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# Discovery of an Organic Trefoil Knot

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Molecular knots remain difficult to produce using the current synthetic methods of chemistry because of their topological complexity. We report here the near-quantitative self-assembly of a trefoil knot from a naphthalenediimide-based aqueous disulfide dynamic combinatorial library. The formation of the knot appears to be driven by the hydrophobic effect and leads to a structure in which the aromatic components are buried while the hydrophilic carboxylate groups remain exposed to the solvent. Moreover, the building block chirality constrains the topological conformation of the knot and results in its stereoselective synthesis. This work demonstrates that the hydrophobic effect provides a powerful strategy to direct the synthesis of entwined architectures.

Molecular knots first observed in DNA (1) and then in proteins (2) have been associated with increased thermal and mechanical robustness. The protein knots are highly conserved throughout evolution and appear to protect active regions by providing resistance against degradation, but the mechanisms involved in the spontaneous threading of a polypeptide chain through a loop to form a knot are not well understood (3).

Chemists are well acquainted with this problem: The synthesis of molecular knots is particularly difficult because it requires precisely defined pathways and transition states that are entropically much more demanding than topologically simpler macrocyclization or catenation processes. Over the past two decades, the only strategy that has provided the necessary well-defined geometry relies on metal coordination (4–10). Only two examples of knot syntheses based on weaker interactions, such as hydrogen bonding (11, 12), have been reported: These knots were formed unexpectedly and in low yields during the process of macrocyclization, and the forces underlying the knotting process remain difficult to rationalize, which limits the scope of the discovery. Trefoil knots are inherently chiral structures, and the previously published syntheses produce, with two notable exceptions (10, 12), only racemic mixtures.

We now report the stereoselective synthesis of a purely organic molecular trefoil knot in water. The knot was assembled almost quantitatively from a readily accessible amino acid-derived dithiol building block, following a dynamic combinatorial approach (13). The knot appears to be the smallest structure that minimizes the solvent-exposed hydrophobic surface. Hydrophobicity has been suggested to be involved in the formation of certain knotted proteins (14), and we propose that our system may offer a simple model of the relation between the chemical structure of an oligomer and its potential to fold into a knot.

The building block is composed of three hydrophobic electron-deficient  $\pi$ -systems (1,4,5,8-naphthalenediimide, NDI) connected by flexible hydrophilic amino acids ( $L$ - $\beta$ -amino-alanine). It is terminated at both ends by  $L$ -cysteine, which provides a thiol for disulfide exchange and additional carboxylate anions for water solubility. The building block was readily synthesized in five steps with a 62% overall yield.

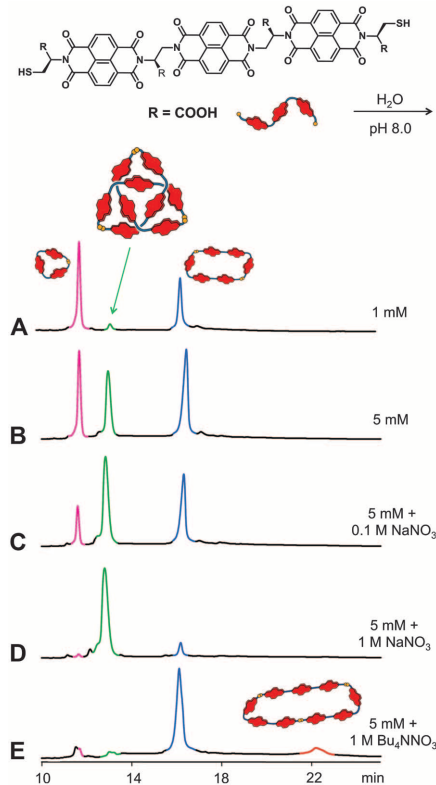
A first dynamic combinatorial library was generated by dissolving the building block at a concentration of 1 mM in water in the presence of two equivalents of NaOH (Fig. 1A). The pH was adjusted to pH 8 using a concentrated stock solution of NaOH (100 mM). The library was stirred for 1 day under air in capped vials to allow slow oxidation of the building block. After full oxidation of the library, liquid chromatography–mass spectrometry (LC–MS) analysis (Fig. 1A) revealed a distribution composed predominantly of the closed monomer (eluted at 12 min) and dimer (at 16 min). A trace amount of a trimeric macrocycle (at 13 min) was also identified, which was amplified, in accordance with Le Chatelier's principle, when the library was rerun at higher concentrations (5 mM) (Fig. 1B).

