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Characterization of drug encapsulation and retention in archaea-inspired tetraether liposomes†

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The passive leakage of small molecules across membranes is a major limitation of liposomal drug formulations. Here, we evaluate the leakage of 3 clinically used chemotherapeutic agents (cytarabine, methotrexate and vincristine) encapsulated in liposomes comprised of a synthetic, archaea-inspired, membrane-spanning tetraether lipid. Liposomes comprised of the pure tetraether lipid exhibited superior retention of both a neutrally and positively charged drug (up to an ~9-fold decrease in the rate of drug leakage) compared to liposomes formed from a commercial diacyl lipid, while exhibiting a similar retention of a negatively charged drug that did not appreciably leak from either type of liposome. We also demonstrate that liposomes made of the archaea-inspired lipid can be used for the delivery of encapsulated small molecules into living cells.

The application of liposomes (and other bioinspired molecular assemblies) as versatile platforms targeting molecular cargo to specific tissues *in vivo* has made a major impact on many biomedical areas such as imaging diagnostics and drug delivery.[1–4] Indeed, liposomes have been successfully used to improve the therapeutic index of potent drugs by improving drug solubility and biodistribution, overcoming obstacles to cellular and tissue uptake, and decreasing systemic toxicity.[5,6] As a result, liposomal formulations have made it possible to clinically evaluate drugs in a range of therapeutic areas, leading to, for instance, the delivery of anti-cancer, anti-fungal and antibiotic drugs, the delivery of gene medicines, and the delivery of anesthetics and anti-inflammatory drugs.[5] For cancer therapy, the benefits of liposomal encapsulation are dependent on the rates of passive drug release from the liposomes prior to reaching the targeted tissue, leading to the need to develop formulations which are stable and exhibit a slow leakage profile.[7] Lipid composition, therefore, becomes crucial for retaining the encapsulated drug. Liposomal formulations are typically made of either solid-phase phospholipid bilayers (*e.g.* hydrogenated soy phosphatidylcholine, HSPC) or fluid-phase phospholipid bilayers (*e.g.* egg phosphatidylcholine, EPC).[8] However, current liposomal formulations still suffer from drug leakage due to the use of highly permeable membranes, especially when the encapsulated drug does not form stable intra-liposomal precipitates (such as the case with encapsulated anthracycline analogs at low intra-liposomal pH).[9] Attempts at reducing membrane permeability include the cross-linking[10] of lipids (with the risk of not being able to release the encapsulated drug when desired), or the use of robust and tightly packed lipid bilayers.[11]

In nature, archaeal organisms have evolved mechanically and chemically robust membrane compositions that allow survival in extreme environments.[12,13] For instance, liposomes made of extracted archaeal lipids, called archeasomes, have been shown to exhibit low permeability, tight membrane packing, and high stability.[14] Whereas archaeal lipids seem to be an ideal candidate to overcome the limitations associated with drug leakage from liposomal drug carriers,[15] reproducibly harvesting large quantities of specific lipid compositions from cultured archaeal species can be challenging. While Kakinuma and coworkers previously reported the total synthesis of a natural archaeal 72-membered macrocyclic tetraether lipid,[16] only a few groups have reported syntheses of archaea-like tetraether lipids that incorporate structural features found in natural lipids such as: ether glycerol linkages, tethering of lipid tails, and the incorporation of small rings within the transmembrane portion of the lipid.[17–22] Among the few reported examples of synthetic archaea-like lipids, only Benvegnu and coworkers have shown that synthetic tetraether lipids could be used for the delivery of genes to cells.[23,24] To the best of our knowledge, however, the evaluation of encapsulation and permeability of small molecules from tetraether lipids has been limited to model fluorophores (*e.g.*, carboxyfluorescein)[19,25,26] and small ions (*e.g.*, H^+, OH^-, Na^+, Cl).[27] There have been no previous reports, for instance, on the encapsulation and retention of small molecule therapeutics from liposomes comprised of tetraether lipids.

