Tryptophan association in water driven by charge-transfer interactions with electron-deficient aromatic haptens

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The ability of a series of electron-deficient aromatic compounds to form charge-transfer complexes with tryptophan in water has been evaluated by X-ray diffraction studies, UV-vis spectra and NMR. As dinitrophenyl (DNP) ligands are well-known to generate antibody-mediated responses and the π–π stacking interactions with tryptophan residues of the antibody Fab fragment have been reported, most of the aromatic receptors studied here are nitro derivatives. Charge-transfer interactions between the rich indole ring of tryptophan and the electron-deficient aromatic receptors have been observed in the solid state, as four crystal structures of the complexes were obtained. The aromatic donor-acceptor interactions in solution were also verified by UV-vis and NMR spectroscopy. The association of the tripeptide Trp-Gly-Trp, a motif found in antigen Ag43, with the electron-deficient aromatic diimide was also studied by UV-vis and NMR spectroscopy. Our results show that these simple electron-deficient molecules could potentially behave as novel haptens and be incorporated in more elaborated drugs targeting protein-protein interactions, due to the synergistic effect of multiple non-covalent interactions.

Introduction

Haptens are low molecular weight molecules (<1000 dalton) which induce an immune response when bounded to a given protein or antibody.[1] Since the pioneer works by Landsteiner and Jacobs[2] to date, antibody-hapten interactions have found many applications and are nowadays employed in daily-used techniques such as affinity chromatography, immunohistochemistry, *in situ* hybridization, and enzyme-linked immunosorbent assays (ELISAs).[3]

Dinitrophenyl (DNP) ligands have long been known to function as haptens against rabbit, guinea pig, goat and horse antibodies. At the present time, polyclonal and monoclonal anti-DNP antibodies and single chain variable fragments against DNP are readily available[4] and DNP-labelled proteins have found important applications.[5] Also, DNP can be incorporated to self-antigens to enhance the immune response of the host and has been applied for several human diseases.[6]

Key for these achievements was the understanding of the interactions between the antibody and the dinitrophenyl haptens at a molecular level. It had been observed that the spectral shifts for DNP haptens bound to anti-DNP antibodies were reproduced in the complexes formed by the same haptens with tryptophan, suggesting the presence of the amino acid tryptophan in the active site of these antibodies. Visible and UV spectroscopy,[7] fluorescence[8] and circular dichroism[9] studies were thus interpreted according to charge-transfer interactions between the dinitrophenyl haptens and the expected tryptophan residues at the antibody.[10] These earlier observations could be fully corroborated when the crystal structure of the Fab fragment of the anti-dinitrophenyl antibody AN02 complexed with its hapten was solved in 1991.[11] This structure shows that the dinitrophenyl ring of the hapten is stacked between two indole rings of two tryptophan residues (Fig. 1a). Today, X-ray structures of many Fab fragments and different haptens have been solved demonstrating that antibodies utilize a variety of intermolecular forces (charge-charge interactions, hydrophobic effects, hydrogen bonding, van der Waals and π-π stacking) to bind haptens.[12]

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†Paper dedicated to Prof. Cruz Caballero Salvador on the occasion of her retirement.
Electronic Supplementary Information (ESI) available: NMR and UV/Vis spectra, titrations, crystallographic data and modelling studies.

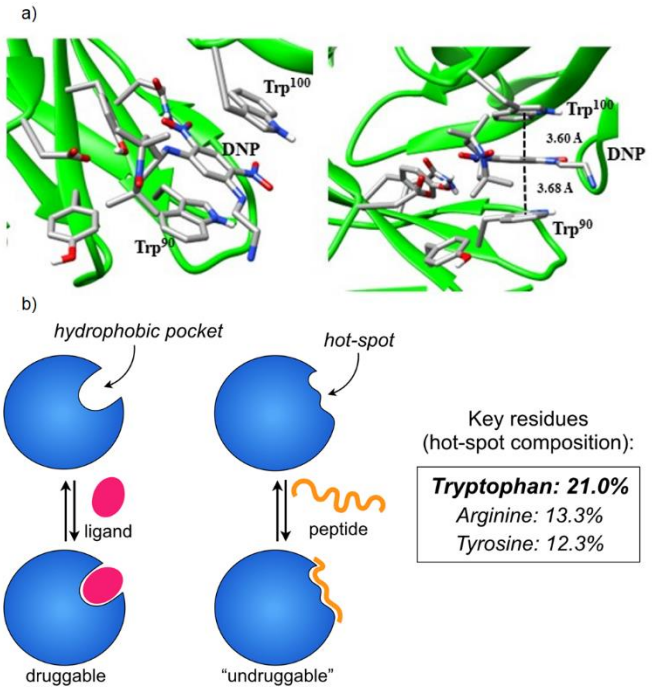

Fig. 1 *(a)* 2.9 Å resolution structure of an anti-dinitrophenyl-spin-label monoclonal antibody Fab fragment with bound hapten (PDB: 1BAF); *(b)* Classical (*druggable*) and protein-protein interaction approach (*undruggable*) for protein targeting, showing the key residues and composition of hot spots.

