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Facilitated Chloride Transport Across Phosphatidylcholine Bilayers by an Acyclic Calixarene Derivative: Structure-Function Relationships

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As the field of anion transport develops, it becomes increasingly important to understand mechanistic and structure-function relationships for those synthetic compounds that facilitate transmembrane anion transport. We define some key structural aspects that control the Cl^- anion transport function of an acyclic calixarene analog, triamide 2.We find that the secondary amide NH groups are necessary, but not sufficient, for activity in a standard base-pulse assay that measures the ability of compounds to dissipate pH via chloride transport. Evidence for self-association of 2 in the liposome is found in comparative studies of H^+/ Cl^- transport in EYPC and DPPC liposomes. Using an assay with a Cl^- sensitive dye, we also report direct evidence for Cl^- transmembrane transport by the acyclic triamide 2 and the *1,3 alternate* calix[4]arene 1.Consistent with the base-pulse assay, the acyclic triamide 2 is more active than calixarene 1 in the lucigenin Cl^- transport assay.

Keywords: Calixarene; Ion transport; Membrane-active; Ionophore; Phenol-formaldehyde oligomer; Anion transport

INTRODUCTION

Transmembrane ion channel proteins help maintain the proper ion concentrations within cells. In the past decade there have been tremendous advances in the molecular level understanding of the structure and function of these proteins, most notably the K^+ ion and Cl^- ion channels [1,2]. This structural knowledge is important since ion balance is essential to cellular function, including maintenance of proper pH, osmotic pressure, and lipid membrane asymmetry. In addition, a detailed understanding of transmembrane ion transport is crucial since impaired transport can lead to disease. For example, cystic fibrosis is caused by a mutation in the CFTR channel, a protein that facilitates transmembrane chloride transport [3].

This desire to better understand natural ion transport processes, coupled with the goal of discovering new therapeutics, has driven the discovery and development of synthetic ion transporters [4]. Supramolecular chemistry has helped spur many of these advances. In just the past few years, a number of groups have described low molecular weight compounds that transport Cl^- across membranes. "Small molecules" that facilitate Cl^- transport are also potentially useful as therapeutics. Tomich et al. used peptides derived from the glycine receptor to form anion-selective channels in planar bilayers [5–8]. Gokel and Schlesinger have described a family of synthetic lipopeptides that effect Cl^- efflux from both liposomes and planar bilayers [9,10]. Regen and colleagues described a sterol whose protonated polyamine chain functioned as a transmembrane "anion slide" [11,12]. Notably, this analog restored Cl^- transport in cystic fibrosis cells [13]. The groups of Smith and A. Davis have designed sterols known as "cholapods" that are effective transmembrane chloride transporters [14,15]. There is also renewed interest in the prodigiosins, tripyrroles with anti-cancer activity isolated from *Streptomyces* and *Serratia* [16]. These natural products bind HCl and transport Cl^- across vesicles and cell membranes [17]. One hypothesis is that prodigiosins trigger apoptosis of cancer cells by lowering intracellular pH [18]. Recently, the groups of Gale, Smith and Sessler have shown that prodigiosin analogs transport Cl^- across

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membranes, opening the door for further advances with these compounds [19,20].

Our contributions in the anion transport field developed from using the venerable calix[4]arene scaffold [21]. While there is a vast literature on calixarenes as receptors for neutral guests, cations, anions, and ion pairs [22], a number of calixarene derivatives have been previously shown to transport ions across phospholipid membranes. Kobuke and colleagues [23–25], and Gokel, deMendoza and co-workers [26], have described synthetic cation channels formed from calixarenes. Our studies on calixarene-guanosine conjugates [27,28], originally designed to be cation transporters, led us to discover that tetrabutylamide 1,3-*alt* calix[4]arene **1** transports Cl^- anions across phospholipid membranes [29]. Solid-state structures confirmed the Cl^- mediated assembly of **1** into channel motifs. Voltage clamp experiments on planar lipid bilayers and HEK cells indicated that calixarene **1** formed transmembrane channels. We later compared the apparent H^+/Cl^- co-transport activity of calixarene **1** with a series of acyclic phenol-formaldehyde analogs in liposomes. The butyl triamide **2** emerged as the most efficient transporter of this group [30–32]. The ability of acyclic triamide **2** to transport Cl^- at μM concentrations, along with its low molecular weight and simple preparation makes such a compound a potential lead in drug development for Cl^- transport malfunctions or as an antibiotic [33].