Although the formation of a trimer was not completely unexpected, its exceptionally short retention time caught our attention. Hydrophobicity and size effects usually give a closed monomer the lowest retention time, followed in succession by dimer and trimer. This irregularity is characteristic of an interlocked macrocycle, such as a catenane (15). However, tandem mass spectrometry MS/MS analyses disclosed a fragmentation pattern characteristic of a noncatenated macrocycle, which suggested that the trimer could be folded into another well-organized and compact structure: the trefoil knot, depicted in Figs. 1 and 2, in which the hydrophobic surfaces are efficiently buried and the hydrophilic side chains are exposed to the solvent.

This hypothesis was reinforced by the observation that addition of high concentrations of salt (NaNO<sub>3</sub>) (Fig. 1, C and D), which enhances the hydrophobic effect, resulted in further amplification to a nearly quantitative assembly (94%) of the knotted trimer. The use of other mineral salts led to an identical library distribution (fig. S3), which confirmed that the assembly is indepen-

dent of the salt chosen and is mainly driven by the increased polarity of the medium. Conversely, addition of acetone reduced both the medium polarity and the yield of the knot (fig. S4). Among the salts used, only tetrabutylammonium nitrate (Bu<sub>4</sub>NNO<sub>3</sub>) (Fig. 1E), which can solvate the NDI surface (16), led to a different library distribution, as it amplified the dimer at the expense of the other macrocycles and thus acted as a negative template for the knot (17). Addition of the Bu<sub>4</sub>NNO<sub>3</sub> also resulted in the amplification, albeit to a lesser extent, of a new peak identified by MS as another trimeric macrocycle, likely to be the trivial trimer, with a retention time of 22 min. The presence of two trimers at different retention times in similar libraries strongly suggests that these two macrocycles are topologically inequivalent.

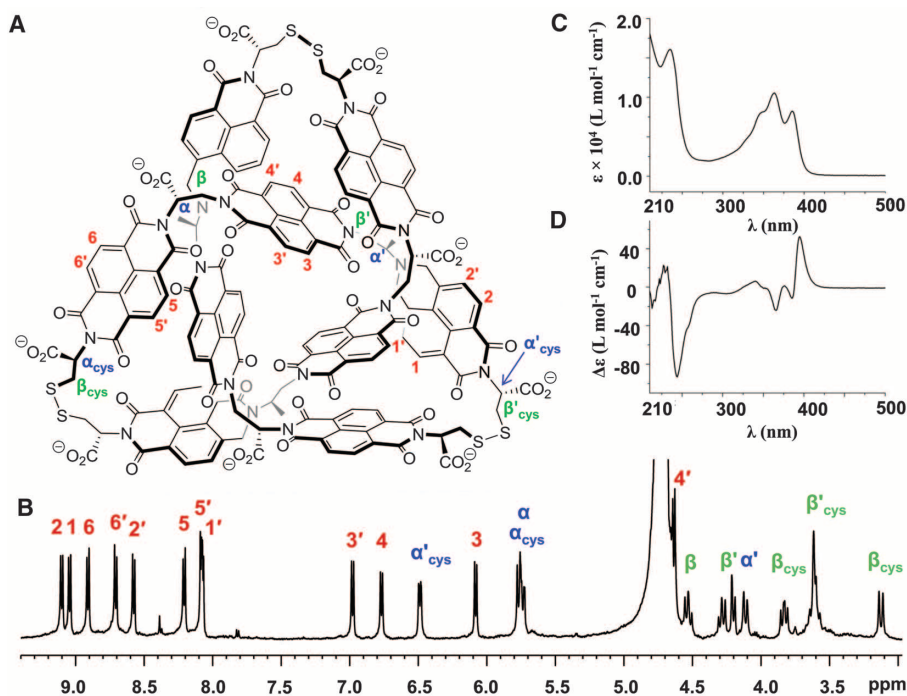
To confirm that the trimeric macrocycle eluting at 13 min was a knot, we isolated it by preparative high-performance liquid chromatography (HPLC) (isolated yield: 92%) (fig. S2) and analyzed the redissolved sample by nuclear magnetic resonance (NMR) and circular dichroism (CD). The trimeric macrocycle exhibits sharp NMR signals in water (500 MHz, 298 K): This suggests that it is not a flexible macrocycle, as these usually exhibit broad NMR signals that sharpen with increase in temperature (15). In D<sub>2</sub>O at 358 K and in 1:1 water/acetone mixture



