Anion Recognition Hot Paper

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# Anion Recognition and Induced Self-Assembly of an α,γ-Cyclic Peptide To Form Spherical Clusters

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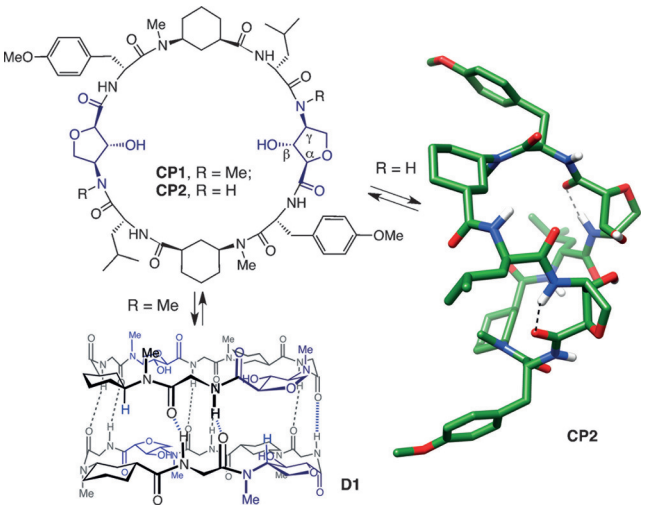
***Abstract:*** *A cyclic octapeptide composed of hydroxy-functionalized γ-amino acids folds in a "V-shaped" conformation that allows the selective recognition of anions such as chloride, nitrate, and carbonate. The process involves the simultaneous self-assembly of six peptide subunits and the recognition of four anions to form a tetrahedral structure, in which the anions are located at the corners of the resulting structure. Each anion is coordinated to three different peptides. The structure was fully characterized by several techniques, including NMR spectroscopy and X-ray diffraction, and the material was able to facilitate the transmembrane transport of chloride ions.*

The ability to construct complex functional materials from a small number of components represents an economical synthetic strategy that allows the rapid generation of novel functions.[1,2] The recognition of anions is quite a difficult task due to their large size compared with isoelectronic cations and which, as a consequence, establish weaker interactions with the receptor.[3] In addition, anions are more susceptible to changes in the pH value and lose their charge at low pH values. Furthermore, anions have a wide range of geometries as well as high free energies of hydration. All of these limitations demand a high level of design and synthesis to produce effective receptors for recognition and this has retarded the development of such systems. Nevertheless, in the last few years, this topic has emerged as a fundamental area in chemistry, with applications in a variety of fields, such as catalysis, ion extraction, and in responsive chemical systems.[4] Anions have an enormous impact upon our lives because of the fundamental roles that they play in biological events.[5] Apart from organic anions (ATP, RNA, or DNA), inorganic anions, such as chloride, carbonate, or phosphate, are found extensively in extracellular fluids and their dysregulation is associated with diseases such as cystic fibrosis and bone mineralization.[6] These factors, amongst others, make it of fundamental importance to understand the nature of the anion-recognition process to improve receptor designs.

In recent years new designs have been developed in which molecular recognition is combined with a self-assembly process to form supramolecular gels, crystals, or metal-organic cages.[7] Despite these advances, more efficient designs are required in which small molecules merge symbiotically with specific guests (anions) to form discrete and well-defined supramolecular entities with novel properties. The use of biologically compatible systems (such as peptides) that can be involved in relevant bioprocesses is an area of great interest.[8] The study described here concerns a new and efficient supramolecular self-assembly of a cyclic peptide templated by specific anion recognition to form discrete spherical aggregates in which four anions are coordinated in a tetrahedral disposition to six cyclic peptides.

In recent years we have been working with cyclic peptides (CPs) composed of γ-amino acids in the search for peptide nanotubes.[9,10] With the aim of tuning the internal properties of the nanotube (Scheme 1),[11] 4-amino-3-hydroxytetrahydrofuran-1-carboxylic acid (Ahf) was prepared.[12] The incorporation of two N-methylated Ahf residues together with two N-methylated γ-amino acids [3-aminocyclohexanecarboxylic acid (Ach)] (**CP1**, R = Me) in a cyclic octapeptide gave the corresponding dimer **D1** (Scheme 1).[11b] In contrast, the CP with the nonmethylated Ahf residues (**CP2**, R = H) did not form the corresponding dimer (**D2**).




