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Anion Complexation and Transport by Isophthalamide and Dipicolinamide Derivatives: DNA Plasmid Transformation in *E. coli*

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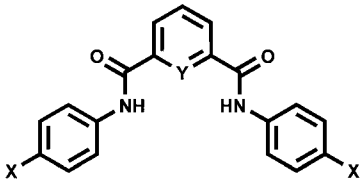
ABSTRACT: Tris-arenes based on either isophthalic acid or 2,6-dipicolinic acid have been known for more than a decade to bind anions. Recent studies have also demonstrated their ability to transport various ions through membranes. In this report, we demonstrate two important properties of these simple diamides. First, they transport plasmid DNA into *Escherichia coli* about 2-fold over controls, where the ampicillin resistance gene is expressed in the bacteria. These studies were done with plasmid DNA (~2.6 kilobase (kb)) in JM109 *E. coli* cells. Second, known methods do not typically transport large plasmids (>15 kb). We demonstrate here that transformation of large pVIB plasmids (i.e., >20 kb) were enhanced over water controls by ~10-fold. These results are in striking contrast to the normal decrease in transformation with increasing plasmid size.

Crabtree and co-workers reported that such simple tris-arenes as N^1,N^3-dimesitylisophthalamide complex anions by dint of their amide NH bonds.[1] During the decade since that report, numerous structural variants have been reported that bind and/or transport an array of anions such as F^-, Cl^-, Br^-, and CH_3COO^-.[2] Since these reports, isophthalic acid dianilides and numerous structural variants have been studied by Gale and co-workers[3] and by others.[4] The receptor has also been used as a module in the construction of ion-pair hosts.[5] Aniline derivatives have been replaced by a range of other residues including aliphatic, aromatic, and heterocyclic amines. A very recent report details a supramolecular interaction between anthracene-substituted pyridinium amides and DNA.[6]

Our interest in anion binding, specifically chloride anion binding, resulted from work to develop a chloride anion transporter that functions in phospholipid bilayer membranes.[7] We called these compounds synthetic anion transporters (SATs) and showed that they are selective for Cl^- over K^+ by more than 10-fold.[8] A comparison of these SATs with the tris-arenes showed that their binding affinities for Cl^- were similar and anion dependent.[9] In more recent work, we showed that at least one tris-arene showed channel-like behavior in phospholipid bilayers[10] and that they are generally good phosphate-complexing agents.[11]

The ability of the 4,4′-dinitrodipicolinanilide to exhibit channel-like behavior and the ability to bind phosphate combined to suggest the possibility of binding and/or transporting DNA. We envisioned that a stack of tris-arenes within the membrane might foster transport of a DNA plasmid. The approximate thickness of a tris-arene is 3.4 Å. Although this is similar to the thickness of the aromatic bases in DNA, the phosphate groups in the backbone are typically separated by about twice that distance. In the structure reported for B-DNA, the phosphate spacing varies but it is generally between 6 and 7 Å (as measured from the crystal structure available in the Protein Data Bank as 1BNA).[12] We show here that the simple tris-arenes mediate the transport of DNA plasmids into *E. coli* and that they are effective transformation agents for plasmids larger than those that can be transported by currently available chemical agents.

Eight simple tris-arenes were prepared for the present study. They include isophthalanilides and dipicolinanilides that are substituted in aniline's (*para*) 4,4′-positions. The substituents are methoxy, hydrogen (no substituent), chloro, and cyano. The structures are shown in Figure 1. Compounds **2**, **6**, and **8** were reported in an earlier study from our laboratory.[10] The substituents reported in this work represent a range of electronic effects from releasing (**1**, **5**) to withdrawing (**4**, **8**). The tris-arenes were prepared by treating either isophthalic (**1**−**4**) or dipicolinic acid (**5**−**8**) with $SOCl_2$, followed by reaction with the appropriate aniline derivative. The products were typically crystalline[14] and isolated as stable solids. Solid-state structures have been reported by Malone et al.[13] for **2** and **6**. The solid-state structures of relatives having hydroxyl,[14] methyl,[15] *n*-butyl,[16] and nitro[17] substituents have all been reported.



1, X = OCH_3; Y = CH **5**, X = OCH_3, Y = N
2, X = H; Y = CH **6**, X = H, Y = N
3, X = Cl, Y = CH **7**, X = Cl, Y = N
4, X = CN, Y = CH **8**, X = CN, Y = N

Figure 1. Structures of tris-arenes **1**−**8**.