To better define the structure-function relationships that are important for efficient transport of Cl^- anions, we are in the process of surveying compounds that are structurally related to *1,3-alt* calixarene **1** and triamide **2**. In this paper, we report results on the transmembrane transport activity of a series of related acyclic phenoxyacetamide analogs with an eye toward firmly establishing the structural components within **2** that are needed for Cl^- ion transport. Another goal of this study was to develop an assay that would be capable of directly monitoring the kinetics of Cl^- transport across a liposomal membrane.

RESULTS AND DISCUSSION

Some Rationale

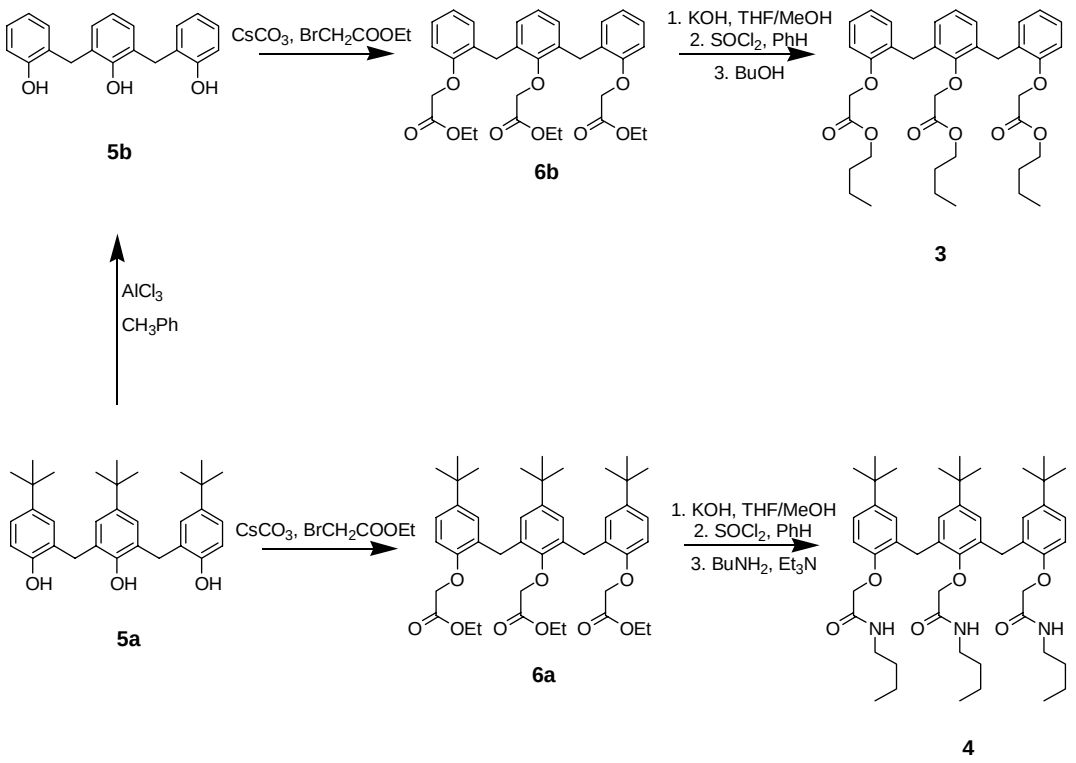
To clarify some important structure-function relationships about membrane transport we first compared three acyclic "calixarene analogs" for their ability to dissipate a pH gradient, in a Cl^- dependent fashion, across EYPC liposomes. The compounds that we evaluated in this study were triamide **2**, the acyclic calixarene analog that we had previously identified to be an efficient Cl^- anion transporter [30], tributyl ester **3** and the t-butyl-substituted triamide **4**. Comparison of triamide **2** and triester **3** was done to demonstrate that the secondary amide sidechains in **2** are essential for transmembrane anion transport, as the NH groups are likely needed to hydrogen bond to Cl^- [34]. Comparison of the transport activity of unsubstituted triamide **2** and the t-butyl triamide **4** was done to determine if transport function is sensitive to subtle structural factors, as we speculated that aromatic substitution with the t-butyl groups might well alter either the acyclic trimer's conformation or its membrane permeability. Indeed, the substitution pattern of related acyclic phenol-formaldehyde oligomers has been shown to strongly influence the formation and stability of inclusion complexes [35].