***Scheme 1.*** Structure of cyclic peptides **CP1** and **CP2** and the expected equilibrium with the corresponding dimers (**D1** and **D2**), side chains were removed for clarity. Right: structure of **CP2** from DFT calculations using experimental structural restrictions.

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The NMR spectra of **CP2** (see Figure S1 in the Supporting Information) recorded in nonpolar solvents led us to study its properties. A downfield shift of the three amide protons ($\delta =$ 9.27, 8.62, and 8.24 ppm) was observed in the $^1$H NMR spectrum of **CP2**. The chemical shift of the Ahf amide proton ($\delta = 8.62$ ppm) and the singlet signals for the two vicinal protons at C2 and C3 suggest that the peptide adopts a folded conformation in which the NH group is hydrogen bonded to its own carbonyl group.[13] DFT calculations, with experimental constrictions such as the aforementioned coupling constants between Hα, Hβ, and Hγ and the participation of the NH group (Ahf) in a hydrogen bond, suggested that **CP2** adopts the folded conformation illustrated in Scheme 1.[14] Variable concentration experiments were carried out on **CP2** and only a small upfield shift was observed for the Ahf NH proton (from $\delta = 8.62$ to 8.55 ppm from 20 to 2.5 mM; see Figure S2). The addition of small amounts of methanol (1–5%) produced a small downfield shift in the resonances of the amide protons. The addition of larger amounts of methanol (>10%) led to the appearance of new signals that correspond to other conformations. These structures were the only ones remaining after 48 h under these conditions (see Figure S3). Unfortunately, the symmetry of **CP2** did not allow the solution structure to be determined due to the lack of clear nOe cross-peaks.

The nature of the structure was investigated by carrying out a crystallization study.[20] It was found that small crystals were formed from a chloroform solution of **CP2** in a hexane-saturated atmosphere. Two different supramolecular units were found (Figure 1). One of the aggregates is a tetrameric structure in which the CP is folded in a "V-shaped" conformation, similar to that predicted by DFT calculations with the aforementioned Ahf hydrogen-bond restriction (Scheme 1). The tetramer consists of two independent molecules, one of which is conformationally disordered (Figure 1B and see Figure S4). The main conformation, with a refined occupancy of 68%, is almost coincident with the other independent molecule of the tetramer (green color in Figure S4). The α-amino acids participate in hydrogen bonds with the neighboring CPs through a β-sheet-type interaction with the Leu residues hydrogen bonded to Tyr (see Figure S5).

Each of these peptides interacts through its concave face with the second supramolecular structure (Figure 1C). This cluster is a hexameric aggregate formed by three independent molecules (see Figure S6). One of the CPs shows conformational disorder at the Tyr(Me) side chain. To our surprise, this hexamer contains four carbonate ions that establish interactions (hydrogen bonds to the Tyr amide proton) with three different CPs (Figure 1D and 1E). As carbonate was not added, it was inferred that **CP2** must scavenge this anion from the drying agent used to dry the chloroform solution before crystallization. The resulting tetrahedral structure has a carbonate anion located in each corner. The NH group of Leu is hydrogen bonded to the carbonyl group of Leu of the closed CP, which also has a trimeric arrangement (Figure 1F). This structure, which is electrically neutral, contains eight sodium ions that occupy sixteen positions (0.5 occupancy each). The resulting "V-shaped" structure is more compact than those