Moreover, tryptophan has been recently shown to be one of the "hot-spots" residues with a major contribution in protein-protein interactions (PPIs) (Fig. 1b).[13] Targeting PPIs has attracted great interest as a new strategy for drug discovery since 80% proteins cannot be addressed with the classical strategy of small molecule-binding pocket approach.[14] These proteins, which have been defined as *undruggable* (Fig. 1b), lack a well-defined, or difficult to access, binding pocked suitable for synthetic inhibitors. The design of small molecules able to bind to a PPI interface is more difficult than that of traditional targets, due to the nature of the PPI interfaces themselves, mainly flat, large and usually water exposed.[15]

However, due to the critical role of some amino acids in the protein interface (Fig. 1b), it is possible to design small molecule drugs by targeting the "hot-spot". And even though blocking a flat protein surface to prevent PPIs is still a challenge, some of these small molecule PPI drugs have already proven promising in clinical trials.[16]

Molecular recognition of biomolecules as amino acids and peptides lies at the heart of supramolecular chemistry and many synthetic receptors have been shown to bind these biomolecules.[17] Although the selective binding of amino acids and small peptides in aqueous solvents remains a difficult task, molecules showing specific interactions with the side chains of the "hot-spot" amino acids might be used to create new drugs. Tryptophan is an essential amino acid which is involved in many biological processes such as protein synthesis, among other metabolic functions.[18] Due to its biological relevance, different synthetic receptors for tryptophan have been published, able to bind the free amino acid in water or transport the zwitterionic tryptophan across U-tube type model membranes. Some of

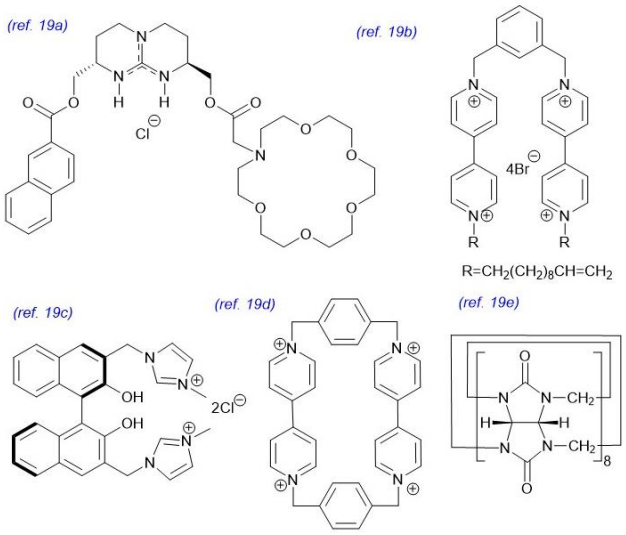

Chart 1 Selected synthetic receptors for the association of tryptophan in aqueous solutions.[19]

them are shown in Chart 1.[19] Acridinium,[20] bipyridinium[21] or imidazolium units[19c] have been commonly used in these receptors, as the positively charged species improve solubility in water and more important, their strongly π-electron deficient units form π-π charge-transfer complexes with the electron-rich tryptophan indole ring. In addition, cholic acid derivatives,[22] functionalized calixarenes and calix[4]resorcinarenes,[23] cyclodextrins[24] and cucurbiturils[25] show a hydrophobic cavity capable of binding tryptophan in water.

In this work, inspired by the X-ray crystal structure of the ANO2 bound to its DNP hapten (Fig. 1a), we exploit the charge-transfer properties of the electron-rich tryptophan indole ring to develop synthetic molecular receptors containing electron-poor aromatic rings (Chart 2, receptors **2**-**6**). Receptors **2**-**5** are dinitrophenyl or trinitrophenyl derivatives, as nitro groups on the benzene ring have proven to be highly immunogenic. To improve water solubility and increase the donor-acceptor interaction with tryptophan, acceptor groups as sulfonic or carboxylic acids were incorporated in these compounds (**2**-**5**). On the other hand, receptor **6** is *N,N'*-bis(glycinyl)-pyromellitic diimide, an aromatic acceptor compound that self assembles forming charge transfer complexes with electron donors.[26] All these compounds must be water soluble as our initial purpose is the association of tryptophan in water. Herein, we would like to report our findings.

Results and discussion

X-ray diffraction analysis

X-ray crystallography has proven to be a powerful tool to obtain structural information about the interactions of a ligand with a pharmacological target and the rational design of drugs relies largely on the prior knowledge of the crystal structures.[27]

Indole compounds are known to form charge-transfer complexes with different aromatic acceptors and the crystal structure of the complex between tryptophan and picric acid (2,4,6-trinitrophenol **1**) has been determined;[28] both aromatic

rings are almost parallelly arranged with the interplanar spacing close to 3.4 Å, suggesting the formation of the charge-transfer complex.