Synthesis

Triamide **2** was prepared according to previous methods [30]. Preparation of triester **3** and the t-butyl triamide **4** is outlined in Scheme 1. The known triphenol 5b [30,35] was converted via S_N2 displacement with ethyl bromoacetate to triester **6b**. The ester was hydrolyzed to the corresponding acid, and after treatment with thionyl chloride, the acid chloride intermediate was then reacted with n-butyl alcohol to give tributyl ester **3** in 39% overall yield after purification. Preparation of the para-substituted triamide **4** was similarly straightforward. Thus, the known triphenol **5a** [30,35] was reacted with ethyl bromoacetate to give triester **6a**. After hydrolysis to the corresponding acid, thionyl chloride was added to give the corresponding acid chloride, which upon acylation with n-butylamine gave triamide **4** in 22% overall yield from **5a**. Both NMR and high-resolution mass spectrometry data were consistent with the structures of **2–4**. Compound **4** was crystallized and its structure was determined from single crystal x-ray analysis [36]. diagram of the t-butyl substituted triamide is **4** is shown in Fig. 1. This structure indicated that each of the three amide NH protons forms an intramolecular NH-O hydrogen bond with the phenoxy O atom on the corresponding aromatic ring, thus precluding any hydrogen bonding between the neighboring n-butylamide chains. Attempts to obtain a crystal structure of the hydrogen substituted triamide **2** for comparison purposes with **4** have, so far, been unsuccessful.

The Secondary Amide Group and the Aromatic Substitution Patterns are Important for Ion Transport Activity of Acyclic Phenoxymethylene Analogs

We compared the ion transport properties of compounds **2–4** in EYPC liposomes by using the so-called "base pulse" assay, a standard protocol in the evaluation of membrane active transporters



SCHEME 1 Synthesis of triester **3** and triamide **4**.

[37–39]. In this assay, a compound's ion transport ability is measured indirectly by monitoring the pH changes inside a liposome. The pH sensor in this particular assay is the fluorescent dye 8-hydroxy-1,3,6-pyrene-trisulfonate (HPTS). Protonated and deprotonated forms of HPTS have different excitation wavelengths (403 and 460 nm), but both species emit at the same wavelength (510 nm). Thus, using dual excitation of the liposome solution one can determine the intravesicular pH by measuring the ratio of protonated/deprotonated HPTS. The potential transporter is added to a suspension of EYPC liposomes containing entrapped dye. Addition of NaOH to the extravesicular solution creates a pH gradient, wherein the intravesicular solution is more acidic than the outer solution. The resulting pH gradient is dissipated only if the transporter promotes either cation influx or anion efflux. The ion transport is necessary to balance the electrostatic potential generated by the efflux of protons (or influx of hydroxide in the case of an anion exchange molecule) as the pH equilibrates. If the compound does not transport ions to relieve this electrostatic potential, then no change in internal HPTS fluorescence is observed. If the compound mediates transmembrane ion transport, then the internal HPTS florescence ratio increases as the intravesicular pH increases.

Our previous studies using the "base-pulse" assay showed that the acyclic triamide **2** (at μM concentrations) changed HPTS fluorescence in the presence of NaCl [30]. Similar experiments with Na_2SO_4 solutions showed little change in HPTS fluorescence; this was strong evidence that it is indeed transmembrane transport of Cl^- anion, as

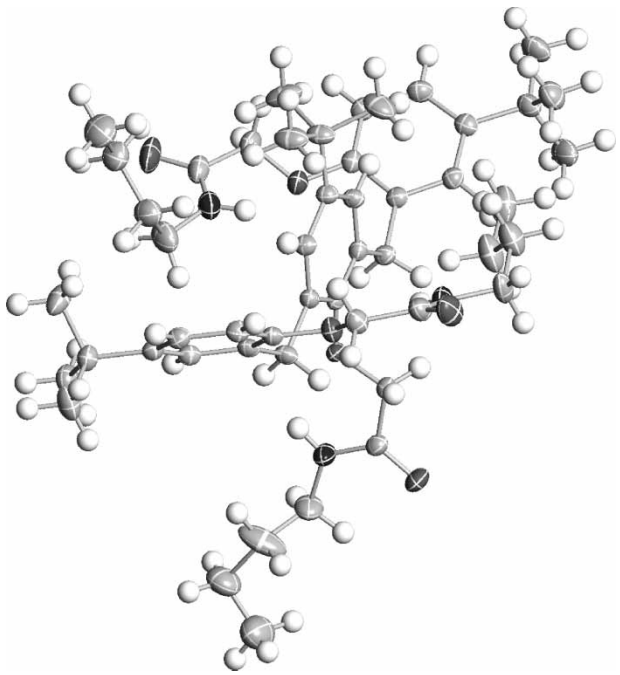


FIGURE 1 The solid-state structure of the t-butyl substituted acylic triamide **4**.

mediated by triamide **2**, that was responsible for the observed pH change [30]. Additionally, no detectable differences in initial rates were seen when experiments were conducted in solutions containing CsCl, KCl, or NaCl, suggesting that the activity of **2** was due to its Cl^- transport properties and not to any M^+ cation transport (data not shown). Indeed, as shown below, assays with the Cl^- sensitive dye, lucigenin, provided direct evidence that triamide **2** transports Cl^- across phospholipid membranes.

