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Selectivity assessment in host-guest complexes from single crystal X-ray diffraction data: The cavitand-alcohols case.

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The determination of selectivity is central to the development of molecular receptors. Competition binding experiments based on the relative binding ratios are commonly used to evaluate relative binding constants. When more than one species binds to the same active site of a receptor, the crystallographic evaluation of the occupancy factors of each ligand could be very informative about their relative affinity. However, in presence of overlapped electron densities, the statistical occupancy factors are hard to retrieve using conventional crystal structure refinement. Here we present an original method to evaluate the relative binding constants based on the direct treatment of the diffraction intensities obtained from isomorphous single crystals grown in presence of binary mixtures of competitive ligands. This method was developed and first applied to evaluate the affinity of a tetraphosphonate cavitand receptor towards short chain alcohols. In the easier cases, wherein the electron densities of the two alcohols are less overlapped, the occupancy factors for guest molecules obtained in conventional structural refinement are in good agreement with the values calculated from the direct comparison of diffraction intensities. The affinity constants were estimated from the calculated occupancy factors, considering the molar ratios of alcohols used in the competition experiments. The congruence of the method has been tested on several binary mixtures using different concentration ratios of alcoholic guests. In general, for a given alcoholic pair the different molar ratio of alcohols, used in the crystallization batch, produces a trend of the occupancy factors in agreement with the binding constants ratio of two alcohols. From 32 data sets collected at the Elettra synchrotron for six short alkylic chain guest molecules, we evaluated the binding constant ratios with a good internal consistency. The relative binding constants for these six alcohols were evaluated from the entire statistical sample (EtOH, 8.8 > 1-PrOH, 2.2 > MeOH, 1.3 > 2-PrOH, 1.00 > 2-BuOH, 0.32 > 1-BuOH, 0.11, using 2-PrOH as reference with arbitrary value of 1), and are in good agreement with the structural parameters of host-guest interactions observed in the corresponding crystal structures. In particular, the binding constant decreases with the increasing of the host-guest H-bond distance, which follows the increase of the length of the alkyl chain of the alcoholic guest. Moreover, quartz crystal microbalance (QCM) measurement and fluorescence data have been compared and discussed with respect to the relative affinity scale obtained by crystallography.

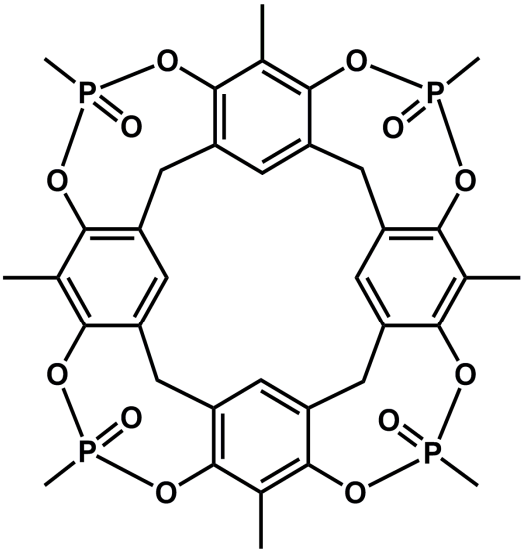
Introduction.

In the last two decades, a remarkable number of synthetic receptors has been designed and prepared for recognition and binding of molecules through non-covalent interactions. Among these, cavitands,[1] synthetic organic compounds with rigid cavities, are extremely interesting and versatile for host-guest complexation.[2-6] The selectivity of the receptor is achieved when affinities for different guests are sensibly different and the binding occurs preferentially with a given guest. Compared with similar molecules having the same functional groups but more flexible structures, the cavitand preorganization adds a determining energetic component to the possibility to form stable and specific complexes, using non-covalent interactions.[2,7-10] Cavitands are also attractive for the wide choice of bridging groups, which affect the properties of the cavity.[11] Bridging groups, indeed, not only influence cavitands solubility,[12,13] but have been also proven to play a determining role in stabilizing transition states when the receptor is applied in catalytic processes, mimicking enzymes.[14] Cavitands featuring phosphonate bridging groups at the upper rim have been considered particularly as receptors for both

cationic[15] and neutral[16] guests due to the basic properties of the oxygen atom. Regarding the latter class of guests, a careful and clever choice of the upper rim substituents allows to obtain interesting and promising results for selective molecular recognition of short-chain alcohols. In particular, a new solid-state fluorescent sensor based on a phosphonate cavitand for selective detection of short-chain alcohols in the gas phase has been recently reported.[17]

The determination of binding constants is very important to the study of selectivity of molecular receptors. Competition binding experiments based on the relative binding ratio are a useful method to evaluate selectivity of the receptors towards different ligands. Competition between different ligands is a difficult issue to address in many complexation reactions, in the field of supramo-lecular chemistry, i.e. host-guest complexes such as those considered in this study. Generally, the widely applied analytical tools for studying competitive phenomena of non-covalent binding are electrospray ionization mass spectrometry (ESI-MS),[18-24] for measurements in gas phase, and NMR techniques,[25-27] in solution. However, practical application of supramolecular receptors, such as chromatographic applications or sensing devices, are applied at the gas-solid or liquid-solid interfaces. Thus, a more thorough characterization in the solid phase is yet an unfulfilled goal. To the best of our knowledge, there are few examples of competitive binding studies in single crystals.[28-30]

