Communications

Synthesis and Characterization of Covalent Mimics of Phosphatidylinositol-4,5-bisphosphate Micelles

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There is increasing evidence that multivalency plays an important role in protein−lipid recognition and membrane targeting in biological systems. We describe here the preparation and characterization of multivalent analogues of the signaling lipid phosphatidylinositol-4,5-bisphosphate (PIP_2). Tetherable analogues of the PIP_2 headgroup were appended to polyamidoamine dendrimers via a squarate linker to afford polymers displaying four or eight headgroup moieties. This class of molecules should provide a powerful tool for the study of protein−lipid interactions.

Introduction

Binding interactions in which multiple ligands simultaneously contact a polyvalent receptor or surface are common in biological systems, and synthetic multivalent ligands have been invaluable for investigating these interactions.[1,2] Multivalent interactions are especially important for protein−carbohydrate recognition, and a large array of linear and branched glycopolymers have been prepared and assayed for biological activity.[1−3]

Several lines of evidence suggest that multivalent interactions may also be important for protein−lipid molecular recognition in various signal transduction pathways. The lipid phosphatidylinositol-4,5-bisphosphate (PIP_2) appears to cluster into lipid domains known as lipid rafts in vivo.[4] A number of structurally distinct PIP_2-binding proteins, including profilin, gelsolin, MARCKS, and FERM domain proteins, have been reported to contact multiple lipid molecules simultaneously.[5−11] In addition, proteins such as myosin X, dynamin, and FYVE domain proteins contain multiple lipid binding domains or require dimerization for productive membrane binding.[12−14]

The study of the molecular basis for protein−lipid interactions is complicated by the mobility of molecules in lipid bilayers. Many, if not most, of the PIP_2-binding proteins listed above use positively charged regions to bind to the surface of the membrane, making the strongest contacts with the negatively charged lipid headgroups.[5−11,14] We therefore reasoned that polymeric scaffolds capable of presenting multiple lipid headgroups in a covalently constrained fashion would provide a powerful tool for the study of protein−lipid recognition. We report here the synthesis of multivalent PIP_2 analogues containing four (**1**) and eight (**2**) copies of the PIP_2 headgroup (Figure 1).

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Results and Discussion

Dendrimers have been used for the surface attachment of a variety of chemical moieties.[15] We chose polyamidoamine (PAMAM) dendrimers as our polymeric scaffold because of their aqueous solubility, extensive structural characterization, and commercial availability.[16] Moreover, the larger branched polymers in this class adopt a spherical shape, providing a micelle-like arrangement of lipid headgroups.[17] The synthesis and characterization of dendrimers containing phosphoinositide moieties present additional challenges to those of carbohydrate ligands because of their insolubility in organic media and negative charge. Anionic polymers are prevalent in nature. However, in marked contrast to glycopolymers,[1−3,18] very few examples of polymers containing anionic lipids[19] have been prepared in the laboratory.

Many efforts have been directed toward the synthesis of chiral phosphoinositide headgroup analogues.[20−22] For our polymer syntheses, the amine-tethered PIP_2 lipid headgroup analogue (**3**) was prepared using modifications of procedures from the groups of Chen and Prestwich.[20,23,24]

Coupling of saccharides to dendrimers has been achieved in the past without protecting groups,[18,25−27] and for our initial studies, this strategy presented the most promising route to artificial micelles. In seeking efficient chemistry, we were limited by the poor solubility of the lipid headgroup **3** in nonaqueous solvents and by its sensitivity to both acidic and basic conditions. Many chemical moieties employed in the cross-linking of amines in aqueous or polar organic solutions, such as water-soluble carbodiimides, activated hydroxysuccinimide esters, and isothiocyanates, therefore proved to be unsuitable for attachment of **3** to the dendrimer scaffold. The optimal solution proved to be the use of the 3,4-diethoxy-3-cyclobutene-1,2-dione moiety as a vinylogous amide cross-linker (Scheme 1).[28,29] This "squarate" moiety is stable to hydrolysis under the desired reaction conditions, and its reactivity can be tuned with