Figure 2 indicates that triamide **2** is relatively active in this base pulse assay, whereas ester **3** shows essentially no activity above background. Clearly, the secondary amide NH groups in **2** are crucial for transport activity, as they presumably bind and facilitate the transport of Cl^- anion across the lipid membrane. Interestingly, formation of **2**·Cl^- must be quite weak, given that the ^1H NMR spectra of triamide **2** did not show any detectable shifting of the amide NH protons when excess Bu_4N^+ Cl^- was added to a solution of **2** in $CDCl_3$ (data not shown). Although triamide **2** binds only weakly to Cl − it is still able to move the anion across the lipid membrane [40]. Fig. 2 also shows that the t-butyl triamide **4**, unlike its unsubstituted analog **2**, is inactive in the base-pulse assay. We speculate that introduction of these para-substituents likely changes the compound's global conformation, and perhaps its self-association properties, so that it becomes an inactive Cl^- transporter.

Importantly, the base-pulse experiments with acyclic analogs **2–4** establish that amide NH groups are necessary, but not sufficient, for transport activity. The amide NH groups in **2** are undoubtedly needed to hydrogen bond to Cl^- anion. It's also possible that these amide sidechains enable self-association of **2** into a membrane active species. Indeed the lack of activity for t-butyl analog **4**, despite its having the same amide sidechains as **2**, supports the hypothesis that monomer conformation (and perhaps the propensity to self-associate into a particular oligomer structure) is crucial for function.

In an effort to probe the role of self-association of triamide **2** in ion transport, the base-pulse assay was repeated for triamide **2** in liposomes composed of DPPC, a lipid having a gel to liquid crystalline phase transition (41°C) higher than that of EYPC (−15°C). Assays in DPPC liposomes are useful in determining the mechanism of ion transport [41–43]. Whereas compounds acting as ion channels show activity in gel state DPPC, those acting as mobile ion carriers demonstrate significantly crippled transport rates, as diffusion through the gel state membrane is much slower than through liquid-crystalline state membranes [41–43].

Thus, DPPC liposomes (100 nm) were prepared containing HPTS dye and 100 mM NaCl in 10 mM sodium phosphate solution (pH 6.4). After removal of the external dye (Sephadex G-25), the isolated liposomes were diluted into a similar solution (100 mM NaCl, 10 mM sodium phosphate, pH 6.4). The fluorescence of HPTS was monitored at 35°C after triamide **2** was added via injection port to a stirring liposome solution giving a 1:100 ligand to lipid ratio. Addition of NaOH afforded a pH gradient of approximately 1 pH unit.

As seen in Fig. 3, the triamide **2** shows activity in the gel-state DPPC that is quite similar to that seen in the liquid-crystalline EYPC. We take this data as strong evidence that triamide **2** self-associates into channel structures within the phospholipid membrane.

EYPC Chloride Gradient Assays: Chloride is Transported across a Chloride Gradient

The HPTS base pulse assays, while very informative, provided only indirect evidence for ion transport activity. Therefore we sought an assay that would give direct evidence for Cl^- anion transport as

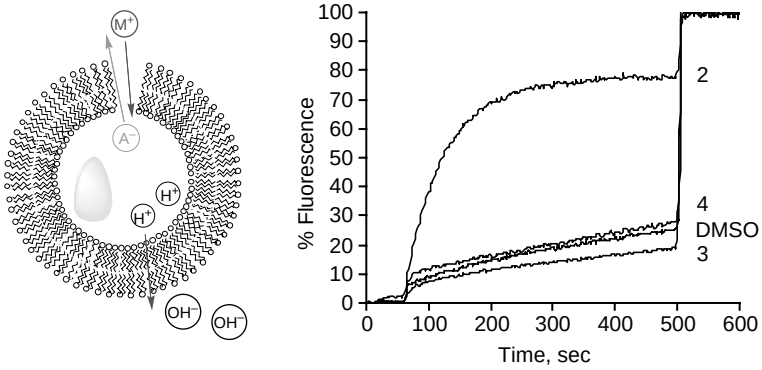


FIGURE 2 Transport of H^+/Cl^- by acyclic calixarene analogs **2–4**, as determined in a pH gradient assay. EYPC liposomes (100 nm) containing HPTS and 100 mM NaCl in 10 mM NaPi, pH 6.4 were suspended in 100 mM NaCl in 10 mM NaPi, pH 6.4. The compounds were added at t = 20 sec as DMSO solutions to give a 1:100 ligand to lipid ratio. The addition of NaOH solution at t = 60 sec established a pH gradient of approximately 1 pH unit. At t = 500 sec the liposomes were destroyed with Triton-X detergent to determine maximal fluorescence. The pH dependant fluorescence of HPTS dye is reported as % of the total change.

