.28 (dd, $^3J_{HH}$ = 8.1 Hz, $^4J_{HH}$ = 1.4 Hz, 1H, C*H*-1); 7.32 (dd, $^3J_{HH}$ = 8.1 Hz, $^3J_{HH}$ = 7.2 Hz, 1H, C*H*-2); 7.38 (ddd, $^3J_{HH}$ = 8.1 Hz, $^3J_{HH}$ = 7.1 Hz, $^4J_{HH}$ = 1.2 Hz, 1H, C*H*-9); 7.68 (ddd, $^3J_{HH}$ = 8.1 Hz, $^4J_{HH}$ = 1.0 Hz, $^5J_{HH}$ = 0.8 Hz, 1H, C*H*-10); 7.88 (dd, $^3J_{HH}$ = 8.3 Hz, $^5J_{HH}$ = 0.4 Hz, 1H, C*H*-6); 8.01 (dd, $^3J_{HH}$ = 8.3 Hz, $^5J_{HH}$ = 0.4 Hz, 1H, C*H*-5); 8.13 (dddd, $^3J_{HH}$ = 7.8 Hz, $^4J_{HH}$ = 1.2 Hz, $^5J_{HH}$ = 0.8 Hz, $^5J_{HH}$ = 0.6 Hz, 1H, C*H*-7); 10.99 (bs, 1H, N*H*-11); 11.11 (bs, 1H, N*H*-12). ^{13}C NMR (100.6 MHz, DMSO-d_6, 25 °C) δ 55.3 (O*C*H$_3$); 100.1 (*C*H-3); 104.6 (*C*H-1); 111.46 (*C*H-10); 111.50 (*C*H-6); 112.9 (*C*-4′); 114.0 (*C*H-5); 118.8 (*C*H-8); 119.4 (*C*-6′); 119.4 (*C*-5′); 119.6 (*C*H-7); 123.8 (*C*-7′); 124.4 (*C*H-9); 124.6 (*C*-11″); 125.3 (*C*-11′); 125.4 (*C*H-2); 138.9 (*C*-10′); 140.3 (*C*-12′); 157.3 (*C*-4). IR (ATR-FT-IRS) $\tilde{\nu}$: 1102, 3385 cm^{-1}. APCI-ICR (*m*/*z*): solvent methanol, [M + H]$^+$ calcd for $C_{19}H_{15}N_2O$ 287.11789, found 287.11782.

Preparation of Compound 11. 2-Trifluoromethylphenylhydrazine hydrochloride (0.37 g, 1.78 mmol) and cyclohexane-1,2-dione (0.05 g, 0.44 mmol) was dissolved in propanol (2 mL), and the reaction mixture was stirred at reflux temperature for 2 h and then concentrated under reduced pressure. The concentrated mass was stirred in glacial acetic acid for 24 h at reflux temperature, then cooled to room temperature, quenched with saturated $NaHCO_3$ solution (50 mL), and extracted with ethyl acetate (2 × 25 mL). The combined

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ethyl acetate layers were washed with water (100 mL). The ethyl acetate solution was dried over anhydrous $MgSO_4$ for 5 min and filtered. The filtrate was evaporated under reduced pressure to obtain a crude product. The crude product was purified by column chromatography (silica 230−400 mesh) eluting with hexane to obtain pure compound: 1,10-di(trifluoromethyl)indolo[2,3-*a*]carbazole **11** (0.049 g, 0.12 mmol, yield 28.5%).