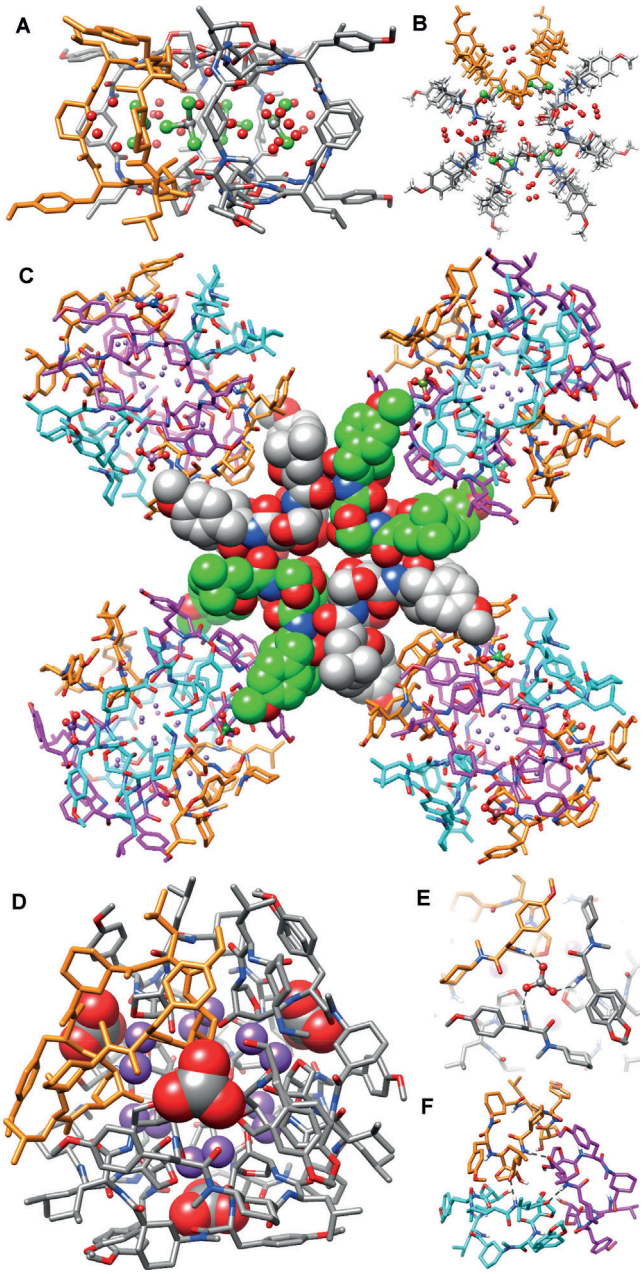


***Figure 1.*** Crystal structure of **CP2**. A) side and B) top view of the tetrameric structure; C) view of the packing of hexamers and tetramers, D) view of the hexameric structure in which each carbonate ion is coordinated to three different CPs, such interactions being based on three hydrogen bonds (E) between amide protons of Tyr and carbonate anions. F) Hydrogen-bonding interaction between Leu residues stabilizing the hexameric structure.

that form the tetrameric aggregate or the DFT-calculated structure (see Figures S7 and S8).

Intrigued by the formation of this anion cluster, we decided to investigate the anion (carbonate) recognition in solution. Thus, NMR studies were carried out with the addition of salts that were soluble in organic solvents. Treatment of a solution of **CP2** in $CDCl_3$ (5 mM) with small portions of bistetrabutylammonium carbonate (BTBAC, Figure 2) led to the downfield shift of some of the signals in

  