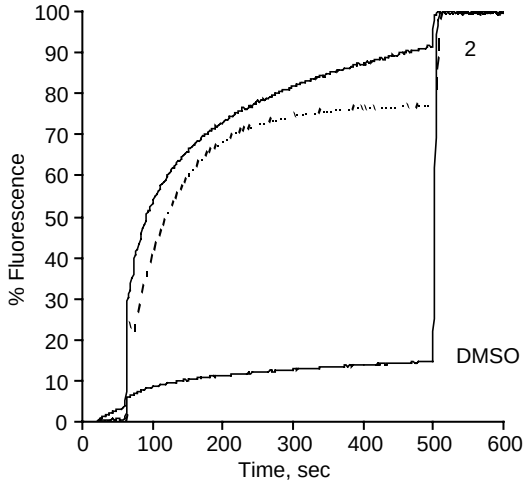


FIGURE 3 Triamide **2** in DPPC liposomes at 35°C. DPPC liposomes (100 nm) contained and were suspended in 100 mM NaCl in 10 mM sodium phosphate, pH 6.4. Triamide **2** was added at t = 20 sec (DMSO solution, 1:100 final ligand to lipid ratio). The addition of NaOH solution at t = 60 sec established a pH gradient of approximately 1 pH unit. At t = 500 sec the liposomes were destroyed with Triton-X detergent to determine maximal fluorescence. The pH dependant fluorescence of pyranine dye is reported as % of the total change. The dotted trace represents data using compound **2** in EYPC (Fig. 2) included here for comparison.

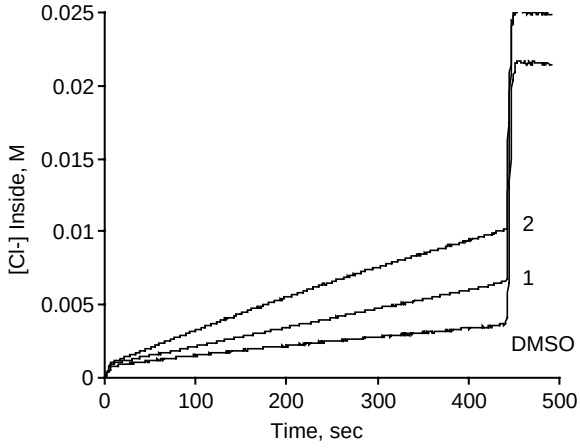


FIGURE 4 EYPC liposomes (100 nm) containing 1 mM lucigenin and 100 mM $NaNO_3$ in 10 mM sodium phosphate (pH 6.4). Chloride transport plotted as internal [Cl^-] vs. time upon application of a chloride gradient (t = 0) such that external [Cl^-] = 25 mM. The liposomes were destroyed with Triton-X at t = 440 sec. Chloride concentration was calculated from fluorescence using the Stern-Volmer constant determined for lucigenin under the conditions of the assay. Concentration of ligand to lipid was 1:100.

mediated by the calixarene **1** and its analogs such as triamide **2**. Therefore, to confirm that these compounds could transport chloride across liposomal membranes, we used the chloride sensitive dye lucigenin [44–46]. This fluorescent dye has recently been used by Smith and coworkers to compare the effectiveness of steroid-derived Cl^- transporters [47], and we essentially adopted Smith's protocol in our own studies using these calixarene based transporters.

Lucigenin was entrapped in EYPC liposomes containing 100 mM sodium nitrate in 10 mM phosphate buffer at pH 6.4. The external lucigenin was removed by passing the liposomes through a Sephadex column. In a typical assay the fluorescence of lucigenin was monitored as a function of time as a sodium chloride gradient was introduced to the solution such that the outer concentration of sodium chloride was 25 mM. As seen in Fig. 4 the relative Cl^- transport activity for the triamide 2 and the *1,3-alt* calixarene **1** is the same as in the pH gradient assay using HPTS. Triamide **2** shows a significantly faster rate of Cl^- transport than does the *1,3-alt* calix **1** under the same conditions.

Although the calculated rate constants in using the Cl^- sensitive probe lucigenin are an order of magnitude lower than those measured for proton transport in the pH gradient assay, one must recognize that the driving force for transport is different in the two cases. In the HPTS assay it is an inwardly directed pH gradient that drives movement, whereas in the lucigenin assays a Cl^- gradient exists at constant pH. Importantly, as shown in Table I, the relative rates for the triamide **2** and calixarene **1** in are similar for both transport assays.

