

Construction of a DNA-Truncated Octahedron

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Received November 1, 1993*

Abstract: A covalently closed molecular complex whose double-helical edges have the connectivity of a truncated octahedron has been assembled from DNA on a solid support. This three-connected Archimedean solid contains six squares and eight hexagons, formed from 36 edges arranged about 24 vertices. The vertices are the branch points of four-arm DNA junctions, so each vertex has an extra exocyclic arm associated with it. The construct contains six single-stranded cyclic DNA molecules that form the squares and the extra arms; in addition, there are eight cyclic strands that correspond to the eight hexagons. The molecule contains 1440 nucleotides in the edges and 1110 in the extra arms; the estimated molecular weight for the 2550 nucleotides in the construct is 790 kDa. Each edge contains two turns of double-helical DNA, so that the 14 strands form a catenated structure in which each strand is linked twice to its neighbors along each edge. Synthesis is proved by demonstrating the presence of each square in the object and then by confirming that the squares are flanked by tetracatenane substructures, corresponding to the hexagons. The success of this synthesis indicates that this technology has reached the stage where the control of topology is in hand, in the sense of both helix axis connectivity and strand linkage.

Introduction

One of the major goals of both biotechnology and nanotechnology^{1,2} is the assembly of novel biomaterials that can be used for analytical, industrial, and therapeutic purposes. In particular, one would like a rational approach to the construction of individual objects, devices, and periodic matter on the nanometer scale,^{3–5} utilizing the informational macromolecules of biological systems. For the past several years, our laboratory has been engaged in the nanoscale construction of stick figures, using branched DNA molecules as building blocks. The edges of these figures consist of double-helical DNA, and the vertices correspond to the branch points of stable DNA branched junctions.^{6,7} We have reported the assembly in solution of molecules whose helix axes have the connectivities of a quadrilateral⁸ and of a Platonic polyhedron, the cube.⁹

Difficulties encountered in the solution synthesis of the cube have stimulated the development of a solid-support-based synthetic procedure that provides control over the formation of a single edge at a time.¹⁰ This is accomplished by protecting with a hairpin loop each double-helical arm designated to participate in edge formation; the loop is removed by a restriction endonuclease, thereby exposing a ligatable sticky end. Each intermediate product is a topologically closed molecule, which can be separated from ligation-failure products by denaturation on the support. Furthermore, intermolecular addition reactions and intramolecular cyclization reactions can be performed separately, in conditions optimized for each type of reaction; cyclizations using symmetric sticky ends would yield a mixture of intra- and intermolecular products in solution, but the isolation of molecules on the support makes them feasible.

We have demonstrated previously that one can use the solid-support-based procedure to assemble polygons from individual

branched junctions.¹⁰ It is ultimately desirable to be able to construct objects of much greater complexity than polygons. Here we demonstrate that solid-support-based assembly can be used to combine individual polygons and polygonal clusters into a complex polyhedral object, whose helix axes have the connectivity of a truncated octahedron (Figure 1a). The truncated octahedron is an Archimedean semiregular polyhedron, containing 14 faces (six squares and eight hexagons), 24 vertices, and 36 edges. It is a three-connected object,^{11,12} meaning that each vertex is connected to three other vertices by an edge of the figure; in principle, it could be built from three-arm branched junctions.⁶ Nevertheless, we have built this object from four-arm branched junctions; the extra arms contain the potential for connections that could produce periodic matter in one, two, or three dimensions, although forming such connections is beyond the scope of the work reported here.

The edges of the polyhedron contain seven specific sites for cleavage by restriction endonucleases; in addition, each extra arm contains a restriction site. These sites facilitate analysis of the ligation reactions, because one can digest both intermediate and final structures to generate well-defined breakdown products. Each edge of the polyhedron is designed to contain 20 nucleotide pairs, corresponding to two full double-helical turns of DNA. Consequently, each of the 14 faces corresponds to a single cyclic DNA molecule,¹³ and the entire polyhedron is a complex 14-catenane. The cyclic molecules corresponding to the hexagons are each exactly 120 nucleotides long, and each hexagon is linked twice to three ‘square’ molecules and to three other ‘hexagons’. The cyclic molecules corresponding to the squares contain 80 nucleotides linked twice to four different hexagons, plus the DNA in the 24 extra arms, which varies from 40 to 50 nucleotides. The entire molecule contains 1440 nucleotides in the edges and 1110 in the extra arms for a total of 2550 nucleotides; the total estimated molecular weight is about 790 kDa.

Schematic views of the truncated octahedron are shown in Figure 1. Figure 1a is a drawing of the Archimedean polyhedron. Figure 1b is a modified Schlegel diagram of the truncated octahedron. In this representation, the square in the center (labeled 5) is closest to the viewer, those flanking it, 2, 3, 4, and

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- Abstract published in *Advance ACS Abstracts*, February 1, 1994.
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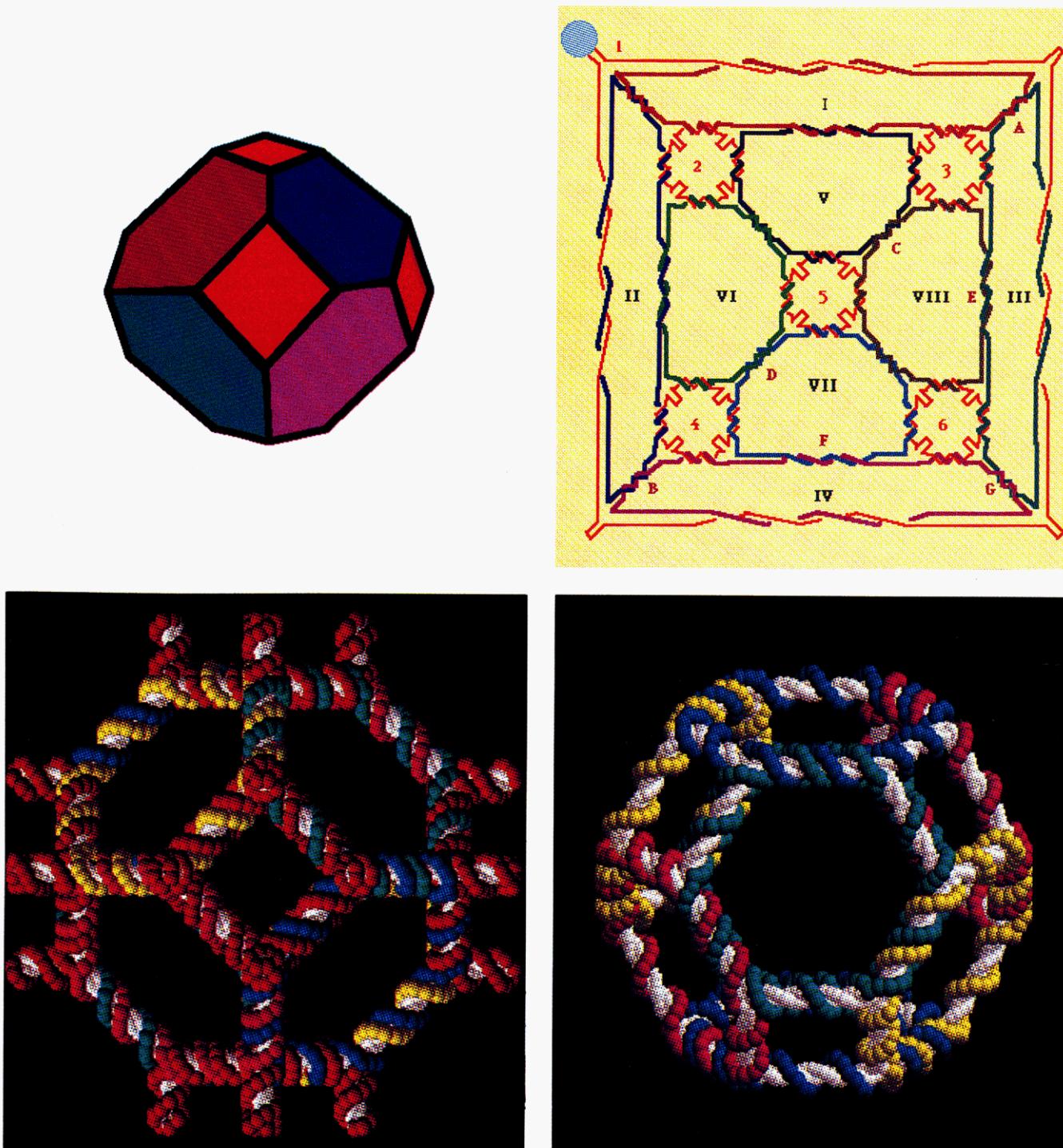