In the solid state, it is quite common to obtain isomorphous crystals from complexes of the same receptor with different guests. This opportunity is usually fulfilled when the binding site is buried in the center of the receptor and the chemical properties of the ligands, such as charge, hydrophobic-hydrophilic properties, etc., are similar. In this case, ligands have an analogous binding mode, a similarly small influence on the crystal packing and, therefore, isomorphous crystals grow even in presence of a mixture of ligands. For such receptor-guest systems, crystallographic competitive-binding studies are very informative about the different affinities, linking the differences in binding strengths of the guests to subtle differences in their mode of binding. However, the crystallographic determination of the occupancy factors of the ligands is often hampered by the electron density overlap in the binding site with an inherent difficulty in the identification of the ligand features. Moreover, refinement of the structural model by the conventional full-matrix least-squares method is affected by the high correlation between the occupancy factors and the thermal parameters of atoms statistically disordered and overlapped in the same site. To overcome this problem, we developed a novel method that does not require the structural refinement of the crystallographic data, but allows the determination of occupancy factors from the direct evaluation of the diffracted intensities of different crystals. For this study of binding competition in crystals, the cavitand Tiiii[H,CH_3,CH_3][31] **1** (Figure 1) was used as model system. The characteristic of this cavitand make it a very suitable candidate for the present crystallographic study. In particular, all Tiiii[H,CH_3,CH_3] complexes with alcoholic guests easily crystallize from 2,2,2-trifluoroethanol (TFE) solutions and give the same isomorphous monoclinic crystal form, previously characterized by X-ray diffraction (Figure 2).[17]



Tiiii[H,CH_3,CH_3]

Figure 1. Tetraphosphonate cavitand Tiiii[H,CH_3,CH_3] **1** used in the described competition experiments.

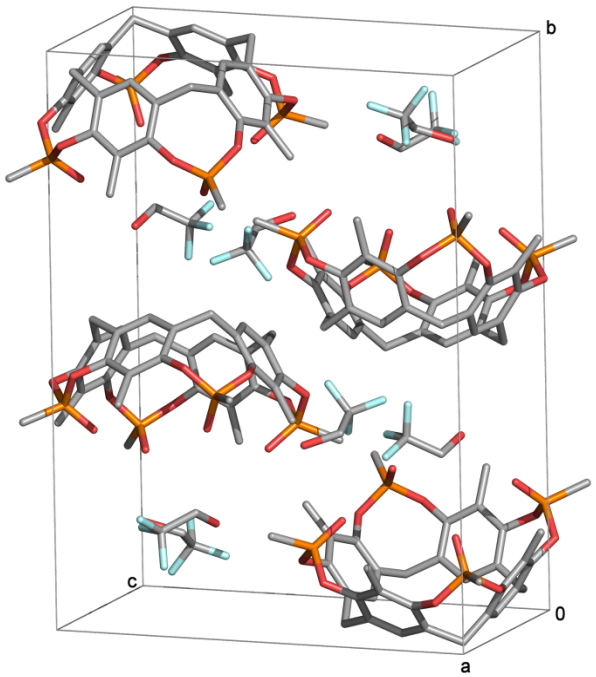


Figure 2. Unit cell of the isomorphous monoclinic crystal form of the cavitand Tiiii[H,CH_3,CH_3], crystallized from a TFE solution in presence of alcoholic guests. The guest molecules are omitted from the picture to underline the constant part of the structure.

Experimental

Co-crystallization competition experiments in micro-batch and data collections.
The synthesis of the phosphonate cavitand **1**, with the P=O groups pointing inward the cavity, was performed as previously described.[32] In order to study the complexation properties of **1** towards short alkyl chain alcohols, crystals were grown in presence of a single alcoholic guest through the vapour diffusion method, in a sitting drop configuration.[17] A similar set up was used for the competition experiments, wherein crystals of **1** were grown in presence of binary mixtures of alcohols in 24-well Linbro plates. As a general procedure, 7 μL of a 8 mM solution of **1** in TFE were sit on a well and 7 μL of the reservoir solution were added. The reservoir solution consisted of 50% TFE v/v and a mixture of two short alkyl-chain alcohols (C_1-C_4) at different molar fractions. In binary mixtures, the volume of each alcohol introduced in the reservoir was determined considering its density and molecular weight, so that the molar fractions were fixed. Particular care was taken in avoiding evaporation of the alcoholic guests from the reservoir solution and Linbro wells were immediately sealed. Evaporation was estimated to be negligible. Data sets were collected to a resolution of 0.85 Å at 100 K using synchrotron radiation (0.9 Å wavelength) and a 165 mm diameter MAR CCD detector at the XRD1 Elettra beamline (Trieste, Italy). Routinely, the crystal dipped in Paratone, as cryoprotectant, was mounted in a loop and flash-frozen by a liquid nitrogen stream. For each data set 60 frames were collected while the crystal rotated of 3°/frame. The detector was located at a fixed distance of 40 mm from the crystal, the minimum allowed by the geometry of the goniometer, in order to increase the maximum diffraction angle collected. Due to the statistical method applied (see below), which requires the highest possible number of observations, the diffraction experiments were set up in order to maximize the number of collected reflections.

Scaling protocol and statistical analysis.
Unit cell dimensions for the isomorphous monoclinic crystals containing mixtures of alcoholic guests are shown in Table S1. Data reduction was performed using MOSFLM[33] and SCALA.[34,35] Since the analysis we performed required the direct comparison of diffraction intensities from different datasets (see below), a specific scaling protocol was applied to obtain on a common scale all datasets. To this purpose a reference dataset was selected. The selection criterion was based on the scaling statistics of each data collection. In particular, the data collection with the best completeness and R_{merge} value was obtained from the crystal grown in presence of 2-PrOH, and thus chosen as the reference dataset for the scaling protocol. A common scaling protocol was applied to each data collection, against this reference dataset. The scale factors were calculated for each image (BATCH option in Scala) and the independent scaling *B* factors were evaluated for batches of 10 images (30° of rotation of the crystal). Moreover, secondary beam corrections expanded to the sixth-order spherical harmonics were applied. In this scaling protocol, the scale constants can be affected by very large differences in the diffraction intensities of isomorphous crystals, such as those between intensities that are mostly influences by the guest molecule. To prevent errors in the scale factors due to this effect, reflections, with a standard deviation of the mean intensity (reference and scaled datasets) 12 times greater than the standard deviation of the reflection intensity, have been omitted from the scale constants calculations. Each dataset was then merged independently using the values of the scale factors as calculated before and rejecting 4σ outliers. Thus, the final values of the standard deviations are based only on the data collected on the single crystal, while the scale factors maintain the merged dataset on the same scale of the reference dataset.

