Supplementary Material (ESI) for Chemical Communications

Limitations of the "tethering" strategy for the detection of a weak noncovalent interaction†

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Contents:

Synthesis and characterization of 2-ethylphosphonoxybenzaldehyde 1 (acid)

A solution of salicylaldehyde (5.8 g, 47.7 mmol) in THF (15 ml) was added dropwise within 1 hour to a suspension of NaH (1.19 g, 49.5 mmol) in THF at 0°C. After the addition, the reaction mixture was stirred for half an hour at rt. After re-cooling at 0 °C, a solution of ethylphosphonic dichloride (5.5g, 47.7 mmol) in THF was added dropwise. The resulting solution was stirred at rt for 2h, after which the solution was cooled to 0 °C and quenched with 2ml of HCl 12N. Subsequently, THF was removed under vacuum and a brown oil was obtained. Water was added and basified using solid $NaHCO_3$. The acqueous phase was extracted with $CHCl_3$ and then acidified with HCl 12N. After extraction with $CHCl_3$, the organic layer was dried over Na_2SO_4 and the solvent was evaporated under reduced pressure to give 1.5 g of **1** as a green oil (15% yield). **^{1}H-NMR** (300 MHz, $CDCl_3$) δ (ppm): 1.20 (td, J = 21.11, 7.55Hz, 3H), 1.90 (qd, J = 18.83, 7.55Hz, 2H), 7.22-7.33 (m, 2H), 7.48-7.53 (m, 1H), 7.84-7.87 (m, 1H), 8.17 (s br, 1H), 10.32 (s, 1H). **^{13}C-NMR** (75 MHz, CDCl3) δ (ppm): 5.98 (d, J_{P-C} = 6.45 Hz), 19.02 (d, J_{P-C} = 145.46 Hz), 121.72, 125.11, 127.68 (d, J = 3.80 Hz), 128.56, 135.32, 151.95 (d, J = 9.31 Hz), 188.51. **^{31}P-NMR** (122 MHz, $CDCl_3$) proton decoupled δ (ppm): 34.43. **ESI-MS(+)** ACN+0,1%HCOOH: $[M+Na]^+$ 237m/z, **ESI-MS(-)** ACN: $[M-H]^-$ 213 m/z

Synthesis and characterization of the tetrabutylammonium salt of 2-ethylphosphonoxybenzaldehyde 1-TBA

2-Ethylphosphonoxybenzaldehyde (531.8 mg, 2.48 mmol) was dissolved in water and the pH of the resulting acidic solution was corrected to pH~7.5 using $NaHCO_3$. The acqueous phase was extracted with a solution of tetrabutylammonium chloride (669 mg, 2.22mmol) in $CHCl_3$. The organic solvent was evaporated under reduced pressure, to give a yellow oil (904.9mg, 1.99mmol, 90 % yield). **^{1}H-NMR** (300 MHz, $CDCl_3$) δ (ppm): 0.94 (t, J = 7.28Hz, 12H), 1.15 (td, J = 18.29, 7.65Hz, 3H) 1.36 (tq, J = 7.36 Hz, J = 7.36 Hz, 8H), 1.53-1.76 (m, 10H), 3.03 (s, 1H), 3.24 (m, 8H), 6.99 (t, 7.47 Hz, 1H), 7.45-7.37 (m, 1H),7.64 (d, J = 8.34 Hz, 1H), 7.74 (dd, J = 7.76, 1.56 Hz, 1H), 10.54 (s, 1H). **^{13}C-NMR** (75 MHz, CDCl3) δ (ppm): 8.41 (d, J_{P-C} = 6.37 Hz),13.59, 19.63, 21.38 (d, J_{P-C} = 137.16 Hz), 23.93, 58.63, 121.68, 122.24 (d, J = 2.92 Hz), 126.98, 127.73 (d, J_{P-C} = 4.20 Hz), 135.04 (d, J_{P-C} = 0.81 Hz), 157.79 (d, J_{P-C} = 7.35 Hz), 191.54. **^{31}P-NMR** (122 MHz, $CDCl_3$) proton decoupled δ (ppm): 24.95. **ESI-MS(-)** ACN: $[M-TBA]^-$ 213 m/z.

Synthesis and characterization of hydrazones 1A, 1B, 2A, and 2B.