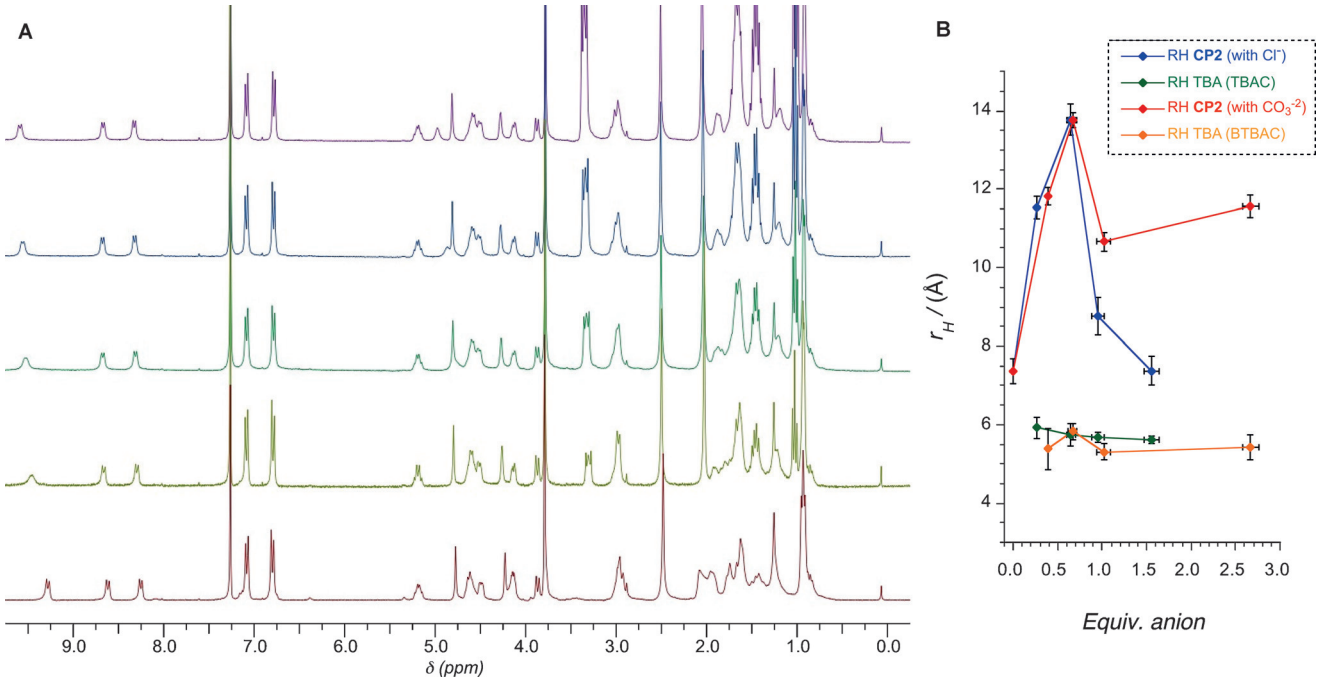


***Figure 2.*** A) $^{1}$H NMR spectra for the addition of BTBAC (0.0, 0.2, 0.4, 0.6, and 1.0 equiv, bottom to top) to a solution of **CP2** in $CDCl_3$ (5 mM). B) Plot of the hydrodynamic radii ($r_H$, Å) measured by DOSY (500 MHz, $CDCl_3$, 25 °C) for samples containing **CP2** (2.55 mM) and different proportions of BTBAC and TBAC. The values for **CP2** are shown in red (BTBAC) and blue (TBAC), while the values for the TBA cation are represented in orange (BTBAC) and green (TBAC).

the $^{1}$H NMR spectrum of **CP2**. Of particular significance was the change observed for the Tyr NH proton ($\delta = 9.27$ to 9.59 ppm), which is responsible for the interaction with the carbonate anions in the crystal structure. The other NH protons were also shifted downfield, albeit to a lesser extent (Leu NH from $\delta = 8.25$ ppm to 8.32 ppm and $\delta = 8.62$ to 8.68 for the Ahf NH). The singlet at $\delta = 4.21$ ppm for the proton at Cβ became a broad singlet at $\delta = 4.29$ ppm, thus suggesting some conformational changes at this position. Only about 0.6 equivalents of BTBAC were required to achieve the maximum downfield shift, and this corresponds to the stoichiometry observed in the solid state. Further addition of carbonate did not change the spectrum. The addition of tetrabutylammonium bicarbonate[15] (TBABC, see Figure S9) gave very different results. Initially, the addition of small amounts of bicarbonate produced the downfield shift of the NH signals, which was followed by the splitting of signals, especially those corresponding to amide protons. Further addition of TBABC led to a reduction in the intensity of the amide proton signals, while the intensity of the chloroform signal increased. These changes correspond to a proton-deuterium exchange between the amide group and chloroform catalyzed by the bicarbonate ions. Definitive evidence for the formation of a supramolecule was not found. Similar results to those with BTBAC were obtained on the addition of chloride (TBAC, see Figure S10) or hydroxide ions (TBAH, see Figure S11). The $^{1}$H NMR spectra showed changes in the chemical shifts of the amide protons, thus confirming their participation in hydrogen-bonding interactions with the amide proton of Tyr. Titration with nitrate (TBAN, see Figure S12) also produced the downfield shift of the amide protons, although to a lesser extent and without saturation, even in the presence of four equivalents of anion, thus suggesting a weaker binding interaction. The addition of softer anions, such as iodide (TBAI, see Figure S13), barely modified the $^{1}$H NMR spectrum of the peptide and this suggests a weaker recognition of these ions.