Crystal structure determination.
In order to validate the statistical method applied to the diffraction intensities, data from different crystals were refined with the conventional methods. For each dataset, the crystallographic structure of the complex between the host and a single guest or two different guests were obtained by direct refinement of a starting model constituted by the coordinates of the empty cavitand. Atoms of the guests were added to the model after the first refinement cycles, to account for the residual electron density observed in the cavity of the host. The refinements were performed using SHELX-97[36] and by direct analysis of the Fourier maps. All non-hydrogen atoms were refined anisotropically and hydrogen atoms were added to the model at calculated positions (*riding model*). As expected, during the refinement cycles severe problems due to the correlation between the occupancy factor of the disordered alcohols and their thermal parameters were encountered. The occupancy factors of the hosted alcohols were then estimated by isotropic refinement of their thermal parameters. For some datasets, a severe overlap of the electron densities of the alcoholic guests was observed. Therefore, it was necessary to introduce some geometrical constraints on bond distances and angles to better define the alcohol geometry. A similar treatment was applied in cases with low populated guest. Nevertheless, a significant inconsistency in the guest thermal parameters was generally observed in the final cycles. The occupancy factors obtained with the conventional refinement are reported in Table S2.

Selectivity constants evaluation from crystallographic data.
Due to the superposition of the electron densities of the alcoholic guests in the cavitand site (Figure 3), the determination of the occupancy factors of guest molecules was very problematic. Thus, the determination of the latter has proven to be unreliable in some cases and a new approach was developed. In this approach, the occupancy factors of the guest molecules are evaluated from the simultaneous analysis of the diffraction intensities of more than one dataset.
For a generic reflection with Miller indexes *h k l*, the structure factor can be factorized in:

$$F_{hkl} = F_{hkl}^{cav,TFE} + F_{hkl}^{guest} \quad (1)$$

with $F_{hkl}^{cav,TFE}$ expressing the constant component of the structure factor related to the cavitand and solvent atoms and F_{hkl}^{guest} representing the component related to the guest atoms.
The structure factors $F_{hkl}^{(A)}$, for a crystal containing only an alcoholic guest *A*, and $F_{hkl}^{(B)}$, for a crystal containing only an alcoholic guest *B*, can be written as:

$$F_{hkl}^{(A)} = F_{hkl}^{cav,TFE} + F_{hkl}^{A} \quad (2)$$

and

$$F_{hkl}^{(B)} = F_{hkl}^{cav,TFE} + F_{hkl}^{B} \quad (3)$$

respectively.
Considering a crystal in which two alcohols, *A* and *B*, compete for the cavitand site, a generic structure factor can be expressed as:

$$F_{hkl}^{(A,B)} = F_{hkl}^{cav,TFE} + x^{A}F_{hkl}^{A} + y^{B}F_{hkl}^{B} \quad (4)$$

where x^A equals to the occupancy factor of the alcohol *A* in the cavitand site, whereas y^B is the occupancy factor of the guest *B*. The crystal structures of the pure complexes[19] suggest that the fraction of

empty cavitand can be neglected due to the high affinity of the cavitand for the alcoholic guests, thus, the statistical population of the alcohol *B* in the cavitand site can be expressed as $y^B = (1 - x^A)$:

$$F_{hkl}^{(A,B)} = F_{hkl}^{cav,TFE} + x^A F_{hkl}^A + (1 - x^A) F_{hkl}^B \quad (5)$$

From the expressions (2), (3) and (5), the value of x can be calculated as:

$$x_{hkl}^A = \frac{F_{hkl}^{(A,B)} - F_{hkl}^{(B)}}{F_{hkl}^{(A)} - F_{hkl}^{(B)}} \quad (6).$$

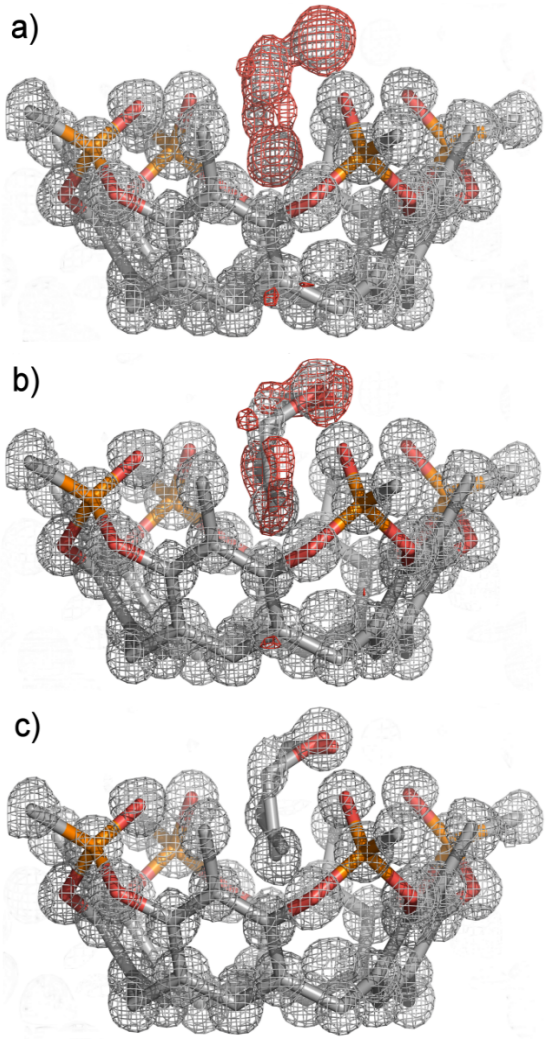


Figure 3: Detail of electron density maps (in grey, contour level: 1σ) and residual electron density maps (in red, contour level: 4σ) in the cavitand site, for a crystal structure of the cavitand **1** and a mixture of guests ethanol and 1-propanol. (a) Maps obtained after refinement without alcoholic guests. (b) An ethanol molecule was fitted in the electron density, but a residue was still observed. A 1-propanol molecule was fitted in the further residual electron density. (c) In the final structure the overlap between the electron densities of the two alcoholic guests is clearly observed.