Studies were undertaken in *E. coli* to determine the abilities of **1**−**8** to mediate the transfer of a DNA plasmid into a microbe. The overall experiment was done as follows. A

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plasmid of specified size range was used that codes for ampicillin resistance. The bacteria were grown on a medium that is rich in ampicillin so that no bacteria will grow in the absence of gene insertion (transformation). After exposure to the plasmid and compounds, the bacteria are permitted to grow and colonies are counted. The number of colonies obtained after exposure to the plasmid and carrier is compared with the number of colonies observed when only the plasmid is present and when both plasmid and tris-arene are present.

Initial studies were done using Promega[18] JM109 competent cells. These cells are commercially prepared so as to be amenable to plasmid transformation experiments. Two plasmids were selected for study. The first was a 2.6 kilobase (kb) ($\sim 1.7 \times 10^6$ Da) plasmid that is expected to readily enter competent *E. coli* cells. The second was a >20 kb ($>1.3 \times 10^7$ Da) plasmid that was expected to be prohibitively large. Note that the molecular weights in both cases were calculated by using an average base pair weight of 660 Da.[19] The smaller plasmid was studied as a control and the larger plasmid was expected to pass through the boundary membranes of the microbe reluctantly if at all. Typically, a plasmid size of 13−15 kb is considered to be too large to traverse the membrane barrier either of bacterial or eukaryotic cells.[20]

Both plasmids examined contained an ampicillin resistance gene.[21] Solutions of the synthetic isophthalic acid dianilides, **1**−**4**, were dissolved in dimethylsulfoxide (DMSO). Aliquots of the resulting solution were added to standard RNase- and DNase-free microcentrifuge tubes containing plasmid DNA dissolved in nuclease-free water. Mixtures were allowed to react at ambient temperature for 10 min. A suspension of the competent cells (as provided by Promega), plasmid, and dianilide or control chemical were mixed as described in the experimental section. In each case, an *E. coli* culture was exposed to a 2 μL aqueous solution containing the plasmid to be tested [0.1 $\mu g \cdot mL^{-1}$] and isophthalic acid dianilide derivative **1**−**4** [2 μL] and plated on ampicillin-containing LB-agar media. Each experiment was repeated a minimum of three times, and the error bars shown on the graph of Figure 2 indicate the reproducibility for compounds **1**−**4**. The controls were sampling solvents: either Milli-Q (18 MΩ) H_2O or 2 μL of DMSO added to Milli-Q H_2O. Successful transformation was revealed by an enhancement of colony-forming units (CFUs) counted after 18−24 h on each plate. Each cell that acquires the ampicillin resistance gene will grow into a colony, hence "colony forming unit". The colonies were counted on the plate under microscopic examination.

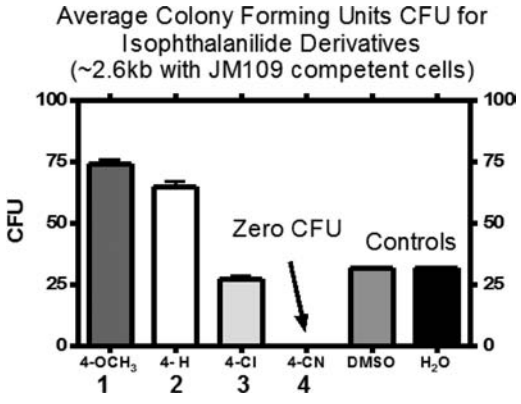

Figure 2. Transformation efficiencies plot for *E. coli* and **1**−**4**. CFU counted after *E. coli* was exposed to a 2 μL aqueous solution containing 0.1 $\mu g \cdot mL^{-1}$ plasmid and 2 μL of isophthalic acid derivative (**1**−**4**) and plated on ampicillin. Figure 1 represents the colony counts resulting from a 100 μL aliquot, taken from a 1 mL solution. The results were verified by using the entire 1 mL solution in a separate experiment.

The vertical bars in the graph of Figure 2 show transformation efficiencies. Both DMSO and H_2O controls gave CFU counts of ~32 with essentially no variation over replicates. The results for transformation by the four isophthalanilides were as follows: **1**, R = OCH_3, CFU ≈ 74; **2**, R = H, CFU ≈ 65; **3**, R = Cl, CFU ≈ 27; and **4**, R = CN, CFU ≈ 0. The results observed in these experiments proved to be both encouraging and surprising. First, a modest enhancement in transformation was apparent for methoxy-substituted **1** and for unsubstituted **2**. The enhancements were ~2.3-fold and ~2-fold, respectively. The bacteria in contact with 4-chloro-substituted **3** showed a decrease in transformation (only 80% of control values), a reduction that is outside of experimental error. Moreover, compound **4** whose *para*-substituent is cyano, showed complete suppression of transformation.