Data for **11**: Mp 304.1 °C. R_f = 0.82 (hexane). ^{1}H NMR (400.1 MHz, DMSO-d_6, 25 °C) δ 7.39 (ddq, $^3J_{HH}$ = 7.8 Hz, $^3J_{HH}$ = 7.5 Hz, $^5J_{HF}$ = 0.9 Hz, 2H, C*H*-3,8); 7.75 (ddq, $^3J_{HH}$ = 7.5 Hz, $^4J_{HH}$ = 1.1 Hz, $^4J_{HF}$ = 0.8 Hz, 2H, C*H*-2,9); 8.07 (bs, 2H, C*H*-5,6); 8.50 (ddqd, $^3J_{HH}$ = 7.8 Hz, $^4J_{HH}$ = 1.1 Hz, $^6J_{HF}$ = 0.8 Hz, $^5J_{HH}$ = 0.6 Hz, 2H, C*H*-4,7); 11.50 (bs, 2H, N*H*-11,12). ^{13}C NMR (100.6 MHz, DMSO-d_6, 25 °C) δ 111.6 (q, $^2J_{CF}$ = 32.2 Hz, *C*-1,10); 112.8 (*C*H-5,6); 118.8 (*C*H-3,8); 120.0 (*C*-5′,6′); 121.9 (q, $^3J_{CF}$ = 4.6 Hz, *C*H-2,9); 124.6 (*C*H-4,7); 125.0 (q, $^1J_{CF}$ = 271.5 Hz, *C*F$_3$-1,10); 125.7 (*C*-11′,11″); 125.5 (*C*-4′,7′); 133.9 (q, $^3J_{CF}$ = 2.0 Hz, *C*-10′,12′). ^{19}F NMR (188.3 MHz, DMSO-d_6, + 25 °C) δ −59.69. IR (ATR-FT-IRS) $\tilde{\nu}$: 1092, 3482 cm^{-1}. APCI-ICR (*m*/*z*): solvent methanol, [M + H]$^+$ calcd for $C_{20}H_{11}F_6N_2$ 393.08209, found 393.08207.

Preparation of Compounds 12 and 13. 2-Chlorophenylhydrazine hydrochloride (1.00 g, 5.58 mmol) and cyclohexane-1,2-dione (0.23 g, 2.05 mmol) were dissolved in glacial acetic acid (50 mL), and the reaction mixture was stirred at reflux temperature for 24 h until disappearance of the starting material (monitored by TLC). Then the reaction mixture was cooled to room temperature, quenched with saturated $NaHCO_3$ solution (100 mL), and extracted with ethyl acetate (2 × 25 mL). The combined ethyl acetate layers were washed with water (200 mL). The ethyl acetate solution was concentrated under reduced pressure to obtain crude product (1.3 g). The crude product was dissolved in toluene (25 mL), and *p*-chloranil (0.28 g, 1.1 mmol) was added. The reaction mixture was stirred for 2 h at reflux temperature. After disappearance of the intermediate as monitored by TLC, the reaction mixture was concentrated under reduced pressure. The concentrated mass was dissolved in ethyl acetate (50 mL), and the solution was washed with saturated $NaHSO_4$ (200 mL) and with water (2 × 100 mL). Ethyl acetate solution was dried over anhydrous $MgSO_4$ for 5 min and filtered. The filtrate was evaporated under reduced pressure to obtain a crude product. The crude product was purified by column chromatography (silica 230−400 mesh) eluting with 1−4% ethyl acetate in hexane to obtain pure compounds with combined yield 78.3%: 1,10-dichloroindolo[2,3-*a*]carbazole **12** (0.40 g, 1.23 mmol, yield 59.9%) and 1-chloroindolo[2,3-*a*]carbazole **13** (0.11 g, 0.37 mmol, yield 18.4%) as a white solid.