The anomalous dispersion component for these crystals is small and can be neglected. Therefore, the structure factor F_{hkl} can be considered as a real number for these centrosymmetric structures. The modulus of F_{hkl} is proportional to the square root of the diffraction intensities, while its sign depends on the phase, which can be either zero or π.

Since the number of electrons of the cavitand and TFE molecules is by far greater than the number of electrons of the guest molecule, the sign of the structure factor is mainly due to the contribution of the constant part of the isomorphous structures. The structure factor can be approximated with the square root of the experimental diffraction intensity, measured for crystals containing only *A* ($I_{hkl}^{(A)}$), only *B* ($I_{hkl}^{(B)}$) and both guests ($I_{hkl}^{(A,B)}$):

$$x^A \approx \frac{\sqrt{I_{hkl}^{(A,B)}} - \sqrt{I_{hkl}^{(B)}}}{\sqrt{I_{hkl}^{(A)}} - \sqrt{I_{hkl}^{(B)}}} \quad (7).$$

By direct measurement of diffraction intensity from a crystal obtained by co-crystallization of the cavitand in presence of guest *A*, guest *B* and a binary mixture *A,B*, the last expression allows the determination of the occupancy factor of the guest *A* in the cavitand site. Expression (7) allows to calculate a value of the occupancy factor *x* for each triplet of *h k l* indexes. A statistical analysis of the calculated values *x* was performed to obtain a final occupancy value. In the protocol applied for the statistical analysis, only the reflections *h k l* for which the intensities were available for all three data sets (*A*, *B*, and *(A,B)*) were considered. The standard deviations of intensities have been taken into account to evaluate a weight factor in the statistical treatment. The standard deviation of the occupancy factor was calculated, for each *h k l* triplet, from the standard deviation of the corresponding intensities:

$$\sigma_{x_{hkl}^A} = \frac{\sqrt{\frac{\sigma^2_{I_{hkl}^{(A,B)}}}{4I_{hkl}^{(A,B)}}\left(\sqrt{I_{hkl}^{(A)}} - \sqrt{I_{hkl}^{(B)}}\right)^2 + \frac{\sigma^2_{I_{hkl}^{(B)}}}{4I_{hkl}^{(B)}}\left(\sqrt{I_{hkl}^{(A,B)}} - \sqrt{I_{hkl}^{(A)}}\right)^2 + \frac{\sigma^2_{I_{hkl}^{(A)}}}{4I_{hkl}^{(A)}}\left(\sqrt{I_{hkl}^{(B)}} - \sqrt{I_{hkl}^{(A,B)}}\right)}}{\left(\sqrt{I_{hkl}^{(A)}} - \sqrt{I_{hkl}^{(B)}}\right)^2} \quad (8).$$

Afterwards, weight factors were calculated for the values obtained for the statistical occupancy factors x_{hkl}:

$$w_{hkl} = \frac{1}{\sigma^2_{x_{hkl}}} \quad (9).$$

The weighted average was obtained from occupancy factors calculated on all available *h k l* reflections:

$$x_w^A = \frac{\sum_{hkl}\left(w_{hkl} \cdot \frac{\sqrt{I_{hkl}^{(A,B)}} - \sqrt{I_{hkl}^{(B)}}}{\sqrt{I_{hkl}^{(A)}} - \sqrt{I_{hkl}^{(B)}}}\right)}{\sum_{hkl} w_{hkl}} \quad (10).$$

The standard deviation of the weighted average was calculated as:

$$\sigma_{x_w^A} = \sqrt{\frac{\sum_{hkl} w_{hkl} \cdot \left(x_{hkl}^A - x_w^A\right)^2}{(N-1) \cdot \sum_{hkl} w_{hkl}}} \quad (11)$$

where *N* is the total number of reflections.

Values obtained for the occupancy factors for the crystals of Tiiii[H,CH_3,CH_3]-alcohol complexes are reported in Table 1. Three co-crystallization experiments were repeated (MeOH/EtOH 0.4:0.6,

EtOH/2-PrOH 0.6:0.4, 1-PrOH/2-PrOH 0.4:0.6) twice to verify the reproducibility of the method.

Table 1. Values of the occupancy factors x_w^A (%) of alcohols in the cavitand binding site, evaluated using the described statistical method, for each pair of alcoholic guests and for each molar ratio χ_A of the first guest in the crystallization solution. The ratios K_A/K_B between complexation constants were calculated using the occupancy factors.