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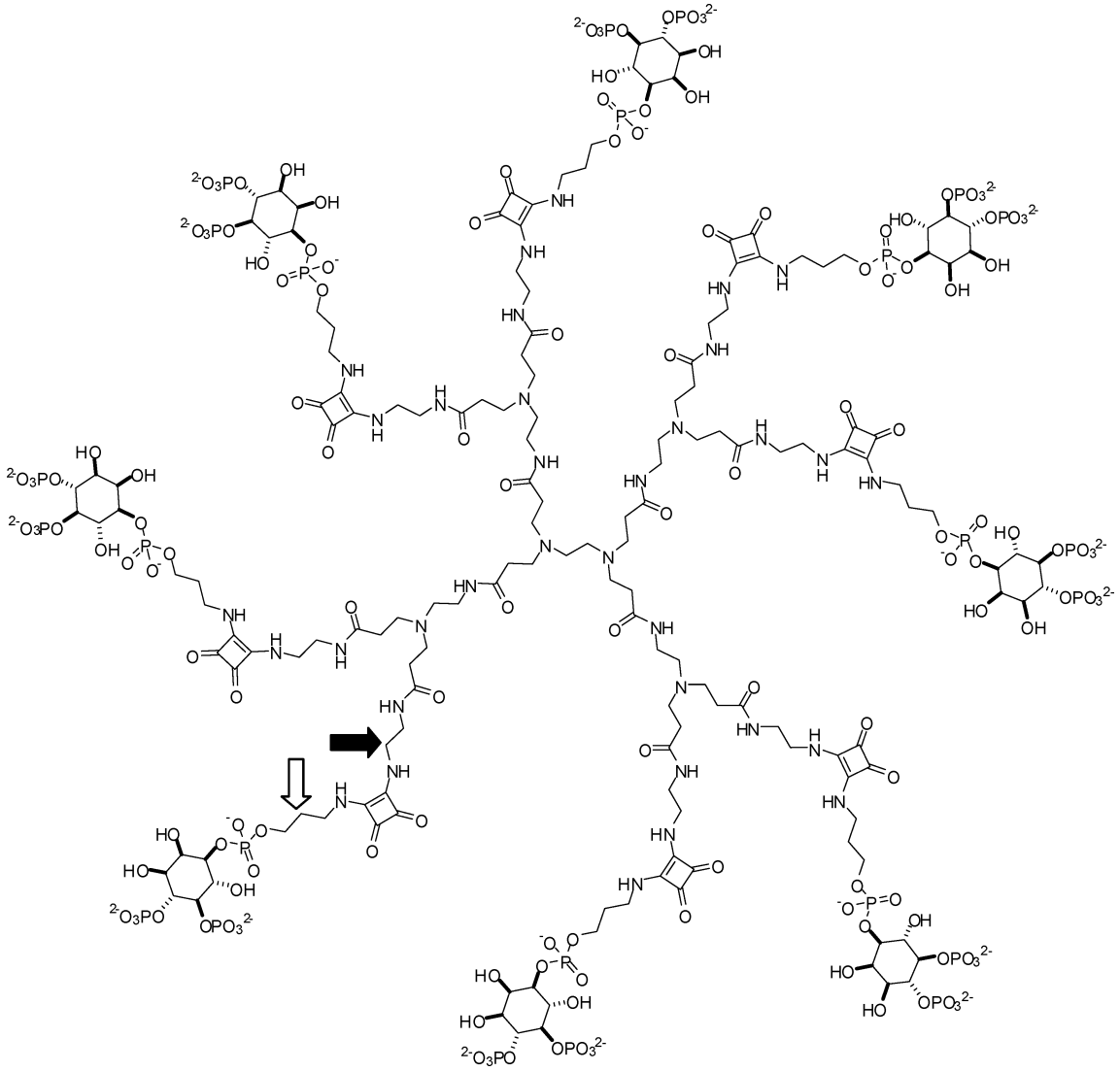
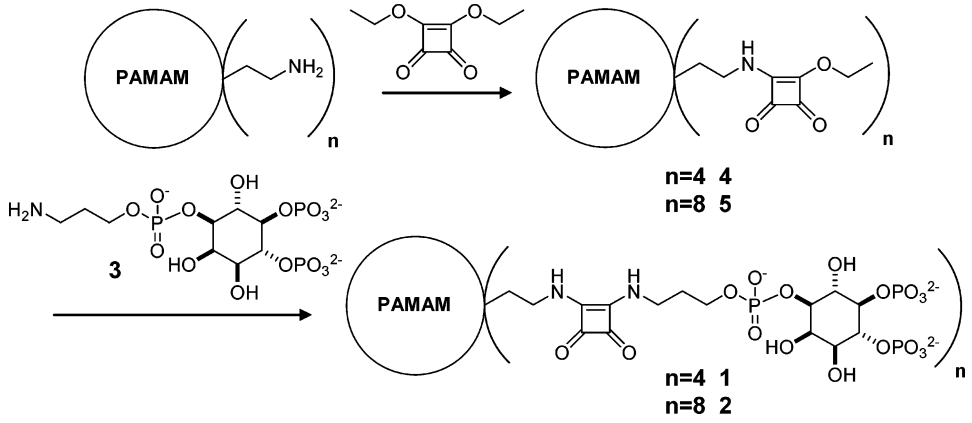