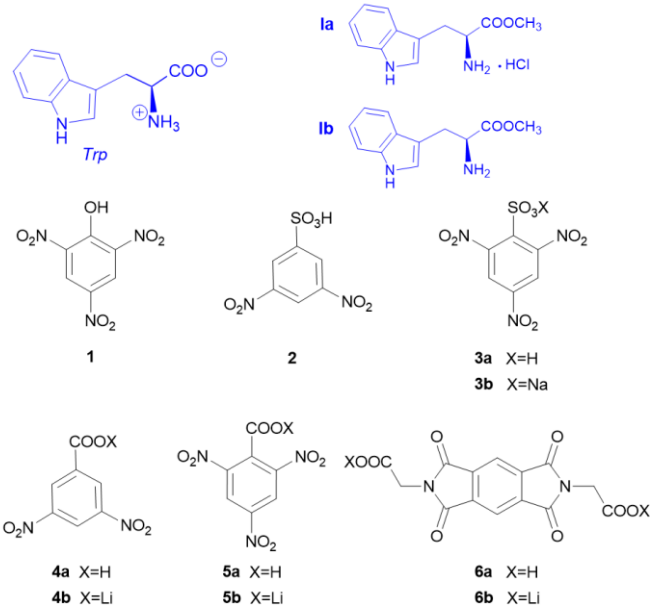

Chart. 2 Aromatic compounds used in this study.

We started our investigation preparing X-ray quality crystals of several electron deficient aromatic rings (Chart 2) in the presence of tryptophan. Receptors **2** and **3** are sulfonic acid derivatives and compounds **4** and **5** are decorated with a carboxylic acid group directly attached to the benzene ring. The presence of the electron-withdrawing groups, such as sulfonic acid or carboxylic acid instead of the electron donating OH group in picric acid (**1**), should make these receptors more electron-deficient favouring the interaction with the tryptophan electron-rich indole. Also, the presence of these functional groups in the haptens might facilitate the covalent linkage of the electron-deficient rings to the antibody or carrier proteins.

Since dinitrobenzene sulfonic acid and trinitrobenzene sulfonic acid derivatives have been shown to act as haptens,[29] these two sulfonic acids were the first to be tested. Equimolar amounts of tryptophan water/THF solutions and the corresponding sulfonic acid derivative were allowed to slowly evaporate to achieve suitable crystals for X-ray analysis. While no crystals were obtained for the dinitrobenzene sulfonic acid (**2**), deep red crystals of the complex were formed with the 2,4,6-trinitrobenzene sulfonic acid (**3a**) and tryptophan. As it is shown in the ORTEP representation (Fig. 2), the six-membered ring of Trp and the benzene ring of trinitrobenzene sulfonic acid are nearly parallelly stacked (3.15°), with a centroid–centroid distance of 3.544 Å. This confirms the expected charge-transfer interaction characterized by the red-coloured crystals. The three S-O bond lengths in the sulfonic group are similar (1.440, 1.442, and 1.437 Å), suggesting proton transfer from the sulfonic group to the tryptophan carboxylate, which indeed shows different C-O bond lengths (1.174 and 1.276 Å). Similar proton transfer has been reported for the complex of Trp and picric acid[28] as predicted from the pK_a values (Trp: 2.38; picric acid: 0.29).[30] Moreover, the crystal structure evidences that the nitro groups in positions 2 and 6 of the trinitrobenzene sulfonic

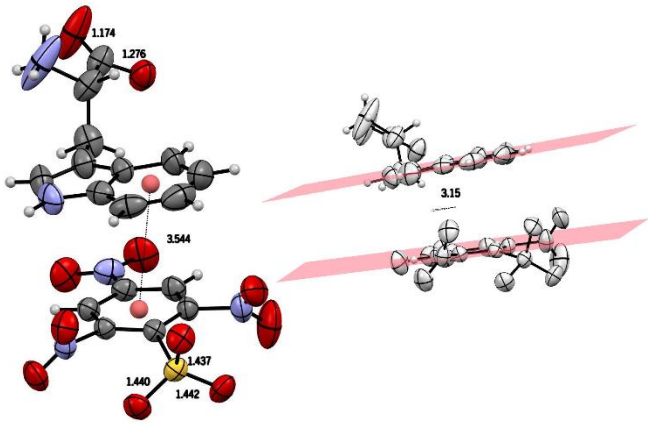

Fig. 2 ORTEP diagram of the complex between tryptophan and trinitrobenzene sulfonic acid (**3a**). Selected distances [Å] are shown: S-O bond lengths for the sulfonic group: 1.440 (2); 1.442 (2); 1.437 (2); C-O bond lengths for the carboxylic group: 1.174 (4) and 1.276 (4); distance between centroids: 3.544. Angle between planes 3.15°.

acid (**3a**) are twisted out of the benzene plane (about 50°) and that three water molecules are included in the unit cell. These water molecules contribute to the hydrogen-bonding network, that also involves the sulfonate group of the trinitrobenzene sulfonic acid and the ammonium and tryptophan carboxyl groups (Fig. S19-S24).