A	*B*	χ_A	$x_w^A \pm \sigma_{x_w^A}$	$K_A/K_B \pm \sigma_{K_A/K_B}$
MeOH	EtOH	0.4	16.31 ± 0.23	0.292 ± 0.003
		0.4	11.44 ± 0.23	0.194 ± 0.003
		0.6	11.99 ± 0.24	0.091 ± 0.001
		0.8	33.72 ± 0.23	0.127 ± 0.001
MeOH	1-PrOH	0.2	22.99 ± 0.20	1.194 ± 0.014
		0.4	36.24 ± 0.24	0.852 ± 0.009
		0.6	35.14 ± 0.23	0.361 ± 0.004
		0.8	66.20 ± 0.32	0.490 ± 0.007
MeOH	2-PrOH	0.2	35.50 ± 0.26	2.201 ± 0.019
		0.4	67.01 ± 0.16	3.047 ± 0.025
MeOH	1-BuOH	0.2	77.18 ± 0.37	13.525 ± 0.291
EtOH	1-PrOH	0.2	55.50 ± 0.25	4.989 ± 0.054
		0.4	77.04 ± 0.21	5.033 ± 0.063
		0.6	83.20 ± 0.22	3.300 ± 0.056
EtOH	2-PrOH	0.2	75.30 ± 0.26	12.191 ± 0.180
		0.4	89.12 ± 0.23	12.285 ± 0.295
		0.6	87.69 ± 0.24	4.749 ± 0.109
		0.6	93.28 ± 0.23	9.251 ± 0.342
1-PrOH	2-PrOH	0.2	38.22 ± 0.17	2.475 ± 0.020
		0.4	58.03 ± 0.26	2.074 ± 0.023
		0.4	63.21 ± 0.19	2.578 ± 0.023
		0.6	76.88 ± 0.17	2.217 ± 0.025
		0.8	85.88 ± 0.24	1.520 ± 0.031
1-PrOH	1-BuOH	0.2	82.19 ± 0.29	18.459 ± 0.377
1-PrOH	2-BuOH	0.2	62.13 ± 0.20	6.562 ± 0.073
		0.4	83.35 ± 0.24	7.510 ± 0.081
2-PrOH	1-BuOH	0.2	56.34 ± 0.24	5.161 ± 0.054
		0.6	96.11 ± 0.13	16.478 ± 0.568
2-PrOH	2-BuOH	0.2	52.47 ± 0.23	4.415 ± 0.044
		0.4	69.81 ± 0.19	3.468 ± 0.034
1-BuOH	2-BuOH	0.4	19.21 ± 0.23	0.357 ± 0.005
		0.6	46.36 ± 0.27	0.576 ± 0.007

Considering the host-guest complexation reactions and their equilibrium constants:

$$T_{iiii} + A \xrightarrow{K_A} T_{iiii} \subset A \qquad K_A = \frac{[T_{iiii} \subset A]}{[T_{iiii}]\cdot[A]}$$
$$T_{iiii} + B \xrightarrow{K_B} T_{iiii} \subset B \qquad K_B = \frac{[T_{iiii} \subset B]}{[T_{iiii}]\cdot[B]} \quad (12)$$

(13)

where [*A*] and [*B*] are the concentrations of the free alcohols in the crystallization drop solution, [T_{iiii}] is the concentration of the free cavitand in the crystallization solution and [$T_{iiii} \subset A$] and [$T_{iiii} \subset B$] are the concentrations of the complexes formed by the cavitand with the alcohol *A* and *B*, respectively, the ratio between the equilibrium constants can be calculated as:

$$\frac{K_A}{K_B} = \frac{[T_{iiii} \subset A]\cdot[B]}{[A]\cdot[T_{iiii} \subset B]} \quad (14).$$

Two hypotheses were considered in the following treatment. (1) The ratio between the free alcohol concentrations in the crystallization drop was assumed to be the same as in the reservoir solution: [B]/[*A*] = $\chi_B/\chi_A = (1-\chi_A)/\chi_A$, where χ_A and χ_A are the molar fractions of alcoholic guests *A* and *B* in the reservoir solution. This consideration is acceptable considering that the drop was obtained by mixing the host solution with the reservoir solution and that, after equilibration of the crystallization batch, the molar fractions in the drop are very close to those in the reservoir solution. Furthermore, considering the volatility of the components, the equilibration process is very fast. (2) The nature of the alcoholic guest has a small influence on the crystallization process of these isomorphous crystals, as discussed above, i.e. the solubility of the two complexes is similar.

Since for each complex the concentration is proportional to the occupancy factor of the alcoholic guest in the cavitand site, $[T_{iiii} \subset A] \propto x_w^A$ and $[T_{iiii} \subset B] \propto y_w^B = 1 - x_w^A$, the ratio between complexation constants of the two alcohols can be calculated for all structures containing binary mixtures of guests:

$$\frac{K_A}{K_B} = \frac{x_w^A}{(1-x_w^A)} \cdot \frac{(1-\chi_A)}{\chi_A} \quad (15).$$

Errors on these selectivity constants were estimated as:

$$\sigma_{K_A/K_B} = \left(\frac{K_A}{K_B}\right) \cdot \sqrt{\frac{\sigma_{x_w^A}^2}{(1-x_w^A)^2 (x_w^A)^2} + \frac{\sigma_{V_A}^2}{V_A^2} + \frac{\sigma_{V_B}^2}{V_B^2}} \quad (16),$$

where V_A and V_B are the volumes of each alcohol dispensed in the reservoir of the crystallization experiment, while σ_{V_A} and σ_{V_B} are the standard deviations on these values. The standard deviations on the volumes of alcoholic guest in the reservoir solution were obtained considering the random errors calculated for the automatic pipettes used during the experimental set up. In particular, depending on the volume of the alcohol required in each crystallization trial, two kinds of pipettes were used: a *Gilson-P200* pipette for volumes between 50 µL and 200 µL (average error: 0.25 µL) and a *Gilson-P1000* pipette for volumes between 201 µL and 500 µL (average error: 1 µL).

A software in Basic was developed to calculate the occupancy factors and to estimate their standard errors from the scaled and merged reflections, as described above. Using the molar ratio of the guest molecules, the software calculates also the selectivity constants, as ratios between complexation constants, and their estimated errors.

Scale of relative binding affinities.

