COMPLEXATION OF CALIX[4]ARENE HYDROXYMETHYL-PHOSPHONIC ACID WITH TRYPTOPHAN AND N-ACETYL-TRYPTOPHAN AMIDE

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 HYDROXYMETHYL COMPLEXATION

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The Host-Guest complexation of calixarene hydroxymethylphosphonic acid with tryptophan and N-acetyltryptophan amide has been investigated by the RP HPLC method in H₂O/MeCN (99/1) solution (column support Hypersil CN, UV-detector, λ = 254 nm). Adsorption of calixarene hydroxymethylphosphonic acid on the Hypersil CN surface has been studied. It has been found that hydroxymethylphosphonic acid is characterized by reversible sorption on the Hypersil CN surface. The binding constants (Kᵢ = 23000 M⁻¹ and 39000 M⁻¹) for tryptophan and N-acetyltryptophan amide, respectively, of the supramolecular complexes have been calculated from the ratio between the capacity factors k' of the Guest and the calixarene hydroxymethylphosphonic acid Host concentration in the mobile phase. The Gibbs free energies of the tryptophan and N-acetyltryptophan amide complexes are -24.84 and -26.15 kJ/mol, respectively. The molecular modelling of calixarene hydroxymethylphosphonic acid and its complexes with tryptophan and N-acetyltryptophan amide (Hyper Chem, version 8, force field PM3) has indicated that the complexes are stabilized by hydrogen bonds, electrostatic, π-π, and solvatophobic interactions. The geometric parameters of the energy minimized calixarene macrocycle and its complexes with tryptophan and N-acetyltryptophan amide have been calculated. According to the calculations it has been shown that the Host-Guest complexation does not change the flattened-cone conformation of calixarene. Finally, the inverse correlation has been found between the Kᵢ values of the complexes and the Log P values of the guest molecules.

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L-Tryptophan is an essential amino acid that is low abundant in proteins (1.4% only). As a consequence, Trp residues frequently play a key role in studying the protein structure and functions. For instance, soluble Trp residues in proteins have been shown to be critical for the specific recognition of nucleic acid sequences [1, 2, 3, 4]. Moreover, it is worth mentioning that Trp has the peculiar property to exhibit a significant intrinsic fluorescence that is environment sensitive, and therefore, can be used to investigate the properties and interactions of proteins with ligands [5].

To characterize the role of the given Trp residue in the protein properties and functions, the common strategy is to site-selectively mutate this residue into another one. To disturb the protein structure minimally the aromatic Phe or Tyr residues are frequently selected as a substitute. Nevertheless, due to the key role of Trp residues in protein folding, these mutations can result in improperly folded proteins, and it does not allow characterizing the specific role of the Trp residues mutated.

To characterize the role of soluble Trp residues in proteins it would be interesting to use complexing agents that can selectively bind these Trp residues as an alternative strategy, and therefore, promote the interaction of the target proteins with their ligands.

Calixarenes [6] contain preorganized bio-affine groups that are able to recognize different biological molecules such as amino acids, dipeptides, proteins, choline and acetylcholine, carbohydrates, riboflavin, vitamin B_{12}, nucleotides, nucleosides and short DNA fragments [7, 8, 9, 10]. Calixarene derivatives can also bind amino acids on the surface of proteins [11, 12].

In this respect, calix[4]arene hydroxymethylphosphonic acids [13, 14, 15] which have been shown to form selectively Host-Guest supramolecular complexes with amino acids [16, 17], appear to be good candidates to bind soluble Trp residues. To test this possibility the Host-Guest complexation of calixarene hydroxymethylphosphonic acid (CPA) with Trp and N-acetyltryptophan amide (NATA) used as models of Trp residues in proteins has been investigated by RP HPLC and molecular modelling (Scheme).

**Experimental Part**

CPA was synthesized by the reaction of formaldehyde with Na salt of ethylphosphate followed by dealklylation of the ester formed by the consecutive action of trimethylbromosilane and methanol in accordance with [13]. Because of its poor solubility in water CPA was analysed as a monosodium salt obtained by addition of one equivalent of sodium methyle to CPA solution in methanol. Trp and NATA were obtained from Sigma-Aldrich (St. Louis, MO, USA), acetonitrile was obtained from Acros Organics (Janssen Pharmaceutical 3A 2440 Geel Belgium).