General procedure

The aldehyde (either compound **1-TBA** or commercially available 2-methoxybenzaldehyde **2**) was dissolved in MeOH and 1 equivalent of the commercially available hydrazides **A** (Girard's reagent T) or **B** (phenylacetic hydrazide) was added. The reaction mixture was refluxed for 5 hours and then evaparated to dryness.

Characterization

^{1}H-NMR spectra were recorded on a Bruker spectrometer operating at 300MHz. Chemical shift are reported in ppm from tetramethylsilane using residual solvent $CDCl_3$ (7.26 ppm) or CD_3OD (3.31 ppm) for calibration. ^{13}C-NMR were recorded at 75 MHz using residual solvent $CDCl_3$ (77.0 ppm) or CD_3OD (49.05 ppm) for calibration. ^{31}P-NMR were recorded at 121 MHz with external calibration on a sample of H_3PO_4 85% in water (0 ppm).

It should be noted that all hydrazones are present as two isomers in a ratio dependent on the type of hydrazide used. The different isomers are due to *E*,*Z*-isomerism around the NH-C(O) bond, based on related studies in the literature (F.V. Bagrov, T.V. Vasil'eva *Russ. J. Org, Chem.* 2002, **38**, 1309-1313.) It should be emphasized that the ratio between the isomers does not depend on the presence of either the phosphonyl-group in **1** or the methoxy-group in **2**, since the isomer ratio for both **1A** and **2A** is 20:80, whereas a ratio of 62:38 is found for both **1B** and **2B**.

Hydrazone 1A.
^{1}H-NMR: Isomer I (80%): (300 MHz, $CDCl_3$) δ (ppm): 1.03 (t, *J* = 7.31Hz, 12H), 1.19 (dt, *J* = 19.02, 7.69 Hz, 3H), 1.42 (m, 8H), 1.62-1.83 (m, 10H), 3.22-3.27 (m, 8H), 3.42 (s, 1H), 4.79 (s, 1H), 7.07-7.12 (m, 1H), 7.32-7.44 (m, 2H), 7.93 (dd, *J* = 7.83, 1.23 Hz, 1H), 8.39 (s, 1H).
^{1}H-NMR: Isomer II (20%): (300 MHz, $CDCl_3$) δ (ppm): 1.03 (t, *J* = 7.31, 7.31 Hz, 12H), 1.19 (dt, *J* = 19.02, 7.69 Hz, 3H), 1.42 (m, 8H), 1.62-1.83 (m, 10H), 3.22-3.27 (m, 8H), 3.32 (s, 1H), 4.20 (s, 1H), 7.07-7.12 (m, 1H), 7.32-7.44 (m, 2H), 8.05 (dd, *J* = 7.68 Hz, 1.13, 1H), 8.64 (s, 1H).
^{13}C-NMR: Isomer I (80%) (75 MHz, CDCl3) δ (ppm): 8.17 (d, J_{P-C} = 6.28 Hz), 13.99, 20.77, 21.84 (d, J_{P-C} = 139.53 Hz), 24.86, 54.83, 59.58, 64.32, 122.44 (d, J_{P-C} = 2.74 Hz), 124.29, 126.61 (d, J_{P-C} = 4.52 Hz), 127.19, 132.57, 144.35, 153.81 (d, J_{P-C} = 7.74 Hz), 166.35.
^{13}C-NMR: Isomer II (20%) (75 MHz, CDCl3) δ (ppm): 8.17 (d, J_{P-C} = 6.28 Hz), 13.99, 20.77, 21.94 (d, J_{P-C} = 139.95 Hz), 24.86, 55.04, 59.58, 64.82, 122.71 (d, J_{P-C} = 2.53 Hz), 124.50, 126.61 (d, J_{P-C} = 4.52 Hz), 127.58, 133.10, 148.55, 154.04 (d, J_{P-C} = 7.98 Hz), 161.70
^{31}P-NMR: Isomer I (80%) (122 MHz, $CDCl_3$) proton decoupled δ (ppm): 26.59
^{31}P-NMR: Isomer II (20%) (122 MHz, $CDCl_3$) proton decoupled δ (ppm): 26.74
ESI-MS: (+) MeOH+0,1%HCOOH: $[M+H]^+$ 328 m/z
HPLC: (Jupiter Proteo 4u 250x4.60x4u, flow=0.8ml/min, 0-15min 10-90% ACN, 15-25min 90%ACN, λ=226nm): 9.5 min