The other significant result obtained in these experiments is that we can now carry out both the HPTS base-pulse assay and the lucigenin Cl^- assay under identical conditions. Such experiments are potentially illuminating for determining the mechanism of anion transport. As mentioned, the HPTS indirectly detect chloride transport by following the dissipation of a pH gradient across the liposomal membrane. Thus, an H^+/Cl^- symport process can not be distinguished from an OH^-/Cl^- antiport mechanism using this base-pulse assay. We hope to overcome such mechanistic ambiguities by monitoring both Cl^- influx and internal liposomal pH as a function of internal anion using separate batches of liposomes prepared under identical conditions.

TABLE I Rate comparison of calixarene **1** and acyclic triamide **2** in the pH gradient and chloride gradient assays.

Compound	Pseudo-first order rate constant in Base Pulse Assay ($x10^{-3}$)	Relative Rate in Base Pulse Assay	Pseudo-first order rate constant in NaCl Gradient Assay ($x10^{-3}$)	Relative Rate in NaCl Gradient Assay
1 (1,3 Alternate)	12.1	1	1.5	1
2 (Acyclic Triamide)	24.6	2	3.2	2.1

In this way, we can simultaneously track Cl^- and H^+ (or OH^-) transport [48].

CONCLUSION

We have confirmed that triamide **2**, an acyclic calixarene analog, is an effective Cl- transmembrane transporter. Direct evidence for Cl^- transport was obtained by using the chloride-selective probe, lucigenin. Also, using the standard base-pulse method, we determined important structure-function relationships regarding triamide **2**. Most importantly, the amide NH groups are necessary, but not sufficient, for transport activity, as demonstrated by the lack of transport activity for ester analog **3** and t-butyl analog **4**. Amide NH groups in **2** are undoubtedly needed to hydrogen bond to Cl^- anion to enable transport. The lack of activity for t-butyl analog **4**, despite its having the same butylamide sidechains as the active transporter **2**, supports the notion that ligand conformation and/or ligand self-association are crucial for transmembrane transport ability. Finally, facially-amphiphilic compounds such as triamide **2** may find use as lead structures in the development of new antibiotics or apoptotic compounds.

METHODS AND MATERIALS

^1H NMR spectra were recorded on a Bruker DRX400 instrument at 400.130 MHz and chemical shifts are reported in ppm relative to the residual solvent peak. ^{13}C NMR spectra were recorded at 100.613 MHz and chemical shifts reported in ppm relative to the solvent peak. Mass spectra obtained using fast atom bombardment ionization and recorded on a JOEL SX-102A magnetic sector mass spectrophotometer. Fluorimetric experiments for the EYPC base-pulse assays were done on an SLM Aminco (Aminco Bowman Series 2) Luminescence Spectrometer. The DPPC fluorimetric assays were performed using a Fluoromax 3 (Jobin-Yvon/Horriba) spectrophotometer with cuvette temperature maintained by a water bath circulator at 35°C (+/− 0.2°C). Chemicals and solvents were purchased from Sigma, Aldrich, Fluka, or Acros. Lucigenin dye was purchased from Molecular Probes, and the EYPC and DPPC lipids were purchased from Avanti Polar Lipids.

Preparation of Liposomes

Large unilamellar vesicles were prepared using EYPC lipid. 50 mg of lipid was dissolved in $CHCl_3$/MeOH and evaporated to give a thin film. The thin film was dried *in vacuo* for 2.5 hours and then hydrated with appropriate solution containing fluorescent dye to give a 50 mg/mL solution of lipid. After 4–5 freeze/thaw cycles, the liposomes were extruded through a 100 nm polycarbonate membrane using a high pressure mini-extruder (Avanti Lipids). The liposomes were then passed through a Sephadex (G-25) column to remove external dye and diluted to appropriate concentration for use in the applied assays.

Base-Pulse Liposome Transport Assays

The base-pulse assays with HPTS were carried out as previously reported [29,30].