Data for **12**: Mp 286.8−288.8 °C. R_f = 0.75 (1% ethyl acetate in hexane). ^{1}H NMR (400.1 MHz, DMSO-d_6, 25 °C) δ 7.24 (dd, $^3J_{HH}$ = 7.8 Hz, $^3J_{HH}$ = 7.7 Hz, 2H, C*H*-3,8); 7.51 (dd, $^3J_{HH}$ = 7.7 Hz, $^4J_{HH}$ = 0.9 Hz, 2H, C*H*-2,9); 7.99 (bs, 2H, C*H*-5,6); 8.17 (ddd, $^3J_{HH}$ = 7.8 Hz, $^4J_{HH}$ = 0.9 Hz, $^5J_{HH}$ = 0.6 Hz, 2H, C*H*-4,7); 11.32 (bs, 2H, N*H*-11,12). ^{13}C NMR (100.6 MHz, DMSO-d_6, 25 °C) δ 112.8 (*C*H-5,6); 115.4 (*C*-1,10); 119.0 (*C*H-4,7); 120.1 (*C*H-3,8); 120.6 (*C*-5′,6′); 124.0 (*C*H-2,9); 125.4 (*C*-4′,7′); 125.6 (*C*-11′,11″); 135.6 (*C*-10′,12′). IR (ATR-FT-IRS) $\tilde{\nu}$: 1324, 3419, 3437 cm^{-1}. APCI-ICR (*m*/*z*): solvent 80% ACN/20% H_2O (0.1% formic acid), [M + H]$^+$ calcd for $C_{18}H_{11}Cl_2N_2$ 325.02938, found 325.02928.

Data for **13**: Mp 250.7−254.2 °C. R_f = 0.68 (4% ethyl acetate in hexane). ^{1}H NMR (400.1 MHz, DMSO-d_6, 25 °C) δ 7.22 (dd, $^3J_{HH}$ = 7.8 Hz, $^3J_{HH}$ = 7.7 Hz, 1H, C*H*-3); 7.23 (ddd, $^3J_{HH}$ = 7.8 Hz, $^3J_{HH}$ = 7.1 Hz, $^4J_{HH}$ = 1.0 Hz, 1H, C*H*-8); 7.41 (ddd, $^3J_{HH}$ = 8.1 Hz, $^3J_{HH}$ = 7.1 Hz, $^4J_{HH}$ = 1.2 Hz, 1H, C*H*-9); 7.48 (dd, $^3J_{HH}$ = 7.7 Hz, $^4J_{HH}$ = 0.9 Hz, 1H, C*H*-2); 7.73 (ddd, $^3J_{HH}$ = 8.1 Hz, $^4J_{HH}$ = 1.0 Hz, $^5J_{HH}$ = 0.8 Hz, 1H, C*H*-10); 7.93 (dd, $^3J_{HH}$ = 8.3 Hz, $^5J_{HH}$ = 0.5 Hz, 1H, C*H*-5); 7.97 (dd, $^3J_{HH}$ = 8.3 Hz, $^5J_{HH}$ = 0.5 Hz, 1H, C*H*-6); 8.15 (ddd, $^3J_{HH}$ = 7.8 Hz, $^4J_{HH}$ = 0.9 Hz, $^5J_{HH}$ = 0.6 Hz, 1H, C*H*-4); 8.17 (dddd, $^3J_{HH}$ = 7.8 Hz, $^4J_{HH}$ = 1.2 Hz, $^5J_{HH}$ = 0.8 Hz, $^5J_{HH}$ = 0.6 Hz, 1H, C*H*-7); 10.90 (bs, 1H, N*H*-11); 11.38 (bs, 1H, N*H*-12. ^{13}C NMR (100.6 MHz, DMSO-d_6, 25 °C) δ 111.7 (*C*H-10); 111.9 (*C*H-5); 115.4 (*C*-1); 112.6 (*C*H-6); 118.8 (*C*H-4); 119.1 (*C*H-8); 119.9 (*C*H-7); 120.06 (*C*H-3); 120.12 (*C*-5′); 120.6 (*C*-6′); 123.5 (*C*-7′); 123.8 (*C*H-2); 124.9 (*C*H-9); 125.6 (*C*-11′); 125.72 (*C*-4′); 125.77 (*C*-11″); 135.7 (*C*-12′); 138.8 (*C*-10′). IR (ATR-FT-IRS) $\tilde{\nu}$: 1111, 3386, 3437 cm^{-1}. APCI-ICR (*m*/*z*): solvent 80% ACN/20% H_2O (0.1% formic acid), [M + H]$^+$ calcd for $C_{18}H_{12}ClN_2$ 291.06835, found 291.06819.