Figure 1. Representations of the truncated octahedron. (a, top left) Solid truncated octahedron. This drawing represents the solid that has been built here. Shown is the front half of an idealized molecule, which has been rotated slightly off a symmetrical view. Each square abuts four hexagons, and each hexagon shares an edge with three squares and three other hexagons. The object has the same 432 symmetry as a cube. This is a three-connected object, because each vertex is connected by edges to three other vertices. (b, top right) Modified schlegel diagram of the truncated octahedron. In this representation, the square at the center is closest to the viewer, and polygons further from the center are further from the viewer. The square on the outside is at the rear of the object. Each edge is represented by two helical strands, all of whose twisting is confined to the center of the edge, for clarity.⁹ The squares are numbered with Arabic numerals, and the hexagons are numbered with Roman numerals. Each hexagon is drawn distorted from ideality. The inner hexagons (V–VIII) are drawn as hexagons with two right angles, and the outer hexagons (I–IV) are drawn as trapezoids, with their inner edge representing three edges of the polygon. Note that the 14 polygons each correspond to a particular strand (1–6 or I–VIII), because there is an integral number of full turns between vertices; nevertheless, each edge is actually composed of two strands that form a double helix. The solid support is indicated at the upper left corner, so this diagram represents the construct just before release and annealing. The capital letters (A–G) indicate the seven edges that are formed by ligation of sticky ends (S1–S7, respectively) exposed by symmetrically cleaving restriction enzyme pairs; each edge contains a restriction site. Note that the tetracatenane flanking every square except square 6 can be released from a squareless molecule by cleavage at two of these sites. (c, bottom left) Double-helical representation of an ideal truncated octahedron. Each edge is colored, with the squares and external hairpin arms red and the hexagonal strands blue, magenta, yellow, and green. The bases are shown as white. The external hairpins have been shortened from those constructed for purposes of clarity; they are shown parallel to the fourfold axes of the molecule. Each pair of vertices is separated by an edge containing two full turns of double-helical DNA. This is a view down a fourfold axis of the molecule. The strands corresponding to the squares are drawn in red, and the four strands corresponding to visible hexagons are drawn (counterclockwise from the upper right) in green, yellow, magenta, and blue; hexagons opposite these through the center of the molecule have the same colors. Base pairs are shown in white. (d, bottom right) Octacatenane formed by the strands that correspond to the hexagons. The view is down one of the threefold axes of the molecule. The strands corresponding to the squares and extra arms have been removed. The hexagon is flanked by three hexagons and three squares. Note that each square is flanked by strands of four different colors. The bases of the square strands have been retained for clarity.

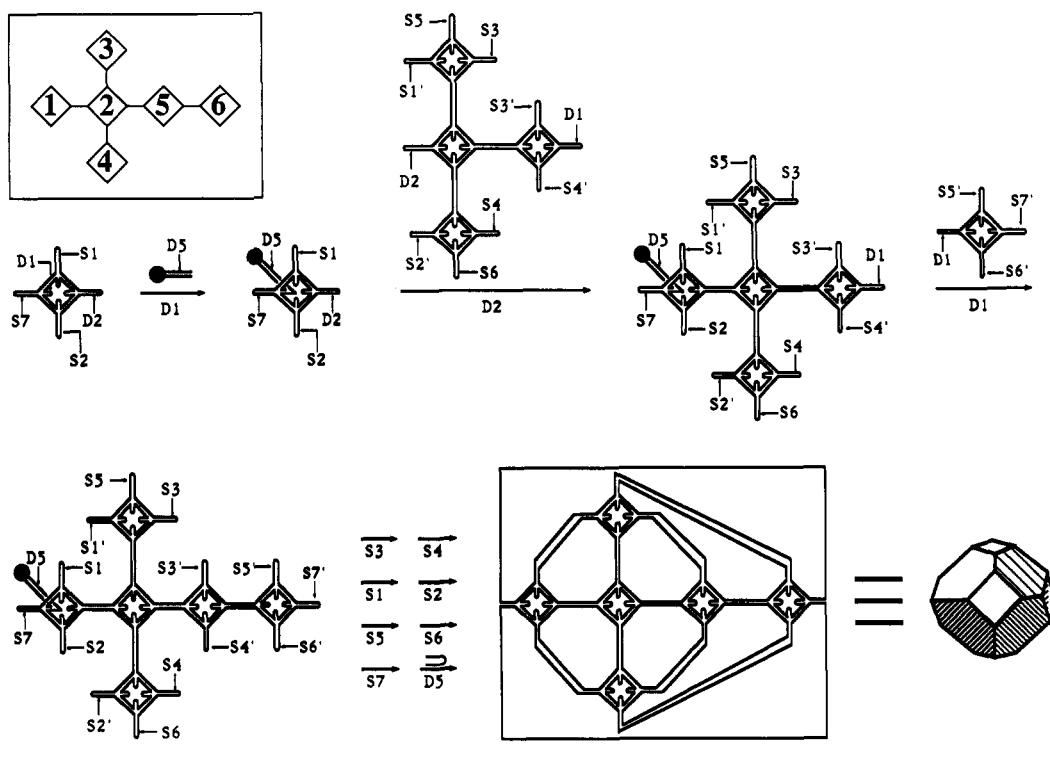
Table 1. Sequences of the Square Components of the Truncated Octahedron^a