The twisting of the two nitro groups respect to the benzene ring might decrease its electron-deficient character, making the associate with tryptophan weaker than expected. The rotation of the nitro groups is strongly reduced in the 3,5-dinitrobenzoic acid (**4a**), with torsion angles ranging from 0.5° to 7.6° (Fig. S27). Bright yellow crystals of the 1:1 complex between 3,5-dinitrobenzoic acid (**4a**) and tryptophan, containing one methanol molecule, were obtained by slow evaporation of a water/methanol solution (Fig. 3). Unlike the previous case, no proton transfer was observed from the 3,5-dinitrobenzoic acid to the zwitterionic tryptophan, evidenced by the similar C-O bond lengths for tryptophan (1.248 and 1.249 Å) and the unequal C-O bond lengths for the carboxylic group of the 3,5-dinitrobenzoic acid (1.220 and 1.306 Å). The ORTEP representation shows the angle between planes defined by both aromatic rings is 2.83°, indicating a nearly parallel arrangement of Trp and 3,5-dinitrobenzoic acid molecules. The complex is also stabilized by multiple H-bonds: there is a strong intermolecular H-bond (2.558 Å) between the tryptophan carboxylate and the donor carboxylic group of the dinitrobenzoic acid; the ammonium group of tryptophan sets up to three additional H-bonds: one with the carbonyl group of 3,5-dinitrobenzoic acid (2.872 Å), another with the carboxylate of a second molecule of tryptophan (2.755 Å) and the third one with a methanol molecule (2.810 Å) from the crystallization solvent. The indole NH only presents a weak interaction with one of the nitro groups (3.139 Å, Fig. S27-S29).

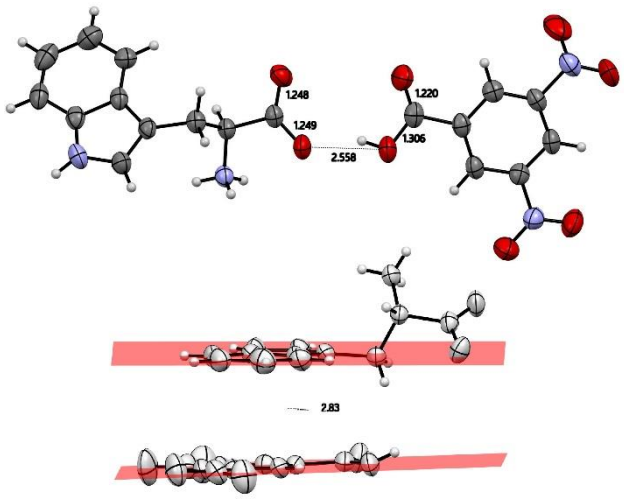

Fig. 3 ORTEP diagram of the complex between tryptophan and 3,5-dinitrobenzoic acid (**4a**). Selected distances [Å] are shown: C-O bond lengths for the 3,5-dinitrobenzoic acid carboxyl group: 1.220 (5); 1.306 (5); C-O bond lengths for the carboxylic group of Trp: 1.248 (5) and 1.249 (4). Angle between planes 2.83º.

Trying to further improve the charge-transfer interaction with tryptophan, 2,4,6-trinitrobenzoic acid (**5a**) was tested. Following the same procedure described above, red crystals of sufficient quality for X-ray structure determination were obtained. A structure first glance reveals significant differences with the previous complexes: the donor and acceptor aromatic rings are no longer parallelly stacked, laying in planes forming an angle of 12.73°. Carboxylic C-O bond lengths indicate that there is a proton transfer from the trinitrobenzoic acid (**5a**) to the tryptophan: similar C-O bond lengths (1.233 Å and 1.244 Å) for trinitrobenzoic acid and unequal C-O bond lengths (1.204 Å and 1.315 Å) for tryptophan. This proton transfer had already been observed for the complex of Trp with trinitrobenzene sulfonic acid (**3a**).

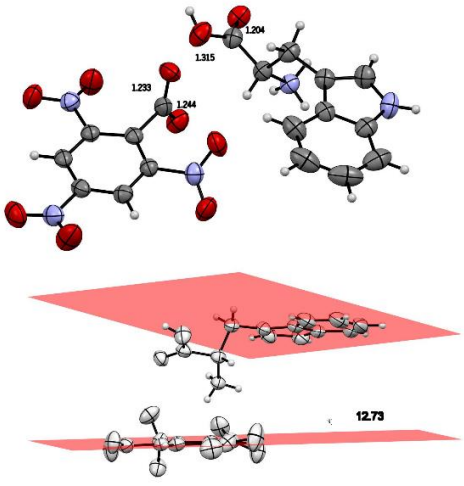

Fig. 4 ORTEP diagram of the complex between tryptophan and 2,4,6-trinitrobenzoic acid (**5a**). Selected distances [Å] are shown: C-O bond lengths for the 2,4,6-trinitrobenzoic acid carboxylic group: 1.233 (3) and 1.244 (2); distances C-O for the carboxylic group of Trp: 1.204 (2) and 1.315 (2). Angle between planes 12.73º.