Preparation of Compounds 14, 15, and 16. 3-Chlorophenylhydrazine hydrochloride (1.00 g, 5.58 mmol) and cyclohexane-1,2-dione (0.23 g, 2.05 mmol) were dissolved in glacial acetic acid (40 mL), and the reaction mixture was stirred at reflux temperature for 24 h. After disappearance of the starting material as monitored by TLC, the reaction mixture was cooled to the room temperature, quenched with saturated $NaHCO_3$ solution (200 mL), and extracted with ethyl acetate (2 × 50 mL). The combined organic layers were washed with water (200 mL). The ethyl acetate solution was concentrated under reduced pressure to obtain crude product (1.6 g). The crude product was dissolved in toluene (150 mL), *p*-chloranil (0.30 g, 1.2 mmol) was added, and the mixture was stirred for 2 h at reflux temperature. After disappearance of the intermediate as monitored by TLC, the reaction mixture was concentrated under reduced pressure. The concentrate was dissolved in ethyl acetate (100 mL) and washed with saturated $NaHSO_4$ (200 mL) and water (2 × 150 mL). The ethyl acetate solution was dried over anhydrous $MgSO_4$ for 5 min, filtered, and evaporated under reduced pressure to obtain a crude product as solid. The crude product was purified by column chromatography (silica 230−400 mesh) eluting with 3−10% ethyl acetate in hexane to obtain pure compounds with yield of 78.6%: 2,9-dichloroindolo[2,3-*a*]-carbazole **14** (0.11 g, 0.33 mmol, yield 16.6%), 2,7-dichloroindolo[2,3-*a*]carbazole **15** (0.26 g, 0.80 mmol, yield 39.3%), and 4,7-dichloroindolo[2,3-*a*]carbazole **16** (0.15 g, 0.46 mmol, yield 22.7%) all as off-white solids.

Data for **14**: Mp 250 °C. R_f = 0.62 (3% ethyl acetate in hexane). ^{1}H NMR (400.1 MHz, DMSO-d_6, 25 °C) δ 7.22 (dd, $^3J_{HH}$ = 8.3 Hz, $^4J_{HH}$ = 1.9 Hz, 2H, C*H*-3,8); 7.79 (dd, $^4J_{HH}$ = 1.9 Hz, $^5J_{HH}$ = 0.5 Hz, 2H, C*H*-1,10); 7.93 (bs, 2H, C*H*-5,6); 8.16 (ddd, $^3J_{HH}$ = 8.3 Hz, $^5J_{HH}$ = 0.6 Hz, $^5J_{HH}$ = 0.5 Hz, 2H, C*H*-4,7); 11.27 (bs, 2H, N*H*-11,12). ^{13}C NMR (100.6 MHz, DMSO-d_6, 25 °C) δ 111.4 (*C*H-1,10); 112.2 (*C*H-5,6); 119.2 (*C*H-3,8); 119.9 (*C*-5′,6′); 121.1 (*C*H-4,7); 122.6 (*C*-4′,7′); 126.0 (*C*-11′,11″); 129.1 (*C*-2,9); 139.6 (*C*-10′,12′). IR (ATR-FT-IRS) $\tilde{\nu}$: 804, 3413 cm^{-1}. APCI-ICR (*m*/*z*): solvent 80% ACN/20% H_2O (0.1% formic acid), [M + H]$^+$ calcd for $C_{18}H_{11}Cl_2N_2$ 325.02938, found 325.02936.