1.1.1:	5'-TAAGTGTGGTATTAGATCTGGAACCTTGTTCAGATCTAAATACCTGAACCT-3'	4.1.2:	5'-ACACCAAGCATCACCATACCACCGCTGGATGGACAGCTGGCTAC-3'
1.1.2:	5'-ACACCAAGGTTACCGGACCGCCTGCTCATT	4.2.1:	5'-TGGTAGCTGGTGGTCAACTCATGAACTCTT-3'
	TTTATGAGCAGGCGCTGGACACTTGGTACCGCAGCTT		TTGAGGTTCATGAGTTGACCTGGACTCAGCCGTT-3'
1.2.1:	5'-TGGTAGCTGGTGGTCAACTTGGTACCGCAGCTT	4.2.2:	5'-GAGTCACCTATCCACTCCTGGAACCGCTT-3'
	TTCGTGGCTTACCGGTGCTGAGCCAAGCCGTT-3'		TTGCTTCAAGGAGTGGATAGGACAGCT-3'
1.2.2:	5'-TGGCTCACCATCACTCGGCGTGTCTTCAATCCTT	4.3.1:	5'-GTGCTGTGGAGCTCTCGAGCTT-3'
	TTGGATTGGAAGACACGCCGAGTGTAGGACGACCT-3'		TTTAATGACTCGAGAAGCTCTGTGACCT-3'
1.3.1:	5'-GTATAGTGGTACTCCATGGCAATCTT	4.3.2:	5'-CGTACGGTACACCTTACCAACCAATGGTCTT-3'
	TTGATTGCCATGGAGTAACCTGTTAC-3'		TTTACGAACCATGGTGGTAAGGACGACACCGCT-3'
1.3.2:	5'-CGTACGGTACACCTCACAGCTCTGCACGCTT	4.4.1:	5'-GTCACGGACAAGTGGCGTATGCCGCTCTATT
	TTGCGTGCAGGAGCTGTGAGGACTATAACCGGCT-3'		TTTAGAGGCGCGCATACGCCCTGGCTCCGTAGCC-3'
1.4.1:	5'-GTCACGGAAAGGTGGTCTATCCAGCAGAGACATTGTT	4.4.2:	5'-GGAGGCCACATTCCACGGTCTGGTTGATT
	TTAACAAATGTCCTGCTGGATAGACCTGTCACCGTAGCC-3'		TTCAAACAGACCGTGGATGGACTTGTC-3'
1.4.2:	5'-GGTACACCAACCAGCCACTGTCCTATT	4.1.2:	5'-ACACCAAGCATCACCATACCACCGCTGGATGGACAGCTAC-3'
	TTAGGACAGTGGCTGGTGGACCTTTC-3'		TTGATTCCAGGGATGGTATGGACAGTGCCTAC-3'
2.1.1:	5'-TGGTAGTGGCAACATGGAGAAGAGAGTTCTT	5.1.1:	5'-GCAGAGTGGTCTACTAGTCTACCTT
	TTGGAACCTCTTCTCGCAATGTTGCTGATACT-3'		TTGGTAGACTAGTGAAGAACCTGATGCT-3'
2.1.2:	5'-ACACCAAGTATCACCTAACGCTCTGCCTGATCATT	5.1.2:	5'-ACACCAAGCATCACCATGGCTGCTGGCCGCTAC-3'
	TTTATGATGCCGAGAGGCTTAGGACTACCAGGCTAC-3'		TTACGGCGGCCAGACAGGCAATGGACTCTGGGCTAC-3'
2.2.1:	5'-TGGTAGGAAAGTGGACCTACGCTGTGAGACTCCAACCT	5.2.1:	5'-TGGTAGGTGGTGGAGCACTGACGACTCGCAGGTATCCTT
	TTGTTGGAGTCTCACAGCTAGGTCTGACAGGCGT-3'		TTGAGGATACTCGAGCTGCTAGTGTCTGACTCCAGCGT-3'
2.2.2:	5'-TCGTCACCCAGCGCTCCTCGGCTCATCTT	5.2.2:	5'-GGAGTCACCTTAGGCCGCTGTGCCGACTT
	TTGATGAGCCGAGGAGGCCGCTGGACTTCCT-3'		TTTAAGTCGGCCACAGGCCCTAAGGACACCT-3'
2.3.1:	5'-GGTCTGTGGTCAACCGACAAGTCTTCATCCGCTT	5.3.1:	5'-GGAACGTGGTGTACCGCTTCACT
	TTGCGGATGAAAGACTTGTGGTGCACCTGTCAAC-3'		TTGTGAAACGCGTGAACACCTGGCTCC-3'
2.3.2:	5'-CGTACGGTACACCTCAGCCACCGTGGCACTGATT	5.3.2:	5'-CGTACGGGACCAACGCCCTCAGGGCTTCATT
	TTTCAGTGGCAGCTGGCTGAGGACAGACCCGGT-3'		TTTATGAAAGGCCGCTGAGGCCGTTGACGTTCAACGGT-3'
2.4.1:	5'-GTCACGGTGTGTCGCTGCTGCAATCGCAGGTGCGCTTATT	5.4.1:	5'-GTCACGGTGGTGGAGCATCCAACCTCGTCTTCAACTGCTT
	TTTACGGGACCTTGCATTGACGAGCACGCCCTGCTTAGTGCCT-3'		TTGCAGTTGAAGACGAGGTTGGATGTCTGTGCGCTAGCC-3'
2.4.2:	5'-TAAGGCACCATGCCCTACCTGGCTTGT	5.4.2:	5'-GGACACACCTCTGCCATCTGCCACCTT
	TTTAACAAAGCCAGGTAGGCATGGACACGAC-3'		TTTACGGTGGCAGATGGCCAGAGGACCGCAC-3'
3.1.1:	5'-GAGATGTGGCTCAACCCGGGATGCCCT	6.1.1:	5'-TGTGGTGGCACTATCCGGAGTTCTT
	TTGGCATCCCGGGTTGAGCCTGATTCT-3'		TTGAAACTCCGGATAGTGCCTGCTCGT-3'
3.1.2:	5'-ACACCAAGAACATCACCGTACCTCGTGGTCAATT	6.1.2:	5'-ACACCAAGCACCTCTCCATCGTCTGGTCAATT
	TTTATGACCAAGGAGGTACTGGACATCTGGCTAC-3'		TTTAATGACCAAGGACATGGAGAGGACCGACAGGCTAC-3'
3.2.1:	5'-TGGTAGTGGAGTGGTCAAGGATCCACACTT	6.2.1:	5'-TGGTAGGTGGTGGAGCATGGTCTGTCTTCAAGTCTT
	TTGTTGTTGGATCCTTGAACCTGAACTCAGCCGT-3'		TTGACTATGAAGACAGAACCGATGCTCTGAACGGAGCCGT-3'
3.2.2:	5'-GAGTTCACCGCTACCGCAGTGTCTCTT	6.2.2:	5'-CCGTTACCATACCATCCACTGGTCTGTT
	TTGAGGACACTCGCTGAGCGGACTCCAT-3'		TTTACGAACCAAGTGGATGGTATGGACCCACT-3'
3.3.1:	5'-GCAAAGTGGCACTACCGCCGAAGAGCTCGCTT	6.3.1:	5'-GTGCTGTGGATTACGTCGACCTCACTT
	TTGCGAAGCTCTCGGGCGTAGTGCCTGCCGT-3'		TTGTGAGGTGACGTAATCTGCATCC-3'
3.3.2:	5'-CGTACGGACGGCACCTAACACTCGGTGGCCGT	6.3.2:	5'-CGTACGGGACCAAGTGGCGTATCGTACGCTCGCTT
	TTTACGGCCACCGAGTGTAGGACTTGTGACCGCT-3'		TTGCGAGCGTAGCATACGCCCTGCCGACCGTAGCC-3'
3.4.1:	5'-GTCACGGAACAGTGGACCTTGTGACCGTATT	6.4.1:	5'-GTACGGGACAAGTGGCGTATCGTACGCTCGCTT
	TTTAATGGCTAGCAAGGTCTGTACGTAGCC-3'		TTGCGAGCGTAGCATACGCCCTGCCGACCGTAGCC-3'
3.4.2:	5'-GATGACACCAACCACCGTGTGCTGAATT	6.4.2:	5'-GGTCCGACCGAACCAATCGCTGGTTGATT
	TTTCAGCACACGGTGGTGGACTGTTCT-3'		TTTCAAGGCCAGCGGATTGGTGGACTTGTC-3'
4.1.1:	5'-GACTGTGGCACCTCGTCGAGCTTCTACAACCTT		
	TTGTTGAGAAGACTCGCGACGGTGCCTGATGCT-3'		

^a The sequences of the individual squares are given as square.side.strand.

6, are on the sides, and square 1, shown on the outside, is at the rear. One extra arm of square 1 is illustrated attached to the support; hence, this diagram represents the truncated octahedron just before cleavage from the support. The hexagons that flank square 5 (V, VI, VII, and VIII) are shown as distorted hexagons. Those that abut square 1 (I, II, III, and IV) are shown as trapezoids, whose inner edges actually represent three edges of the hexagons. The double-helical twisting of the object is shown

for clarity^{8,9} only in the central portion of each edge, even though it extends to the vertices. Figure 1c is a diagram that attempts to illustrate the structure the molecule would assume if it adopted the geometry of an ideal truncated octahedron, whose faces are all regular polygons. Such an object has 432 symmetry. The view in Figure 1c is down one of the fourfold axes of the molecule. Figure 1d shows the molecule as it would appear if stripped of the strands that correspond to the squares and the extra arms.