The interactions showed by the trinitrobenzoic acid carboxylate might explain why the aromatic sheets of donor and acceptor are not completely parallel since the trinitrobenzoic acid carboxylate forms two strong H-bonds with two tryptophan molecules: a H-bond with the NH of the indole ring of one Trp molecule (O3-N1: 2.891 Å) and a second H-bond with the carboxylic group of another tryptophan molecule (O4-O1: 2.534 Å), which probably fix this geometry (Fig. S33). The intermolecular hydrogen bonding array in the crystal is completed with the ammonium three H-bonds: one with a carbonyl group of another Trp molecule (N2-O2: 2.852 Å); another with a carboxylate of a trinitrobenzoic acid molecule (N2-O4: 2.776 Å) and the third one with one nitro group (N2-O6: 2.958 Å) (Fig. S34).

As evidenced by the torsion angles, the three nitro groups of trinitrobenzoic acid show a reasonable planarity with the benzene aromatic ring (torsion angles less than 29°), while the carboxylate is essentially perpendicular (torsion angle about 82°), and therefore may render no conjugation (Fig. S35).

As *N*,*N´*-bis(glycinyl)pyromellitic diimide (**6a**) is well known to form complexes with aromatic hydrocarbons (as anthracene, phenanthrene or perylene)[26] stabilized by π-π stacking interactions, its complex with tryptophan was studied. Crystallization from water/methanol of a stoichiometric mixture of Trp and the diimide **6a** afforded coloured crystals with a 2:1 stoichiometry. The ORTEP representation (Fig. 5) shows the expected π-π stacking, with the electron-deficient aromatic ring (**6a**) intercalated between two molecules of tryptophan and centroid-centroid distances of 3.664 Å and 3.665 Å. Both distances are in the expected range for charge-transfer interactions. The angles between the planes are 11.58° and 10.85°, showing that the stacking is not exactly parallel for the six-membered rings of tryptophan and diimide. Proton transfer was observed from the diimide **6a** carboxylic groups (similar C-O bond lengths for both: 1.272 Å and 1.242 Å) to the two tryptophan carboxylate groups (unequal C-O bond lengths: 1.207 and 1.333 Å) (Fig. S38). The ionic interaction between the positively charged Trp molecules and the receptor **6** dianion might explain the observed stoichiometry. Finally, the crystal structure is stabilized by an extended array of H-bonds. The ammonium group of each tryptophan sets three hydrogen bonds (2.784 Å; 2.850 Å and 2.897 Å), the carboxyl group of Trp forms another three H-bonds (2.521 Å; 2.887 Å; 2.971 Å) and the indole NH is also involved in a H-bond (3.036 Å) (Fig. S39-S40).

UV-vis absorption studies

X-ray crystallographic studies have yielded a picture of how tryptophan and haptens are successfully bound by intermolecular forces in the solid state. The binding is generally based on a combination of noncovalent interactions, including π–π interactions, electrostatic interactions, hydrogen bonds and charge-transfer interactions. As we were especially interested in charge-transfer interactions such as the ones showed by the DNP haptens, UV–vis absorption spectroscopy was used.

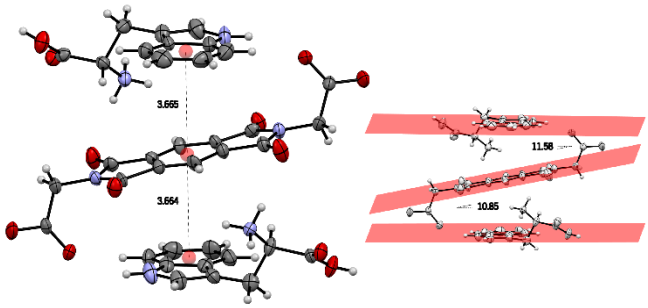

Fig. 5 ORTEP diagram of the 2:1 complex between tryptophan and *N,N´*-bis(glycinyl)pyromellitic diimide (**6a**). Centroid-centroid distances [Å] are shown: 3.664 and 3.665. Angle between planes 11.58° and 10.85°.