Considering the $^{1}$H NMR titration results obtained with carbonate and chloride anions, we attempted to gain further insights into the supramolecular structures formed in solution. To this end, diffusion ordered spectroscopy (DOSY) was carried out on samples containing **CP2** (2.55 mM in $CDCl_3$) with increasing proportions of the target anions (as the TBA salts). The self-diffusion rates obtained in DOSY experiments were compared with those measured for tetrakis(trimethylsilyl)silane (TMSS). The results were used to estimate the apparent hydrodynamic radii ($r_H$) of the species formed in solution (see the Supporting Information for details). The apparent $r_H$ value in the absence of anions was measured to be $7.4 \pm 0.3$ Å, which is compatible with the size of **CP2** (Figure 2B). Interestingly, the size of the molecule increased during the titration experiment, reaching a maximum value at about 0.67 equivalents of anion. This value corresponds to a **CP2**/anion ratio of 3:2 and was obtained for both carbonate and chloride ions (red and blue symbols, respectively, in Figure 2B). The maximum $r_H$ value obtained with 0.67 equivalents of anion was $13.8 \pm 0.4$ Å, which is in good agreement with the size of the hexameric assembly observed in the solid state, with a calculated radius of gyration of 13.9–14.2 Å. The addition of an excess of anion led to a decrease in the apparent size of the CP in solution. These observations suggest that the hexameric structure was formed in solution in

  

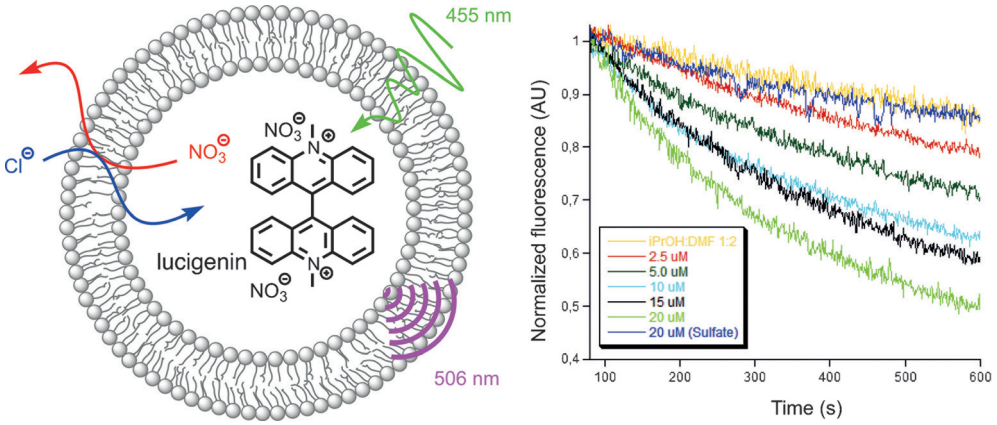


***Figure 3.*** Transport activities were measured in synthetic vesicles using the lucigenin assay, in which fluorescence quenching as a result of incoming chloride was detected. Right: representative normalized fluorescence kinetics of transport-mediated reduction of fluorescence following addition of **CP2** at concentrations between 2.5 and 20 μM in a cuvette (0.6–5 mol% CP to lipid ratio).

the presence of the appropriate amount of anion, but a large excess produce species with smaller CP/anion ratios. In any case, the clear maximum observed (Figure 2B) implies the anion-templated assembly of **CP2** has a high stability. The apparent molecular size of the TBA cation was also monitored during the titration and this remained constant at $r_H \approx 5.4–5.9$ Å, which is consistent with its dimensions, and inferring that it is not interacting with the hexameric aggregate.