SQUARE ASSEMBLY



TETRASQUARE ASSEMBLY

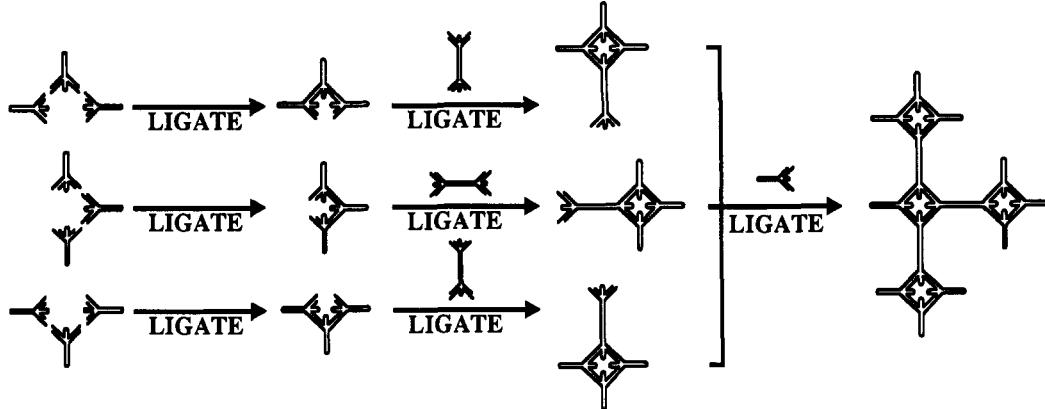


Figure 2. Synthetic schemes used to synthesize the truncated octahedron. (a, top) Solid-support construction of the polyhedron. The boxed diagram in the upper left corner indicates the numbering of the individual squares. Each square in the rest of the diagram is shown with its restriction sites indicated. Symmetrically cleaving restriction sites (decoded in Table 2) are named S, indicated in pairs, with one member of the pair being primed; restriction sites that are cut distally are named D; restriction sites on the exocyclic arms are not indicated. The arms that will eventually be combined to form edges of the object are drawn on the outside of each square, and the exocyclic arms are drawn on the inside of the square. A reaction is indicated by a line above a restriction site name: This means that the restriction enzyme (or enzyme pair for those labeled S) is added, protecting hairpins are removed, and then the two sticky ends are ligated together. In the first step, square 1 is ligated to the support. In the second step, the tetrasquare complex is added to square 1. In the final intermolecular step, square 6 is ligated to the restricted pentasquare complex. The next seven intramolecular closure steps entail seven individual pairs of restrictions, as indicated: S3 and S3', S4 and S4', S1 and S1', S2 and S2', S5 and S5', S6 and S6', and S7 and S7'. The S1 and S2 reaction sets have also been done preceding the S3 and S4 reaction sets. The product is shown in two forms. On the left, the S1-S6 closures are shown as triple edges, to emphasize their origins; the two strands of the edge formed by the S7 closure are separated to maintain the symmetry of the picture. On the right, a slightly rotated front view of a polyhedral representation of a truncated octahedron is shown without the exocyclic arms; the 432 cubic symmetry of the ideal object is evident from this view. (b, bottom) Solution syntheses of the individual components. The assemblies of squares 1 and 6 are shown on the top part of this figure. Every outside strand of square 6 is radioactively labeled. The lower part of the figure illustrates the construction of the tetrasquare complex. The outermost three quarters of each of squares 3, 4, and 5 are annealed in pairs and ligated together. The four-strand complexes that constitute the linkers and closest sections of square 2 are then added to them. The three squares, attached to fragments of square 2 are then combined, along with the missing portion of square 2, to make the final intermediate product. The labels are on the outer strand portions of both left edges of square 2 and both left edges of square 5; hence, combined with the labels on all outer strands on the edges of square 6, every hexagonal strand is labeled.

Table 2. Restriction Enzymes Used for Synthesis and Analysis^a

code name	name	mate code	mate name	union	union code
D1	<i>BbsI</i>				
D2	<i>BsmAI</i>				
D5	<i>BbvI</i>				
S1	<i>BglII</i>	S1'	<i>BamHI</i>	<i>DpnII</i>	A
S2	<i>NcoI</i>	S2'	<i>BspHI</i>	<i>nlaIII</i>	B
S3	<i>NheI</i>	S3'	<i>SpeI</i>	<i>RmaI</i>	C
S4	<i>MluI</i>	S4'	<i>BssHII</i>	<i>BstUI</i>	D
S5	<i>XmaI</i>	S5'	<i>BspEI</i>	<i>MspI</i>	E
S6	<i>XbaI</i>	S6'	<i>SalI</i>	<i>TaqI</i>	F
S7	<i>Acc65I</i>	S7'	<i>BsiWI</i>	<i>RsaI</i>	G

^a Restriction enzymes Si and Si' have different six-base recognition sites that both expose the same four-base symmetric sticky end. The union is the restriction enzyme that cleaves the four-base symmetric site formed by ligation of the sites.

This is an octacatenane, viewed down a threefold axis; its components are the strands that correspond to the hexagons.

Synthetic Scheme

The sequence of the truncated octahedron has been designed using the program SEQUIN.¹⁴ The squares are the basic units used in the assembly, and the hexagons are derived by ligating the squares together. Each square consists of two cyclic strands, an 'inner strand' corresponding to a square of the truncated octahedron and four extra arms and an 'outer strand' that will be used for ligation to other squares; the nucleotides of the outer strand will ultimately be part of the hexagons in the final product. The sequence of each of the square units has been designed separately and, insofar as possible, in accord with the basic principles of sequence symmetry minimization,⁶ using sequence elements of length 6. The program has been modified from its reported version¹⁴ to enable absolute exclusion of specified sequences, so that restriction sites cannot occur adventitiously at unwanted positions. The sequence of the squares is shown in Table 1. Each branch point is flanked by the same sequence that surrounds the branch point of the well-characterized junction J1.¹⁵ Each edge of each square is formed by ligating six-nucleotide sticky ends, two of which are 5' overhangs and two of which are 3' overhangs, as done previously;⁸ the same set of sticky ends is used for each square. The exocyclic arms of each square contain the same interrupted restriction site (square 1, *A/wNI*; 2, *BglI*; 3, *DraIII*; 4, *PstMI*; 5, *SfiI*; 6, *BstXI*), except for square 1. One of its exocyclic arms contains a *BbsI* site, used to attach it to the support. Each of the five intermolecular ligation sites is designed to be deprotected by a restriction enzyme that cleaves distally from its recognition site on the hairpin loop. Two of these restriction enzymes (*BspMI* and *Earl*) have been found to be ineffective when used to digest DNA attached to the solid support, forcing us to modify the planned synthetic scheme. The seven intramolecular ligation sites are composed of pairs of six-base symmetric sites that expose four-base sticky ends. Ligation results in the destruction of these sites¹⁰ but produces four-base symmetric sites that can be used for product analysis.

Whereas the solid-support procedure permits one to perform ligations in a stepwise fashion, we initially planned to ligate the squares together one at a time. The square numbering is shown in Figure 2a. The positions of distal-cutting restriction enzymes (D1, D2, and D5) used in intermolecular linkages are shown, as well as those of symmetrically cleaving restriction enzymes (S1, S1', ..., S7, S7'), used to form edges from intramolecular ligations. The correspondence between our notation and restriction enzyme names is shown in Table 2. The planned synthesis entailed the attachment of square 1 to the support and of square 2 to square 1, and then the attachment of squares 3–5 to square 2, and finally the attachment of square 6 to square 5. The failure of *BspMI*

and *Earl* to cleave DNA attached to the solid support has forced us to condense the five-ligation intermolecular synthesis to the two-ligation process shown in Figure 2a: squares 2–5 are combined with preformed connecting edges in solution and added to square 1; square 6 is then added to this intermediate. The assembly schemes of individual squares and of the tetrasquare complex (2–3–4–5) are shown in Figure 2b. Following construction of the six-square intermediate, the seven intramolecular closures are done sequentially, followed by removal from the support and the addition of an annealing hairpin¹⁰ to make a covalently closed molecule (Figure 2a). Thus, except for square 1, the attachment site, the cyclic strands corresponding to the squares of the truncated octahedron (the inner strands), is already intact when particular fragments are ligated to the support. The hexagons are derived by restriction and ligation of the long 'outer strand' of the heptacatenane visible in the lower left corner of Figure 2a.