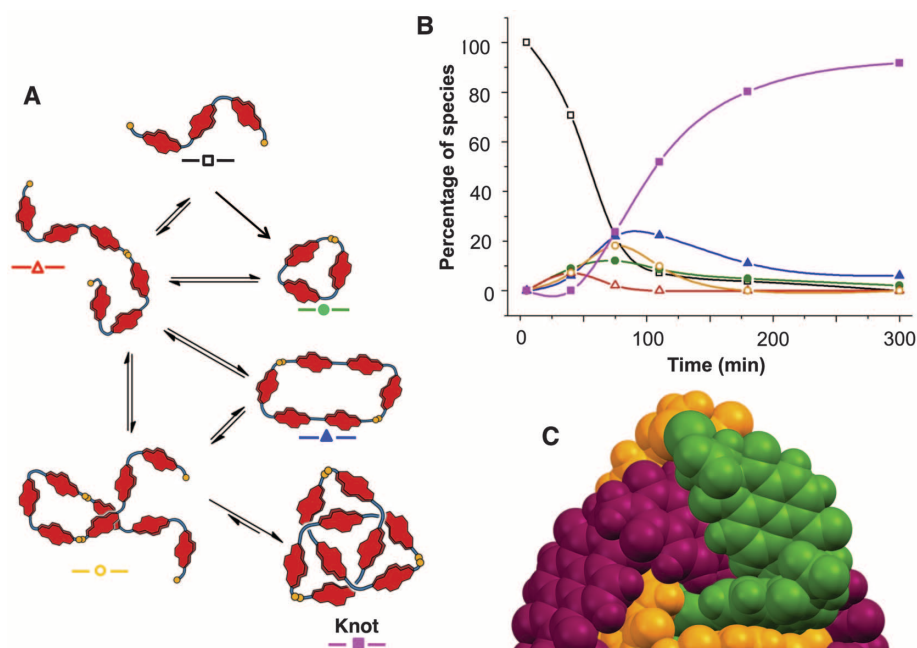
**Fig. 1.** Reversed-phase HPLC analysis of libraries at 1 mM (A) and 5 mM of building block, in the absence (B) and presence (C to E) of salts. Absorbance was recorded at 383 nm.

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**Fig. 2.** (A) Chemical structure of the right-handed knot. (B)  $^1\text{H-NMR}$  spectrum of the knot (500 MHz,  $\text{D}_2\text{O}$ , 298 K). The protons are labeled according to the scheme shown above. (C) UV-Vis and (D) CD spectra of the knot in water at 298 K.