Chloride Transport Assay in Liposomes

In a typical experiment, 0.1 mL of the stock liposome solution was diluted into 2 mL of a solution of 10 mM sodium phosphate (pH 6.4, 100 mM $NaNO_3$) to give a solution that is 0.5 mM in lipid. The compounds were added at t = 20 sec as DMSO solutions to give a 1:100 ligand to lipid ratio (1 mol %). Fluorescence was monitored at excitation 372 nm and emission at 504 nm for 500 s. At t = 20 s, .02 mL of 4.0 M NaCl was added to the cuvette through an injection port to give a final concentration of 25 mM chloride outside of the liposomes. After 470 s, 0.04 mL of 10% Triton-X detergent was added to destroy the liposomes and determine maximal fluorescence quenching of lucigenin by Cl. Experiments were repeated in triplicate and all traces reported are the average of the three trials. Lucigenin fluorescence was converted to chloride concentration using the Stern-Volmer constant determined under the assay conditions. To measure the Stern-Volmer constant, liposomes were prepared as above, except that the liposomes were lysed immediately with Triton-X. Then, 5 μL of 4.0 M NaCl was titrated in every 30 s via the injection port. The titration was completed twice. A plot of f_0/f vs. chloride concentration was generated, the slope of which is taken to be the Stern-Volmer constant.

Synthesis

Compounds **1** and **2** synthesized as previously described [29,30].

Tris-(butyl Phenoxyacetate) 3

Tri- phenoxyacetic acid [30,35] (720 mg, 1.5 mmol) was activated with $SOCl_2$ (6.7 mL in 15 mL of benzene) and heated to reflux for 4.5 hours. The solvent and excess thionyl chloride was then evaporated under reduced pressure and the resulting acid chloride was further dried *in vacuo* for 2 hours. 15 mL dry CH_2Cl_2 was added and the solution stirred as butanol (15 mL) was added

dropwise and the reaction stirred at room temperature overnight. After suspending in chloroform-water, the organic layer was dried with sodium sulfate and evaporated. The crude solid was submitted to column chromatography (silica gel, 5:1 Hexane/EtOAc) to obtain compound **3** (250 mg, 39% yield over 2 steps). ^{1}H NMR ($CDCl_3$) 7.14 (2H dt $J = 8.4, 1.6$), 7.11 (2H d $J = 7.6$), 6.92 (5H m), 6.71 (2H d $J = 8$), 4.60 (4H s), 4.38 (2H s), 4.16 (4H t $J = 6.4$), 4.14 (2H t $J = 6.4$) 4.10 (4H s), 1.51 (6H m), 1.31 (6H m), 0.89 (3H t $J = 7.2$), 0.88 (6H t $J = 7.2$) ^{13}C NMR (DMSO-d6) 13.8, 13.9, 18.8, 18.9, 29.5, 30.4, 64.5, 65.2, 70.1, 79.6, 112.1, 121.4, 124.6, 127.8, 128.8, 129.1, 130.9, 133.8, 154.9, 155.8, 169.0, 169.2 FAB + MS estimated *m/z* 648.3298, found 648.3284

Tris-(ethyl p-tertbutylphenoxyacetate) 6a

Tri-p-tertbutylphenol [30,35] (1.19 g, 2.5 mmol) and Cs_2CO_3 (1.44 g, 3 equivalents) was suspended in 60 mL acetone. Ethyl bromoacetate (1.25 mL, 4.5 equivalents) was added dropwise at room temperature. The reaction was heated to reflux and stirred 8 hours. After evaporating the acetone under reduced pressure, the resulting solid was suspended in chloroform-water. The organic layer was dried with sodium sulfate and evaporated under reduced pressure. Upon column chromatography (silica gel, 5:1 Hex/EtOAc), pure Tris-(ethyl p-tertbutylphenoxyacetate) was obtained (1.26 g, 68% yield) ^{1}H NMR ($CDCl_3$) 7.12 (d, 2), 7.10 (s, 2), 6.95 (s, 2H), 6.62 (d, 2H), 4.60 (s, 4H), 4.38 (s, 2H), 4.16 (q, 4H $J = 6.4$), 4.12 (q, 2H $J = 6.4$), 4.10 (s, 4), 1.1-–1.3 (m, 27H), 1.1 (s, 9) ^{13}C NMR ($CDCl_3$) 169.7, 154.2, 153.5, 147.1, 144.5, 132.9, 129.8, 128.5, 126.6, 123.9, 111.5, 70.7, 66.5, 61.6, 61.3, 34.7,34,5, 31.9, 30.3, 14.6 FAB + MS estimated *m/z* 739.4397, found 739.4442.