Materials and Methods

Synthesis and Purification of DNA. All DNA molecules used in this study are synthesized on an Applied Biosystems 380B automatic DNA synthesizer, removed from the support, and deprotected using routine phosphoramidite procedures.¹⁶ All strands greater than 30 nucleotides in length are purified by polyacrylamide gel electrophoresis. Shorter strands are purified by preparative HPLC on a DuPont Zorbax BioSeries oligonucleotide column at room temperature, using a gradient of NaCl in a solvent system containing 20% acetonitrile and 80% 0.02 M sodium phosphate. Fractions from the major peak are collected, desalted, and evaporated to dryness.

Solution Syntheses of Individual Squares 1 and 6. Each strand is phosphorylated as described previously¹⁰ for 2–3 h at 37 °C. One hundred eighty picomoles of each of the eight strands of the squares are combined in partially complementary pairs, heated to 90 °C for 10 min, slowly cooled down, and ligated with 20 units of T4 DNA ligase (USB) at 16 °C or at room temperature. The square is purified on a 7% denaturing polyacrylamide gel, electroeluted for 2 h at 200 V, and ethanol precipitated. The final yield is about 10%.

Solution Synthesis of the Tetrasquare Complex. The outermost three quarters of each of squares 3, 4, and 5 are annealed in pairs and ligated together. The four-strand complexes that constitute the linkers and closest sections of square 2 are then added to them. The three squares, attached to fragments of square 2, are then combined, along with the missing portion of square 2, to make the final intermediate product. The tetrasquare is purified on a 4.5% denaturing gel, electroeluted, and ethanol precipitated. The synthesis is conducted on the 350 pmol scale and proceeds with a final yield of 1%, about 3.5 pmol.

Intermolecular Addition of Components to the Support. In the first step, 20 pmol of square 1 is ligated in 30% yield to 10 nM of support, prepared as described previously,¹⁰ containing about 50 pmol of linker. The linker on the support consists of a psoralen-crosslinked 26-mer ligated on one end to a 30-mer deoxythymidine strand synthesized on the support and on the other end to an 18-mer linker containing a *HindIII* site and a *BbvI* site. All restriction enzymes are purchased from New England Biolabs. The square is added by incubating it in 1000 μL of buffer 1 (New England Biolabs) with 50 units of restriction enzyme D1 (*BbsI*) overnight at 37 °C, adding a complement to the restricted hairpin, incubating 15 min at 37 °C, and ligating to the support with 30–40 units of T4 DNA ligase (USB) overnight at room temperature.

In the second step, 3.5 pmol of the tetrasquare complex is added to the support by incubating in the presence of 50 units of restriction enzyme D2 (*BsmAI*) overnight at 50 °C. The tetrasquare is incubated with an excess of the complement to the restricted hairpin for 15 min at 37 °C, added to the support, and then ligated overnight at room temperature; the yield is about 1–2 pmol, 30–50% efficiency. The complement to the hairpin restricted from the support need not be added, because it can be washed off the support readily. All restrictions on the support are done in 200-μL volumes with 100 units of restriction enzyme and proceed overnight.

In the final intermolecular step, square 6 is restricted with enzyme D1 (*BbsI*), incubated with a complement to the hairpin, and then ligated to restricted pentasquare complex overnight at room temperature. About 2 pmol of the six-square heptacatenane is formed.

Intramolecular Closure Reactions, Release from Support, and Annealing. The closure ligations are carried out sequentially overnight at 37 °C. All

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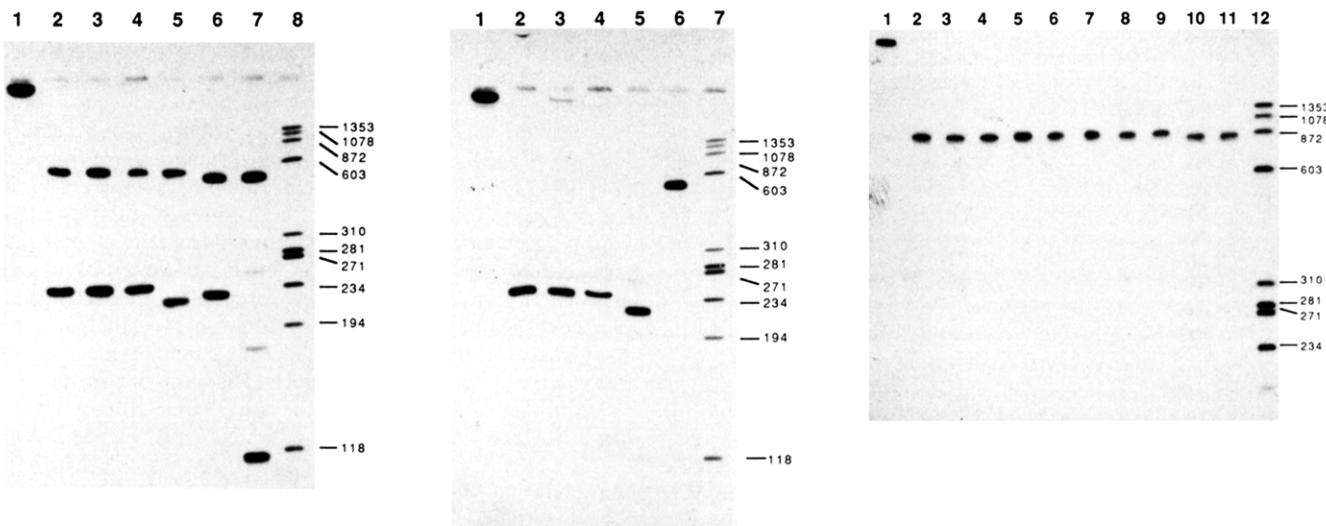


Figure 3. Synthesis of the components of the polyhedron. (a, left) Square 1. This is an autoradiogram of a 7% (19:1 acrylamide/bisacrylamide) denaturing gel. Both strands of square 1 are labeled for this demonstration, although neither is labeled in the synthesis of the final product. Lane 1 contains the intact molecule, and lanes 2–5 contain the digestion products of the outer circle with restriction enzymes S1 (*Bgl*II), S2 (*Nco*I), S7 (*Acc*65I), and D2 (*Bsm*AI). Lane 6 contains the cleavage products of D1 (*Bbs*I), and lane 7 contains the *Alw*NI digestion products from three sites on the inner circle. Lane 8 contains a series of linear length markers. (b, center) Square 6. This is an autoradiogram of a 7% (19:1 acrylamide/bisacrylamide) denaturing gel. Only the outside strand is labeled on square 6. As in part a, lane 1 contains the intact square, and the last lane, lane 7, contains a series of linear length markers. Lanes 2–5 contain the results of digesting the outer circle respectively with restriction enzymes S5' (*Bsp*EI), S6' (*Sall*), S7' (*Bsi*WI), and D1 (*Bbs*I). Linear strands of the expected lengths, 236, 236, 236, and 220 nucleotides, are produced. Lane 6 contains the visible digestion product of treating the square with *Bst*XI, the labeled outside circle. (c, right) Tetrasquare. This is an autoradiogram of a 4.5% (40:1 acrylamide/bisacrylamide) denaturing gel. Lane 1 contains the intact tetrasquare, and lanes 2–9 contain respectively the digestion products of symmetric enzymes S1' (*Bam*HI), S5 (*Xma*I), S3 (*Nhe*I), S3' (*Spe*I), S4' (*Bss*HII), S4 (*Mlu*I), S6 (*Xho*I), and S2' (*Bsp*HI). Lane 10 contains the D2 (*Bsm*AI) digest, and lane 11 contains the D1 (*Bbs*I) digest. Lane 12 contains a series of linear length markers. The strands corresponding to the squares are not visible in these gels, because they are unlabeled.

ligations are carried out in 200–300 μ L of the ligation buffer described previously.¹⁰ The seven intramolecular closure steps entail seven individual pairs of restrictions: S3 and S3', S4 and S4', S1 and S1', S2 and S2', S5 and S5', S6 and S6', and S7 and S7'. The S1 and S2 reaction sets have also been done preceding the S3 and S4 reaction sets. The seven intramolecular reactions proceed with about 50% yield each, for a final yield of about 15 fmol. The final step entails releasing the polyhedron from the support by means of enzyme D5 (*Bbv*I) and ligating on an annealing hairpin to close the central strand of square 1.