Figure 1. Generation 1 PIP_2 micelle mimic. Arrows indicate the positions of the methylene groups used to determine the ratio of headgroup molecules per dendrimer.

Scheme 1. General Strategy for Synthesis of PAMAM PIP_2-Functionalized Dendrimers



modification of buffer pH, allowing for efficient synthesis of the desired vinylogous amide ester or diamide products.[28,29]

Generation 0 (G0) and generation 1 (G1) PAMAM dendrimers were derivatized with an excess of 3,4-diethoxy-3-cyclobutene-1,2-dione (1.1−1.4 equiv per amino terminus) to afford amide esters **4** and **5** (Scheme 1). Removal of residual squaric acid diester was accomplished by dialysis.

Reaction of polymers **4** and **5** with excess **3** (2 equiv per terminus) afforded G0 and G1 PAMAM PIP_2 dendrimers **1** and **2**, respectively. The extent of the reaction was monitored by ^1H NMR (Supporting Information). For both the G0 and the G1 PAMAM PIP_2 dendrimers, conversion from the squarate dendrimer to the desired phosphatidylinositol dendrimers was quantitative as judged by NMR. However, loss of product

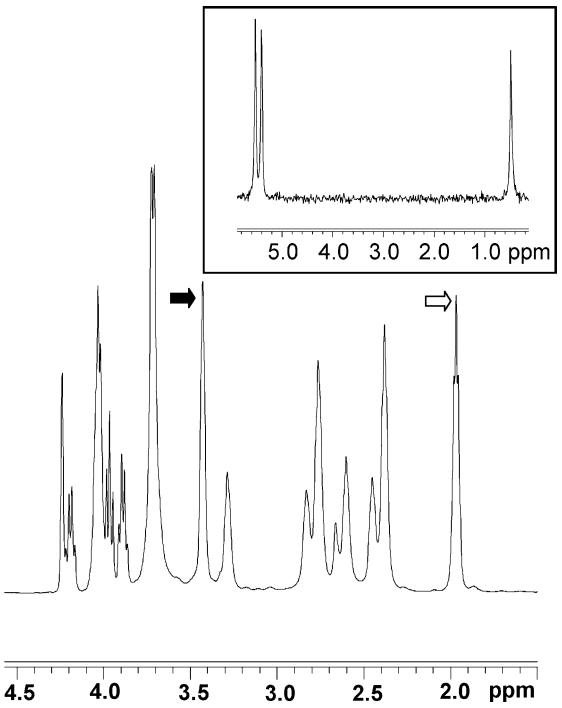


Figure 2. ^{1}H and ^{31}P (inset) NMR spectra for the generation 1 PIP_2 micelle mimic (**2**). The well-resolved peaks used to determine the number of headgroup moieties coupled to the dendrimer are indicated by open and filled arrows, respectively. Arrows correspond to those shown in Figure 1.

occurred as excess **3** was removed by dialysis, especially for the smaller dendrimer **1**. Isolated reaction yields (53% and 78% for G0 and G1 PAMAM PIP_2 dendrimers respectively) were determined using a colorimetric assay for moles of phosphate.[30,31]