Data for **15**: Mp 315.8- 318.3 °C. R_f = 0.50 (7% ethyl acetate in hexane). ^{1}H NMR (400.1 MHz, DMSO-d_6, 25 °C) δ 7.24 (dd, $^3J_{HH}$ = 8.3 Hz, $^4J_{HH}$ = 1.9 Hz, 1H, C*H*-3); 7.26 (dd, $^3J_{HH}$ = 7.7 Hz, $^4J_{HH}$ = 0.9 Hz, 1H, C*H*-8); 7.40 (dd, $^3J_{HH}$ = 8.1 Hz, $^3J_{HH}$ = 7.7 Hz, 1H, C*H*-9); 7.70 (dd, $^3J_{HH}$ = 8.1 Hz, $^4J_{HH}$ = 0.9 Hz, 1H, C*H*-10); 7.84 (dd, $^4J_{HH}$ = 1.9 Hz, $^5J_{HH}$ = 0.5 Hz, 1H, C*H*-1); 7.98 (dd, $^3J_{HH}$ = 8.5 Hz, $^5J_{HH}$ = 0.6 Hz, 1H, C*H*-5); 8.18 (ddd, $^3J_{HH}$ = 8.3 Hz, $^5J_{HH}$ = 0.6 Hz, $^5J_{HH}$ = 0.5 Hz, 1H, C*H*-4); 8.29 (dd, $^3J_{HH}$ = 8.5 Hz, $^5J_{HH}$ = 0.5 Hz, 1H, C*H*-6); 11.21 (bs, 1H, N*H*-12); 11.54 (bs, 1H, N*H*-11. ^{13}C NMR (100.6 MHz, DMSO-d_6, 25 °C) δ 110.6 (*C*H-10); 111.5 (*C*H-1); 112.0 (*C*H-5); 113.8 (*C*H-6); 118.9 (*C*-6′); 119.2 (*C*H-3); 119.6 (*C*H-8); 119.8 (*C*-5′); 120.4 (*C*-7′); 121.2 (*C*H-4); 122.4 (*C*-4′); 125.3 (*C*H-9); 125.6 (*C*-11″); 125.9 (*C*-11′); 126.5 (*C*-7); 129.2 (*C*-2); 139.6 (*C*-12′); 140.2 (*C*-10′). IR (ATR-FT-IRS) $\tilde{\nu}$: 721, 796, 3393 cm^{-1}. APCI-ICR (*m*/*z*): solvent 80% ACN/20% H_2O (0.1% formic acid), [M + H]$^+$ calcd for $C_{18}H_{11}Cl_2N_2$ 325.02938, found 325.02950.

Data for **16**: Mp 300 °C (dec). R_f = 0.30 (10% ethyl acetate in hexane). ^{1}H NMR (400.1 MHz, DMSO-d_6) δ 7.27 (dd, $^3J_{HH}$ = 7.7 Hz, $^4J_{HH}$ = 0.8 Hz, 2H, C*H*-3,8); 7.41 (dd, $^3J_{HH}$ = 8.1 Hz, $^3J_{HH}$ = 7.7 Hz, 2H, C*H*-2,9); 7.72 (dd, $^3J_{HH}$ = 8.1 Hz, $^4J_{HH}$ = 0.8 Hz, 2H, C*H*-1,10); 8.33 (bs, 2H, C*H*-5,6); 11.57 (bs, 2H, N*H*-11,12). ^{13}C NMR (100.6 MHz, DMSO-d_6, 25 °C) δ 110.7 (*C*H-1,10); 119.0 (*C*-5′,6′); 113.8 (*C*H-5,6); 120.4 (*C*-4′,7′); 119.7 (*C*H-3,8); 125.6 (*C*H-2,9); 125.7 (*C*-11′,11″); 126.7 (*C*-4,7); 140.4 (*C*-10′,12′). IR (ATR-FT-IRS) $\tilde{\nu}$: 728, 3385, 3438 cm^{-1}. APCI-ICR (*m*/*z*): solvent 80% ACN/20% H_2O (0.1% formic acid), [M + H]$^+$ calcd for $C_{18}H_{11}Cl_2N_2$ 325.02938, found 325.02928.