**HPLC analysis**

Chromatographic analysis was performed in isocratic conditions using a Hitachi liquid chromatogra-
The phy system (Hitachi, Ltd, Tokyo, Japan) equipped with a high-pressure pump, a Rheodyne Model Sample 7120 injector (20 μl) and an UV-detector. The column (250×4.6 mm i.d.) was packed with Hypersil CN (Merck, Germany, Darmstadt). The samples of CPA for RP HPLC analysis were prepared by dissolution in the mobile phase (H₂O/MeCN, 99/1 v/v). The choice of the solvent was dictated by solubility of CPA. Trp and NATA under the same conditions. The flow rate of the mobile phase was 0.8 ml/min. The final CPA concentrations were in the range of 0.10-0.70×10⁻⁴ M. The ultraviolet detector was operated at 254 nm. The Trp and NATA samples for HPLC analysis were prepared in the same solvent (C = 0.05×10⁻⁴ M). The amount of the sample injected was 20 μL. Each of the samples was analyzed five times. The mobile phase that contained the CPA as an additive was equilibrated for 3 h before analysis. Under these conditions the column was saturated with the CPA additive. All chromatograms were obtained at 32°C.

Molecular modelling

The initial molecular modelling of CPA and its complexes with Trp or NATA was carried out by the molecular mechanics MM+ method (the force field PM3). The structures obtained were optimized by the semi-empirical method (the HyperChem software package, version 8) [http://www.hyper.com/Download/AllDownloads/tabid/470/Default.aspx].

Results and Discussion

Calixarene CPA, Trp and NATA in the given analysis conditions were registered on the chromatograms as sharp peaks (Fig. 1-3).
The binding constants of Host-Guest complexes of CPA with Trp or NATA were determined by the RP HPLC method as previously described [18, 19]. The method is based on determination of the Guest retention time, $t_R$, and the capacity factor, $k'$, before and after CPA addition to the mobile phase. The binding constants $K_A$ of the CPA complexes with the Guest molecules were calculated by equation (1):

$$1/k' = 1/k'_0 + K_A \times [CA]/k'_0 \quad (1)$$

where $k'_0$ and $k'$ are the capacity factors of the Guest molecule determined in the absence and the presence of CPA in the mobile phase; $[CA]$ is the concentration of CPA in the mobile phase.

CPA (monosodium salt), Trp and NATA appear on the chromatograms as sharp symmetrical peaks (Fig. 1, 2) with the chromatographic characteristics given in Table 1.

The linear character of the adsorption isotherm of CPA ($R^2 = 0.99$) indicates its reversible sorption on the Hypersil CN surface. Addition of CPA to the mobile phase decreases the capacity factor values of Trp and NATA. The linear plots of their $k'$ values vs the calixarene concentration (Tab. 2, Fig. 4) clearly show the formation of Host-Guest supramolecular complexes with 1:1 stoichiometry and allows calculating the $K_A$ values of the complexes by equation (1).

The binding constants $K_A$ and free Gibbs energies $\Delta G (\Delta G = -RT \ln K_A)$ for the CPA complexes are given in Tab. 2.

The binding constant $K_A$ of the CPA-NATA complex (39000 M$^{-1}$) is 1.66-fold higher than the $K_A$ value of the CPA-Trp complex (23000 M$^{-1}$). The binding constant $K_A$ values of the complexes increase with decrease of $Log P$ values of the Guest (0.93 for Trp and -0.11 for NATA).

It should be noted that the CPA-NATA complex is more stable than complexes of CPA with such aminoacids as Ala (21200 M$^{-1}$), Phe (26600 M$^{-1}$), Arg (27500 M$^{-1}$), Asp (28800 M$^{-1}$), Hist (31200 M$^{-1}$), Lys (32500 M$^{-1}$) [17]. Moreover, comparison of Trp and NATA indicates that changing the $-OH$ group of Trp to the $-NH_2$ group and acylation of its alpha-amino group significantly increase the interaction with CPA.

To clarify the nature of the Host-Guest interaction, the molecular modelling study was carried out. The conformational search of the optimum geometry of CPA, Trp and NATA was performed by the method of molecular mechanics and the semi-empirical method.

### Table 1

| Compound | $t_R$ (min) | $k'$ | $K_A$ |
|----------|-------------|------|-------|
| CPA      | 3.34        | 0.67 | 1.00  |
| Trp      | 9.71        | 3.86 | 1.27  |
| NATA     | 6.12        | 2.06 | 1.20  |

Fig. 3. Chromatograms of NATA (a) and Trp (b) obtained after CPA addition in the mobile phase.
Then the structures of the CPA complexes with the least energies were calculated (Fig. 5).