UV-vis spectra of aqueous solutions of the free receptors and their complexes with Trp were run at 0.01M concentration. This large concentration is needed to identify the broad charge-transfer bands due to their small extinction coefficients. Receptor **3** was the first to be tested and since the X-ray crystal structure of its complex with Trp (Fig. 2) showed proton transfer from the sulfonic group to the zwitterionic amino acid, we preferred to use the sodium salt, **3b**. In this way, shifts in absorption might be solely attributed to aromatic donor-acceptor interactions. Colour change from pale yellow (**3b**) to intense orange (mixture 1:1 of Trp and **3b**) was observed with the naked eye and a new absorption band appeared at 385 nm (Fig. 6, left). This new band is indicative of the formation of a charge-transfer complex. UV-vis titration of receptor **3b** (0.01M) with increasing amounts of Trp revealed an increase in absorbance with a gradual bathochromic shift of the absorption band, from 372 nm (0.2 equiv.) to 393 nm (1.7 equiv.) (Fig. 6, right). To determine the binding constant the data were analysed over a spectral range (λ = 375-445 nm) with the aid of the website: http://supramolecular.org[31] and an association constant of 87 M^{-1} was obtained (Fig S45). Although the concentrations used during the titration are far from the micromolar concentrations commonly employed in UV-vis spectrophotometric titrations, the dissociation constant $K_d = K_a^{-1} \approx [H]_0 = 0.01M$, indicating an acceptable level of reliability.[31c]

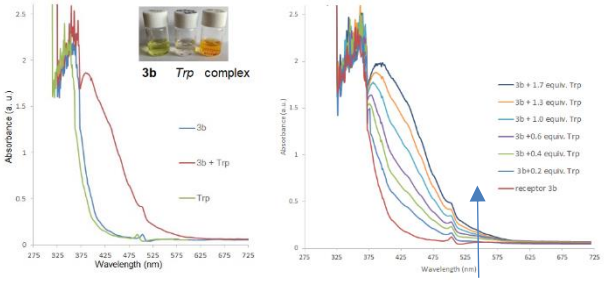

Fig. 6 (*a*) UV-vis spectra in H_2O: receptor **3b**, Trp and 1:1 complex (0.01M); Insert: photographs of the three solutions; (*b*) UV-vis titration spectra of receptor **3b** (0.01M) with increasing amounts of Trp.

The UV-vis spectrum for the 1:1 complex of dinitrobenzoic acid (**4a**) and Trp in water could not be obtained; a bright yellow solid, highly insoluble in water, was immediately formed upon mixing both solutions (Fig. S48). However, the stoichiometric mixture of tryptophan (0.01M) and trinitrobenzoic acid (**5a**, 0.01M) displayed a deep orange colour characteristic of the formation of charge-transfer complexes and the appearance of a new band (partially overlapped with the receptor absorption band) was observed (Fig S49).

Due to the low solubility of the diimide (**6a**) in water, the UV-vis studies were run in a lithium phosphate buffered solution (pH=6.9), where the predominant species is **6b**. Titration of Trp (0.01M) with increasing amounts of **6b** (0.01M) showed the presence of a band in the region where neither the acceptor **6b** nor the donor Trp absorbs. Data were analyzed[31] to determine the binding constant, but no reliable values could be obtained (Fig. S50-S51)

To test the influence of coulombic interactions[19e,32] between the electron-deficient receptors and tryptophan (zwitterionic at pH=7), we explored binding of Trp with neutral derivatives of acid receptors, as esters or amides, but their water solubility was significantly reduced. Fortunately, tryptophan derivatives like methyl ester hydrochloride (**Ia**) and the corresponding amino acid ester **Ib** showed the required solubility in water. Both derivatives (**Ia** and **Ib**) lack a negative charge and amino acid ester **Ib** also lacks a positive charge, so these compounds were used to assess the effects of electrostatic charge on the binding of tryptophan. Sodium sulfonate **3b** was chosen as the receptor to compare UV-vis results, as electrostatic charges on Trp and derivatives are not affected by the receptor. When receptor **3b** (0.01M) was titrated against increasing amounts of **Ia**, similar spectral changes to those with Trp were observed: the pale-yellow solution (**3b**) turned orange and appeared the same absorption band at 385 nm, indicative of the formation of the charge-transfer complex (Fig. S46). From these results, similar charge transfer complexes are formed either with Trp (NH_3^+ and COO^-groups) or with its methyl ester hydrochloride (NH_3^+, COOMe). The possible effect of the positively charged ammonium group could not be examined in a comparable titration of **3b** with tryptophan methyl ester **Ib** (NH_2, COOMe), as gelation took place (Fig. S47). The gelation promoted by charge-transfer interaction has been reported by different groups.[33]

As conclusion, UV-vis studies confirm the donor-acceptor interactions of the electron-deficient receptors with tryptophan or derivatives.

^{1}H NMR titrations

NMR spectra can provide additional information on the aromatic donor–acceptor interaction. As receptors **2**-**6** are either sulfonic or carboxylic acids and the guest is an amino acid, ^{1}H NMR titrations were run in both non-buffered and buffered solutions. Proton chemical shifts of the complexes could then be attributed to proton-transfer processes, charge-transfer interactions, or combination of both. ^{1}H NMR titrations were performed keeping a constant concentration for one component (about 5 mM) and adding increasing amounts of the other. Where buffer was used, this was a phosphate solution (pH=6.9; comprised of Li_2HPO_4 and LiH_2PO_4).

Table 1 Binding constants[a] for the association of tryptophan determined from ^{1}H NMR titrations in D_2O at 20°C.