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Herein, we present a novel synthetic route to an archaea-inspired lipid, namely a glycerol monoalkyl glycerol tetraether lipid with phosphocholine head groups (GMGTPC), which made it possible to prepare this lipid at practical scales for studies on the encapsulation and retention of small molecule therapeutics. We previously reported that GMGTPC lipids exhibit low permeability to small ions, and, therefore, appeared to be a suitable tetraether lipid for the initial drug encapsulation and retention studies reported here.[19] To evaluate whether GMGTPC lipids can be used in liposomal drug delivery formulations with high drug retention, we first prepared three liposomal preparations of GMGTPC lipids that encapsulated three known hydrophilic drugs with different physicochemical properties: cytarabine (Ara-C), vincristine (VCR) and methotrexate (MTX) (Fig. 1A). Drug retention within the liposome was then measured *in vitro* and compared to liposomal formulations comprised of EggPC lipids (EPC). Folic acid-targeted liposomal formulations of GMGTPC were also prepared and incubated with KB cells (a HeLa-derived cell line[28]) to probe for (1) cell-uptake using membrane-labeled rhodamine liposomes, and (2) liposomal content delivery using dye- and drug-encapsulated liposomes.

In previous work, we described the preparation of a set of archaea-inspired lipids (including GMGTPC) synthesized through a series of Wittig and S_N2 reactions.[19] Unfortunately, this strategy has been hampered by inconsistent yields for the Wittig reactions, and low yields for the formation of the tetraether core through Williamson etherification due to elimination-derived side reactions (resulting, on average, in a 3.5% overall yield from commercial starting materials). In an effort to improve the overall yield for preparing GMGTPC lipids, we optimized the chemical synthesis of this lipid while keeping dibromooctacosane **4** as a key intermediate for the preparation of GMGTPC. First, we sought for a better access to 1,28-dibromooctacosane **4** through dimerization of an acetylenic derivative **2** under Glaser coupling conditions (Fig. 1B).[29] The iodo derivative **1** was then prepared in good yield in two steps from 12-bromododecan-1-ol through halide exchange under Finkelstein conditions and the protection of the alcohol with a tetrahydropyranyl group.[30] The reaction of intermediate **1** with trimethylsilylacetylene followed by treatment of the protected alkyne intermediate under basic conditions afforded terminal alkyne **2**. Homo-coupling of acetylene **2** under Glaser conditions[29] led to the formation of 1,3-diyne **3**, which was converted to 1,28-dibromooctacosane **4** through a hydrogenation/bromination[31] sequence. This dibromide was reacted with glycerol scaffold **5** in the presence of superbase KOH/DMSO[32] to form tetraether core **6** in moderate yield, but without substantial formation of side products. After deprotection of the dibenzylated derivative **6** under reductive conditions, the final GMGTPC lipid was generated by reaction of diol **7** with 2-bromoethyl dichlorophosphate, followed by nucleophilic displacement of the bromine with trimethylamine. This new synthetic strategy resulted in approximately a 5-fold increase in the overall yield of GMGTPC synthesis compared to our previous reported synthesis.[19]