Results

Synthesis of the Squares and the Tetrasquare. Figure 3a demonstrates the successful assembly of square 1, and Figure 3b similarly illustrates the synthesis of square 6. These are autoradiograms of denaturing gels, showing that the cleavage of exocyclic arms by restriction enzymes yields specific predicted linear and cyclic molecules. Lane 1 of Figure 3a contains the intact square, the lanes 2, 3, 4, and 5 contain respectively the S1 (*Bgl*II), S2 (*Nco*I), S7 (*Acc*65I), and D2 (*Bsm*AI) digestion products, corresponding to cleavage of the outer strand in Figure 2a. Each of these cleavages leaves the inner circle (262 nucleotides) intact and results in a 232-nucleotide linear fragment, except for D2, which leaves a 220-nucleotide fragment. Lane 6 contains the cleavage products of D1 (*Bbs*I), on the inner circle, which leaves the 252-nucleotide outer circle and a 226-nucleotide linear fragment. Lane 7 contains the cleavage products of *Alw*NI, which has three sites on the inner circle and which also leaves a 252-nucleotide outer circle. Figure 3b illustrates the same analysis for square 6. It is clear that the products expected from the restriction of properly formed squares are obtained.

Figure 3c contains a denaturing gel that demonstrates the assembly of the tetrasquare molecule, by showing that the digestion products of each of its ten outside arms leave a linear molecule of the expected length. Lane 1 shows the intact tetrasquare, labeled only on the outside strand. Cleavage of this strand by the symmetrically cleaving enzymes (S1', S5, S3, S3', S4', S4, S6, S2') generates a molecule containing 848 nucleotides, whereas cleavage by D2 produces a molecule with 836 nucleotides and

cleavage by D1 generates a molecule with 832 nucleotides. The products expected from the complete outer strand are clearly present. We demonstrate the presence of each inner strand below.

Intermolecular Ligation on the Solid Support. The product of intermolecular ligation on the solid support is the six-square heptacatenane intermediate seen at the lower left of Figure 2a. There are two parts to the demonstration that this molecule has been formed: (1) We confirm that restriction with any of the 14 symmetric restriction sites on the outer circle produces the same predicted linear molecule; (2) we show that the intact object increases in mobility as we successively restrict and remove each of the inner squares. Parts a and b of Figure 4 demonstrate the first point, and part c illustrates the second point. The circle is predicted to contain 1220 nucleotides, when restricted by any of the 14 enzymes. It is clear from parts a and b that the same fragment, migrating as expected relative to standards, results from cleavage with each of the 14 enzymes.

Figure 4c contains the results of removing stepwise each of the squares from the heptacatenane. Lane 1 contains intact heptacatenane corresponding to the six-square intermediate. Lane 2 contains the products of cleaving square 1 with *Alw*NI, to produce a hexacatenane. Lane 3 contains the products of cleaving the hexacatenane in square 6 with *Bst*XI to produce a pentacatenane. Lane 4 contains the products of cleaving the pentacatenane in square 5 with *Sfi*I to produce a tetracatenane. Lane 5 contains the products of cleaving the tetracatenane in square 4 with *Pfl*MI to produce a triple catenane. Lane 6 contains the products of cleaving the triple catenane in square 3 with *Dra*III to produce a double catenane. Lane 7 contains the products of cleaving the double catenane in square 2 with *Bgl*II to produce the outer circle. Lane 8 contains the results of cleaving the outer circle with *Bgl*II to produce the 1220-nucleotide linear molecule. This gel clearly demonstrates that each of the inner squares is indeed a component of the intermediate.

Intramolecular Ligation on the Solid Support. It is necessary to demonstrate that each of the strands corresponding to the hexagons is contained within the structure. In the absence of