Hydrazone 1B
^{1}H-NMR: Isomer I (62%) (300 MHz, $CDCl_3$) δ (ppm): 1.01 (t, *J* = 7.31 Hz, 12H), 1.17 (dt, *J* = 19.02, 7.69 Hz, 3H), 1.41 (m, 8H), 1.59-1.79 (m, 10H), 3.18-3.24 (m, 8H), 3.63 (s, 1H), 7.05-7.46 (m, 8H), 8.08 (dd, *J* = 7.82, 1.05 Hz, 1H), 8.60 (s, 1H)

^{1}H-NMR: Isomer II (38%) (300 MHz, $CDCl_3$) δ (ppm): 1.01 (t, *J* = 7.31 Hz, 12H), 1.17 (td, *J* = 19.02, 7.69 Hz, 3H), 1.41 (m, 8H), 1.59-1.79 (m, 10H), 3.18-3.24 (m, 8H), 4.07 (s, 1H), 7.05-7.46 (m, 8H), 7.95 (dd, *J* = 7.79, 1.10 Hz, 1H), 8.40 (s, 1H)
^{13}C-NMR: Isomer I (62%) (75 MHz, CDCl3) δ (ppm): 8.18 (d, J_{P-C} = 6.18 Hz), 13.98, 20.75, 21.84 (d, J_{P-C} = 139.61 Hz), 24.81, 42.42, 59.53, 122.15 (d, J_{P-C} = 2.52 Hz), 124.23, 126.72, 127.10 (d, J_{P-C} = 4.67 Hz), 127.56, 127.72, 128.08, 129.66, 130.14, 132.48, 136.49, 146.28, 153.87 (d, J_{P-C} = 7.73 Hz), 170.42
^{13}C-NMR: Isomer II (38%) (75 MHz, CDCl3) δ (ppm): 8.18 (d, J_{P-C} = 6.18 Hz), 13.98, 20.75, 21.84 (d, J_{P-C} = 139.61 Hz), 24.81, 40.46, 59.53, 122.32 (d, J_{P-C} = 2.50 Hz), 124.23, 126.72, 127.10 (d, J_{P-C} = 4.67 Hz), 127.56, 127.72, 127.94, 129.47, 130.52, 131.97. 136.91, 142.47, 153.61 (d, J_{P-C} = 7.72 Hz), 175.64
^{31}P-NMR: Isomer I (62%) (122 MHz, $CDCl_3$) proton decoupled δ (ppm): 26.47
^{31}P-NMR: Isomer II (38%) (122 MHz, $CDCl_3$) proton decoupled δ (ppm): 26.54
ESI-MS: (-) MeOH: $[M-H]^-$ 345 m/z
HPLC: (Jupiter Proteo 4u 250x4.60x4u, flow=0.8ml/min, 0-15min 10-90% ACN, 15-25min 90%ACN, λ=226nm): 16.8 min

Hydrazone 2A
^{1}H-NMR: Isomer I (80%) (300 MHz, $CDCl_3$) δ (ppm): 3.42 (s, 9H), 3.88 (s, 3H), 4.79 (s, 2H), 6.96-7.06 (m, 2H), 7.38-7.44 (m, 1H), 7.92 (dd, *J* = 7.77, 1.65 Hz, 1H), 8.38 (s, 1H)
^{1}H-NMR: Isomer II (20%) (300 MHz, $CDCl_3$) δ (ppm): 3.41 (s, 9H), 3.88 (s, 3H), 4.30 (s, 2H), 6.96-7.06 (m, 2H), 7.38-7.44 (m, 1H), 8.00 (dd, *J* = 7.78, 1.63 Hz, 1H), 8.65 (s, 1H)
^{13}C-NMR: Isomer I (80%) (75 MHz, CDCl3) δ (ppm): 54.79, 64.30, 112.54, 121.84, 123.14, 127.13, 133.30, 141.39, 143.54, 147.90, 160.07
^{13}C-NMR: Isomer II (20%) (75 MHz, CDCl3) δ (ppm): 55.06, 56.26, 64.30, 112.55, 121.94, 123.14, 127.68, 133.85, 142.34, 147.83, 160.07
ESI-MS: (+) MeOH+0,1%HCOOH: $[M]^+$ 250 m/z
HPLC: (Jupiter Proteo 4u 250x4.60x4u, flow=0.8ml/min, 0-15min 10-90% ACN+0,1%TFA, 15-25min 90%ACN, λ=280nm): 11.4 min