Measurements of Relative Binding Constants. Spectrophotometric titrations were carried out at (25.0 ± 0.2) °C. All solutions were prepared in acetonitrile with 0.5% water (m/m). Acetonitrile solvates

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anions weakly, and traces of water can have a significant effect on results.[1] Therefore 0.5% of water was added for better reproducibility in order to ensure that all anions are solvated in similar conditions. Such water content has been used in several works.[16,36] Receptor molecule stock solutions were approximately 0.001−0.002 M. The working concentration of the receptors during the measurements (in the spectrophotometer cells) was in the range of 2×10^{-4} to 7×10^{-5} M. The concentration of the tetrabutylammonium acetate titrant stock solution was in the range of 0.08−0.1 M. It was further diluted to 0.005−0.007 M. Both 97% and 99% purity tetrabutylammonium acetate batches were used for measurements. The purity of the salts was confirmed with low and high resolution MS analysis. In the course of the titration diluted titrant was used to obtain degrees of dissociation at different levels. The relationship between formation of the complex and amount of anion added is nonlinear. Therefore when nearing the end point higher and higher amounts of anion have to be added so that essentially all molecules of the receptor are in the receptor-anion complex form. This becomes especially important when $\log K_{ass}$ is not very high ($\log K_{ass} < 5$). The medium used was not buffered because the introduction of additional anionic species would interfere with the binding process. For relative binding strength measurement compounds R_1H and R_2H were chosen, which had sufficiently different spectra. It was necessary that the two compounds have similar binding strength. The relative measurement method allowed us to obtain reasonably accurate results when $\Delta\log K_{ass} < 1$. During the experiment the absorption spectra of titration of the individual receptors with acetate were recorded first over the course of titration with acetate. At least 4−7 spectra from different parts of the titration curve were recorded in addition to the free receptor and receptor-anion complex to confirm the spectral purity of the receptors using isosbestic points. Next, a mixed solution containing both receptors was titrated with the anion titrant. On the average 16−20 spectra were recorded. From the spectra degrees of dissociation for both receptor-anion complexes were found using multilinear regression. $\Delta\log K_{ass}$ was calculated according to eq 10. The measurement method is schematically presented in Figure 1. The Job's plot approach was used to confirm that the binding occurs in 1:1 ratio (see the Supporting Information).

Calculation Method. The calculation method was very similar to the one used earlier by our group for pK_a measurements in nonaqueous solvents.[3,5] By replacing the equilibrium concentration in eq 5 with α_1 and α_2, which are the degrees of dissociation of R_1HA^- and R_2HA^-, $\Delta\log K_{ass}$ values were found using following equation:

$$\Delta\log K_{ass} = \log \frac{\alpha_2(1-\alpha_1)}{(1-\alpha_2)\alpha_1} \tag{10}$$

Deprotonation Studies by Spectrophotometric Titration. Deprotonation studies were carried out using spectrophotometric titration. Working conditions were the same as were used in binding studies. Tetrabutylammonium hydroxide was used as titrant. Several spectra were recorded over the course of titration until the end-point was reached.

Determination of Relative Acidities. Relative acidities (ΔpK_a values) of the compounds with respect to acetic acid in the solvent used for binding studies were determined using essentially the same method as described in ref 5. Direct measurements of acidities between acetic acid and receptors under study were not possible because acetate, which is produced during deprotonation of acetic acid, would have bound to the receptor molecules, resulting in a complex system of equilibria. Therefore reference acids, which on deprotonation give stable anions with delocalized charge and do not bind to receptor molecules (mostly CH acids), were used as "intermediate links" for comparison of acidities between acetic acid and the receptor molecules. 1,3-Bis(4-NO_2-phenyl)urea (**22**) showed both complexation and deprotonation characteristics in the spectra during titration with the phosphazene base (*tert*-butylimino-tris(pyrrolidino)-phosphorane) (see the spectrum in Figure 2). Possible complexation between the respective urea and hydroxide anion produced by deprotonation of water with the phosphazene base was assumed. In the used solvent where water concentration is relatively high, water molecules can have remarkably high acidity due to hydrogen bonds formed between hydroxide anion and other water molecules (the acidity of water increases with increasing its concentration due to the cooperative effect). This effect makes water a sufficiently strong acid to protonate the used phosphazene base. The other receptor molecules, whose acidities were studied, did not show such effects.