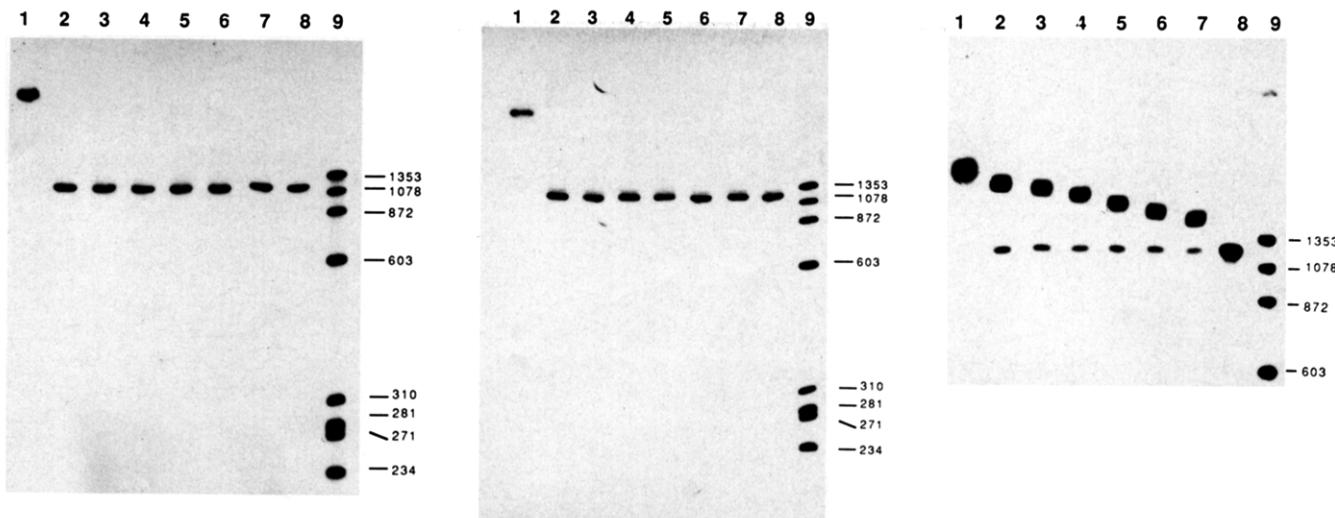


Figure 4. Restriction analysis of the six-square heptacatenane. (a, left, and b, center) Restriction of the outer circle. The construct was cleaved from the support with *Hind*III. The label in the molecule is only in the outer circle, which is destined to form all of the hexagons. These two gels are autoradiograms of 4% (40:1 acrylamide/bisacrylamide) denaturing gels. Each shows the same linear band that results upon cleavage with a symmetrically cleaving restriction endonuclease. Both gels have the same format, in which lane 1 contains uncleaved six-square product and lane 9 contains linear length markers. In part a, lanes 2-8 contain respectively the results of digestion with S5' (*Bsp*EI), S6' (*Xba*I), S6' (*Sall*), S3' (*Spe*I), S2' (*Bsp*HI), S7 (*Acc*65I), and S7' (*Bsi*WI). In part b, lanes 2-8 contain respectively the results of digestion with S1 (*Bgl*II), S1' (*Bam*HI), S2 (*Nco*I), S3 (*Nhe*I), S4 (*Mlu*I), S4' (*Bss*HII), and S5 (*Xma*I). In all cases, the band corresponds to the 1220-nucleotide predicted product. (c, right) Restriction of the squares. The six-square construct was cleaved from the support with *Bbv*I, and the missing hairpin was ligated to square 1 to complete the structure of the intermediate. This panel shows an autoradiogram of a 3% (40:1 acrylamide/bisacrylamide) denaturing gel showing the digestion of the construct by successively greater numbers of restriction enzymes that cleave the exocyclic arms of the cyclic molecules that correspond to the squares of the truncated octahedron. Lane 9 contains a series of linear length markers. Lane 1 contains the intact six-square intermediate product. Lane 2 contains the results of digesting that material with *Alw*NI, the enzyme that cleaves square 1. Lane 3 contains the results of digesting the material in lane 2 with *Bst*XI, the enzyme that cleaves square 6. Lane 4 contains the results of digesting the material in lane 3 with *Sfi*I, the enzyme that cleaves square 5. Lane 5 contains the results of digesting the material in lane 4 with *Pfl*MI, the enzyme that cleaves square 4. Lane 6 contains the results of digesting the material in lane 5 with *Dra*III, the enzyme that cleaves square 3. Lane 7 contains the results of digesting the material in lane 6 with *Bgl*II, the enzyme that cleaves square 2. Lane 8 contains the results of cleaving the molecule with *Bgl*II, which cleaves the outer strand. A certain amount of material corresponding to breakdown of the outer strand is visible in each lane. It is clear from this gel that each square is contained in the six-square product.

standards or of standard analyses for this type of molecule, we feel that the following demonstrates that the 14-catenane molecule has indeed been made. In analogy to proof of the synthesis of the cube-like molecule,⁹ we believe that the degradation of the final product to a characterizable oligocatenane standard will suffice to demonstrate the synthesis: We degrade the 14-catenane to the cyclic arrangement of four-hexagon tetracatenanes that flank each square. Three of these tetracatenanes are visible in part in Figure 1d. For example, the square on the upper right is flanked by red, blue, green, and yellow strands; the top hexagon has an edge that leads to the red-blue corner of the square, showing the catenation of those strands; the front (green) hexagon shows the catenation of the blue and green strands and the catenation of the green and yellow strands; and the hexagon on the lower right shows the catenation of the yellow and red strands. It is possible to prepare a standard four-hexagon cyclic tetracatenane with this system, by omitting the addition of square 6, closing the edges corresponding to sites S1, S2, S3, and S4, releasing from the support without annealing, and then digesting at the restriction sites in squares 2-5 (data not shown). The presence of two of these tetracatenanes flanking opposite squares constitutes minimal proof of the simultaneous presence of all eight hexagonal strands. We show here that five of the six possible tetracatenanes are produced by the appropriate restrictions; the inadvertent omission of restriction sites on the edges between hexagons I and II and between hexagons V and VI (Figure 1b) prevents us from demonstrating the presence of the tetracatenane flanking square 6.

The restriction sites available to demonstrate the intramolecular closure of the object are those on the extra arms of the squares, as well as those sites shown as the capital letters A-G in Figure 1b. These sites are located on the seven edges formed by intramolecular closure (Table 2). They correspond respectively to the four-base restriction sites that remain after ligating the

arms that had contained restriction sites S1-S7 and their primed complements. Figure 5a illustrates the presence of the tetracatenanes that flank squares 1-3, and Figure 5b illustrates the presence of the tetracatenanes that flank squares 4 and 5. In each case, the completed, annealed polyhedron is digested in a mixture of the restriction enzymes that digest all the exocyclic arms of the squares. This mixture is then divided into five aliquots and digested by the restriction enzymes corresponding to sites that liberate tetracatenanes: (1) C and D (*Rma*I and *Bst*UI) to liberate the tetracatenane that flanks square 1; (2) E and F (*Msp*I and *Taq*I) to liberate the tetracatenane that flanks square 2; (3) B and D (*Nla*III and *Bst*UI) to liberate the tetracatenane that flanks square 3; (4) A and C (*Dpn*II and *Rma*I) to liberate the tetracatenane that flanks square 4; and (5) A and B (*Dpn*II and *Nla*III) to liberate the tetracatenane that flanks square 5. In each case, the final digestion products comigrate with the cyclic tetracatenane marker on the same gel. When the tetracatenanes (including the standard) are treated with exonuclease III, certain amount of material that comigrates with dicatenane appears, perhaps due to a topoisomerase I-like strand passage activity that frequently contaminates preparations of this enzyme; treatment of DNA knots with commercial preparations of exonuclease III produces circles (S. M. Du and N.C.S., unpublished), and treatment of catenanes produces smaller catenanes (Y.Z. and N.C.S., unpublished). The integrity of the multistrand band on a denaturing gel and the persistence of the tetracatenane band while much longer amounts of linear material (at the bottom of the gel) are digested suggest that such disappearance of the tetracatenane as is seen results from this contaminating activity and that the material is covalently closed.

Discussion

Construction of the Truncated Octahedron. The truncated octahedron constructed here is the most complex object built to