Inclination (dihedral angles) of the calixarene benzene rings A, B, C, D in relation to the main macrocycle plane formed by CH$_2$ links for CPA and its complexes in the structures calculated is presented in Tab. 3.

The macrocyclic skeleton of CPA shows a flattened-cone conformation. The aromatic rings with phenolic OH groups are almost “coplanar” with the main macrocycle plane, but the propylated rings are “perpendicular” to the plane. The dihedral angle between the “coplanar” rings A and C is 110°, while the angle between “perpendicular” rings B and D is 3°. As seen from Table 3, the Host-Guest complexation does not almost change the flattened cone conformation of the calixarene skeleton.

For the structure of the Trp complex calculated an electrostatic contact of the negatively charged oxygen atom of the calixarene phosphonic group with the positively charged nitrogen atom of the amino acid is obvious (P-O-H-N distance is 2.3 Å). Additionally, the complex is stabilized by an intermolecular hydrogen bond between the indole NH group and the oxygen atom at the calixarene lower rim (NH-O distance is 3.0 Å).

Table 2

| CPA conc. x 10$^{-4}$ M | Trp 1/$k'$ | CPA-Trp $K_a$ | CPA-Trp ΔG | NATA 1/$k'$ | CPA-NATA $K_a$ | CPA-NATA ΔG |
|------------------------|-----------|---------------|-------------|-------------|----------------|-------------|
| 0                      | 0.259     | 23000±200     | -24.84      | 0.485       | 39000±400      | -26.15      |
| 0.336                  | 0.336     | 23000±200     | -24.84      | 0.485       | 39000±400      | -26.15      |
| 0.369                  | 0.369     | 23000±200     | -24.84      | 0.485       | 39000±400      | -26.15      |
| 0.432                  | 0.432     | 23000±200     | -24.84      | 0.485       | 39000±400      | -26.15      |
| 0.577                  | 0.577     | 23000±200     | -24.84      | 0.485       | 39000±400      | -26.15      |

* (RSD = 8-12%).

Fig. 5. Energy minimized structures of CPA (a) and the complexes with Trp (b) and NATA (c).
Similar to the Trp complex an intermolecular hydrogen bond (2.604 Å) between the indole NH group and the oxygen atom at the calixarene lower rim is also observed. However, in contrast to the Trp complex, three intermolecular hydrogen bonds with the phosphonic group are formed in the NATA complex calculated: POH=O=CH₂(2.247 Å), CH₂C(O)NH–O=P (1.753 Å) and C(O)NH–O=P (1.957 Å). It is possible to predict that under experimental conditions hydrophobic and π–π interactions are also involved in stabilization of both NATA and Trp complexes. As is evidenced by the calculation data with the help of the molecular modelling, the structure of the CPA – NATA complex (the relative CPA – NATA complex energy ΔE = -14.9 kcal/mol) is more stable comparatively with the structure CPA – Trp complex (ΔE = -11.7 kcal/mol). These data are in a good agreement with the data obtained in the chromatographic calculations of the binding constants of the CPA – NATA complex (Kₐ = 39000 M⁻¹, ΔG = -6.25 kJ/mol) and the CPA – Trp complex (Kₐ = 23000 M⁻¹ and ΔG = -5.94 kJ/mol).

Conclusions

Summarizing all above-mentioned information it should be noted that experimental measurements of the complex stabilities show that CPA binds more effectively NATA than Trp or other aminoacids, such as Ala < Phe < Arg < Asp < His < Lys in the aqueous solution. It can be explained by formation of three intermolecular hydrogen bonds between the phosphonic group of the CPA-Host and the CNHC(O)CH₃-C(O)NH₃ fragment of the NATA-Guest. The investigation of the molecular recognition and binding of calixarene phosphonic acids to Trp residues in proteins is in progress.

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Table 3

Inclination of benzene rings A, B, C, D in respect to the main macrocycle plane in CPA and its complexes

| Compound     | Dihedral angles, ° | A   | B   | C   | D   |
|--------------|--------------------|-----|-----|-----|-----|
| CPA          |                    | 147 | 92  | 143 | 91  |
| CPA-Trp      |                    | 154 | 98  | 147 | 98  |
| CPA-NATA     |                    | 152 | 99  | 153 | 97  |

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26