The extent of derivatization was determined from the relative integrations at well-separated resonances from the aminoalkyl linker region of the PIP_2 headgroup and a methylene group from the PAMAM dendrimer core (Figure 2). The observed dendrimer-to-linker relative integrations of 1.01:1 for **1** and 0.96:1 for **2** suggest complete derivatization with PIP_2 lipid headgroup analogue **3** for both dendrimers. As expected, the ^{31}P NMR spectrum of G1 PAMAM PIP_2 dendrimer **2** (Figure 2, inset) indicates three separate peaks at δ 0.49, δ 5.39, and δ 5.51 (1:1:1), consistent with one phosphodiester, and two nonequivalent phosphate monoesters. Similar results were observed for G0 PAMAM PIP_2 dendrimer **1** (Supporting Information).

The complete derivatization of the dendrimers observed by NMR was confirmed by mass spectrometry. Because of the high negative charge density on these molecules, electrospray ionization mass spectrometry (ESI-MS) in the negative ion mode was utilized.[32] The charge density of dendrimers **1** and **2** led to the observation of higher-order charge states (-2 to -6) for these compounds. Isotopic resolution within these charge states, along with the observed Na^+ atom envelope, allowed the unambiguous assignment of charges and masses. The calculated mass-to-charge ratio of 911.12 for the triply charged species of **1** is consistent with its observed mass-to-charge ratio of 911.11 (Figure 3). Peaks corresponding to the expected mass-to-charge ratio for sodium adducts containing one, two, and three sodium ions are also observed. Data for G1 dendrimer **2** are also consistent with the expected mass (Supporting Information). Moreover, no dendrimer species with incomplete modification are observed, even though these species are clearly detectable in partially modified samples (Supporting Information). Thus,

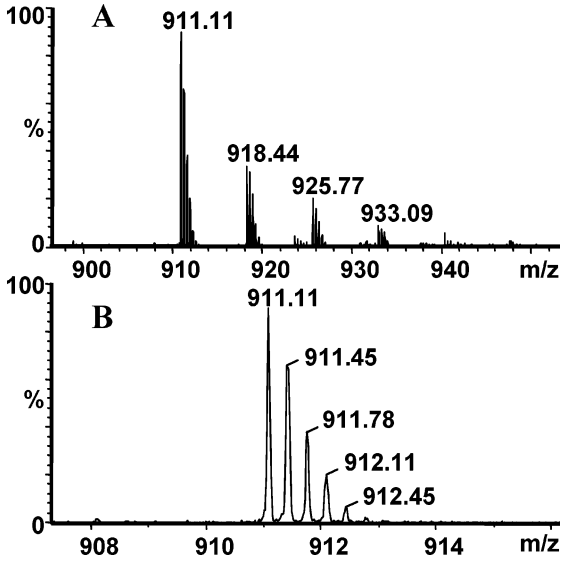


Figure 3. ESI-MS of G0 PAMAM PIP_2 dendrimer (**1**). (A) The observed mass-to-charge peak of 911.11 corresponds to the calculated mass-to-charge ratio of 911.12 for $C_{74}H_{125}N_{14}O_{72}P_{12}$ for the triply charged species of **1**. Multiple adducts formed between **1** and sodium ions are observed; the spacing allows unambiguous assignment of the ion charge state. **(**B) An expanded view of part A distinctly shows isotopic spacing for the highest intensity species in the sodium envelope.

mass spectrometry data are consistent with a series of sodium adducts for the fully derivatized G0 and G1 PAMAM PIP_2 dendrimers.

Conclusions

We have described the successful preparation of multivalent lipid analogues displaying four or eight PIP_2 headgroups. We predict that this class of molecules will be invaluable for studying the molecular basis of protein−phosphoinositide recognition and the means by which these lipids modulate protein function in signal transduction pathways. In addition to their biological utility, these molecules represent progress in the synthesis and characterization of highly anionic biomimetic polymers.