Entry	Receptor	Association constants (K_a) M^{-1}
1	**1**	47 (±2.3%)
2	**3b**	62 (±3.9%)
3	**4a**	72 (±7.4%)
4	**4b**[b]	22 (±4.4%)
5	**5a**	93 (±5.8%)
6	**5b**[b]	13 (±2.5%)
7	**6b**[b]	38 (±3.3%)

[a] Values are determined for the 1:1 binding model; errors are shown between parenthesis; [b] Titrations in phosphate buffered D_2O.

Data were analysed with the aid of the website http://supramolecular.org[31] and association constants (Table 1) have been mostly determined from the upfield chemical shifts of the receptor aromatic protons (S52-S63)

A first glance at this table shows weak association in water, with binding constants for the formation of the 1:1 complexes ranging from 10 to 100 M^{-1}. This is not unexpected, as low to moderate association constants have been reported for the case of intermolecular donor-acceptor complexes.[33a] K_a values for the binding in non-buffered solutions are larger than in the buffered ones (see entries 3 *vs* 4; 5 *vs* 6). Formation of charge-transfer complexes results in upfield shifts of the aromatic protons, but also aromatic protons of the acid receptors are shielded due to proton transfer to Trp. In buffered solutions (pH=6.9), Trp is zwitterionic and the upfield chemical shifts might be due mainly to charge-transfer interactions. Comparison of the association constants for the acid receptors in non-buffered solutions (entries 1, 3, 5) reveals affinity for Trp increases from picric acid (**1**, K_a= 47 M^{-1}) to 3,5-dinitrobenzoic acid (**4a**, K_a= 72 M^{-1}) and 2,4,6-trinitrobenzoic acid (**5a**, K_a= 93 M^{-1}). This trend cannot be justified considering only the acid strength of the receptors: pK_a values for picric acid 0.29; trinitrobenzoic acid 0.65; dinitrobenzoic acid 2.82.[30] But if the formation of a charge-transfer complex contributes to binding, based on the more electron-deficient ring, one would expect **5a** to bind more tightly. In buffered titrations (entries 4, 6 and 7), where no proton transfer takes place, association constants are reduced, and the highest affinity is shown by diimide **6b** (K_a= 38 M^{-1}) suggesting better charge-transfer than dinitrobenzoate **4b** (K_a= 22 M^{-1}) or trinitrobenzoate **5b** (K_a= 13 M^{-1}).

Regarding the stoichiometry of the complexes, it is known that the analysis of the aromatic donor-acceptor interaction (D-A) is difficult since several models of binding are possible,[34] not only 1:1 (A-D) but also 1:2 (D-A-D) and 2:1 (A-D-A) complexes. Although we have only considered 1:1 binding for the data shown in Table 1, 1:2 and 2:1 binding models were also explored with Thordarson´s website.[31] Attempts to fit data to other models than 1:1 resulted in a worse fit, except for diimide **6b**. These data were fitted to the 1:2 model (1 molecule **6b** with 2 Trp molecules) with an improvement, compared to the 1:1, in the cov_{fit} (>2.8 fold) for three of the four variants of the 1:2 binding model (Tables S5-S6). Considering 1:2 binding, the stepwise constants K_1 (ranging from 137 to 472 M^{-1}) and K_2 (in the range of 118-214 M^{-1}) are appreciably higher than the association constant for the 1:1 binding model (Tables S5-S6). This stoichiometry also matches the one shown by X-ray for the complex (Fig 5).

Trp-Gly-Trp

Encouraged by these results, we explored binding to tryptophan-containing peptides. To show this possibility the tripeptide Trp-Gly-Trp **10** was prepared as a model to test the targeting of different peptides/proteins using charge-transfer receptors.

Recently, the Trp-Gly-Trp has been found in antigen Ag43,[35] which is a surface-displayed autotransporter protein that mediates bacterial self-association and pathogenicity. This and other binding peptides enriched in Ar-Ar and Ar-X-Ar motifs (with Ar standing for any aromatic residue and X for any other residue) have been suggested to play an important role in the association of Ag43 and chaperone SurA.[36] Therefore, molecules able to bind the tripeptide Trp-Gly-Trp with a high association constant could disrupt the interaction between Ag43 and SurA, which may avoid auto aggregation and biofilm formation of antibiotic-resistant bacteria.

Attempts to produce X-ray quality crystals of the complex between Trp-Gly-Trp and **6** were not successful. However, the charge-transfer interaction between receptor **6b** and tripeptide **10** could be confirmed by UV-vis spectroscopy (Fig. S64-S66): a new broad absorption band appeared for the 1:1 mixture of receptor and tripeptide **10**. An UV-vis titration of tripeptide **10** (0.005M) with increasing amounts of receptor **6b** allowed to estimate a binding constant K_a =156 (±4.1%) M^{-1} for the 1:1 binding model (Fig. S66) in the spectral range (λ = 400-410 nm).