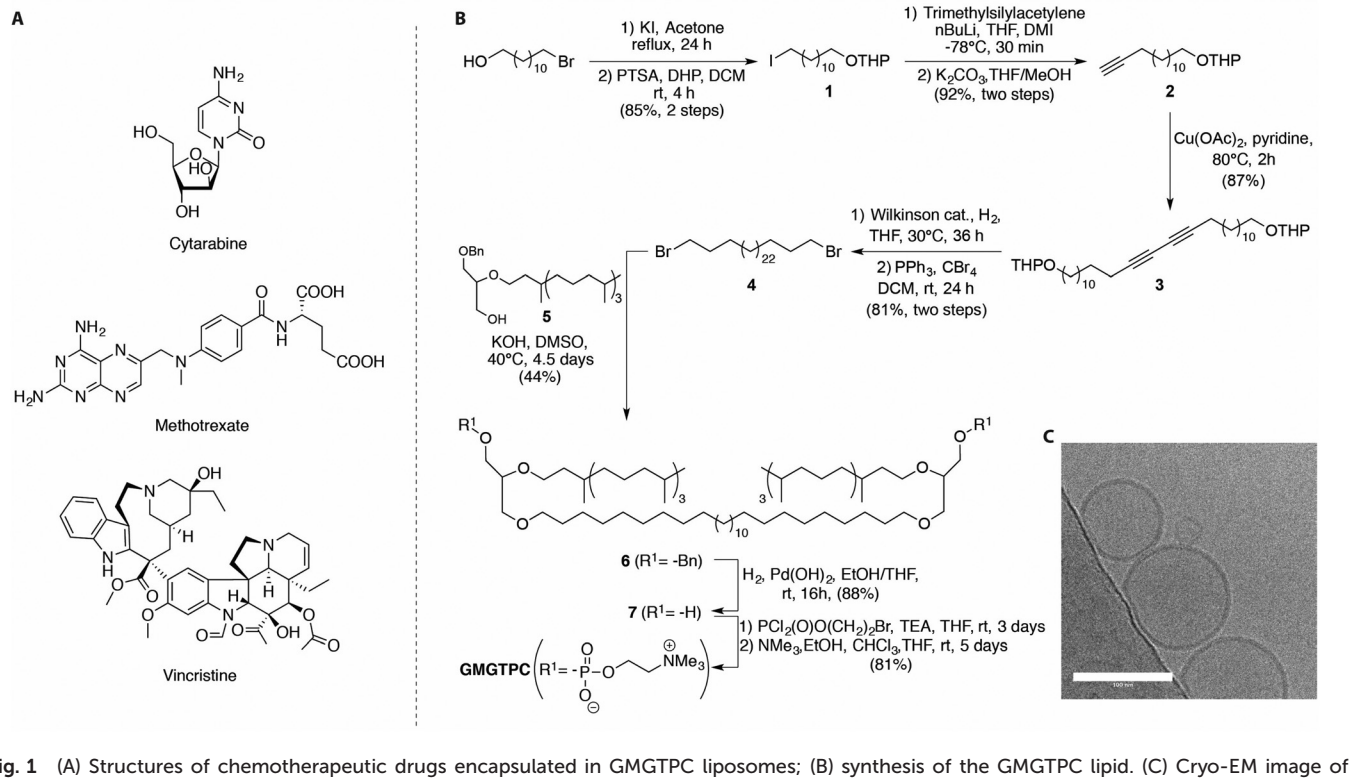
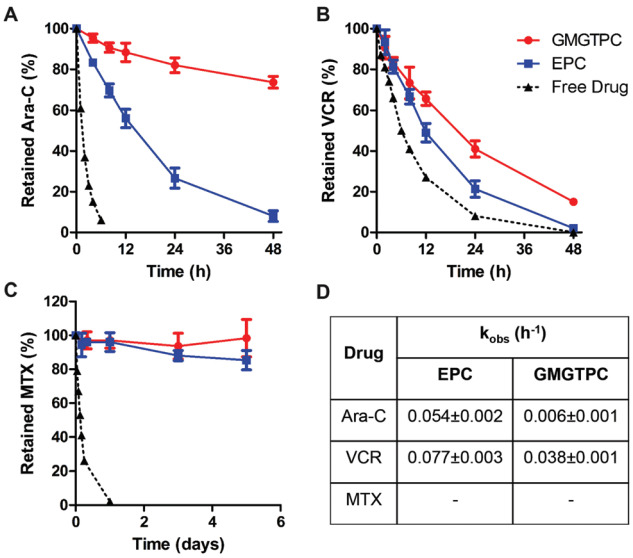


Fig. 1 (A) Structures of chemotherapeutic drugs encapsulated in GMGTPC liposomes; (B) synthesis of the GMGTPC lipid. (C) Cryo-EM image of liposomes made of GMGTPC in PBS (pH 7.4). Scale bar: 100 nm.