Tris-(N-butyl-2-p-tertbutylphenoxyacetamide) 4

Tris-(ethyl p-t-butylphenoxyacetate) **6a** (1.26 g, 1.7 mmol) and 5.1 mL 45% aqueous KOH solution was stirred in 25 mL methanol/THF (1:1 by volume) at room temperature 5 hours. After the solvent was evaporated under reduced pressure the resulting solid was dissolved in a minimum amount of water, acidifid with 6 N HCl and then the water evaporated under reduced pressure. The organic acid was dissolved in acetone and the KCl salt removed by filtration. The acetone was evaporated to give the desired acid as a white solid. Without further purification, the acid was activated with $SOCl_2$ (3.4 mL in 10 mL of benzene) by the same procedure as described for compound **3**. The solvent and excess thionyl chloride was evaporated under reduced pressure and the resulting acid chloride was further dried *in vacuo* for 2.5 hours. 15 mL dry CH_2Cl_2 was added and the solution stirred as butylamine (0.92 mL, 9.2 mmol) and triethylamine (0.96 mL, 6.9 mmol) were added dropwise at room temperature. The reaction continued to stir at room temperature for 12 hours. After suspending in chloroform-water, the organic layer was dried with sodium sulfate and evaporated. The crude solid was submitted to column chromatography (silica gel, 3% MeOH in $CHCl_3$) to obtain compound **4** (440 mg, 32% yield over 3 steps). ^{1}H NMR (DMSO-d6) 7.94 (1H t NH), 7.68 (2H t NH), 7.14 (2H dd $J = 8.4, 2.4$), 7.05 (2H d $J = 2$), 6.99 (2H s), 6.78 (2H d $J = 8.4$), 4.39 (4H s), 4.08 (2H s), 4.04 (4H s), 3.09 (6H m), 1.35 (6H m), 1.22–1.17 (6H m), 1.15 (18H s), 1.10 (9H s), 0.82 (9H t) ^{13}C NMR (DMSO-d6) 14.5, 20.4, 30.5, 31.9, 32.0, 34.5, 34.8, 38.9, 68.1, 72.9, 112.1, 124.5, 126.7, 128.0, 129.1, 133.1, 143.8, 146.8, 153.3, 154.2, 168.3, 168.4 FAB + MS estimated *m/z* 814.5734, found 814.5742.

Crystallographic Experimental

A colorless block of the t-butyl substituted triamide **4** with dimensions 0.489 x 0.396 x 0.196mm^3 was centered on the Bruker SMART CCD system at −100°C. The initial unit cell was indexed using a least-squares analysis of a random set of reflections collected from three series of 0.3° wide ω-scans, 10 seconds per frame, and 25 frames per series that were well distributed in reciprocal space. Data frames were collected [MoKα] with 0.3° wide ω-scans, 20 seconds per frame and 606 frames per series. Five data series were collected at varying angles (α = 0°, 72°, 144°, 216°, 288°). The crystal to detector distance was 4.397 cm, thus providing a complete sphere of data to $2\theta_{max} = 55.0°$. A total of 41158 reflections were collected and corrected for Lorentz and polarization effects and absorption with 11,255 unique reflections [R(int) = 0.0277]. The SHELXTL program was used to determine the probable space group and set up the initial files [49]. System symmetry, lack of systematic absences and intensity statistics indicated the centrosymmetric triclinic space group P-1 (no. 2). The structure was determined by direct methods with the successful location of nearly the entire ensemble using the program XS [50]. The structure was refined with XL [51]. A single least-squares difference-Fourier cycle was required to locate the remaining non-hydrogen atoms. All non-hydrogen atoms were refined anisotropically. Hydrogen atoms were placed in calculated positions. Various disorders were optimized in the three side chains. The final structure was refined to convergence [Δ/σ 0.001] with R(F) = 6.23%, wR(F^2) = 14.96%, GOF = 1.086 for all 11255 unique reflections [R(F) = 4.85%, wR(F^2) = 13.79% for those 8945 data with Fo > 4σ(Fo)]. The final difference-Fourier map was featureless indicating that the structure is both correct and complete.

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[36] , Crystal data for $C_{50}H_{75}N_3O_6$ M_r 814.13, crystal dimensions 0.489 x 0.396 x 0.196 mm^3, triclinic, space group P-1, a=13.524(2) Å, b=13.962(2) Å, c=15.014(2) Å, V=2449.5(6) $Å^3$, Z=2, D_x 1.104 mg/m3, MoK 0.071 mm^{-1}. Data were collected on a Bruker SMART 1000 CCD diffractometer at 173(2) K. The structure refined to convergence [(/((0.001] with R(F) =6.23%, wR(F^2)=14.96%, GOF =1.086 for all 11,255 unique reflections [R(F) =4.85%, wR(F^2)=13.79% for those 8945 data with $F_o > 4((F_o)]$. Crystallographic data for the structure reported in this paper have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication no.CCDC-282326. Copies of the data can be obtained free of charge on application to CCDC, 12 Union Road, Cambridge CB21EZ, UK e-mail: deposit@ ccdc.cam.ac.uk)
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