As NMR studies provide additional information, we carried out a ^{1}H NMR titration in phosphate buffered solution (pH=6.9) at a fixed concentration of the diimide **6b** (0.0048 M) and increasing amounts of tripeptide **10** (Fig. S67). Previously, dilution experiments in D_2O had proven that self-association of Trp-Gly-Trp does not exist in the concentration range of the titration (Fig. S70). ^{1}H NMR titration data were fitted to a 1:1 binding model with an estimated association constant K_a =112 (±1.9%) M^{-1} (Table 2, entry 1). The diimide aromatic protons shift from 8.147 ppm (free receptor) to 7.886 ppm, as expected in a charge-transfer complex. Attempt to fit data to other binding models (as 1:2 or 2:1) resulted in non-reliable results (Tables S7-S8). A Job plot analysis[37] was used as additional positive confirmation of the 1:1 stoichiometry (Fig. S69).

To explore the possible geometry of the complex, 2D NMR spectra were performed (ESI). Cross correlation peaks among the aromatic tryptophan rings and pyromellitic diimide protons were observed (ROESY, Fig S74). Also, there is a third correlation between the methylene diimide protons (4.20 ppm) and both tryptophan aromatic rings (7.26 ppm and 6.99 ppm) (Fig. S75).

Table 2 Binding constants for the association of Trp-Gly-Trp determined from NMR at 20°C.

Entry	Receptor	Solvent	Association constants (K_a) M^{-1}
1	**6b**[a]	D_2O	112 (±1.9%)
2	**6a**	DMSO-d_6	2108 (±13.7%)

[a] Phosphate buffer solution.

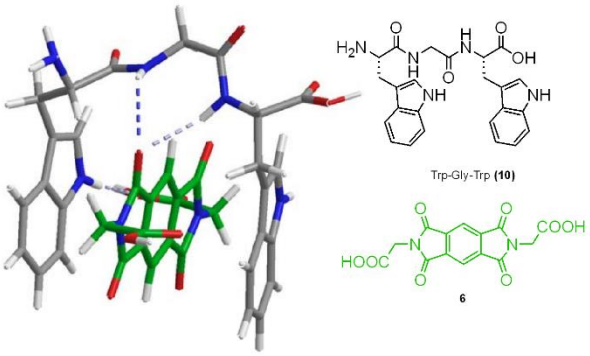

Fig. 7 Proposed geometry for the association of diimide **6** and Trp-Gly-Trp, showing the folding of the tripeptide (Chem3D, GAMESS interface, AM1, Hartree-Fock).

These results show that the aromatic rings of the Trp and **6b** are in close contact, and probably in a parallel arrangement.

The binding receptor affinity of diimide **6a** for the tripeptide **10** was also evaluated in DMSO-d_6; in this case, as expected, a higher association constant was obtained, K_a = 2108 M^{-1}(Table 1, entry 2) as charge-transfer and hydrogen bonding interactions occur. 2D NMR spectra in DMSO-d_6 showed similar correlations as above.

Finally, figure 7 shows a possible geometry for the associate between receptor **6** and Trp-Gly-Trp: receptor **6** might be encapsulated by the folded tripeptide **10**, to maximize hydrophobic and charge-transfer interactions.

Conclusions

In this work, the association of several electron-deficient aromatic rings have been evaluated with tryptophan in water. These receptors are differentiated either by their acceptor properties or by their surface area. X-ray structures of four complexes have been obtained and proved the formation of a charge-transfer interaction in the solid state. Complexes are also formed in aqueous solutions and, charge-transfer interactions have been proved by UV-vis and ^{1}H NMR spectroscopy. Association constants in the range 10-400 M^{-1} have been determined from the complexes in water, showing different stoichiometries.

Interestingly, diimide **6b** has proved its ability to bind either the amino acid Trp or the tripeptide Trp-Gly-Trp by charge-transfer interaction in water. In the former case (Trp), the associate shows a 1:2 binding ($K_{11} = 471\ (\pm 4\%) M^{-1}$; $K_{12} = 118\ (\pm 4\%) M^{-1}$ non-cooperative model) while for tripeptide **10**, stoichiometry is 1:1 ($K_1 = 112\ (\pm 2\%) M^{-1}$). So far, the small values for the association constants obtained in water are not necessarily a discouraging result since the rational design of a selective drug is based on the sum of many cooperative effects, and our models, hitherto, explore mainly the charge-transfer interactions.

Author Contributions

J. R. M., J. J. G. G. and V. A. M. designed the project. E. S. S. and L. F. R. S. carried out the NMR titrations. E. S. S., A. L. F. A and A. H. contributed to the tripeptide synthesis. J. J. G. G. and J. R. M. were in charge of the modelling studies. F. S. obtained X-ray structures. A. L. F. A. and E. M. M. V. are responsible for the funding acquisition. All authors approved the final version of the manuscript for submission. J. R. M. and V. A. M. co-wrote the paper and E. S. S. organized the ESI.

Conflicts of interest

There are no conflicts to declare.

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