We then encapsulated three different drugs (cytarabine Ara-C, vincristine VCR and methotrexate MTX) in GMGTPC liposomes, which have been used in liposomal formulations for the treatment of cancer (Fig. 1A).[5] For instance, DepoCyte® and Marquibo® represent two FDA-approved liposomal formulations containing cytarabine and vincristine, respectively, which have been used for the treatment of leukemia. Similar to diacyl lipids such as EPC, ~100 nm diameter unilamellar liposomes of GMGTPC can be readily prepared upon rehydrating lipid films followed by extrusion with 100 and 50 nm pore membranes (Fig. 1C, and see ESI Fig. S2†), and liposomal drug loading can be achieved by either passive[33,34] or active[35,36] methods. For both EPC and GMGTPC lipids, neutral and anionic drugs were passively loaded during lipid film hydration using buffered solutions of Ara-C (200 mM) or MTX (10 mM), respectively, whereas cationic VCR was actively loaded into liposomes in response to a transmembrane pH gradient (drug/lipid ratio of 0.1 : 1 w/w). Non-encapsulated drugs were removed *via* size exclusion chromatography and the drug and lipid concentrations were measured by HPLC titration and the Bartlett assay,[37] respectively. Passive loading of Ara-C and MTX afforded a low but similar drug-to-lipid molar ratio for both GMGTPC (0.32 for Ara-C and 0.016 for MTX) and EPC (0.29 for Ara-C and 0.012 for MTX) (also summarized in ESI Fig. S3†). In contrast, the tetraether GMGTPC lipid was superior to the diacyl lipid for the active loading of VCR. The liposomal encapsulation efficiency[38] of VCR after 10 min of incubation at 60 °C was 84 ± 9% for GMGTPC liposomes, whereas a 56 ± 6% encapsulation efficiency was measured for EPC liposomes. While both lipids formed liposomes with a similar size (and, thus, internal volume), the difference in the encapsulation efficiency could be explained by the difference of proton permeability between membranes comprised of pure GMGTPC or EPC.[19] We hypothesize that the reduced permeability of the GMGTPC membrane for small ions (*e.g.* H^+) should help to maintain a pH-gradient across the liposome membrane, allowing a higher encapsulation efficiency compared to highly proton permeable membranes formed from EPC.

We next measured the leakage of the drugs from liposomes using a modified dialysis assay. Briefly, suspended liposomes in HEPES buffered saline (HBS) solutions were placed in a dialysis chamber with a molecular weight cutoff of 20 kDa, and dialyzed against HBS buffer at 37 °C. At various time points, aliquots were withdrawn from the dialysis chamber and the retention of the encapsulated drug was measured by HPLC. As a control, the diffusion profiles for free drugs (*i.e.*, in the absence of lipids) from the dialysis chamber were also measured under the same conditions as the liposomal suspensions. Compared to the control data, the rates of leakage of all drugs across the dialysis membrane from liposomal formulations were slower (Fig. 2A–C). Leakage profiles for neutrally charged Ara-C suggested that GMGTPC liposomes provide better retention than EPC liposomes for encapsulated Ara-C (Fig. 2A). We observed a nearly complete leakage of Ara-C (>95%) from EPC liposomes after 48 hours, while GMGTPC



Drug	k_{obs} (h^{-1})	
	EPC	GMGTPC
Ara-C	0.054±0.002	0.006±0.001
VCR	0.077±0.003	0.038±0.001
MTX	-	-

Fig. 2 *Ex vitro* drug retention profiles of cytarabine (A), vincristine (B) and methotrexate (C) encapsulated in GMGTPC liposomes (red lines) or EPC liposomes (blue lines). Liposome suspensions were dialyzed in 20 kDa cutoff Slide-A-Lyzer mini dialysis units (at 37 °C in HBS) and the remaining encapsulated drug was measured by HPLC at different time points. Dashed lines represent the transport of the free drugs across the dialysis membrane. (D) Average observed rates of drug leakage in liposomes made of EPC or GMGTPC. All measurements were recorded in triplicate and are represented as a mean ± SEM (n = 3).

liposomes displayed less than 20% of leakage of Ara-C over the same time period. Analysis of the data revealed that liposomes composed of GMGTPC lipids exhibited an ~9-fold decrease in the observed rate of membrane leakage of Ara-C compared to liposomes comprised of EPC (Fig. 2D). A similar trend was observed for VCR, but the leakage rate was only ~2-fold slower from GMGTPC liposomes compared to EPC liposomes for this positively charged drug (Fig. 2B). For MTX, we did not observe any significant drug leakage from liposomal formulations using either EPC or GMGTPC lipids within 5 days.