Hydrazone 2B
^{1}H-NMR: Isomer I (62%) (300 MHz, $CDCl_3$) δ (ppm): 3.60 (s, 2H), 3.86 (s, 3H), 6.92-7.03 (m, 2H), 7.20-7.40 (m, 5H), 8.00 (dd, *J* = 7.78, 1.55 Hz, 1H), 8.55 (s, 1H)
^{1}H-NMR: Isomer II (38%) (300 MHz, $CDCl_3$) δ (ppm): 3.86 (s, 3H), 4.05 (s, 2H), 6.92-7.03 (m, 2H), 7.20-7.40 (m, 5H), 7.89 (dd, *J* = 7.73, 1.53 Hz, 1H), 8.34 (s, 1H)
^{13}C-NMR: Isomer I (62%) (75 MHz, CDCl3) δ (ppm): 39.80, 55.69, 111.26, 121.16, 122.48, 126.32, 126.92, 127.71, 128.63, 129.64, 129.72, 131.56, 135.26, 140.22, 158.22, 173.83
^{13}C-NMR: Isomer II (38%) (75 MHz, CDCl3) δ (ppm): 42.84, 55.59, 111.00, 121.01, 122.48, 126.32, 126.92, 127.80, 128.63, 129.28, 129.72, 132.26, 135.26, 144.26, 158.22, 173.83
ESI-MS: (+) MeOH+0,1%HCOOH: $[M+Na]^+$ 291 m/z
HPLC: (Jupiter Proteo 4u 250x4.60x4u, flow=0.8ml/min, 0-15min 10-90% ACN+0,1%TFA, 15-25min 90%ACN, λ=280nm): 17.3 min

General procedure used for the amplification experiments.

NMR

Concentrated mother solutions of the scaffold molecule (**1** or **2**, 100 mM in CD_3CN, Note 1) and the hydrazides (**A** and **B**, 500 mM in CD_3OD) were always freshly prepared. Appropriate amounts of the hydrazide stocksolutions were transferred to an NMR tube, CD_3OD was added to dilute and finally the scaffold molecule was added. The sample was kept at 50 °C and monitored by ^1H NMR spectroscopy until no additional changes were observed in time. Integration of the hydrazone signals yielded the relative concentrations of the two hydrazones.

Note 1. For the amplification studies with [**1**]=50 mM, a stocksolution of 500 mM was used.

HPLC

The HPLC study was performed starting from preformed hydrazone **1B** instead of the free scaffold **1**. This was motivated by the observation that the equilibration times were very long. Starting from the unfavorable hydrazone **1B** changes in the HPLC spectrum were more easy to detect, allowing a better evaluation whether the thermodynamic equilibrium was reached. In addition, it made calculation of the thermodynamic composition (via fitting) more reliable for the mixtures with a small excess of hydrazides. Mixtures were prepared as before (see NMR) and kept at 50 °C. Samples were injected directly in the HPLC at regular time intervals. The concentration of hydrazone **1A** was calculated by comparing the integrated area with a calibration curve.

Fingerprint part of the ^{1}H NMR spectra obtained for the mixtures 1:A:B (2:6:6 mM) and 2:A:B (2:6:6 mM) at thermodynamic equilibrium.

In Figure SI-1 parts of the ^{1}H NMR spectra (300 MHz, CD_3OD) are shown for the mixtures **1:A:B** (2:6:6 mM, a) and **2**:**A:B** (2:6:6 mM, b) at thermodynamic equilibrium. Notably, each hydrazone is present as a mixture of 2 isomers in a ratio of 20:80 for **1A** (*) and **2A** (♦) and 62:38 for **1B** (o) and **2B** (□) (see characterization data). The ratio's **1A**:**1B** (68:32) and **2A**:**2B** (51:49) are based on integration of the respective hydrazone signals. The ratio **1A**:**1B** is based on integration of the two signals at lower field, corrected for the relative amount of that isomer present.