The transport of anions across phospholipid bilayers is crucial to maintain the concentration gradients used for signaling and cellular regulation.[16] The ability of **CP2** to transport anions across phospholipid bilayers was assessed by fluorescence techniques. These experiments were carried out in large unilamellar vesicles (200 nm diameter) and using the anion-selective probe lucigenin (Figure 3).[17] The vesicles enclosed $NaNO_3$ and the halide-sensitive dye lucigenin (1.0 mM) were suspended in an isosmotic solution containing NaCl (24 mM). After 70 seconds, **CP2** was added and the decay in the lucigenin fluorescence upon the influx of chloride was measured over time. As expected, the transport rate increased upon addition of **CP2**. At CP concentrations above 20 μM, the transport experiments were quite erratic and the formation of a white precipitate was observed. Unfortunately, this low solubility did not allow a Hill analysis to be carried out.[18] Control studies, whereby the release of internal dye (carboxyfluorescein) was monitored, indicated that the changes in the fluorescence intensity was not due to rupture of the vesicles (liposome leakage; see Figure S14).[19] The anion transport (Figure 3) was expected to be antiporter, with chloride exchanged by intravesicular nitrate. In an effort to confirm this hypothesis, the nitrate was replaced by hydrophilic sulfate. Under these conditions the rate of fluorescence decay was negligible compared to the background. Therefore, the inward flow of anions ($Cl^-$) cannot be balanced under these conditions. This result confirms that the transport is an antiporter process, in which **CP2** can transport chloride or nitrate but is unable to transport sulfate.

In summary, a new supramolecular-based anion-recognition process is reported in which self-assembly and molecular recognition combine to form a hexameric cluster. The process is based on a cyclic peptide (**CP2**) containing two 4-amino-3-hydroxytetrahydrofuran-1-carboxylic acid residues that induce the peptide to fold in a "V-shaped" conformation. This conformation recognizes biologically relevant anions that induce self-assembly to form a supermolecule composed of six CPs and four anions. The data clearly demonstrate that **CP2** has an extremely high affinity for carbonate and other anions, such as chloride, nitrate, or hydroxide. The high anion affinity and the hydrophobicity of the resulting cluster enable efficient anion transport through biological membranes. The peptidic character of this cluster might allow its implementation in relevant processes of biological interest. Efforts in this direction will be reported in due course.

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[1] A. C. Mendes, E. T. Baran, R. L. Reis, H. S. Azevedo, *WIREs Nanomed. Nanobiotechnol.* **2013**, *5*, 582–612.
[2] J. W. Steed, *Nat. Chem.* **2011**, *3*, 9–10.
[3] P. A. Gale, *Chem. Commun.* **2010**, *47*, 82–86; N. Busschaert, C. Caltagirone, W. Van Rossom, P. A. Gale, *Chem. Rev.* **2015**, *115*, 8038–8155; S. Kubik, *Chem. Soc. Rev.* **2010**, *39*, 3648–3663.
[4] N. H. Evans, P. D. Beer, *Angew. Chem. Int. Ed.* **2014**, *53*, 11716–11754; *Angew. Chem.* **2014**, *126*, 11908–11948.
[5] F. M. Ashcroft, *Ion Channels and Disease*, Academic Press, San Diego, USA, **2000**.

  