All three drugs present important differences for liposomal retention based on their physicochemical properties. For instance, neutrally charged Ara-C (pK_a of the anilinium group is 4.2)[39] was more rapidly released from liposomes compared to negatively charged MTX (pK_as of the two carboxylate groups are 4.8 and 5.6).[40] The results from drug leakage studies shown in Fig. 2 are in agreement with reports claiming that the membrane permeability of neutral molecules is expected to be orders of magnitude faster than the membrane permeability of charged species.[41] The observed leakage profile of Ara-C from GMGTPC liposomes, in particular, highlights a potential advantage of using GMGTPC liposomes over liposomal formulations comprised of conventional diacyl lipids such as EPC for the encapsulation and retention of neutrally charged drugs. The rate of release of VCR (which has been shown to contain 2 protonated amine groups in water with pK_as of 5.0–5.5 and 7.4)[36] from liposomes is dependent on the ability of the membrane to maintain a transmembrane

pH gradient, since only the neutral form of VCR can cross the liposomal membrane and not a positively charged form.[42] Therefore, the observed difference in the leakage of VCR from liposomes of GMGTPC and EPC can be attributed to the capability of GMGTPC lipids to form membranes with reduced permeability to small ions such as protons.[19]

In order to gain some initial insight into the biocompatibility of GMGTPC lipids, we evaluated the toxicity of empty liposomes made entirely of GMGTPC lipids. We used a standard SRB assay[43] to determine the relative effect of different concentrations of liposomes (up to 100 μM total lipid) on the viability of KB cells. We did not observe any toxicity after a 24 h incubation of cells in the presence of GMGTPC liposomes at all concentrations examined (see ESI Fig. S4†).

In order to determine whether these liposomes could be directed to targeted cells, we next incorporated 0.5 mol% of a folic acid-conjugated lipid (FA-PEG_{2000}-DSPE) and 1% of a rhodamine-conjugated lipid (Rh-PE) into GMGTPC liposomes to probe for the cell uptake of liposomes. We chose to use folic acid as a targeting molecule in these studies since KB cells are known to express folate receptors on their surface and facilitate cell attachment and internalization of folate-containing drug delivery systems.[28] We prepared targeted and untargeted rhodamine-labeled liposomes composed of GMGTPC/Rh-PE/FA-PEG_{2000}-DSPE (ratio 98.5/1/0.5) and GMGTPC/Rh-PE (ratio 99/1), respectively (see the ESI† for details). We exposed KB cells to either liposomal formulation (10 μM, 5 h), washed and stained the cells with a Hoechst® 33342 nuclear stain, and then imaged them by confocal microcopy after washing with PBS. As shown in Fig. 3A, liposomes containing folic acid-conjugated lipids (+FA-PEG-PE) bound strongly to the surface of KB cells in a folate-free medium, as evidenced by the observed strong red fluorescence located on the cell surface. Additionally, the red fluorescent puncta located near the nuclei in Fig. 3A supports that some lipids also internalized in the cells. In contrast, liposomes that did not contain folic acid-conjugated lipids (−FA-PEG-PE) did not show detectable red fluorescence signals on the surface or inside the cells after treatment under the same conditions (Fig. 3B).

In order to demonstrate that GMGTPC liposomes could also direct the encapsulated cargo into cells, we passively loaded GMGTPC liposomes with membrane-impermeable calcein (10 mM), with or without the incorporation of a folic acid-conjugated lipid (see the ESI† for details). Both liposomal formulations were then incubated for 6 hours with KB cells. As shown in Fig. 3C and D, only the folic acid-targeted liposomes resulted in a strong green fluorescence signal of calcein on the cell surface and within the cells, suggesting that these liposomes were capable of facilitating the internalization of calcein in cells. As a control, incubation of free calcein with the same KB cells did not result in any observed calcein on the surface or inside the cells (see ESI Fig. S5†). These proof-of-concept studies demonstrate that liposomes comprised of GMGTPC lipids possess the capability of delivering small organic molecules to cells.