**Fig. 3.** (A) Proposed mechanism for the synthesis of the trefoil knot. (B) Kinetic profile of a library (5 mM, 1 M  $\text{NaNO}_3$ , water, pH 8, absorption monitored at 383 nm), showing (black open square) the consumption of the building block and (magenta square) the formation of the knot. The formation and disappearance of the macrocyclic intermediates can also be traced: (green filled circle) closed monomer, (red open triangle) open dimer, (blue filled triangle) closed dimer, (yellow open circle) open trimer. The percentages were evaluated from the HPLC peak areas. (C) Energy-minimized model of the knot from molecular modeling at a semiempirical level using PM6-DH+ (20, 21) force field with COSMO (22) parameters for expressing the dielectric medium.

at 298 K, the  $^1\text{H-NMR}$  spectrum remained essentially unchanged, which indicates the absence of flexibility or gross conformational change (figs. S5 and S6).

The  $^1\text{H-NMR}$  spectrum at 298 K (Fig. 2B) is entirely consistent with the suggested trefoil knot drawn in Fig. 2: a structure that exhibits  $C_3$  symmetry, with an inequivalent environment for each proton of an individual building block but overall equivalence of the three building blocks composing the knotted macrocycle. The NDI protons span a wide range of chemical shifts from  $\delta = 9.2$  to 4.6 ppm. The occurrence of an NDI proton signal (4' in Fig. 2) at 4.6 ppm fortifies the hypothesis of a knot structure where at least one hydrophobic NDI aromatic core is deeply buried within the molecule and so experiences shielding from neighboring NDIs. The  $^1\text{H-NMR}$  could be fully rationalized by matching the structure of the proposed knot and the observed nuclear Overhauser effect (NOE) crosspeaks, which allows the assignment of each proton of the molecule as labeled in Fig. 2A (figs. S7 to S11).

A trefoil knot is topologically chiral, and in this case, the right- and left-handed knots would be diastereomers, because the building block is chiral. However, only one diastereomer of the knot was observed by LC-MS and NMR, which implies that the building block, composed of all L-amino acids, is dictating the chirality of the knot. The enantiomeric building block derived from all D-amino acids leads to the formation of the enantiomeric knot. The racemic mixture of all-L- and all-D building blocks mainly self sorts to give the single diastereomer of the knot in racemic form (figs. S19 and S20), which further demonstrates that this structure is exceptionally efficient in its packing and, therefore, thermodynamically stable. By contrast, the diastereomeric building block containing L-cysteine and D- $\beta$ -amino-alanine does not form the knot but instead gives mainly the dimer (fig. S18).

The CD signal of the knot (Fig. 2D) consists of an intense positive band in the NDI absorption region (385 nm) and a strong negative band in the imide region (245 nm); this matched a Zerner's intermediate neglect of differential overlap-random phase approximation (ZINDO/RPA)-predicted CD spectrum (fig. S17) based on the right-handed knot depicted in Fig. 2A. This induced CD signal is orders of magnitude stronger than that in related catenanes and macrocycles reported in earlier publications by our group (fig. S15), which indicates rigid geometrical relations between the NDI units.

The library is fully oxidized after only 4 hours and may not reach thermodynamic equilibrium; therefore, kinetics plays an important role in the knot formation and allows us to explore possible mechanistic pathways. The oxidation of a 5 mM library containing 1 M  $\text{NaNO}_3$  was monitored by LC-MS every 35 min (Fig. 3B). The formation and disappearance of intermediate species (macrocycles and open oligomers) highlight the reversibility of the system in the early



stages of oxidation and show the conversion of the less stable macrocycles into the knot, which is presumably the smallest oligomer that can maximize hydrophobic stabilization. Although other pathways may be involved, the kinetic profile shown in Fig. 3 suggests that knot formation predominantly occurs via the formation and folding of the open trimer. Once formed, the open trimer folds into condensed structures that bury the hydrophobic NDI surfaces. Presumably only one of the structures places the terminal thiols in sufficiently close proximity to allow the ring closure that results in a knot. If the ring were opened by cleavage of a disulfide link, closure would be rapid, whereas unfolding would require unfavorable and slow reexposure of hydrophobic surfaces. The formation of the trefoil knot is, therefore, kinetically and thermodynamically favored and closely mimics the mechanism of knot formation in naturally occurring  $\alpha/\beta$  methyl transferases (18).