Measurements of Absolute Binding Constants. The working conditions and solvents used were the same as in measurements of relative binding constants. Concentrations for the receptor molecules were similar to the measurements of the relative binding constants. The concentrations of TBAA in the concentrated solution were approximately 0.07 M and in diluted solutions approximately 0.0015−0.002 M for indolocarbazole (**1**) and 1,10-dichloroindolocarbazole (**12**) $\log K_{ass}$ value measurements. For 3,4,4′-trichlorodiphenylurea (**23**) the titrant concentrations were approximately 0.07 and 0.0008−0.0015 M. During titration the spectrophotometric cell was weighed before and after each addition of titrant. Over the course of titration around 12−17 spectra were recorded. The spectrum of the free receptor was obtained before the first addition of titrant. The spectrum of the receptor-anion complex was obtained by adding a large amount of titrant. From the weighing data exact amounts of titrant added were found. The dissociation degree of the complex is expressed through *a*:

$$\alpha = \frac{[RH]}{[RH] + [RHA^-]} = \frac{A^\lambda - A^\lambda_{RHA^-}}{A^\lambda_{RH} - A^\lambda_{RHA^-}} \tag{11}$$

where A^λ is absorbance at the titration step, and A^λ_{RH} and A^λ_{RHA} are the absorbances of free receptor and receptor-anion complex accordingly. Three methods were used for calculation of the binding constants. The assigned values for each run were averaged from the results of the three calculation methods taking into account their internal consistency.

Calculation from Every Individual Titration Point. The amount of free anion added and the concentration of the free anion for each titration point were found from titration data. Equation 2 was modified to obtain the equation for finding the $\log K_{ass}$ values:

$$\log K_{ass} = \log \frac{(1-\alpha)\gamma_{RHA^-}}{\alpha\cdot[A^-]\gamma_{A^-}} \tag{12}$$

γ_{RHA^-} and γ_{A^-} are the activity coefficients of the receptor-anion complex and the anion of interest, respectively. The activity coefficients were calculated according to the Debye−Hückel equation, which for acetonitrile reads as follows:[37]

$$\log\gamma = -\frac{1.64z^2\sqrt{I}}{1 + 0.48a\sqrt{I}} \tag{13}$$

where *I* is the ionic strength, *z* is the ion charge, and *a* is the effective radius of the ion.

This is a crude approximation under the conditions of our work because the acetonitrile used contains a considerable amount of water. Also, the ionic species in this work are not really spherical (which is an assumption of the Debye−Hückel theory).

Least Square Fitting of the Isotherm, without Linearization. Based on eqs 2 and 11 it is possible to arrive at the equation of the binding isotherm:

$$\Delta A = \Delta A_{max} \frac{K_{ass}\frac{[A^-]\gamma_{A^-}}{\gamma_{RHA^-}}}{1 + K_{ass}\frac{[A^-]\gamma_{A^-}}{\gamma_{RHA^-}}} \tag{14}$$

K_{ass} was found by fitting the experimental data to this isotherm using the least-squares approach and taking ΔA_{max} (equal to $A^\lambda_{RHA^-} - A^\lambda_{RH}$) and K_{ass} as adjustable parameters.

Least Square Fitting of the Isotherm, with Linearization. Equation 14 was linearized as described by Benesi and Hildebrand[38] to arrive at the following:

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$$\frac{1}{A^{\lambda} - A^{\lambda}_{RH}} = \frac{\gamma_{RHA^-}}{K_{ass}(A^{\lambda}_{RHA^-} - A^{\lambda}_{RH})[A^-]\gamma_{A^-}} + \frac{1}{A^{\lambda}_{RHA^-} - A^{\lambda}_{RH}} \quad (15)$$

By plotting $(1/(A^{\lambda} - A^{\lambda}_{RH}))$ versus $((\gamma_{RHA^-})/([A^-](\gamma_{A^-})))$, K_{ass} can be found from the slope.

■ ASSOCIATED CONTENT

Supporting Information

Compound characterization data (NMR, IR, and HRMS data) and Job plots of representative compounds, examples of fitting curves of absolute binding constant measurements, titration spectra of receptor molecules and example titration spectra of mixtures, examples of calculation files (in .xlsx format) of relative binding measurements and uncertainty estimation procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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