A scale of relative affinity constants of cavitand towards the six selected alcohols has been obtained by a weighted combination of

the overall values of constant ratio K_A/K_B calculated for the 32 experiments. The values of affinity constant, K_A are normalized with respect to the affinity constant of 2-PrOH (already already used as the reference alcohol in the scaling protocol of the diffraction data), according to the equation (17):

$$K_A = K'_A * \frac{1}{K'_{2-\mathrm{Pr}OH}} \qquad (17)$$

where K'_A and $K'_{2-\mathrm{Pr}OH}$ are the weighted mean of the affinity constants for the generic alcohol *A* and for the reference 2-propanol, calculated as:

$$K'_A = \frac{\sum_{i=1}^{n} w_i (K_B^0 * K_{AB_i})}{\sum_{i=1}^{n} w_i} \qquad (18)$$

where the summation extends over all ***n*** constants derived from crystals containing the alcohol A with various competitive guest B in different concentration ratios, K_{AB_i} is the K_A/K_B ratio obtained from the statistical analysis of diffraction intensities from the ***i*** crystal, K_B^0 is the normalized affinity constant calculated (eq. 17) for the various alcoholic species *B* in the previous cycle of the iterative procedure (unity values in the starting cycle) and w_i is the weight factor calculated according to the equation:

$$w_i = \frac{1}{(K_B^0 * \sigma_{K_{AB_i}})^2 + (K_{AB_i} * \sigma_{K_B^0})^2} \qquad (19)$$

where $\sigma_{K_{AB_i}}$ is the standard deviation of the K_{AB_i} and $\sigma_{K_B^0}$ is the standard deviation of the normalized affinity constant for species *B* calculated in the previous cycle of the iterative procedure.
The standard deviation of the relative affinity constant is calculated as:

$$\sigma_{K_A} = \sqrt{\frac{\sum_{i=1}^{n} w_i [(K_{AB_i} * K_B^0) - K_A^0]^2}{(n-1)\sum w_i}} \qquad (20)$$

where K_A^0 is the value of the normalized affinity constant obtained in the previous cycle.
The K_A^0 and the corresponding sigma values are then replaced by the calculated K_A and σ_{K_A} values, in iterative cycles till reaching convergence. Typically, in the initial cycles the normalized relative constants K_A start to oscillate with decreasing oscillation amplitudes and in about 5 cycles assume constant values, having differences from the previous cycle within the standard deviations. The final K_A values are reported in Table 2 and plotted in the histogram shown in Figure 4.

Table 2: Values of the relative binding affinity constants for the cavitand Tiiii[H,CH_3,CH_3], calculated for each alcoholic guest and relevant geometric parameters describing the host-guest interaction [Å] in the alcohol-cavitand complexes.[a]

Guest	K_A	H-bond	O_{alc}···oop	$CH_{3\ alc}$···oop
MeOH	1.3 ± 0.2	2.704(4)	0.039(3)	-1.264(4)
EtOH	8.8 ± 0.9	2.765(9)	1.150(3)	-1.028(4)
1-PrOH	2.2 ± 0.2	2.730(3)	1.409(3)	-1.341(4)
2-PrOH	1.00 ± 0.08	2.816(9)	0.926(3)	-0.964(4)
1-BuOH	0.11 ± 0.02	2.85(1)	1.926(7)	-1.521(7)
2-BuOH	0.32 ± 0.03	2.75(1)	1.357(8)	-1.43(1)

[a] Hydrogen-bond distances ($O_{alcohol}\cdots O{=}P$) and distances of the hydroxyl and methyl groups from the mean plane of oxygen atoms of P=O groups (out-of-plane, oop) are given. Atoms inside the cavity have a negative sign for the oop distances.

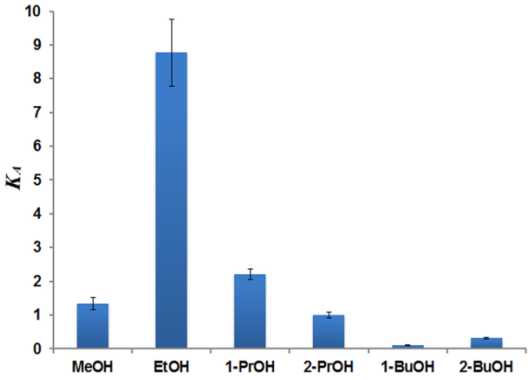


Figure 4. Relative binding affinity constants of C_1-C_4 alcohols for the cavitand Tiiii[H,CH_3,CH_3].

Results and discussion

Cavitand **1**, with the P=O groups pointing inward the cavity, easily crystallizes in few minutes by sitting drop vapour diffusion method from TFE solutions in presence of a short chain linear and branched alcohols. The isomorphous monoclinic crystals (Space Group $P2_1/c$, a = 12 Å, b = 22 Å, c = 18 Å, β = 104°) of six different inclusion complexes, i.e. with MeOH, EtOH, 1-PrOH, 2-PrOH, 1-BuOH and 2-BuOH, were obtained and the molecular structures are shown in Figure 5. Crystallographic analysis showed that they have the same asymmetric unit composition: a single host-guest complex and two co-crystallized solvent molecules of TFE.[17] The whole series of host–guest complexes presents the same pattern of intermolecular interactions: a H-bond between the OH group of the guest and the P=O moieties of the cavitand, and several CH···π contacts between a methyl group of the alkyl chain of the guest and the aromatic host cavity. The relevant geometric parameters describing the host–guest interaction are reported in Table 2. It is worth noting that in the case of the longest guest, 1-BuOH, the simultaneous formation of both interactions, H-bond and CH···π contacts, requires a deeper insertion of the alcohol methyl group into the host cavity. Due to steric hindrance of the cavitand lower rim, the alkyl group protrudes from the cavity, weakening the H-bond with the upper rim. When even a single methylene unit is added to the alkyl chain of alcohol, the

matching in size and shape between host and guest is dramatically modified, as confirmed by crystallization trials with 1-pentanol. In this case, the formation of the host-guest complex in the solid state was completely inhibited.[17]