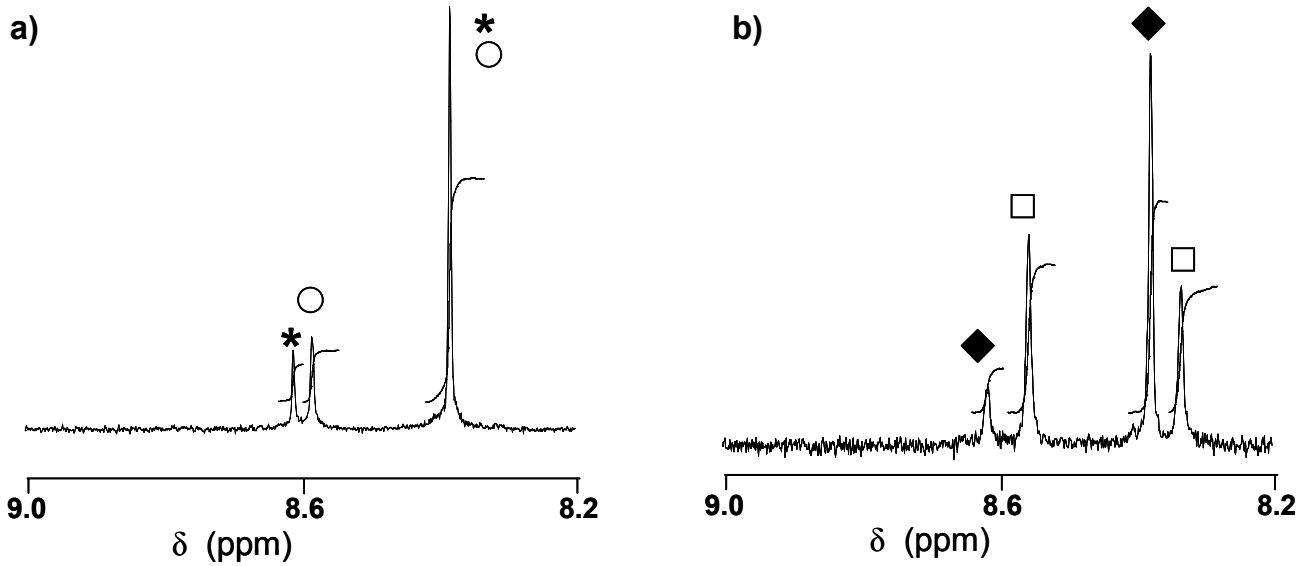


Figure SI-1 Parts of the ^{1}H NMR spectra (300 MHz, CD_3OD) for the mixtures **1:A:B** (2:6:6 mM, a) and **2**:**A:B** (2:6:6 mM, b) at thermodynamic equilibrium. The following symbols are used: * = **1A**, O=**1B**, ♦=**2A**, □=**2B**.

Amplification curve obtained for 2 as a function of the number of equivalents A and B.

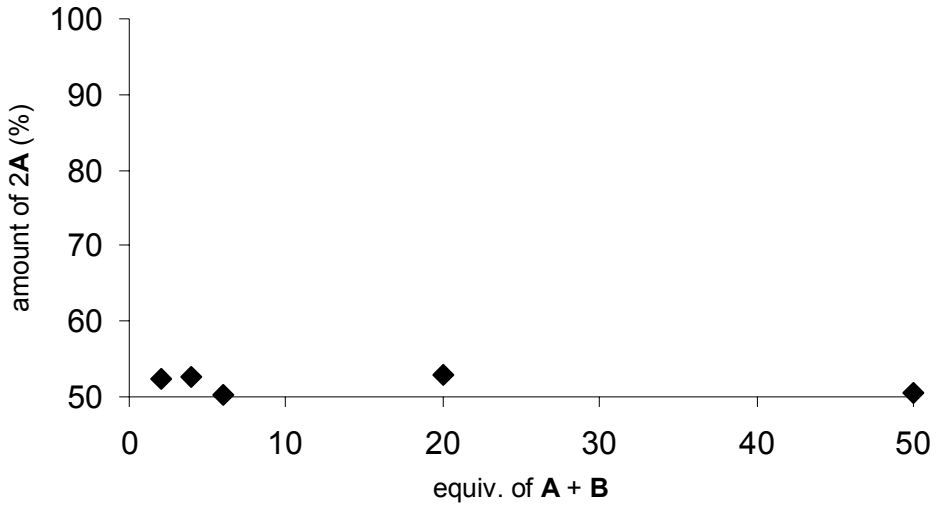


Figure SI-2. (a) Amplification of **2A** as a function of the number of equivalents of hydrazides **A** and **B** present with $[\mathbf{2}]_0$ = 2 mM.