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Supporting Information Available. Experimental procedures for the preparation of compounds **1**−**5** and details of the characterization of these compounds, including NMR spectra for **1** and mass spectral data for **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Kiessling, L. L.; Gestwicki, J. E.; Strong, L. E. Synthetic multivalent ligands as probes of signal transduction. *Angew. Chem., Int. Ed.* **2006**, *45*, 2348−2368.

(2) Mammen, M.; Seok-Ki, C.; Whitesides, G. M. Polyvalent interactions in biological systems: Implications for design and use of multivalent ligands and inhibitors. *Angew. Chem., Int. Ed.* **1998**, *37*, 2754−2794.

(3) Lundquist, J. J.; Toone, E. J. The cluster glycosidic effect. *Chem. Rev.* **2002**, *102*, 555−578.
(4) Czech, M. P. Dynamics of phosphoinositides in membrane retrieval and insertion. *Annu. Rev. Physiol.* **2003**, *65*, 791−815.
(5) Lassing, I.; Lindberg, U. Specific interaction between phsophatidylinositol 4,5-bisphosphate and profilactin. *Nature* **1985**, *314*, 835−838.
(6) Goldschmidt-Clermont, P. J.; Machesky, L. M.; Baldassare, J. J.; Pollard, T. D. The actin-binding protein profilin binds to PIP2 and inhibits its hydrolysis by phospholipase C. *Science* **1990**, *247*, 1575−1578.
(7) Feng, L.; Mejillano, M.; Yin, H. L.; Chen, J.; Prestwich, G. D. Full-contact domain labeling: Identification of a novel phosphoinositide binding site on gelsolin that requires the complete protein. *Biochemistry* **2001**, *40*, 904−913.
(8) Wang, J.; Arbuzova, A.; Hangyas-Mihalyne, G.; McLaughlin, S. The effector domain of myristolated alanine-rich C kinase substrate binds strongly to phosphatidylinositol 4,5-bisphosphate. *J. Biol. Chem.* **2001**, *276*, 5012−5019.
(9) Wang, J.; Gambhir, A.; Hangyas-Mihalyne, G.; Murray, D.; Golbebiewska, U.; McLaughlin, S. Lateral sequesteration of phosphatidylinositol 4,5-bisphospate by the basic effector domain of myristolated alanine-rich C kinase substrate is due to nonspecific electrostatic interaction. *J. Biol. Chem.* **2002**, *277*, 34401−34412.
(10) Barret, C.; Roy, C.; Montcourrier, P.; Mangeat, P.; Niggli, V. Mutagenesis of the phosphatidylinositol 4,5-bisphosphate (PIP_2) binding site in the NH_2-terminal domain of ezrin correlates with its altered cellular distribution. *J. Cell Biol.* **2000**, *151* (5), 1067−1080.
(11) Bompard, G.; Martin, M.; Roy, C.; Vignon, F.; Freiss, G. Membrane targeting of protein tyrosine phosphatase PTPL1 through its FERM domain via binding to phosphatidylinositol 4,5-biphosphate. *J. Cell Sci.* **2003**, *116*, 2519−2530.
(12) Berg, J. S.; Derfler, B. H.; Pennisi, C. M.; Corey, D. P.; Cheney, R. E. Myosin-X, a novel myosin with pleckstrin homolgy domains, associates with regions of dynamic actin. *J. Cell Sci.* **2000**, *113*, 3439−3451.
(13) Klein, D. E.; Lee, A.; Frank, D. W.; Marks, M. S.; Lemmon, M. A. The pleckstrin homology domains of dynamin isoforms require oligomerization for high affinity phosphoinositide binding. *J. Biol. Chem.* **1998**, *273*, 27725−27733.
(14) Lemmon, M. A. Phosphoinositide binding domains. *Traffic* **2003**, *4*, 201−213.
(15) Bosman, A. W.; Janssen, H. M.; Meijer, E. J. About dendrimers: Structure, physical properties, and applications. *Chem. Rev.* **1999**, *99*, 1665−1688.