A similar study was conducted with the ~2.6 kb plasmid and *E. coli* using the 2,6-dipicolinic acid analogues (**5**−**8**) of **1**−**4** under otherwise identical conditions. The results are shown in the graph of Figure 3. The results generally parallel those

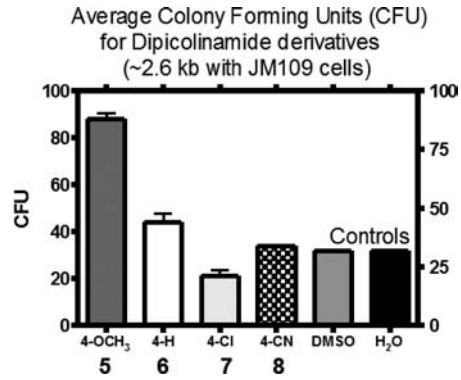

Figure 3. Transformation efficiencies plot for *E. coli* and **5**−**8**. CFU counted after *E. coli* was exposed to a 2 μL aqueous solution containing 0.1 $\mu g \cdot mL^{-1}$ plasmid and 2 μL of dipicolinic acid derivatives (**5**−**8**) and plated on ampicillin.

observed with the corresponding isophthalic acid derivatives. The relative transformation efficiencies were as follows: **5**, R = OCH_3, CFU ≈ 88; **6**, R = H, CFU ≈ 44; **7**, R = Cl, CFU ≈ 21; and **8**, R = CN, CFU ≈ 34. Although there is some difference in magnitude comparing **1** to **5**, **2** to **6**, and **3** to **7**, the major difference is that the cyano-substituted dipicolinic acid tris-arene **8** showed transformation similar to controls whereas **4** was not only inactive, it apparently suppressed activity.

Two conclusions can be reached from the data shown in Figures 2 and 3. First, both isophthalanilides and dipicolinanilides function as transformation agents effectively in *E. coli*. The enhancement over controls is modest, but real and reproducible. Second, there is a general correlation between the electronic effect of the *para* substituent and transformation efficiencies for methoxy, chloro, and hydrogen. Indeed, plots of **1**, **2**, and **3** or **5**, **6**, and **7** vs Hammett[22] σ_p gave straight lines with r^2 values of 0.86 and 0.98, respectively (data not shown). The relationship obviously breaks down in both cases for **4** and **8**, which are cyano-substituted. A plausible explanation for the poor transformation efficiencies of tris-arenes having electron-

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withdrawing groups is posed below. Notwithstanding, methoxy-substituted **1** (isophthalic acid) and **5** (dipicolinic acid) show transformation enhancements over control of ∼2.3-fold and ∼2.8-fold. These increases expand the versatility of the large family of tris-arenes, the results are clearly of both practical and of mechanistic interest, but the extent by which transformation has been improved is modest.

When similar experiments were undertaken using "large" (>20 kb) plasmids, the results obtained were far more dramatic. Transformation of such large plasmids currently presents a significant challenge in biology generally and in genetics in particular. Plasmids >20 kb are inherently valuable because of the number of genes they encode. The dianilides described above, namely **1**, **2**, **4**, and **5** were chosen for study because they were successful with the smaller plasmid. We thus reproduced the experiments described above using a >20 kb, rather than a 2.6 kb, plasmid. We did so being fully aware that the efficacy of typical chemical transformation agents is limited to 13−15 kb DNA.[20] The results of the experiments conducted with *E. coli*, large plasmids, and compounds **1**, **2**, **4**, and **5** are shown in Figure 4.

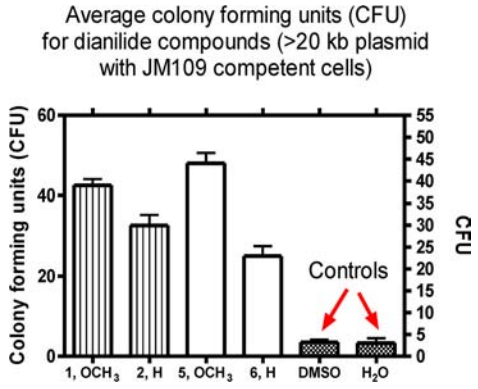

Figure 4. Transformation efficiencies plot. CFU after *E. coli* was exposed to a 2 μL aqueous solution containing 0.1 $\mu g \cdot L^{-1}$ plasmid (pVIB) and 2 μL of isophthalic acid and dipicolinamide derivative and plated on ampicillin. Bar: **1**, R = OCH_3, CFU ≈ 43; **2**, R = H CFU ≈ 33; **5**, R = OCH_3, CFU ≈ 48; **6**, R = H, CFU ≈ 25. Control: 2 μM DMSO, CFU ≈ 4; 2 mL of Milli-Q (18 MΩ) H_2O, CFU ≈ 3.