The two TFE solvent molecules were found in the same positions in all the isomorphous structures, supporting the idea that, even if not completely buried in the receptor site, the guests are 'shielded' by solvent molecules in a similar way, thus giving a comparable contribution to the packing interactions. On the other hand, the isomorphism of the crystals highlights that the crystal packing has the same influence on the analysed structures and the small differences in the intermolecular contacts are not to be considered as determining factors in the formation and crystallization of the host-guest complexes. Therefore, the phenomenon of molecular recognition of tetraphosphonate cavitands towards short chain alcohols can be analyzed by single crystal X-ray diffraction of complexes obtained by experiments of binding competition. This crystallographic study allows the evaluation of the relative affinity constants of the artificial receptor **1** towards different alcohols. In particular, the occupancy factors of the guests in the receptor site of an isomorphous crystal grown in presence of a pair of guests, can be used to estimate the binding constants ratio of the guests for the receptor site. The following analysis starts from the hypothesis that the statistical occupation of the cavitand site is only dependent on the affinity of the cavitand for the guests and on the relative concentration of the two alcohols introduced in the crystallization solution, and not by crystallization related effects. Moreover, due to the high affinity of this receptor for alcoholic guests, the concentration of unbound cavitand is considered to be negligible.

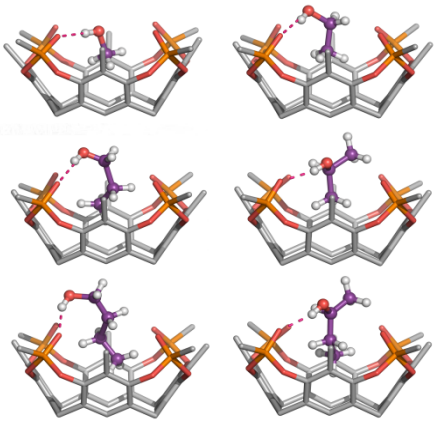


Figure 5. Side view of the six host-guest complexes of cavitand Tiiii[H,CH_3,CH_3].

For a systematic study of the competition between different guests in the cavitand site, a micro-batch procedure has been implemented in order to co-crystallize **1** in presence of binary mixtures of alcohols with different molar ratios.

The relative affinity of a pair of guests is related to the occupancy factors for each guest in the host cavity, for a crystal grown in presence of both alcohols. However, the conventional structure refinement methods show very high correlations between *B*-thermal factors and occupancy factors of disordered guests located inside the cavity, reflecting the strong overlap of the electron densities of alcohols observed in the Fourier maps. For this reason an original method has been developed to evaluate the selectivity constants from a direct statistical treatment of diffraction intensities obtained from isomorphous single crystals grown in presence of binary mixtures of competitive ligands, and from crystals grown in presence of the single guest molecules.

In order to validate the novel statistical method, the structures of 32 crystals of the cavitand with two alcoholic guests were refined with the conventional methods, determining the occupancy factor for each alcohol. The results of the refinement, shown in Table S2, are in good agreement with the occupancy factors directly obtained from the diffraction intensities for structures where the electron densities of the alcohol pair are not dramatically overlapped, i.e. for the pair MeOH:2-PrOH with molar ratio 0.20:0.80 (Figure S1). On the contrary, when the electron densities of the alcohols strongly overlap, a larger disagreement has been observed between the occupancy factors calculated with the two methods. Moreover, a substantial predominance of one alcoholic guest over the other often leads to inconsistent values of the thermal parameters in the structural refinement. The average difference of 0.13 between the occupancy factors determined by structural refinement and those calculated by statistical comparison of diffraction intensities for the 32 structures evidences a substantial general agreement between the two methods.

With the aim of verifying the reproducibility of the method, three co-crystallization experiments of the cavitand **1** were repeated twice. The three independent statistical analyses were conducted on the diffraction intensities collected from these crystals, and the occupancy factors show an average difference of 0.05, indicating a good reproducibility.

Furthermore, to verify the crucial hypothesis that the occupancy factors are proportional to the relative affinity of the cavitand towards alcohols, and that the crystal packing has a negligible influence, the method has been tested on several binary mixtures with different ratios of alcoholic guests. In general, for a given alcoholic binary mixture, different molar ratios of alcohols in the crystallization trials yield different occupancy factors in agreement with a given binding constants ratio of two alcohols (Table 1). In particular, an excellent linear correlation between the ratio of occupancy factors and the ratio of molar fractions is observed for the alcoholic mixture 1-PrOH / 2-PrOH (Figure 6).

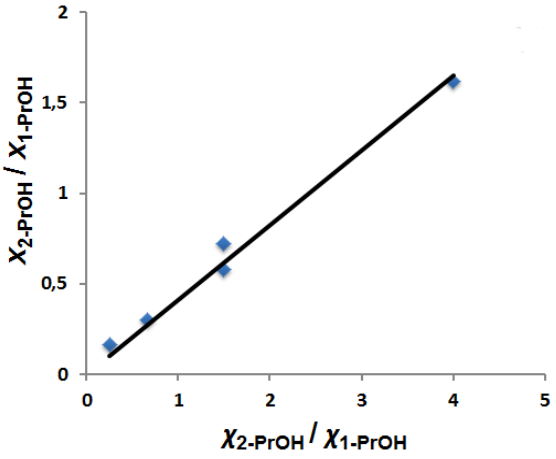


Figure 6. Correlation between the ratio of occupancy factors and the ratio of molar fractions for the alcoholic mixture 1-PrOH / 2-PrOH. The linear regression equation is $x_B / x_A = 0.413\ \chi_B / \chi_A$ with $r^2 = 0.986$.