The conclusions drawn from the experimental results were corroborated by molecular modeling calculations, which showed that the knot was relatively more stable and has a smaller surface area and volume than its topological isomers (the closed trimer and the [2]catenane) (19).

Although the cyclic monomer and dimer are transiently formed in the library, the reversible nature of the disulfide bond allows their reorganization into the more ordered, but more stable, trimeric knot, highlighting the dual conflicting role of entropy at the different stages of the folding process: On one hand, the formation of small molecules is preferred, whereas the formation and

stability of the larger knot result from the necessity of burying hydrophobic surfaces. Overall, the design of the building block offers an efficient way to access purely organic knotted macrocycles in aqueous media. First, the alternation of rigid hydrophobic NDIs, which can stack and form the core of the knot and bury most hydrophobic surfaces, and flexible hydrophilic loops supplies an ideal scaffold for the knitting of organic oligomers into complex knots. Second, the choice of the amino acid chirality is crucial: The all-L and all-D building blocks induce the directionality of the knot; whereas in the D/L building block, the mismatch of chiralities prevents knot formation. The folding of a linear species into a knot, driven by the hydrophobic effect, may provide a simple model for the formation of knots in proteins.

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#### Supplementary Materials

www.sciencemag.org/cgi/content/full/338/6108/783/DC1  
Materials and Methods  
Figs. S1 to S22  
Tables S1 to S4  
References

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## Tissint Martian Meteorite: A Fresh Look at the Interior, Surface, and Atmosphere of Mars

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Tissint (Morocco) is the fifth martian meteorite collected after it was witnessed falling to Earth. Our integrated mineralogical, petrological, and geochemical study shows that it is a depleted picritic shergottite similar to EETA79001A. Highly magnesian olivine and abundant glass containing martian atmosphere are present in Tissint. Refractory trace element, sulfur, and fluorine data for the matrix and glass veins in the meteorite indicate the presence of a martian surface component. Thus, the influence of in situ martian weathering can be unambiguously distinguished from terrestrial contamination in this meteorite. Martian weathering features in Tissint are compatible with the results of spacecraft observations of Mars. Tissint has a cosmic-ray exposure age of  $0.7 \pm 0.3$  million years, consistent with those of many other shergottites, notably EETA79001, suggesting that they were ejected from Mars during the same event.

**D**emonstration in the early 1980s that an important group of meteorites was of martian origin represented a breakthrough in attempts to understand the geological evolu-

tion of Mars (1–3). Unfortunately, most of the samples were collected long after their arrival on Earth and thus have experienced variable degrees of terrestrial weathering (4). Even the few martian

meteorites that were collected shortly after their observed fall to Earth have been exposed to organic and other potential contaminants during storage. Here, we report on the Tissint martian meteorite, which fell on 18 July 2011 in Morocco (figs. S1 and S2). This is only the fifth witnessed fall of a meteorite from Mars and therefore provides an opportunity to improve our understanding of processes that operated on that planet at the time the meteorite was ejected from its surface.

The largest recovered stones from the Tissint fall are almost fully covered with a shiny black fusion crust (Fig. 1). Internally the meteorite consists of olivine macrocrysts set in a fine-grained matrix of pyroxene and feldspathic glass (maskelynite) (5) (figs. S3 to S6 and tables S1 to S6). The matrix is highly fractured and penetrated by numerous dark shock veins and patches filled with black glassy material enclosing bubbles (fig. S7). The petrology of Tissint shows similarities to that of other picritic shergottites (an important group of olivine-rich martian basaltic rocks), in particular, lithologies A and C of EETA79001 (2). The grain density and magnetic properties of Tissint (fig. S8) also match previous results from basaltic and picritic shergottites (6).

Tissint is an Al-poor ferroan basaltic rock, rich in MgO and other compatible elements (Ni,