The transformation experiment, conducted in the presence of DMSO but in the absence of any dianilide, resulted in the observation of ∼4 CFUs. In contrast, isophthalanilides substituted by OCH_3 (**1**), unsubstituted, (**2**), dipicolinanilide substituted by OCH_3 (**5**) and the unsubstituted dipicolinanilide (**6**) resulted in 43, 33, 48, and 25 CFUs, respectively. The range of enhancements is **1**, 10-fold; **2**, 8-fold; **5**, 12-fold; and **6**, 6-fold. When these observations are coupled with the fact that the plasmids tested were larger than are typically transported through bilayers by known reagents, the results are both significant and remarkable.

The transformation efficacy appears to follow a Hammett relationship, diminishing roughly in the order of the substituent sigma parameters.[22] In all of the cases studied thus far, 4-methoxy-substituted tris-arenes were better transformation agents than were those having electron-withdrawing groups on the arenes. Our current hypothesis is that the DNA phosphate residues are bound by individual tris-arenes. This is shown schematically in Figure 5. The array of tris-arenes thus envelop the plasmid in a hydrophobic coat that is—possibly by

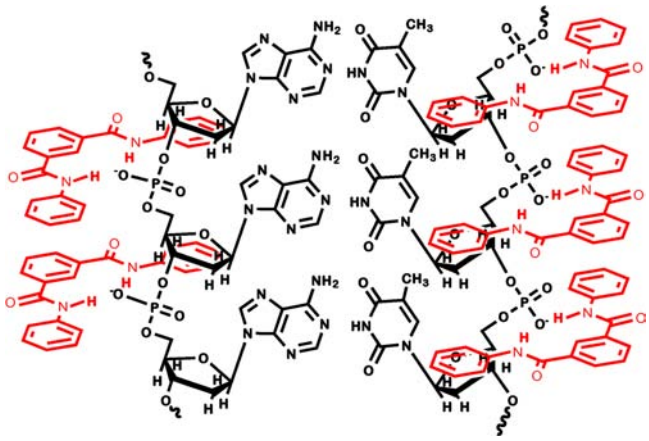

Figure 5. Schematic illustration of the postulated tris-arene:DNA complexation model.

passive diffusion—transported through the bilayer. In support of this hypothesis, we have varied the stoichiometry of DNA:tris-arene over a wide range (data not shown). Optimal transformation, as reflected in the highest colony counts, was achieved with a ratio of 1:1 between receptor and DNA phosphate. Of course, endocytosis may play a role.[23]

The complex must be strong enough to support transport but unless the plasmid is released from the complexing agent within the cell, no genetic modification can take place and the phenotype will remain unexpressed. If binding to the phosphate occurs, it will presumably be strongest when the aniline substituent is most electron-withdrawing. The acidity of the corresponding aniline will correlate with the amide acidities and binding strengths. The pK_A values for protonated anilines increase for *para* substituents in the order NO_2 < CN < Cl < H < OCH_3. The transformations shown in the graphs of Figures 3 and 4 show that rates decreased in the order OCH_3 > H > Cl > CN. Note that the decrease observed in the dipicolinamides was similar to that of the isophthalamides except that Cl^- was less active than CN, which was equal to controls.

An issue that could affect the observation of colony growth is the cytotoxicity of the tris-arenes. No difference in toxicity was observed between any of the tris-arenes and DMSO alone in Kirby−Bauer tests.[24] Even so, minimum inhibitory concentrations (MICs, *E. coli*) were determined for compounds **1**−**8**. Note that the higher the MIC value, the lower is the compound's toxicity. In all cases, the MIC values were >1 mM. The maximum concentration of any tris-arenes used in any experiment reported here was 40 μM. The concentration of tris-arenes exposed to JM109 *E. coli* cells during most of the experiment is only 2 μM.

The work reported here is not only a new application of the broad family of tris-arenes, it represents a breakthrough in the size restriction normally experienced in chemical vector transformation or transfection. Additional studies are underway to determine the broad scope of this application and to better understand the mechanism by which these compounds function.

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Notes

The authors declare no competing financial interest.

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