A total of 32 data sets were collected at Elettra synchrotron from crystals of the cavitand **1** with six alcohols with short alkyl chains. The computed ratios between the complexation constants for each alcoholic pair are reported in Table 1 and show a good internal

consistency. In all mixtures containing ethanol, this alcohol results the favorite guest. A similar trend is observed for 1-propanol, except for the mixture containing ethanol. Conversely, the alcohol with the longest alkyl chain 1-butanol, is the guest with the lower affinity for the receptor cavity, showing the lowest affinity constant in all pairs. The analysis of the competition experiments allows the determination of the following general trend of affinity of the cavitand for the alcoholic guests:

EtOH > 1-PrOH > MeOH > 2-PrOH > 2-BuOH > 1-BuOH

A quantitative scale of the relative affinity constants was obtained by combining all 32 selectivity constants of alcoholic pairs (see the Experimental Part Section) and assuming an arbitrary binding constant value of **1** for the 2-propanol guest. These normalized affinity constants are plotted in Figure 4. As previously described,[17] the strength of complexation depends on the synergy between hydrogen bonding and cavity inclusion – namely dispersive CH···π interactions – between the guest alkyl chain and the host aromatic cavity walls. The quantitative scale shows that the encapsulation is a phenomenon with a pronounced size and shape selectivity. Indeed, selectivity of **1** towards alcoholic guests is not only influenced by the length of the alcohol alkyl chain, but also by the shape. The complementarity in size between the guest and the host cavity contributes to maximize the van der Walls contacts within the cavity. Both factors determine the strength and the number of interactions that the guest can establish with the receptor once enclosed in the cavity. According to the data summarized in Figure 4, ethanol has the best compromise between length and shape of the alkyl chain. In the relative scale the second best alcohol is the 1-propanol, with an affinity slightly higher than methanol. Indeed, although among all alcoholic guests the methanol hydroxyl group forms the most efficient H-bond, with the shortest O–H···O=P distance, it is too small to fill entirely the volume of the cavity. On the contrary, 1-propanol, having an alkyl chain longer than methanol, is locked inside the cavity also by further CH···O=P and CH···π interactions. In order to understand the lower affinity of 2-propanol, it should be noted that the branched alkyl chain sterically hinders a deep encapsulation in the cavity, limiting the number of interactions with the receptor. The comparison between the affinities of the C4-alcohols, 1-butanol and 2-butanol, shows that the branched guest matches more closely the cavity of **1**. In the 2-butanol, the methyl group in position 2 does not constitute a hindrance because it protrudes out from the cavity. On the contrary, the presence of this methyl group allows an energetic gain due to the formation of further weak hydrogen bonding interactions with the P=O groups.

The results of this competition analysis have been compared with results obtained with sensing techniques, operating at the gas-solid interface. In particular, Quarz Crystal Microbalance (QCM) measurements using phosphonate cavitands[16,32] indicate a general enhancement of the responses of receptors associated with increasing number of carbon atoms in the alcohol series. Previous adsorption isotherm data showed that this behaviour is due to the increased number of purely dispersive interactions, directly related to chain length, and not to any strengthening of the specific CH···π and H-bond interactions.[37,38] If these results are normalized considering the response of a non-specific polymer (polyepichlorohydrin, PECH), the QCM shift frequency is higher for shorter chain alcohols due to a progressive dilution of the specific responses as the analyte chain length increases.[32] These non-specific responses conceal the inherent complexation properties of cavitands determined by crystallographic analyses.

Instead, once the sensor response is directly correlated to the recognition event, like in fluorescence experiments carried out with a fluorescent phosphonate cavitand,[17] the correlation between crystallographic and sensing results is consistent. In this case, the trend of affinity obtained considering both linear and branched alcohols is similar to the one described in this study. In particular, fluorescence measurements highlight the same trend for the pair 2-butanol and 1-butanol consistent with a limited complexation of the last longer linear alcohols. The negligible sensor response to 1-pentanol mirrors the absence of complexation in the crystal phase. What is not fully reflected in the fluorescent sensor responses is the difference in complexation among the other alcohols. This is due to the low site occupancy of the cavitands in the film to avoid response saturation. In the presence of an excess of cavitand receptors, all suitable alcohols are complexed with the same efficiency.

Conclusions

In this work, we studied the binding affinities of the tetraphosphonate cavitand 1 for a series of alcohols using X-ray crystallographic data. The crystallographic analysis allowed to identify the structural features that determine selectivity and affinity for a specific alcohol. Moreover, the relative binding constants were determined using an original analysis of results of X-ray diffraction experiments, since traditional structure refinement reveals its weaknesses when statistical occupancy factors of disordered competitive ligands are to be determined in presence of a strong electron density overlap. The relative binding constants for six alcohols (EtOH, 8.8 > 1-PrOH, 2.2 > MeOH, 1.3 > 2-PrOH, 1.00 > 2-BuOH, 0.32 > 1-BuOH, 0.11), obtained from the elaboration of crystallographic data, are in good agreement with the structural parameters of host-guest interactions observed in the corresponding crystal structures. In particular, the binding constant decreases with the increase of the host-guest H-bond distance and the increase of the length of the alkyl chain of the alcohol.

This methodology allows to uncover the intrinsic recognition properties of a given receptor in the absence of concealing effects like solvation in solution and non-specific adsorption at the solid-gas interface. Its predictive value is pivotal for developing devices for analytical, environmental and medical applications. Moreover, a similar issue is encountered in drug design studies, where measurement of affinities allows the selection of possible candidates for clinical trials, as well as the optimization of the structural features in order to increase selectivity and strength of the binding.

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Notes and references

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† Electronic Supplementary Information (ESI) available: Table of unit cell parameters for the 32 isomorphous crystals; Table of occupancy

factors obtained by conventional refinement for the alcoholic guests and comparison with those calculated by the statistical method; Table of the ratios between binding constants for each alcoholic binary mixture; Structure and electron density map for the complex obtained in presence of MeOH/1-PrOH 0.20:0.80.

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