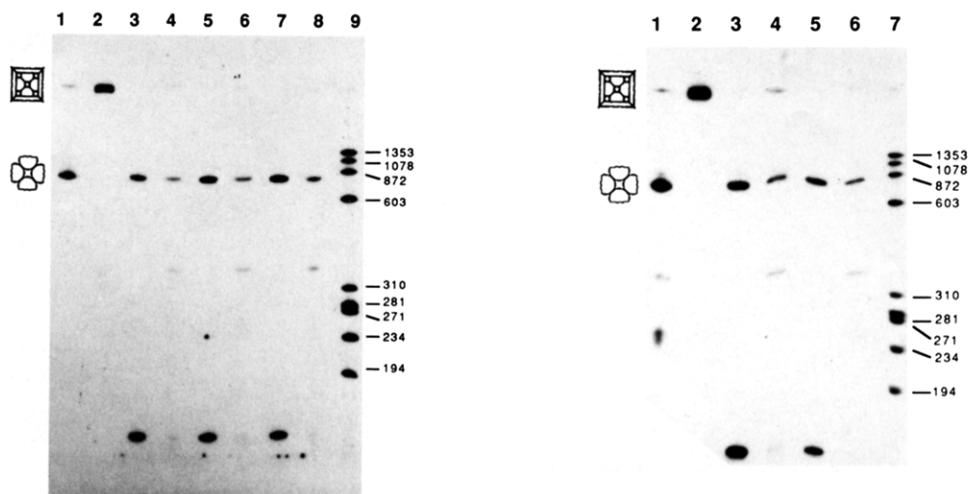


Figure 5. Restriction analysis to demonstrate the presence of all the hexagonal strands. The entire closed and annealed polyhedron is purified on a 3.5% (40:1 acrylamide/bisacrylamide) denaturing gel and digested in 100 μ L of New England Biolabs buffer 3 with a mixture containing *Bgl*I (which also cleaves *Sfi*I sites), *Dra*III, *Alw*NI, *Pst*MI, and *Bst*XI. This produces a squareless octacatenane that is then divided into five tubes containing 20 μ L each for further digestion. The positions of the tetracatenane and of the intact molecule are indicated to the left of each gel. (a, left) Digestion to release the cyclic tetracatenanes that flank squares 1, 2, and 3. This is an autoradiogram of a 5% (40:1 acrylamide/bisacrylamide) denaturing gel. Lane 1 contains a cyclic tetracatenane marker prepared by closing the edges corresponding to S1, S2, S3, and S4 in the five-square hexacatenane shown on the upper right portion of Figure 2a, followed by digestion of its constituent squares; this tetracatenane has been characterized by successive restriction (data not shown). Lane 2 contains undigested target truncated octahedron. Lane 3 contains the results of treating the squareless octacatenane with restriction enzymes C and D (*Rma*I and *Bst*UI) to liberate the tetracatenane (I, II, III, IV) that flanks square 1. Lane 4 contains the results of treating the material in lane 3 with exonuclease III (US Biochemical). Lane 5 contains the results of treating the squareless octacatenane with restriction enzymes E and F (*Msp*I and *Tag*I) to liberate the tetracatenane (I, II, V, VI) that flanks square 2. Lane 6 contains the results of treating the material in lane 5 with exonuclease III. Lane 7 contains the results of treating the squareless octacatenane with restriction enzymes B and D (*Nla*III and *Bst*UI) to liberate the tetracatenane (I, III, V, VIII) that flanks square 3. Lane 8 contains the exonuclease III digestion products of the material in lane 7. Lane 9 contains a series of linear length markers. (b, right) Digestion to release the cyclic tetracatenanes that flank squares 4 and 5. This is an autoradiogram of a 5% (40:1 acrylamide/bisacrylamide) denaturing gel. As in part a, lane 1 contains the tetracatenane marker, and lane 2 contains undigested material. Lane 3 contains the results of digesting the squareless octacatenane with restriction enzymes A and C (*Dpn*II and *Rma*I) to liberate the tetracatenane (II, IV, VI, VII) that flanks square 4. Lane 4 contains the results of digesting the material in lane 3 with exonuclease III. Lane 5 contains the results of digesting the squareless octacatenane with restriction enzymes A and B (*Dpn*II and *Nla*III) to liberate the tetracatenane (V, VI, VII, VIII) that flanks square 5. Lane 6 contains the results of digesting the material in lane 5 with exonuclease III. Lane 7 contains a series of linear length markers. Note that, in both parts a and b, substantial quantities of linear DNA are digested by exonuclease III, but only small quantities of the target catenanes are degraded by this enzyme. Whereas this is a denaturing gel, associations of linear molecules are unlikely to persist on it.

date from branched DNA components. The construction of the square components in solution proceeds readily with 10% yields, but synthesis of the tetrasquare component in solution is only 1% efficient. This reflects the experience of constructing a cube-like molecule in solution, which also proceeds in 1% yield. Ligations of components to the solid support are fairly efficient, with 30–50% yields. Surprisingly, the intramolecular closures on the support do not proceed well, ca. 50% yield each, in contrast to nearly quantitative yields seen in intramolecular closures of a quadrilateral on the support.¹⁰ Nevertheless, a molecule with three times the number of edges contained in the cube has been constructed in similar yield to the cube by means of the solid-support methodology.

The ultimate synthetic schemes of both the cube and the truncated octahedron have incorporated modifications necessitated by unanticipated experimental difficulties: It was impossible to purify the key intermediates of the cube under native conditions, so a denaturation–reconstitution step was included; the inability of *Ear*I or *Bsp*MI to cleave DNA on the support required the low-yield tetrasquare intermediate to be used, instead of a series of individual squares. The reasons for the failure of these enzymes on the support are unclear but may be related to a need for multiple substrates to be available simultaneously.^{17–19} It is possible to connect up six squares in the required order using only two distally cutting enzymes, but whereas a new sequence would have needed to be designed and synthesized, we tried the tetrasquare approach first. It is clear that further developments in the synthetic

methodologies will be required before syntheses can be planned and automated routinely.

Structure of the Molecule. The earlier construction of a cube-like molecule entailed the closure of a belt-like intermediate.⁹ One of the uncertainties associated with such a procedure involves the determination of the inside and outside of the polyhedron, because a belt can close in two structurally inequivalent directions. It is likely that this uncertainty has been overcome in the synthesis of the truncated octahedron, because one of the exocyclic arms is attached to the support at the start of the synthesis. We believe that this feature has selected those molecules that have their exocyclic arms facing outward rather than inward.

As in the case of the cube-like molecule, we have established the connectivity of the product catenane, rather than the 3-D structure of the molecule. We assume that each edge indeed constitutes a double linking of two cyclic single strands. The method of synthesis suggests that this type of linking structure would be most probable; however, there is no direct evidence that a link somewhere is not missing. In contrast to the techniques used in the solution synthesis of the cube,⁹ one of the strengths of the solid-support methodology is that no single strand is required to hybridize intermolecularly to three separate strands, possibly missing a link to the central strand. Here, we have had to do triple hybridizations only with the edge intermediates in the synthesis of the tetrasquare (Figure 2b); whereas the edges are the dominant portions of these objects and no strain appears to be involved, we feel that missing links are unlikely in these molecules.

We do not know the shape that the molecule assumes. Indeed, the high flexibility of the four-arm junction²⁰ makes it likely that many different conformers of the molecule exist. If the molecule

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were to adopt the form of an ideal semiregular truncated octahedron, it would resemble the shape shown in Figure 1c. In this idealized structure, the vertices constitute a macromolecular-scale version of the arrangement of Si(Al) atoms in sodalite; extending this structure in three dimensions by joining the external arms of numerous cages in the directions indicated would create an expanded version of a material similar to zeolite A (e.g. ref 21). Given a 68-Å edge, the distance between opposite squares of a polyhedron inscribed inside the DNA would be 192 Å, and the distance between opposite hexagons would be 166 Å. The volume of the inscribed polyhedron would be 3557 nm³.

Suggestions for Improved Syntheses. The synthesis of an object from DNA as complex as a truncated octahedron with approximately two worker years of labor demonstrates that DNA is a tractable medium for nanoscale construction. The successful assembly of the truncated octahedron from DNA demonstrates that the technology has attained control of DNA topology, in terms of both strand linking and helix-axis connectivity. It seems reasonable to suggest that any Platonic, Archimedean, or Catalan solid could probably be constructed from this system. The fact that we have been able to construct a polyhedron from polygons augurs well for the chances of building 3-D arrays from polyhedra. Nevertheless, the methodology could benefit from several improvements: (1) Means must be found to render a complex synthetic design less sensitive to the failure of biologically derived components, such as the problems encountered here with restriction enzymes. Possibly, chemical cleavage^{22,23} or more general enzymatic^{24,25} or mixed chemical–enzymatic²⁶ techniques

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can be developed as emergency measures to use at specific steps to produce asymmetric sticky ends when routine restriction fails. (2) Before incorporation into a synthetic scheme, all enzymes should be tested for their activity on the support, preferably in the presence of branched DNA molecules. In addition, enzymes should be free of strand-passage activities, as well as of nonspecific endonuclease activities. (3) The use of more rigid components, such as those identified recently by Leontis and his colleagues,²⁷ might permit the incorporation of multiple steps in the synthesis. More rigid components are probably necessary for the assembly of periodic matter. (4) If DNA is to be used as scaffolding to direct the associations of other macromolecules,^{3–5} the enzymatic cleavage process used here will need to be replaced with selective chemical procedures that will not be sensitive to the presence of bulky molecules tethered to the DNA. (5) Likewise, chemical ligation^{28,29} might prove more effective than enzymatic ligation. However, one must be aware that DNA ligase is capable of transducing the energy of its ATP cofactor to generate strained target products that are not seen when chemical ligation is used (S.M. Du and N.C.S., unpublished). (6) The synthesis of complex objects and periodic matter would be simplified by the automation of the process. It is to be hoped that the implementation of these improvements will lead to even more effective chemistry on the nanometer scale.

Acknowledgment. We thank Francis Barany for invaluable advice about restriction enzymes. This research has been supported by Grants N00014-89-J-3078 from the Office of Naval Research and GM-29554 from the NIH to N.C.S. and by a Dean's Dissertation Fellowship from New York University to Y.Z. The support of Biomolecular Imaging on the NYU campus by the W. M. Keck Foundation is gratefully acknowledged.

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