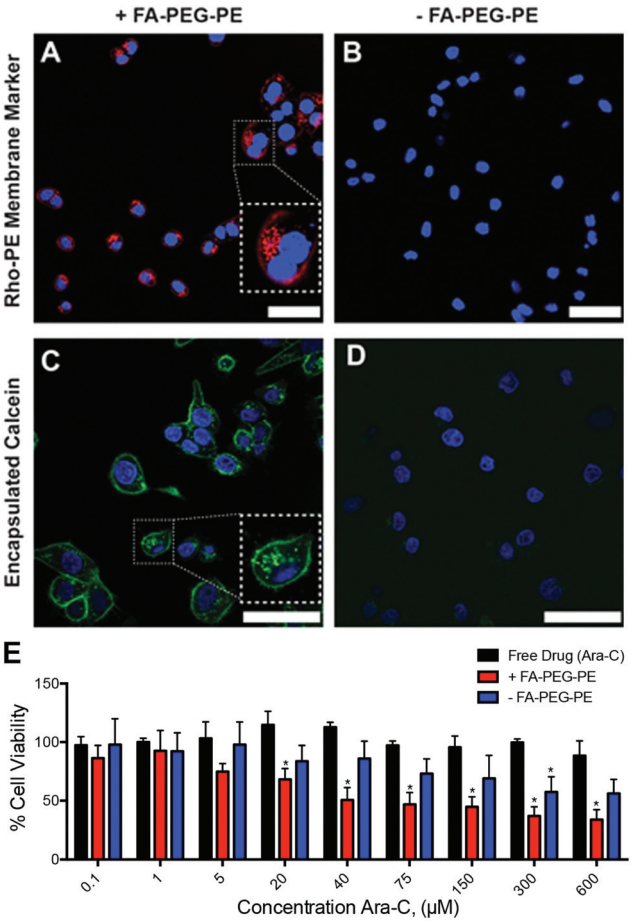


Fig. 3 Uptake of folate-targeted (GMGTPC + FA-PEG-lipid 0.5 mol%) and non-targeted liposomes (GMGTPC only) in KB cells. Fluorescence images of KB cells incubated with targeted (A) and non-targeted (B) rhodamine-phosphatidyl-ethanolamine (Rh-PE)-labeled (1 mol%) liposomes (red). Fluorescence images of KB cells incubated with targeted (C) and non-targeted (D) calcein-loaded liposomes (green). Nuclei are stained with Hoechst and are shown in blue. The insets represent magnified images of individual cells. Scale bar: 20 μm. (E) Graph of cell viability *versus* the total concentration of cytarabine (Ara-C) to KB cells. KB cells were incubated for 2 h with either the free drug (Ara-C), or Ara-C-encapsulated GMGTPC liposomes with (+FA-PEG-PE) or without (−FA-PEG-PE) the presence of a folate targeting lipid in the membrane, followed by removal of excess drug and incubation for an additional 48 h prior to analysis of cell viability. Data expressed as mean values ± SD, $n \geq 3$ for each concentration. Statistical analyses were performed using an unpaired *t*-test comparing to the results from free drug. * indicates a *p*-value ≤0.05.

Finally, we investigated the potential of using GMGTPC liposomes to enhance the cellular delivery of drugs. Cytotoxicity studies were carried out in KB cells using FR-targeted GMGTPC liposomes encapsulating either Ara-C or VCR (Fig. 3E and ESI Fig. S7,† respectively).[44,45] As shown in Fig. 3E, Ara-C-encapsulated liposomes containing folic acid-conjugated lipids (+FA-PEG-PE) exhibited more potent toxicity compared to both untargeted liposomes (−FA-PEG-PE) or free Ara-C. No toxicity was observed in KB cells treated with empty GMGTPC liposomes at lipid concentrations up to 4 mM under the same experimental conditions (see ESI Fig. S6†).

FR-targeted, GMGTPC-encapsulated vincristine was also more toxic in KB cells compared to untargeted liposomes or free vincristine (see ESI Fig. S7†), thus, supporting the potential advantage of using targeted GMGTPC liposomes to deliver and release cytotoxic drugs to cells.

In conclusion, we have successfully encapsulated three anti-cancer drugs in liposomes made of pure GMGTPC tetraether lipids using either passive or active loading methods. These liposomal formulations showed similar or superior retention of hydrophilic drugs compared to EPC-based liposomes. Liposome formulations of GMGTPC lipids appear particularly advantageous for the encapsulation and retention of small neutrally charged drugs such as cytarabine (Ara-C). Using folic acid-targeted liposomes, we also demonstrated that GMGTPC liposomes have the potential to deliver and release small organic molecules (calcein, cytarabine and vincristine) to specific cells. Current efforts are focused on further evaluating the use of GMGTPC lipids in drug formulations for the delivery of various hydrophilic drugs to targeted cells and tissues.

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