(16) Tomalia, D. A.; Naylor, A. M.; Goddard, W. A., III. Starburst dendrimers: Molecular-level control of size, shape, surface chemistry, topology, and flexibility from atoms to macroscopic matter. *Angew. Chem., Int. Ed. Engl.* **1990**, *29*, 138−175.
(17) Naylor, A. M.; Goddard, W. A., III.; Kiefer, G. E.; Tomalia, D. A. Starburst dendrimers. 5. Molecular shape control. *J. Am. Chem. Soc.* **1989**, *111*, 2339−2341.
(18) Cloninger, M. J. Biological application of dendrimers. *Curr. Opin. Chem. Biol.* **2002**, *6*, 742−748.
(19) Ferguson, C. G.; James, R. D.; Bigman, C. S.; Shepard, D. A.; Abdiche, Y.; Katsamba, P. S.; Myszka, D. G.; Prestwich, G. D. Phosphoinositide-containing polymerized liposomes: Stable membrane-mimetic vesicles for protein−lipid binding analysis. *Bioconjugate Chem.* **2005**, *16*, 1475−1483.
(20) Prestwich, G. D. Touching all the bases: Synthesis of inositol polyphosphate and phosphoinositide affinity probes from glucose. *Acc. Chem. Res.* **1996**, *29*, 503−513.
(21) Potter, B. V. L.; Lampe, D. Chemistry of inositol lipid mediated cellular signaling. *Angew. Chem., Int. Ed. Engl.* **1995**, *34*, 1933−1972.
(22) Conrad, R. M.; Grogan, M. J.; Bertozzi, C. R. Stereoselective synthesis of myo-inositol via ring-closing metathesis: A building block for glycosylphosphatidylinositol (GPI) anchor synthesis. *Org. Lett.* **2002**, *4*, 1359−1361.
(23) Prestwich, G. D.; Marecek, J. F.; Mourey, R. J.; Theibert, A. B.; Ferris, C. D.; Danoff, S. K.; Snyder, S. H. Tethered IP_3. Synthesis and biochemical applications of the 1-*O*-(3-aminopropyl) ester of inositol 1,4,5-triphosphate. *J. Am. Chem. Soc.* **1991**, *113*, 1822−1825.
(24) Gou, D.-M.; Liu, Y.-C.; Chen, C.-S. An efficient chemoenzymic access to optically active myo-inositol polyphophates. *Carbohydr. Res.* **1992**, *234*, 51−64.
(25) Jayaraman, N.; Stoddart, J. F. Synthesis of carbohydrate-containing dendrimers. 5. Preparation of dendrimrs using unprotected carbohydrates. *Tetrahedron Lett.* **1997**, *38*, 6767−6770.
(26) Woller, E. K.; Cloninger, M. J. Mannose functionalization of a sixth generation dendrimer. *Biomacromolecules* **2001**, *2*, 1052−1054.
(27) Kieburg, C.; Lindhorst, T. K. Glycodendrimer synthesis without using protecting groups. *Tetrahedron Lett.* **1997**, *38*, 3885−3888.
(28) Tietze, L. F.; Arlt, M.; Beller, M.; Glusenkamp, K.; Jahde, E.; Rajewsky, M. F. Squaric acid diethyl ester: A new coupling reagent for the formation of drug biopolymer conjugates. Synthesis of squaric acid ester amides and diamides. *Chem. Ber.* **1991**, *124*, 1215−1221.
(29) Kitov, P. I.; Sadowska, J. M.; Mulvey, G.; Armstrong, G. D.; Ling, H.; Pannu, N. S.; Read, R. J.; Bundle, D. R. Shiga-like toxins are neutralized by tailored multivalent carbohydrate ligands. *Nature* **2000**, *403*, 669−672.
(30) Chen, P. S., Jr.; Toribara, T. Y.; Warner, H. Microdetermination of phosphorus. *Anal. Chem.* **1956**, *28*, 1756−1758.
(31) Fiske, C. H.; Subbarow, Y. The colorimetric determination of phosphorus. *J. Biol. Chem.* **1925**, *66*, 375−400.
(32) Wenk, M. R.; Lucast, L.; Di, P. G.; Romanelli, A. J.; Suchy, S. F.; Nussbaum, R. L.; Cline, G. W.; Shulman, G. I.; McMurray, W.; De Camilli, P. Phosphoinositide profiling in complex lipid mixtures using electrospray ionization mass spectrometry. *Nat. Biotechnol.* **2003**, *21*, 813−817.

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