Control experiments that prove that thermodynamic equilibrium is reached for mixtures 1A-1B and 2A-2B.

Scaffold **1** (5mM) was mixed with hydrazide **A** (5 equivalents) and kept at 50 ºC in CD_3OD till the aldehyde signal of **1** had disappeared, indicating a quantitative formation of hydrazone **1A**. At that point, hydrazide **B** (5 equivalents) were added and the mixture was allowed to equilibrate at 50 ºC in CD_3OD till no further changes in the ^1H NMR spectrum was detected (Figure SI-1a).
The experiment was repeated starting from scaffold **1** and hydrazide **B**, to which hydrazide **A** was added after formation of hydrazone **1B** was complete. The ^1H NMR spectrum (Figure SI-1b) after equilibration was identical to the one for the first experiment, proving that the distribution reflects the thermodynamic distribution.
The control experiments was also performed using the control scaffold 2-methoxybenzaldehyde **2**, giving an identical result (Figures SI-1c and SI-1d).

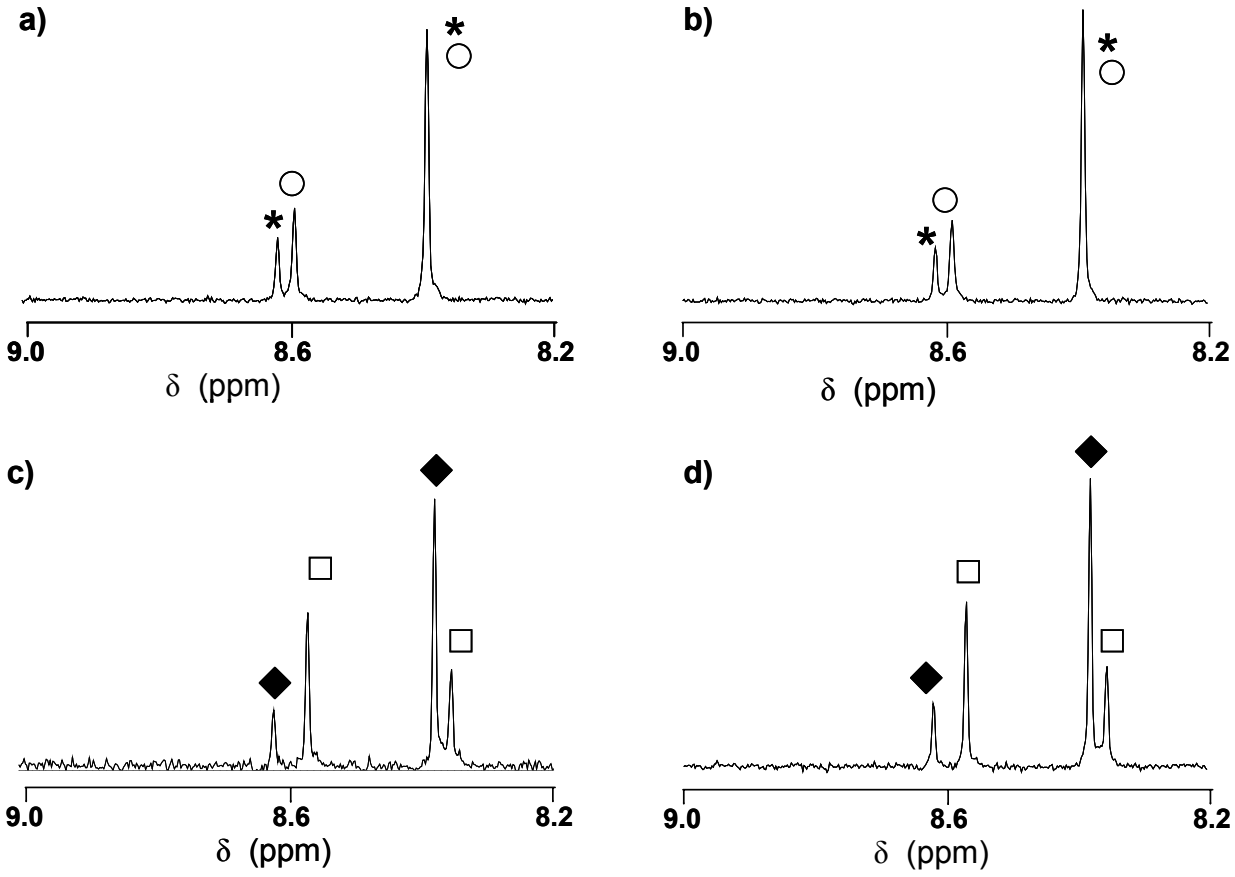


Figure SI-3 Fingerprint parts of the ^1H NMR spectra (300 MHz, CD_3OD) obtained after equilibrating the mixtures obtained by adding **B** to **1A** (a), **A** to **1B** (b), **B** to **2A** (a), and **A** to **2B** (d). The following symbols are used: * = **1A**, O=**1B**, ♦=**2A**, □=**2B**. Note that all hydrazones are present as two isomers (see before).

Fingerprint parts of the ^{1}H NMR spectra of hydrazone 1A at 2 and 25 mM.

The ^{1}H NMR spectra of hydrazone **1A** at 2 (a) and 25 (b) mM are superimposable and show no shift indicative of dimer formation.

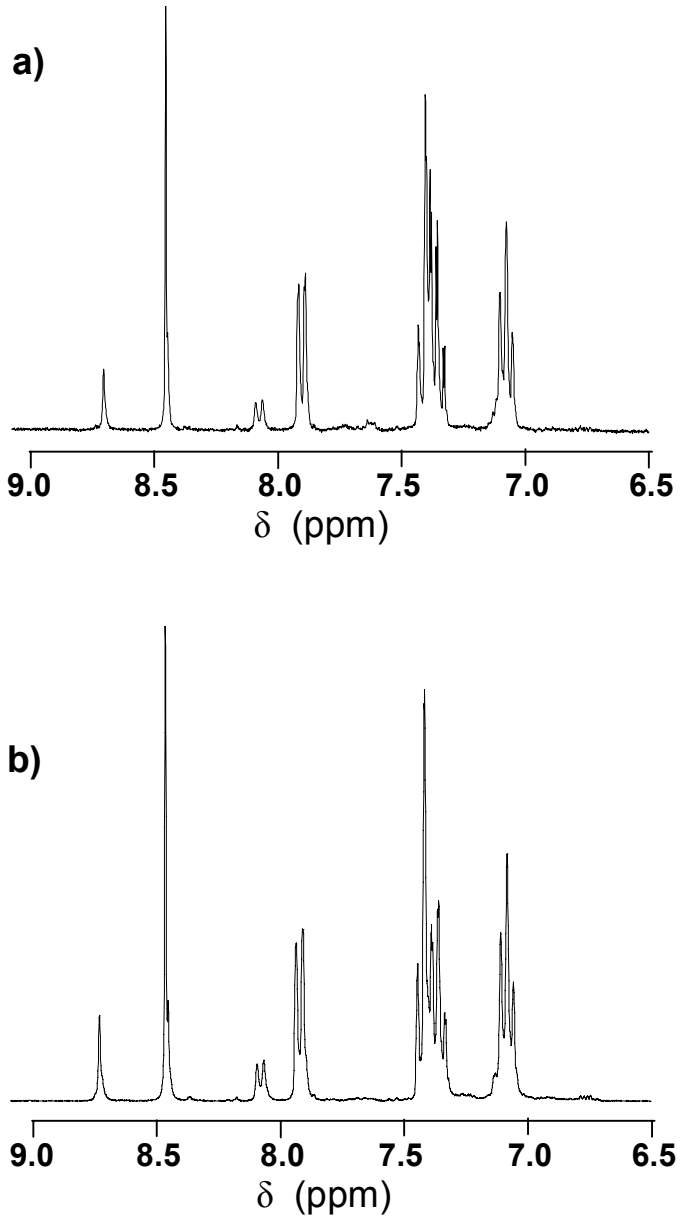
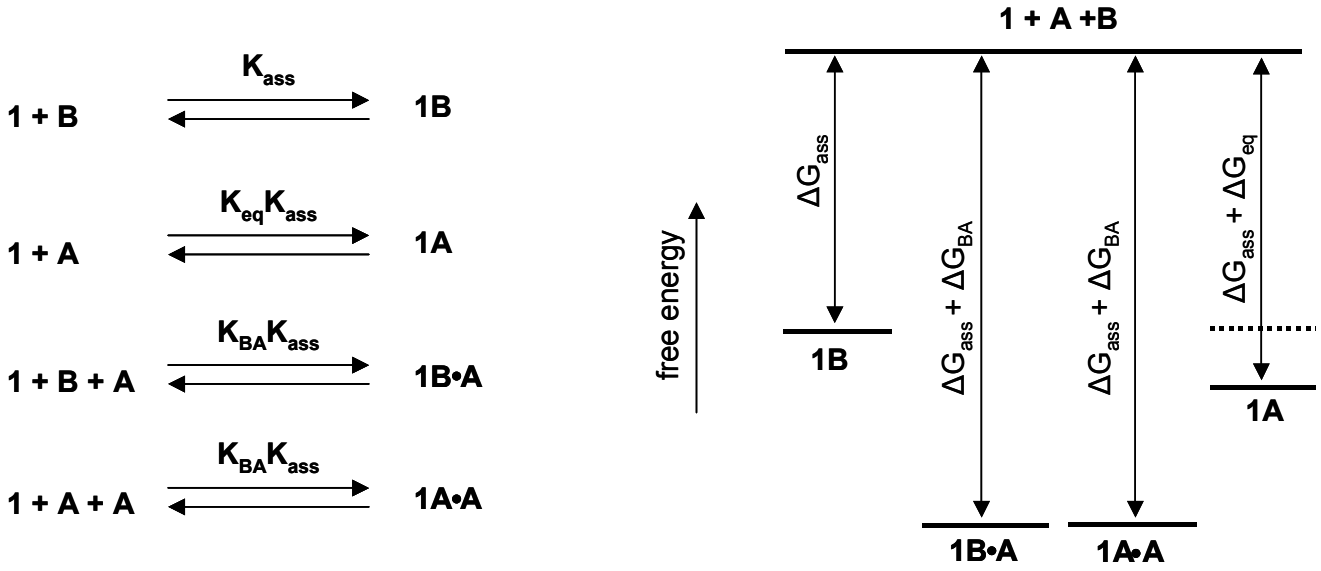