[6] S. M. Rowe, S. Miller, E. J. Sorscher, *N. Engl. J. Med.* **2005**, *352*, 1992 – 2001; P. A. Gale, N. Busschaert, C. J. E. Haynes, L. E. Karagiannidis, I. L. Kirby, *Chem. Soc. Rev.* **2014**, *43*, 205 – 241.
[7] R. Custelcean, *Chem. Soc. Rev.* **2010**, *39*, 3675 – 3684; M.-O. M. Piepenbrock, G. O. Lloyd, N. Clarke, J. W. Steed, *Chem. Rev.* **2010**, *110*, 1960 – 2004; I. A. Riddell, M. M. J. Smulders, J. K. Clegg, Y. R. Hristova, B. Breiner, J. D. Thoburn, J. R. Nitschke, *Nat. Chem.* **2012**, *4*, 751 – 756.
[8] R. B. P. Elmes, K. A. Jolliffe, *Chem. Commun.* **2015**, *51*, 4951 – 4968; S. Kubik, R. Goddard, R. Kirchner, D. Nolting, J. Seidel, *Angew. Chem. Int. Ed.* **2001**, *40*, 2648 – 2651; *Angew. Chem.* **2001**, *113*, 2722 – 2725.
[9] M. Amorín, L. Castedo, J. R. Granja, *J. Am. Chem. Soc.* **2003**, *125*, 2844 – 2845; M. Amorín, R. J. Brea, L. Castedo, J. R. Granja, *Org. Lett.* **2005**, *7*, 4681 – 4684; R. J. Brea, M. Amorín, L. Castedo, J. R. Granja, *Angew. Chem. Int. Ed.* **2005**, *44*, 5710 – 5713; *Angew. Chem.* **2005**, *117*, 5856 – 5859.
[10] R. García-Fandiño, M. Amorín, L. Castedo, J. R. Granja, *Chem. Sci.* **2012**, *3*, 3280 – 3285; J. Montenegro, C. Vázquez-Vázquez, A. Kalinin, K. E. Geckeler, J. R. Granja, *J. Am. Chem. Soc.* **2014**, *136*, 2484 – 2491; M. Cuerva, R. García-Fandiño, C. Vázquez-Vázquez, M. A. López-Quintela, J. Montenegro, J. R. Granja, *ACS Nano* **2015**, *9*, 10834 – 10843.
[11] a) C. Reiriz, M. Amorín, R. García-Fandiño, L. Castedo, J. R. Granja, *Org. Biomol. Chem.* **2009**, *7*, 4358 – 4361; b) N. Rodríguez-Vázquez, R. García-Fandiño, M. Amorín, J. R. Granja, *Chem. Sci.* **2016**, *7*, 183 – 187.
[12] N. Rodríguez-Vázquez, S. Salzinger, L. F. Silva, M. Amorín, J. R. Granja, *Eur. J. Org. Chem.* **2013**, 3477 – 3482.
[13] A. A. Edwards, G. J. Sanjayan, S. Hachisu, G. E. Tranter, G. W. J. Fleet, *Tetrahedron Lett.* **2006**, *62*, 7718 – 7725.
[14] R. García-Fandiño, L. Castedo, J. R. Granja, S. Vázquez, *J. Phys. Chem. B* **2010**, *114*, 4973 – 4983; W. J. Hehre, L. Radom, P. von R. Schleyer, J. A. Pople, *Ab Initio Molecular Orbital Theory*, 1st ed., Wiley, New York, **1986**.
[15] C. T. Yang, Y. Fu, Y. B. Huang, J. Yi, Q. X. Guo, L. Liu, *Angew. Chem. Int. Ed.* **2009**, *48*, 7398 – 7401; *Angew. Chem.* **2009**, *121*, 7534 – 7537.
[16] N. Busschaert, P. A. Gale, *Angew. Chem. Int. Ed.* **2013**, *52*, 1374 – 1382; *Angew. Chem.* **2013**, *125*, 1414 – 1422; J. T. Davis, O. Okunola, R. Quesada, *Chem. Soc. Rev.* **2010**, *39*, 3843 – 3862.
[17] B. A. McNally, A. V. Koulov, B. D. Smith, J. B. Joos, A. P. Davis, *Chem. Commun.* **2005**, 1087 – 1089; M. Lisbjerg, H. Valkenier, B. M. Jessen, H. Al-Kerdi, A. P. Davis, M. Pittelkow, *J. Am. Chem. Soc.* **2015**, *137*, 4948 – 4951.
[18] T. Takeuchi, N. Sakai, S. Matile, *Faraday Discuss.* **2009**, *143*, 187 – 203.
[19] J. N. Weinstein, S. Yoshikami, P. Henkart, R. Blumenthal, W. A. Hagins, *Science* **1977**, *195*, 489 – 492.
[20] CCDC 1443051 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre.

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