Figure SI-4 Fingerprint parts of the ^{1}H NMR spectra (300 MHz, CD_3OD) of hydrazone **1A** at (a) 2 and (b) 25 mM.

Implementation of the proposed model (Scheme 1) in MicroMath Scientist for Windows, version 2.01.

Implementation of the model in Scientist as discussed in the manuscript requires the equilibria to be rewritten as shown in Scheme SI-1. In this scheme, all species are in equilibria with the free components **1**, **A**, and **B** with the relative binding constants as shown. Since experimentally, the concentration of **1** is below the detection limit, the value of K_{ass} is given a very high value (10^8 M^{-1}). The relative stabilities of the four species present at thermodynamic equilibrium (**1A**, **1B**, **1AA**, and **1BA**) are defined by the equilibrium constants K_{eq} and K_{AB}. These parameters are used to fit the experimental data via a least-square algorithm. The Scientist model file is given below:



Scheme SI-1 Equilibria and representation of the relative energies as implemented in Scientist.

```
// MicroMath Scientist Model File
IndVars: H0
DepVars: ratio, perc, PA, PB, PAA, PBA, P, A, B, PTOT, ATOT, BTOT
Params:Kass, Keq, Kba, P0
A0=H0/2
B0=H0/2
PA=Kass*Keq*P*A
PB=Kass*P*B
PAA=Kass*Kba*P*A*A
PBA=Kass*Kba*P*A*B
P=P0-PA-PB-PAA-PBA
A=A0-PA-2*PAA-PBA
B=B0-PB-PBA
PTOT=P+PA+PB+PAA+PBA
ATOT=A+PA+2*PAA+PBA
BTOT=B+PB+PBA
ratio=(PA+PAA)/(PB+PBA)
perc=(PA+PAA)/(PA+PAA+PB+PBA)*100
//constraints
0<PA<P0
0<PB<P0
0<PAA<P0
```

0<PBA<P0
0<A<A0
0<B<B0
0<P<P0
***

with:
H0: total amount of hydrazide present
A: hydrazide **A**
B: hydrazide **B**
P: scaffold molecole **1**
PA: hydrazone **1A**
PB: hydrazone **1B**
PAA: complex **1A▪A**
PBA : complex **1B▪A**

Parameters PTOT, ATOT, and HTOT are used as controls to verify that the total concentrations of P, A, and B, remain constant during the simulation. Parameters ratio and perc are the amplification factors.